THE PHYTOCHEMICAL INVESTIGATION ON THE SURFACE EXUDATE OF
Microglossa pyrifolia: CASE STUDY AS ANTI-PLASMODIAL, ANTI-OXIDANT, ANTI-LEISHMANIAL AND ANTI-MICROBIAL

AGGREY AKIMANYA

A thesis Submitted to the Graduate School in Partial Fulfillment for the Requirements of the Award of the Degree of Master of Science in Chemistry of Egerton University

EGERTON UNIVERSITY
OCTOBER, 2014

DECLARATION AND RECOMMENDATION

DECLARATION

This thesis is my original work and has not been submitted wholly or in part for award in any institution.

Signature _____ Date_____

Aggrey Akimanya

RECOMMENDATION

This thesis has been prepared under our supervision and has our recommendation to be presented for examination as per the Egerton University regulations.

Prof. J. Matasyoh

Egerton University

Signature _____ Date_____

Dr. F. Okanga

Egerton University

Signature _____ Date_____

Prof J O Midiwo

University of Nairobi

Signature _____ Date_____

ACKNOWLEDGEMENTS

ABSTRACT

Malaria is a global disease prevalent in the tropics caused by Plasmodium species. It affects 300-500 million people and kills 1.5-2.7 million people annually. To date, a large number of herbal remedies are used to treat malaria and manage related fever; nevertheless, in most of these cases of plant therapies, efficacies have not been proven nor have their active components been identified. Most malaria episodes are accompanied by clinical complications attributable to radical oxygen species. In an effort to address the problem of malaria and the associated complications, phytochemical analysis of *Microglassa pyrifolia* (Asteraceae), used to treat malaria ethnomedically, was investigated for compounds with anti-plasmodial, antimicrobial, antileishmanial and anti-oxidant activities. In the preliminary investigation, the crude extracts of aerial parts of the plant [dichloromethane: methanol (1:1) extract] had an anti-plasmodial activity against D6 and W2 strains of *Plasmodium falciparum* of IC_{50} value of 8.0 and 13.0 μg/ml respectively. In this study crude leaf surface extract was obtained by dipping a handful of fresh leaves into ethyl acetate for a short period of 15 seconds to avoid the extraction of internal tissue components. Separation of compounds was carried out using chromatographic methods and their identification done by spectroscopic methods. A total of seven compounds were isolated, namely; 5,7,4'-trihydroxy-3,8,3'-trimethoxyflavone (**81**) 5,7,4'-trihydroxy-3,8,3',5'-tetramethoxyflavone (**82**), 5,3'4'-trihydroxy-7-methoxyflavanone (83), 5,7,3'-trihydroxy-3,8,2',5'-tetramethoxyflavone (**84**), 5,3'4'-trihydroxy-3,7,8-trimethoxyflavone (**85**), 8-acetoxyisochiliolide lactone (**86**) and 7,8-epoxyisocholiolide lactone (**87**) out of which compounds **82**, **84** and **86** were new. The *in vitro* anti-plasmodial tests were done by a colorimetric assay that determined the parasitic lactate dehydrogenase (pLDH) activity in 96-well microplate. Anti-oxidant activity was performed by UV-VIS spectrometry method based on the stable radical compound DPPH. The compounds showed no anti-plasmodial, anti-bacterial, anti-fungal and anti-leishmanial activities except 5,3,4'-trihydroxy-7-methoxyflavone (**83**) which exhibited mild anti-leishmanial activity (IC_{50} 13.13 μg/ml). Anti-oxidant activities were exhibited, with IC_{50} of 6.02 ±.0.2 for 5,7,4'-trihydroxy-3,8,3'-trimethoxyflavone (**81**), 6.45 ± 0.3 for 5,7,4'-trihydroxy-3,8,3'5'-tetramethoxyflavone (**82**), 8.5 ± 0.3 for 5,3'4'-trihydroxy-7-methoxyflavonone (**83**), 8.79 ± 0.3 for 5,7,3'-trihydroxy-8,4'5'-trimethoxyflavone (**84**) and 6.02 ± 0.2 for 5,3',4'-trihydroxy-3,7,8-trimethoxyflavone (**85**)

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

LIST OF APPENDICES

LIST OF ABBREVIATIONS AND ACRONYMS

ACT   Artemisinin Combination Therapies

DCM   Dichloromethane

DDT   Dichlorodiphenyltrichloroethane

DEPT   Distortionless Enhancement by Polarization Transfer

DMSO   Dimethylsulfoxide

DPPH   2, 2-Diphenyl-1-picrylhydrazyl

EIMS   Electron Impact Mass Spectroscopy

HMBC   Heteronuclear Multiple Bond Correlation

HMQC   Heteronuclear Multiple Quantum Correlation

HR-EIMS   High Resolution Electron Impact Mass Spectrometry

HSQC   Heteronuclear Single Quantum Correlation

IC_{50} Inhibition Concentration-50: Concentration of substance that produce 50% inhibition of certain process

LC_{50} Lethal Concentration-50: Concentration that kills 50% of test animal

MIC   Minimum Inhibition Concentration

NMR   Nuclear Magnetic Resonance

pLDH   Plasmodium lactate dehydrogenase

PTLC   Preparative Thin Layer Chromatography

RBM   Roll Back Malaria

ROS   Reactive Oxygen Species

TLC   Thin Layer Chromatography

UNICEF   United Nation Children Fund

UV-VIS   Ultra Violet-Visible

WHO   World Health Organization

CHAPTER ONE
INTRODUCTION

1.1   Background information

Plants through an integrated network of enzymes and carefully regulated chemical reactions, synthesize crucially important molecules including carbohydrates, proteins, fats, and nucleic acids which are called primary metabolites. Despite the extremely varied characteristics of living organisms, the pathways for modifying and synthesizing these metabolites are found to be essentially the same in all organisms, apart from minor variations. These processes demonstrate the fundamental unity of all living matter, and are collectively described as primary metabolism, with the compounds involved in the pathways being termed primary metabolites (Dewick, 2002) In contrast to these primary metabolic pathways, there also exists an area of metabolism concerned with compounds which have a much more limited distribution in nature. Such compounds, called secondary metabolites, are found in only specific organisms, or groups of organisms, and are an expression of the individuality of species or groups of species called taxons. Secondary metabolites are not necessarily produced under all conditions, and in the vast majority of cases the function of these compounds and their benefit to the organism is not clear. These compounds sometimes have important ecological functions, providing protection against attack by herbivores and microbes and serving as attractants for pollinators and seed-dispersing agents. They may also contribute to competition and invasiveness by suppressing the growth of neighboring plant species, a phenomenon known as allelopathy (Osbourn and Lanzotti, 2009). In addition to their function in plants, the natural products have great impact on both human and animal life. Plants have been an integral part of life in many indigenous communities, and Africa is no exception (Sidigia *et al*., 1995). Mankind has throughout the ages used natural products for various purposes including flavors, fragrances, dyes and most important, for medicine.

The use of plants for medical purposes dates back to antiquity (Sofowara, 1982). Most of Africa's biodiversity play major specific roles in the cultural evolution of human societies (Mugabe and Clark, 1998). The use of natural products for treatment of parasitic diseases is well known and documented since ancient times, and stems from the fact that some of these natural products are biosynthesized as defense agents against plant pathogens (Kaur *et al*., 2009). Initially these medicines took a form of crude extracts contained in herbal formulations like teas, tinctures, poultices and powders (Balunas and Kinghorn, 2005).

But with the enormous advancements in separation and isolation chemistry and pharmacological testing, natural products have continued to be significant sources of drugs and leads. Their dominant role is evident in the approximately 60% of anticancer compounds and 75% of drugs for infectious diseases that are either natural products or natural product derivatives (Newman *et al.*, 2003; Cragg *et al.*, 2005).

With developments in synthetic and computational chemistry, some drugs are designed through molecular modeling (Meyer *et al.*, 2000). However, despite all these developments, natural products and their derivatives represent more than 50% of all the drugs in clinical use in the world, including the treatment of malaria (Gurib-Fakim, 2006). Historically, majority of antimalarial drugs have been derived from medicinal plants or from structures modeled on plant lead compounds (Klayman, 1985). Quinine, artemisinin, derivatives and analogues, the drugs of choice for treatment of malaria, were either obtained directly from plants or developed using chemical structures of plant derived compounds as templates (Basco *et al.*, 1994; Kayser *et al.*, 2003). Many more compounds with significant anti-plasmodial activities have been isolated from plants and efforts are being made to develop some of these into future drugs (Kaur *et al.,* 2009). In underdeveloped communities, crude natural products made in the form of herbal formulations are still used for malaria therapy. Several plants with traditional use in curing malaria by different communities have been documented (Gessler *et al.*, 1995; Kvist *et al.*, 2006; Muthaura *et al.*, 2007a).

The recent emergence of multi-drug resistant malaria parasites and insecticide resistant mosquitoes, coupled with the limited number of chemotherapeutic agents available to treat and control the disease has triggered an alarming increase in the incidence and spread of the disease therefore highlighting the need for new chemically diverse effective drugs (Pamisha *et al.*, 2007). Another challenge encountered in the use of natural product-derived drugs and drugs from other sources in combating malaria has been the complications arising from oxidative stress during malaria infections. Oxidative stress has been linked to several malaria complications such as cerebral and pulmonary oedema, anemia and poor eyesight (Taoufiq *et al.,* 2006). The use of natural product-derived drugs from other sources in combating malaria has however been faced with several challenges, including the emergence of drug resistant parasites to drugs world-wide as well as complications arising from oxidative stress during malaria infections.

The increase in resistance to the anti-malarial drugs has necessitated continued search for alternative drugs to chloroquine and other commonly used anti-malaria drugs (Muthaura *et al*., 2007a). In this regard, several plants are being screened for anti-plasmodial activity.

In a collaboration programme between University of Nairobi and University of Mississippi, 140 plants were screened out of which six plants namely *Clerodendrum eriophyllum* (Verbenaceae), *Sphaeranthus bullatus* (Asteraceae), *Microglossa pyrifolia* (Asteraceae), *Vernonia galamensis* (Asteraceae), *Albizia gummifera (Fabaceae)* and *Alibizia schimperiana* (*Fabaceae*) showed activities as indicated in Table 1:1. These activities were interpreted as good to moderate activities, based on WHO guidelines on activity, which stipulates that extracts with IC_{50} of less than 10 µg/ml have promising activity, whereas those with IC_{50} of 11-50 µg/ml have modearate activity. The ethanol extact of *M.pyrifolia* showed antimicrobial effects which were poor to moderate with MIC values no less than 500 µg/mL as indicated in table 1.2. The same ethanol extract exhibited antioxidant properties with IC_{50} value of 47.31µg/mL for free radical scavanging activity (Dickson *et al.,* 2006). The active ingredients in the extracts of these plants were therefore to be sought through chromatographic fractionation and further bioassay.

Table 0.1: Anti-plasmodial activities of the selected plant extracts towards D6 (Chloroquin sensitive) and W2 (Chloroquin resistant) strains of *Plasmodium falciparum*

Plant	*In vitro* activity (IC_{50},µg/ml)	
	Pf D6	Pf W2
Microglossa pyrifolia (whole plant)	8.0	13
Albizia gummifera (root bark)	1.1	1.0
Sphaeranthus bullatus (whole plant)	9.7	15
Alibizia schimperiana (stem bark)	0.97	1.5
Vermonia galamensis (aerial parts)	9.0	9.2
Clerodendrum eriopyllum (aerial parts)	8.8	8.8

Table 0.2: Mean minimum inhibitory concentration (MIC; µg/mL) of *M. pyrifolia* on various microorganisms

Test organism	*M.pylifolia*	Tetracycline	Miconazole
Bacteria			
Staphylococcus aureus	>1000	2.5	NT
Bacillus subtilis	500	5	NT
Micrococcus flavus	>1000	1	NT
Streptococcus faecali	1000	5	NT
Escherichia coli	0	10	NT
Pseudomonas aeruginosa	0	20	NT
Salmonela abony	0	2.5	NT
Klebsiella aerogenes	0	5	NT
Fungi			
Candida albicans		NT	10
Saccharomyces cerevisae		NT	10
Trichophyton interdigitale		NT	20
Microsporum floccosum		NT	20

0, no inhibition observed; NT, not tested. All tests were done in triplicates. Tetracycline and miconazole served as positive control for antibacterial and antifungal assays respectively.

1.2   Statement of the problem

Malaria is the world's deadliest parasitic disease. It is transmitted by female *Anopheles* mosquitoes, and is a serious significant impediment to socioeconomic development in poor countries. It is one of the most important diseases of the African and Asian countries, killing 1-3 million people and causing disease in 300-500 million people in 107 countries annually.

Of these deaths, over 90% occur in tropical Africa. Some of the fatalities are caused by eleveted production of radicals in host cells. Efforts to combat the disease are hampered by growing resistance of malaria parasites to the readily available drugs. There is, therefore, a need for continued efforts in the search for antioxidants and compounds that are active against malaria parasites. These can be developed to new and more effective drugs.

1.3 Objectives

1.3.1 General objective

To identify phytochemicals with anti-plasmodial antimicrobial antileishmanial and antioxidant activities from the plant *Microglossa pyrifolia*

1.3.2 Specific objectives

1. To prepare a crude extract of *M. pyrifolia*
2. To isolate secondary metabolites from *M. pyrifolia* crude extract and elucidate the structures of the isolated pure compounds using spectroscopic methods.
3. To determine the *in vitro* anti-plasmodial, antimicrobial, antileishmanial and antioxidant activities of pure compounds of *M. pyrifolia*

1.4 Justification

In more recent times, natural products have continued to be significant sources of drugs and leads for drug development through structural modification. Some of the most important antimalarial compounds that have been isolated from traditional medicinal plants include quinine (**1**) from *Cinchona succiriba* (Rubiaccae) and artemisinin (**4**) from the *Artemesia annua* (Asteraccae). The ethno pharmacological approach for the search of new antimalarial agents from plant sources has proved to be predictive and very significant due to the success of the antimalarial drug quinine and the recent discovery of artemisinin. In Kenya, several plants have been in use traditionally for the treatment of malaria. Around Lake Victoria, Western Kenya, and in some West African countries, *M. pyrifolia* is used for the treatment of malaria. Although a lot of research has been carried out on this plant to establish its antimicrobial, antiplasmodial and antioxidant activities, phytochemical analysis to investigate the compound(s) behind these biological activities have not been undertaken. It is thus important to undertake further investigations on this plant for metabolites and to establish the compounds that are antiplasmodial (*in vitro*) and anti-oxidant.

CHAPTER TWO
LITERATURE REVIEW

2.1   Malaria problem

Malaria is a life-threatening parasitic disease transmitted by several species of *Anopheles* mosquitoes and is predominant in the tropics particularly in Africa and Asian countries (Korenromp, 2004; Geissbühler *et al* 2007). It is the female sex which is the vector. Malaria is caused by protozoa of the genus *Plasmodium*. The four human-pathogenic *Plasmodium* species *P. falciparum, P. vivax, P. ovale* and *P. malariae* differ in pathogenesis and global distribution (Odugbemi *et al*., 2007). *P. falciparium* causes most of the severe and fatal cases of malaria and is the predominant species throughout sub-Saharan Africa. Since *P. vivax* can develop in mosquitoes at a lower temperature than *P. falciparum*, its geographical distribution is wider. *P. vivax* and *P. ovale* can form resting stages in the liver (hypnozoites) which may cause clinical relapses many months after the first attack (Hetzel *et al*., 2007).

In humans, malaria parasites are found in the liver and in the blood where they invade, grow and replicate in erythrocytes. Once sexual forms (gametocytes) of the parasites have developed and circulated in the blood stream, they may be taken up by a female *Anopheles*, and the parasite's sexual reproduction can take place in the gut of the mosquito. Once in the mosquito gut, they undergo extensive replication and when the mosquito bites another human for blood meal, the parasites can pass via the salivary glands into the blood stream of the victim usually 10-14 days after the initial blood meal (Warrel and Giles, 2002; Greenwood *et al*., 2005). Clinical malaria is manifested by a range of symptoms. In semi-immune populations, malaria often results in a symptomatic parasitaemia; which is manifested by symptoms such as headaches, cough, body pains, chills, vomiting and diarrhea (Warrel and Gilles, 2002; Nkuo-Akenji and Menang, 2005). Severe malaria is a complex multi-system disorder, characterized by one or more of the following clinical features; Severe anaemia, prostration, impaired consciousness, respiratory disease, multiple convulsions, circulatory collapse, pulmonary oedema, abnormal bleeding, jaundice and haemoglobinuria (WHO, 2000b; Mackintosh *et al*., 2004).

Cerebral malaria and severe anaemia are responsible for long term developmental impairments and is the cause of most deaths in children (Carter *et al*., 2005). Severe anaemia results from the destruction of red blood cells, either directly by the malaria parasite or indirectly by immune mechanisms.

In children it may lead to long-term neurological, cognitive and developmental impairments while in pregnant women it may lead to low birth-weight, increase in infant and child mortality (Breman, 2001; Schellenberg *et al*., 2003b; Marchant, 2004.). Where malaria prospers most, human societies have prospered least. Malaria imposes economic costs on households and states. In some African countries, it is estimated that malaria is responsible for an economical decline of up to 13% per year (Sachs and Malaney, 2002). There is a strong link between malaria and poverty. Due to the heavy financial burden it imposes on the family and the loss of workdays and income, it leads to poverty and consequently, poverty makes the family more vulnerable to it.

In terms of economy, malaria is known to be both a disease and a cause of poverty. It is estimated that malaria is responsible for a GDP economic growth penalty of up to 1.3% per year in some African countries (Sachs and Malaney, 2002). When compounded over the years, this penalty leads to substantial differences, totaling US\$ 12 billion annually in GDP between countries without malaria and countries with malaria, and severely restrains the economic growth of entire Africa region [Kamau, 2006].

The problem of controlling malaria has been aggravated by inadequate health structures and poor socio-economic conditions. The recent emergence of multi-drug resistant malaria parasites and insecticide resistant mosquitoes has compounded the problem further and triggered an alarming increase in the incidence and spread of the disease. (Olliaro and Yuthavong, 1999; Berry, 2000). Efforts to overcome the problem are hindered by two obstacles. One is the growing resistance of malaria parasite *Plasmodium falciparum* to chloroquine and other commonly used cheap synthetic drugs, as well as the growing resistance of the vector *Anopheles* to DDT and other insecticides (Bilia, 2006). The second obstacle is limited availability, coupled with higher cost and greater toxicity of alternative drugs (Saidu *et al*., 2000).

In recognition of the gravity of the malaria problem, WHO and UNICEF launched the Roll Back Malaria (RBM) partnership in 1998, with the aim of reducing malaria burden by at least 50% by the year 2010 through application of evidence based interventions and strengthening health delivery services (Kamau, 2006). This was supported by the Abuja declaration in 2000 where African heads of state agreed on a concerted effort to reduce malaria burden on the continent and endorsed the goals of RBM partnership of halving the number of malaria deaths by the year 2010 (Hetzel *et al*., 2007). Currently, efforts to promote three core malaria control interventions are being undertaken concurrently: use of insecticide treated nets, intermittent preventive treatment during pregnancy and infancy, and effective treatment of clinical cases with artemisinin based combination therapies (ACTs) (Kamau, 2006).

In Kenya, like other tropical countries, malaria is endemic and resistance of malaria parasites to readily available and cheap drugs like chloroquine is growing fast. According to the Ministry of Health, twenty four million Kenyans are at risk of malaria infections and majority cannot afford the costs of the adopted artemisinin based combination therapy. The government therefore found it necessary to provide free ACT therapy in health facilities alongside expansion of healthcare provision (Muthaura *et al*., 2007b; World Report, 2007). Despite this effort however, many regions remain underserved and subsequently the communities use herbal remedies and other cheaply available alternatives (Kirira *et al*., 2006; Muthaura *et al*., 2007b). Amid the malaria deaths and suffering, progress is being made. Though for too long the global community has been reluctant to invest sufficient resources in fighting malaria, leaving it near the bottom of the world's health agenda, there is gradual increase in international awareness of the problem and malaria is now on the agenda of the health community, political arena and international financial institutions. In 1997, the multi-lateral initiative of malaria (MIM), an alliance of agencies, institutions and governments, was formed to maximize the impact of scientific research on malaria through capacity building in Africa and global collaboration.

2.2   The use of plants and natural products in chemotherapy of malaria

Natural products have been investigated and utilized to alleviate disease since early human history. In early 1900s, before the "synthetic Era", 80% of all medicines were obtained from roots, barks and leaves (James *et al*., 2006). Thus plants and natural products have had a great contribution to the fight against malaria since time immemorial.

In Kenya and other developing countries, the use of herbal remedies in treatment of malaria is well known as evidenced by overwhelming literature on plants used traditionally to cure malaria (Kokwaro, 1976; Muregi *et al*., 2003; Koch *et al*., 2005; Kirira *et al*., 2006; Muthaura *et al*., 2007a; Muthaura *et al*., 2007b). Apart from their use in traditional medicine, plants and natural products have also contributed to modern treatment of malaria, being the source of some commonly used anti-malarial drugs like quinine (**1**) and artemisinin (**4**) from *Cinchona* and *Artemisia* respectively and their synthetic modifications and analogues (Figure 2.1).
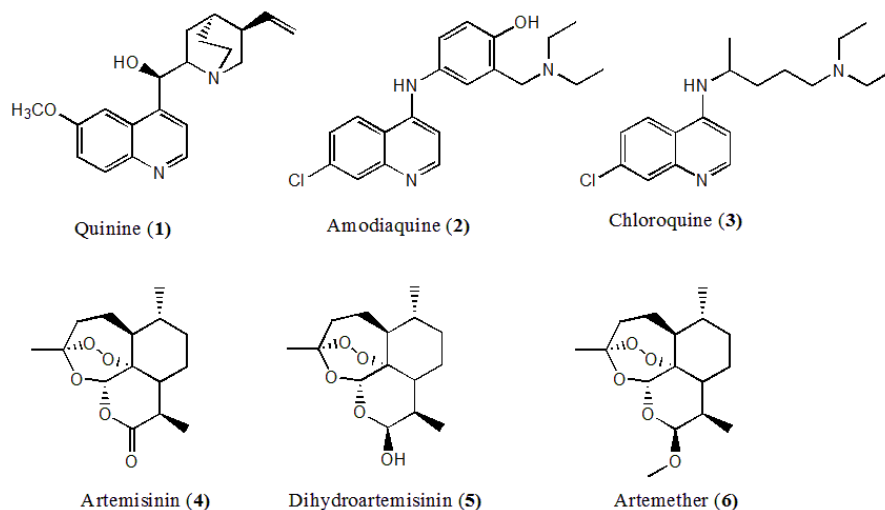
Figure 2.1: Some Natural Products and their synthetic analogues used against malaria.

2.3   Malaria and oxidative stress

Normal physiological processes in the body result in the production of Reactive Oxygen Species (ROS), which are also produced by the influence of exogenous chemicals. In the absence of parasitic attack, numerous enzymatic and non enzymatic antioxidant systems in cells and biological fluids are able to counter these ROS and their subsequent adverse effects.

However, in malaria patients, there is an increase in the production of ROS and the antioxidant defense system is weakened. This therefore results in oxidative tissue damage leading to oxidative stress associated with several pathological phenomena such as cerebral and pulmonary oedema, poor eyesight, atherosclerosis, cardiac ischemies, rheumatic diseases and cancer (Bahorun *et al*., 1996). Antioxidants are thought to act by suppressing the enzymes responsible for superoxide production, chelating metal ions that induce free radicals and reduction of radicals by donation of hydrogen atoms (Pieta, 2000), as is illustrated in figure 2.2
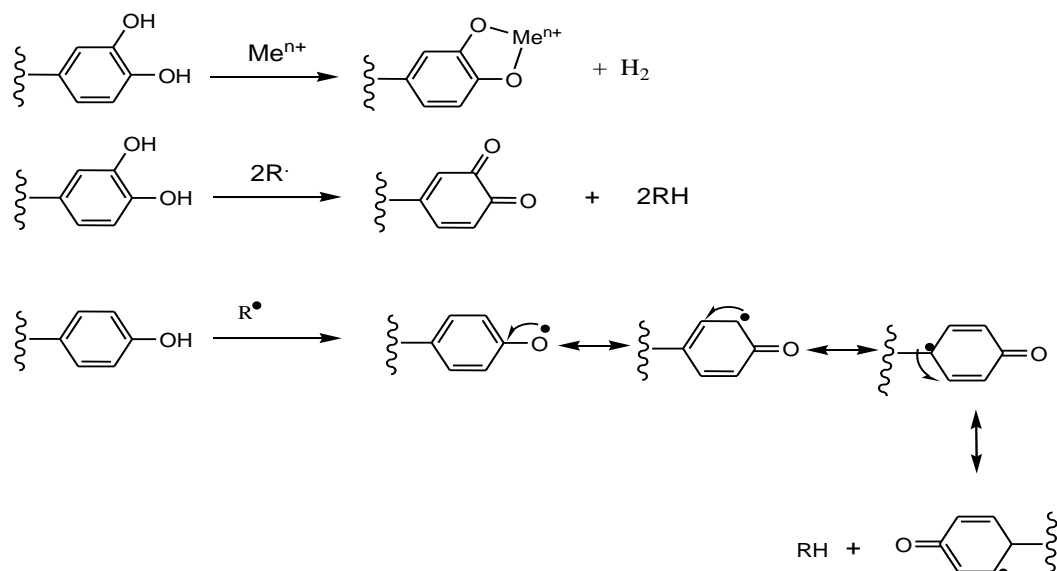
Figure 2.2: Mechanisms of antioxidant activity through chelation of metal ions and reduction by hydrogen donation.

As malaria parasites proliferate in malaria patients, large quantities of ROS are generated which damage macromolecules leading to several malaria complications. The major source of ROS is heme, a by-product of hemoglobin digestion degradation by *Plasmodium*, whereby the iron II in heme is oxidized to iron III releasing an electron that is used to ionize oxygen molecules and subsequently form peroxides which are easily broken to radicals (Figure 2.3).
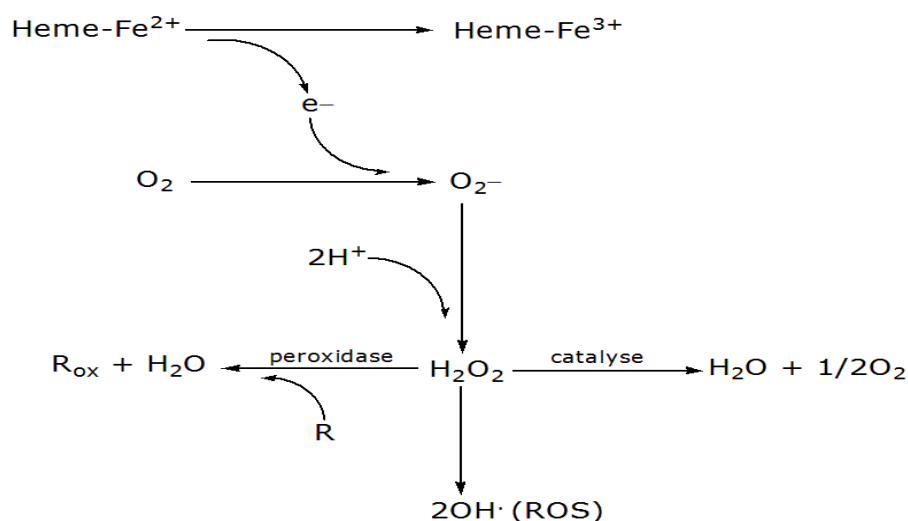
Figure 2.3: Generation of ROS from heme.

ROS are also produced by host immune system for the purpose of eradicating the parasite, as the parasite is highly susceptible to oxidative burden (Kawazu *et al*, 2008). Malaria parasites are therefore equipped with anti-oxidant defenses that are meant to establish redox equilibrium for their survival. Such defenses are the ones targeted in malaria control strategies, some of the anti-malarials like chloroquine and artemether are known to act by increasing the production of ROS (Taoufiq *et al*., 2006).

The effect of ROS to malaria patient therefore, though beneficial as it suffocates the parasite, is also detrimental since it leads to the damage of some cells and hence bringing about more complications to the patient. It is therefore important to investigate if some of anti-plasmodial compounds can also have anti-oxidant effect.

2.4   Leishmaniasis

Leishmaniasis is a protozoan disease that affects many people in the world; 12 million are currently infected and around 350 million others are threatened (Croft *et al*., 2003). It is transmitted by a bite of female phlebotomine sand flies which infect the blood with parasites of the genus *Leishmania* (Tonui, 2006). Two common forms of leishmaniasis are known; cutaneous leishmaniasis (CL) which causes sore (ulcers) at the bite site (Figure 2.4) and visceral leishmaniasis (VL) which affects vital organs. Sandflies *Phlebotomus martini* and *Phlebotomus orientalis*, are known vectors for *Leishmania donovani*, the causative agent for visceral leishmaniasis (VL), also known as Kalaazar. *Phlebotomus duboscqi* and *Phlebotomus guggisbergi* are vectors for *Leishmania major* and *Leishmania tropica* respectively, the aetiological agents for cutaneous leishmaniasis (CL).

Figure 2.4: Cutaneous leishmaniasis ulcer on the hand of an infected adult human being.

Leishmaniasis is found in 88 countries hosting 350 million people, being widespread in tropical and sub-tropical regions of the world where it is a serious health problem (Kigondu *et al*., 2009). More than 90% of the global cases of visceral leishmaniasis (VL) occur in India, Bangladesh, Nepal, Brazil and Africa (particularly East and North Africa including Sudan). Globally, the number of new cases of cutaneous leishmaniasis (CL) and visceral leishmaniasis (VL) is estimated at 1.5 million and 500,000 annually, respectively. People of all ages are at risk of infection by leishmaniasis if they live or travel where the disease is endemic.

In Kenya, the disease has been reported in Baringo, Kitui, Machakos, Meru, West Pokot, Elgeyo Marakwet and Turkana districts, which are known to be endemic foci for kala-azar (VL) (Kigondu *et al*., 2009). Currently, the recommended drugs for both VL and CL are pentavalent antimonials: sodium stibogluconate (pentosam®) and meglumine antimoniate (glucantime®). Both drugs require long courses of parenteral administration and have toxic side effects (Kigondu *et al*., 2009).

2.5 Ethno-botanical, ethnopharmacological and phytochemical information on *Microglossa pyrifolia*

Microglossa pyrifolia (Figure 2.5) which belongs to the family Asteraceae is a climbing shrub indigenous in Africa and tropical Asia (Schmidt *et al*., 2003). The plant is also called *Conyza pyrifolia* or *Microglossa volubilis* and is in the genus *Microglossa*, with about ten species (Kuiate *et al*., 1999). It is traditionally used against fever and malaria in Kenya and Ghana (Kokwaro, 1976; Kohler *et al*., 2002). It is also used as an abortifacient, as an analgesic to relieve headaches and stomach pains as well as for treatment of colds, wounds and eyes (Akah and Ekekwe, 1995; Johns *et al*., 1990; Kuiate *et al*., 1999; Schmidt *et al*., 2003). Its organic solvent extract showed anti-plasmodial activity (Kohler *et al*., 2002).

Figure 2.5: Photo of *Microglossa pyrifolia*

2.6 Phytochemistry of the genus *Microglossa*

Microglossa pyrofolia is the only species reported to have medicinal uses and biological activities amongst *Microglossa* species. Phytochemical information however is available on this plant together with *M. angolensis, M. mespilifolia, M. pyrrhopappa* and *M. zeylanica*. Clerodane and *seco*-clerodane diterpenoids have been reported from *M. pyrifolia* (**7 – 16**, Figure 2.6) (Zdero *et al.*, 1990a; Kohler *et al.*, 2002), M. *pyrrhopappa* and *M. angolensis* (**17 – 43**, Figure 2.7) (Zdero *et al.*, 1990b; Tene *et al.*, 2005).
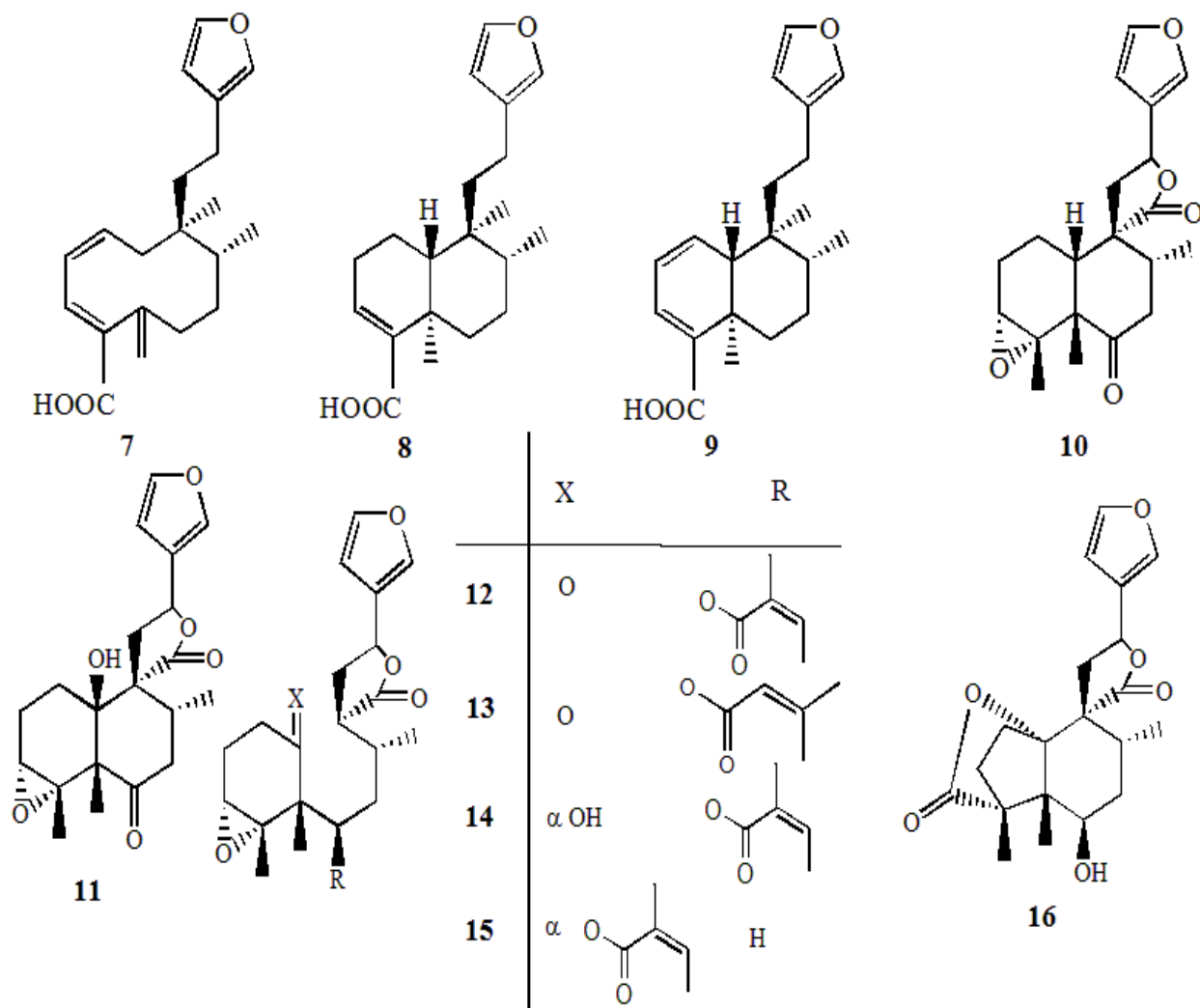
Figure 2.6: Clerodane and seco-clerodane diterpenoids from *M.pyrifolia*

	R_1	R_2
18	H	H
19	Ac	H
20	Ac	Ac

17

21

22

	R_1	R_2
23	H	OH
24	HO (angelate)	OH
25	(2-methylbutanoate)	OH
26	HO (angelate)	H

27

28

	R_1	R_2
29	H	H
30	(methacrylate)	H
31	(butanoate)	OH
32	H	OH

33

R = H : **34**
R = OAc: **35**

	R
36	H
37	OH
38	(acrylate)
39	(senecioate)
40	(tigloate)
41	(2-methylbutanoate)

R = H : **42**
R = Ac: **43**
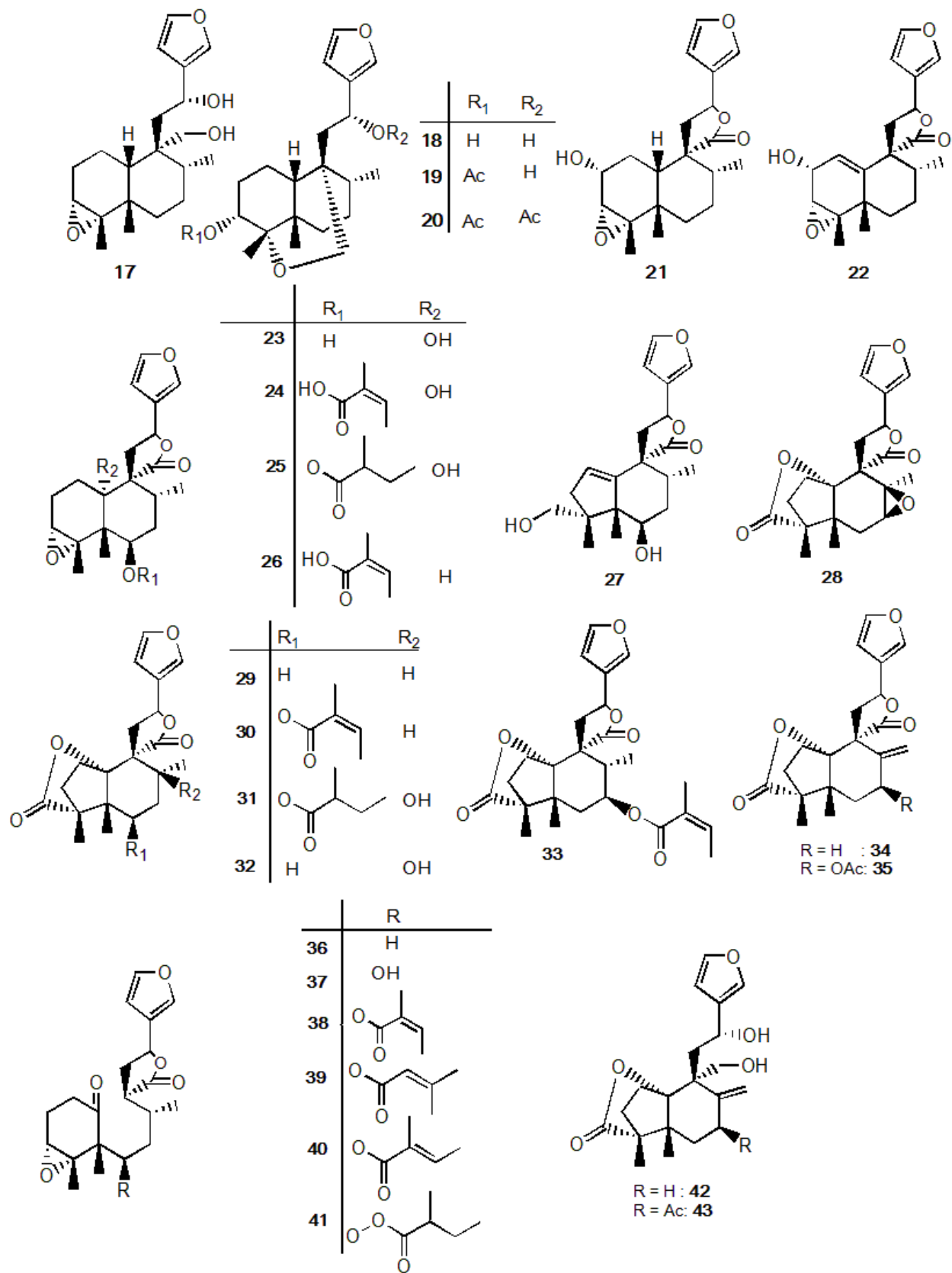
Figure 2.7: Clerodane and seco-clerodane diterpenoids from *M. pyrrhopappa* and *M. angolensis*

14

Another class of compounds isolated from the genus *Microglossa* is phytane diterpenoids. These have been isolated from *M. pyrifolia* (**44 –49**, Figure 2.8) (Kohler *et al*., 2002; Zdero *et al*., 1990a), *M. pyrhopappa* (**48 –50**, Figure 2.8) (Zdero *et al*., 1990b) and *M. zeylanica* (**51 – 52**, Figure 2.8) (Gunatilaka *et al*., 1987). Out of these, phytol (**44**) and 6*E*-geranylgeraniol-19-oic acid (**46**) exhibited antiplasmodial activities of 8.5 and 12.9 µM respectively against chloroquine sensitive strains of *Plasmodium falciparum* (PoW). Compounds **44** and **46** also exhibited antiplasmodial activities of 11.5 and 15.6 µM respectively against chloroquine resistant strains of *Plasmodium falciparum* (Dd2) (Kohler *et al*., 2002).

	46	**47**	**48**	**49**	**50**
R_1	H	Ac	H	Ac	H
R_2	H	H	H	H	H
R_3	CH_3	CH_3	CH_2OH	CH_2OH	COOH
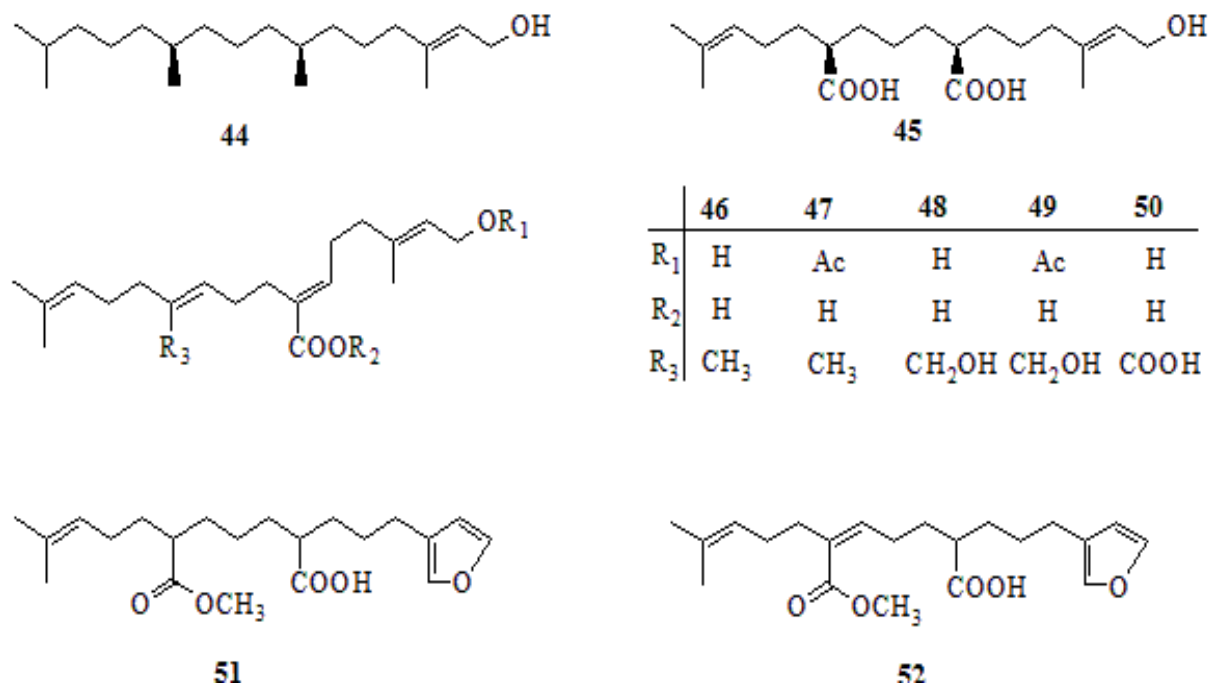
Figure 2.8: Phytane diterpenoids from *M. pyrifolia*, *M. Pyrhopappa* and *M. zeylanica*

Other compounds reported from *Microglossa pyrifolia* are dihydrobenzofurans and aurone flavonoids (**53 – 60**, Figure 2.9), (Schmidt *et al*., 2003; Rucker *et al*., 1992), triterpenoids (**61 – 67**, Figure 2.10), (Schmidt *et al*., 2003), sesquiterpenes (**68-72**, figure 2.11), (Zdero *et al*., 1990a; Kohler *et al*., 2002) as well as acetylenes and acetylenic glucosides (**73 –80**, Figure 2.12) (Rucker *et al*., 1992). Compound **74**, namely 2-*β-D*-glucopyranosyloxy-1-hydroxytrideca-3,5,7,9,11-pentayne exhibited antibacterial activities against the bacteria *Escherichia coli*, *Pseudomonas aeruginosa*, *Enterococcus faecalis* and *Staphylococcus aureus* (Rucker *et al*., 1992).
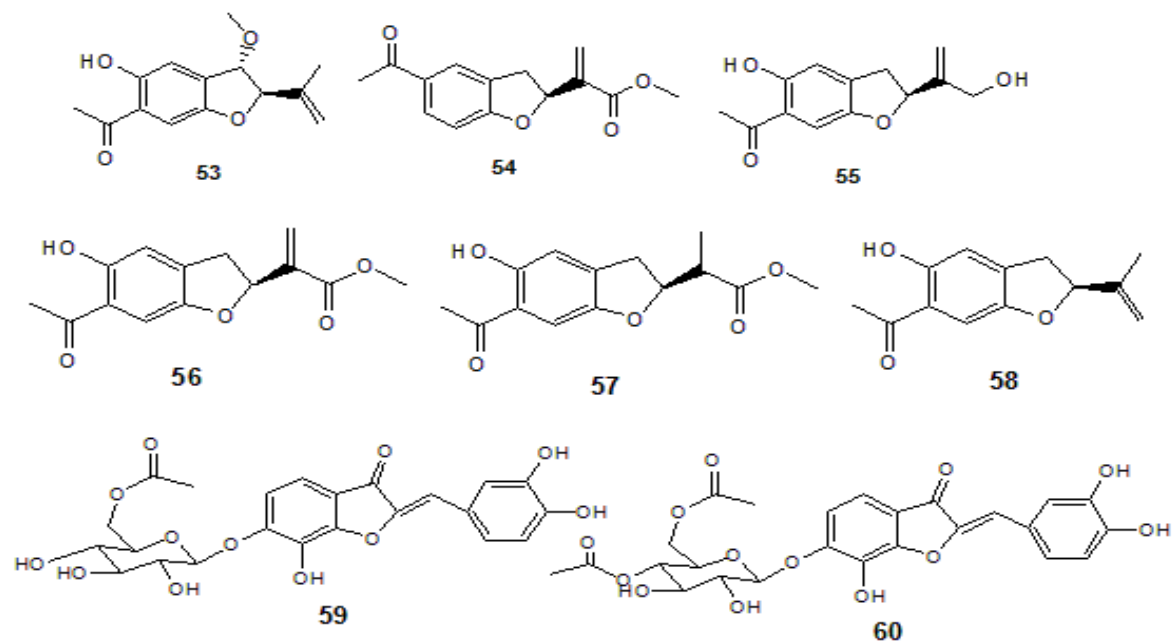
Figure 2.9: Dihydrobenzofurans and aurone flavonoids from *M. pyrifolia*
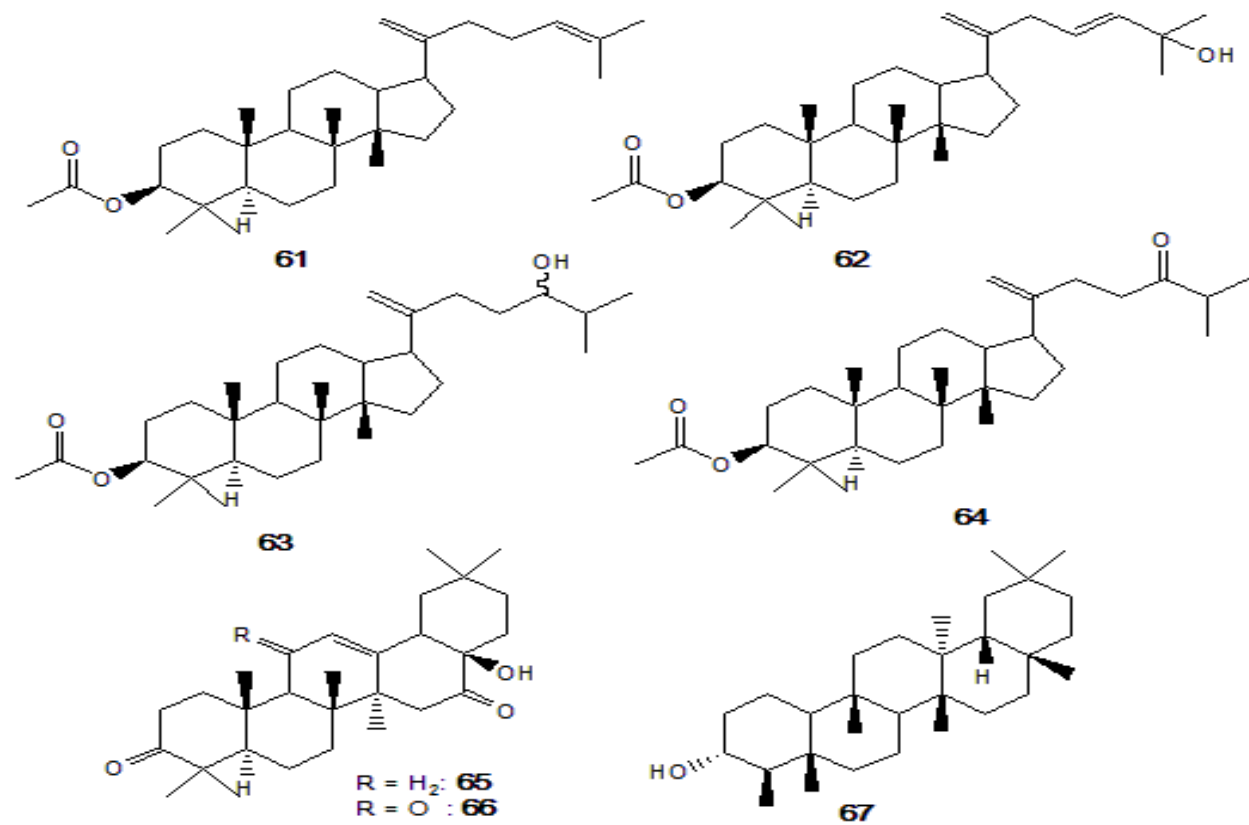
Figure 2.10: Triterpenoids from *Microglossa pyrifolia*
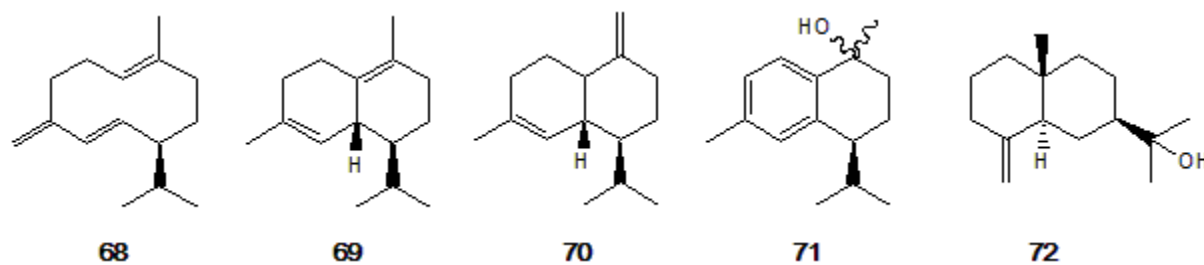
Figure 2.11: Sesquiterpenes from *Microglossa pyrifolia*
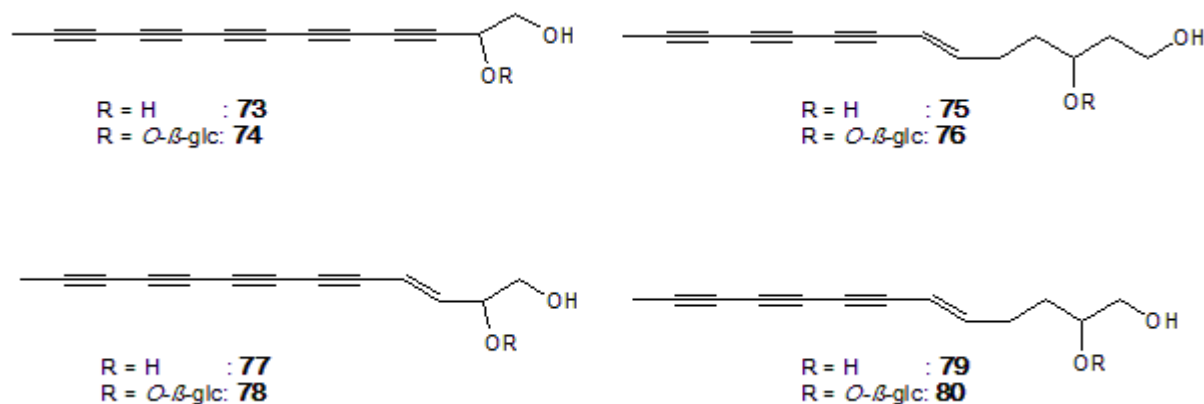
Figure 2.12: Acetylenes and acetylenic glucosides from *Microglossa pyrifolia*

2.7 Secondary metabolites

Secondary metabolites are metabolic intermediates or products, found as differentiation products in restricted taxonomic groups, not essential to growth and the life of the producing organism. They are biosynthesized from one or more general metabolites by a wider variety of pathways than are available in general metabolism. Secondary metabolites tend to be strain specific and have a wide range of chemical structures and biological activities (Rosenthal and Berenbaum, 1992).

The ability to synthesize secondary metabolites has been selected through the course of evolution in different plant lineage when such compounds address specific needs. Floral scent volatiles and pigments have evolved to attract insect pollinators and thus enhance fertilization. Synthesis of toxic chemicals has evolved to ward off pathogens and herbivores or to suppress the growth of neighboring plants (Bennet and Wallsgrove, 1994; Dixon *et al.,*. 1996; Mitchel-Olds *et al.,* 1998; Trossat *et al.,* 1998; Harborne, 1999).

Chemicals found in fruits prevent spoilage and act as signals (in the form of color, aroma, and flavor) of the presence of potential rewards (sugars, vitamins and flavor) for animals that eat the fruit and thereby help to disperse the seeds. Other chemicals serve cellular functions that are unique to the particular plant in which they occur (e.g. resistance to salt or drought) (Dudareva and Pichersky, 2000; Mol *et al.,* 1998; Mert-Turk, 2002). Secondary metabolites are formed in only specific organisms, or groups of organisms, and are an expression of the individuality of species. They have a very restricted distribution than primary metabolites in the whole plant kingdom i.e. they are often found only in one plant species or a taxonomically related group of species.

High concentrations of secondary metabolites might result in a more resistant plant. Their production is thought to be costly and reduces plant growth and reproduction (Simms and Fritz, 1992; Karban and Baldwin, 1997; Stotz *et al*., 2000; Siemens *et al*., 2002). Therefore, defense metabolites can be divided into constitutive substances, also called prohibitions or phytoanticipins and induced metabolites formed in response to an infection involving *de novo* enzyme synthesis, known as phytoalexins (Van Etten *et al*., 1994; Woodall and Stewart, 1999). Phytoanticipins are high energy and carbon consuming and exhibit fitness cost under natural conditions (Mauricio, 1998), but recognized as the first line of chemical defense that potential pathogens have to overcome. In contrast, phytoalexin production may take two or three days, as by definition first the enzyme system needs to be synthesized (Woodall and Stewart, 1999). The majority of secondary metabolites belong to one of a number of families, each of which has particular structural characteristics arising from the way in which they are built up in nature (biosynthesis). The three major classes of secondary metabolites are, polyketides and fatty acids, terpenoids and steroids,phenylpropanoids and alkaloids (Rosenthal *et al*., 1992).

2.7.1 Surface exudates

Plants represent a rich source of nutrients for many organisms including bacteria, fungi, protists, insects, and vertebrates. Although lacking an immune system comparable to animals, plants have developed a stunning array of structural, chemical, and protein-based defenses designed to detect invading organisms and stop them before they are able to cause extensive damage (Brian and Gwyn, 2008).

Trichomes are simple or glanded epidermal appendages that occur on most plants. Glandular secreting trichomes are found on approximately 30% of vascular plants (Dell and McComb, 1978; Fahn,1988), and they produce surface-accumulated exudates that usually contain hydrophobic isoprenoids and phenylpropanoids, the latter including flavonoids, phenolics, tannins, quinones, etc. (Wagner *et al*.,1991). In some plants, amphipathic sugar esters are also commonly found in glandular trichome exudates (Wagner *et al*., 2004). Such compounds have been associated with insect resistance in many plants, and pest resistance is often correlated with glandular trichome density (Spring, 2000). Two well-studied cases of glandular trichome–based insect resistance are found in the plant family Solanaceae. Sugar esters produced by tall glandular trichomes (TGSTs) of primitive tomato (*Lycopersicon pennellii*) and potato (*Solanum berthaultii*) species, and the diterpenoid cembratriene-ol produced by tobacco TGSTs, have been shown to inhibit aphid infestation (Goffreda *et al*., 1990; Wang *et al*., 2004). Antimicrobial activities of trichome exudate compounds (particularly monoterpenoids and sesquiterpenoids) have also been reported, but these are less studied than insect resistance (Wagner *et al*., 2004).

Considerable recent research has focused on glandular secreting trichomes (GSTs) to understand and exploit their ability to secrete phytochemicals that might improve resistance to insects, microbes and herbivores. This research is also looking at the possibility of modifying gland metabolism towards improving properties of exudates (e.g. flavour and aroma in herbs) and allows commercial production of useful compounds (molecular farming). Plant sources are increasingly being exploited for new drug development, and there is increased interest in validating traditional medicines and herbal remedies (Wagner, 1991). Many of the phytochemical principals involved are surface secretions. Leaf surface protuberances include simple trichomes, glandular secreting trichomes, hydathodes, and other structures that extend above the epidermal plane, and the morphologies of these vary widely between plant species (Wagner *et al*., 2004). In general, surface structures increase the distance between invaders and the leaf interior, and because of this they are mechanical deterrents to pathogens (Bennet and Wallsgrove, 1994). Trichomes were among the first anatomical features of plants to be recognized by early microscopists, and they have played a key role in plant taxonomy (Bennet and Wallsgrove, 1994). Simple trichomes are present on aerial surfaces of most angiosperms and on some gymnosperms and bryophytes (Uphof, 1962; Johnson, 1975).

In Angiosperms, trichomes may occur on leaves, petals, stems, petioles, peduncles and seed coats, depending on the species. GSTs are found on perhaps 30 % of vascular plants (Dell and McComb, 1978; Fahn, 1988). The amount of exudate produced by GSTs may reach 30 % of mature leaf dry weight (Dell and McComb, 1978). Terpenoids (mono- terpenes, sesquiterpenes, diterpenes and triterpenes) are clearly the most common and structurally diverse compound group found in GST exudates (Kellogg, 2001). Certain plants produce copious amounts of mixed-terpenoids, such as the terpenophenolic cannabinoids of *Cannabis* (Mahlberg and Kim, 1992). Flavonoids occur as aglycones in trichome exudates of a number of species, sometimes as crystalline deposits (Wang *et al*., 2004). The occurrence of flavonoids as aglycones is not surprising since they are lipophylic and would be highly soluble in mixed exudates containing terpenoids.

In contrast, flavonoid glycosides, as accumulated in many plants, occur in vacuoles and chromoplasts. Examples of trichome-secreted phenolics include primin (*Primula sp*.), chlorogenic acid and rutin (*Solanum sp*.). The accumulation of toxic compounds at the surface allows their direct contact with insects, pathogens and herbivores. Thus trichome exudates are ideally positioned to provide a first line of defence against attacking organisms, perhaps providing time for activation of induced defences (Harborne, 1999; Wagner *et al*., 2004).

2.7.2   Biosynthesis of Flavonoids

Flavonoids are synthesized by extension of *p*-hydroxycoumaroyl CoA with three molecules of malonyl CoA in a head-to-tail manner forming a tetraketide intermediate (Figure 12.14). This step is catalyzed by the enzyme chalcone synthase (CHS). The intermediate then folds and condenses further to give a chalcone. This reaction is the first step in flavonoid biosynthesis and is also catalyzed by chalcone synthase (CHS). Chalcone isomerase (CHI) is the second enzyme involved in the biosynthesis of flavonoids and it catalyzes the stereospecific intramolecular cyclization of the chalcones isoliquiritigenin and naringeninchalcone into the flavanones (2*S*)-liquiritigenin and (2*S*)-narigenin, respectively (Winkel-Shirley, 2001).
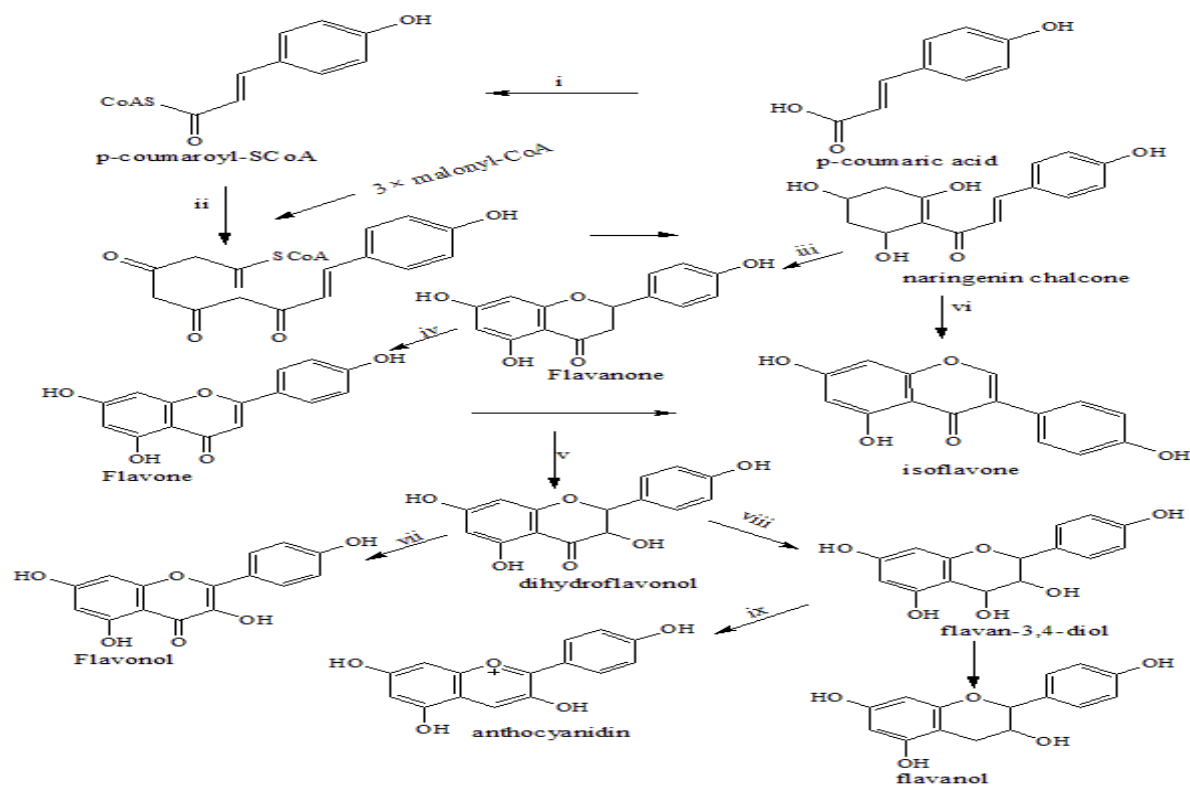
Figure 2.13: General Biosynthetic pathway of flavonoids

Enzymes: (i) p-coumarate-CoA ligase, (ii) chalcone synthase, (iii) chalcone isomerase,(iv) flavone synthase II, (v) flavanone 3-β-hyroxylase, (vi) isoflavone synthase, (vii) flavonol synthase, (viii) dihydroflavonol 4-reductase, (ix) anthocyanidin synthase (Davies and Schwinn, 2005)

2.7.3   Biosynthesis of Terpenoids

Terpenoids constitute the largest family of natural plant products with over 30,000 members (Sacchettini and Poulter, 1997; Dewick, 2002). Over 30,000 terpene hydrocarbons and oxidized or modified derivatives, called terpenoids, have been isolated to date from a wide variety of biological sources. These metabolites serve crucial roles in primary metabolism and ecological interactions of their organisms as well as display medically and economically valued characteristics (Sacchettini and Poulter, 1997).

For instance, several monoterpenes and sesquiterpenes are important volatile organic compounds that are important in the food and perfume industries (Schulz and Dickschat, (2007), whereas some microbial terpenoids are phytotoxic metabolites that have significant impact on agriculture (Collado *et al*., 2007).

Terpenoids are classified by the homologous series of number of five carbon isoprene units in their structure: hemiterpenes C_5 (1 isoprene unit), monoterpenes C_{10} (2 isoprene units), sesquiterpenes C_{15} (3 isoprene units), diterpenes C_{20} (4 isoprene units), tri-terpenes C_{30} (6 isoprene units), tetraterpenes C_{40} (8 isoprene units), polyterpenes $(C_5)_n$ where 'n' may be 9–30,000 (McGarvey and Croteau, 1995). Monoterpenes (C_{10}) and sesquiterpenes (C_{15}), the constituents of essential oils, are important flavouring and fragrance agents in foods, beverages, cosmetics, perfumes, soaps and exhibit specific biological, pharmaceutical and therapeutical activities as well (Singh *et al.,* 1989; Mahmoud and Croteau, 2002).

Metabolically speaking, the biosynthesis of isoprenoids can be divided into three stages:

(i) formation of the C_5 isoprenoid unit, (ii) condensation of the C_5 units and chain elongation,

and (iii) cyclization and modification of linear isoprenoid precursors. The extensive family of isoprenoid compounds is derived from two universal precursors, isopentenyl diphosphate (IPP) and dimethylallyl diphosphate (DMAPP). The mevalonate (MVA) pathway and the methylerythritol phosphate (MEP) pathway are the two independent pathways associated with the biosynthesis of these C_5 building blocks.

The classical mevalonate pathway (Fig: 2.14A and 2.16) begins with acetate molecules activated as acetyl-CoA, forming 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA) which is reduced to mevalonate. Mevalonate is then activated via three phosphorylation reactions followed by a decarboxylation and elimination to yield IPP (Qureshi and Porter, 1981). DMAPP is generated subsequently from IPP in an equilibration reaction catalyzed by IPP-DMAPP isomerase that exploits a protonation-deprotonation mechanism (Leyes, *et al.,* 1999). Studies of this pathway have led to the development of the statins, or HMG-CoA reductase inhibitors, which have been widely used as therapeutics and preventive medicine for human cardiovascular diseases by lowering blood cholesterol levels (Stancu and Sima, 2001). An alternative mevalonate independent pathway, called the MEP pathway or deoxyxylulose phosphate pathway was discovered in certain bacteria and the plastids of both lower and higher plants in the 1990's (Rohmer, 1999; Eisenreich, *et al.,* 2004). The pathway starts with the condensation of pyruvate and glyceraldehyde-3-phosphate to form 1-deoxyxylose-5-phosphate which undergoes a rearrangement coupled with a reduction resulting in MEP.

MEP is converted into its cyclic diphosphate, 2-C-methyl-D-erythritol-2, 4-cyclodiphosphate (MECDP), by sequential phosphocytidyl transfer and phosphorylation. MECDP is then reduced twice and finally transformed into IPP and DMAPP (Fig: 2.14 B and 2.15) (Eisenreich, *et al.,* 2004).
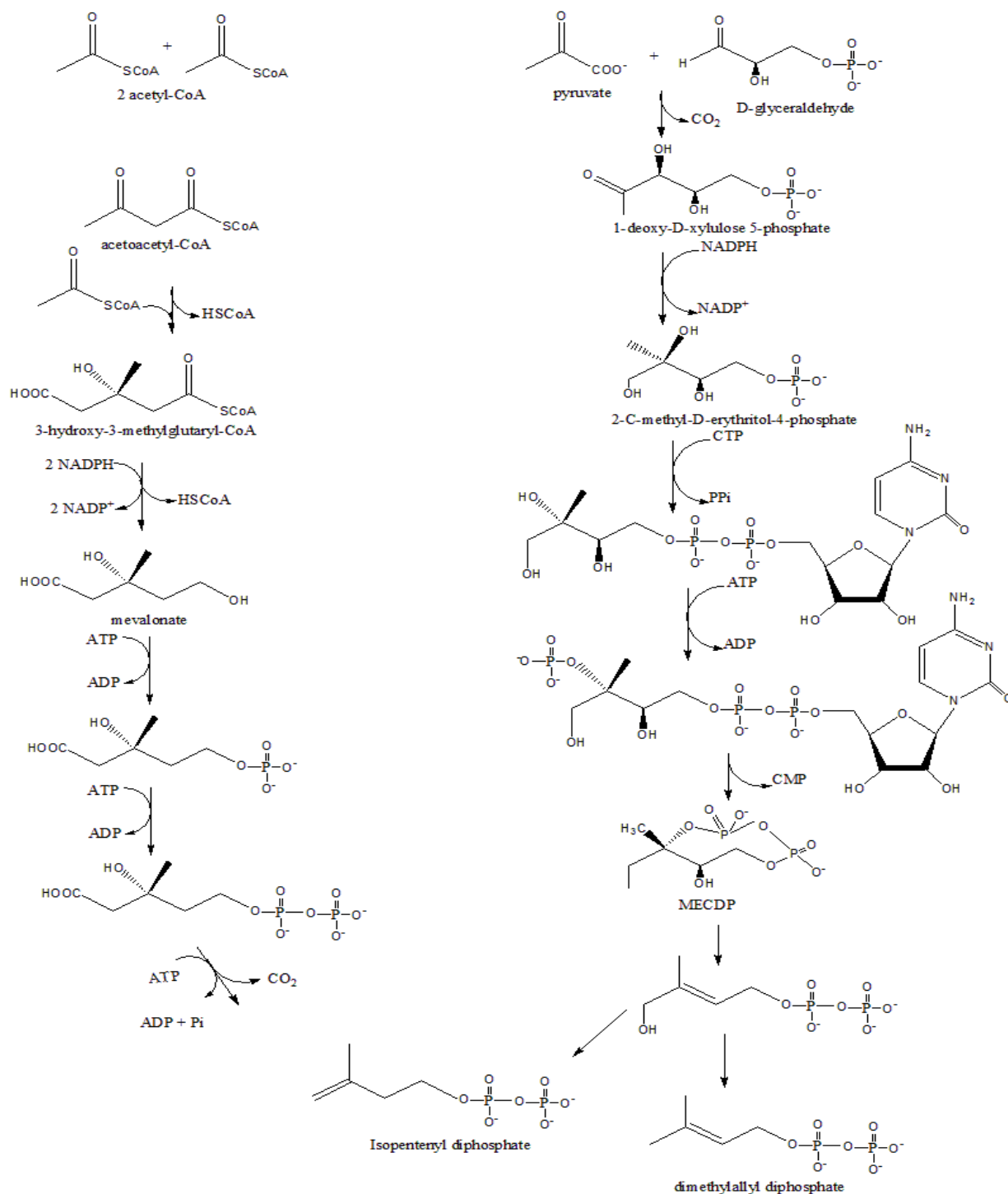
Figure 2.14: Two independent pathways for biosynthesis of IPP and DMAPP in plants
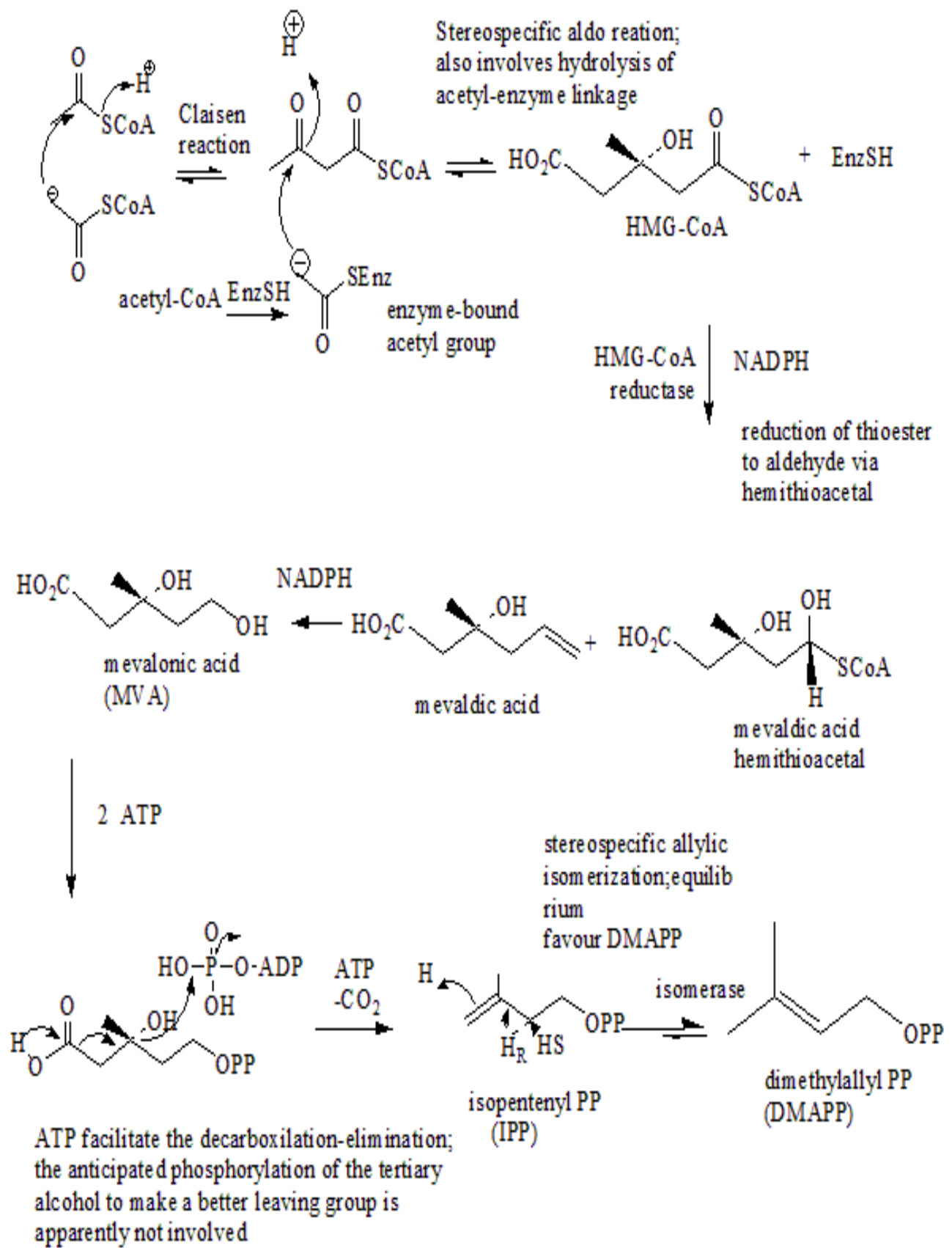
Figure 2.15: The mechanistic details of mevalonic acid pathway
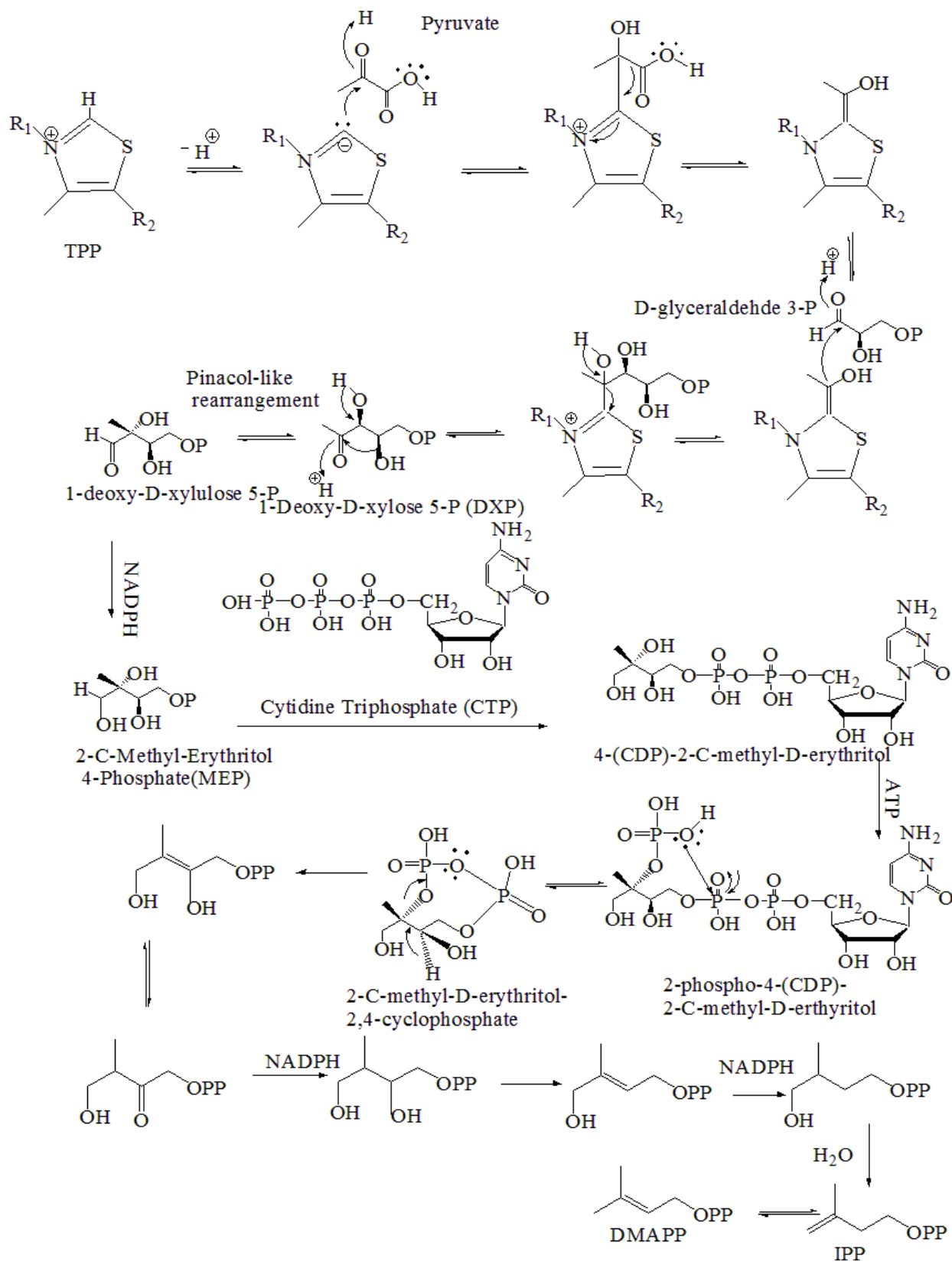
Figure 2.16: The mechanistic details of Deoxyxylulose phosphate (Methylerythrol Phosphate) pathway

All carbon skeletons of linear isoprenoids are assembled by repetitive head-to-tail condensation of IPP with an allylic diphosphate through the catalysis of prenyltransferases, a group of prenyl chain elongating enzymes (Takahashi and Koyama, 2006). Coupling of IPP to DMAPP by geranyl diphosphate synthase generates geranyl diphosphate (GPP, C_{10}) while addition of IPP to GPP by farnesyl diphosphate synthase gives farnesyl diphosphate (FPP, C_{15}), which can again couple to IPP by geranylgeranyl diphosphate synthase to yield geranylgeranyl diphosphate (GGPP, C_{20}), although FPP synthase and GGPP synthase can also utilize only IPP and DMAPP as the initial substrates in multistep elongation sequences via bound intermediates (Poulter and Rilling, 1981).

The reaction proceeds through an ionization-condensation-elimination mechanism, which involves an initial ionization of the allylic diphosphate ester substrate to form a charge delocalized carbocation which undergoes C4'-C1 coupling via the terminal double bond of IPP to generate a tertiary carbocation followed by deprotonation to complete the reaction (Fig.2.17). (Poulter and Rilling,1978). The resulting acyclic intermediates are the key precursors from which monoterpenes (from GPP), sesquiterpenes (from FPP), and diterpenes (from GGPP) are derived. Terpenoid synthases, also called cyclases since the reaction products are most often cyclic, are the family of enzymes that catalyzes the conversion of the three acyclic branch point intermediates to the parent skeletons of the various monoterpene, sesquiterpene, and diterpene types and largely contributes to the great structural diversity encountered in this class of natural products. (Davis and Croteau, 2000). Reaction of terpenoid synthases may be considered as the intramolecular analogue of the intermolecular electrophilic coupling reaction catalyzed by the prenyltransferases, although the former case comprises far more diversity in type and mechanistic complexity (Davisson *et al* 1985). Terpenoid cyclases, responsible for catalyzing some of the most complex chemical reactions occurring in nature, are functionally soluble, modestly lipophilic and acidic enzymes with native sizes in the 35–80 kDa range; a divalent metal ion is the only cofactor required for enzyme activity, which exhibits Michaelis constants for the prenyl substrate that rarely exceed 10 μM and turnover numbers typically ranging from 0.03 to 0.3 s^{-1}(Davisson *et al* 1985). About two-thirds of the carbon atoms of a linear terpenoid substrate undergo changes in bonding, hybridization, or configuration during the course of an elaborated cyclization cascade (Christianson, 2006).

Formation of a highly reactive carbocation intermediate is the initiation step of a terpenoid cyclase reaction which is either ionization-dependent or protonation-dependent.

Typical ionization-dependent cyclizations involve metal-triggered ionization of the prenyl diphosphate substrate yielding an allylic carbocation-pyrophosphate ion pair followed by an intramolecular attack from a double bond on the prenyl chain to generate a cyclic carbocation intermediate. Subsequent cationic transformations may involve further cyclizations and rearrangements, including hydride shifts, methyl migrations and Wagner-Meerwein rearrangements, which can create a potentially vast and diverse array of products. The reactions are ultimately terminated by quenching of the positive charge by deprotonation or capture of an external nucleophile such as water or the original pyrophosphate anion.
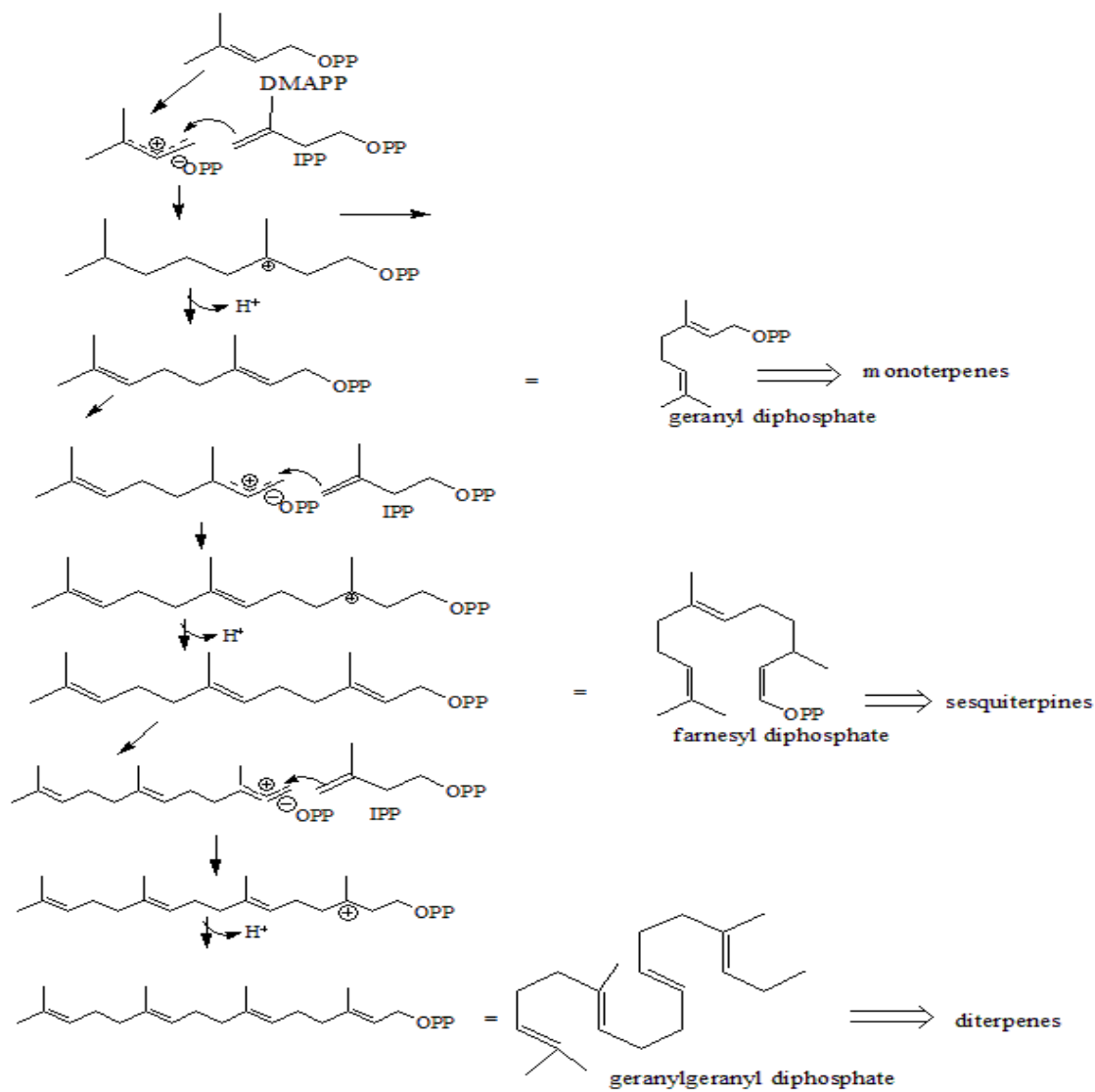
Figure 2.17: Chain elongations by prenyltransferases

27

CHAPTER THREE
MATERIALS AND METHODS

3.1   General

Column chromatography used Merck Silica gel 60 (0.063-0.200 mm) and Fluka Sephadex LH-20 as stationary phases; PTLC on Merck Silica gel 60 PF254+366, coated on glass plates (20 x 20 cm) to make 1.0 mm layers; Analytical TLC was carried out using factory prepared aluminum plates (0.25 mm) coated with Silica gel (60 F254, Merck) and spots visualized by observing under UV light at 254 or 365 nm, followed by spraying with 1% vanillin-H_2SO_4 spray reagent. These were carried out at the University of Nairobi, department of Chemistry.

1D data were acquired on Bruker Avance 600 MHz or Bruker BioSpin instrument 400 MHz for ^{1}H and Bruker Avance 100 MHz or Varian-Mercury 150 MHz for ^{13}C, while 2D data were acquired on Bruker Avance 600 MHz or Bruker BioSpin instrument 400 MHz for HSQC and HMBC. NMR measurements were done in deuterated solvents using the residual undeuterated chemical shifts as internal standard. HREI-MS spectra were recorded on GC-MS TRACE DSQII single quadrupole mass.spectrometer. These were done at the National Center for Natural Product Research, School of Pharmacy, University of Mississippi.

3.2   Plant material

The aerial parts of *Microglossa pyrifolia* were collected from Ngong forest Nairobi in February 2010. A Voucher specimen was deposited at the University Herberium, Department of Botany, University of Nairobi (No AAI/2010/10 *Microglossa pyrifolia*).

3.3   Extraction and isolation

Crude leaf surface extract was obtained by dipping about 400 g of fresh leaves into about 5 litres of ethyl acetate for a short period of 15 seconds thus avoiding the extraction of internal tissue components (Midiwo *et al*., 1990). This process was repeated until the colour of ethyl acetate changed to yellowish at which fresh ethyl acetate was used. The surface extract was then filtered and evaporated off in a rotary evaporator under reduced pressure at a temperature not exceeding 45°C to give 190 g of yellowish black gummy extract.

Of this, 180 g portion of the extract was adsorbed on 200 g of silica gel and subjected to column chromatography over Silica gel (2 kg, 110 x 90 cm column), eluted with n-hexane/CH_2Cl_2 mixtures (100:0, 16 L; 9:1, 14 L; 4:1, 12 L; 3:2, 12 L; 1:1, 16 L; 1:3, 12 L; 0:100, 18 L) and CH_2Cl_2/MeOH mixtures (99:1, 14 L; 98:2, 12 L; 97.5:2.5, 12 L; 97:3, 10 L) to give a total of 127 fractions each 500 mL. The fractions were monitored by thin layer chromatography and similar fractions were combined to give a total of twelve fraction summarized in Table 3.1.

Table 3.1: Combined fractions and their weights

Fraction No	New fraction Code	Weight of Fraction (g)
1-3	1A	13.88
4-6	12B	13.28
7-16	12C	2.60
17-21	12D	5.87
22-25	12E	2.23
26-30	12F	5.08
31-36	12G	2.6
37-60	12H	3.17
61-69	12I	1.9
70-88	12J	7.68
89-103	12K	6.88
104-127	12L	16.75

Fraction 12E which was eluted with n-hexane/CH_2Cl_2 mixtures (100:0, 116 L) yielded epoxyisochiliolide lactone (**86**) (80 mg) which crystallized in 1:1 n-hexane/CH_2Cl_2. Fraction 12I (1.9g) was subjected to column chromatography over silica gel (20 g, 2.0 x 30 cm) eluted with n-hexane/CH_2Cl_2 mixture (9:1,0.1 L; 4:1, 0.1 L; 7:3, 0.1 L; 1:1, 0.15 L; 1:3, 0.12 L; 0:100, 0.15 L) to give 36 fractions of 20 ml each. Combined fractions 22-29 (120 mg) were chromatographed over sephadex LH-20 (20 g, 2.5 x 30 cm; eluent: 1:1 CH_2Cl_2/MeOH) to give 8-acetoxyisochiliolide lactone (**87**) (210 mg).

Fraction 12L (16.76 g) was subjected to column chromatography on a silica gel column (200 g, 4×30 cm) eluted with increasing gradient of ethyl acetate in n-hexane (100:0, 1.6 L; 9:1, 1.4 L; 8.5:1.5, 1.2 L; 8:2, 1.2 L; 7.5:2.5, 1.6 L; 7:3, 1.2 L; 6.5:3.5, 1.8 L) to give 48 fractions each 20 mL.

These gave compounds **81** (80 mg), **82** (46 mg), **83** (35 mg) and **84** (52 mg) that crystallized from fractions 1-4, 5-6, 7-15 and 19-34 respectively. The other fractions contained mixtures of compounds with very close R_f values. Fraction 12K (6.88 g) was adsorbed onto 12 g of silica gel and subjected to column chromatography (120 g, 4×30 cm) eluted with increasing gradient of ethyl acetate in n-hexane to (100:0, 1.0 L; 9:1, 0.9 L; 8.5:1.5, 0.8 L; 8:2, 0.7 L; 7.5:2.5, 0.5 L; 7:3, 0.5 L; 6.5:3.5, 0.5 L) give thirty fractions of each 150 mL. Fractions 1-2 (58 mg) were combined and passed through sephadex LH-20 column (20 g, 2.5 x 30 cm) eluted with 1:1 CH_2Cl_2/MeOH and the fractionated eluents concentrated and re-crystallized in n-hexane/CH_2Cl_2 (1:1) to give compound **85** (47 mg).

3.4 *In vitro* **anti-plasmodial assay**

The *in vitro* anti-plasmodial activities were measured at the National Center for Natural Products Research of School of Pharmacy, University of Mississippi. All the compounds isolated (**81**, **82**, **83**, **84**, **85**, **86**, and **87**) were tested using the colorimetric assay method that determines the parasitic lactate dehydrogenase (pLDH) activity (Makler *et al*., 1993; Samoylenko *et al*., 2009). The assay was performed in 96-well microplate and included two *P. falciparum* strains [Sierra Leone D6 (chloroquine-sensitive) and Indochina W2 (chloroquine-resistant)].

For the assay, a suspension of red blood cells infected with *P. falciparum* (D6 or W2) strains (200 μL, with 2% parasitemia and 2% hematocrit in RPMI - 1640 medium supplemented with 10% human serum and 60 μg/mL amikacin) was added to the wells of a 96-well plate containing 10 μL of test samples at various concentrations. The plate was flushed with a gas mixture of 90% N_2 , 5% O_2, and 5% CO_2 , in a modular incubation chamber (Billups-Rothenberg, 4464 M) and incubated at 37 °C, for 72 h. Plasmodial LDH activity was determined by using Malstat™ reagent (FlowInc., Portland, OR). The IC_{50} values were computed from the dose response curves generated by plotting percent growth against test concentrations. DMSO, artemisinin and chloroquine were included in each assay as vehicle and drug controls, respectively.

The selectivity index (SI) of anti-plasmodial activity was determined by measuring the cytotoxicity of samples towards mammalian cells (VERO; monkey kidney fibroblasts).

3.5   Cytotoxicity Assay

All the compounds isolated (**81**, **82**, **83**, **84**, **85**, **86**, and **87**) were tested. These were measured at the National Center for Natural Products Research of School of Pharmacy, University of Mississippi. The activity was determined against monkey kidney fibroblasts (VERO) obtained from the American Type Culture Collection (ATCC, Rockville, MD). The assay was performed in 96-well tissue culture-treated microplates. Cells were seeded at a density of 25000 cells / well and incubated for 24 hours. Samples at different concentrations were added and plates were again incubated for 48 hours. The number of viable cells was determined using Neutral Red (Borenfreund *et al*., 1990). IC_{50} values were determined from dose response curves of percent growth inhibition against test concentrations. Doxorubicin was used as a positive control, while DMSO was used as the negative (vehicle) control.

3.6   Antioxidant test

A preliminary anti-oxidant test was done by spotting the compounds (**81, 82, 83, 84, 85, 86** and **87**) on a TLC plate followed by spraying with 0.2 mg/ml DPPH solution to view the active compounds which displayed white or yellowish sports on a purple background. The active compounds were quantitatively analysed by UV-VIS spectrometry method adopted from Hou *et al*., 2002 with modifications made on the concentrations of the samples. For each compound, the concentration of the sample was varied by serial dilutions to give concentrations of 160, 80, 40, 20, 10, 5.0, 2.5, 1.25 and 0µg/ml while the concentration of DPPH was kept constant at 100 µg /ml (Figure 3.1). The reaction mixture consisted of adding 0.5 mL of sample, 3 mL of absolute ethanol and 0.3 mL of 100µg/mL DPPH radical solution in ethanol. These solutions were then measured for UV-VIS absorbance at DPPH absorbing wavelength (517 nm) half an hour after adding the DPPH.

The absorbance measured at each of these intervals were converted into percentages of scavenged DPPH radicals using the following equation

$$\% \text{ of scavenged DPPH} = \left(\frac{A_{blank} - A_{sample}}{A_{blank}} \right) \times 100\%$$

Where A_{blank} is the absorbance of DPPH solution without sample. The percentages of scavenged DPPH were then plotted against concentration of the compound to give graphs from which concentrations at half inhibition (IC_{50}) were determined. The tests were done in triplicates.

Figure 3.1: Samples with varied concentration of compounds but constant concentration of DPPH.

3.7   Antimicrobial Assay

The organisms were obtained from the American Type Culture Collection (Manassas, VA) and include the fungi *Candida albicans* ATCC 90028, *Candida glabrata* ATCC 90030, *Candida krusei* ATCC 6258, *Cryptococcus neoformans* ATCC 90113 and *Aspergillus fumigatus* ATCC 90906 as well as the bacteria *Staphylococcus aureus* ATCC 29213, methicillin-resistant *Staphylococcus aureus* ATCC 43300 (MRS), *Escherichia coli* ATCC 35218, *Pseudomonas aeruginosa* ATCC 27853 and *Mycobacterium intracellulare* ATCC 23068. Susceptibility testing was performed at the National Center for Natural Products Research, School of Pharmacy, University of Mississippi, using a modified version of the CLSI methods (Samoylenko *et al*., 2009). All the isolated compounds were tested. Drug controls ciprofloxacin (ICN Biomedicals, Ohio) for bacteria and amphotericin B (ICN Biomedicals, Ohio) for fungi were included in each assay. The number of microbial cells of 10^7 CFU/ml was prepared by using McFarland standards.

This was done by adjusting the absorbance at 550 nm to 0.125 of microbial cells suspension with sterile saline and making an additional 10-fold diluted cells suspension of it with sterile saline. The number of microbial cells was 10^7 CFU/ml. The compounds, drug controls ciprofloxacin (ICN Biomedicals, Ohio) for bacteria and amphotericin B (ICN Biomedicals, Ohio) for fungi were serially diluted to obtain concentrations of 64, 32, 16, 8 , 4, 2, 1, 0.5, 0.25, 0.12, 0.06 µg / ml in Mueller-Hinton broth. Of the serially diluted compounds and drug controls, 180 µl of each were dispensed to each well and then 10 µl of the cell suspension (10^7 CFU/ml) was inoculated to each well (final cell density: 10^4 CFU/ml). The plate was incubated at 47^0C for 6 hours, after which 10 µl of Coloring reagent was added to each well and the microplate incubated for an additional 2 hours. Absorbance was measured at 450 nm using the microplate reader

3.8   Anti-leishmanial Assay

Antileishmanial activity of the compounds was tested *in vitro* on a culture of *Leishmania donovani* promastigotes at the National Center for Natural Products Research of School of Pharmacy, University of Mississippi. In a 96 well microplate assay compounds with appropriate dilution were added to the *Leishmania* promastigotes culture (2×106 cells/mL). The plates were incubated at 26°C for 72 hours and growth of *Leishmania* promastigotes was determined by Alamar blue assay (Mikus and Steverding, 2000). All the compounds isolated were tested. Pentamidine and amphotericin B were used as standard antileishmanial agents. 10^6cells/ml promastigotes were seeded in 96-well flat-bottom plates in final volume of 200 µl. Various concentrations of experimental compounds and pentamidine and amphotericin B drugs were made in three-fold dilutions and 180 µl of each were then added to triplicate wells and plates incubated for 72 h at 22°C. At the end of incubation, cell viability was measured by counting the number of motile cells using a haemocytometer. For each drug, the concentration-response curve was plotted from which IC_{50} values (the concentration of the agent that reduce cell viability by 50% compared to controls) were determined. All experiments were repeated at least three times. IC_{50} values for each compound were computed from the growth inhibition curve.

CHAPTER FOUR

RESULTS AND DISCUSSION

4.1 Secondary metabolites isolated from surface exudates of *Microglossa pyrifolia*

Chromatographic separation of the surface exudates of *Microglossa pyrifolia* led to the isolation and characterization of five flavonoids and two diterpeniods, of which three (**82, 84** and **86**) are new compounds.

4.1.1 Flavonoids

In this study five flavonoids were isolated and successfully characterized. Of these flavonoids, four were trihydroxylated and methoxylated flavones while one was trihydroxylated and methoxylated flavanone. The characterization of these compounds are discussed as follows.

4.1.1.1 5, 7, 4'-Trihydroxy-3, 8, 3'-trimethoxyflavone (81)

Compound **81** was isolated as a yellow solid with HR-EIMS molecular ion at *m/z* 359.1009 [M-H]$^+$ (Appendix1E) consistent with the molecular formula $C_{18}H_{16}O_8$. The HR-EIMS fragmentation pattern (Figure 4.2) had fragment at *m/z* 344 (M-CH$_3$) and the C-ring degraded by cleavage of bonds 0, 2 and produced reto Diels-Alder (RDA) fragment $^{0,2}A^+$ which lost a CO to produce a peak at m/z 154.98. This fragment then lost a CH$_3$ resulting to a peak at m/z 112.99 (Tsimogiannis *et al.*, 2007) (Appendix1E). Another cleavage of ring A resulted in a peak at m/z 248.97. Its ^{1}H NMR (Table 4.1) (Appendix 1A) revealed the presence of four aromatic protons of which one is singlet at δ_H 6.27 on ring A and the other three showed an ABX spin system [δ_H 6.98 (1H, *d*, *J* = 8.4 Hz), δ_H 7.26 (1H, *dd*, *J* =8, 1.6 Hz) and δ_H 7.66 (1H, *d*, *J* =2 Hz)]. The singlet at δ_H 6.27 showed HMBC correlations with two oxygenated carbons δ_C 157.4 and 156.3 (Appendix1B) and long range HMBC correlation with the α,β-conjugated ketone (δ_C 178.5, C-4) as shown by the blue and yellow arrows respectively in figure 4.1, suggesting that the proton be assigned to H-6. In agreement with this, 5-hydroxy group did not correlate with any of the carbons carrying the methoxyl groups and hence any of the methoxyl could not be placed at position C-6. This led to exclusion of the possibility of 5, 6, 7-oxygenation pattern in ring A. According to the HSQC spectrum, we had three methoxyl groups attached to carbons resonating at δC 55.9, 60.1 and 60.4.

The downfield chemical shift of one of the hydroxyl group proton at δ_H 12.32 was due to its *peri* position to the carbonyl carbon and hence the hydroxyl group was at C-5. The HMBC correlations between H-6 and C-10 (δ_C 104.47) and a weak ^2J interaction between H-6 and C-5 (δ_C 157.4) were all evident from HMBC spectrum (Appendix 1D).

The presence of two oxygenated carbons at δ_C 149.0 and 150.2 was in agreement with 1,2 oxygenation pattern in ring B of flavone skeleton and the three aromatic protons with ABX spin pattern showed HMBC cross peaks with these oxygenated carbons (Table 4.1) which was in agreement with the placement of the two oxygenated carbons at C-3',4' positions.

One of the methoxyl protons at δ_H 3.80/δ_C 55.2(Appendix 1C) showed HMBC correlation with one of the above oxygenated carbons at δ_C 149.0 and hence assigned to be at position C-3'. If ring A and B are tri- and di-substituted respectively, then the remaining methoxyl group δ_H 3.85/δ_C 60.1 which showed HMBC correlation with the carbon at δ_C 138.2, is at C-3 position. Thus, the above NMR data in comparison with literature NMR data (Horie *et al.*, 1998) suggested that the compound is 5, 7, 4'-trihydroxy-3, 8, 3'-trimethoxyflavone (**81**) as shown in figure 4.1.

Figure 4.1: Structure of 5, 7, 4'-Trihydroxy-3, 8, 3'-trimethoxyflavone (**81**)

Table 4.1: ^{1}H/^{13}C NMR (400/100 MHz) of Compound **(81)**

Position	δ_H	δ_C	HMBC
2	-	155.6	-
3	-	138.2	-
4	-	178.5	-
5	-	157.4	-
6	6.27, *s*	99.2	C-4,5,6,7,8,10
7	-	156.3	-
8	-	127.9	-
9	-	147	-
10	-	104.5	-
1'	-	122.5	-
2'	7.66 (*d, J* =2)	112.0	C-1',3', 4',5', 6'
3'	-	149.0	-
4'	-	150.2	-
5'	6.98 (*d, J* = 8.4)	116.2	C-1',3',4',5',6'
6'	7.26 (*dd, J* =8, 1.6)	121.5	C-1', 2', 4',5',6'
3'-OCH$_3$	3.80, *s*	55.2	C-3'
3-OCH$_3$	3.82, *s*	60.4	C-3
8-OCH$_3$	3.85, *s*	60.1	C-8,9
5-OH	12.33, *s*	-	C-5,6,10
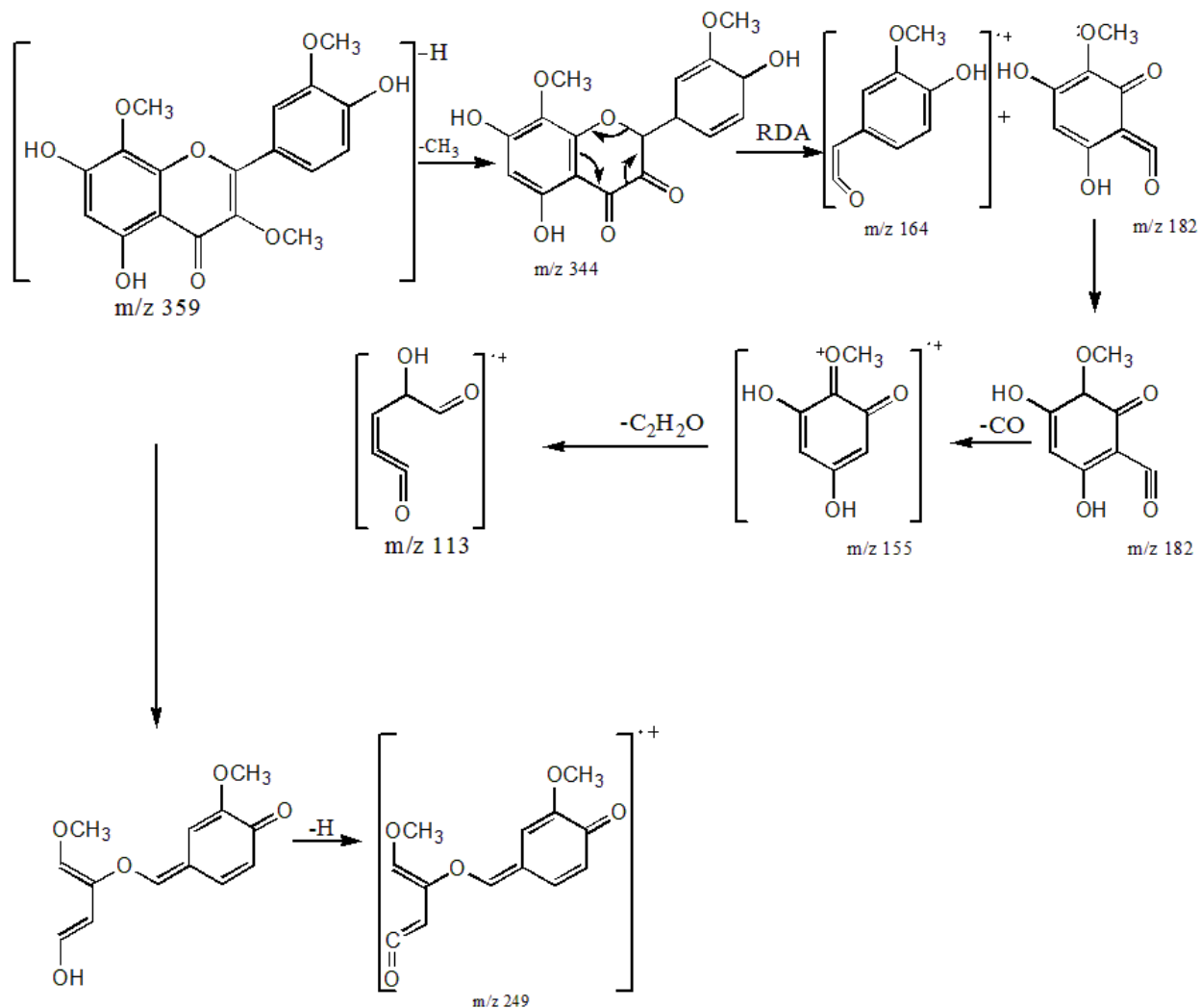
Figure 4.2: Fragmentation pattern for 5, 7, 4'-trihydroxy-3, 8, 3'-trimethoxyflavone (**81**)

4.1.1.2 5, 7, 4'-Trihydroxy-3, 8, 3', 5'-tetramethoxyflavone (82)

Compound **82** was isolated as a yellow solid and gave a molecular ion peak of 389.102 on HR-EIMS analysis (Appendix2E). The HR-EIMS fragmentation pattern (Figure 4.4) showed fragments at m/z 374 [M-CH$_3$]$^+$ and m/z 290.95 [M-3CH$_3$-CO-CHO+H]$^+$.

The fragment at m/z 290.95 lost a CH$_2$COH to form a peak at 248.96, which then underwent a series of cleavages to form a fragment at m/z 112.98. (Tsimogiannis *et al.*, 2007) (Appendix2E).

Its ^{1}H-NMR (Table 4.2) displayed the presence of a singlet at δ_H 6.27 (1H, *s*) and δ_H 7.42 (2H, *s*). The singlet proton at δ_H 6.27 showed HSQC correlation (Appendix 2C) with carbon with δ_C 99.2 and HMBC correlations with carbon δ_C 104.4, 127.9, 157.5 and δ_C 178.4 (Appendix2B) suggesting that this proton be assigned to H-6 (Appendices 2C and 2D).

37

Furthermore, the above spectroscopic data suggests that ring A of flavone skeleton is tri-substituted.

The presence of two protons appearing as singlet at δ_H 7.42 suggest that there is symmetry in ring B of the flavone skeleton. The presence of δ_C 138.4 and 139.4 suggested the possibility of a methoxy placed at C-3 and a 1, 2, 3 tri-oxygenated pattern in ring B. The methoxy resonating at δ_H 3.81/δ_C 60.1 showed HMBC correlation with the quaternary carbon at δ_C 138.4 suggesting that ring B is tri-substituted and taking into consideration the symmetry and HMBC correlation of the two protons with δ_C 106.1, 120.1, 139.4 and 155.3 suggesting that the two protons (at δ_H 7.42) belong to H-2' and 6'. Furthermore, the two methoxyl of ring B were placed on C-3' and C-5'. Thus, the above spectroscopic NMR data suggests that the compound is 5, 7, 4'-trihydroxy-3, 8, 3', 5'-tetramethoxyflavone (**82**) as shown in figure 4.3. This compound was found to be a new natural product.
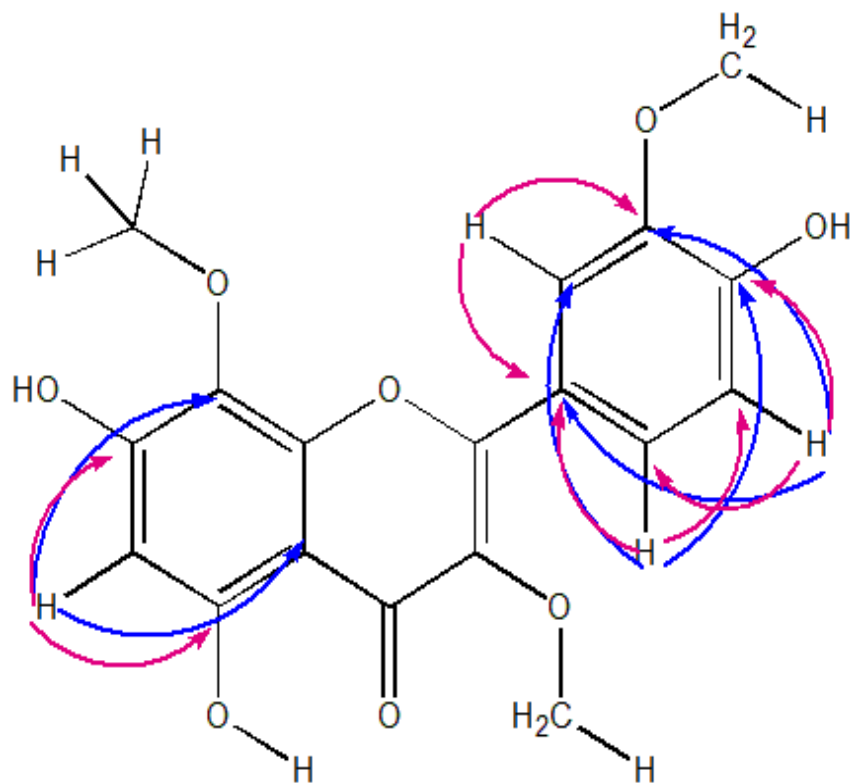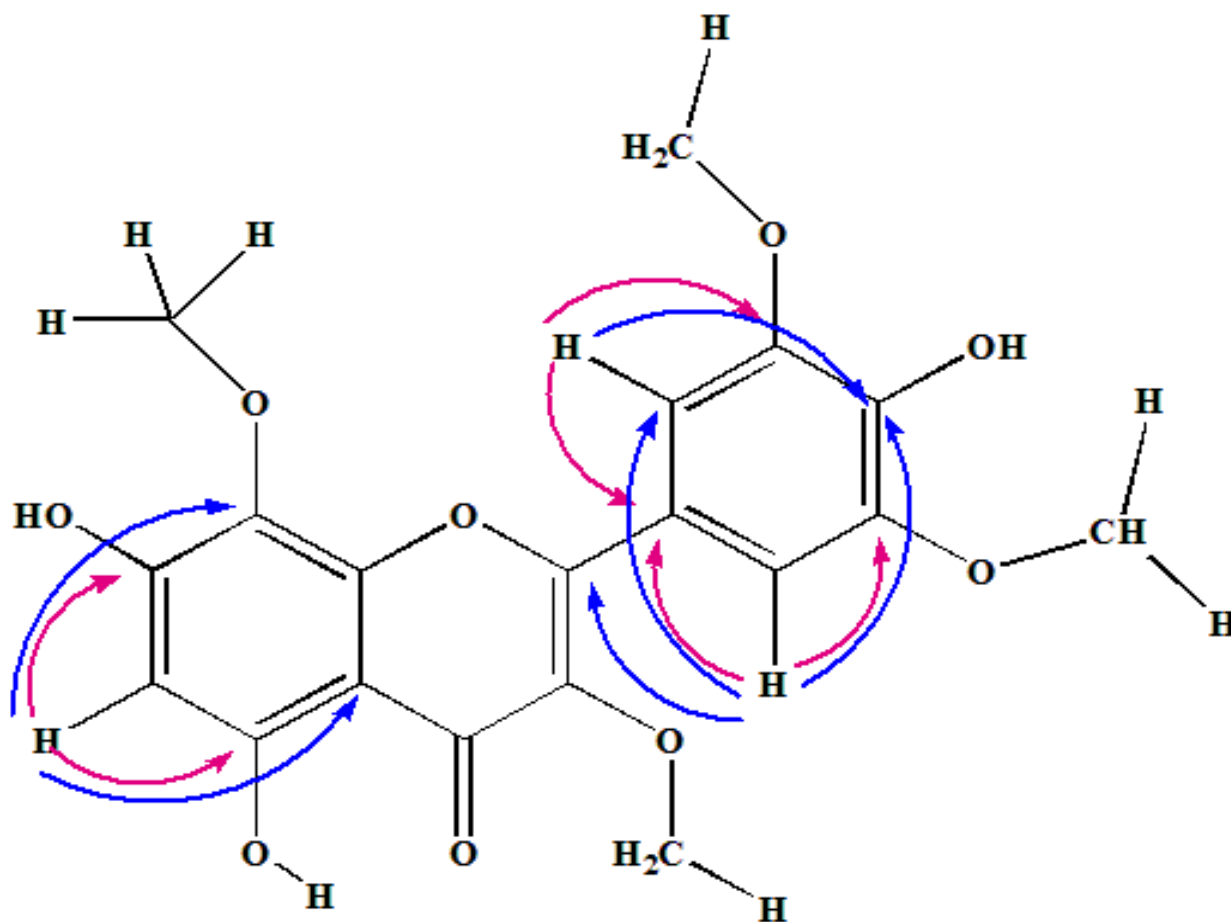

Figure 4.3: Structure of 5, 7, 4'-trihydroxy-3, 8, 3', 5'-tetramethoxyflavone (**82**).

Table 4.2: $^1H/^{13}C$ NMR (400/100 MHz) of Compound (82).

Position	δ_H	δ_C	HMBC
2	-	155.3	-
3	-	138.4	-
4	-	178.5	-
5	-	157.5	-
6	6.26, *s*	99.2	C-4,5,6,7,8,10
7	-	156.3	-
8	-	127.9	-
9	-	148.9	-
10	-	104.4	-
1'		120.0	-
2'	7.42, *s*	106.1	C-1',2',3',4',6'
3'	-	148.2	-
4'	-	139.4	-
5'	7.42, *s*	148.2	-
6'	-	106.1	C-1', 2',4',5',6'
5-OH	12.39(5-OH)	-	C-5,6,10
3',5'-OCH$_3$	3.82, *s*	58.2	C-3',5'
3-OCH$_3$	3.81, *s*	60.1	C-3
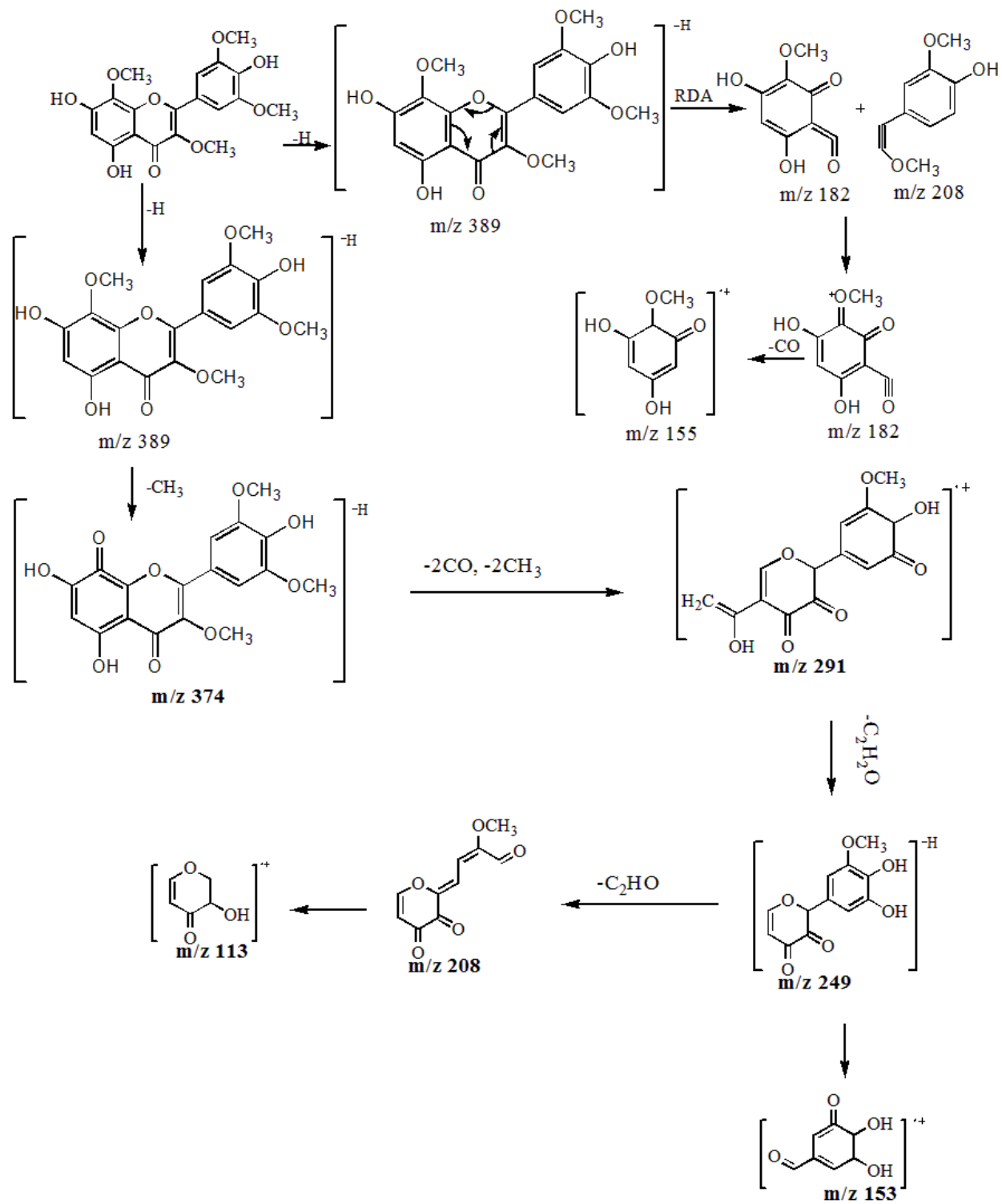
Figure 4.4: Fragmentation pattern for 5, 7, 4'-trihydroxy-3, 8, 3'5'-tetramethoxyflavone (**82**)

4.1.1.3 5, 3', 4'-Trihydroxy-7-methoxyflavanone (83)

Compound **83** was isolated as a light yellow solid and gave a molecular ion peak of 303.08 [M+H]$^+$ on HR-EIMS analysis ((Appendix 3E). The protonated flavanone dehydrated to give [M+H-H$_2$O]$^+$ (m/z= 253) and also may have lost the whole B-ring to produce a [M+H-B-ring]$^+$ (m/z= 192) (Figure 4.6) (Hughes *et al*., 2001).

The HRMS fragmentation pattern (Figure 4.6, Appendix 3E) further confirmed the structure with molecular ion peak at m/z 303.08 (C$_{16}$H$_{14}$O$_6$), a loss of a the C-ring and methyl giving a peak at *m/z* 191.08, loss of a methoxyl and water molecules giving a fragment at *m/z* 253.06

Its ^{1}H-NMR (Table 4.3) (Appendix 3A) displayed a pair of doublet aromatic protons at δ_H 6.08 (1H, *d*, *J*=2.0 Hz) and δ_H 6.06 (1H, *d*, J=2.0 Hz) being placed meta to each other. Based on HMQC correlations (Appendix 3C), these two protons belonged to δ_C 94 and δ_C 95 respectively. The proton at δ_H 6.06 showed HMBC correlations with the quaternary carbons at δ_C 103.0, 163.3 and 167.8 and the carbonyl carbon (197.4) (Appendix 3B) and hence the two protons were assigned to H-6 (δ_H 6.06) and H-8 (δ_H 6.08). The ^{1}H NMR further revealed that ring B of flavanone skeleton is di-substituted. The presence of two aromatic protons at δ_H 6.07 (1H, *d*, *J*=8.0) and 6.77(1H, *d*, *J*=8.0) coupling to each other and showing HMBC correlations with C-5' and C-6' suggest that ring B is di-substituted at C-3' and C-4'. Based on HSQC correlations (Appendix 3c), two diastereotopic protons at δ_H 3.23(1H, *dd*, *J*=17.2Hz, 12.8Hz) and δ_H 2.71 (1H, *dd*, *J*=17.2Hz, 3.2Hz) were assigned to carbon at δ_C 42.6 (C-3). Likewise, the proton at δ_H 5.41 (1H, *dd*, J=12.8Hz, 3.2Hz) was assigned to carbon at δ_C 79.1 (C-2). The HMBC correlations further revealed that the methylene protons (H-3) and the methine proton (H-2) showed HMBC correlations with the carbonyl carbon (C-4) and a quaternary carbon δ_C 129.7 (C-1'). The ^{13}C NMR (Table 4.3) spectrum showed two oxygenated carbons in a 1,2-dioxygenation pattern at δ_C 145.6 and 146.2 and the HMBC correlation of all the three protons (ring B) with these two carbons further support that ring B is di-oxygenated at C-3' and C-4' positions. Thus, based on the above spectroscopic evidence the compound was identified to be 5, 3', 4'-trihydroxy-7-methoxyflavanone (**83**). The NMR data matched those reported in literature (Ibrahim *et al*., 2003).
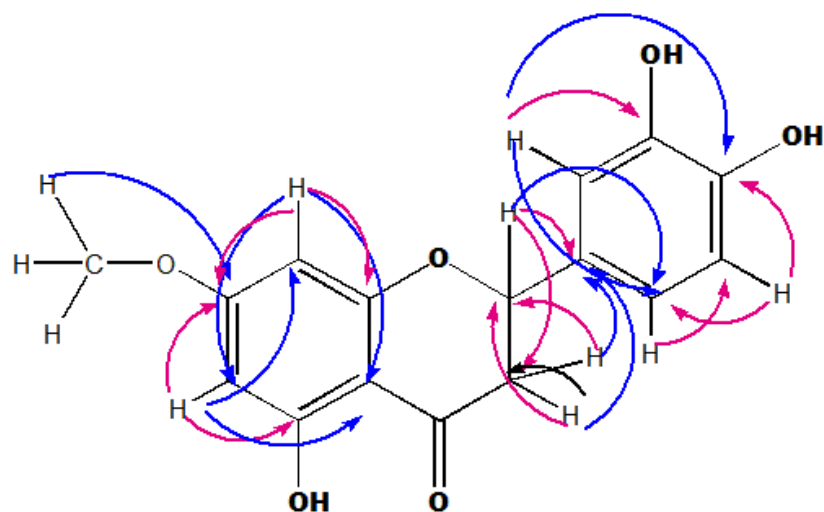
Figure 4.5: Structure of 5, 3', 4'-Trihydroxy-7-methoxyflavanone (**83**)

Table 4.3: ^{1}H/^{13}C NMR (400/100 MHz) of Compound (**83**)

Position	δ_H	δ_C	HMBC
2	5.41, *m*	79.1	C-2',6',3,1'
3	2.71, 3.22,*dd*	42.6	C-2,3,1',6'
4	-	197.4	-
5	-	163.3	-
6	6.07 (*d*)	95.0	C-4,5,6,7,8,9,10
7	-	167.8	-
8	6.08 (*d*)	94.2	-
9	-	163.6	-
10	-	103.0	-
1'		129.7	-
2'	6.77	114.8	C-2',3',4', 6'
3'		146.2	-
4'		145.6	-
5'		115	C-5',6',4'
6'		118	C-1', 2', 5',6'
5-OH	12.11(5-OH)	-	C-5
7-OCH$_3$	3.78, s	56.2	C-7
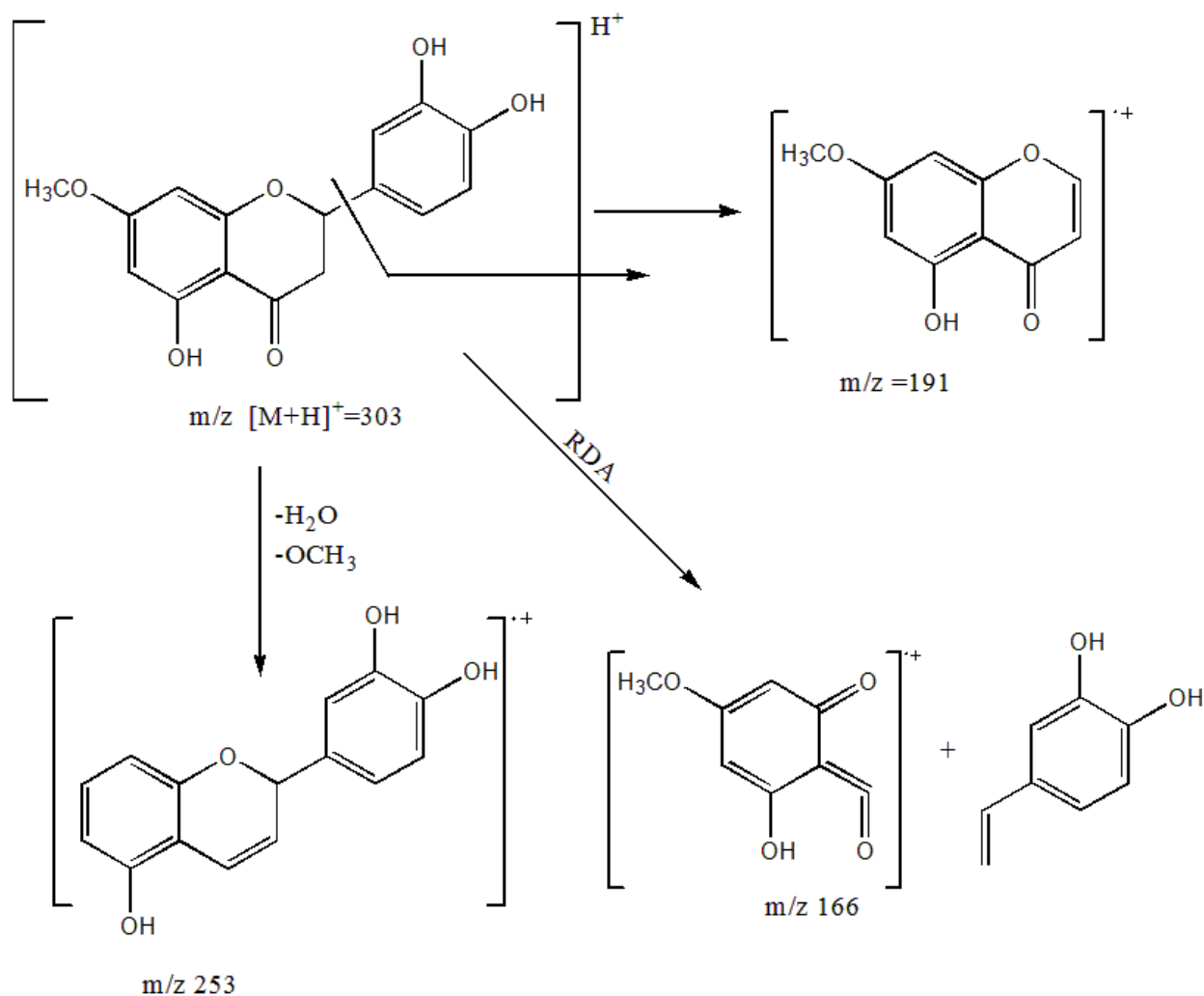
Figure 4.6: Fragmentation pattern for 5, 3', 4'-trihydroxy-7-methoxyflavanone (**83**)

4.1.1.4   5, 7, 3'-Trihydroxy-3, 8, 4', 5'-tetramethoxyflavone (84).

Compound **84** was isolated as yellow solid. Its HR-EIMS molecular ion (Appendix 4E) was observed at m/z 389.12 [M-H]$^+$ corresponded to the molecular formula of $C_{19}H_{18}O_9$. The HR-EIMS fragmentation pattern (Figure 4.8) had fragment at *m/z* 359 [M-CH$_3$]$^+$ and the C-ring degraded by cleavage of bonds 0,2 to produce RDA fragment $^{0,2}A^+$ which subsequently lost a CO to produce a peak at m/z 154.98. The other peaks were at m/z 248.98 and m/z 112.99 which were due to a series of cleavages (figure 4.8). Its ^{1}H NMR (Appendix 4B and Table 4.4) gave seven signals of which three (δ_H 7.35, 7.21, 6.14) were singlet aromatic protons (Appendix4A) and four (δ_H 3.84, 3.79, 3.76) were attributed to methoxyl groups (Appendix 4C).

Its ^{13}C NMR (Table 4.4) revealed nineteen signals of which fifteen were attributed for a flavonoid basic skeleton whereas the remaining four are methoxyl groups.

HMBC (Appendix D) correlation of the singlet proton at δ_H 6.14 with C-4, C-5, C-7, C-8, and C-10 suggested that this proton belong to H-6 and carbons 5, 7 and 8 are oxygenated quaternary carbons. The HMBC correlation (Appendix 4D) of the two methoxyl groups with oxygenated carbons at δ_C 129.1 (C-8) and δ_C 138.5 (C-8) suggested that the oxygenated quaternary carbons at C-3 and C-8 have methoxy substituents.

The presence of two singlet protons at δ_H 7.35 and δ_H 7.21, suggested that ring B of flavones skeleton is tri-substituted. The two singlet aromatic protons at δ_H 7.35/δ_C 110.2 and δ_H 7.21/δ_C 103.5 were attributed to H-4' and H-6' based on HMBC correlations of H-4' (δ_H 7.35) with carbons C-2', C-3', C-5', C-6' and H-6' (δ_H 7.21) with C-1', C-2', C-4' and C-5'. Furthermore, the HMBC correlation of the two methoxyl groups at C-2' and C-5' allowed the assignment of the two methoxyl groups at C-2' and C-5' positions. Thus, based on the above spectroscopic evidence the compound was identified to be 5, 7, 3'-trihydroxy-3, 8, 2', 5'-tetramethoxyflavone (**84**). This compound was found to be a new natural product.
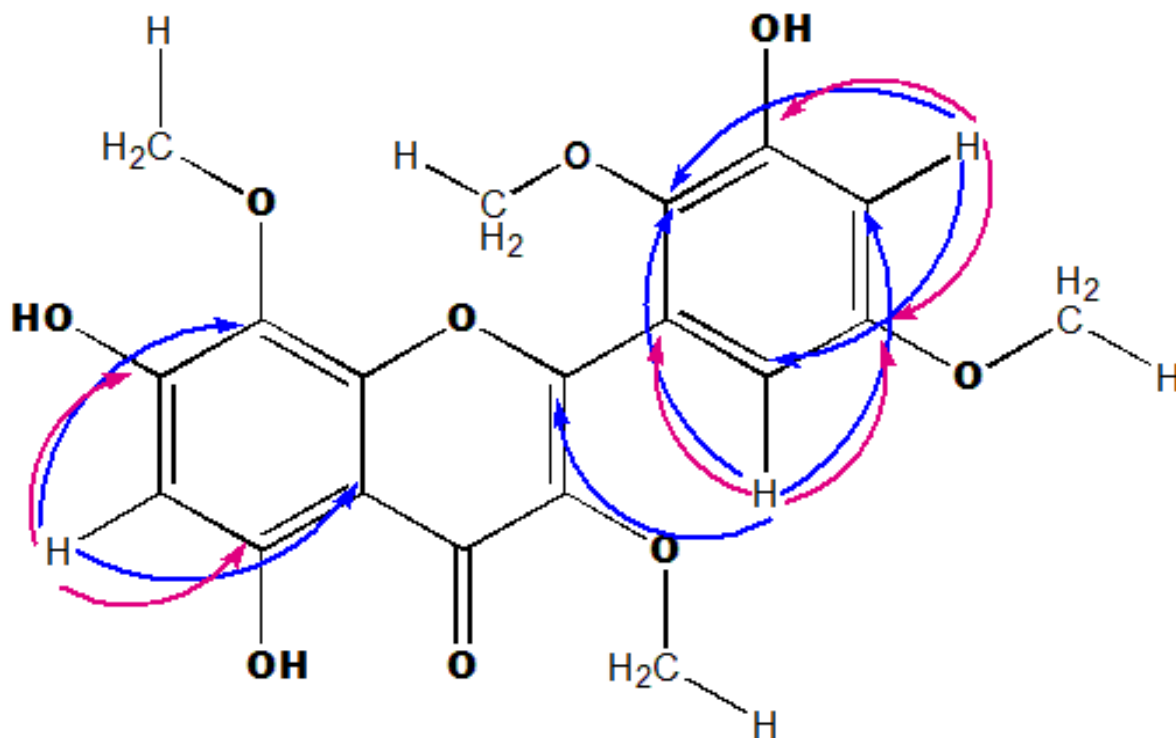
Figure 4.7: Structure of 5, 7, 3'-trihydroxy-3, 8, 2', 5'-tetramethoxyflavone (**84**)

Table 4.4: $^1H/^{13}C$ NMR (400/100 MHz) of Compound (**84**).

Position	δ_H	δ_C	HMBC
2	-	153.7	-
3	-	138.5	-
4	-	177.5	-
5	-	165	-
6	6.14, *s*	101.2	C-4,5,6,7,8,10
7	-	156.7	-
8	-	129.1	-
9	-	149.0	-
10	-	101.7	-
1'	-	126.0	-
2'	-	139.0	-
3'	-	151.5	-
4'	7.35, *d*	110.2	C-2', 3',4' 5', 6'
5'	-	153.4	-
6'	7.21, *d*	103.5	C-1', 2',3' 4', 5'
8-OCH$_3$	3.79, *s*	60.25	C-8
2'-OCH$_3$	3.81, *s*	60.7	C-2'
5'-OCH$_3$	3.76, *s*	56.1	C-5'
3-OCH$_3$	3.83, *s*	60.4	C-3
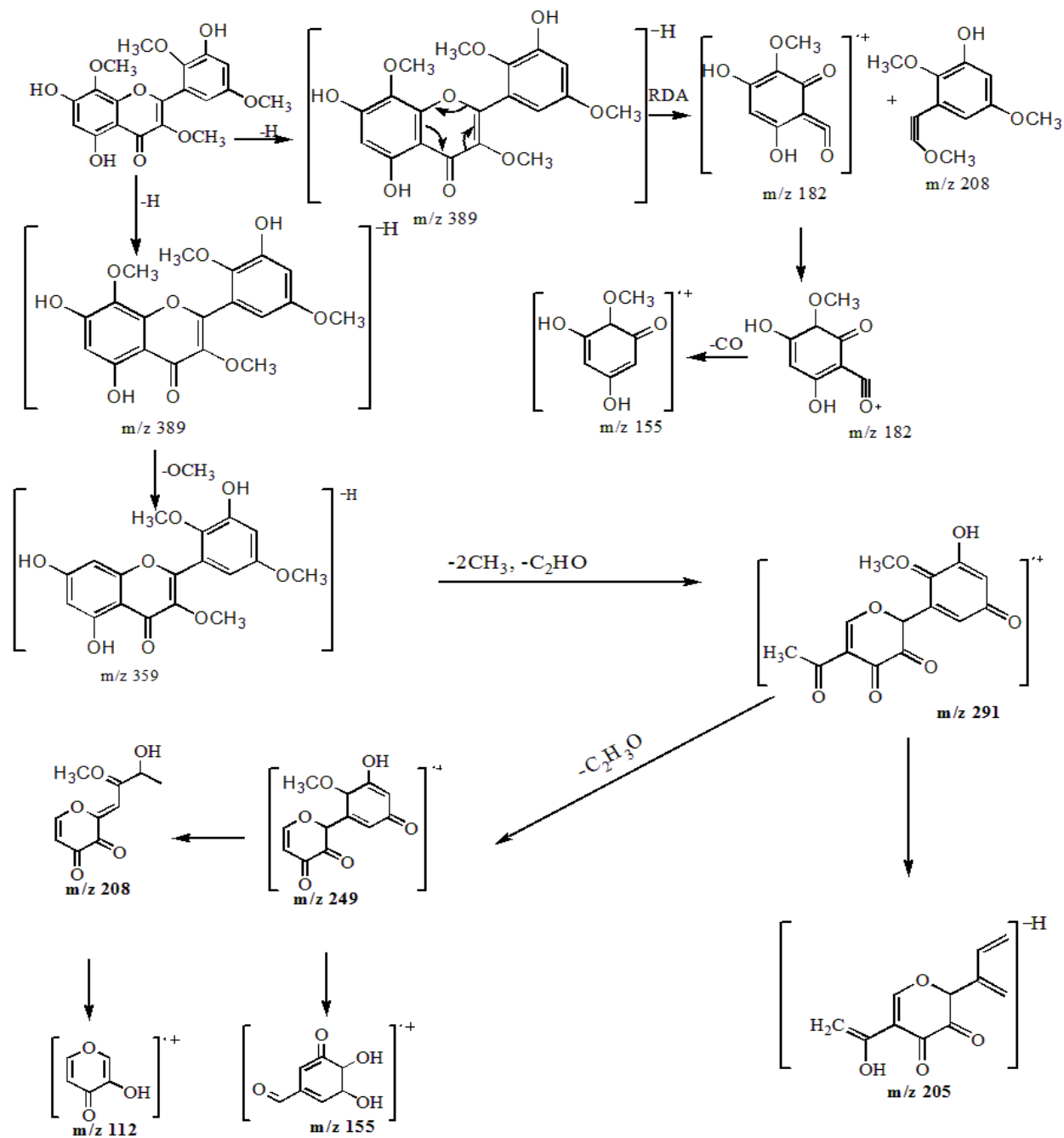
Figure 4.8: Fragmentation pattern for 5, 7, 3'-trihydroxy-3, 8, 4', 5'-tetramethoxyflavone (**84**)

4.1.1.5   5, 3', 4'-Trihydroxy-3, 7, 8-trimethoxyflavone (85)

Compound **85** was isolated as a yellow compound. Its HR-EIMS (Appendix 5E) molecular ion was observed at m/z 361 $[M+1]^+$ corresponded to the molecular formula of $C_{18}H_{16}O_8$ and the C-ring degraded by cleavage of bonds 0,2 to produce RDA fragment $^{0,2}A^+$ which subsequently lost a CO to produce a peak at m/z 169 (Figure 4.10).

Its ^{1}H NMR (Table 4.5 and Appendix 5A) spectrum revealed four aromatic signals δ_H 6.89 (*d*, *J*=8.4 Hz), δ_H (7.47, *dd*, *J*=8.4, 1.6 Hz) δ_H 7.58, (*d*, *J*=1.6 Hz) and a singlet δ_H 6.95. Its ^{13}C NMR (Table 4.5 and Appendix 5B) spectrum revealed a total of eighteen signals out of which fifteen belong to the flavonoid skeleton whereas the remaining three were attributed to methoxyl groups (Appendices 5A and 5B).

The downfield chemical shift of the hydroxyl groups at δ_H 12.62 suggests its *peri* position to the carbonyl carbon at C-4 and hence the hydroxyl group be assigned at C-5 position. The singlet aromatic proton at δ_H 6.95 showed HMBC correlation with C-4, C-5, C-7, C-8 and C-10 suggesting that this proton be assigned to H-6 (Appendices 5C and 5D). The above spectroscopic evidence suggests that positions C-5, C-7 and C-8 were oxygenated carbons belonging to δ_C 152.1, 159.0, 131.9 respectively. The HMBC correlation of the methoxy groups with C-8 (δ_C 131.9) and C-7 (δ_C 159.0) suggest that positions C-7 and C-8 are substituted with methoxy groups. Furthermore, the methoxyl group at δ_H 3.77/δ_C 60.1 was placed at C-3 based on HMBC correlation with C-3 (δ_C 138.1).

The presence of two oxygenated quaternary carbons at δ_C 145.7 and 149.2 suggest that ring B of flavones skeleton is di-substituted. Taking into consideration the ABX spin pattern observed in ring B and HMBC correlation of these protons with the two oxygenated quaternary carbons suggest that ring B is oxygenated at C-3' and C-4' positions. The HRMS fragmentation pattern (Figure 4.10, Appendix 5E) showed a protonated molecular ion peak at *m/z* 361 and a fragment with m/z 169 (Wu *et al.*, 2003). Thus, based on the above spectroscopic evidence the compound was identified as 5, 3', 4'-trihydroxy-3, 7, 8-trimethoxyflavone (**85**) as shown below. It was previously reported from *Ricinocarpos muricatus* (Henrick and Jefferies, 1965).
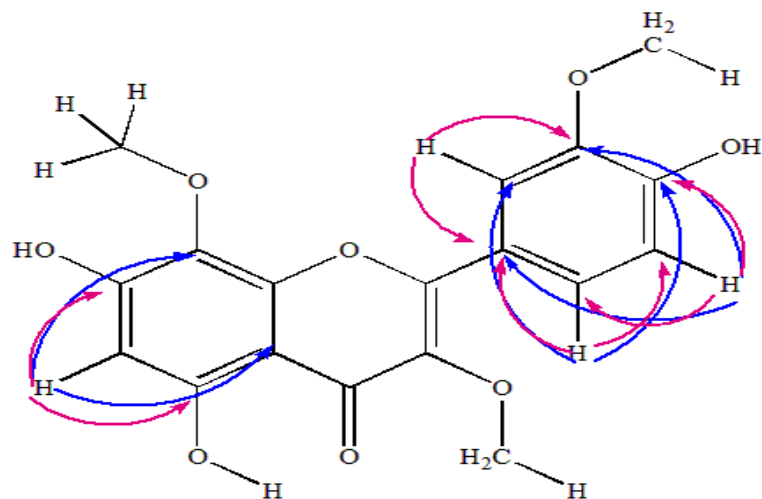
Figure 4.9: Structure of 5, 3', 4'-trihydroxy-3, 7, 8-trimethoxyflavone (**85**)

Table 4.5: $^{1}H/^{13}C$ NMR (400/100 MHz) of Compound (**85**).

Position	δ_H	δ_C	HMBC
2	-	156.4	-
3	-	138.1	-
4	-	178.6	-
5	-	152.1	-
6	6.95	91.7	C-4,5,6,7,8,10
7	-	159.0	-
8	-	131.9	-
9	-	149.2	-
10	-	105.9	-
1'	-	121.0	-
2'	7.58	115.9	C-1',2',3',4', 6'
3'	-	145.7	-
4'	-	149.2	-
5'	6.89	116.1	C-1',3', 4',5' 6'
6'	7.47	121.0	C-1', 2', 4', 5',6'
3-OCH$_3$			C-3
7-OCH$_3$			C-7
8-OCH$_3$			C-8

Figure 4.10: Fragmentation pattern for 5, 3', 4'-trihydroy-3, 7, 8-trimethoxyflavone (**85**)

4.1.2 Rearranged Clerodane diterpenoids

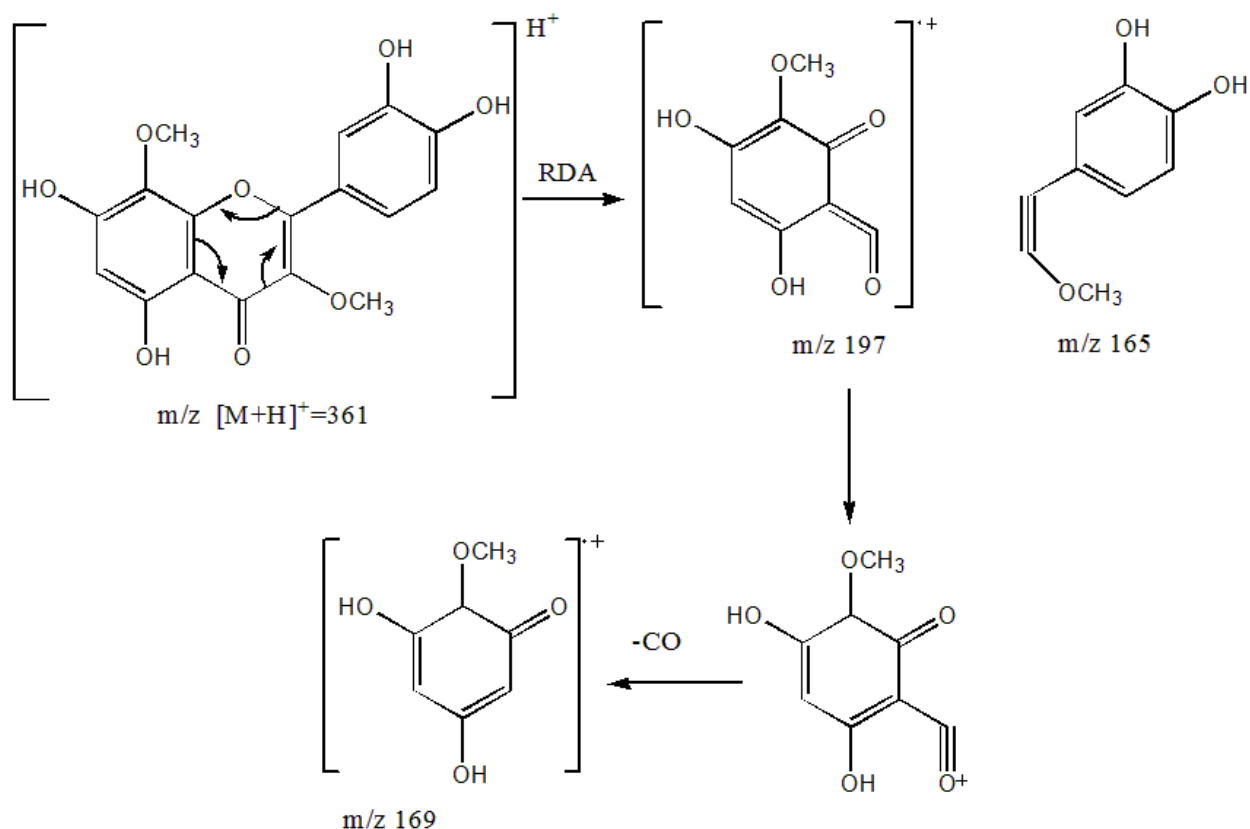From the surface exudates of the aerial parts of *Microglossa pyrifolia* two rearranged clerodane diterpenoids, 8-acetoxyisochiliolide lactone (**86**) and epoxyisochiliolide lactone (**87**) were isolated of which **86** was found to be new compound.

4.1.2.1 8-Acetoxyisochiliolide lactone (86)

Compound **86** was isolated as white crystals. Its HR-EIMS (Appendix 6F) provided a molecular ion peak at 402.1700 which is attributed to $C_{22}H_{26}O_7$ from the (calc for 402.1679). Its ^{13}C NMR (Appendix 6B) revealed four olefinic carbons (δ_C 108.5, 126.0, 140.0, 144.5), three ester carbonyls (δ_C 169.6, 172.5, 177.5). The presence of two olefinic carbons, one ester carbonyl and five rings is in agreement with the DBE of 10 derived from its molecular formula.

The presence of two olefinic protons at δ_H 6.45 (*d*, 1.5 Hz) and 7.45 (*d*, 1.5 Hz) coupled with the singlet proton at 7.49 (*s*) and four olefinic carbons suggest the presence of a furan ring.

Two of the ester carbonyls (δ_C 172.5 and 177.5) were attributed to two lactone rings where as the one at δ_C 169.6 was an acetoxy carbonyl based on its HMBC (Appendix 6D) correlation with a methyl singlet at δ_H 1.79.

Three more methyl singlets were observed, two of which being vicinal methyls at δ_H 1.08 and 1.13 and the third methyl at δ_H 1.93.According to the HSQC, these methyl singlets were attached to carbons resonating at δ_C 9.4, 16.9 and 22.4 respectively (Appendices 6D and 6E) The presence of four olefinic carbons for a furanyl rings, two lactone carbonyls and three methyls suggests a clerodane diterpenoid of isochiliolide lactone skeleton (Zdero *et al*., 1990b). Furthermore, these NMR data matched those reported for the pentacyclic isochiliolide lactone diterpenoid, 8-hydroxyisochiliolide lactone reported from *M. pyrrhopappa* (Zdero *et al.*, 1990b). Thus, based on the above spectroscopic evidence the compound was identified to be 8-acetoxyisochiliolide lactone (**86**) as in figure 4.12.

4.1.2.2   7, 8-Epoxyisocholiolide lactone (87)

Compound **87** was isolated as white crystals from fraction 12I. The ^{13}C-NMR (appendix 7B) had twenty signals implying that it was a diterpenoid with twenty carbons. Among the carbons were four olefinic carbons at δ_C 144.8, 140.8, 124.5, and 108.5.[for a furanyl ring, δ_H 7.58 (*d,* J = 1.2 Hz), δ_H 7.51 (br *s*) and δ_H 6.51 (br *s*)], two lactone carbonyls at δ_C 172.2, 178.1 and three methyls at δ_C 9.7, 17.4 and 20.8, suggesting a clerodane diterpenoid of isochiliolide lactone skeleton (Zdero *et al*., 1990b). The adjacent oxygen bearing carbons at positions 7 and 8 gave peaks at δ_C 62.6 and δ_C 60.8 and were deduced to be forming an epoxide from the EIMS molecular ion at *m/z* 360 (Appendix 7E). Compound **87** lacks one of the acetoxy moiety in the NMR spectrum and instead epoxy groups at positions 7 and 8 appeared at δ_C 62.6 and 60.8. The above spectroscopic data for compound **87** is the same as the data reported for 7, 8-epoxyisocholiolide lactone isolated from *M. pyrrhopappa* (Zdero *et al*., 1990b). So it is concluded that the compound is 7, 8-epoxyisocholiolide lactone (**87**) as shown in figure 4.11.

Table 4.6: ^{1}H/^{13}C NMR (400/100 MHz) of Compounds (**86**) and (**87**).

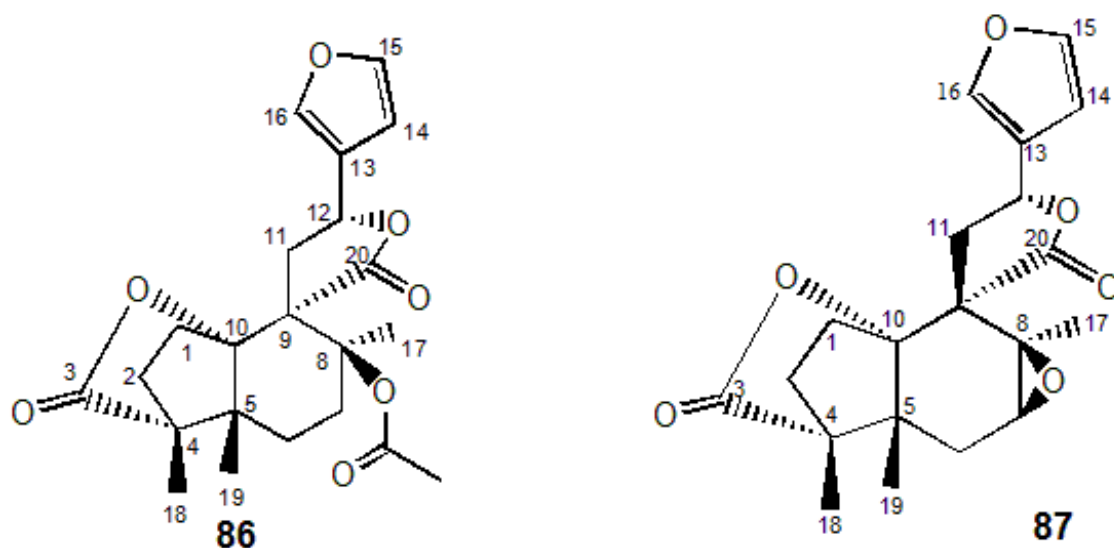|	86			87	
	^{13}C	^{1}H	HMBC	^{13}C	^{1}H
1	28.8	2.23, *ddd* (13.6, 10.5, 3.8) 1.84, *ddd* (13.6, 9.2, 4.2)	C-2 C-2, C-5	28.8	1.90, *m* 1.72, *m*
2	29.4	1.94, *m* 1.71, *ddd* (13.0, 9.2, 3.8)	C-1 C-1	28.5	1.90, *m* 2.26, *m*
3	177.5			178.4	
4	54.4			55.6	
5	51.2			50.0	
6	26.1	1.44, *td* (14.0, 3.2) 1.39, *dt* (14.0, 3.8)	C-5, C-7	28.4	1.93, br *d* (18.0) 1.87, *m*
7	31.5	2.54, *dt* (14.0, 3.6) 1.75, *m*	C-5, C-6, C-17	62.6	3.35, br *s*
8	86.0			60.8	
9	54.4			52.0	
10	93.3			91.0	
11	34.4	2.69, *dd* (13.8, 8.2) 2.61, *dd* (13.8, 8.8)	C-8, C-9, C-10, C-12, C-13 C-9, C-10, C-12, C-13, C-20	37.6	2.63, *dd* (14.4, 7.8) 2.67, *dd* (14.4, 9.4)
12	70.7	5.37, *dd* (8.8, 8.2)		72.1	5.40, *dd* (9.4, 7.8)
13	126.0			124.5	
14	108.5	6.45, *d* (1.5)	C-13, C-15, C-16	108.5	6.51, br *s*
15	144.5	7.47, *t* (1.5)	C-13, C-16	144.8	7.51, *d* (1.2)
16	140.0	7.49, *s*	C-14, C-15	140.8	7.58, br *s*
17	21.2	1.93, *s*	C-7, C-8, C-9	20.8	1.52, *s*
18	9.4	1.08, *s*	C-2, C-3, C-4, C-5	9.7	1.12, *s*
19	16.9	1.13, *s*	C-4, C-5, C-6, C-10	17.4	1.13, *s*
20	172.5			172.2	
OAc- CO	169.6				
OAc- Me	22.2	1.79, *s*	C-OAc CO		

Figure 4.11: Structures of rearranged clerodane diterpenoids, 8-acetoxyisocholiolide lactone (**86**) and 7, 8-epoxyisocholiolide lactone (**87**)

4.2 Anti-plasmodial, anti-oxidant, anti-microbial and anti-leishmanial activities

All the compounds isolated were subjected to bio assay tests to establish their potential as antiplasmodial, antioxidant, antimicrobial and antileishmanial as follows.

4.2.1 Anti-plasmodial activity of compounds

As a follow up to the antiplasmodial activities shown by the extract of the investigated plants (Table 1.1), *in-vitro* antiplasmodial tests were done for the compounds isolated from the plant. The tests aimed at identifying active components which contributed to the observed activities of crude extract. The compounds tested were 5, 7, 4'-trihydroxy-3, 8, 3'-trimethoxyflavone (**81**), 5, 7, 4'-trihydroxy-3, 8, 3', 5'-tetramethoxyflavone (**82**), 5, 3', 4'-trihydroxy-7-methoxyflavanone (**83**), 5, 7, 3'-trihydroxy-3, 8, 4', 5'-tetramethoxyflavone (**84**), 5, 3', 4'-trihydroxy-3, 7, 8-trimethoxyflavone (**85**), 8-acetoxyisochiliolide lactone (**86**) and 7, 8-epoxyisocholiolide lactone (**87**). Two *P. falciparum* strains were used, Sierra Leone D6 (chloroquine-sensitive) and Indochina W2 (chloroquine-resistant). Cytotoxicities of the compound were measured simultaneously. These results are summarized in table 4.7.

Generally, a compound is considered to be inactive when it shows an $IC_{50} > 200$ μM, whereas those with an IC_{50} of 100-200 μM have low activity; IC_{50} of 20-100 μM, moderate activity; IC_{50} of 1-20 μM good activity; and $IC_{50} < 1$ μM excellent/potent antiplasmodial activity (Batista *et al*., 200). All the compounds under test except crude extract did not show antiplasmodial activities. This can be interpreted that the activity of their extracts are either due to synergistic effects or contributed by trace compounds which were not isolated.

Table 4.7: In-vitro antiplasmodial activity and cytotoxicity

Compound	*In-vitro* antiplasmodial activity (IC_{50})		Cytotoxicity (IC_{50})
	D6 (μg/ml)	W2 (μg/ml)	(μg/ml)
5, 7, 4'-Trihydroxy-3, 8, 3'-trimethoxyflavone (**81**)	NA	NA	NC
5, 7, 4'-Trihydroxy-3, 8, 3', 5'-tetramethoxyflavone (**82**)	NA	NA	NC
5, 3', 4'-Trihydroxy-7-methoxyflavanone (**83**)	NA	NA	NC
5,7,3'-Trihydroxy-3,8,4',5'-tetramethoxyflavone (**84**)	NA	NA	NC
5, 3', 4'-Trihydroxy-3, 7, 8-trimethoxyflavone (**85**)	NA	NA	NC
8-Acetoxyisochiliolide lactone (**86**)	NA	NA	NC
7, 8-Epoxyisocholiolide lactone (**87**)	NA	NA	NC
Crude extract	8.00	13.00	NC
Chloroquine (**standard**)	<0.026	0.14	NC

NA = Not Active (IC_{50} values > 50 μg/ml); NC = Not Cytotoxic (up to the maximum dose tested 5.0 μg/ml).

4.2.2 Anti-oxidant activities of compounds

All the compounds isolated were tested for anti-oxidant activity. The compounds were first subjected to preliminary qualitative testing on a TLC, out of which Compounds **86** and **87** (rearranged clerodane diterpenoids) were inactive while compounds **81**, **82**, **83**, **84** and **85** (flavonoids) were active. Compounds which showed activities on TLC assays were quantitavely analysed by UV-VIS absorption measurements on the compound solutions on reaction with DPPH. The results are presented in Table 4.8 and Figure 4.8.

Table 4.8: Anti-oxidant activity of flavonoids with DPPH experiment

Compound	IC_{50}	
	µg/ml	µM
5, 7, 4'-Trihydroxy-3, 8, 3'-trimethoxyflavone (**81**)	6.02±0.2	17.3
5, 7, 4'-Trihydroxy-3, 8, 3', 5'-tetramethoxyflavone (**82**)	6.45±0.3	16.5
5, 3', 4'-Trihydroxy-7-methoxyflavanone (**83**)	8.54±0.3	28.3
5, 7, 3'-Trihydroxy-3,8, 4', 5'-tetramethoxyflavone (**84**)	8.79±0.3	22.5
5, 3', 4'-Trihydroxy-3, 7, 8-trimethoxyflavone (**85**)	6.2±0.2	17.2
Quercetin (**standard**)	6.02 ± 0.2	19.9

The most active compound was 5, 7, 4'-trihydroxy-3, 8, 3'-trimethoxyflavone **(81).** Its radical scavenging activity of 6.2µg/ml (17.3 µM) was less than that of the standard used, quercetin (IC_{50} 6.0 µg/ml; 19.9 µM).

All of the compounds found to have radical scavenging activities had phenolic moiety oxygenated at either *ortho* or *para* positions, explained by the ability of hydroxyl groups at these positions to reduce radicals and form quinones (Chen *et al*., 1996), as illustrated for compound **81** in figure 4.13.
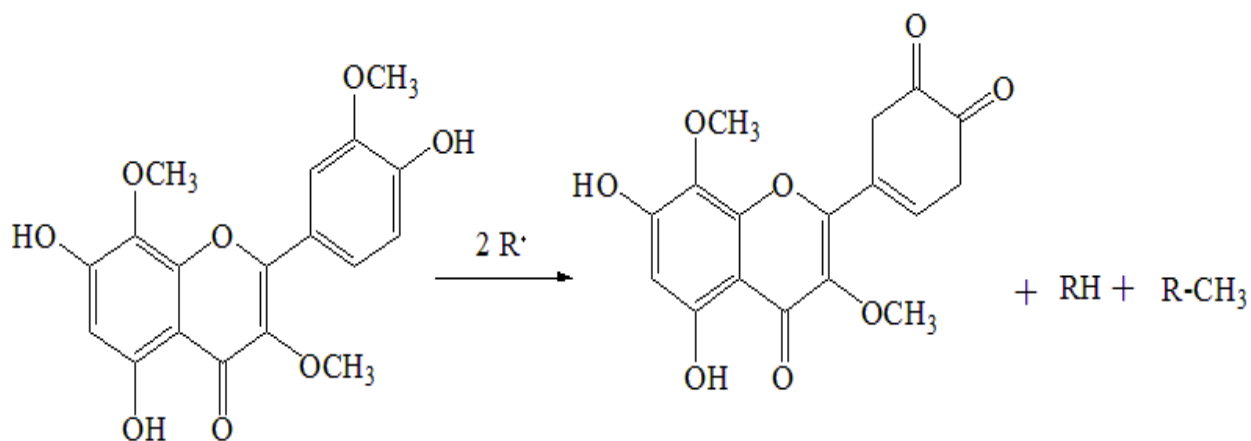
Figure 4.12: Reduction of radicals by a compound with phenolic ortho oxygenation.

4.2.3 Antimicrobial activities

The isolated compounds were tested for antifungal and antibacterial activities, using fungi *Candida albicans*, *Candida glabrata*, *Candida krusei*, *Cryptococcus neoformans*, *Aspergillus fumigatus* as well as the bacteria *Staphylococcus aureus*, methicillin-resistant *Staphylococcus aureus* (MRS), *Escherichia coli*, *Pseudomonas aeruginosa* and *Mycobacterium intracellulare*. Compounds showing % Inhibition < 50 are considered inactive at 15.9 µg/m. All the compounds tested showed no activity against any fungi or bacteria.

4.2.4 Anti-leishmanial activities

Antileishmanial activities of the compounds were tested *in vitro* on a culture of *Leishmania donovani* promastigotes. 5, 3', 4'-Trihydroxy-7-methoxyflavanone (**83**) exhibited mild antileishmanial activity (IC_{50} 13.13µg/ml). Compounds which exhibited antileishmanial activities of IC_{50} >50 µg/ml were considered inactive. Results for antileishmanial activity are summarized in Table 4.11.

Table 4.9: Antileishmanial activities

Compound	Activity against *L. donovani* (μg/ml)	
	IC$_{50}$	IC$_{90}$
5, 7, 4'-Trihydroxy-3, 8, 3'-trimethoxyflavone (**81**)	NA	NA
5, 7, 4'-Trihydroxy-3, 8, 3', 5'-ratetmethoxyflavone (**82**)	NA	NA
5, 3', 4'-Trihydroxy-7-methoxyflavanone (**83**)	13.13	NA
5, 7, 3'-Trihydroxy-3,8, 4', 5'-tetramethoxyflvone (**84**)	NA	NA
5, 3', 4'-Trihydroxy-3, 7, 8-trimethoxyflavone (**85**)	31.13	38.15
8-Acetoxyisochiliolide lactone (**86**)	NA	NA
7, 8-Epoxyisocholiolide lactone (**87**)	38	NA
Pentamidine (standard)	0.85	1.75
Amphotericin B (standard)	0.12	0.15

NA = Not Active

CHAPTER FIVE
CONCLUSIONS AND RECOMMENDANTIONS

5.1   Conclusions

Chromatographic separation of the surface exudates of *Microglossa pyrifolia* led to the isolation and characterization of five flavonoids and two diterpeniods, of which three of them, 5, 7, 4'-trihydroxy-3, 8, 3', 5'-tetramethoxyflavone (**82**), 5, 7, 3'-trihydroxy-3, 8, 4', 5'-tetramethoxyflavone (**84**) and 8-epoxyisocholiolide lactone (**86**), are new compounds.

In-vitro anti-plasmodial testing revealed that all compounds were found to be inactive against D2 and W6 strains of *Plasmodium falciparum.* The internal tissues of the aerial parts of *M* pyrifolia studied previously by Zdero *et al* afforded clerodanes, sec-clerodane, rearranged clerodane and geranylgeranol derivatives. During the preliminary investigations, the crude extract was found to have IC_{50} of 8.0 µg/ml (against *P.falciparum* D6) and 13.0 µg/ml (against *P.falciparum* W2). In this analysis the compounds isolated were from surface exudates. None of these compounds resembled those isolated by Zdero *et al* and the bulk of them were flavonoids. This therefore suggests that either the preliminary result was due to the compounds found in the internal tissues which are different from those on the surface or that it was due to synergistic effect or to trace compounds which were not isolated.

Anti-oxidant testing revealed that phenolic compounds with the phenolic hydroxyl groups and other hydroxyl or methoxyl groups at ortho or para positions were reasonably active. These were the flavonoids and the most active of them was 5, 7, 4'-trihydroxy-3, 8, 3'-trimethoxyflavone (**81**) with IC_{50} of 6.02 µg/ml (16.7 µM) which was less than that of quercetin (IC_{50} 6.0 µg/ml; 19.9 µM), the standard used. Other compounds that had good anti-oxidant activities were, **82** (5, 7, 4'-trihydroxy-3, 8, 3', 5'-tetramethoxyflavone) and compound **85** (5, 3', 4'-Trihydroxy-3, 7, 8-trimethoxyflavone) which had IC_{50} of 6.45 ± 0.3 µg/ml (16.7 µM) and 6.2 ± 0.2 µg/ml (17.2 µM) respectively. This gives a revelation for a possibility of having an anti-malarial which is also an anti-oxidant, and hence giving the possibility of fighting malaria while minimizing the chances of developing complications from oxidative stress.

All the compounds were inactive to the *Leishmania donovani promastigotes*, except for 5, 3', 4'-Trihydroxy-7-methoxyflavanone (**83**) which exhibited mild antileishmanial activity (IC_{50} 13.13µg/ml).

5.2 Recommendations

i. In taxonomic terms, other related species within the genus have to be phytochemically investigated so as to establish detailed botanical grouping of the genus.

ii. Careful analysis of the antiplasmodial activity of all compounds of the genus and their structure-activity relationship followed by rational synthetic modifications has potential for identifying useful agents with low cytoxicity and high anti-plasmodial activity in the fight against malaria.

iii. The anti-plasmodial activity of the mixture of compounds should be performed to establish which combination would give a better activity than the individual compounds

iv. This mixture of non-polar compounds (flavonoids and diterpenoids) could be playing an ecological role for *M. pyrifolia*. The methoxylation of flavonoid hydroxyl groups is an established anti-herbivore, anti-microbiol feature in other species. They also play a role in sunscreen. The existence of the modified clerodanes, **86** and **87** are an interesting feature of this exudate and the potential activities of these compounds should be pursued to help speculate their potential biological function

REFERENCES

Akah, P. A., Ekekwe, R. K. (1995). Ethnopharmacology of some *Asteraceae* family used in Nigerian traditional medicine. *Fitoterapia* **66**, p. 351-355.

Bahorun, T., Gressier, B., Trotin, F., Brunet, C., Dine, T., Luycks, M., Vasseur, J., Cazin, J., Pinkas, M. (1996). Oxygen species scavenging activity of phenolic extracts from Hawthorn fresh plant organs and pharmaceutical preparations. *Arzneimittel-Forschung Drug Resarch* **46**, p. 1086-1089

Balunas, M.J., Kinghorn, A. D. (2005). Drug discovery from medicinal plants. *Life Sciences* **78**, p. 431-441.

Basco, L.K., Mitaku, S., Skaltsounis, A.L., Ravelomanaintsoa, N., Tillequin, F., Koch, M., Le Bras, J. (1994). *In vivo* activities of acridone alkaloids against *Plasmodium falciparum. Antimicrobial Agents and Chemotherapy* **5**, p. 1169–1171.

Bennet, R.N., Wallsgrove, R.M. (1994). Secondary metabolism in plant defense mechanisms. *New phytologist*, **127**(72), p. 617-633.

Batista, R.; Silva, A.D.J.; de Oliveira, A.B. (2009). Plant-derived antimalarial agents: new leads and efficient phytomedicines. Part II. Non-alkaloidal natural products. *Molecules* 2009, **14**, p.3037-3072.

Berry, C. (2000). Plasmepsins as antimalarial targets. *Current Opinion in Drug Discovery and Development* **3**, p. 624-629.

Bilia, A.R. (2006). Non-nitrogenous plant-derived constituents with antiplasmodial activity. *Natural Product communications* **1**, p. 1181-1204.

Borenfreund, E., Babich, H., Martin-Alguacil, N. (1990). Rapid chemosensitivity assay with human normal and tumor cells *in vitro*: *In vitro* cellular and developmental biology. *Journal of the Tissue Culture Association* **26**, p.1030-1034.

Breman, J. (2001). The ears of the hippopotamus: manifestations, determinants, and estimates of the malaria burden. *American Journal of Tropical Medicine and Hygiene* **64,** p. 1-11.

Brian, C. F., Gwyn, A. B (2008). An Overview of Plant Defenses against Pathogens and Herbivores. *The Plant Health Instructor***10**, p.1094

Carter, J.A., Ross A.J., Neville, B.G., Obiero, E., Katana, K., Mung'ala-Odera, V., Lees, J.A., Newton, C.R. (2005). Developmental impairments following severe falciparum malaria in children. *Tropical Medicine & International Health* **10** (1), p. 3–10.

Chen, Z. Y., Chan P. T., Ho K. Y., Fung K. P., Wang J. (1996). Antioxidant activity of natural flavonoids governed by number and location of their hydroxyl groups. *Chemistry and Physics of Lipids* **79**, p. 157-163.

Christianson, D. W., (2006) Structural Biology and Chemistry of the Terpenoid Cyclases, *Chemical. Review* **106**, p. 3412-3442.

Collado, I.G., Sanchez, A. J., Hanson, J. R. (2007) Fungal Terpene Metabolites: Biosynthetic Relationships and the Control of the Phytopathogenic Fungus *Botrytis cinerea*, *Natural Product. Reports* **24,** p 674-686.

Cragg, R. A., Phillips, S. R., Piper, J. M., Varma J. S., Campbell, F. C., Mathers, J. C., Ford, D. *(*2005*).* Homeostatic regulation of zinc transporters in the human small intestine by dietary zinc supplementation. *The international journal of gastroenterology and hepatology.* **54**, p. 469–478.

Croft, S. L., Coombs, G. H. (2003). Leishmaniasis-Current chemotherapy and recent advances in the search for novel drugs. *Trends in Parasitology* **19** (11), p. 502-503.

Davies, K. M., Schwinn, K. E. (2005) Molecular Biology and Biotechnology of Flavonoid Biosynthesis. Flavonoids: Chemistry, Biochemistry and Applications. Andersen, O. M. and Markham, K. R. (eds). CRC Press, Boca Raton, FL. p. 143-218.

Davis, E.M., Croteau, R. (2000) Cyclization Enzymes in the Biosynthesis of Monoterpenes, Sesquiterpenes, and Diterpenes. *Current Topics in Medicinal Chemistry* **209**, p. 53-95.

Davisson, V. J., Neal, T. R., and Poulter, C. D. (1985) Farnesylpyrophosphate Synthetase- A Case for Common Electrophilic Mechanisms for Prenyltransferases and Terpene Cyclases. Journal of American Chemical Society**107**, p. 5277-5279.

Dell, B., McComb, A.J. (1978). Biosynthesis of resin terpenes in leaves and glandular hairs of *Newcastelia viscida*. *Journal of Experimental Botany* **29**, p. 89–95.

Dewick, P.M. Medicinal Natural Products; A Biosynthetic Approach. (2002). Chichester, New York: John Wiley and Sons, p. 5-9.

Dickson, R. A., Houghton, P. J., Hylands, P. J., Gibbons, S. (2006). Antimicrobial, Resistance-Modifying Effects,Antioxidant and Free Radical ScavengingActivities of Mezoneuron benthamianum Baill., *Securinega virosa* Roxb. And Wlld. And *Microglossa pyrifolia* Lam. *Phytotherapy Research* **20**, p. 41-45

Dixon, R.A., Lamb, C.J., Masoud, S., Sewalt, V.J., Paiva, N.*L. (*1996*).* Metabolic engineering*:* prospects for crop improvement through the genetic manipulation of phenylpropanoid biosynthesis and defense responses-a review. *Gene 179* (1), p. 61–71.

Dudareva, N. and Pichersky, E. (2000). Biochemical and molecular genetic aspects of floral scent. *Journal of plant physiology* **122,** p. 627–633.

Eisenreich, W., Bacher, A., Arigoni, D., Rohdich, F. (2004) Biosynthesis of Isoprenoids via the Non-mevalonate Pathway, *Cellular and Molecular Life Science*. **61**, p 1401-1426.

Fahn, A. (1988*).* Secretory tissues in vascular plants. *New Phytologist* **108**, p. 229–257.

Fritz, S.R., Simms, L .E**.** (1992). Plant Resistance to Herbivores and Pathogens: Ecology, Evolution, and Genetics. University of Chicago Press. Chicago.

Geissbühler, Y., Chaki P., Emidi B., Govella N. J., Shirima R., Mayagaya V., Mtasiwa D., Mshinda H., Fillinger U., Lindsay S. W., Kannady K., De Castro M. C., Tanner M., Killeen Gerry F. (2007). Interdependence of domestic malaria prevention measures and mosquito-human interactions in urban Dar es Salaam, Tanzania. *Malaria Journal* **6**, p. 126-129.

Gessler, M. C., Msuya, D. E., Nkunya, M. H. H., Mwasumbi, L. B., Schar, A., Heinrich, M., Tanner, M. (1995). Traditional healers in Tanzania: the treatment of malaria with plant remedies. *Journal of Ethnopharmacology* **48,** p.131- 144.

Goffreda, J.C., Steffens, J.C., Mutschler, M.A. (1990). Association of epicuticular sugars with aphid resistance in hybrids with wild tomato. *Journal of the American Society of Horticultural Science* **115**, p. 161–165.

Greenwood, B. M., Bojang, K., Whitty, C. J., Targett, G. A. (2005*). Malaria Lancet* **365***,* p.1487-1498.

Gunatilaka, A. A. L., Dhanabaladinogham, B., Paredes, L., Jakupovic, J., Bohlmann, F., Adikaram, N. K. B. (1987). Microglossic acid, an alicyclic diterpene and other constituents of *Microglossa zeylanica*. *Phytochemistry* **26**, p. 2408-2409.

Gurib-Fakim, A. (2006). Medicinal plants: Traditions of yesterday and drugs of tomorrow. *Molecular Aspects of Medicine* **27**, p. 1 – 93

Harborne, J.B. (1999). The comparative biochemistry of phytoalexin induction in plants. *Biochemical Systematics and Ecology* **l27,** p.335–367.

Harborne, J.B. (1999). Phytochemical dictionary: Handbook of bioactive compounds from plants 2nd (Edn.). Taylor and Francis, London, p. 221-234.

Henrick, C. A., Jefferies, P. R. (1965). Chemistry of euphorbiaceae. XIII. Flavones and minor terpenes from *Ricinocarpos muricatus*. *Tetrahedron*.**21**(11), p. 3219-3228.

Hetzel, M. W., Iteba N., Makemba A., Mshana C., Lengeler C., Obrist B., Schulze A., Nathan R., Dillip A, Alba S., Mayumana I., Khatib R. A., Njau J. D., Mshinda H. (2007). Understanding and improving access to prompt and effective malaria treatment and care in rural Tanzania: the ACCESS Programme. *Malaria Journal* **6,** p. 83 – 97.

Horie, T., Ohtsuru, Y., Shibata, K., Yamashita, K., Tsikamaya, M., Kawamura, Y. (1998).^{13}C NMR spectral assignment of the A-ring of polyoxygenated flavones. *Phytochemistry* **47**, p. 865–874.

Hou, W., Hsu F., Lee M. (2002). Yam (*Dioscorea batatas*) tuber mucilage exhibited anti-oxidant activities in vitro. *Planta Medica* **68**, 1072-1076

Hughes, J.R., Croley, R.T., Metcalfe, D.C., March, E.R. (2001). A tandem mass spectrometric study of selected characteristic flavonoids. *International journal of Mass Spectrometry* 210-211, p 371-385.

Ibrahim, A.R., Galal, A.M., Ahmed, M.S., Mossa, G.S. (2003). O-demethylation and sulfation of 7-methoxylated flavanones by Cunninghamella elegans, *Chemical and Pharmaceutical Bulletin* **51**, p. 203–206

James, D. M., Syles, K. V., John, T. H. (2006). Plant natural products: Back to the future or extinction? *Phytochemistry* **68** (14), p. 2015-2022.

Johns, T., Kokwaro, J. O., Kimanani, E. K. (1990). Herbal remedies of the Luo of Siaya district, Kenya: Establishing quantitative criteria for consensus. *Economic Botany* **44**, p. 369-381.

Johnson, H.B. (1975). Plant pubescence: an ecological perspective. *Botanical Review* **41**, p. 233-253.

Kamau, E. M. (2006). Roll Back Malaria and the new partnership for Africa's development: Is there potential for synergistic collaboration in partnership? *African Journal of Health Sciences* **13**, p. 22-27.

Karban, R., Baldin, T.I. (1997). Induced responses to herbivory. University of Chicago press. Chicago.

Kaur, K., Jain, M., Kaur, T., Jain, R. (2009). Antimalarials from nature. *Bioorganic and Medicinal Chemistry* **17**, p. 3229–3256.

Kawazu, S., Komaki-Yasuda, K., Oku, H., Kano, S. (2008). Peroxiredixins in malaria parasites: Parasitologic aspect. *Parasitology International* **57**, p. 1-7.

Kayser, O., Kiderlen, A.F., Croft, S.L. (2003). Natural products as antiparasitic drugs. *Parasitology Research* **90**, p. S55–S62.

Kellogg, E.A. (2001). Root hairs, trichomes and the evolution of duplicate genes. *Trends in Plant Science* **6**, p. 550-552

Kigondu, E. V. M., Rukunga, G. M., Keriko, J. M., Tonui., W. K., Gathirwa, J. W., Kirira, P. G., Irungu, B., Ingonga, J. M., Ndiege I. O. (2009). Anti-parasitic activity and cytotoxicity of selected medicinal plants from Kenya. *Journal of Ethnopharmacology* **123**, p. 504-509.

Kirira, P.G., Rukunga G.M., Wanyonyi A.W., Muregi F.M., Gathirwa J.W., Muthaura C.N., Omar S.A., Tolo F., Mungai G.M., Ndiege I.O. (2006). Anti-plasmodial activity and toxicity of extracts of plants used in traditional malaria therapy in Meru and Kilifi Districts of Kenya. *Journal of Ethnopharmacology* **106**, p. 403–407.

Klayman, D.L., (1985). Qinghaosu (artemisinin): an antimalarial from China. *Science* **31**, p. 1049–1055.

Koch, A., Tamez, J., Pezzuto, J., Soejarto, D. (2005). Evaluation of plants used for antimalarial treatment by the Maasai of Kenya. *Journal of Ethnopharmacology* **101**, p. 95–99.

Kohler, I., Janett Siems, K., Kraft, C., Siems, K., Abbiw, D., Bienzle, U., Aiche, E. (2002). Herbal remedies traditionally used against malaria in Ghana; Bioassay guided fractionation of *Microglossa pyrifolia* (Asteraceae). *Zeitschrift fur Naturforschung C* **57c,** p. 1022-1027.

Kokwaro, J.O. (1976). Medicinal Plants of East Africa. East Africa literature bureau. Nairobi, Kenya.

Korenromp, E. (2004). World Malaria Report: Roll Back Malaria.World Health Organization: Geneva.

Kvist, L. P., Christensen, S. B., Rasmussen, H. B., Mejia, K., Gonzalez, A. (2006). Identification and evaluation of Peruvian plants used to treat malaria and leishmaniasis. *Journal of Ethnopharmacology* **106**, p. 390–402.

Kuiate, J.R., Amvam Zollo P.H, Bessière J.M, Lamaty G, Menut C.(1999). Composition of the essential oils from the leaves of *Microglossa pyrifolia* (Lam.) O. Kuntze and Helichrysum (L;) Less. Growing in Cameroon. *Flavour and Fragrance Journal* **14,** 87-89.

Leyes, A. E., Baker, J. A., Hahn, F. M., Poulter, C.D. (1999) Biosynthesis of Isoprenoids in Escherichia coli: Stereochemistry of the Reaction Catalyzed by Isopentenyl Diphosphate: Dimethylallyl Diphosphate Isomerase, *Chemical. Communications*.**8**, p 717-718.

Mackintosh, C.L., Beeson, J. G., Marsh, K. (2004). Clinical features and pathogenesis of severe malarial. *Trends in Parasitol*ogy **200**, p. 597-603.

Makler, M. T., Ries, J. M., Williams, J. A., Bancroft, J. E., Piper, R. C., Gibbins, B. L, Hinriches, D. J. (1993). Parasite lactate dehydrogenase as an assay for *Plasmodium falciparum* drug sensitivity. *American Journal of Tropical Medicine Hygiene* **48**, p. 739-741.

Mahlberg, P.G., Kim, E.S. (1992). Secretory vesicle formation in glandular trichomes of Cannabis sativa (Cannabaceae). *American Journal of Botany* **79**, p. 166-173.

Mahmoud, S. S., Croteau, R (2002) Strategies for transgenic manipulation of monoterpene biosynthesis in plants; *Trends in Plant Science* **7,** p 366 – 373

Marchant, T. (2004). Anaemia in pregnancy and infant mortality in Tanzania. *Tropical Medicine and International Health* **9**, p. 262-266.

Mauricio, R. (1998). Costs of Resistance to Natural enemies in Feld Populations of the Annual Plant *Arabidopsis thaliana*. *American Naturalist* **151**, p. 20-28.

McGarvey, D J., Croteau, R (1995) Terpenoid metabolism. *Plant Cell* **7** p 1015 – 1026

Mert-Türk, F. (2002). Phytoalexins: Defence or just a response to stress? *Journal of Cell and Molecular Biology* **1**, p. 1-6.

Meyer, E. F., Swanson, S. M., William, J. A. (2000).Molecular modeling and drug design. *Pharmacology and Therapeutics* **85**, p. 113-121.

Midiwo, J., Matasi, J., Wanjau, O., Mwangi, R., Waterman, P., Wollenweber, E. (1990) Anti-feedant effects of surface accumulated flavonoids of Polygonum senegalense. Bulletin of the *Chemical Society of Ethiopia* **4**, p.123-127

Mikus, J., Steverding, D. (2000). A simple colorimetric method to screen drug cytotoxicity against Leishmania by using the dye Alamar Blue. *Parasitology International* **48**, p. 265-269.

Mitchell-Olds, T., Gershenzon, J., Baldwin, I.T., Boland, W. (1998*)*. Chemical ecology in the molecular era. *Trends in Plant Scienc*e **9**, p. 362-365.

Mol, J., Cornish, E., Mason, J., Koes, R. (1998). How genes paint flowers and seeds. *Trends in Plant Scienc*e **3,** p. 212–217.

Mugabe, J., Clark, N. (1998). Managing Biodiversity: The National systems of Conservation and Innovation in Africa. Acts Press, Nairobi.p 66-69.

Muregi, F.W., Chhabra, S.C., Njagi, E.N.M., Lang'at-Thoruwa, C.C., Njue,W.M., Orago, A.S.S., Omar, S.A., Ndiege, I.O.(2003). In vitro antiplasmodial activity of some plants used in Kisii, Kenya against malaria and their chloroquine potentiation effects. *Journal of Ethnopharmacology* **84**, p. 235–239.

Muthaura, C. N., Rukunga, G. M., Chhabra, S. C., Mungai, G. M., Njagi, E. N. M. (2007a). Traditional antimalarial phytotherapy remedies used by the Kwale community of the Kenyan Coast. *Journal of Ethnopharmacology* **114**, p. 377-386.

Muthaura, C. N., Rukunga, G. M., Chhabra, S. C., Omar, S. A., Guantai, A. N., Gathirwa, J. W., Tolo, F. M., Mwitari, P. G., Keter, L. K., Kirira, P. G., Kimani, C. W., Mungai, G. M., Njagi, E. N. M. (2007b). Antimalarial activity of some plants traditionally used in treatment of malaria in Kwale district of Kenya. *Journal of Ethnopharmacology* **112**, p. 545-551.

Newman, M.P., Yaman, P., Dennison, J., Rafter, M., Billy, E. (2003). Fracture resistance of endodontically treated teeth restored with composite posts. *The Journal of Prosthetic Dentistry* **89** (4*),* p. 360-367.

Nkuo-Akenji, T.K., Menang, O.N. (2005). Prevelance of falciparum malaria together with acute diarrhoea in children residing in a malaria endemic zone. *Africa Journal of Traditional, Complementary and Alternative Medicine* **12**, p. 26-30.

Odugbemi, T.O.**,** Akinsulire, O.R., Aibinu, I.E., Fabeku, P.O. (2007). Medicinal plants useful for malaria therapy in Okeigbo Ondo state, Southwest Nigeria. *Africa Journal of Traditional, Complementary and Alternative Medicine* **4**, p.191-198.

Olliaro, P. L., Yuthavong, Y. (1999*).* An overview of chemotherapeutic targets for antimalarial drug discovery. *Pharmacology and Therapeutics* **81(2),** p. 91-110.

Osbourn, A.E., Lanzotti, V. (2009) Plant derived natural products; synthesis, function and application. Springer Dordrecht Heidelbeg, London and New York, p. 3-5.

Pamisha, P., Robert, V., Vinesh, J. M., Peter, J. S., Carmen, A. L., Frank C., Kelly, C. (2007).Antiplasmodial hirsutinolides from Vernonia staehelinoides and their utilization towards a simplified pharmacophore. *Phytochemistry* **68**(8), p. 1200-1205.

Pieta, P. (2000). Flavanoids as antioxidants. *Journal of Natural Products* **63**, p. 1035-1042.

Poulter, C. D., and Rilling, H. C. (1978). The Prenyltransfer Reaction. Enzymatic and Mechanistic Studies of the 1'-4 Coupling Reaction in the Terpene Biosynthetic Pathway, *Accounts of Chemical Research* **11**, p. 307-313

Poulter, C. D., Rilling, H. C. (1981). Prenyl transferases and isomerase. In: Porter JW, Spurgeon SL (eds) Biosynthesis of isoprenoid compounds, volume 1, John Wiley, New York, p. 161-224.

Qureshi, N., Porter J. W. (1981). Conversion of acetyl-coenzyme A to isopentyl pyrophosphate. In: Porter J. W. and Spurgeon S. L. (eds), Biosynthesis of Isoprenoid Compounds, volume **1**, John Wiley, New York, p. 47–94.

Rosenthal, G.A, Berenbaum, M.R. (1992). Herbivores: Their interaction with secondary plant metabolites, Vol II Ecological and evolutionary processes, 2nd edition. Academic press, San Diego.

Rohmer, M. (1999). The discovery of a mevalonate independent pathway for isoprenoid biosynthesis in bacteria, algae and higher plants. *Natural Product Reports* **16**, p. 565–574

Rucker, G., Kehrubau, S., Sakulas H., Lawong B., Goeltenboth F. (1992). Acetylated aurone glycosides from *Microglossa pyrifolia*. *Planta Medica* **60**, p. 288-289.

Rugemalila, J.B., Wanga, C.L., Kilama W.L. (2006). Sixth Africa malaria day in 2006: how far have we come after Abuja declaration? *Malaria Journal* **12**, p. 102-106.

Sacchettini, J C., Poulter, C. D. (1997). Creating isoprenoid diversity; *Science* **277**, p.1788–1789.

Sachs, J., Malaney, P. (2002). The economic and social burden of Malaria. *Nature* **415**, p. 680-685.

Saidu, K., Onah, J., Orisadipe, A.S., Olusola, A., Wambebe, C., Gamaniel, K., (2000). Antiplasmodial, analgesic, and anti-inflammatory activities of the aqueous extract of the stem bark of *Erythrina senegalensis*. *Journal of Ethnopharmacology* **71**, p. 275-280.

Samoylenko, V., Jacob, M. R., Khan, S. I., Zhao, J., Tekwani, B. L., Midiwo, J. O., Walker, L. A., Muhammad, I. (2009). Antimicrobial, Antiparasitic and Cytotoxic Spermine Alkaloids from *Albizia schimperiana*. *Natural product communications* **4**, p. 791-796.

Schellenberg, D., Armstrong, S. J., Mushi, A., de Savigny, D., Mgalula, L.,Mbuya, C., Victora, C.G. (2003b). The silent burden of anaemia in Tanzanian children: a community-based study. *Bull World Health Organ* **81**, p. 581-590.

Schulz, S. and Dickschat, J. S. (2007) Bacterial Volatiles: the Smell of Small Organisms, *Natural Product Reports* **24**, p 814-842.

Schmidt, T. J., Hildebrand, M. R., Willuhn, J. (2003). New dihydrobenzofurans and triterpenoids from roots of *microglossa pyrifolia*. *Planta Medica* **69**, p. 258-264.

Siemens, D. H. S. H., Gardbner, T., Mitchell-Olds R., Callaway, M. (*2002)*. Cost of defense in the context of plant competition: *Brassica rapa* may grow and defend. *Ecology* **83***, p.* 505-517.

Sidigia, I., Nyaigotti-Chacha, C., Kanunah, M.P. (Eds.), 1995. Traditional Medicine in Africa. East African Educational Publishers, Nairobi.

Simms, E. L., Fritz, R. S., (1992). Plant Resistance to Herbivores and Pathogens. University of Chicago Press: 1427 E. 60th Street Chicago, USA

Singh, N., Luthra, R., Sangwan, R S., Thakur, R S. (1989). Metabolism of monoterpenoids in aromatic plants. *Curr. Res.Med. Arom. Plants* **11,** p. 174–197

Spring, O. (2000). Chemotaxonomy based on metabolites from glandular trichomes. *Advances in Botanical Research* **31,** p. 153–175.

Sofowora, A., 1982. Medicinal Plants and Traditional Medicine in Africa. JohnWiley and Sons Ltd., Chichester, New York/Brisbane, Toronto/Singapore, p. 177.

Stancu, C., Sima A. (2001) Statins: mechanism of action and effects. *Journal of Cellular and Molecular Medicene*.**5**, p 378–387.

Stotz, H.U., Pittendrigh, BR., Kroymann, J., Weniger, K., Fritsche, J., Bauke, A., Mitchell-Olds, T. *(2000).* Induced plant defense responses against chewing insects. Ethylene signaling reduces resistance of Arabidopsis against Egyptian cotton worm but not diamondback moth. *Plant Physiology* **124,** p. 1007–1018.

Takahashi, S., Koyama, T. (2006) Structure and Function of cis-Prenyl Chain Elongating Enzyme, *The Chemical Record* **6**, p194-205.

Taoufiq, Z., Pino, P., Dugas, N., Conti, M., Tefit, M., Mazier, D., Vouldoukis, I. (2006). Transient supplimentation of superoxide dismutase protects endothelial cells against *Plasmodium falciparum*-induced oxidative stress. *Molecular and Biochemical Palasitology* **150**, p. 166-173.

Tene, M., Tane, P., Sondengam, B. L., Connolly, J. D. (2005). Clerodane diterpenoids from *Microglossa angolensis*. *Tetrahedron* **61**, p. 2655-2658.

Tonui, W. K. (2006). Situational Analysis of *Leishmania*ses Research in Kenya. *African Journal of Health Sciences* **13**, p. 7-21.

Trossat, C., Rathinasabapathi, B., Weretilnyk, E.A., Shen, T.L., Huang, Z.H., Gage, D.A., Hanson, A.D. (1998). Salinity promotes accumulation of 3-dimethylsulfoniopropionate and its precursor S-methylmethionine in chloroplasts. *Plant Physiology* **116**, p. 165-171.

Tsimogiannis, D., Samiotaki, M., Panayotou, G., Oreopoulou, V. (2007). Characterization of Flavonoids subgroups and hydroxyl substitution by HPLC-MS/MS. *Molecules* **12**, p 593-606

Uphof, J.C.T. (1962). Plant hairs. Encyclopedia of Plant Anatomy IV, **5,** p.1206

Van Etten, L., Folman, C. C., Eggelte, T. A., Kremsner, P. G., Deelder,A. M. (1994). Rapid diagnosis of schistosomiasis by antigen detection in urine with a reagent strip. J*ournal of. Clinical Microbiology* **32**, p. 2404-2406.

Wagner, GJ. (1991). Secreting glandular trichomes: more than just hairs. *Plant Physiology* **96**, p. 675-679

Wagner, G. J., Wang, E., Shepherd, R. W. (2004). New Approaches for Studying and Exploiting an Old Protuberance, the Plant Trichome. *Annals of Botany* **93,** p.3-11.

Wang, E., Hall, JT., Wagner, GJ. (2004). Transgenic *Nicotiana tabacum L.* with enhanced trichome exudate cembratrieneols has reduced aphid infestation in the field. *Molecular Breeding* **13**, p. 49–57.

Warrell, D. A., Gilles, H. M. (2002): Essential Malariology. 4th ed, Arnold publishers, London, p. 191-205.

WHO. (2000b): Global strategy for the prevention and control of non communicable diseases.

Woodall, G.S., Stewart, G.R..(1999). Do anthocyanins play a role in UV protection of the red juvenile leaves of Syzygium ?. *Journal of Experimental Botany* **49**, p. 1447–1445

World Report (2007). Malaria returns to Kenya highlands as temperature rise. W.W.W. *thelancet-com* **370**, p. 925-926.

Winkel-Shirley, B. (2001). Flavonoid biosynthesis: a colourful model for genetics, biochemistry, cell biology, and biotechnology. *Plant Physiol*ogy**126**, p. 458-493.

Wu, Q., Wang, M., Simon, E.J. (2003). Determination of isoflavonones in red clover and related species by high-performance liquid chromatography combined with ultraviolet and mass spectrometric detection. *Journal of Chromatography* A**1016**, p. 195-209

Zdero, C., Ahmed A. A., Bohlmann F., Mungai G. M. (1990a). Diterpenes and sesquiterpene xylosides from East African *Conyza* species. *Phytochemistry* **29**, p. 3167-3172.

Zdero, C., Bohlmann, F., Mungai, G.M., (1990b). Rearranged clerodanes and other diterpenes from *Microglossa pyrrhopappa*. *Phytochemistry* **29**, p. 3233-3241.

Appendix 1A: ^{1}H NMR (400 MHz) spectrum of 5, 7, 4'-trihydroxy-3, 8, 3'-trimethoxyflavone (**81**) in DMSO-d$_6$
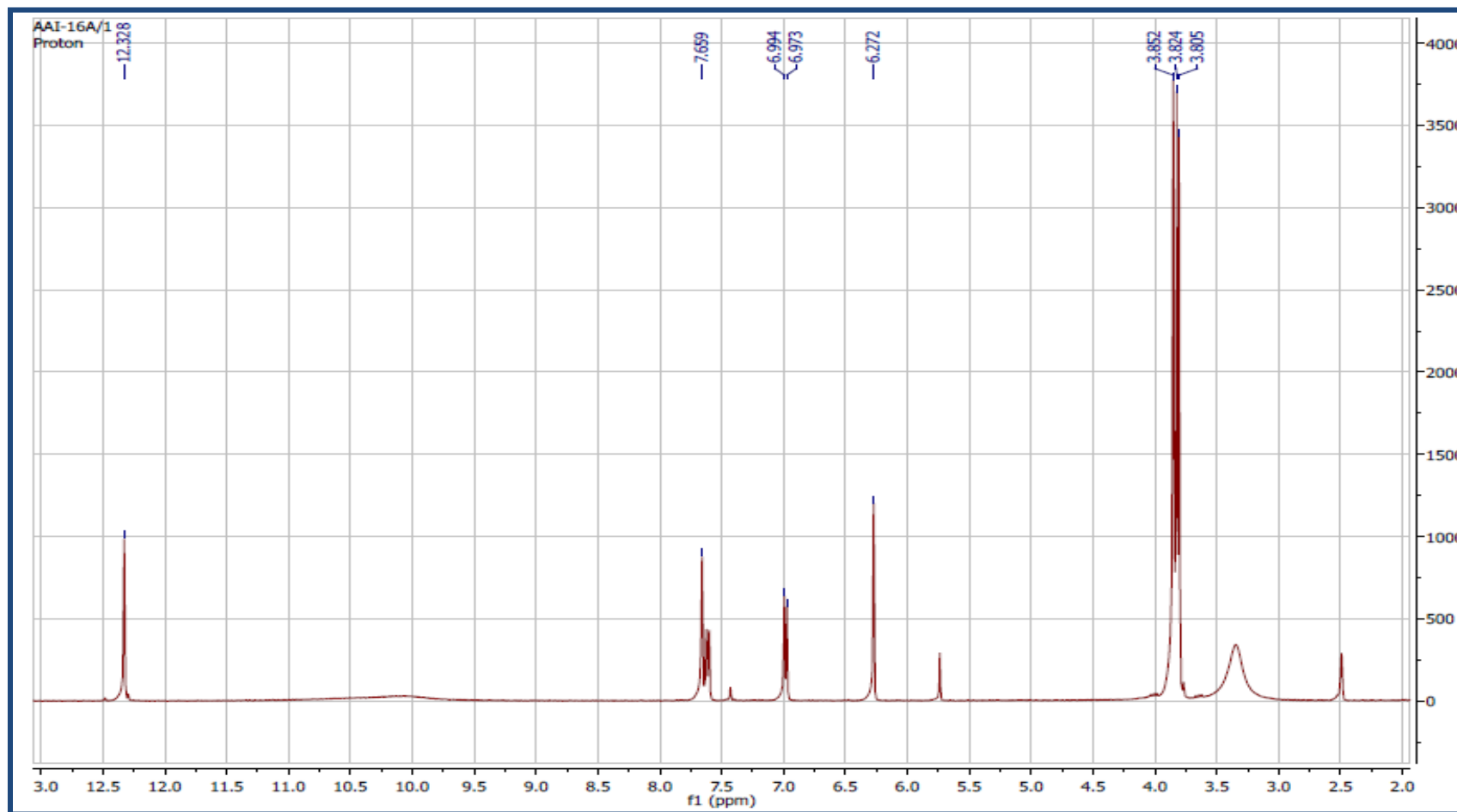
Appendix 1B: ^{13}C NMR (100 MHz) spectrum 5, 7, 4'-trihydroxy-3, 8, 3'-trimethoxyflavone (**81**) in DMSO-d$_6$
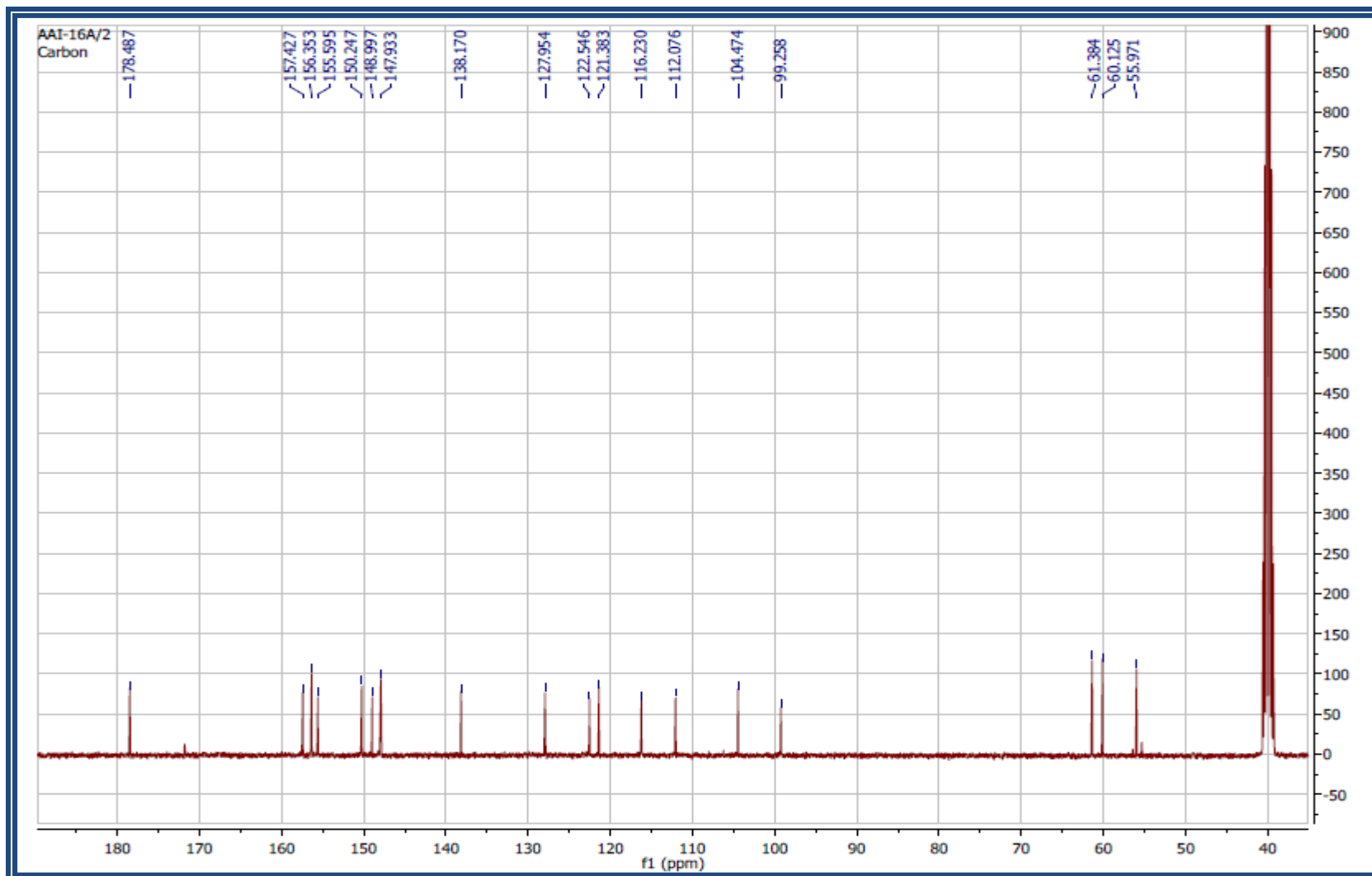
Appendix 1C: HSQC (400 MHz) spectrum of 5, 7, 4'-trihydroxy-3, 8, 3'-trimethoxyflavone (**81**) in DMSO-d$_6$


AAI-16A/2
Ghmqc

f2 (ppm)
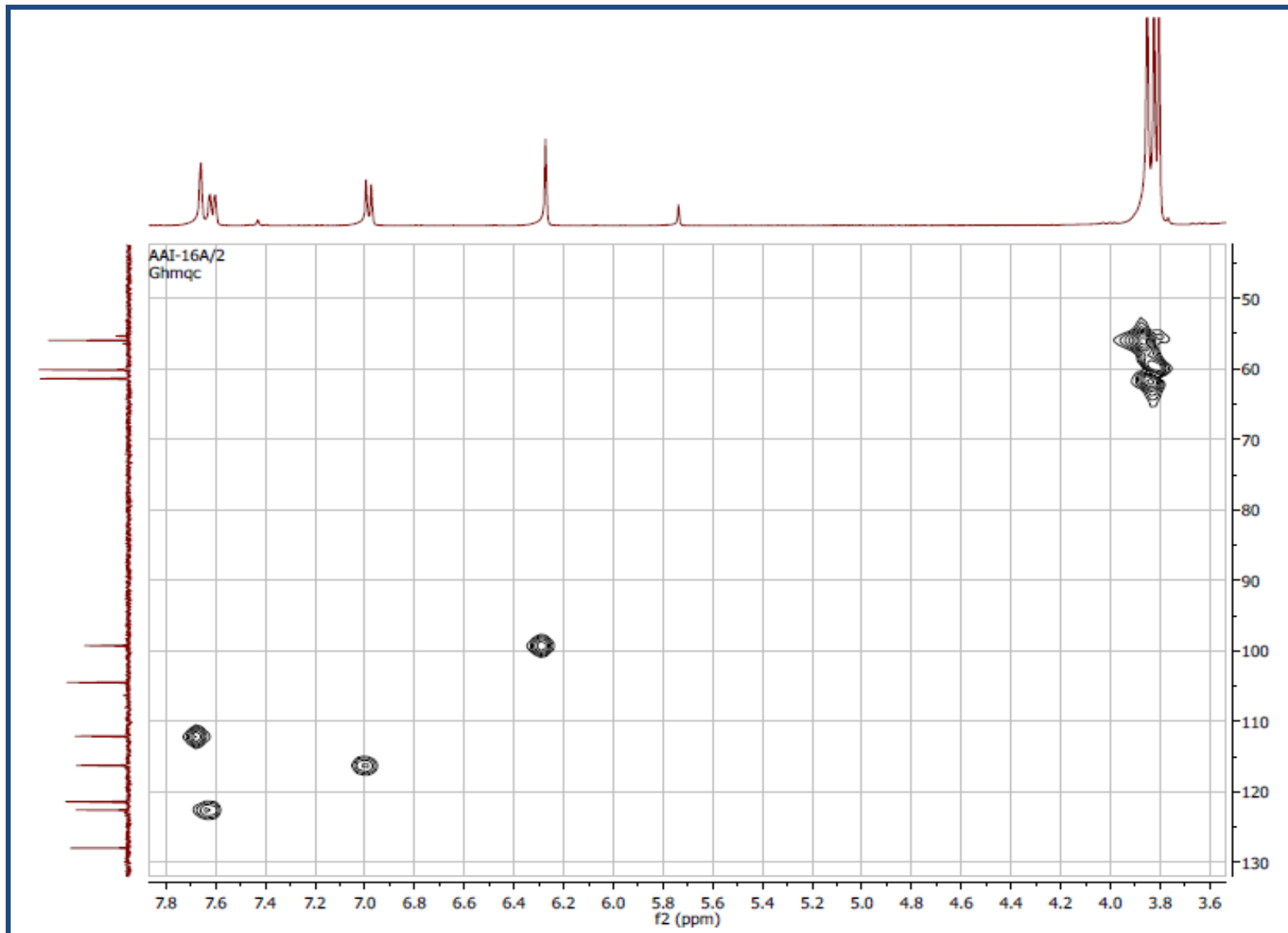
Appendix 1D: HMBC (400 MHz) spectrum of 5, 7, 4'-trihydroxy-3, 8, 3'-trimethoxyflavone (**81**) in DMSO-d$_6$
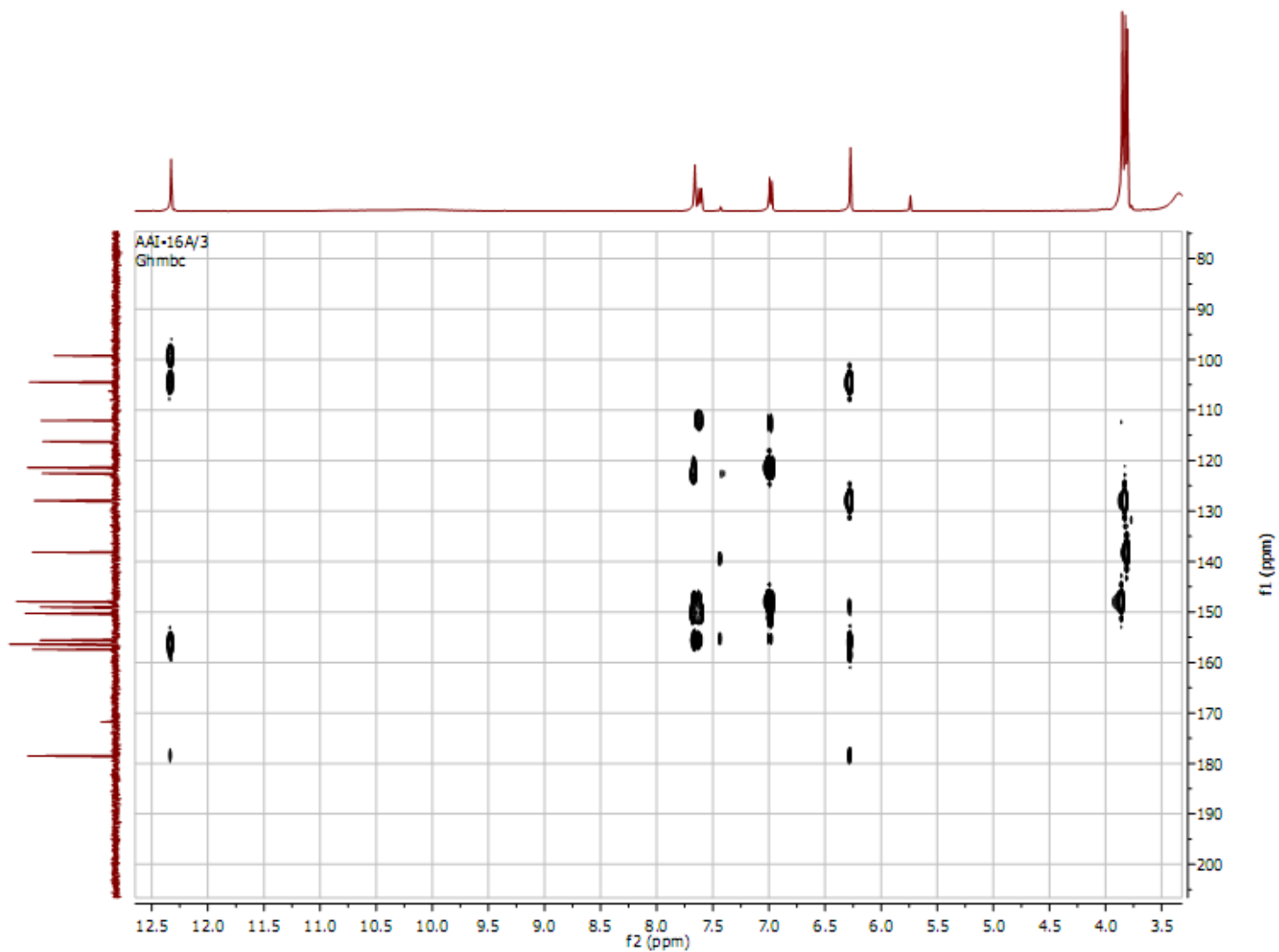
Appendix 1E: HR-EIMS spectrum of 5, 7, 4'-trihydroxy-3, 8, 3'-trimethoxyflavone (**81**)
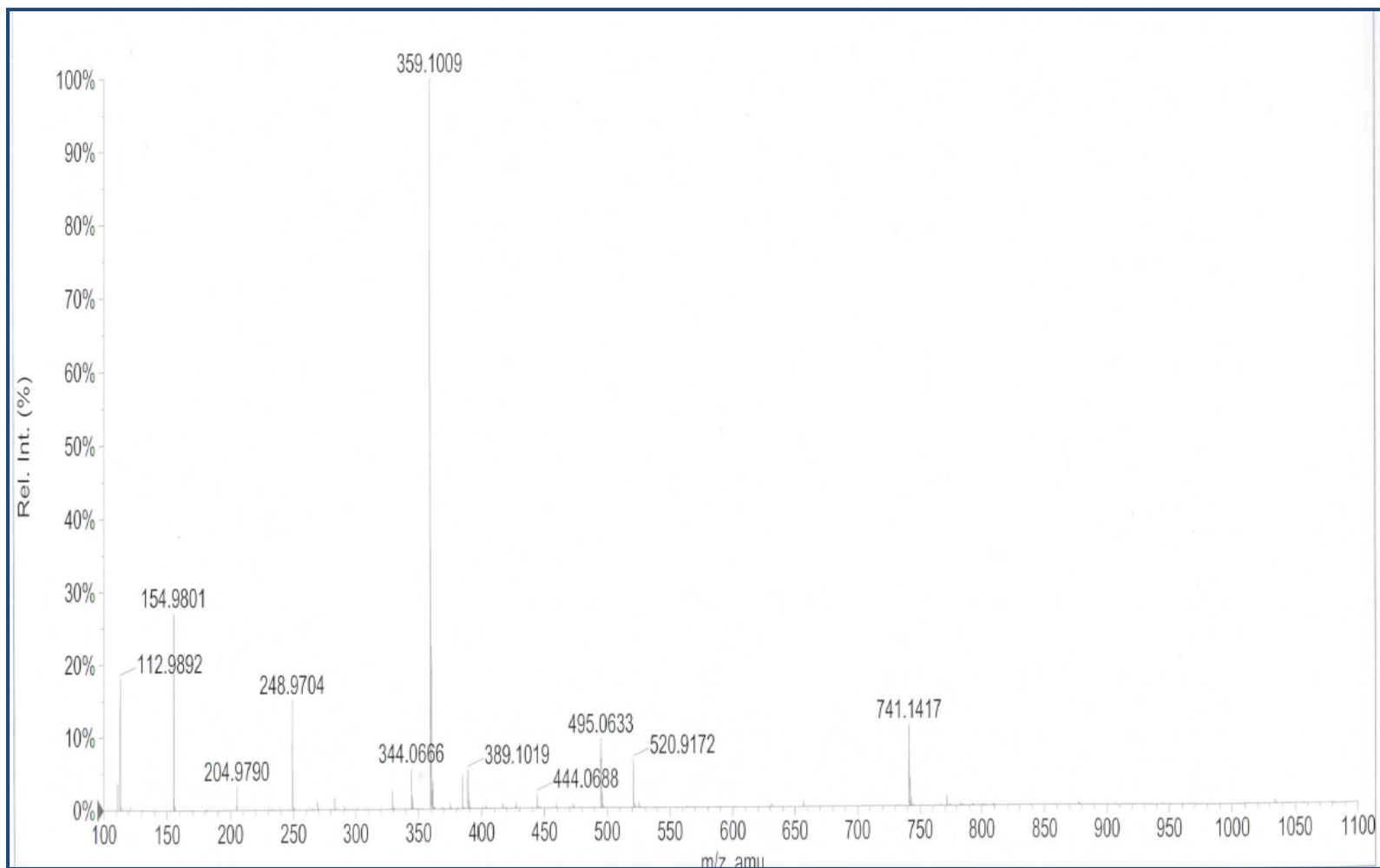
Appendix 2A: ^{1}H NMR (400 MHz) spectrum of 5, 7, 4'-trihydroxy-3, 8, 3', 5'-tetramethoxyflavone (**82**) in DMSO-d6
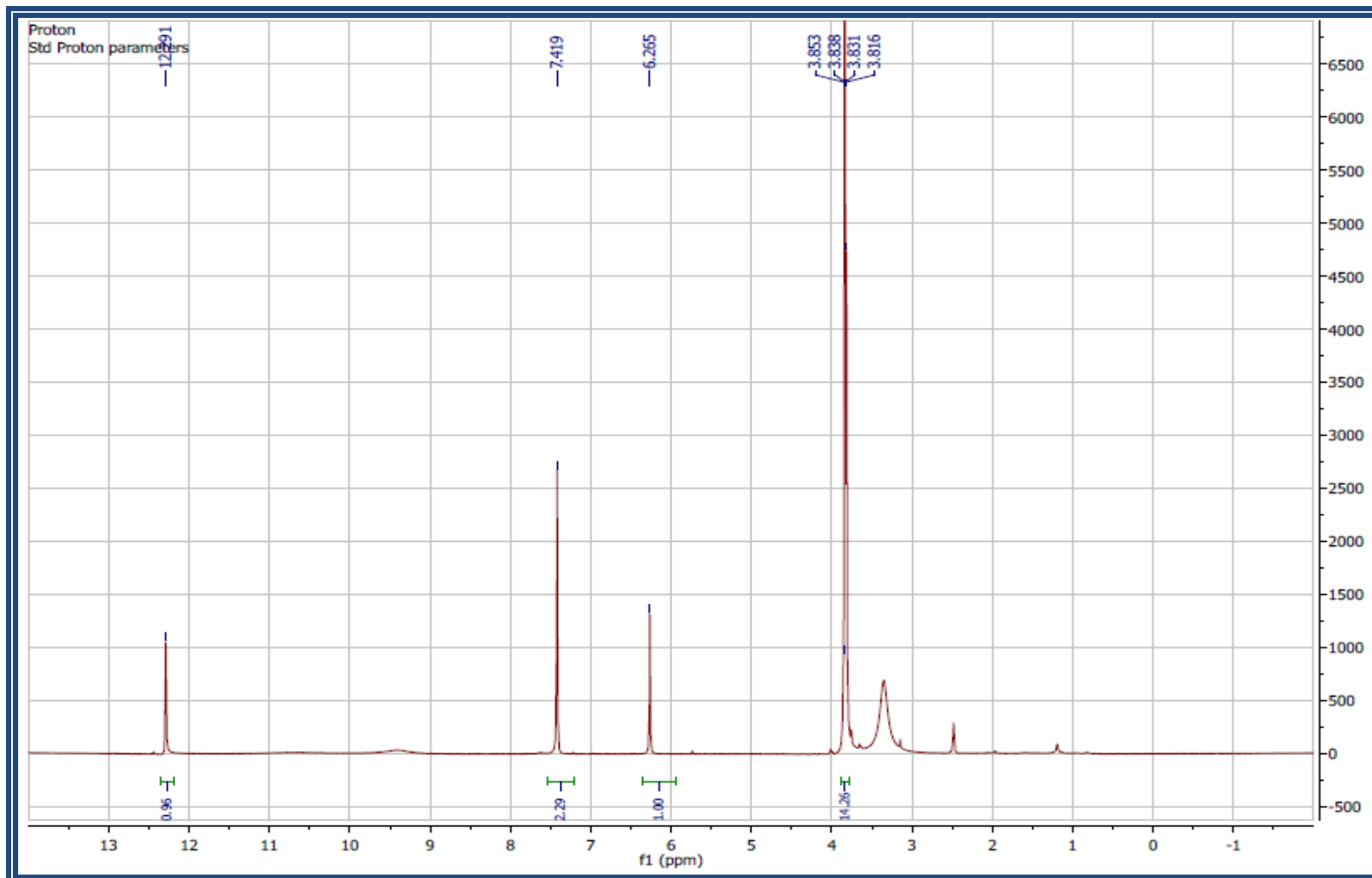
Appendix 2B: ^{13}C NMR (100 MHz) spectrum of 5, 7, 4'-trihydroxy-3, 8, 3', 5'-tetramethoxyflavone (**82**) in DMSO-d$_6$
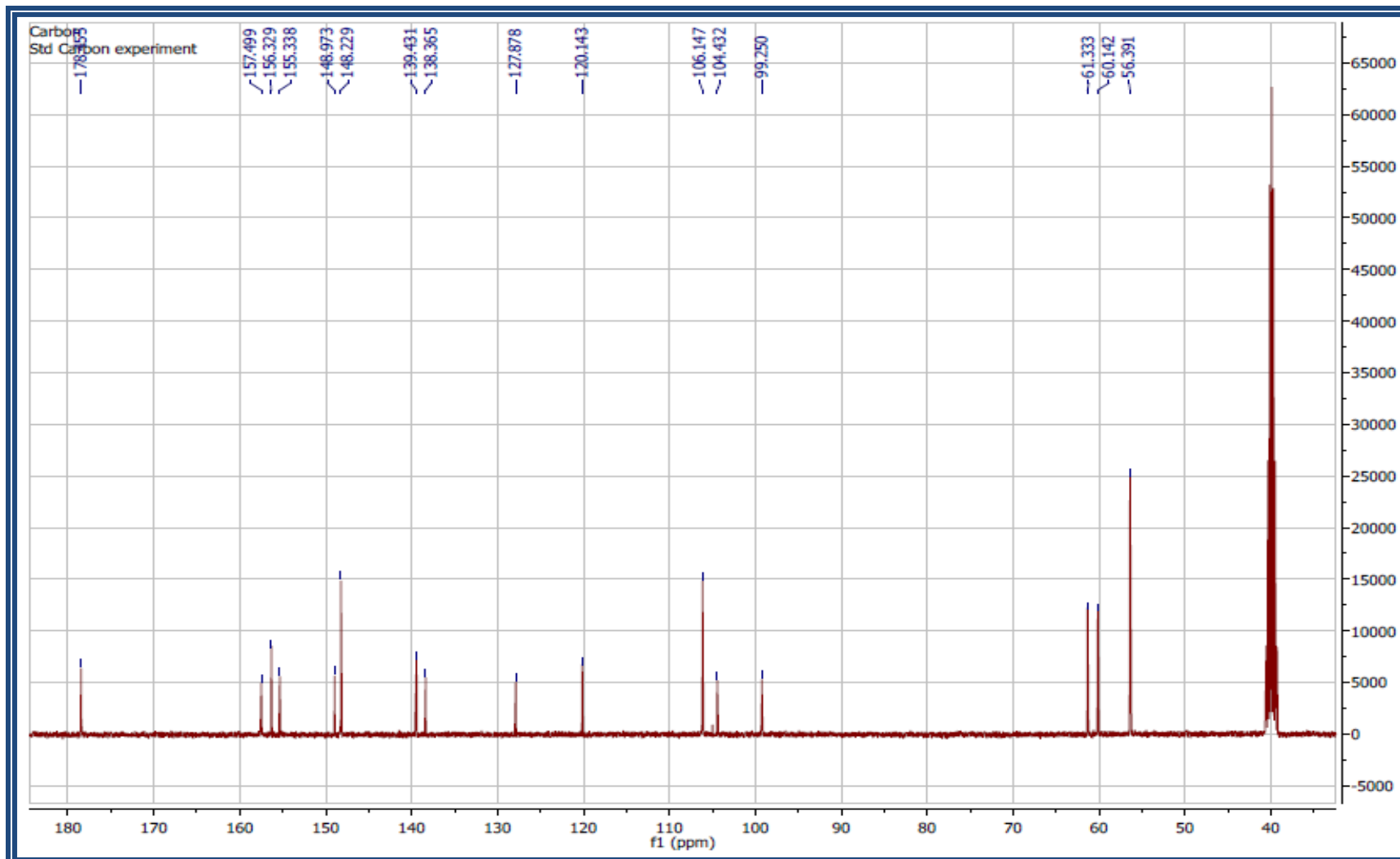
Appendix 2C: HSQC (400 MHz) spectrum of 5, 7, 4'-trihydroxy-3, 8, 3', 5'-tetramethoxyflavone (**82**) in DMSO-d₆
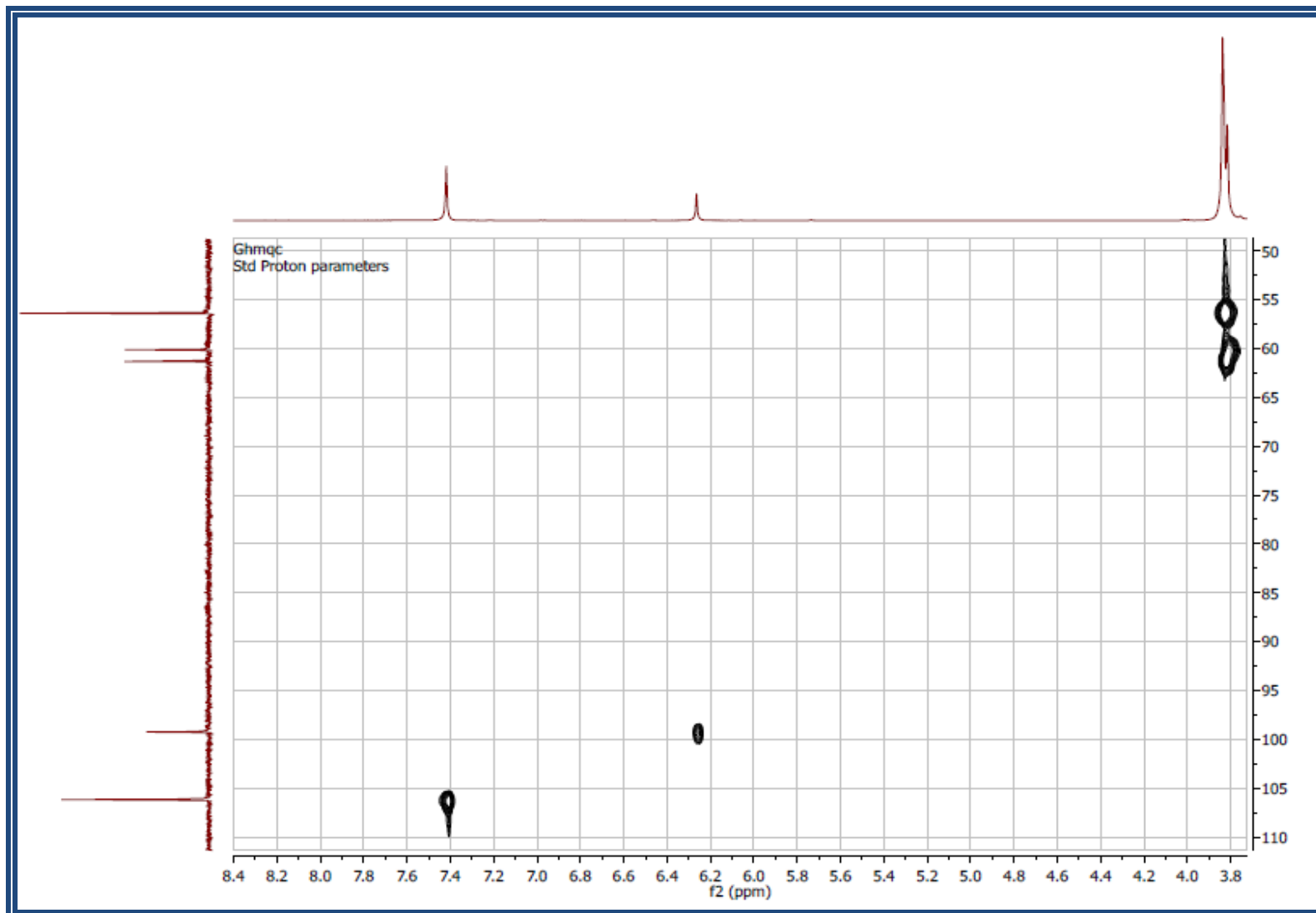
Appendix 2D: HMBC (400 MHz) spectrum of 5, 7, 4'-trihydroxy-3, 8, 3', 5'-tetramethoxyflavone (**82**) in DMSO-d$_6$
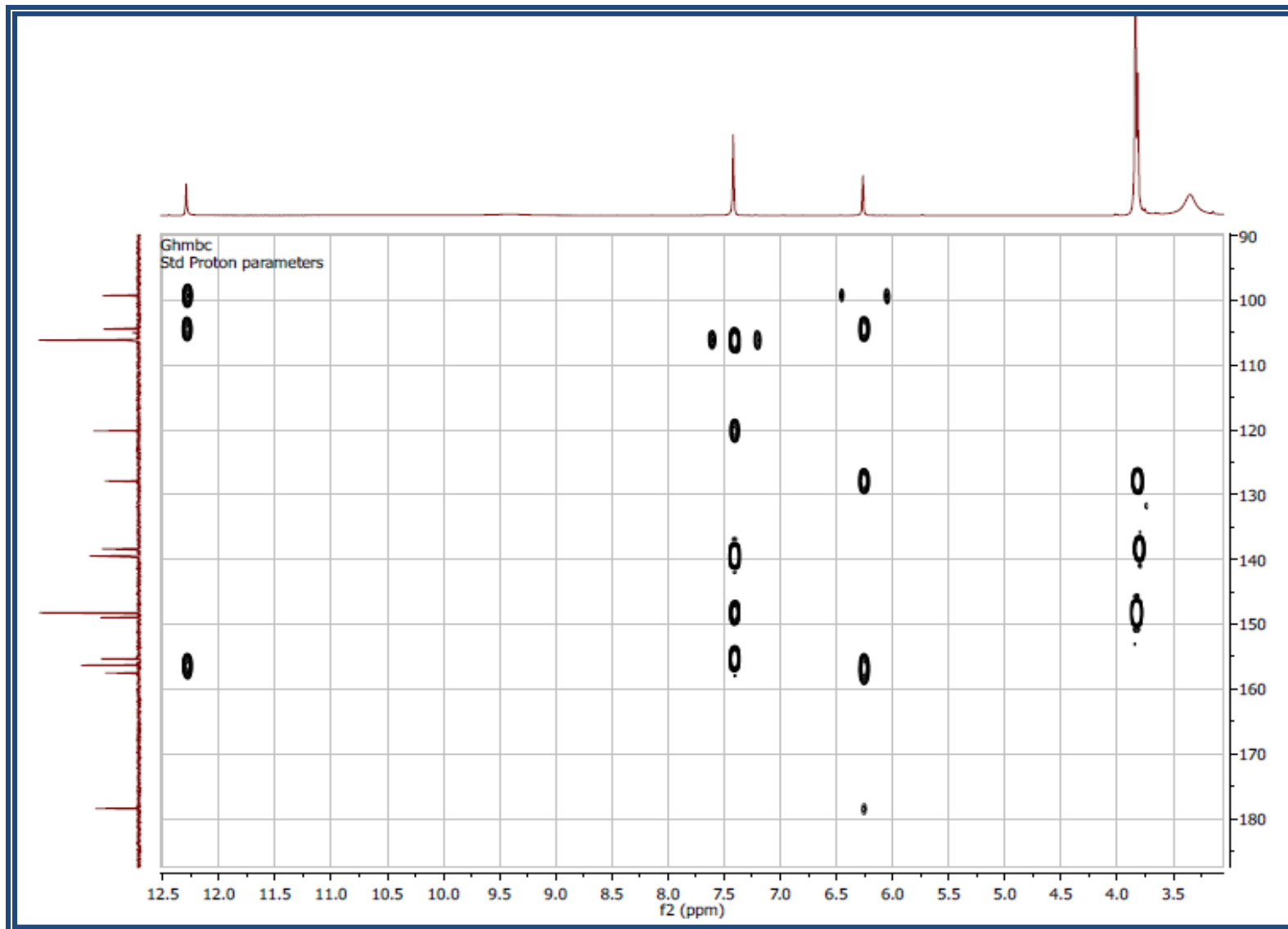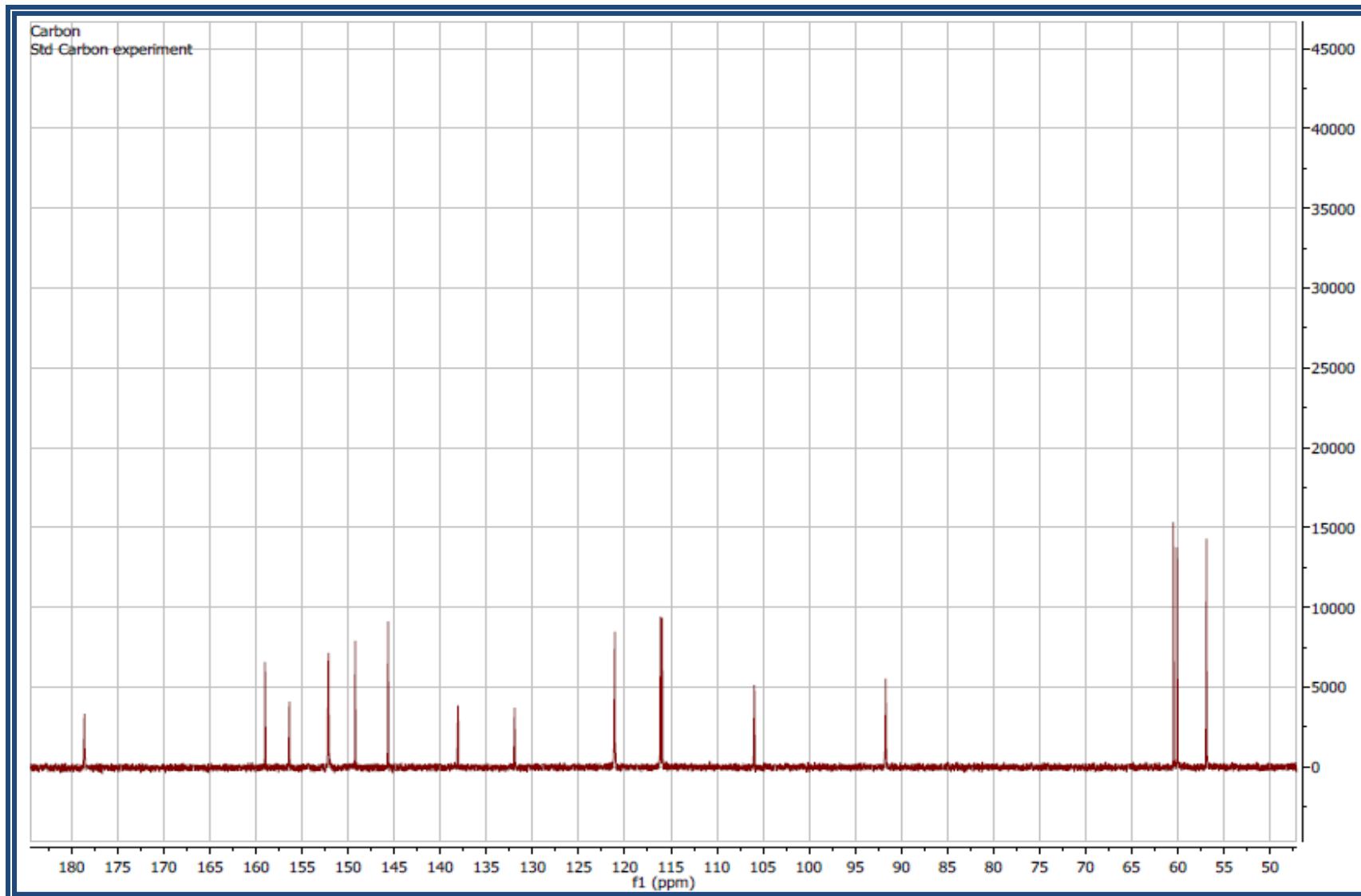
Appendix 2E: HR-EIMS spectrum of 5, 7, 4'-trihydroxy-3, 8, 3', 5'-tetramethoxyflavone (**82**)
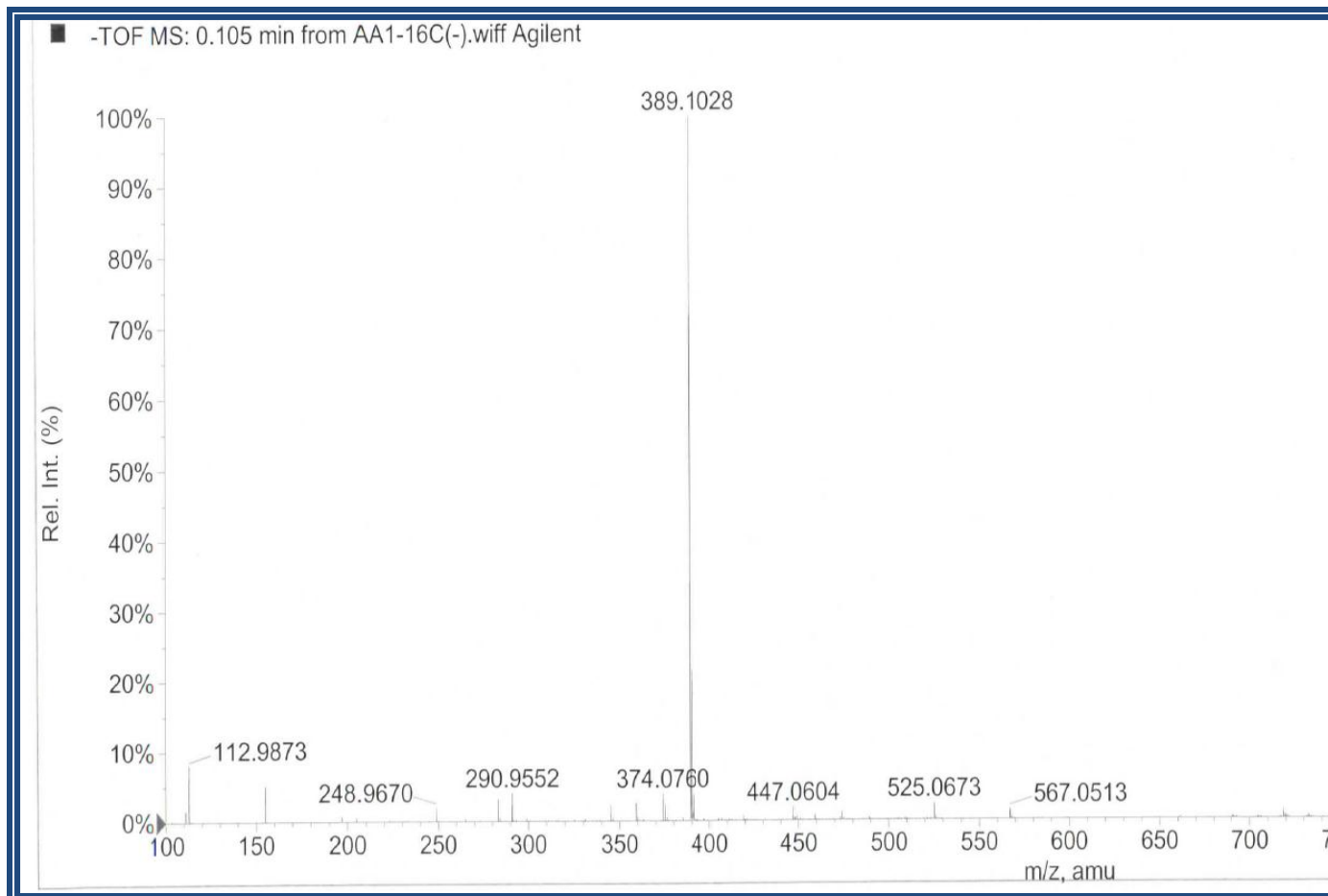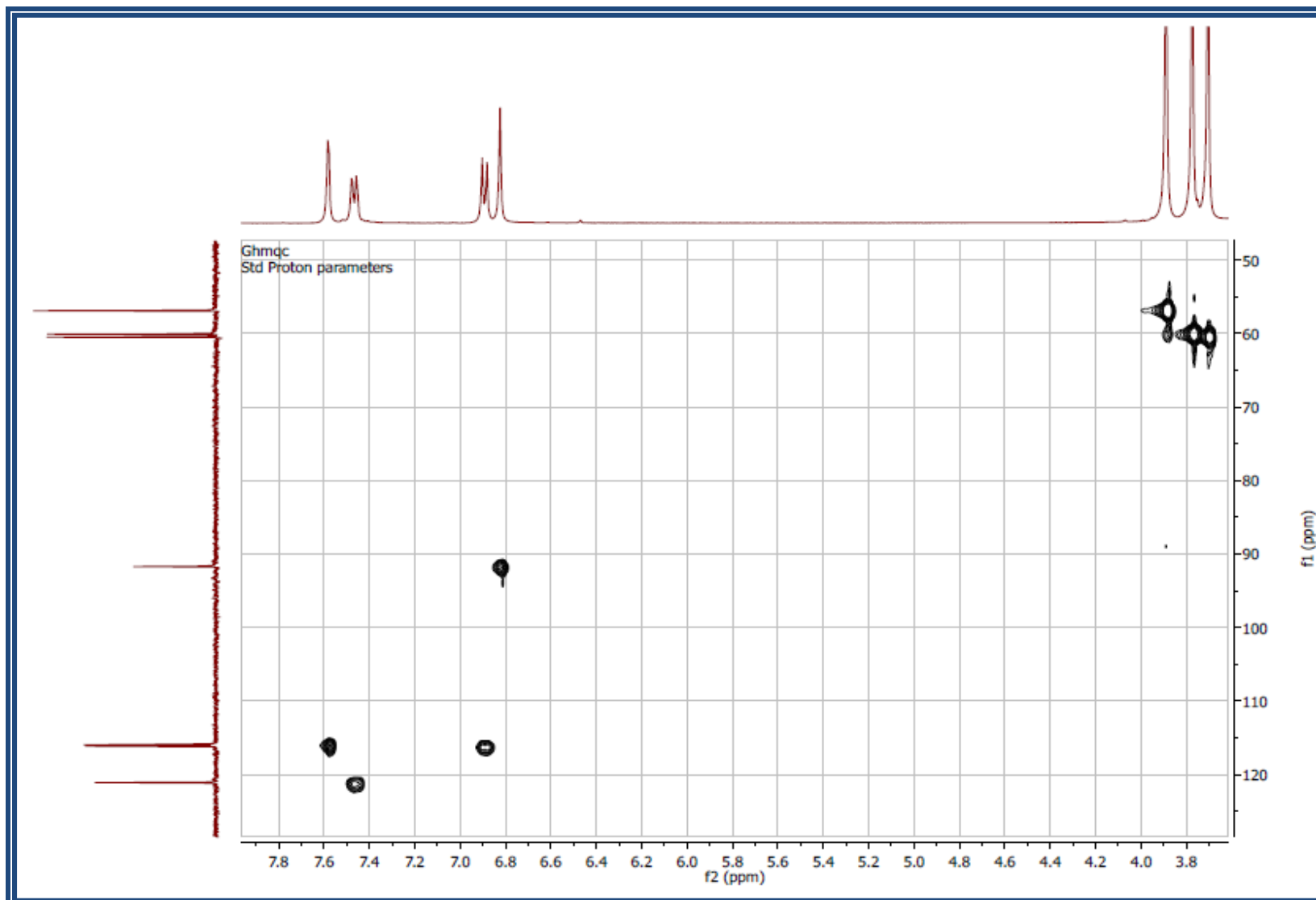
Appendix 3A: ^{1}H NMR (400 MHz) spectrum of 5, 3', 4'-trihydroxy-7-methoxyflavanone (**83**) in DMSO-d$_6$
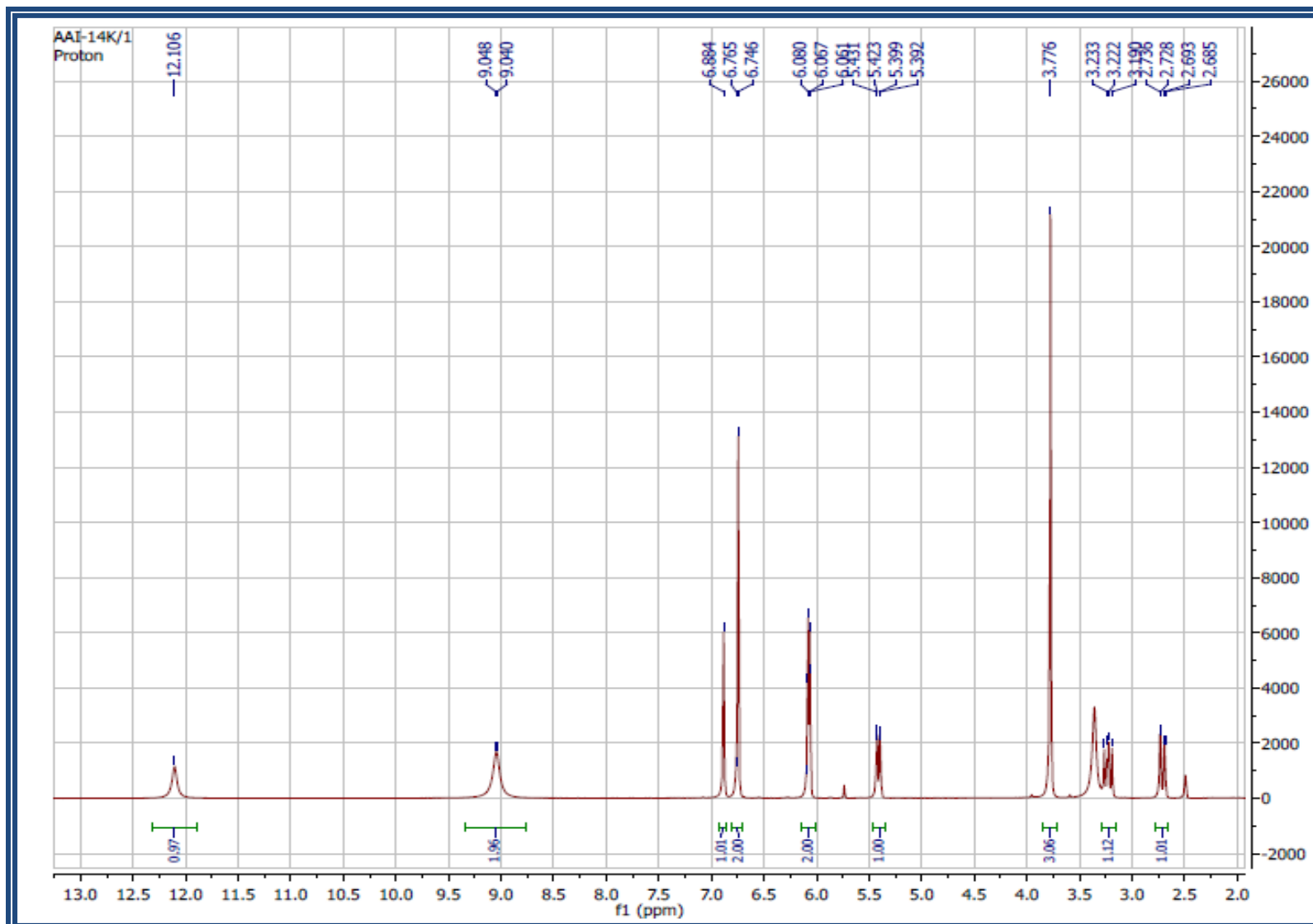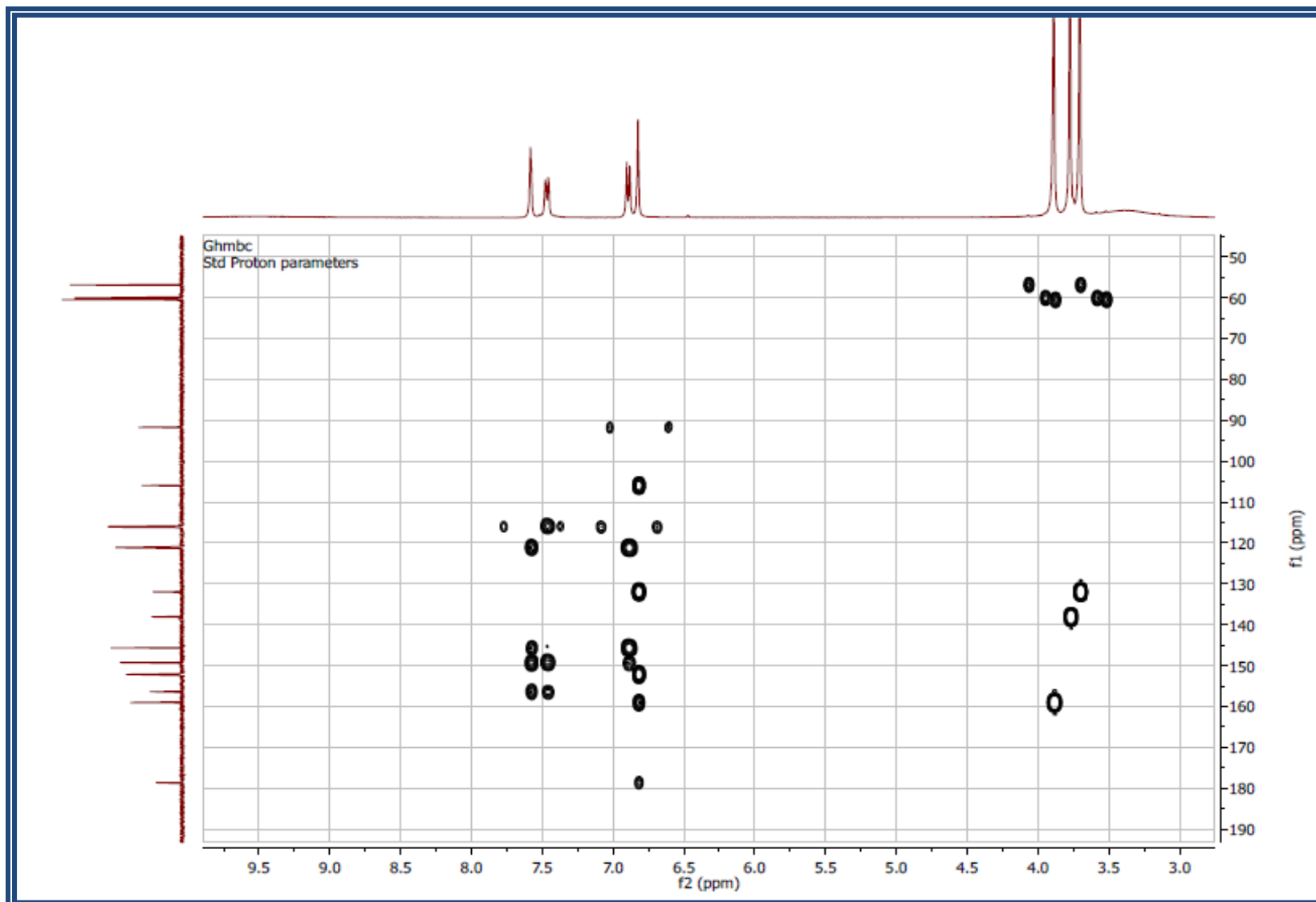
Appendix 3B: ^{13}C NMR (100 MHz) spectrum of 5, 3', 4'-trihydroxy-7-methoxyflavanone (**83**) in DMSO-d$_6$
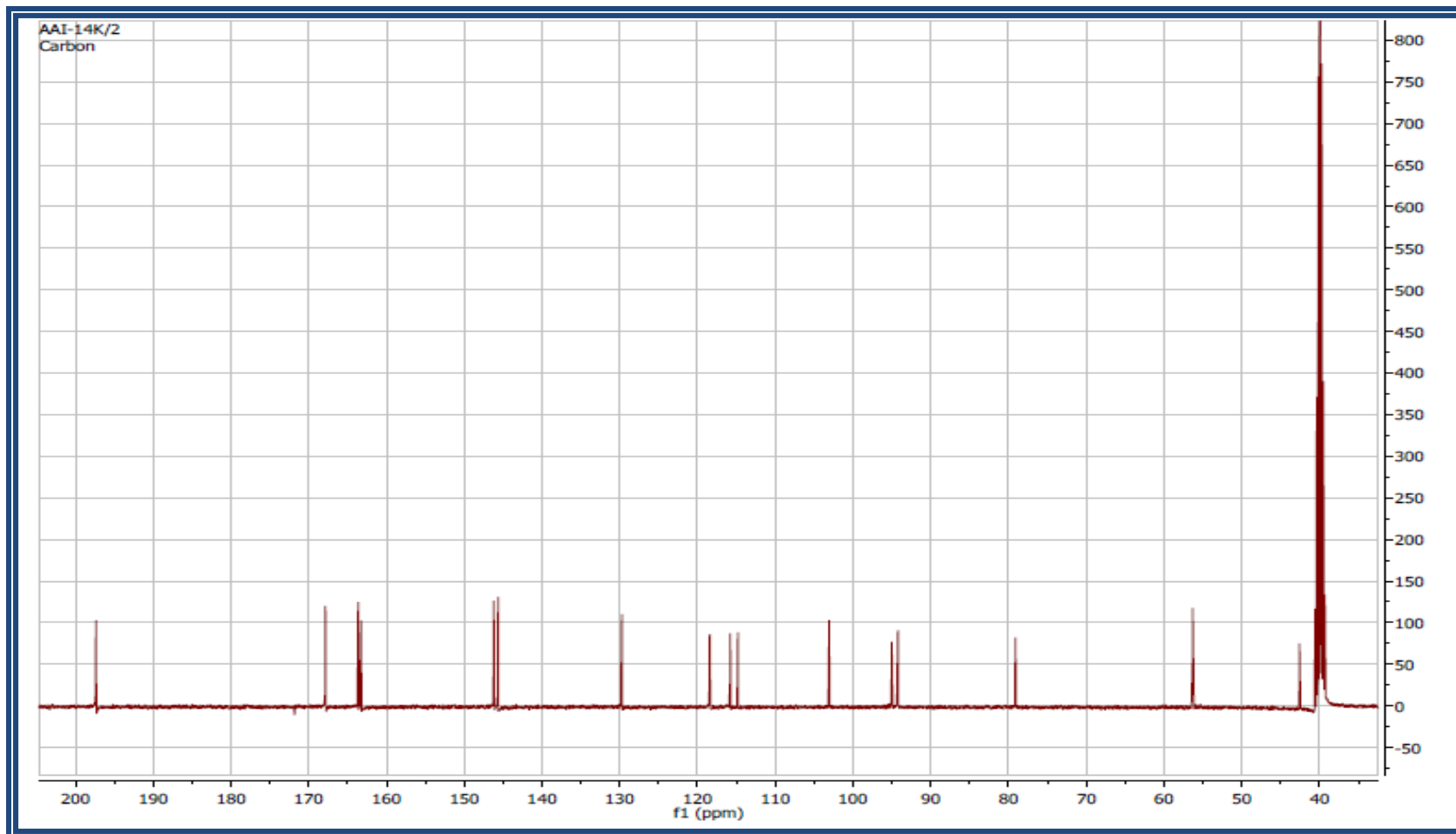
Appendix 3C: HSQC (400 MHz) spectrum of 5, 3', 4'-trihydroxy-7-methoxyflavanone (**83**) in DMSO-d$_6$
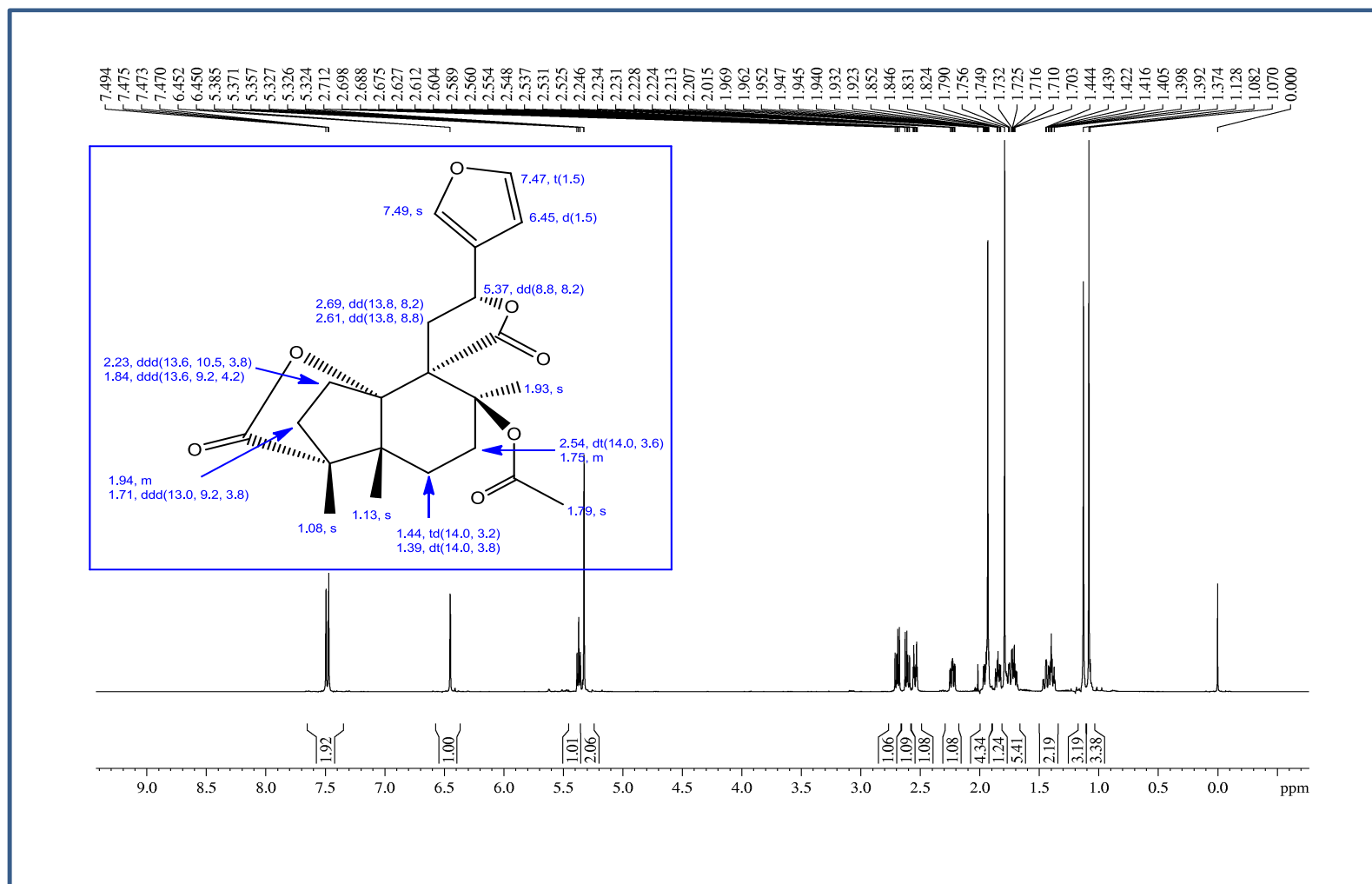

Ghmqc
Std Proton parameters
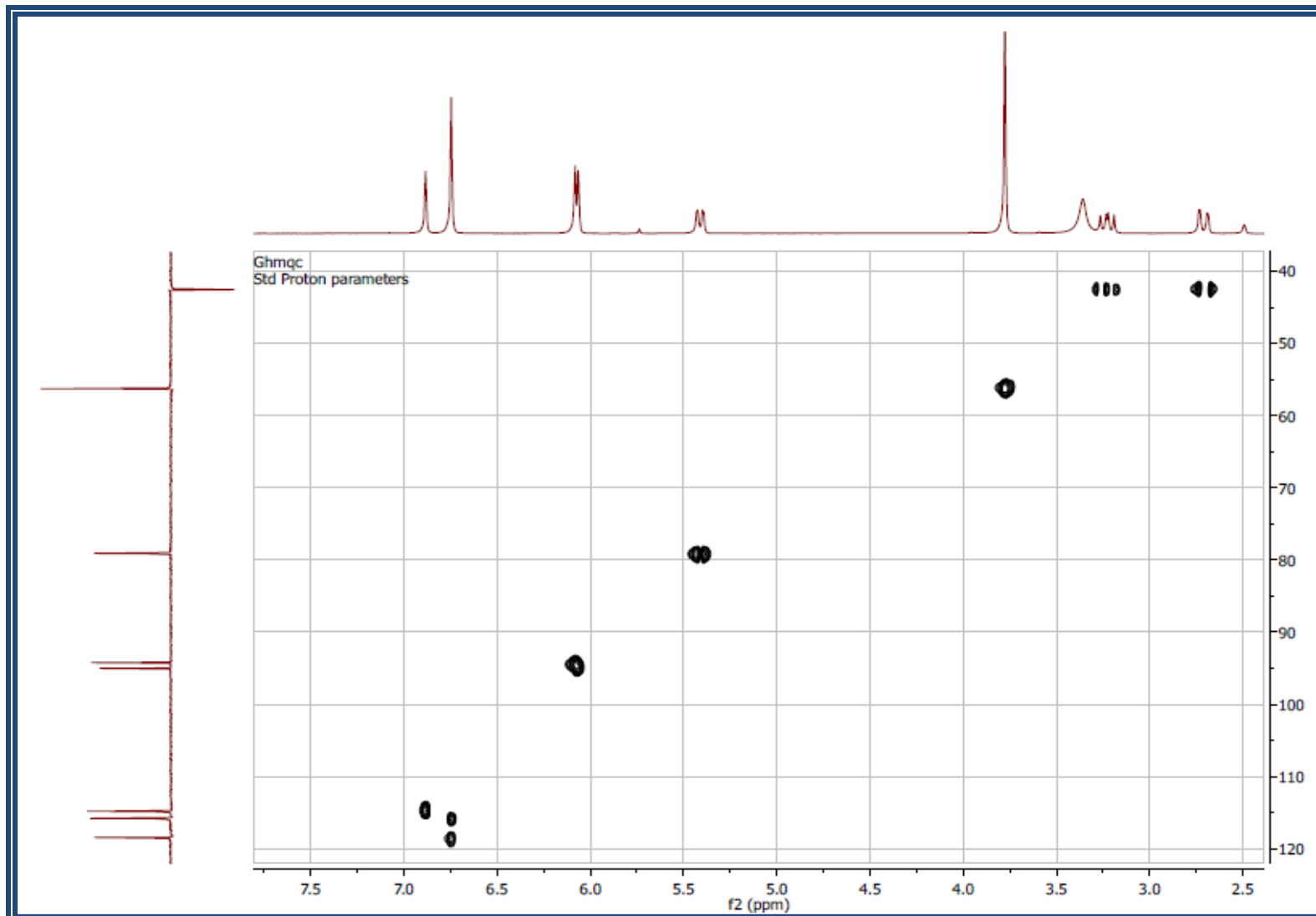
Appendix 3D: HMBC (400 MHz) spectrum of 5, 3', 4'-trihydroxy-7-methoxyflavanone (**83**) in DMSO-d₆
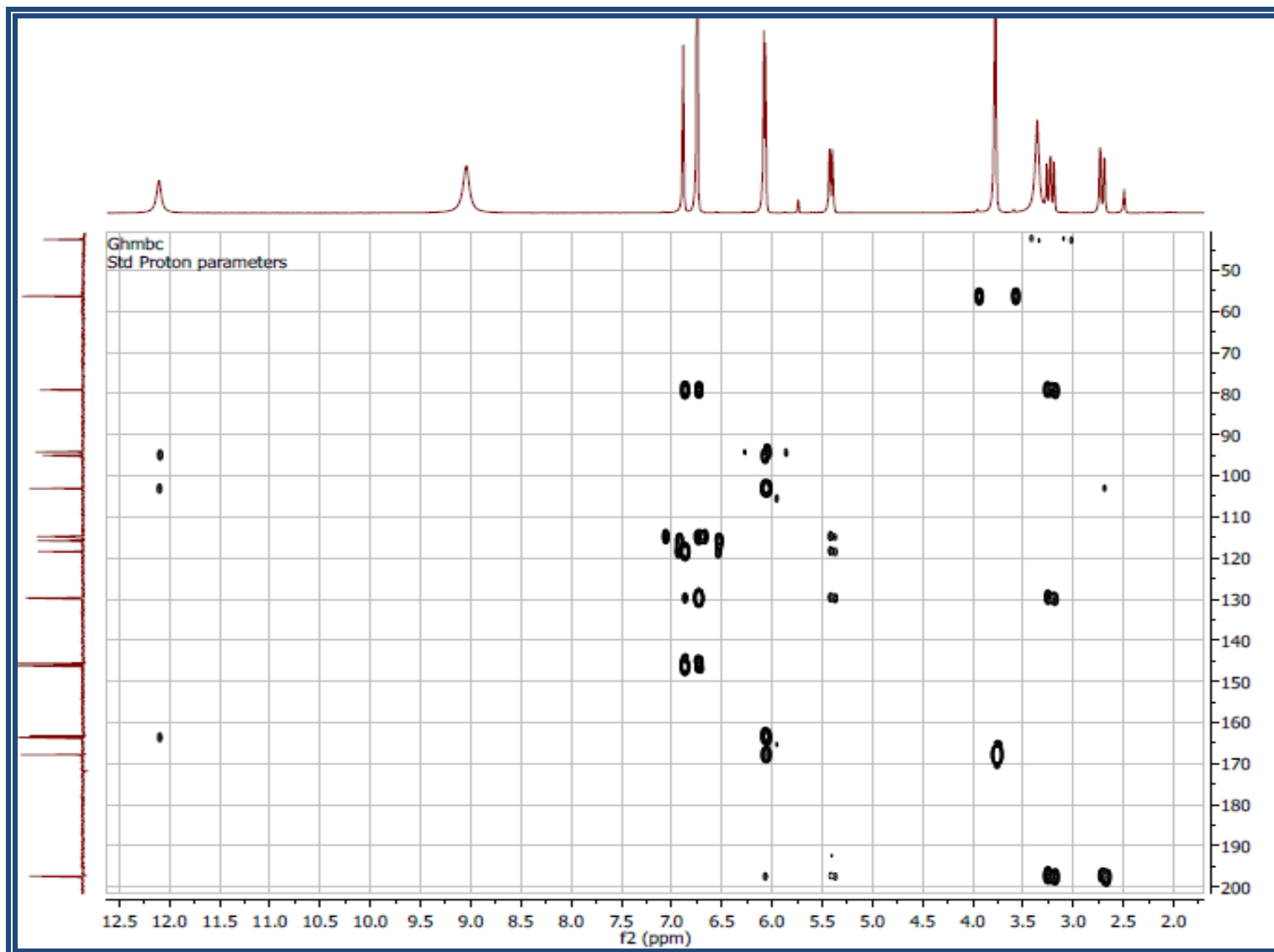
Appendix 3E: HR-EIMS spectrum of 5, 3', 4'-trihydroxy-7-methoxyflavanone (**83**)
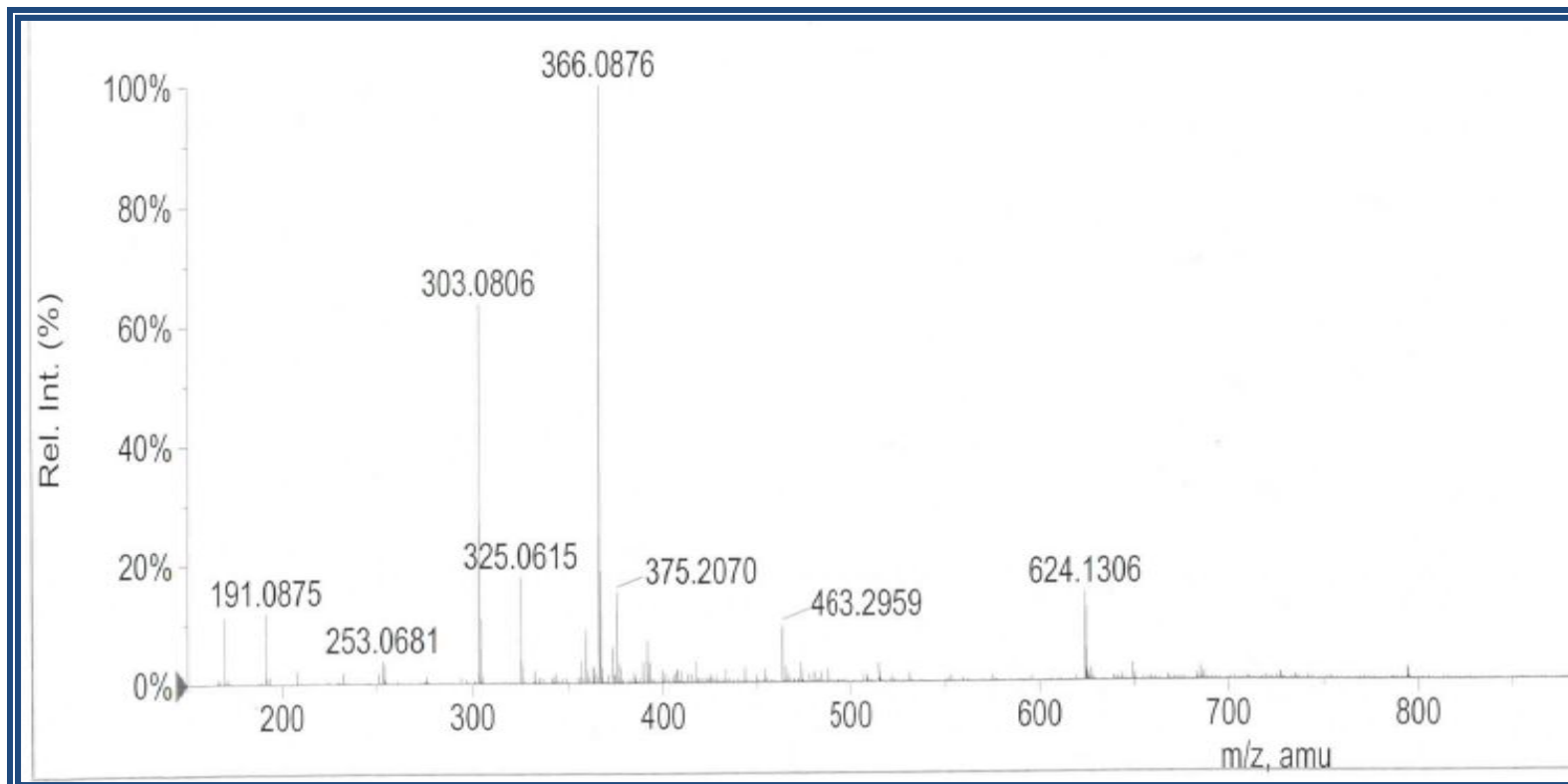
Appendix 4A: ^{1}H NMR (400 MHz) spectrum of 5, 7, 3'-trihydroxy-3, 8, 4', 5'-tetramethoxyflavone (**84**) in DMSO-d$_6$
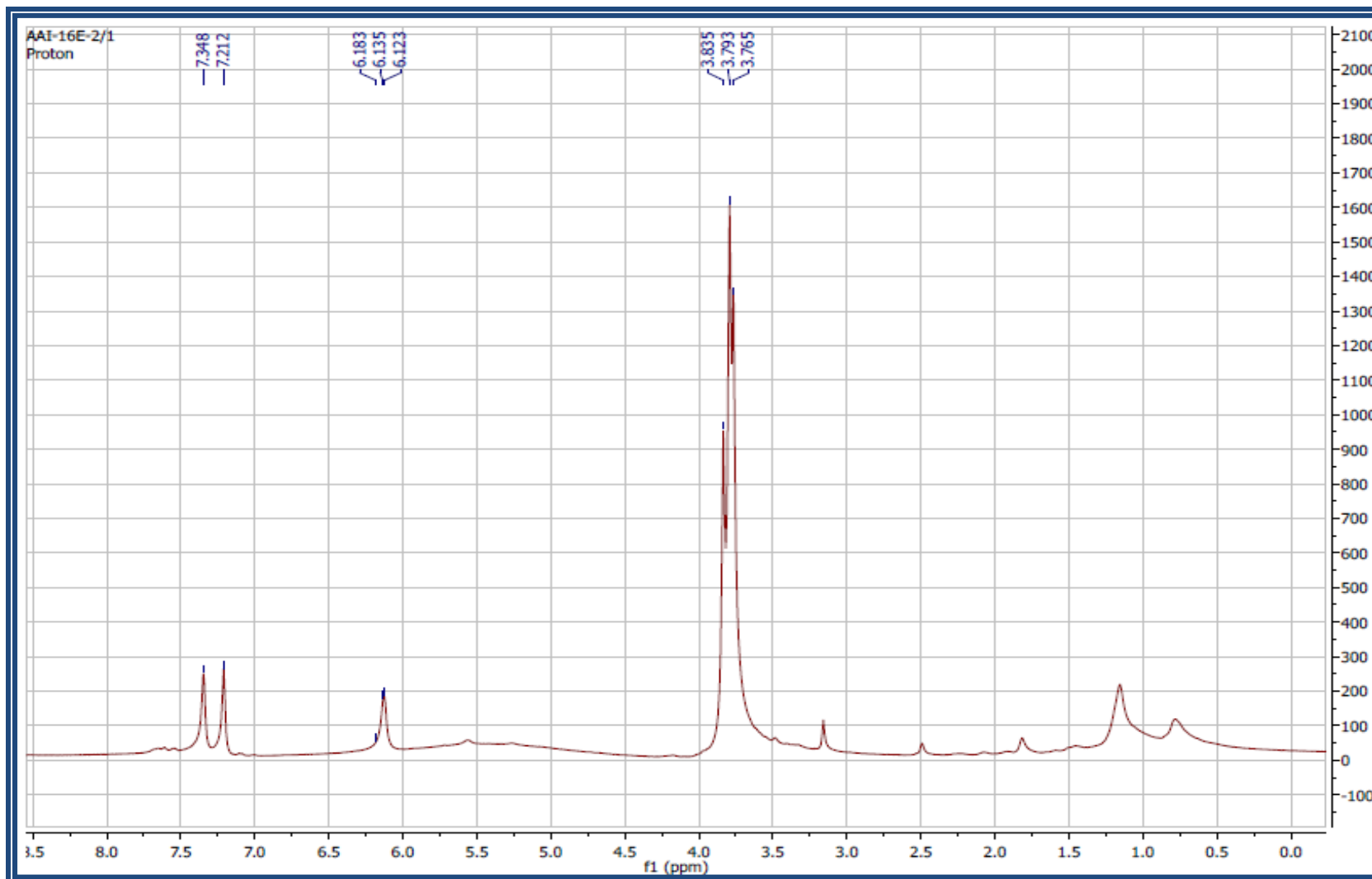
Appendix 4B: ^{13}C NMR (100 MHz) spectrum of 5, 7, 3'-trihydroxy-3, 8, 4', 5'-tetramethoxyflavone (**84**) in DMSO-d$_6$
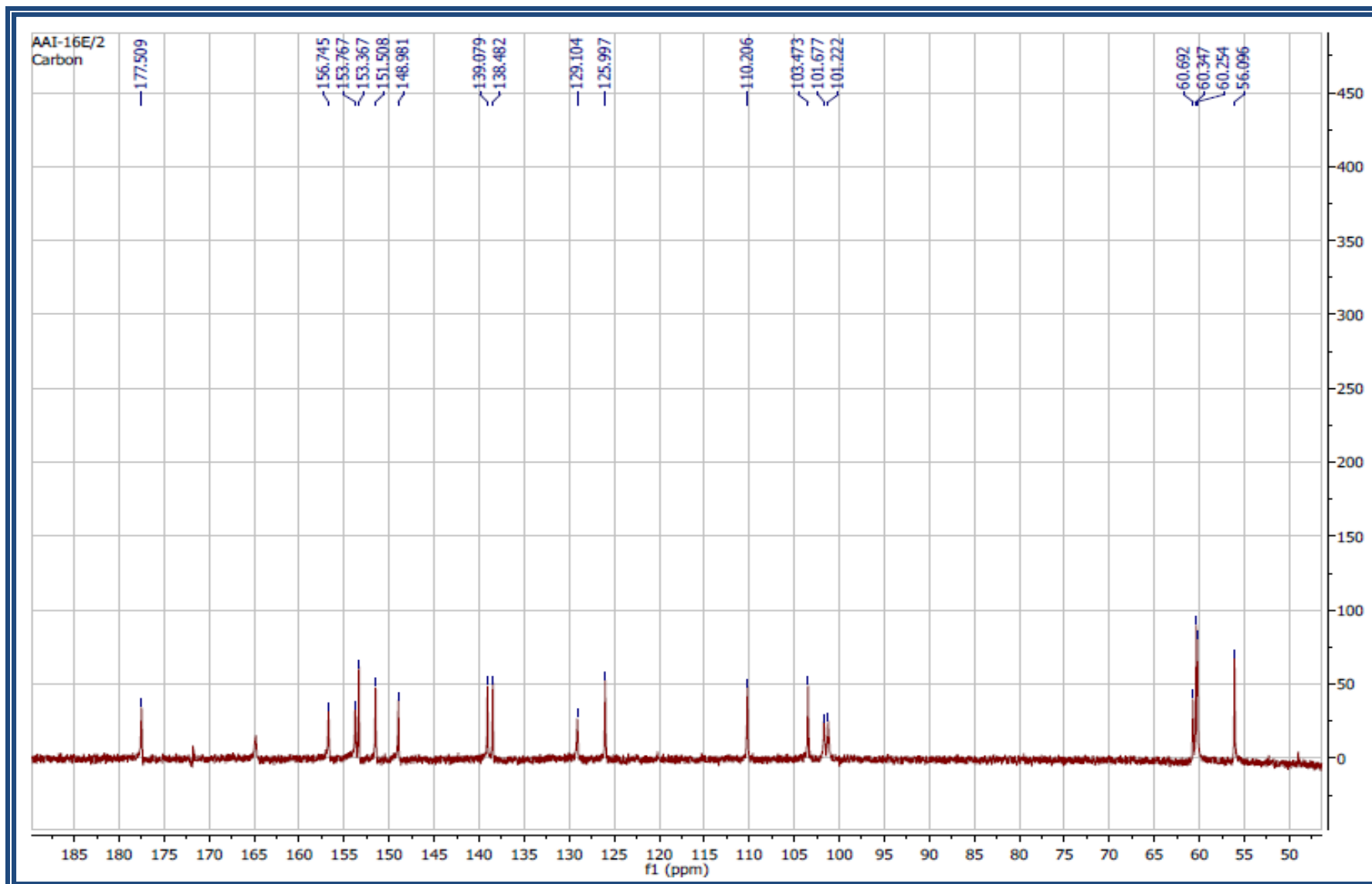
Appendix 4C: HSQC (400 MHz) spectrum of 5, 7, 3'-trihydroxy-3, 8, 4', 5'-tetramethoxyflavone (**84**) in DMSO-d$_6$
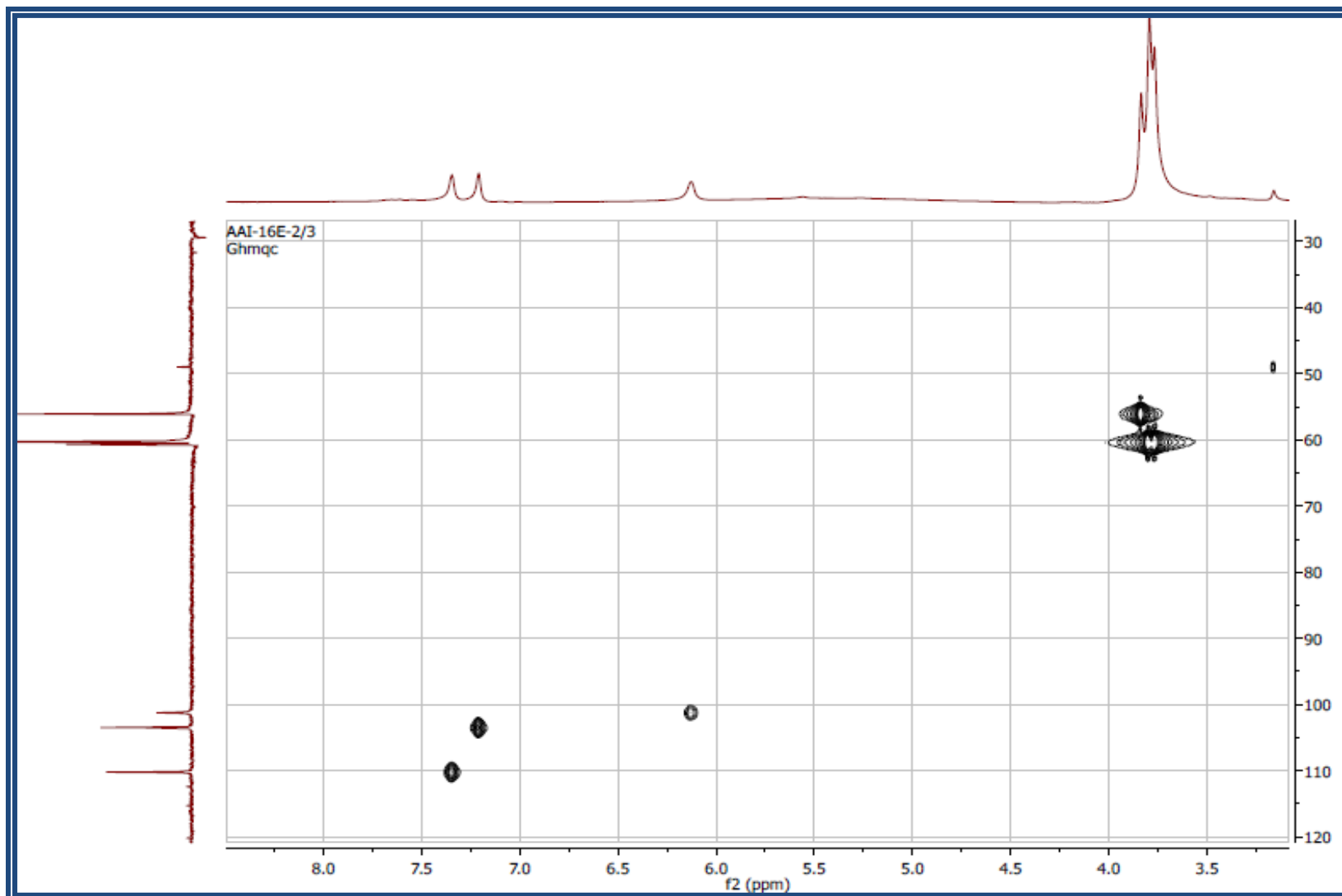
Appendix 4D: HMBC (400 MHz) spectrum of 5, 7, 3'-trihydroxy-3, 8, 4', 5'-tetramethoxyflavone (**84**) in DMSO-d6
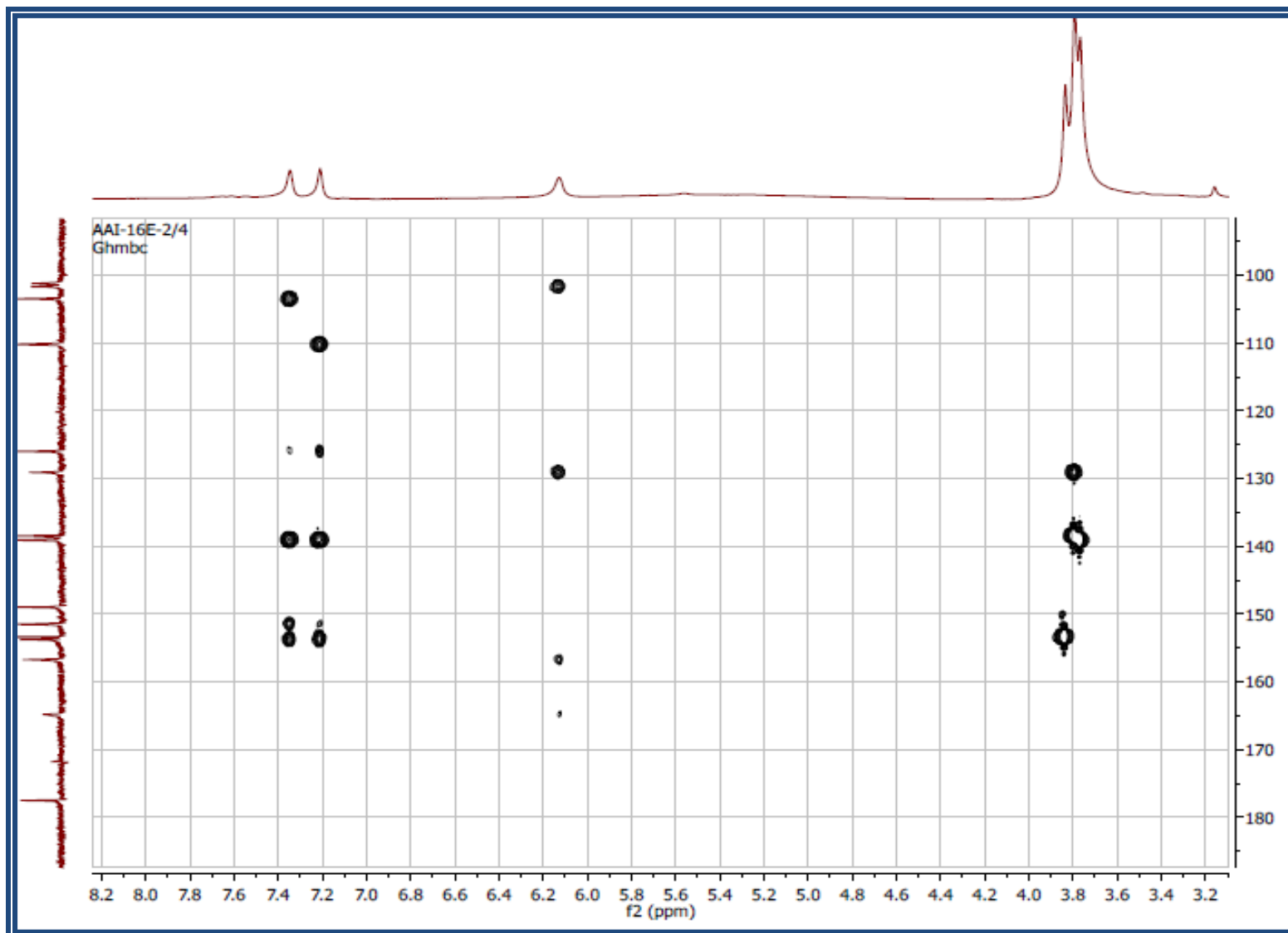
Appendix 4E: HR-EIMS spectrum of 5, 7, 3'-trihydroxy-3, 8, 4', 5'-tetramethoxyflavone (**84**)
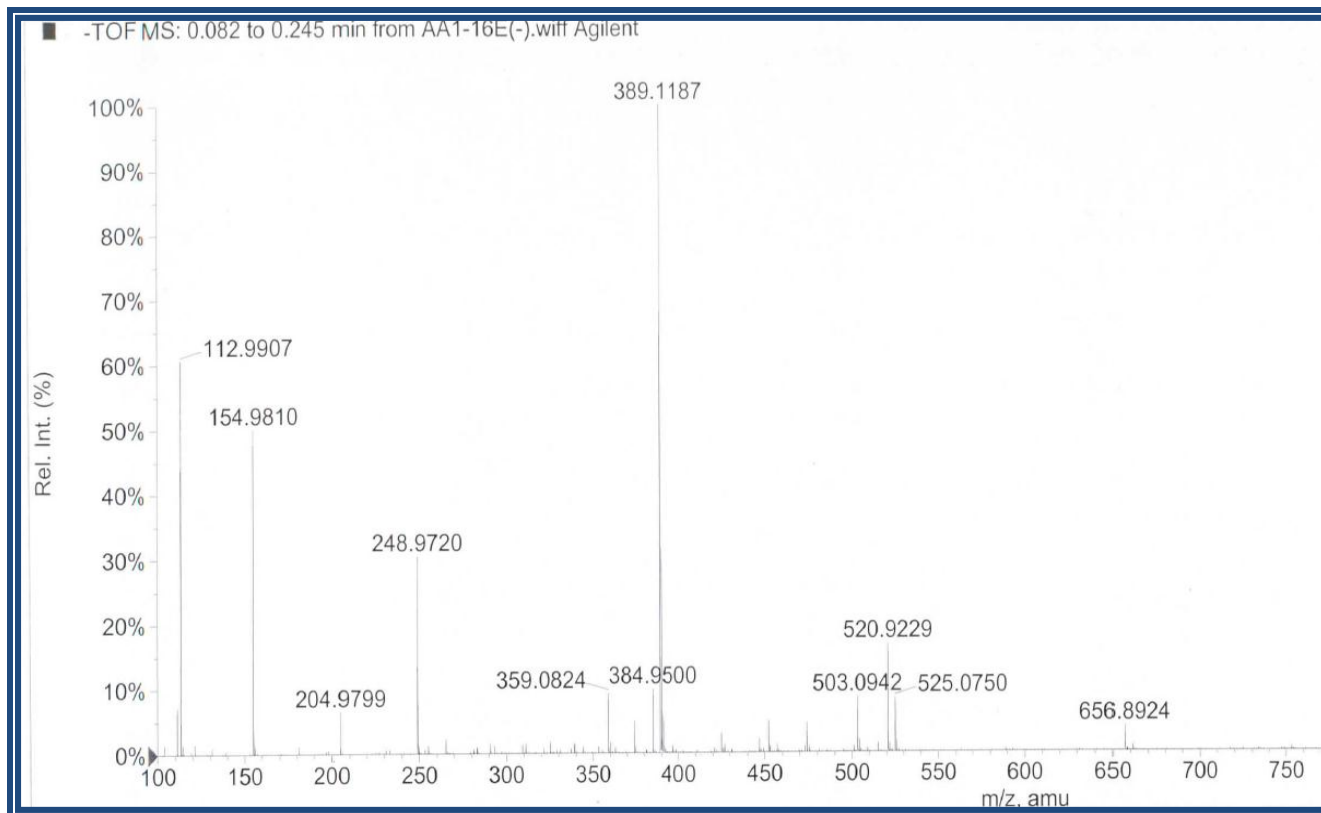
Appendix 5A: ^{1}H NMR (400 MHz) spectrum of 5, 3', 4'-trihydroxy-3, 7, 8-trimethoxyflavone (**85**) in DMSO-d$_6$

Proton
Std Proton parameters
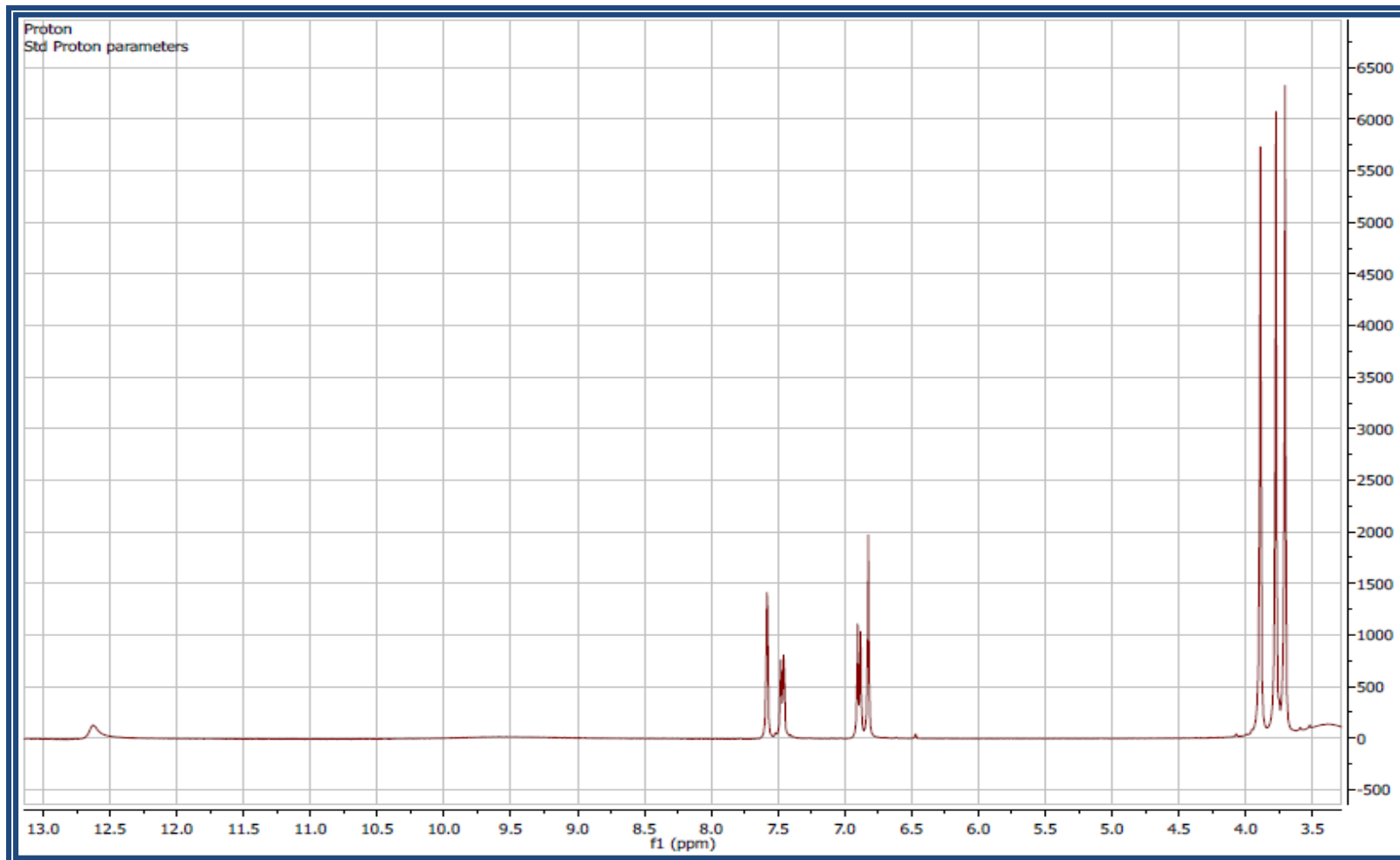
Appendix 5B: ^{13}C NMR (100 MHz) spectrum of 5, 3', 4'-trihydroxy-3, 7, 8-trimethoxyflavone (**85**) in DMSO-d$_6$

Appendix 5C: HSQC (400 MHz) spectrum of 5, 3', 4'-trihydroxy-3, 7, 8-trimethoxyflavone (**85**) in DMSO-d$_6$

Appendix 5D: HMBC (400 MHz) spectrum of 5, 3', 4'-trihydroxy-3, 7, 8-trimethoxyflavone (**85**) in DMSO-d$_6$

Appendix 5E: HR-EIMS spectrum of 5, 3', 4'-trihydroxy-3, 7, 8-trimethoxyflavone (**85**)
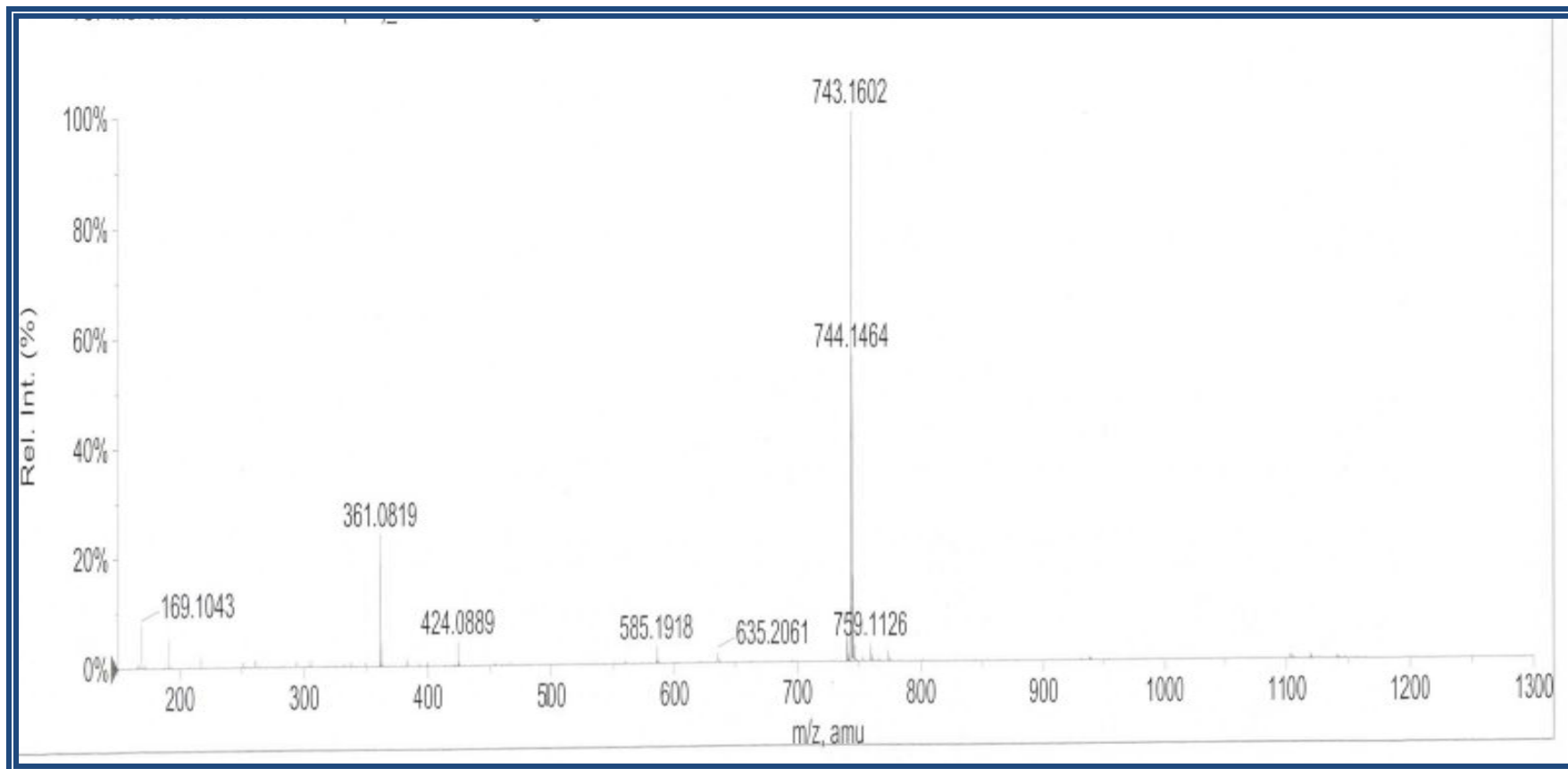
Appendix 6A: ^{1}H-NMR (600 MHz) spectrum of 8-acetoxyisochiliolide lactone (**86**), CD$_2$Cl$_2$.

7.47, t(1.5)
7.49, s
6.45, d(1.5)
5.37, dd(8.8, 8.2)
2.69, dd(13.8, 8.2)
2.61, dd(13.8, 8.8)
2.23, ddd(13.6, 10.5, 3.8)
1.84, ddd(13.6, 9.2, 4.2)
1.93, s
2.54, dt(14.0, 3.6)
1.75, m
1.94, m
1.71, ddd(13.0, 9.2, 3.8)
1.79, s
1.08, s
1.13, s
1.44, td(14.0, 3.2)
1.39, dt(14.0, 3.8)

Appendix 6B: ^{13}C NMR (150 MHz) spectrum of 8-acetoxyisochiliolide lactone (**86**), CD$_2$Cl$_2$**.**
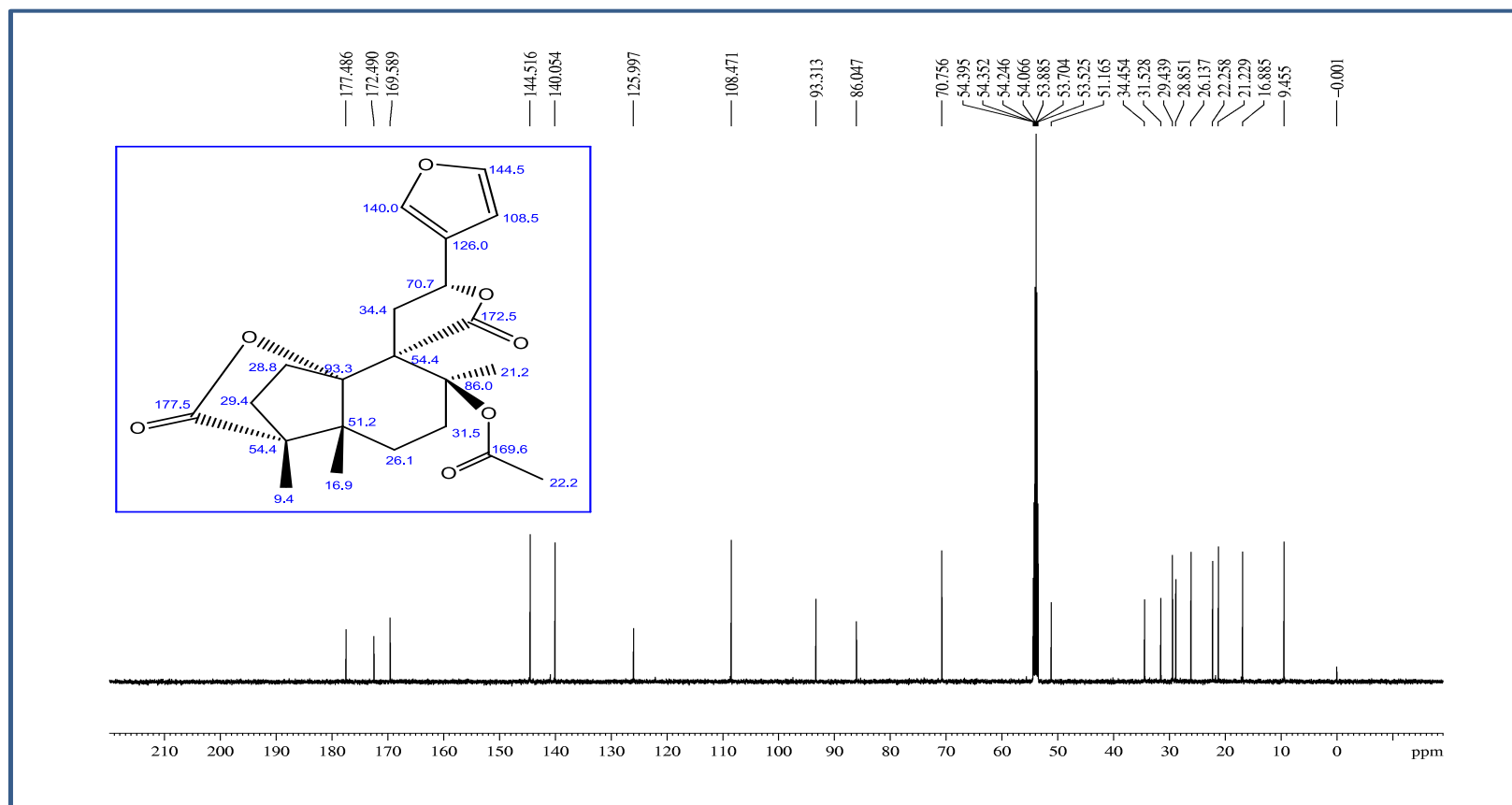
Appendix 6C: HSQC (600 MHz) spectrum of 8-acetoxyisochiliolide lactone (**86**), CD$_2$Cl$_2$.
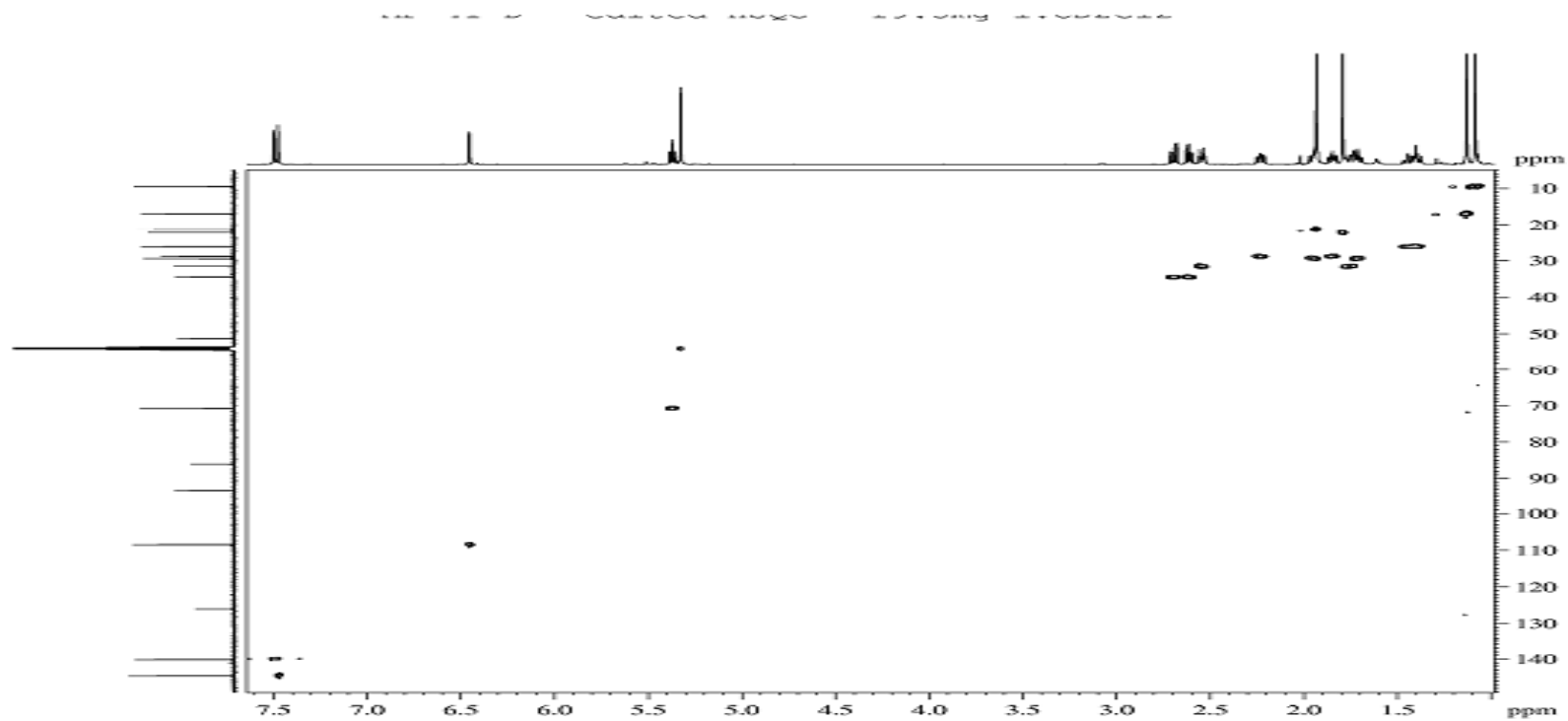
Appendix 6D: HMBC (400 MHz) spectrum of 8-acetoxyisochiliolide lactone (**86**), CD$_2$Cl$_2$.
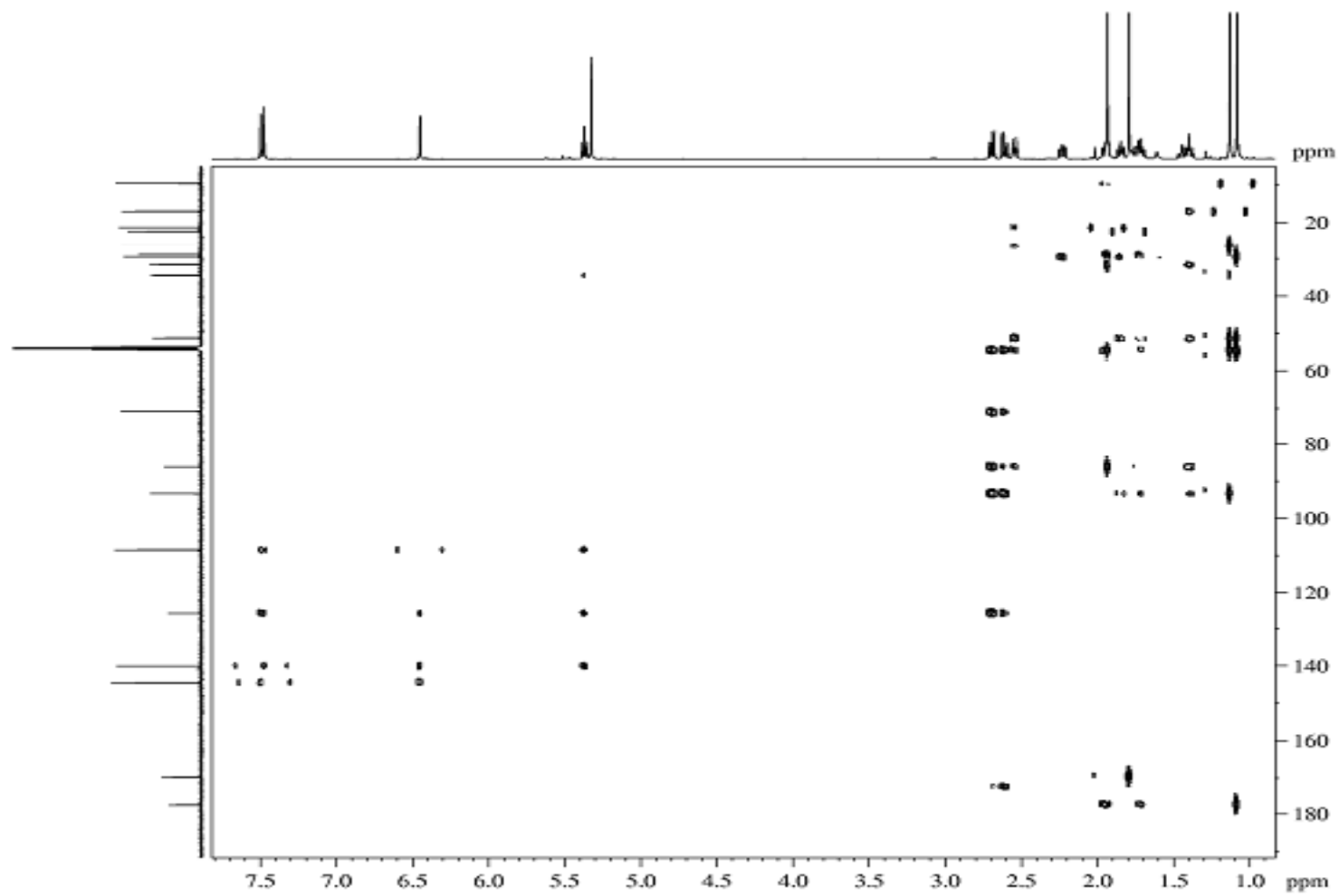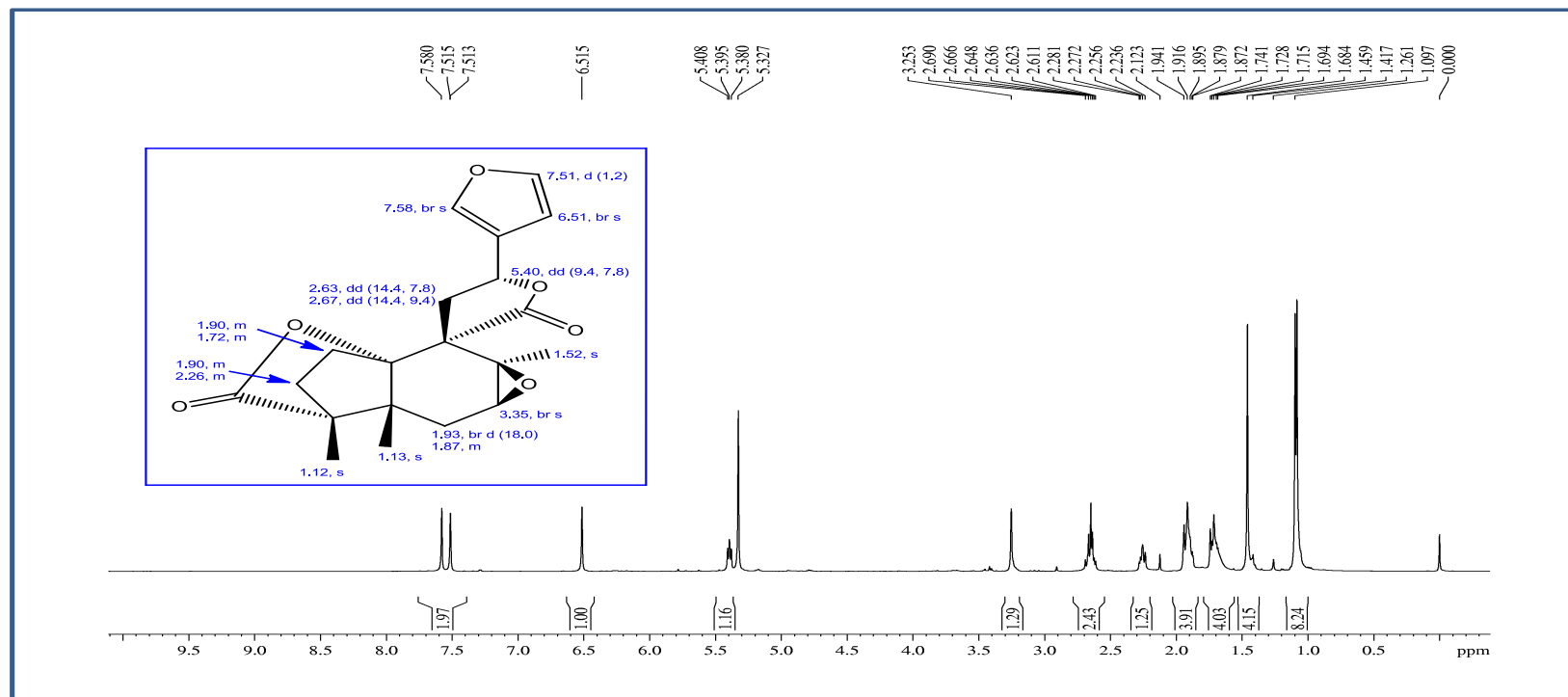
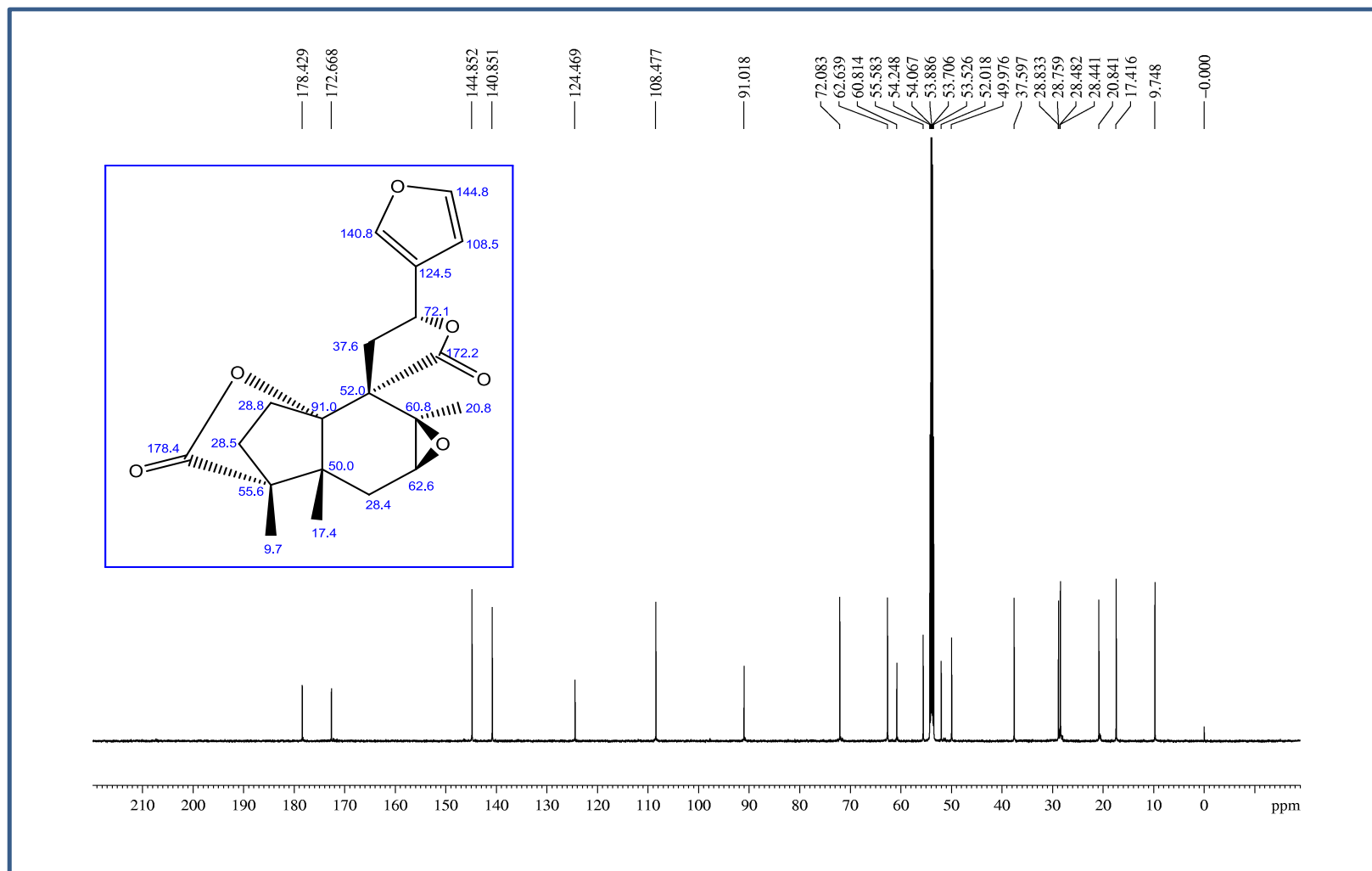**Appendix 6E**: HSQC (400 MHz) spectrum of 8-acetoxyisochiliolide lactone (**86**), CD$_2$Cl$_2$.
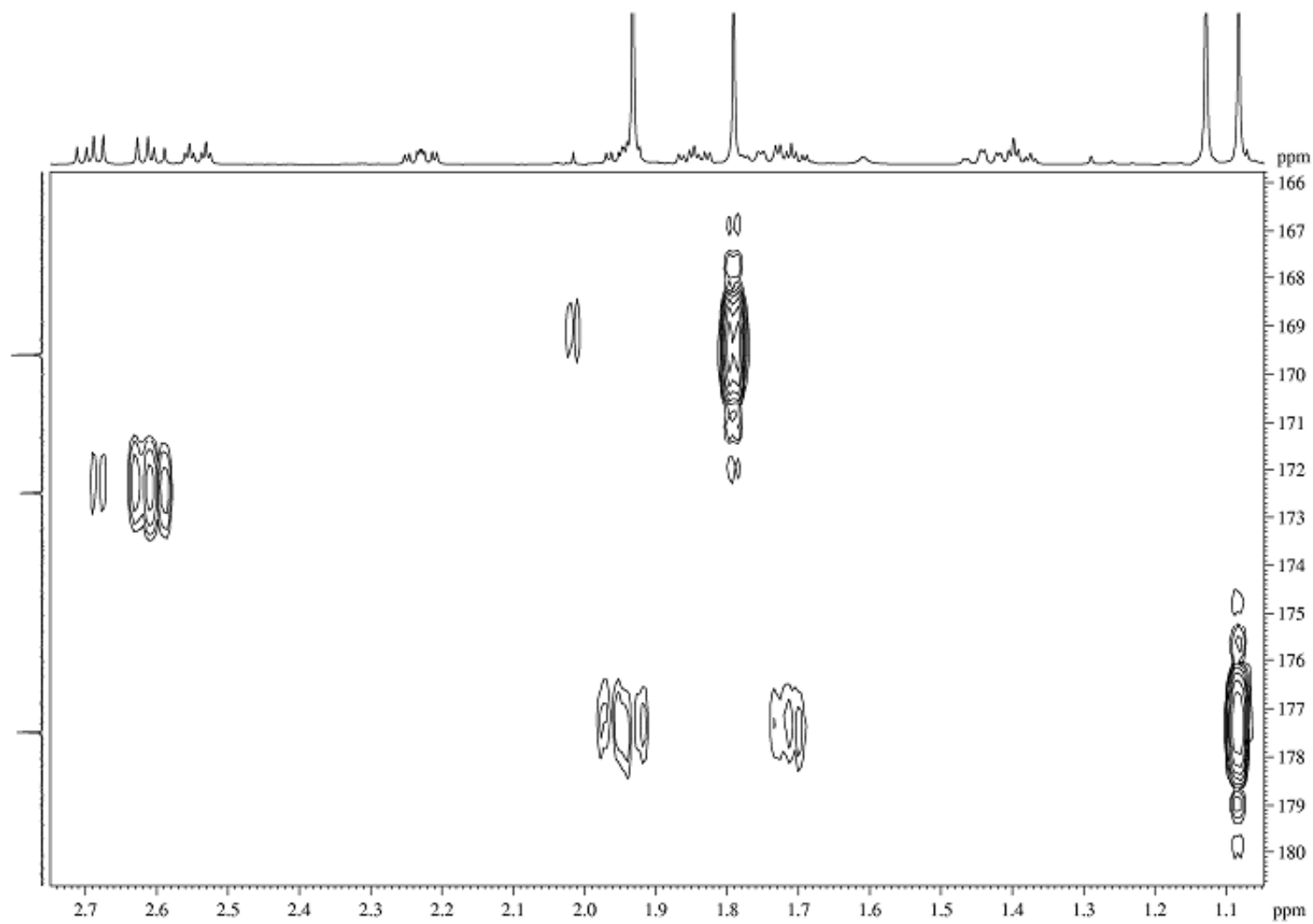
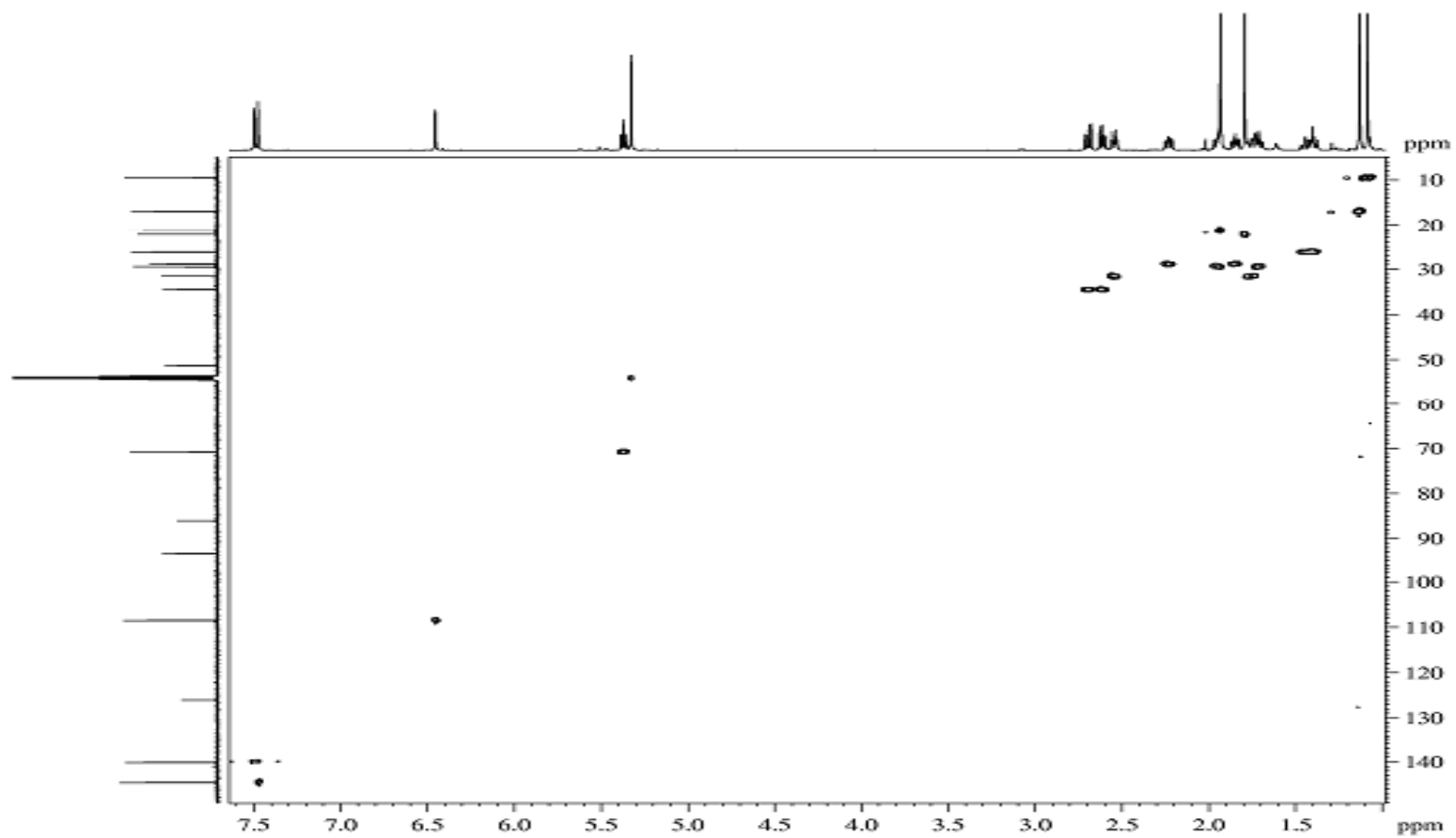**Appendix 6F**: HRE-IMS (600 MHz) spectrum of 8 acetoxyisochiliolide lactone (**86**), CD₂Cl₂.
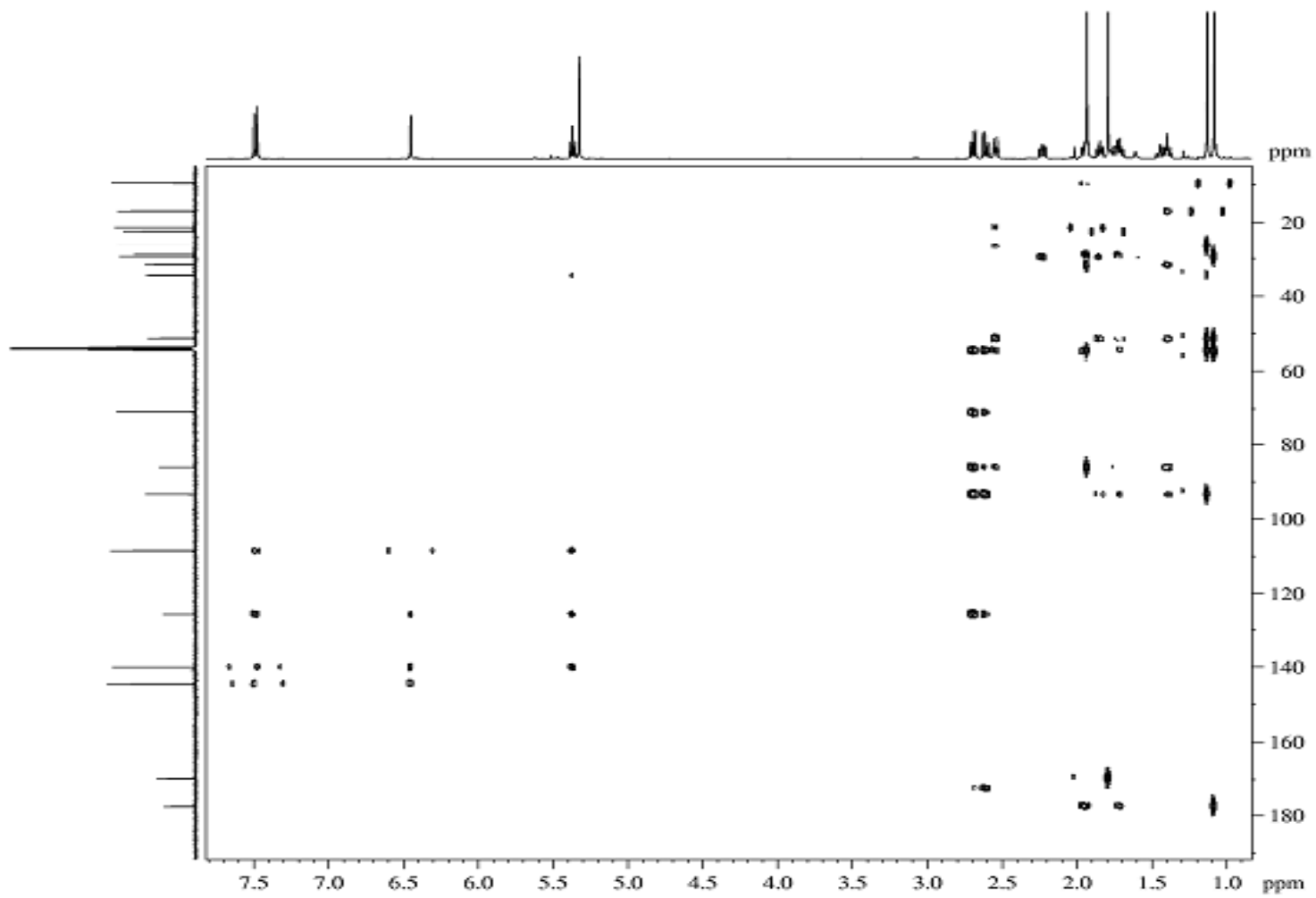

m/z = 402.1700; 403.1866 [M+H]
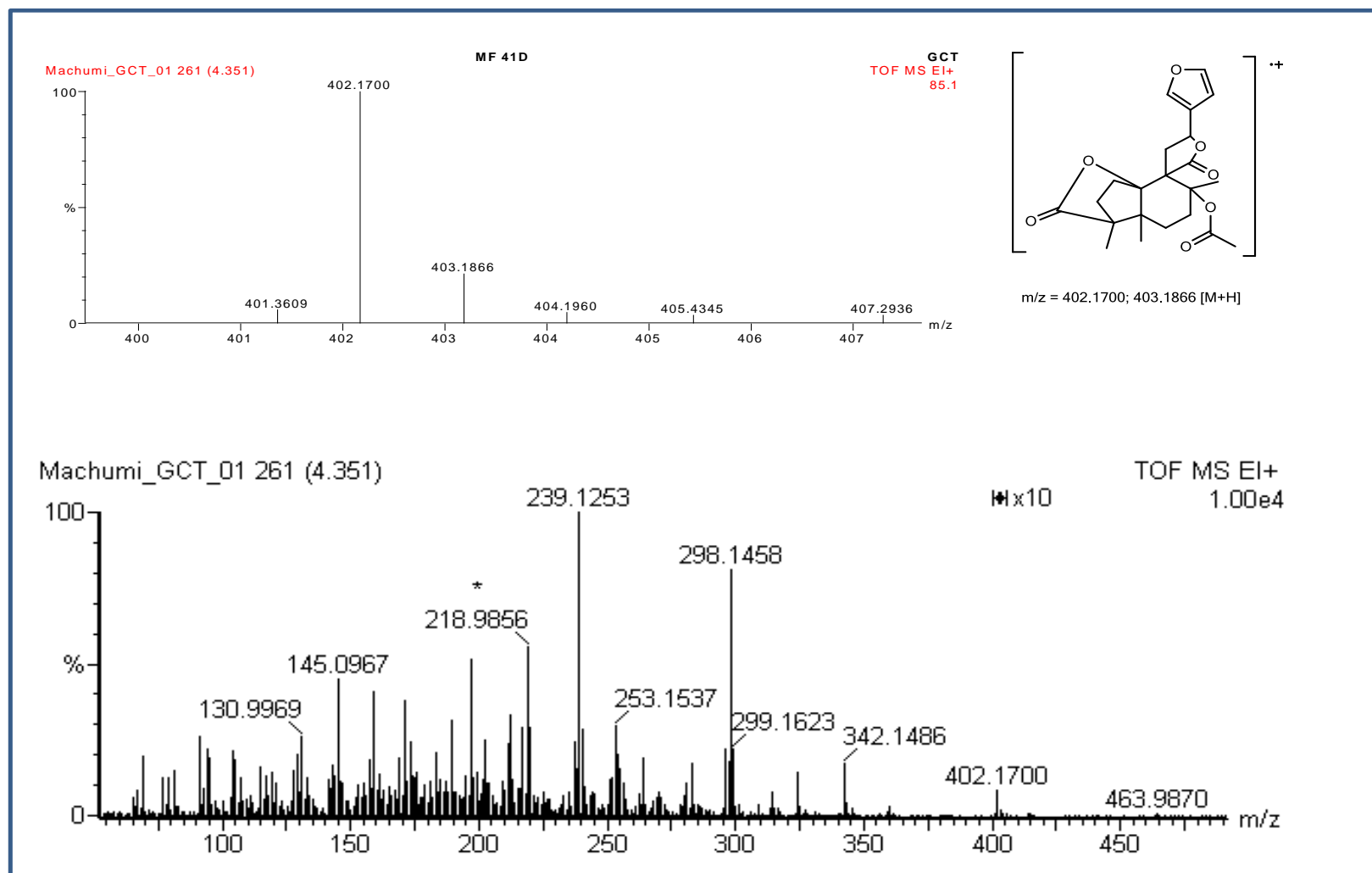
Appendix7A: ^{1}H-NMR (600 MHz) spectrum of 7,8-epoxyisocholiolide lactone (**87**), CD$_2$Cl$_2$.

Appendix 7C: HMBC (400 MHz) spectrum of 7,8-epoxyisocholiolide lactone (**87**), CD$_2$Cl$_2$.

Appendix 7D: HMBC (400 MHz) spectrum of 7,8-epoxyisocholiolide lactone (**87**), CD$_2$Cl$_2$.

Appendix 7E: EI-MS spectrum of 7,8- epoxyisochiliolide lactone (**87**)