Faculteit Farmaceutische, Biomedische en Diergeneeskundige Wetenschappen

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Phytochemical and Biological Investigations on Medicinal Plants from Pakistan

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Promotors

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Perfection is not attainable, but if we chase perfection we can catch excellence.

Vince Lombardi

To My Mother "*Naseem Akhtar*"
May Allah keep her soul in peace and rest Aameen

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LIST OF ABBREVIATIONS

AAPH	2,2′-azobis(2-methylpropionamidine) dihydrochloride
AGEs	Advanced glycation end products
AMG	Aminoguanidine
AP-1	Activator protein-1
BBI	Broadband inverse
BSA	Bovine serum albumin
BSTFA	N,O-Bis(trimethylsilyl) trifluoroacetamide
CDC	Centres for disease control and prevention
CEL	Carboxyethyl-lysine
CML	Carboxymethyl-lysine
COSY	Correlation spectroscopy
CPRG	Chlorophenol red-β-D-galactopyranoside
DAD	Diode array detector
DEPT	Distortionless Enhancement by Polarization Transfer
3-DG	3-deoxyglucosone
D.I.Khan	Dera Ismail Khan
DPPH	2,2-diphenyl-1-picrylhydrazyl
DOLD	Deoxyglucosone-derived lysine dimers
DMSO	Dimethyl sulphoxide
ECM	Extracellular matrix
ELSD	Evaporative light scattering detector
FAS	Fatty acid synthesis
FCS	Fetal calf serum

FFI	2, 2(furyl), 4-(5) 2 (furanyl)1H-imidazole
GAE	Gallic acid equivalents
GO	Glyoxal
GOLD	Glyoxal lysine dimer
GC	Gas chromatography
HMBC	Heteronuclear multiple bond correlation
HPLC	High performance liquid chromatography
HSQC	Heteronuclear single quantum coherence
KPK	Khyber pakhtoon khawa
15-LOX	15-lipoxygenase
MBC	Minimum Bactericidal Concentration
MEM	Minimum Essential Medium
MGO	Methyl glyoxal
MHB	Mueller Hinton Broth
MIC	Minimum Inhibitory Concentration
MOLD	Methylglyoxal lysine dimers
MRC-5	Medical Research Council -5 (human lung fibroblast)
MS	Mass spectroscopy
NMR	Nuclear magnetic resonance
NP	Normal phase
NBT	Nitro blue tetrazolium
NF-IL6	Nuclear factor interleukin-6
PBS	Phospahte buffer saline
PC-3	Human prostate cancer cell line

PES	Phenazine ethosulphate
Pnp	*p*-nitrophenyl-α-D-glucopyranoside
QTOF-MS	Quadrupole-time-of-flight mass spectrometer
RAGE	Receptor for AGEs
ROS	Reactive oxygen species
RPMI	Roswell Park Memorial Institute
RiOS	Reverse osmosis
RUE	Rutin equivalents
SP-1	Specificity protein-1
TCA	Trichloroacetic acid
TFC	Total flavonoid contents
TLC	Thin layer liquid chromatography
TPC	Total phenolic contents
TM	Traditional medicine
TMCS	Trimethylchlorosilane
TQD-MS	Triple quadrupole mass spectrometer
UV-VIS	Ultraviolet-visible light
WHO	World Health Organization

CHAPTER 1

INTRODUCTION

AND AIM OF THE WORK

1.1 Traditional medicine

Medicinal plants are intrinsic components of traditional medicinal systems in treating and preventing an array of ailments. Traditional medicine (TM) is a broad term that refers to “systems” such as Traditional Chinese Medicine (TCM), Indian Ayurveda and Arabic Unani medicine, or multiple forms of indigenous medicine. However, in some countries TM is termed as "alternative" or "complementary" medicine (WHO, 2002).

The history of traditional medicine is as old as human civilization. Earliest records confirm that herbal medicines have been used and documented in Roman, Greek, Egyptian, Chinese and Indian medicinal systems for about 5000 years. Traditional herbal medicine has also been practised from ancient times in American, Arabian countries and Japan. The records of traditional medicine systems in the Indian subcontinent (India, Pakistan, Bangladesh, Nepal, Bhutan, Sri Lanka and the Maldives) include Ayurveda, Rigveda, Siddha and Yunani. Here the correlation between TM and biodiversity is demonstrated by an old custom of “healing powers” associated with the “earth's natural systems” that involves medicinal plants and animal species, the ambient healthy air and spring water (Good, 1980; Gesler, 1992). For example, Ayurveda of India has a documented history dating back to 1500 BCE (Sharma, 1994; Dwarakanath,1952) and its authenticity is proven most recently in a genome-wide SNP (single nucleotide polymorphism) analysis (Affymetrix, 6.0) suggesting that India’s traditional medicine has a genetic basis, and its Prakriti-based (constitutional types of every human) practice in vogue for many centuries resonates with personalized medicine (Govindaraj *et al.,* 2015).

In the Indian subcontinent, practice of TM is mainly accomplished by “herbal practitioners” that are mainly referred to as “Sadhoo” or “Hakeems”. Their practice is mainly based on secret herbal recipes. These recipes were formulated through traditional knowledge transferred from generation to generation. The safety, accessibility, cheapness and sociological acceptance are major factors in the popularity of traditional medicine in developing nations (Mesia, 2009). This trend continues even in our modern era and about 4 billion people, 80% of the world's population, presently use plant-based medicine for some aspects of their primary health care (WHO, 2008).

More interestingly, in a review covering anticancer drugs for instance, it has been reported that of 175 small molecules, approximately 48.6% of drugs were actually being either natural products or directly derived therefrom (between 1981 to 2010)(Newman and Cragg, 2012). WHO has compiled a comprehensive list of 21, 000 medicinal plant species (including synonyms), that are used worldwide, based on the data collected from 91 countries (WHO, 2002a).

1.2 Traditional medicine and the 21st century medicine market.

Indeed, immense progress has been made in the discovery of synthetic medicines for the treatment or prevention of several life threatening diseases; however, excessive and prolonged use of such drugs may produce toxic side effects that are considered as a continuous hazard in modern health systems (Alves and Rosa, 2007; Dubey *et al*., 2004). In the 21st century, medicinal herbs are gaining importance in the mainstream healthcare system in developed countries. A large number of people quest for relatively safe remedies and approaches to healthcare. The demand for herbal medicines, herbal pharmaceuticals, herbal health products, nutraceuticals, food supplements and herbal cosmetics, etc. is boosting globally. The main reason for this trend is thriving appreciation of herbal products as showing less side effects, showing improved compatibility with physiological flora, and being available at reasonable prices (Dubey *et al*., 2004; Sharma *et al*., 2008). Statistics demonstrate that traditional herbal medicine includes 30–50% of the total medicinal usage in China. In North America, Europe and other well-developed regions over 50% of the population have used traditional preparations at least once. In 2003, it was reported that the world market for herbal medicines raised to over $60 billion(US) per year (WHO, 2003) and estimates suggest that global medicinal plant business will reach $5 trillion (US) by 2050 (Shinwari, 2010).

1.3 Ethnomedicinal assessment and drug discovery

In modern times ethnomedicinal assessment of medicinal plant species is considered as a primary step in the discovery of herbal drugs (Vitalini *et al.*, 2013), followed by isolation of bioactive principles, their pharmacological screening and finally ending up in finding new therapeutically active drugs. However, the approval of such a plant based drug or formulation on the market in modern times needs a series of procedures and measures. A number of approaches can be adopted in this regard; however, the scheme proposed by Pieters and Vlietinck (Fig. 1.1) is of particular importance. The scheme proposes two possible operational modes i.e working with standardised extracts or the isolation of pure active components. In both approaches finally toxicity and safety studies, as well as clinical trials are needed for final drug approval (Pieters and Vlietinck, 2005).

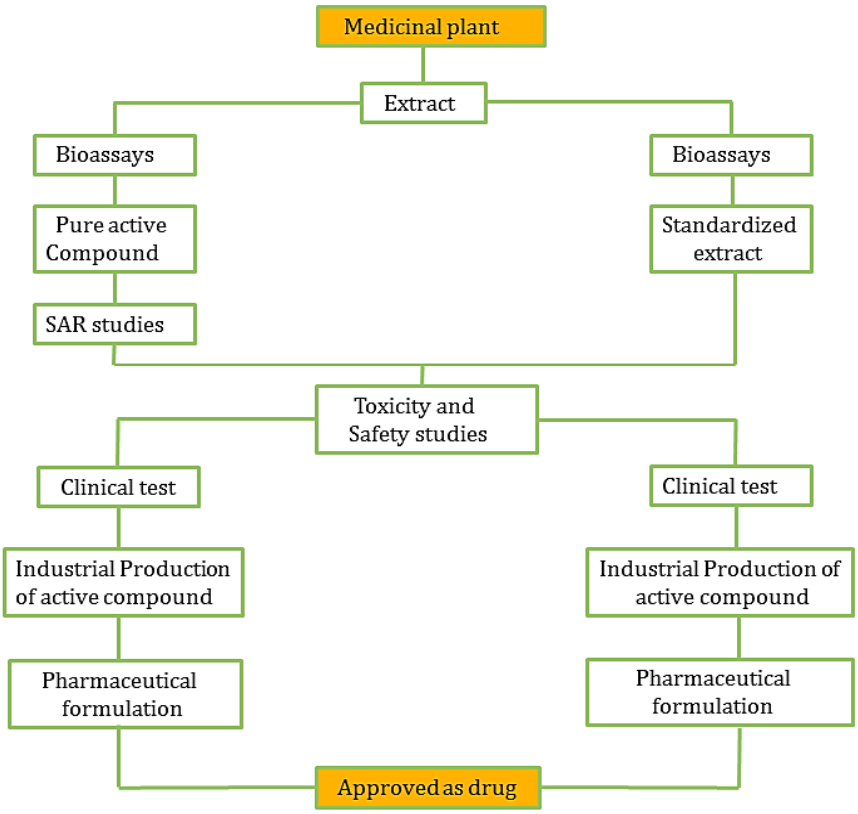


Figure 1.1. Scheme for the study of medicinal plants proposed by Pieters and Vlietinck, 2005.

1.4 Traditional medicine in Pakistan

Pakistan has an area of 80,943 km^2 and lies between 60°55' to 75° 30' E. longitude and 23° 45' to 36° 50' N. latitude. It has an altitude ranging from 0 to 8611 m, with nine major ecological zones and a unique biodiversity.

The country has a subtropical climate and a total of 1572 genera and 5521 species of flowering plants have been identified till now, out of which 400–600 are medicinally important (Bibi *et al*., 2015). Pakistan has four phytogeographical regions including the Saharo-Sindian (9.5% of species), Sino-Himalayan (10%), Irano-Turanian (45%) and Indian element (6%) (Shinwari *et al*., 2000). The plant "hotspots" of Pakistan are spread over 13 natural regions from alpine pastures to mangrove forest (Shinwari *et al*., 2002).

The traditional healthcare system of Pakistan is mainly based on indigenous home-based herbal remedies and experienced local healers called "Hakeems" that use their centuries old traditional medicinal knowledge. Although the indigenous knowledge about medicinal uses of plants has been recorded widely in Pakistan in recent years (Kayani *et al*., 2015; Rehman *et al*., 2015; Bibi *et al*., 2014; Abbasi *et al*., 2013; Qureshi, 2012; Ahmad *et al*., 2014; Shinwari *et al*., 2000; Shinwari *et al*., 2002) and efforts have been made in isolation of active chemical constituents from medicinal plants (Bibi *et al*., 2010; Bibi *et al*., 2011; Choudhary *et al*., 2011; Irshad *et al*., 2011; Amin and Khan, 2011; Jamil *et al*., 2012; Ejaz *et al*., 2014), such efforts need to be broadened especially to less explored plants.

Therefore, the current project was designed to isolate bioactive phytochemicals from some less explored but commonly used medicinal plants from Pakistan, as a part of running programmes in the host laboratory on antimicrobial (especially antiprotozoal) and antidiabetic properties (inhibition of α-glucosidase activity; antioxidant activity; inhibition of Advanced Glycation Endproducts-AGEs) of medicinal plants and natural products (Upadhyay *et al*., 2014a, Upadhyay *et al.,* 2014b; Mesia *et al*., 2008).

1.5 Diabetes mellitus

Diabetes mellitus is a multifarious metabolic disorder (involving carbohydrate, fat and protein metabolism) that results from either insufficiency or dysfunction of insulin, ending up in elevated fasting and post-prandial blood sugar levels. It is generally categorized as Type I and II diabetes (primary forms) (Fig. 1.3). The Type I (also referred to as insulin dependent) or juvenile onset diabetes is characterized by cell mediated, autoimmune destruction of the pancreatic cells, whereas in Type II (also referred to as non-insulin dependent), the patients are unable to respond to insulin (insulin resistance). Type II is the most common form and constitutes up to 90% of diabetes worldwide, whereas Type I accounts for 5–10% of patients. According to recent estimations, 387 million people worldwide are affected by the disease (IDF (International Diabetes Federation), 2014). According to WHO the major burden is in developing countries. Pakistan is one of the 19 countries and territories of the IDF MENA (Middle-East and North-Africa) region. It has been estimated that 415 million people have diabetes in the world and more than 35.4 million people in the MENA region; by 2040 this will rise to 72.1 million. There were over 7 million cases of diabetes in Pakistan in 2015 (IDF, 2015).

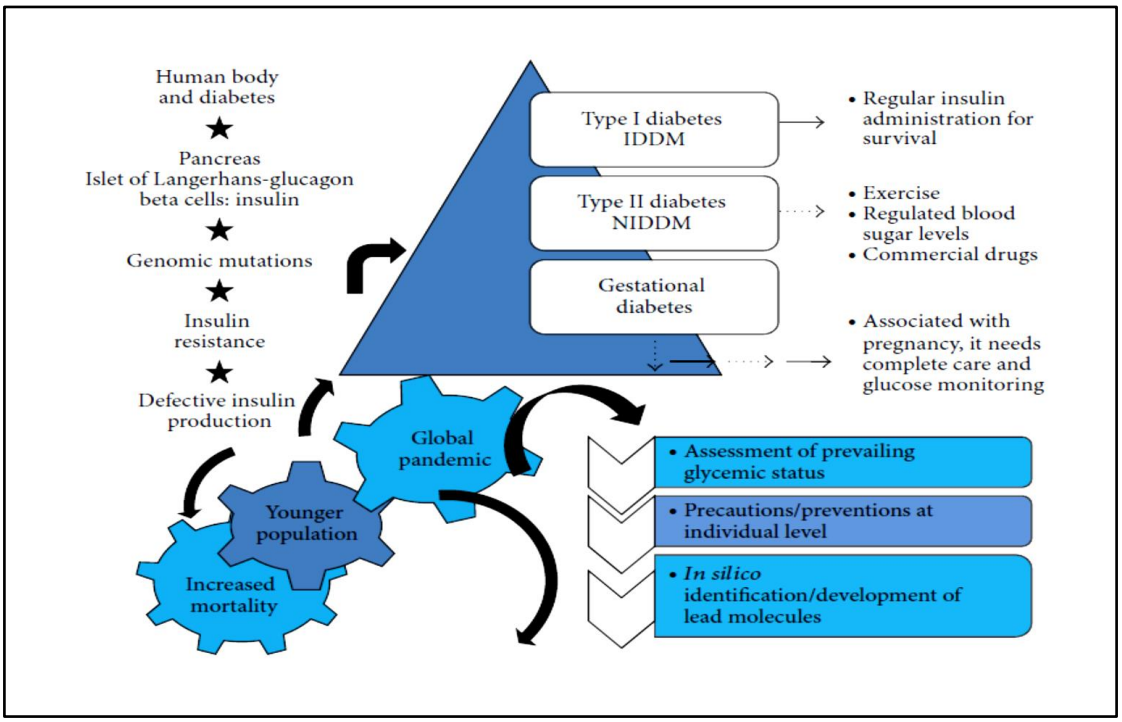


Figure 1.3. Occurrence and management of diabetes (Adopted from Tiwari, 2015).

The development of diabetes can be prevented or delayed in people with impaired glucose tolerance by implementing lifestyle changes or the use of therapeutic agents. Indeed, the pathophysiology of diabetes needs more investigations. However, it has been proven that free radicals and oxidative stress are associated with the development and pathogenesis of diabetes (Matteucci and Giampietro, 2000; Lipinski, 2001). In diabetic patients, extra-cellular and long living proteins such as laminin, collagen, elastin, several plasma proteins, including haemoglobin, serum albumin and transferrin are the major targets of free radicals. As a result of continuous and long term exposure to glucose, plasma proteins endure glycation and chemical modifications, which results in the formation of glycoproteins (Austin *et al*., 1987; Glugliano *et al*., 1996) that finally form "advanced glycation endproducts" (AGEs) after a series of rearrangements.

1.6 Advanced glycation end product (AGEs)

Glycation is a non-enzymatic reaction between the carbonyl group of reducing sugars and a free amino group of proteins, leading to the formation of a Schiff's base. The Schiff's base is the first product of the glycation reaction that is fast and highly reversible (Ulrich and Cerami, 2001). Subsequently thermodynamically unstable Schiff's bases are converted to a stable and reversible Amadori product. Proteins bearing an Amadori product are then referred to as glycated proteins or Maillard reaction products. Finally the Amadori product undergoes a series of dehydration and fragmentation reactions, resulting in a variety of carbonyl compounds including methylglyoxal (MGO), glyoxal (GO), glucosones, 3-deoxyglucosone (3-DG) and so on (Thornalley *et al*., 1999). These carbonyl compounds are more reactive than the original sugar and act as reaction propagators, leading to the formation of advanced glycation end-products (AGEs) (Fig.1.4).

The AGEs formed are known to be quite heterogeneous, depending on the glycating agent. For example, glyoxal-derived AGEs include carboxymethyl-lysine (CML) and glyoxal lysine dimer (GOLD), while methylglyoxal-derived AGEs are carboxyethyl-lysine (CEL), argpyrimidine and methylglyoxal lysine dimers (MOLD), and 3-deoxyglucosone-derived AGEs are pyrraline and deoxyglucosone-derived lysine dimers (DOLD) (Thornalley *et al*., 1999).

The glycation reaction mainly occurs at the terminal amino groups of lysine, arginine and histidine, whereas glycation-mediated protein crosslinking is associated with arginine residues (Munch *et al.*, 1999). The dicarbonyl compounds, like glyoxal, deoxyglucosone or methylglyoxal, favour the formation of intracellular AGEs (Wells-Knecht, 1995). Various proteins, including collagen, interact with AGEs or reactive dicarbonyl compounds and form protein crosslinks (Reddy, 2004). Quantitative analysis of AGEs have shown that intracellular proteins are more heavily AGEs-modified than extracellular proteins (Thornalley *et al.*, 2003)(Fig.1.4).

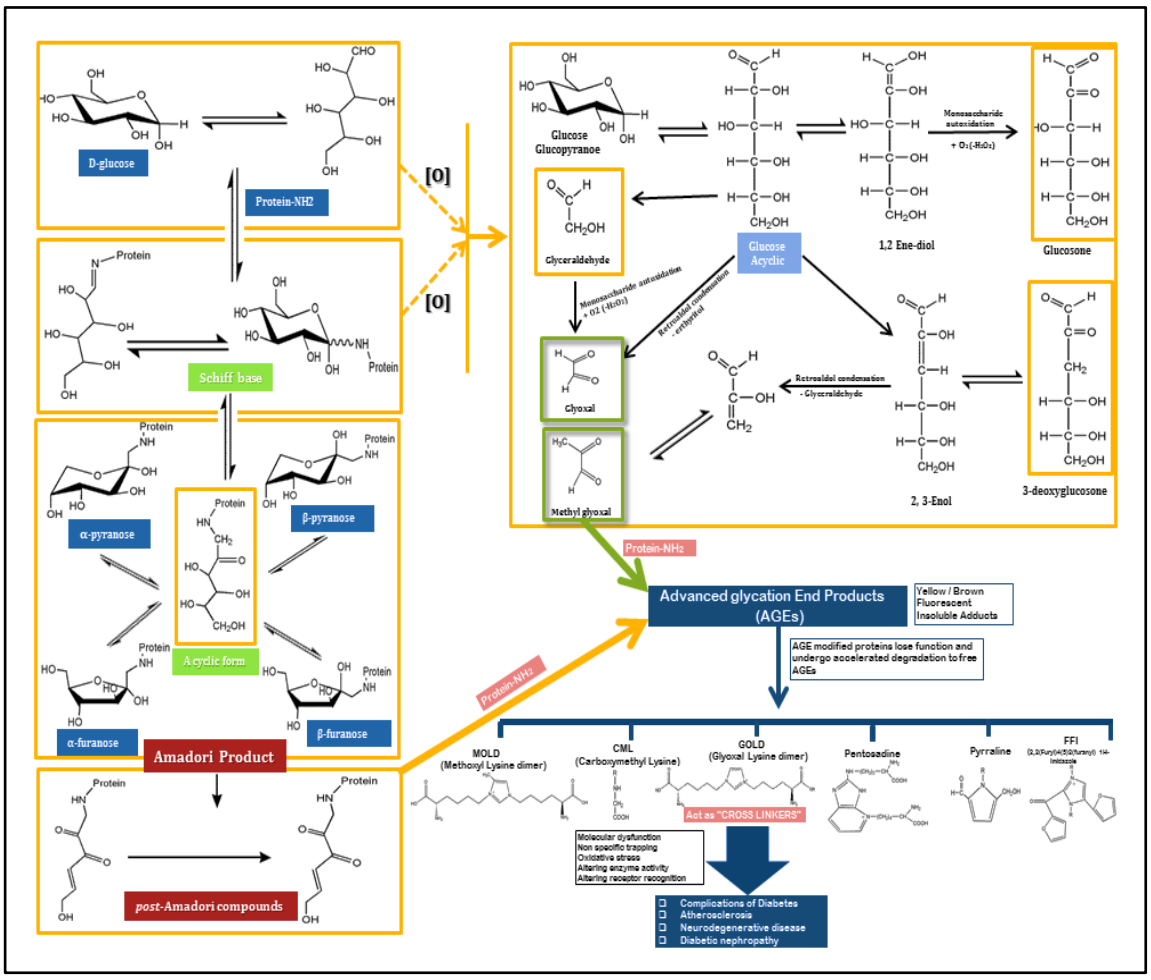
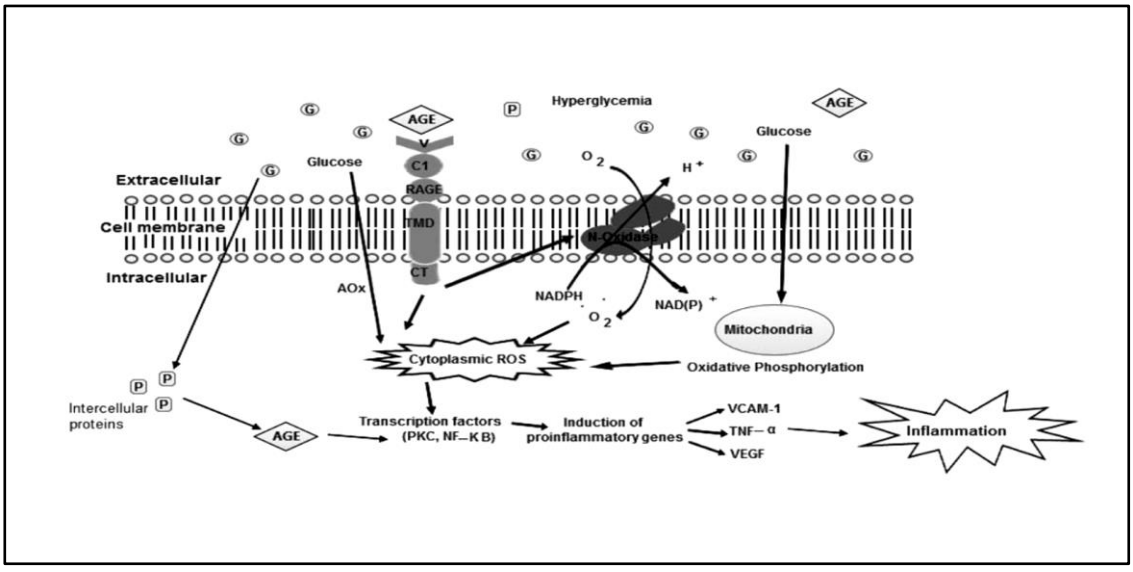


Figure 1.4. AGEs formation and complications (adopted from Adrover *et al.*, 2008 and Thornalley *et al.*, 1999).

1.6.1 AGEs and pathogenesis of diabetic complications

AGEs are mainly involved in pathogenesis of numerous diseases depending on their mode of interaction with target cells. In general AGEs cause modification in the structure and function of intracellular proteins, extracellular matrix (ECM) and cell receptors, and initiate a series of altered cellular processes. In addition the AGE modified protein activates a downstream signalling cascade by binding to receptor for AGEs (RAGE) leading to nuclear translocation of transcription factors such as NF-kB, specificity protein-1 (SP-1), activator protein-1 (AP-1) and nuclear factor interleukin-6 (NF-IL6). All these then contribute to various cellular processes including pro-inflammatory responses, overexpression of RAGE and generation of reactive oxygen species (ROS), etc. (Brownlee, 2001). Other complications of AGEs include atherosclerosis, nephropathy, uremia, poor wound healing, diabetic retinopathy, neurodegenerative diseases, cancer, and the normal ageing processes (Schmidt *et al*., 1994; Basta *et al*., 2004; Heijst *et al*., 2006) (Fig.1.5)



RAGE; receptors for AGEs, V, C1 and C2(extracellular domains for RAGE), TMD; Transmembrane domain, CT; cytoplasmic tail, ROS; reactive oxygen species, AOx; Autoxidation of glucose, PKC; protein kinase C, NF-KB; nuclear factor kappa light chain enhancer of activated B cells, VCAM-1; Vascular cell adhesion protein 1, TNF-α; tumour necrosis factor α, VEGF; Vascular endothelial growth factor, NADPH nicotinamide adenine dinucleotide phosphate.

Figure 1.5. Simplified mechanisms of the pathogenesis of AGEs in hyperglycemic condition (Adopted from Khangholi *et al*., 2016)

1.6.2 Natural products as AGEs-inhibiting compounds

A number of plant species and plant products have been shown to exhibit antiglycation activity. Some biologically important phytochemicals, particularly flavonoids and polyphenols, terpenoids, alkaloids and iridoids have been studied extensively for their antioxidant and anti-glycation activities (Fig. 1.6).

1.6.3. Polyphenols

The polyphenolic compounds are main constituents of plants and are known for a variety of biological activies. A number of polyphenolics have been reported for their antiglycation potential in literature; for instance: vescalagin (**1**), a hydrolyzable tannin recently isolated from the fruit of *Syzygium* sp. It showed a protective effect against methylglyoxal-induced inflammation and carbohydrate metabolic disorder in rats. The effect was considered to be caused by an increase in concentration of D-lactate that inhibited AGE formation and a decrease in cytokine release to prevent damage to β-cells of the pancreas (Chang *et al*., 2013).

Similarly several natural stilbenoids such as resveratrol and its derivatives have been reported to exhibit a broad-spectrum of biological activities, including anti-glycation activity. For example, oxyresveratrol (**2**) isolated from the heartwood of *Artocarpus lakoocha* showed anti-glycation activity with IC_{50} 8.1 μM (Povichit *et al*., 2010).

Nevertheless, a number of different types of polyphenolic compounds were isolated from *Rhus verniciflua* and tested for their antiglycation effect. It was reported that butein (**3**) (IC_{50} 0.7 μM) and sulfuretin (**4**) (IC_{50} 1.3 μM) showed a remarkable inhibitory effect on recombinant human aldose reductase and the accumulation of AGEs (IC_{50} 200μM) and (IC_{50} 1240 μ M) respectively (Lee *et al*., 2008).

1.6.4. Flavonoids

The flavonoids are generally known for their antiglycation effects. Flavones are more active than the corresponding flavanones, isoflavones, and flavonols. In general, some structural features of flavonoids are considered as essential for the inhibition of AGEs formation, such as hydroxyl groups at C-3, C-5 and C-7 . Methylation or glucosylation of the hydroxyl groups alters the activity of flavones, flavonols and flavanones (Matsuda *et al*., 2003).

A series of natural flavonoids has been investigated for their potential as an inhibitor of protein glycation. Quercetin (**5**) is an important flavonol and posesses a broad spectrum of biological activities. It is known as a potent antiglycation agent. *Psidium guajava* leaf extract was found to inhibit glycation processes in an albumin/glucose test system and quercetin was identified as one of the active principles. It has been found to exhibit a strong inhibitory effect on glycation of albumin in various assays (Wu *et al*., 2009a).

Interestingly, the natural flavonol glycosides are reported as effective anti-glycation agents. The Korean medicinal plant *Artemisia capillaris* was explored for its potential antiglycation activities. A series of compounds were isolated. Some flavonol glycosides i.e isorhamnetin 3-robinobioside (**6**), hyperoside (**7**) and isorhamnetin 3- galactoside-7-rhamnoside (**8**) exhibited moderate antiglycation activity (IC_{50} 48 μM, 82 μM and 155 μM, respectively) compared to the positive control aminoguanidine (IC_{50} 920 μM)(Jung *et al*., 2012). Although the exact mechanism for this inhibition is not known yet, it is generally accepted that strong antioxidant capacities, free radical scavenging and/or metal ion trapping activities are mainly involved (Peng *et al*., 2008). Likewise, silymarin (**9**) is an example of a complex of flavonoids, which acts as a specific RAGE blocker and therefore could reduce the burden of AGEs in diabetic patients. Silymarin has also been identified as an antioxidant with antiglycation properties in both *in vitro* and *in vivo* systems (Wu *et al*., 2009b).

1.6.5 Terpenoids

Terpenoides hold a wide structural diversity and reveal a wide spectrum of biological activities. The terpenoids are classified into a number of different types including sesquiterpenes, diterpenes, triterpenes, etc.

The sesquiterpenes are commonly found in many medicinal plants and are considered as anti-AGEs agents. β-Caryophyllene (**10**), a sesquiterpene lactone, is reported as having a protective role against plasma and tissue glycoprotein components in streptozotocin-induced hyperglycemic rats (Basha and Sankaranarayanan, 2015). Similarly, labdane diterpenes and labdadiene (**11**) were isolated from the rhizomes of *Alpinia zerumbet.* These labdadienes exhibited inhibitory activities on the formation of fructosamine adducts and dicarbonyl compounds with IC_{50} 168.0 μM and this activity was comparable with the reference flavonoids quercetin and rutin (Chompoo *et al*., 2011).

1.6.6 Alkaloids

Alkaloids are effective anti-AGEs compounds. A number of alkaloids from different classes have been tested for AGE inhibition over the past few years. Cyclopeptide alkaloids including nummularine-R (**12**), and hemsine-A (**13**) were isolated from *Ziziphus oxyphylla* Edgw. (Rhamnaceae) and tested for AGEs inhibition. Nummularine-R (IC_{50} 720.2 μM) and hemsine-A (IC_{50} 277.7 μM) presented moderate anti-glycation activity. All compounds were non-toxic to the PC-3 cell line (Choudhary *et al*., 2011).

Wang *et al*. (2007) have isolated sinomenine (**14**) from the stem and root of *Sinomenium acutum*. In an attempt to know the molecular mechanism of inhibition of this compound, retinal microglial were activated by AGEs treatment. It was noticed that sinomenine weakened ROS production in a dose-dependent way and reduced the nuclear translocation of NF-kB p65 in AGEs-activated cultured retinal cells.

More recently in a review by Abbas *et al*. (2016) new potent inhibitors of protein glycation belonging to different classes such as flavonoids, alkaloids and terpenes, have been identified. Some interesting activities were highlighted with regard to small compounds such as carnosine (**15**) (IC_{50} 16.3 μM) and nicotinamide (**16**)(IC_{50} 17.8 μM) as potential AGE inhibitors.

1.6.7 Iridoids

Iridoids are monoterpenes based on the cyclopenta [c] pyranoid skeleton, which are known for a number of pharmacological activities including the inhibition of protein glycation (Ghisalberti *et al*., 1998; West *et al*., 2014).

Catalpol (**17**), a common iridoid, was isolated from *Rehmannia glutinosa*, and tested for anti-inflammatory effects on AGE-stimulated THP-1 cells. It was noticed that catalpol causes a considerable decrease in the expression of pro-inflammatory mediators. Moreover, the effective anti-AGEs potential was shown by the decrease in transcriptional activation of NF-κB (Choi *et al*., 2013).

More recently iridoids obtained from dietary sources including *Morinda citrifolia*, Asian cornelian cherry (*Cornus officinalis*), European cornelian cherry (*Cornus mas*) and olive (*Olea europaea*) were tested for their potential antiglycation activities. The major iridoids from *Morinda citrifolia*, deacetylasperulosidic acid (**18**) and loganic acid (**19**), were tested for AGE inhibition *in vitro*. Both inhibited glycation in a concentration-dependent manner (West *et al*., 2014).

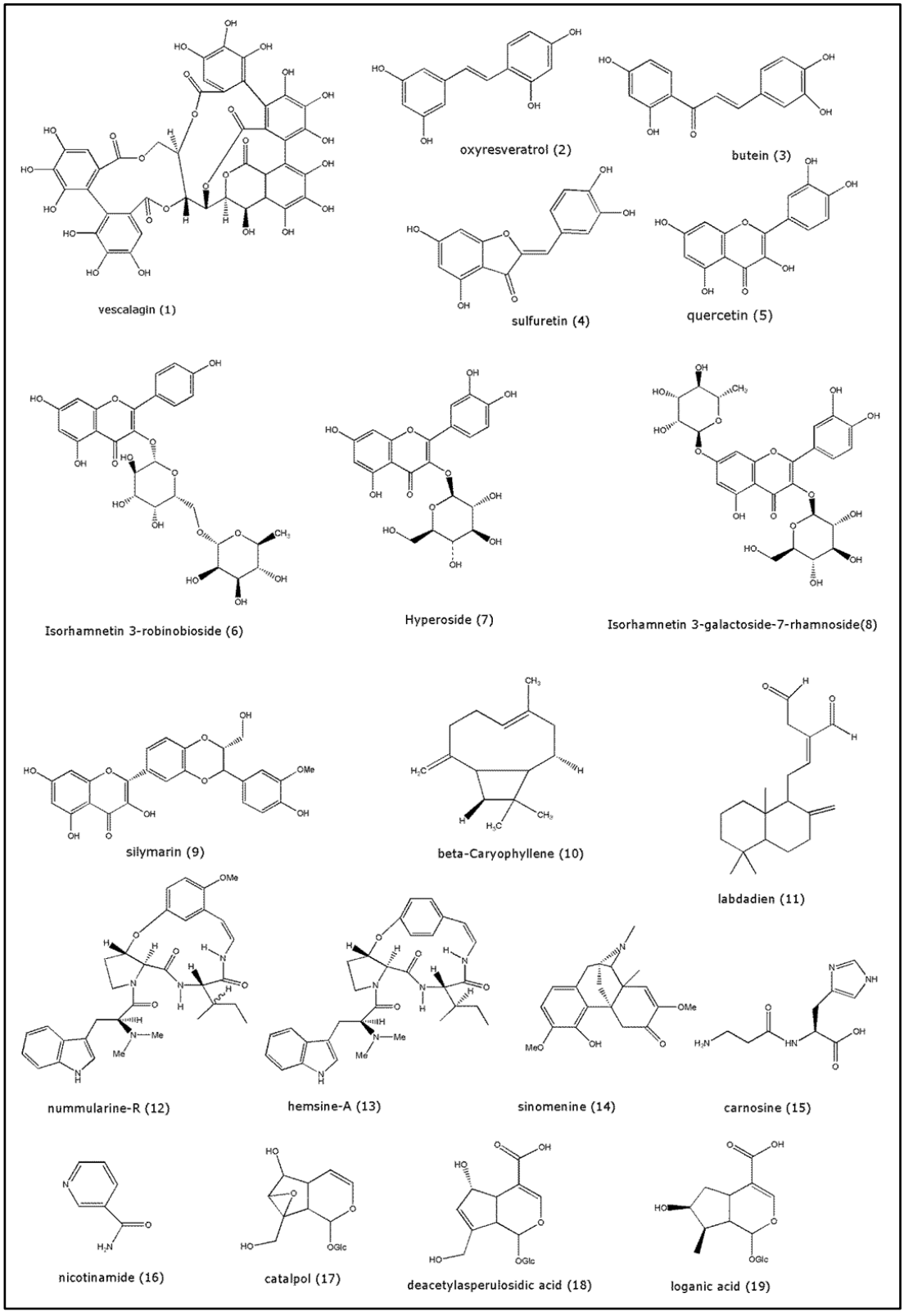


Figure 1.6. Antiglycation compounds isolated from medicinal plants

1.7. Inhibition of α-glucosidase

Inhibition of alpha-glucosidase enzyme activity in the gastro-intestinal tract is a common therapeutic approach to reduce glucose-uptake in diabetic patients. Through a competitive inhibition of α-glucosidase, these drugs help to inhibit the fast breakdown of polysaccharides and thus control the blood sugar level.

Numerous α-glucosidase inhibitors (Fig 1.7) have been isolated from natural sources, for instance acarbose (**20**) and voglibose (**21**) that are clinically used as α- glucosidase inhibitors in the treatment of diabetes mellitus (Playford *et al*., 2013). Commercially available α-glucosidase inhibitors (**20**, **21, 22**), mainly contain nonsugar moieties, are associated with serious side effects (Yin *et al*., 2014). Therefore, there is a need to look for promising molecules as an alternative for existing α-glucosidase inhibitors. In the literature a number of constituents, for instance terpenes, alkaloids, flavonoids and polyphenols have been reported as α-glucosidase inhibitors.

In an investigation by Mbaze *et al*., (2007), a number of compounds were isolated from methanolic extracts of *Fagara tessmannii* (Rutaceae). Among these 3β-acetoxy-16β-hydroxybetulinic acid (**23**) presented a strong inhibition of α-glucosidase with an IC_{50} of 7.6 μM against α-D-glucosidase and IC_{50} 397 μM against β-D-glucosidase compared to acarbose (IC_{50} of 780 μM) (Mbaze *et al*., 2007).

Similarly the crude bark extract of *Uncaria laevigata* was explored for potential α-glucosidase inhibitors. A number of triterpenes were isolated from the crude extract and tested for α-glucosidase inhibitory activity. Among all ursolic acid (**24**) and 3β-hydroxy-30-methoxy-6-oxo-urs-12,19(20)-dien-28-oic acid (**25**) showed interesting inhibitory activity with IC_{50} values of 49 μM and 16 μM, respectively, as compared with that of genistein (IC_{50} 36 μM), used as positive control (Wang *et al*., 2013).

Alkaloids have been reported as interesting inhibitors of α-glucosidase. In a study aimed at the isolation of promising α-glucosidase inhibitors, methanolic extracts from the medicinal parts of 40 traditional Chinese herbs were assessed. From the methanol

extract of the leaves of *Adhatoda vasica* Nees (Acanthaceae), two alkaloids, vasicine (**26**) and vasicinol (**27**) were isolated. Both compounds inhibited rat intestinal α-glucosidase activity (IC_{50}= 125 and 250 μM, respectively), and they were both found to be competitive inhibitors (*Ki*= 82 and 183 μM, respectively) (Gao *et al*., 2008).

Similarly in another investigation two new phenylpropanoyl amides, namely chaplupyrrolidones A (**28**) and B (**29**), were isolated from the leaf extract of *Piper sarmentosum*. Chaplupyrrolidone B (**29**) (IC_{50} 430.0 μM) revealed α-glucosidase inhibitory activity and was 18-fold more active compared to its demethylated congener chaplupyrrolidone A (**28**) (IC_{50} 7800 μM). Kinetic evaluation studies indicated that chaplupyrrolidones B inhibited α-glucosidase in a non-competitive manner (*Ki*= 1.04 mM) (Damsud *et al*., 2013).

Flavonoids are widely distributed in plants and are known for a number of activities including α-glucosidase inhibition. In an investigation by Feng *et al*., (2011), the ethanolic leaf extract of *Aquilaria sinensis* (Lour.) Gilg.b. was explored in a quest for α-glucosidase inhibitors. Four new compounds were isolated namely aquilarisinin (**30**), aquilarisin (**31**), hypolaetin 5-*O*-β-*D*-glucuronopyranoside (**32**), and aquilarixan-thone (**33**) in addition to other (known) compounds. These were further tested for α-glucosidase inhibition, and interesting results were obtained for quilarisinin (**30**)(IC_{50} 273.7 μM, aquilarisin (**31**)(IC_{50} 634.7 μM), hypolaetin 5-*O*-β-*D*-glucuronopyranoside (**32**)(IC_{50} 298.9 μM), and aquilarixanthone (**33**)(IC_{50} 678.1 μM), that were comparable with acarbose (IC_{50} 576.2 μM) (Feng *et al*., 2011).

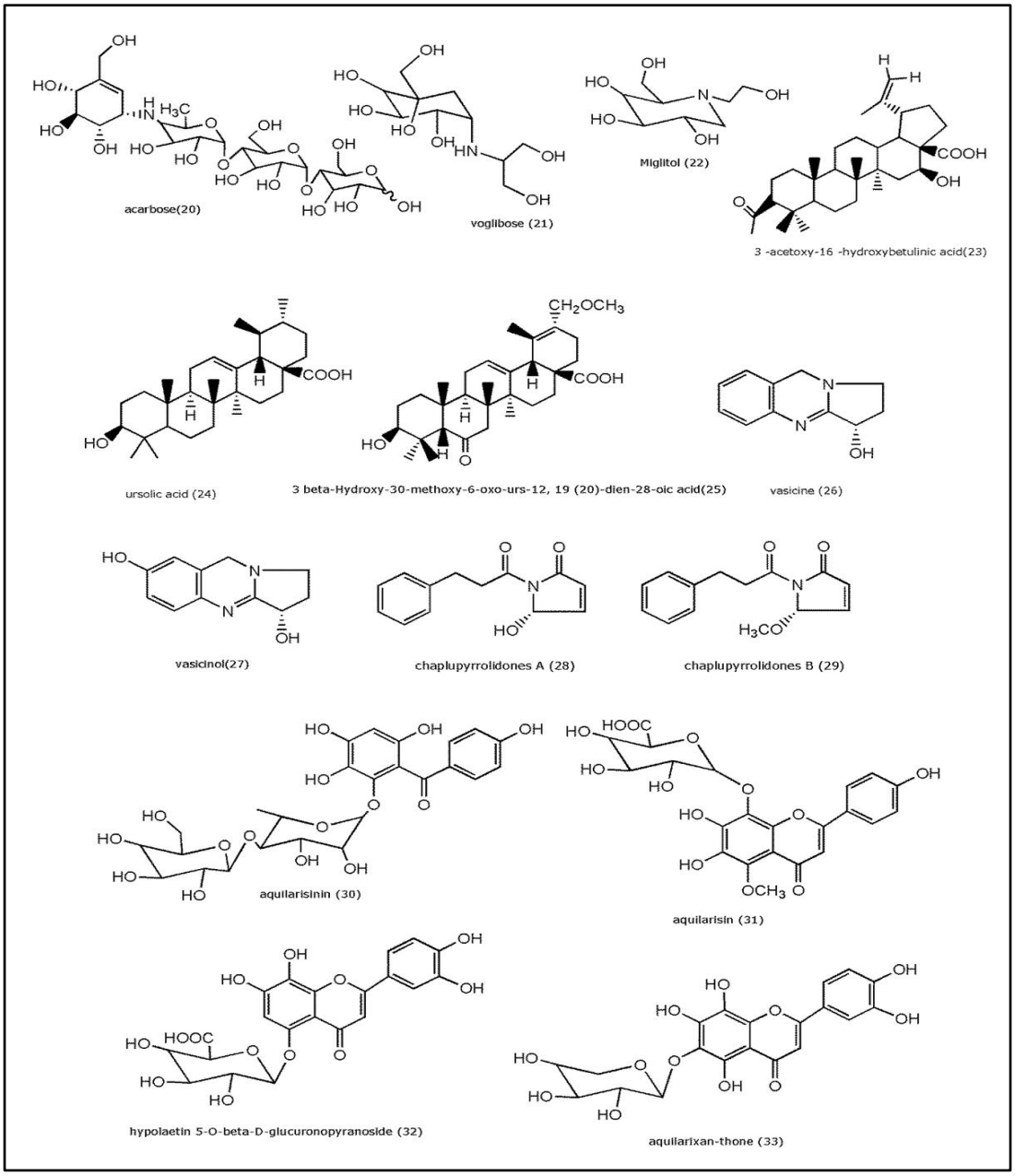


Figure 1.7. Alpha-glucosidase inhibitors isolated from medicinal plants

1.8 Microbial infections

Microbial infections are one of the important health hazards in developing countries including Pakistan, where they account for 12% of deaths (CDC, 2010). Both Gram-positive and Gram-negative bacterial strains including *Bacillus*, *Staphylococcus*, *Salmonella* and *Pseudomonas* are mainly involved in such infections (Ahameethunisa and Hoper, 2010). Poor hygiene, lack of proper health facilities and lack of knowledge are major reasons for this widespread health concern in developing countries. The antibiotics are mainly used as a remedy for bacterial infection; however, the cost of therapy, severe side effects like hypersensitivity, allergic reactions and immune suppression limit their usage. Furthermore prolonged and over-usage of antibiotics that generally leads towards development of resistance is of special concern these days (Alder, 2005; Walsh, 2004).

The increasing incidence of drug-resistant pathogens has drawn the attention of the pharmaceutical and scientific communities towards studies on the potential antimicrobial activity of plant-derived substances based on traditional use (Savoia, 2012). Literature surveys show that plant-based medicines play a promising role in the treatment of infectious diseases (Ahmad and Beg, 2001; Bibi *et al*., 2010; Obeidat, 2011). Some antimicrobial compounds isolated from plants are shown in (Fig 1.8).

Also in Pakistan medicinal plants are considered as primary sources of medicines for the treatment of bacterial infections. A number of ethnopharmacological investigations conducted in various parts of the country have demonstrated the impact of traditional plants as sources of antimicrobial agents (Mahmood *et al*., 2012_b; Ullah *et al*., 2013; Ahmad *et al*., 2015; Barkatullah *et al*., 2015; Yaseen *et al*., 2015). It has been reported that simple phenols, phenolic acids, quinones, flavonoids, tannins, alkaloids, coumarins, terpenoids, lectins, polypeptides and polyacetylenes are the major classes of antimicrobial compounds from plants (Cowan, 1999; Bibi *et al*., 2011; Jamil *et al*., 2012; Ejaz *et al*., 2104; Ashraf *et al*., 2015).

In recent years a number of interesting antibacterial metabolites have been isolated from different plant sources that exhibited significant activities. A naturally occurring 2,2′: 5′,2″ terthiophene (**34**) presented promising inhibitory activity against *S. aureus* (MIC = 0.08 μM) and this activity mainly occurs in the presence of UV light (Ciofalo *et al*., 1996).

The flavonoids and flavonones are well known for their antimicrobial potential. Some compounds including sophoraflavanone G (a geranylated flavonone) (**35**) also act synergistically with other molecules like vancomycin. In Thailand sophoraflavanone G was isolated from *Sophora exigua* Criab (Leguminosae), and presented promising activity against *S. aureus* (MIC = 7.3–14.7 μM) (Tsuchiya *et al*., 1996).

In a recent study methanolic extracts of *Oncoba spinosa* Forssk. (Salicaceae) were investigated and five flavonoids were isolated. Out of all, quercetin 3-O-α-L-rhamnopyranosyl-β-D-glucopyranoside (**36**) and quercetin (**37**) were the most active compounds against bacteria (MIC = 10.5- 21.1 μM) and fungi (MIC = 212 – 423 μM), respectively (Djouossi *et al*., 2015).

In an investigation by Nissanka *et al*.,(2001) two alkaloids i.e 8-acetonyl-dihydronitidine (**38**) and 8-acetonyl-dihydroavicine (**39**) were isolated from the stem bark of *Zanthoxylum tetraspermum*. In the antimicrobial assays, 8- acetonyl-dihydronitidine (**38**) and 8- acetonyl-dihydroavicine (**39**) showed significant inhibition of bacterial growth, i.e MIC 3.8 μM and 8.0 μM, respectively, and MBC 61.6 μM and 32.1 μM, respectively (Nissanka *et al*., 2001).

Coumarins are important secondary metabolites that possess a broad spectrum of pharmacological properties including antimicrobial activities. The dichloromethane extract of the fruits of *Heracleum mantegazzianum* Sommier & Levier (Apiaceae) was investigated for its antimicrobial activities and the isolation of active compounds. From the most active fraction xanthotoxin (**40**) and bergapten (**41**) were isolated. Xanthotoxin (**40**) showed a very high activity against gram-positive bacteria with very low MIC values (13.8 μM), followed by bergapten (**41**) (MIC = 57.8 μM).

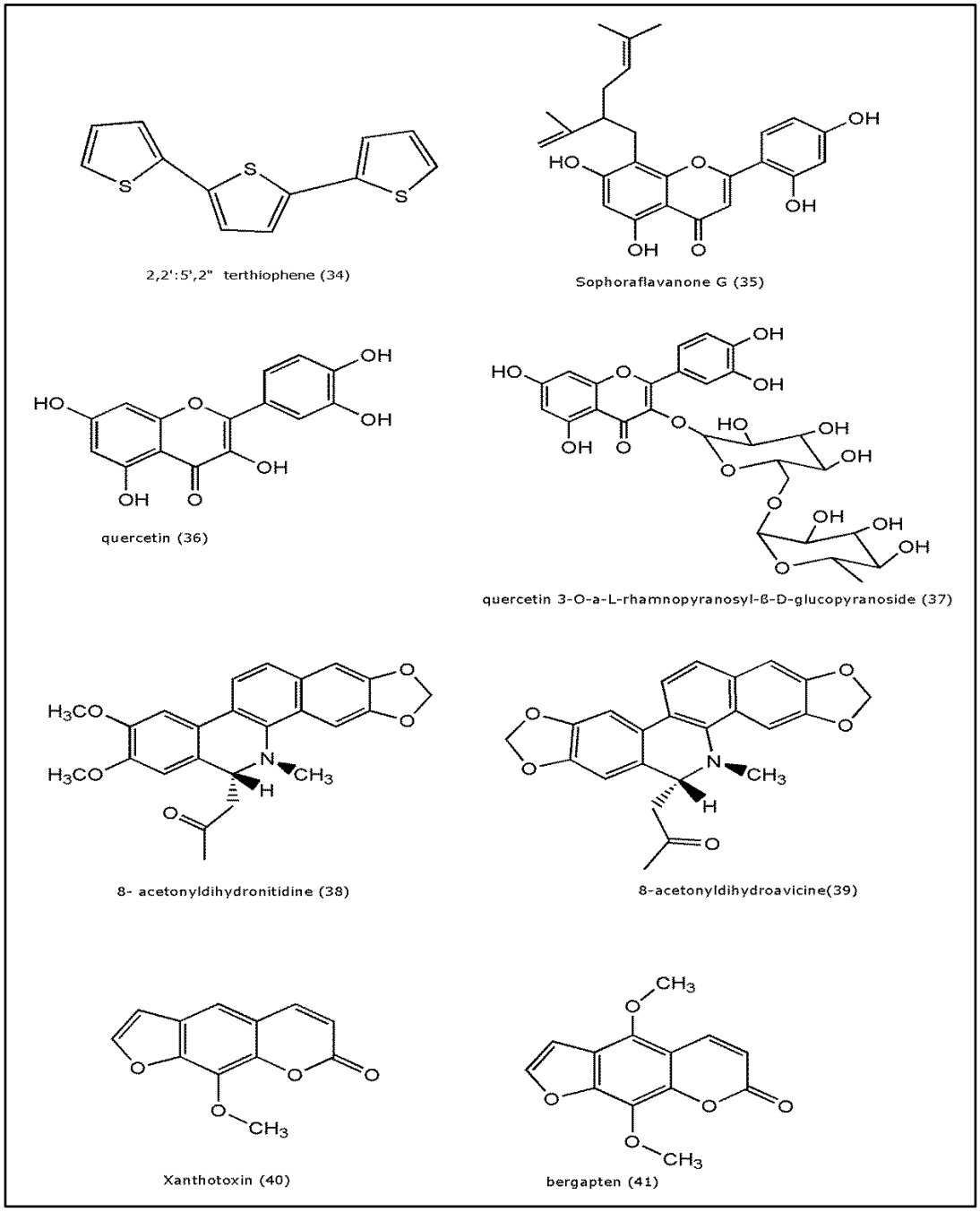


Figure 1.8. Antibacterial compounds isolated from medicinal plants

1.9 Leishmaniasis

Leishmaniasis, common in tropical and sub-tropical parts of the world, is a protozoal infection caused by single cellular, haemoflagellate protozoan parasites of the genus *Leishmania* (*Leishmania major, Leishmania tropica, Leishmania aethiopica, Leishmania mexicana, Leishmania amazonensis* and *Leishmania braziliensis*), transmitted by the bite of an infected female sandfly *Lutzomyia* or *Phlebotomus* (Handman and Bullen, 2002) (Fig. 1.9). Leishmaniasis is endemic in over 98 countries with more than 350 million people at risk. It is estimated that 1.3 million new cases of leishmaniasis (0.3 million visceral leishmania and 1.0 million cutaneous leishmania) occur every year (WHO, 2014a). Also in Pakistan leishmaniasis is reported as an emerging dermal disorder (Iqbal *et al*., 2016).

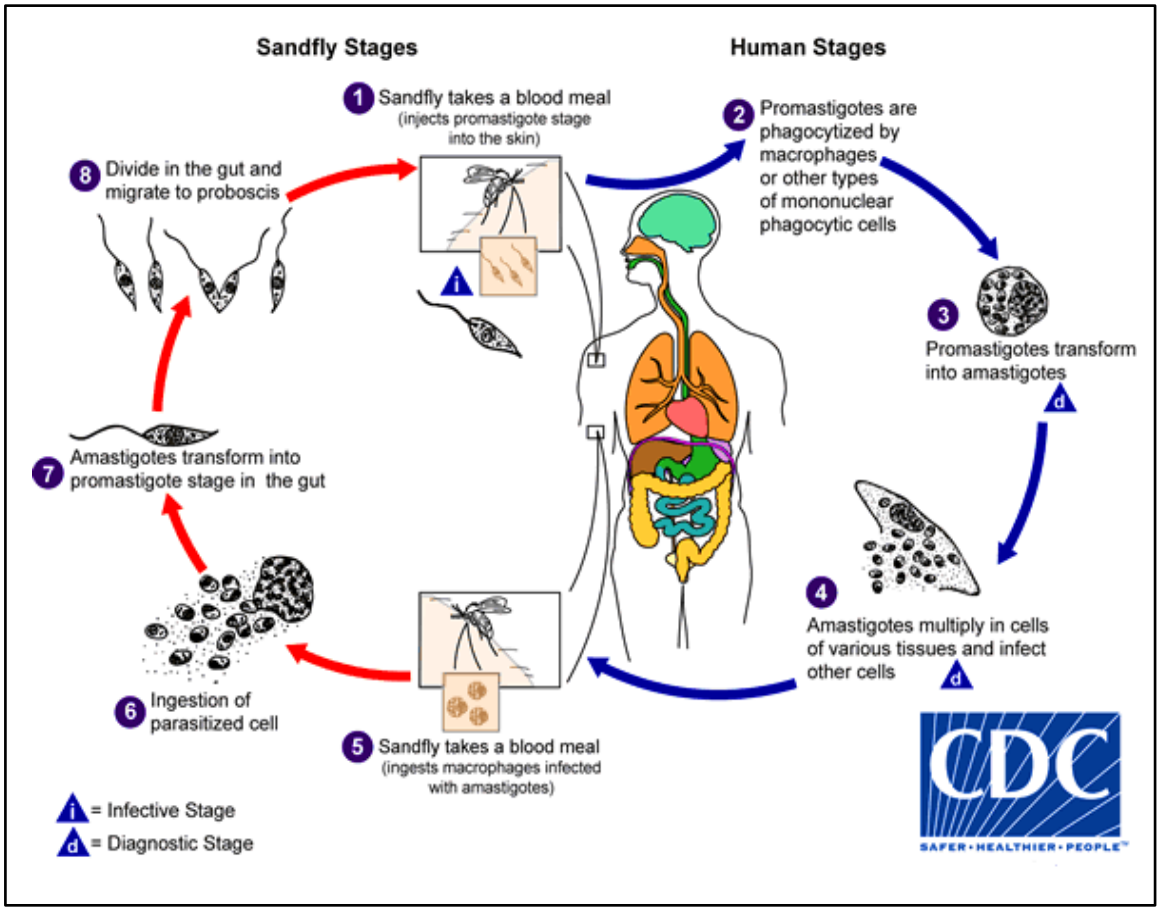


Figure.1.9 Life cycle of *Leishmania* sp. (Adopted from CDC, 2015)

In general the first-line treatment against leishmaniasis is still based on pentavalent antimonial drugs, such as sodium stibogluconate and meglumine antimoniate. However, these are associated with severe undesirable effects (Choudhary *et al*., 2010). Plants are promising sources for new drug candidates. More than hundred plant species have been reported to possess antileishmanial activity (Kvist *et al*., 2006).

The quest for new antileishmanial compounds is still continuing. For instance, the sesquiterpene lactone dehydrozaluzanin C (**42**) was isolated from the petroleum ether extract of leaves of *Munnozia maronii* (Asteraceae) and it inhibited the growth of eleven species of *Leishmania* promastigotes at concentrations between 2.5 and 10.0 μg/mL (Fournet *et al*., 1993). More recently two new sesquiterpenes fnarthexol (**43**) and fnarthexone (**44**) were isolated from *Ferula narthex* in addition to some known compounds. Conferol (**45**) was found to be the most potent compound with IC_{50} value of 30.1 μM, whereas newly isolated compounds i.e fnarthexol (**43**) (IC_{50} 114.2μM) and fnarthexone (**44**) (IC_{50} 123.4 μM) presented moderate activities (Bashir *et al*., 2014) (Fig 1.10).

Flavonoids have a broad spectrum of activities including antileishmanial properties. Quercitrin (quercetin 3-O-α-L-rhamnopyranoside) (**46**) was isolated from *Kalanchoe pinnata* and tested for its antileishmanial activity. The compound presented a potent activity (IC_{50} approximately 2.23 μM) with a low toxicity profile. This was the first demonstration of antileishmanial activity for a flavonoid glycoside (Muzitano *et al*., 2006). In another investigation by Tasdemir *et al*. (2006) more than 100 compounds were screened for antileishmanial potential. It was reported that fisetin (**47**), 3-hydroxyflavone (**48**), luteolin (**49**) and quercetin (**50**) were the most potent compounds with IC_{50} values of 2.2, 2.9, 2.7, and 3.3 μM, respectively.

In a recent study the antileishmanial activity of extracts and phyto-constituents of *Moringa oleifera* Lam. was investigated. The ethyl acetate fraction was found to be the most active with an IC_{50} value of 27.5 μg/mL. From the ethyl acetate fraction, among other isolated compounds, niazinin (**51**), a thiocarbamate glycoside, was reported as highly active (IC_{50} 5.25 μM) (Kaur *et al*., 2014).

Quinones are well known for their antileishmanial activity. In a report by Mori *et al*. (2008) antileishmanial activity of cordiachromes (quinone derivatives) isolated from *Cordia fragrantissima* was determined. Among all the tested samples, cordiachrom A (**52**) and cordiachrom B (**53**) presented interesting leishmanicidal activity with IC_{50} 17.0 µM and 10.2 µM respectively.

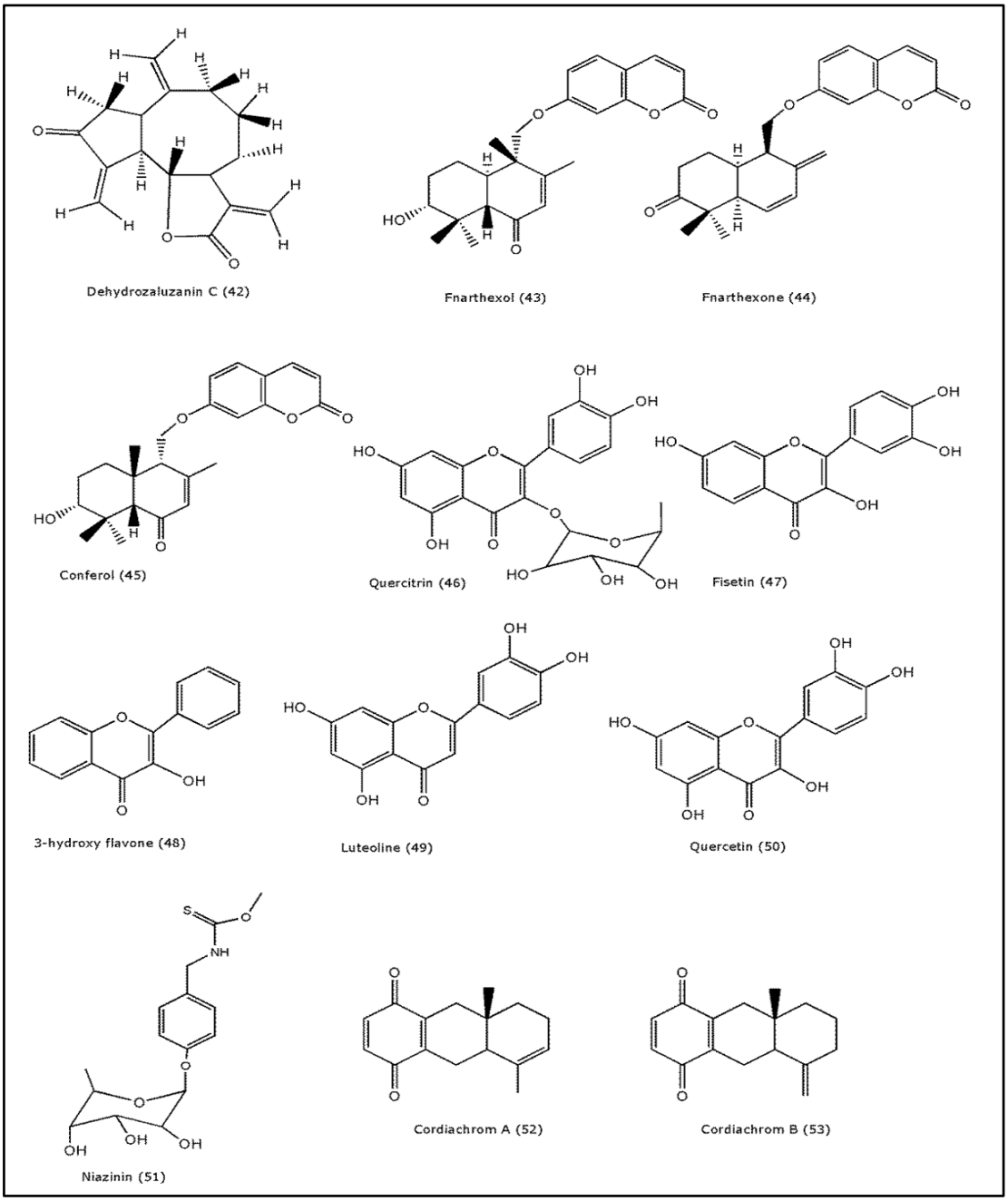


Figure.1.10 Antileishmanial compounds isolated from medicinal plants

1.10 Malaria

Malaria is caused by protozoan parasites from the genus *Plasmodium*, including *P. falciparum*, *P. vivax*, *P. malariae*, *P. ovale* and more recently the hitherto monkey malaria parasite *P. knowlesi* (White, 2008). Amongst all, *P. falciparum* is considered as the most lethal form. All *Plasmodium* parasites are transmitted to humans through female *Anopheles* mosquitoes (Diou *et al*., 2009) (Fig. 1.11).

In Pakistan mainly *P. vivax* (responsible for approximately 64% of infections) and *P. falciparum* (causing 36% of infections) are involved in spreading malaria (Asif, 2008; WHO, 2012). Indeed, malaria accounts for 16% of the total disease burden of the country and therefore has become a serious health concern these days in both in rural and urban areas (Shah *et al*., 2014). Regional statistics of WHO are even more alarming. It is estimated that the annual incidence of malaria is 1.5 million cases and Pakistan is placed in the "group 3 countries" of the WHO "Eastern Mediterranean" region, based on prevalence of malaria (WHO, 2014_b). Keeping in mind the severity of the issue, WHO has initiated a number of malaria control programmes in various parts of Pakistan especially the Khyber Pakhtunkhwa (KPK) and Balochistan Provinces.

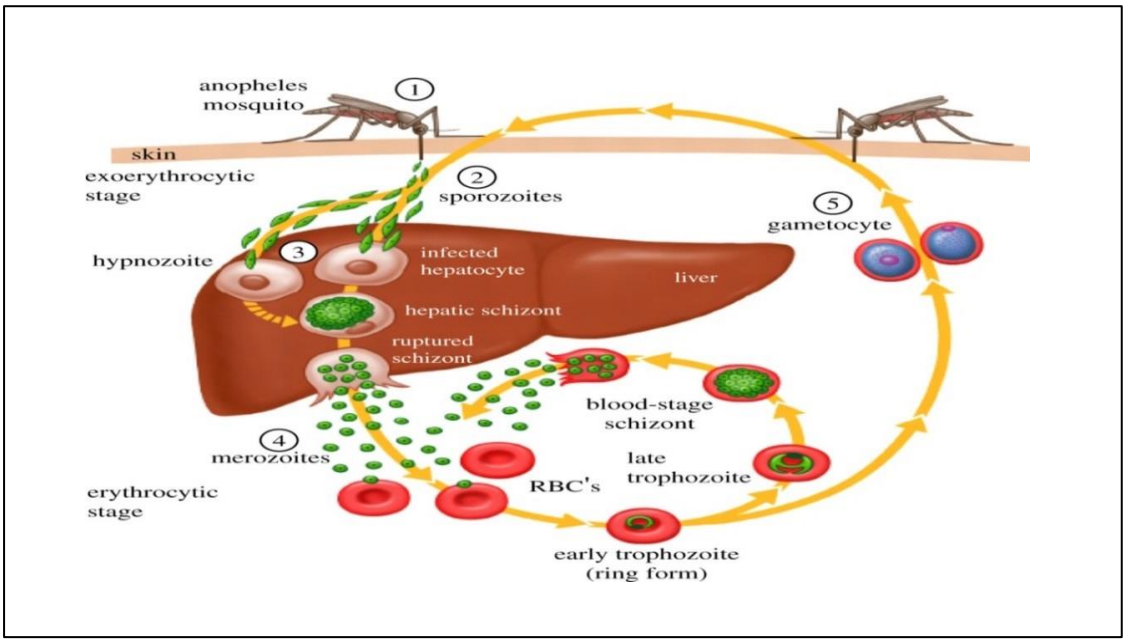


Figure. 1.11 Life cycle of malarial parasite (Adopted from Hill, 2011)

In general, a large number of anti-malarial drugs are commercially available to treat malaria all over the world. However, they are associated with dose-related side effects such as vomiting, nausea, fatigue, pulmonary toxicity, neuropsychiatric effects, blindness and neurotoxicity. In addition, drug resistance against the malarial protozoan is a serious concern these days, which is responsible for failure of therapy in many cases (Ruiz-Irastorza *et al*., 2010, Gravani *et al*., 2013).

Medicinal plants and their constituents have a long history of effective use in prevention and treatment of malaria in various parts of the world, and are still considered as promising sources for new antimalarials (Chiyaka *et al*., 2009). Also a wide majority of rural communities in Pakistan still rely on medicinal plants and herbal formulations for the treatment of malaria (Irshad *et al*., 2011; Marwat *et al*., 2011a; Shah *et al*., 2014; Sher *et al*., 2015). From literature it is obvious that diterpenes, alkaloids, flavonoids, coumarins, steroids and phenolics are mainly responsible for antimalarial activity of many plant extracts (Fig. 1.12)

Abietane type diterpenes were isolated from an apolar *n*-hexane fraction of *Plectranthus barbatu* and tested against erythrocytic schizonts of *Plasmodium falciparum*, intracellular amastigotes of *Leishmania infantum* and *T. cruzi* and free trypomastigotes of *T. brucei*. The compound 5,6-didehydro-7-hydroxy-taxodone (**54**) showed promising activity with acceptable selectivity against *P. falciparum* (IC_{50} 9.2 µM, SI 10.4) and *T. brucei* (IC_{50} 1.9 µM, SI 50.5) supporting its effectiveness against important protozoans (Mothana *et al*., 2014).

Also several plant-derived alkaloids have shown very interesting antimalarial activities and thus have a great potential for anti-malarial drug development. The indole alkaloids are famous for their antimalarial activities. Nkunya *et al.* (2004) explored *Monodora angolensis* and *Isolona cauliflora* (Annonaceae) for their antimalarial activities. They finally isolated a series of prenylated indole alkaloids from both plants. It was reported that 6-(3-methyl-but-2-enyl)-1,3-dihydro-indol-2-one(**55**) and annonidine F (**56**) showed promising antimalarial activities against the multidrug resistant strain K1 of *P. falciparum* (IC_{50} 104 µM and IC_{50} 57.0 µM for each compound).

Naphthoisoquinolines are characterised by a unique structure with a linkage between the naphthalene and the isoquinoline portions. It is hypothised that this characteristic structure is mainly responsible for antimalarial activity. In Kenya naphthoisoquinoline alkaloids were isolated from the stems and the leaves of *Ancistrocladus robertsoniorum*. All isolated compounds were found active, however ancistrocladisine (**57**) (IC_{50} 1.4 μM), cis-dihydro ancistrocladisine (**58**) (IC_{50} 1.7 μM) and ancistrobrevine B (**59**) (IC_{50} 2.0 μM) presented significant activities against the *P. falciparum* K-1 strains (Bringmann *et al*., 1999).

The flavonoids are commonly found in medicinal plants and account for an array of biological activities including antimalarial. It is considered that flavonoids act by inhibiting the fatty acid biosynthesis (FAS II) of the parasite. Several antimalarial flavonoids have been isolated from the stem bark of *Erythrina abyssinica* by Yenesew *et al*.(2004). These include chalcones, prenylated and non-prenylated isoflavones and flavones, pterocarpenes and flavenes. All compounds exhibited moderate antimalarial activity against the D6 and W2 strains of *P. falciparum*. Abyssinone V (IC_{50} 4.9 μM) (**60**) and sigmoidin A (**61**)(IC_{50} 5.8 μM) presented significant antimalarial activities.

Bioassay-guided fractionation of the ethanolic extract of the leaves and twigs of *Piptocoma antillana* (Asteraceae) was performed and a number of compounds were isolated. All compounds were tested for their antiproliferative and antimalarial potential. Two new sesquiterpene lactones i.e 5-O-methyl-5-epiisogoyazensolide (**62**) and 15-O-methylgoyazensolide (**63**) displayed significant (IC_{50} 6.2 μM) and (IC_{50} 2.2 μM) activity against *Plasmodium falciparum.*

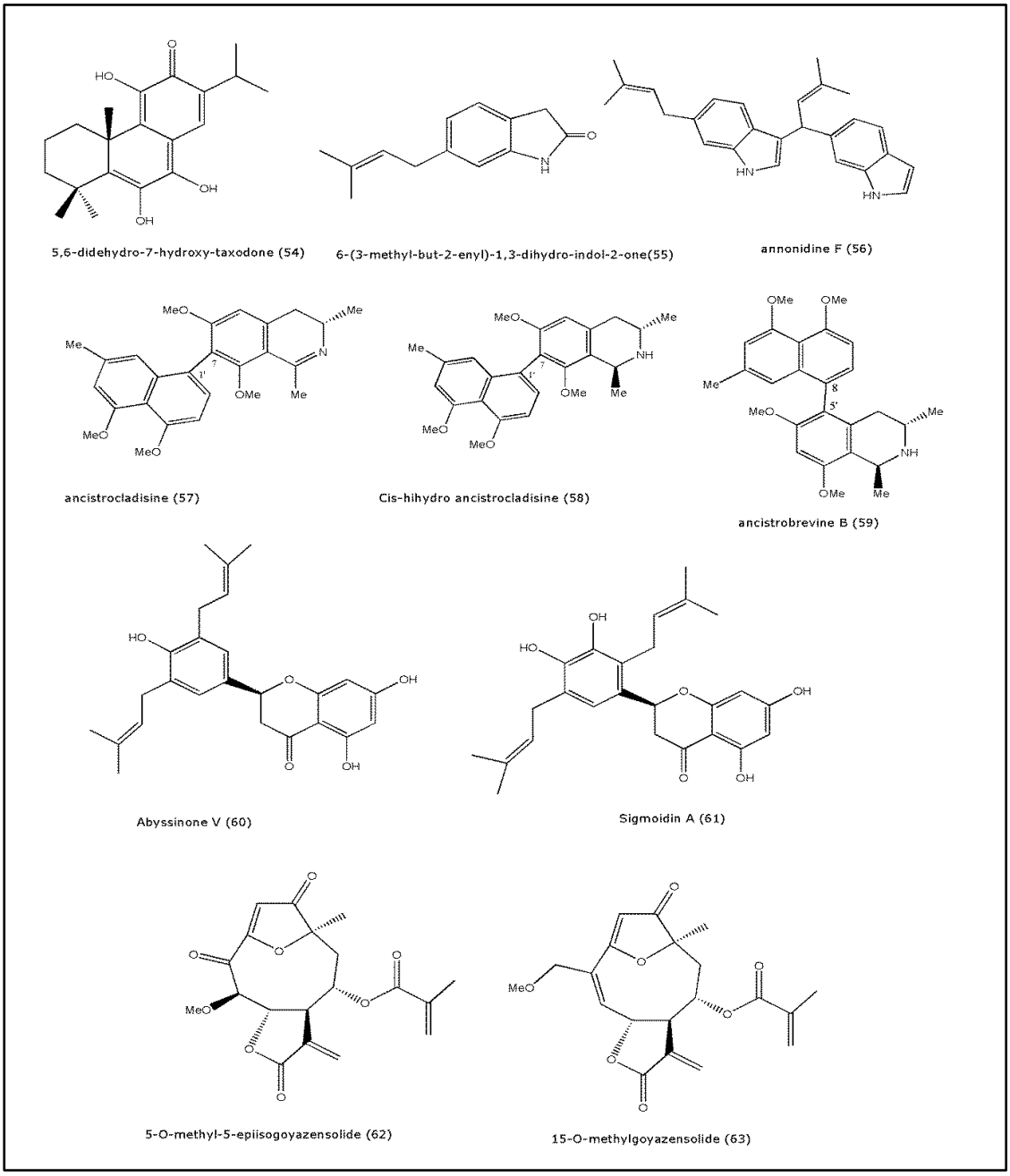


Figure 1.12 Antiprotozoal agents from plant origin

1.11 AIM OF THIS WORK

The aim of this thesis was to perform phytochemical and biological investigations on some selected medicinal plants from Pakistan.

Based on the importance of traditional medicine in Pakistan and various ethno-pharmacological surveys, selected medicinal plants were collected from different phytogeographical regions of Khyber Pakhtunkhwa (KPK) province of Pakistan, including Dera Ismail Khan (wetlands), District Karak (hilly and sandy area) and Chitral (Himalaya region) (Fig 1.13).

D.I.Khan has an area of about 7326 km^2 and is situated between 70°.11′ and 71°.20′ E. longitude and between 31°.15′ and 32°.32′N. latitude. D.I.Khan has mostly flat dry plains, commonly called Daman, about 80% of the total area, where a large number of streams and hill torrents discharge water (Chaudhry, 1998). The city D.I.Khan is located on the river Indus. Aquatic and xerophytic vegetation are commonly found in this study area, which is part of the country's richest biodiversity centre and a source of ethnobotanical knowledge. A number of ethnomedicinal surveys have reported the occurrence of important medicinal plants in this area (Marwat *et al*., 2008; Marwat *et al*., 2011a; Marwat *et al*, 2011_b; Mussarat *et al*., 2014).

The district **Karak** covers an area of 3372 km^2 and is located at 33° 7'12N. latitude and 71° 5'41E. longitude. It is a semi-arid area that mainly consist of xerophytes and meso-phytes. The study area is comprised of mountainous and plain area with mostly sandy soils. A number of ethnomedicinal surveys have been carried out in this area regarding the occurrence of medicinal plants (Khan *et al*., 2013; Murad *et al*., 2013; Khattak *et al*., 2015, Adnan *et al*., 2015; Rehman *et al*., 2015).

The **Chitral** is the largest district (14850 km^2) covering 20% of the total KPK province. It lies within 35°15′06" to 36°55′32"N. latitude and 71°11′32" to 73° 51′ 34"E. longitude. The elevation of the area varies from about 1,070 m (about 3500 ft) in the extreme south in Arandu to 7,690m (25,230 ft) at the summit of Tirich Mir in the Hindu

Kush. The local community of Chitral has a centuries old tradition of using plants for the ailments of various medical problems and therefore people have typical methods of preparation of herbal medicines (Ali and Qaiser, 2009). Despite the ethnomedicinal importance of the area, only limited information is available (Awan *et al*., 2001; Hussain *et al*., 2007; Ali and Qaiser 2009; Khan *et al*., 2011; Shah and Hussain, 2012; Abbasi *et al* 2013; Hadi *et al*., 2013).

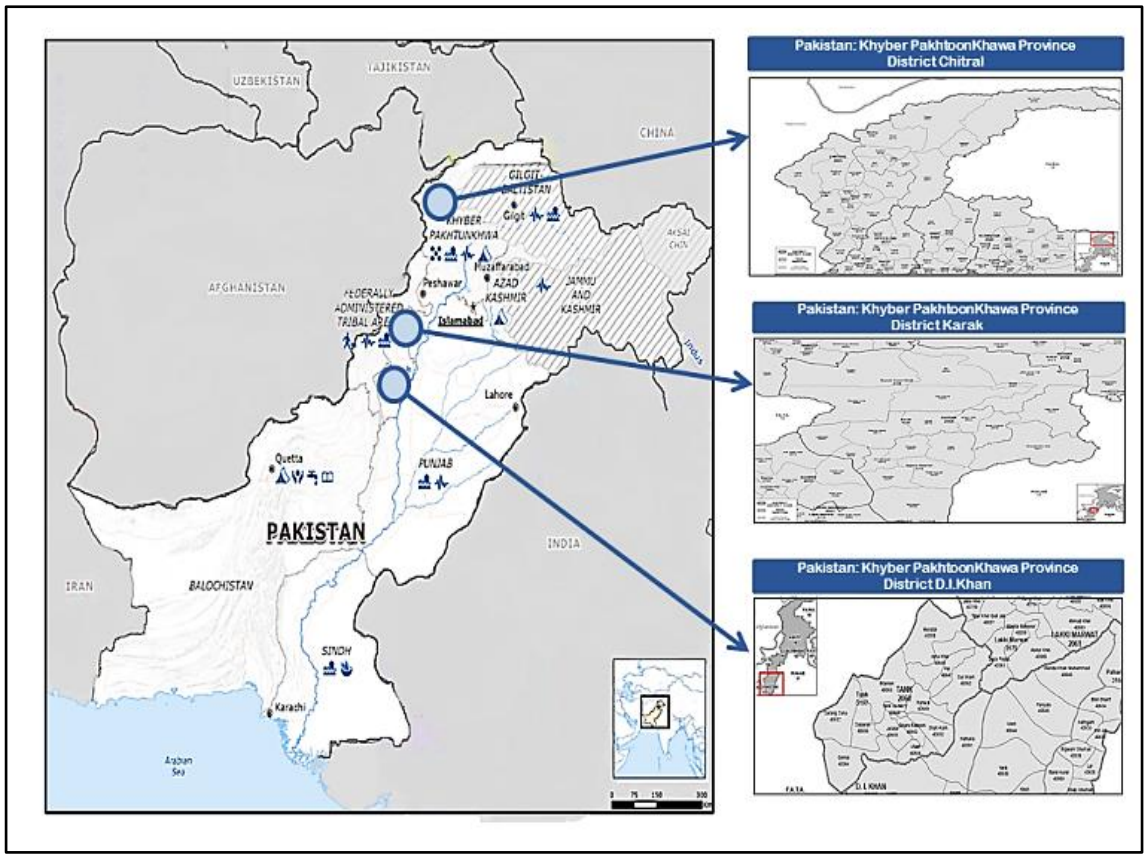


Figure 1.13. Map of Pakistan showing the sampling areas (Adopted from OCHA, Pakistan, http://www.unocha.org/pakistan)

In the present work some less evaluated medicinal plants from Pakistan have been selected in order to investigate their phytochemical constituents and biological properties. This selection was based on their extensive traditional use and on most recent ethnopharmacological surveys. The pharmacological focus was on their potential therapeutic usefulness in diabetic conditions, and on their antimicrobial properties

(antibacterial, antifungal, antileishmanial and antimalarial), according to running programmes in the host laboratory. In this way the following plant species were selected: *Nymphoides indica, Kickxia ramosissima* and *Ferula narthex*.

***Nymphoides indica* (L.) Kuntze** (Menyanthaceae) **(Chapter 3)** is a free floating leaved aquatic herb. It is commonly used as vegetable in local communities. A number of ethnomedicinal surveys reported its medicinal importance in various communities. In general it is used as one of the important ingredients of some traditional formulations. Most commonly *N. indica* is used for treatment of dysentery, scabies, jaundice and as antiproliferative, indicating the potential presence of antimicrobial and liver-protective constituents (Kensa, 2011; Panda and Misra, 2011; Kitdamrongtham *et al*., 2013). Previously flavonoids (Bohm *et al*., 1986) were reported from *N. indica;* however, no comprehensive phytochemical and pharmacological data was available in view of its medicinal importance.

***Kickxia ramosissima* (wall.) Janch**(Scrophulariaceae) **(Chapter 4)** is a small perennial herb, mainly found on rocky areas with shady places. It is commonly used for a number of ailments; for instance, as diuretic, to remove kidney stones, against fever, in rheumatism and in the management of snake and scorpion bites (Jain *et al*., 2008; Bole and Pathak, 1988; Pandya *et al*., 2012). In addition, ethnopharmacological surveys suggest that traditionally the plant has been used as an effective remedy for diabetes mellitus (Ahmad *et al*., 2009; Qureshi *et al*., 2010; Patel and Sachdeva, 2014). Limited phytochemical and pharmacological work has been published in the past and a few flavonoids, iridoids and mannitol were isolated (Khan *et al*., 1991; Khan and Aqil, 1993). In view of its use against fever and in diabetic conditions, the potential presence of antimicrobial, alpha-glucosidase and AGEs inhibitors, respectively, can be hypothesised.

***Ferula narthex* Boiss.** (Umbelliferae) (**Chapter 5**) mainly grows on high altitude areas. The oleo-gum resin obtained from this plant species is used as traditional medicine. It is a very important part of Ayurveda formulations like Hingvaday and Ghrita. It has been used as a spice and as well as folk phytomedicine for centuries for the treatment of gastro-intestinal disorders, nervous disorders, respiratory problems, as anti-diabetic and to treat impotence (Kapoor, 2001; Abuzaitoon, 2010). A number of compounds

have been previously isolated from *Ferula* species including coumarins, various types of terpenes (sesquiterpenes and other terpenoids) and sulfur-containing compounds (Lee *et al*., 2009). However, in the case of *Ferula narthex* limited work is available. More recently antileishmanial compounds have been isolated from this plant (Bashir *et al*., 2014). Therefore in the current project *Ferula narthex* was selected to isolate potentially antidiabetic and antimicrobial compounds.

The investigations accomplished in this project on different medicinal plants of Pakistan embraced:

a) Phytochemical characterization, i.e isolation and identification of constituents by using various chromatographic and spectroscopic techniques;
b) *In vitro* antidiabetic (inhibition of alpha-glucosidase, antioxidant activity, inhibition of AGEs) studies of extracts and isolated compounds;
c) *In vitro* antimicrobial studies of extracts and isolated compounds.

REFERENCES

Abbas, G, Al-Harrasi, AS, Hussain H, Hussain J, Rashid R, Choudhary MI. 2016. Antiglycation therapy: Discovery of promising antiglycation agents for the management of diabetic complications. *Pharm Biol* **54**: 198-206.

Abbasi AM, Mir AK, Munir HS, Mohammad MS, Mushtaq A. 2013. Ethnobotanical appraisal and cultural values of medicinally important wild edible vegetables of Lesser Himalayas Pakistan. *Ethnobiol Ethnomed* **9**: 66. **doi:** 10.1186/1746-4269-9-66

Abu-Zaiton, AS. 2010. Anti-diabetic activity of *Ferula assa foetida* extract in normal and alloxan-induced diabetic rats. *P J Biol Sci* **13**: 97–100.

ADA, 2010. American Diabetes Association, "Diagnosis and classification of diabetes mellitus," *Diabetes Care* **33:** S62–S69, 2010.

Adnan M, Tariq A, Mussarat S, Begum S, Naseer M. Salam AI Ullah R. 2015. Ethno-gynaecological assessment of medicinal plants in Pashtun's Tribal Society. *Bio Med Res Int*, doi: http://dx.doi.org/10.1155/2015/196475

Adrover M, Vilanova B, Frau J, Munoz F, Donoso J. 2008. The pyridoxamine action on Amadori compounds: A re-examination of its scavenging capacity and chelating effect. *Bioorg Med Chem* **16:** 5557-5569.

Ahameethunisa AR, Hoper W. 2010. Antibacterial activity of *Artemisia nilagirica* leaf extract against clinical and phytopathogenic bacteria. BMC *Complement Alt Med* **10**:6. **doi:** 10.1186/1472-6882-10-6.

Ahmad I, Beg AZ. 2001. Antimicrobial and phytochemical studies on 45 Indian medicinal plants against multi-drug resistant human pathogens. *J Ethnopharmacol* **74**: 113–123.

Ahmad VA, Kousar F, Zubair M, Khan A, Ali MS, Choudhary MI, Sener B. 2006. A new iridoid glycoside from *Linaria genestifolia*. *Fitoterapia* **77**: 12–14

Ahmad M, Shazia S, Hadi FI, Hadda TB, Rashid S, Zafar M, Khan MA, Khan MPZ, Yaseen G. 2014. An ethnobotanical study of medicinal plants in high mountainous region of Chail valley (District Swat-Pakistan). *J Ethnobiol Ethnomed* **10**: 1–36.
doi: 10.1186/1746-4269-10-36.

Ahmad N, Mahmood A, Ashraf A, Bano A, Tahir SS, Mahmood A. 2015. Ethnopharmacological relevance of indigenous medicinal plants from district Bahawalnagar, Punjab, Pakistan*. J Ethnopharmacol* **175**: 109–123.

Alder JD. 2005. Daptomycin, a new drug class for the treatment of Gram positive infections. *Drugs Today* (Barc) **41**:81-90.

Ali H, Qaiser M. 2009. The ethnobotany of Chitral Valley, Pakistan, with particular reference to medicinal plants.. *Pak J Bot* **41**: 2009-2041.

Alves, RRN, Rosa LML. 2007. Biodiversity, traditional medicine and public health: where do they meet ? *J Ethnobiol Ethnomed* **3**:14. doi:10.1186/1746-4269-3-14.

Ashraf A, Sarfraz RA, Rashid MA, Shahid M. 2015. Antioxidant, antimicrobial, antitumor, and cytotoxic activities of an important medicinal plant (*Euphorbia royleana*) from Pakistan. *J Food Drug Anal* **23**: 109–115.

Asif SA, 2008. Departmental audit of malaria control programme 2001–2005 North West Frontier Province (NWFP). *J Ayub Med Colleg* **20**: 98–102.

Austin GE, Mullins RH, Morin LG.1987. Non-enzymic glycation of individual plasma proteins in normo-glycemic and hyperglycemic patients. *Clin Chem* **33**: 2220-2224.

Awan MR, Shah M, Akbar G, Ahmad S. 2001. Traditional uses of economically important plants of Chitral District, Malakand division, NWFP, Pakistan. *Pak J Bot* **33**: 587-598.

Barkatullah, Ibrar M, Rauf A, Hadda Ta B, Mubarak MS, Patel S. 2015. Quantitative ethnobotanical survey of medicinal flora thriving in Malakand Pass Hills, Khyber Pakhtunkhwa, Pakistan. *J Ethnopharmacol* **169**: 335-346.

Basha RH, Sankaranarayanan C.2015. Protective role of β-caryophyllene, a sesquiterpene lactone on plasma and tissue glycoprotein components in streptozotocin induced hyperglycemic rats. *J Acute Med* **5**: 9–14.

Bashir S, Alam M, Adhikari A, Shrestha RM, Yousaf S, Ahmad B, Parveen S, Aman A, Choudhary MI. 2014. New antileishmanial sesquiterpene coumarins from *Ferula narthex* Boiss. *Phytochem Lett* **9**: 46–50.

Basta G, Schmidt AM, Caterina RD. 2004. Advanced glycation end products and vascular inflammation: implications for accelerated atherosclerosis in diabetes. *Cardiovasc Res* **63**: 582–592.

Bedoya, LM, Bermejo P, Abad MJ. 2009. Anti-infectious activity in the *Cistaceae* family in the Peninsula. *Mini Rev Med Chem* **9**: 519–525.

Bohm BA, Nicholls KW, Ornduff R. 1986. Flavonoids of the *Menyanthaceae* intra and interfamilial relationships. *Amer J Bot* **73**:204-213.

Bibi T, Ahmad M, Tareen NM, Jabeen R, Sultana S, Zafar M, Zain-ul-Abidin S. 2015. The endemic medicinal plants of Northern Baluchistan, Pakistan and their uses in traditional medicine. *J Ethnopharmacol* **173**: 1–10.

Bibi Y, Nisa S, Waheed A, Zia M, Sarwar S, Ahmed S, Chaudhary MF. 2010. Evaluation of *Viburnum foetens* for anticancer and antibacterial potential and phytochemical analysis. *Afr J Biotechnol* **9**: 5611-5615.

Bibi Y, Nisa S, Chaudhary FM, Zia M. 2011. Antibacterial activity of some selected medicinal plants of Pakistan. BMC *Complement Altern Med*. **11**:52.

doi: 10.1186/1472-6882-11-52

Bibi T, Ahmad M, Tareen RB, Tareen NM, Jabeen R, Rehman SU, Sultana S, Zafar M, Yaseen G, 2014. Ethnobotany of medicinal plants in district Mastung of Balochistan province-Pakistan. *J Ethnopharmacol* **57**: 79–89.

Bole P V, Pathak JM. 1988. Flora of Saurashtra. The Director Botanical Survey of India, New Delhi: Deep Printer.

Bringmann G, Teltschik F, Michael M, Busemann S, Ruckert M, Haller R, Bar S, Robertson SA, Kaminsky R. 1999. Ancistrobertsonines B, C, and D as well as 1,2 di-dehydro-ancistrobertsonine D from *Ancistrocladus robertsoniorum*. *Phytochemistry* **52**: 321–332.

Bringmann G, Dreyer M, Faber JH, Dalsgaard PW, Jaroszewski JW, Ndangalasi H, Mbago F, Brun R, Christensen SB. 2004. Ancistrotanzanine C and related 5,1′- and 7,3′-coupled naphthylisoquinoline alkaloids from *Ancistrocladus tanzaniensis*. *J Nat Prod* **67**: 743–748.

Brownlee M. 2001. Biochemistry and molecular cell biology of diabetic complications. *Nature* **414**: 813-820**.**

CDC, 2010. http://www.cdc.gov/globalhealth/countries/pakistan/sourceGBDcompare 2010.

CDC, 2015. http://www.cdc.gov/parasites/leishmaniasis/biology.html

Chang WC, Shen SC, Wu JS. 2013. Protective effects of vescalagin from pink wax apple (*Syzygium samarangense* (Blume) Merrill and Perry] fruit against methylglyoxal-induced inflammation and carbohydrate metabolic disorder in rats. *J Agric Food Chem*. **61**: 7102-7109.

Chiyaka C, Garrira W, Dube S. 2009. Effects of treatment and drug resistance on the transmission dynamics of malaria in endemic areas. *Theor Popl Biol* **75**: 14–29.

Choi, HJ et al. 2013. Catalpol suppresses advanced glycation end-products-induced inflammatory responses through inhibition of reactive oxygen species in human monocytic THP-1 cells. *Fitoterapia* **86**: 19–28.

Chompoo J, Upadhyay A, Kishimoto W, Makise T, Tawata S. 2011. Advanced glycation end products inhibitors from *Alpinia zerumbet* rhizomes. *Food Chem* **129**: 709–715

Chaudhry KL. 1998. "District census report of Dera Ismail Khan," Census Publication 50, Population Census Organization Statistic Division Government of Pakistan, Islamabad, Pakistan, 1998.

Choudhary MI, Adhikari A, Samreen, Rahman A. 2010. Antileishmanial Steroidal Alkaloids from Roots of *Sarcococca coriacea*. *J Chem Soc Pak* **32**: 799–802.

Choudhary MI, Adhikari A, Rasheed S, Marasini BP, Hussain N, Kaleen WA, Rahman AU. 2011. Cyclopeptide alkaloids of *Ziziphus oxyphylla* Edgw as novel inhibitors of α-glucosidase enzyme and protein glycation. *Phytochem Lett* **4**: 404–406.

Ciofalo M, Petruso S, Schillaci D. 1996. Quantitative assay of photo induced antibiotic activities of naturally-occurring 2,2′:5′,2-terthiophenes. *Planta Med* **62**: 374-5.

Cowan MM. 1999. Plant Products as Antimicrobial Agents. *Clin Microbiol Rev* **12**: 564–582.

Damsud T, Adisakwattana S, Phuwapraisirisan P. 2013. Three new phenylpropanoyl amides from the leaves of *Piper sarmentosu* and their *a*-glucosidase inhibitory activities. *Phytochem Lett* **6:** 350–354.

Desjeux P. 2001. The increase in risk factors for leishmaniasis worldwide. *Trans R Soc Trop Med Hyg* **95**: 239-243.

Dubey NK, Kumar R, Tipathi P. 2004. Global Promotion of herbal medicine: India's opportunity. *Curr Sci* (India) **86**: 37-41.

Diou J, Gauthier S, Tardif MR, Fromentin R, Lodge R, Sullivan DJ, Tremblay MJ. 2009. Ingestion of the malaria pigment hemozoin renders human macrophages less permissive to HIV-1 infection. *Virology* **395**: 56–66.

Djouossi MG, Tamokou JDD, Ngnokam D, Kuiate JR, Tapondjou LA, Harakat D, Nazabadioko LV. 2015. Antimicrobial and antioxidant flavonoids from the leaves of *Oncoba spinosa* Forssk (Salicaceae). BMC *Complement Altern Med* **15**: 134.
doi: 10.1186/s12906-015-0660-1.

Dwarakanath, C. The Fundamental Principles of Ayurveda. (Krishnadas Academy, Varanasi, India, 1952).

Ejaz R, Ashfaq UA, Usman A. Idrees S.2014. Antimicrobial potential of Pakistani medicinal plants against multi-drug resistance *Staphylococcus aureus. J Coast Life Med* **2**: 714-720.

Elosta A, Ghous T, Ahmed N. 2012. Natural products as anti-glycation agents: possible therapeutic potential for diabetic complications. *Curr Diabetes Rev* **8**: 92-108.

Feng J, Yang XW, Wang RF. 2011. Bio-assay guided isolation and identification of *a*-glucosidase inhibitors from the leaves of *Aquilaria sinensis*. *Phytochemistry* **72:** 242–247.

Fournet A, Muñoz V, Roblot F, Hocquemiller R, Cavé A, Gantier JC. 1993. Antiprotozoal activity of dehydrozaluzanin C, a sesquiterpene lactone isolated from *Murinozia maronii* (Asteraceae). *Phytother Res* **7**: 111-115.

Gao H, Huang YN, Gao B, Li P, Inagaki C, Kawabata J.2008. Inhibitory effect on alpha glucosidase by *Adhatoda vasica* Nees. *Food Chem* 108: 965–972.

Gesler WM.1992. Therapeutic landscapes: medical Issues in Light of the new cultural geography. *Soc Sci Med* **34**:735-746

Ghisalberti EL. 1998. Biological and pharmacological activity of naturally occurring iridoids and secoiridoids. *Phytomedicine* **5**: 147-163.

Glugliano D, Ceriello A, Paolisso G. 1996. Oxidative stress and diabetic vascular complications. *Diabetes Care* **19**: 257–267.

Good C. 1980. Ethno-medical Systems in Africa and the LDCs: Key Issues in Medical Geography. In Conceptual and Methodological Issues in Medical Geography Edited by: Meade MS. University of North Carolina at Chapel Hill, *Studies in Geography*: 93-116.

Govindaraj P, et al., 2015. Genome-wide analysis correlates *Ayurveda Prakriti. Sci Rep* **5**: 15786: doi: 10.1038/srep15786.

Gravani A, Gaitanis G, Zioga A, Bassukas ID. 2013. Synthetic antimalarial drugs and the triggering of psoriasis – do we need disease-specific guidelines for the management of patients with psoriasis at risk of malaria? *Int J Dermatol* **53**: 327-330.

Hadi F, Razzaq A, Rahman AR, Rahman Au, Rashid A. 2013. Ethnobotanical notes on woody plants of Rech Valley, Torkhow, District Chitral, Hindu-Kush range, Pakistan. *Schol J Agri Sci* **3**: 468-472.

Handman E, Bullen DV.2002. Interaction of *Leishmania* with the host macrophage. *Trends Parasitol* **18**: 332-334.

Heijst JWJV, Niessen HWM, Hoekman K, Schalkwijk CG.2006. Advanced glycation end products in human cancer tissues. *Ann N Y Acad Sci* **1043**: 725–733.

Hill, AVS. 2011. Vaccines against malaria. *Phil Trans R Soc B* **366:** 2806–2814.

Hussain F, Shah SM, Sher H. 2007. Traditional resource evaluation of some plants of Mastuj, District Chitral, Pakistan. *Pak J Bot* **39**: 339-354.
IDF, 2014. International Diabetes Federation, 2014, http://www.idf.org/Diabetes atlas.

IDF, 2015. International Diabetes Federation, http://www.idf.org/membership/mena /pakistan.

Iqbal H, Ishfaq M, Wahab A, Abbas MN, Ahmad I, Rehman A, Zakir M. 2016. Therapeutic modalities to combat leishmaniasis, a review. *Asian Pac J Trop Dis* **6**: 1-5

Irshad S, Mannan A, Mirza B. 2011. Antimalarial activity of three Pakistani medicinal plants. *Pak J Pharm Sci* **24**: 589-591.

Jain A, Katewa SS, Galave P and Nag A. 2008. Some therapeutic uses of biodiversity among tribes of Rajhistan. *Ind J Trad Med* **7**:256-262

Jamil M, Haq IU ,Mirza B, Qayyum M. 2012. Isolation of antibacterial compounds from *Quercus dilatata* L. through bioassay guided fractionation. *Annals of Clinical Microbiol Antimicr* **3**:11. doi: 10.1186/1476-0711-11-11.

Jung HA, Park JJ, Nurul Islam Md, Jin SE, Min BS, Lee JH, Sohn HS, Choi JS. 2012. Inhibitory Activity of Coumarins from *Artemisia capillaris* against Advanced Glycation End product Formation. *Arch Pharm Res* **35:** 1021-1035.

Lee CL, Chiang LC, Cheng LH, Liaw CC, Abd El-Razek MH, Chang FR, Wu YC, 2009. Influenza A (H1N1) antiviral and cytotoxic agents from *Ferula assa-foetida*. *J Nat Prod* **72**: 1568–1572.

Kapoor LD, 2001. Handbook of Ayurvedic Medicinal Plants, first ed. CRC Press, New York.

Kaur A, Kaur PK, Singh S, Singh IPZ Naturforsch C. 2014. Antileishmanial compounds from *Moringa oleifera* Lam. *Z Naturforsch C* **69**: 110-116.

Kayani S, Ahmad S, Zafar M, Sultana S, Khan MPZ, Ashraf MA, Javid Hussain J, Yaseen G, 2015. Ethnobotanical uses of medicinal plants for respiratory disorders among the inhabitants of Gallies-Abbottabad, Northern Pakistan. *J Ethnopharmacol* **164**: 186–202.

Kensa M, 2011. Floristic study in a Vembanur wetland, Kanyakumari District, Tamilnadu, South India. *Plant Sci Feed* **1**: 194–199.

Khan N, Ahmed M, Ahmad A , Shaukat SS, Wahab M, Ajaib M, Siddiqui MF, Nasir M. 2011. Important medicinal plants of Gol national Park (CGNP), Pakistan. *Pak J Bot* **43**: 797-809

Khan M, Hussain F, Musharaf S. 2013. Ethnobotanical profile of Tehsil Takht-e-Nasratti, District Karak, Pakistan. *J Med Plant Res* **7**: 1636-1651.

Khan, I.Z., Aqil, M., 1993. Isolation and identification of Pectolinarine and mannitol from Kickxia ramosissima (Wall). *Chem Environ Res* **2**: (3&4), 287–289.

Khan, I.Z., Aqil, M., Kolo, B.G., 2001. A new flavone glycoside from *Kickxia ramosissima* (Wall). *Ultra-Physical Sci* 13: 112–115.

Khangholi S, Fadzilah AAMF, Jabbar ABN, Ahmad F, Abd Aziz RA. 2016. The Mechanisms of Inhibition of Advanced Glycation End Products Formation through Polyphenols in Hyperglycemic Condition. *Planta Med* **82**: 32–45.

Khattak NS, Nouroz F, Rahman IU, Noreen S. 2015. Ethno veterinary uses of medicinal plants of district Karak, Pakistan. *J Ethnopharmacol* **171**: 273–279.

Kitdamrongtham W, Manosroi A, Akazawa H, Gidado A, Stienrut P, Manosroi W, Warangkana L, Toshihiro A, Manosro J. 2013. Potent anti-cervical cancer activity: synergistic effects of Thai medicinal plants in recipe N040 selected from the MANOSROI III database. *J Ethnopharmacol* **149**: 288-296.

Kronenberg HM, Melmed S, Polonsky KS, Larsen PR. 2008., *Williams Textbook of Endocrinology*, Saunders, Philadelphia, Pa, USA, 11th edition.

Kvist LP, Christensen SB, Rasmussen HB, Mejia K, Gonzalez A. 2006. Identification and evaluation of Peruvian plants used to treat malaria and leishmaniasis. *J Ethnopharmacol* **106**: 390-402.

Lee EH, Song DG, Lee JY, Pan CH, Um BH, Jung SH. 2008. Inhibitory effects of compounds isolated from *Rhus verniciflua* on Aldo-reductase and advanced glycation end products. *Pharm Bull* **31**: 1626-1630.

Lipinski B. 2001. Pathophysiology of oxidative stress in diabetes mellitus. *J Diabetes Complications* **15**: 203–210.

Mbaze LM, Poumale HMP, Wansi JD, et al. 2007. α-Glucosidase inhibitory pentacyclic triterpenes from the stem bark of *Fagara tessmannii* (Rutaceae). *Phytochemistry* **68**: 591–595.

Mahmood A, Shaheen H, Qureshi RA, Sangi Y, Gilani SA, 2011_b. Ethnomedicinal survey of plants from district Bhimber Azad Jammu and Kashmir, Pakistan. *J Med Plant Res* **5**: 2348–2360.

Maridass M, De Britto AJ. 2008. Origins of Plant Derived Medicines. *J Ethnobot Leaflet* **12**: 373-387.

Marwat SK, Rehman UR, Khan MA, Ahmad M, Zafar M, Ghulam S. 2011_a. Medicinal folk recipes used as traditional phototherapies in District dear Ismail Khan, KPK, Pakistan.. *Pak J Bot* **43**: 1453-1462.

Marwat SK, Khan MA, Ahmad M, Zafar, Rehman UR. 2008. Ethanophytomedicine for treatment of various Diseases in D.I.khan District. *Sarhad J Agri* **24**:305-315.

Marwat SK., Khan MA, Rehman UR, , Ahmad M, Zafar M.2011$_b$. Biodiversity and importance of floating weeds of Dera Ismail Khan District of KPK, Pakistan. *Afr J Tradit Complement Altern Med* **8**: 97-107.

Matsuda H, Wang T, Managi H, Yoshikawa M. 2003. Structural requirements of flavonoids for inhibition of protein glycation and radical scavenging activities. *Bioorg Med Chem* **11**: 5317–5323.

Matteucci E, Giampietro, O. 2000. Oxidative stress in families of type 1 diabetic patients. *Diabetes Care* **23**: 1182–1186.

Mesia GK. 2009. Study on safety and efficacy of an oral standardized extract from *Nuclea pobeguinii* used in the treatment of uncomplicated malaria in Democratic Republic of Congo. Doctoral dissertation, Universiteit Antwerpen, Department Farmaceutische Wetenschapen, Antwerpen, Belgium.

Mori K, Kawano M, Fuchino H, Ooi T, Satake M, Agatsuma Y, Kusumi T, Sekita S. 2008. Antileishmanial Compounds from *Cordia fragrantissima* Collected in Burma (Myanmar) *J Nat Prod* **71**: 18–21.

Mosihuzzman M, Naheed S, Hareem S, Talib S, Abbas G, Khan SN., Choudhary MI, Sener B, Tareen RB, Israr M. 2013. Studies on α-glucosidase inhibition and anti-glycation potential of *Iris loczyi* and *Iris unguicularis*. *Life Sci* **92**: 187-192.

Mothana RA, Al-Said MS , Al-Musayeib NM , El Gamal AA , Al-Massarani SM , Al-Rehaily AJ, Abdulkader A, Maes L. 2014. In Vitro Antiprotozoal Activity of Abietane Diterpenoids Isolated from *Plectranthus barbatus* Andr. *Int J Mol Sci* **15**: 8360-8371.

Munch GD, Schicktanz A, Behme M, Gerlach P, Riederer PD, Chinzel RS. 1999. Amino acid specificity of glycation and protein AGE crosslinking reactivities determined with a dipeptide SPOT library. *Nat Biotechnol* **17**: 1006-1010.

Murad W, Azizullah, Adnan M, Tariq A ,Ullah K, Waheed S., Ahmad A. 2013. Ethnobotanical assessment of plant resources of Banda Daud Shah, District Karak, Pakistan. *J Ethnobiol Ethnomed* **9**: 77 **doi:** 10.1186/1746-4269-9-77

Mussarat S. Nasser M. Salam AE., Akash T, Wazir SM, Ullah R., Adnan M. 2014. Use of Ethnomedicinal Plants by the People Living around Indus River. *Evid Based Complement Alternat Med*. Article ID 212634. doi:10.1155/2014/212634

Muzitano MF, Cruz EA, de Almeida AP, Da Silva SA, Kaiser CR, Guette C, Rossi-Bergmann B . 2006. Quercitrin: an antileishmanial flavonoid glycoside from *Kalanchoe pinnata*. *Planta Med* **72**: 81-83.

Nissanka APK, Karunaeatne V, Bandara BMR, Kumar V, Nakanishi T, Nishi M, Inada A, Tillekaratne LMV, Wijesundara DSA, Gunatilaka AAL. 2001. Antimicrobial alkaloids from *Zanthoxylum tetraspermum* and *caudatum*. *Phytochemistry* **56**: 857-861.

Nkunya MH, Makangara JJ, Jonker SA. 2004. Prenylindoles from Tanzanian *Monodora* and *Isolona* species. *Nat Prod Res* **18**: 253–258.

Newman DJ, Cragg GM. 2010. Natural Products As Sources of New Drugs over the 30 Years from 1981 to 2010. *J Nat Prod* **75**; 311−335.

Obeidat M. 2011."Antimicrobial activity of some medicinal plants against multidrug resistant skin pathogens," *J Med Plant Res* **5**: 3856–3860.

Odjakova M, Popova E, Al Sharif M, Mironova R. 2012. Glycosylation, Biochemistry, Genetics and Molecular Biology, ed. S. Petrescu, 2012, ISBN 978-953-51-0771-2, doi:10.5772/48186.

Ulrich P, Cerami A. 2001. Protein glycation, diabetes, and aging. *Recent Prog Horm Res* **56**: 1-21.

Panda A, Misra MK. 2011. Ethnomedicinal survey of some wetland plants of South Orissa and their conservation. *Ind J Trad Knowl* **10**: 296–303.

Pandya PN, Aghera HB, Ashok BK. 2012. Diuretic activity of *Linaria ramosissima* (Wall.) Janch leaves in albino rats. *J AYU* **33**: 576-578

Patel BI and Sachdeva PD. 2014. Antidiabetic activity of *linaria ramosissima* (wall) Janch in Streptozotocin induced Diabetic Rats. *Int J Pharm Pharm Sci* **6**:166-171.

Peng Xi, Zheng Z, Cheng KW, Shan F, Ren GX, Chen F, Wang M. 2008. Inhibitory effect of mung bean extract and its constituents vitexin and isovitexin on the formation of advanced glycation Endproducts. *Food Chem* **106**: 475–481.

Pieters L, Vlietinck AJ. 2005. Bio-guided isolation of pharmacologically active plant components, still a valuable strategy for the finding of new lead compounds? *J Ethnopharmacol* **100**: 57–60.

Playford RJ, Pither C, R. Gao, et al. 2013. Use of the α-glucosidase inhibitor acarbose in patients with 'Middleton syndrome': normal gastric anatomy but with accelerated gastric emptying causing postprandial reactive hypoglycemia and diarrhea, *Can J Gastroenterol* **27:** 403–404.

Povichit N, Phrutivorapongkul A, Suttajit M, Leelapornpisid P. 2010. Antiglycation and antioxidant activities of oxyresveratrol extracted from the heartwood of *Artocarpus lakoocha* Roxb. *Maejo Int J Sci Technol* **4**: 454-461.

Qureshi R, Bhatti GR. 2008. Ethnobotany of plants used by the Thari people of Nara Desert, Pakistan. *Fitoterapia* **79**: 468–473.

Qureshi R. 2012. Medicinal flora Of Hingol National Park, Baluchistan, Pakistan. *Pak J Bot* **44**: 725–732.

Reddy GK. 2004. Cross-linking in collagen by non-enzymatic glycation increases the matrix stiffness in rabbit a chilles tendon. *Exp Diabesit Res* **5**: 143-153.

Rehman K, Mashwani ZUR , Khan MA, Ullah Z ,Chaudhary HJ. 2015. An ethno botanical perspective of traditional medicinal plants from the Khattak tribe of Chonthra Karak, Pakistan. *J Ethnopharmacol* **165**: 251–259.

Ruiz-Irastorza G, Ramos-Casals M, Brito-Zeron P, Khamashta MA.2010. Clinical efficacy and side effects of antimalarials in systemic lupus erythematosus: a systematic review. *Ann Rheum Dis* **69**:20-28.

Sakai R, Higa T, Jefford CW, Bernardinelli G. 1986. Manzamine A, a novel antitumor alkaloid from a sponge. *J Am Chem Soc* **108:** 6404-6405.

Savoia D. 2012. Plant-derived antimicrobial compounds: alternatives to antibiotics. *Future Microbiol* **7**: 979-90.

Schmidt AM, Hori O, Brett J, Yan SD, Wautier JL, Stern D. 1994. Cellular receptors for advanced glycation end products. Implications for induction of oxidant stress and cellular dysfunction in the pathogenesis of vascularlesions. *Arterioscler Thromb* **14**: 1521–1528.

Shah GM, Abbasi AM, Khan N, Guo X, Khan MA, Hussain M, Bibi S, Nazir A, Ahmad AT. 2014. Traditional uses of medicinal plants against malarial disease by the tribal communities of Lesser Himalayas–Pakistan. *J Ethnopharmacol* **155**: 450–462.

Shah SM, Hussain F. 2012. Ethnomedicinal plant wealth of Mastuj valley, Hindukush range, District Chitral, Pakistan. *J Med Plant Res* **6**: 4328-4337.

Sharma A, Shanker C, Tyagi LK, Singh M, Rao CV. 2008. Herbal medicine for market potential in India: an overview. *Academic J Plant Sci* **1**:26–36.

Sharma PV. 1994. *Caraka Samhita*. Chaukhamba Orientalia, Varanasi, India.

Sher H, Abbasi A, Ali A, de Boer HJ. 2015. Indigenous knowledge of folk medicines among tribal minorities in Khyber Pakhtunkhwa, north western Pakistan. *J Ethnopharmacol* **166**: 157–167.

Shinwari ZK, Gilani SS, Kohjoma M, Nakaike T. 2000. Status of medicinal plants in Pakistani Hindukush Himalayas. Proc. Nepal – Japan Joint Symposium, pp. 235-242.

Shinwari ZK. 2010. Medicinal plants research in Pakistan. *J Med Plants Res* **4**: 161–176.

Shinwari, Z.K., S.S. Gilani and M. Shoukat. 2002. Ethnobotanical resources and implications for curriculum, In: Proc. Workshop *Curr Develop Appl Ethnobot* pp. 21-33.

Tasdemir D, Kaiser M, Brun R, Yardley V, Schmidt TJ, Tosun F, Ruedi P. 2006. Antitrypanosomal and Antileishmanial Activities of Flavonoids and Their Analogues: In Vitro, In Vivo, Structure-Activity Relationship, and Quantitative Structure-Activity Relationship Studies. *Antimicrob Agents Chemother* **50**: 1352–1364.

Thornalley PJ, Battah S, Ahmed N, Karachalias N, Agalou S, Babaei-Jadidi R, Dawnay N. 2003. Quantitative screening of advanced glycation endproducts in cellular and extracellular proteins by tandem mass spectrometry. *Biochem J* **375**: 581-592.

Thornalley PJ, Langborg A, Minhas HS. 1999. Formation of glyoxal, methylglyoxal and 3-deoxyglucosone in the glycation of proteins by glucose. *Biochem J* **344**: 109-16.

Tiwari P. 2015. Recent Trends in Therapeutic Approaches for Diabetes Management: A Comprehensive Update. *J Diabetes Res*. Article ID 340838.
doi.org/10.1155/2015/340838.

Tsuchiya H., Sato M., Miyazaki T., Fujiwara S., Tanigaki S., Ohyama M., Tanaka T., Linuma, M. 1996. Comparative study on the antibacterial activity of phytochemical flavanones against methicillin resistant Staphylococcus aureus. *J Ethnopharmacol* **50:** 27-34.

Ullah M, Khan MS, Mahmood, Malik RN, Hussain, M, Wazir SM, Daud M, Shinwari ZK. 2013. An ethnobotanical survey of indigenous medicinal plants in Wana district south Waziristan agency, Pakistan. *J Ethnopharmacol* **150**: 918–924.

Vitalini S, Iriti M, Puricelli C, Ciuchi D, Segale A, Fico G. 2013.Traditional knowledge on medicinal and food plants used in Val-San-Giacomo (Sondrio, Italy), an alpine ethnobotanical study. *J Ethnopharmacol* **145**: 517–529.

Walsh C. 2003. Where will new antibiotics come from? *Nat Rev Microbiol* **1**: 65-70.

Wang AL, Li Z, Yuan M, Yu, ACH, Zhu XA, Tso MOM. 2007. Sinomenine inhibits activation of rat retinal microglia induced by advanced glycation end products. *Int Immunopharmacol* **7**: 1552–1558.

Wang ZW, Wang JS, Luo J, Kong LY. 2013. α-Glucosidase inhibitory triterpenoids from the stem barks of *Uncaria laevigata*, *Fitoterapia* **90**: 30–37.

Wells-Knecht KJ, Zyzak DV, Litch JE, Thorpe SR, Baynes JW. 1995. Mechanism of autoxidative glycosylation: identification of glyoxal and arabinose as intermediates in the autoxidative modification of proteins by glucose. *Biochemistry* **34**: 3702-3709.

West BJ, Uwaya A, Isami F, Deng S, Nakajima S, Jensen CJ. 2014. Antiglycation Activity of Iridoids and Their Food Sources. *Int J Food Sci* Article ID 276950.
doi:/10.1155/2014/276950.

White NJ. 2008. *Plasmodium knowlesi*: the fifth human malaria parasite. *Clin Infect Dis* **46**: 172–173.

WHO, 1999. Definition, Diagnosis and Classification of Diabetes Mellitus and Its Complications, World Health Organization, Department of Non Communicable Disease Surveillance, Geneva, Switzerland, 1999.

World Health Organization (WHO), 2002$_a$. WHO Traditional Medicine Strategy, WHO, Geneva (2002).

WHO, 2012$_a$. World malaria report 2012. Geneva: 2012.

WHO, 2002$_b$. World Health Organization: *Traditional medicine strategy 2002– 2005.* 2002 [http://whqlibdoc.who.int/hq/2002/ WHO_EDM_TRM_2002.1.pdf].

WHO, 2008. World Health Organisation.(2008, December). Traditional Medicine. Retrieved 14 January, 2016, from World Health Organisation. http://www.who.int/mediacentre/factsheets/2003/fs134/en/.

WHO. 2014. Strategic framework for leishmaniasis control in the WHO European Region 2014–2020. http://www.euro.who.int/_data/assets/pdf_file/0017/245330/Strategic-framework-for-leishmaniasis-control-in-the-WHO-European-Region-20142020.pdf.

WHO, 2015. Regional Office for Eastern Mediterranean. Malaria control and elimination. Available at: http://www. emro.who.int/pak/programmes/roll-back-malaria.html. (Accesseed 20 january 2016).

Wu JW, Hsieh CL, Wang HY, Chen HY. 2009$_a$. Inhibitory effects of guava (*Psidium guajava* L.) leaf extracts. *Food Chem* **113**: 78-84.

Wu CH, Lin JA, Hsieh WC, Yen GC. 2009$_b$. Low-density-lipoprotein (LDL)-bound flavonoids increase the resistance of LDL to oxidation and glycation under pathophysiological concentrations of glucose in vitro. *J Agric Food Chem* **57**: 5058-5064.

Yaseen G, Ahmad M, Sultana S, Alharrasi AS, Hussain J, Zafar M, Rehman SU. 2015. Ethnobotany of Medicinal Plants in the Thar Desert (Sindh) of Pakistan. *J Ethnopharmacol* **163**: 43–59.

Yenesew A, Induli M, Derese S, Midiwo JO, Heydenreich M, Peter MG, Akala H, Wangui J, Liyala P, Waters NC. 2004. Anti-plasmodial flavonoids from the stem bark of Erythrina abyssinica. *Phytochemistry* **65**: 3029–3032.

Yin Z, Zhang W, Feng F, Zhang Y, Kang W. 2014. α-Glucosidase inhibitors isolated from medicinal plants. *Food Sci Human Wellness* 3: 136–174.

CHAPTER 2

GENERAL EXPERIMENTAL METHODS

2.1 CHROMATOGRAPHIC METHODS

2.1.1 Solvents and reagents

All solvents including *n*-hexane, ethyl acetate, *n*-butanol, methanol, absolute ethanol (99.99%), isopropyl alcohol and methylene chloride were analytical grade and obtained from Fisher Scientific (Leicestershire, UK) and Acros Organics (Geel, Belgium). All reagents such as acetic acid glacial (≥97%), sulphuric acid (≥95%), hydrochloric acid (HCL) (≥95%), potassium hydrogen phosphate (≥98%), potassium dihydrogen orthophosphate (≥95%), disodium hydrogen phosphate(≥99%), sodium hydrogen phosphate (≥95%), sodium hydroxide (≥98%) sodium carbonate (99%), formic acid (≥98%), dimethyl sulfoxide (DMSO) (≥95%) and trichloroacetic acid (TCA) (≥99%) were purchased from Acros Organics or Sigma-Aldrich (St. Louis, MO, USA). The solvents for HPLC i.e methanol (MeOH) and acetonitrile were HPLC grade and purchased from Fisher Scientific (Leicestershire, UK). RiOS water was prepared by reverse osmosis and water for HPLC was dispensed by a Milli-Q system, both from Millipore (Bedford, MA, USA). Water was passed through a 0.22 μm membrane filter before usage. Solvents used for GC-MS were purchased from Sigma-Aldrich including BSTFA + TMCS, 99:1 (N,O-bis (trimethylsilyl) trifluoroacetamide and trimethylchloro silane) (Supelco, USA), pyridine (dry, 99.8%) (Seccosolv).

The reference materials such as aminoguanidine (≥99%), quercetin (≥98%), glucose (≥99%), 2,2′-azobis(2-methylpropionamidine) dihydrochloride (AAPH) (≥97%), Trolox (≥97%), linoleic acid (99%), bovine serum albumin (BSA) (≥98%), methylglyoxal (MGO) (≥99%), 2,2-diphenyl-1-picrylhydrazyl (DPPH) (≥99%), α-glucosidase (≥100 units/mg protein), Pnp (*p*-nitrophenyl-α-D-glucopyranoside (≥99%), acarbose(≥95%), sodium azide (99%) and 15-lipoxigenase from *Glycine max* (soybean) (≥50,000 units/mg solid) were purchased from Sigma-Aldrich (St. Louis, MO, USA).

2.1.2 General extraction scheme

The standard extraction scheme used during the project is shown below (Fig. 2.1).

2.1.3 Thin layer chromatography

Analytical plates for thin layer chromatography (TLC) were silica gel 60 F_{254} plates (20 x 20 cm) for normal phase (Merck, Darmstadt, Germany). The spraying reagent *p*-anisaldehyde was prepared by mixing 0.5 mL *p*-anisaldehyde (Sigma-Aldrich) with 10 mL glacial acetic acid, 85 mL methanol and 5 mL sulphuric acid.

Dragendorff's reagent was prepared by mixing solution A and B. Solution A was prepared by adding of 1.7 g bismuth subnitrate in 100 mL water/acetic acid (4:1). Solution B was prepared by dissolving 40 g potassium iodide in 100 mL water. Solution A and B were combined, as follows: 5 mL A + 5 mL B + 20 mL acetic acid + 70 mL water.

The cerium sulphate reagent was prepared by mixing 1g $CeSO_4$ and 10g of trichloroacetic acid (TCA) with 4 mL of 15% H_2SO_4 initially. This was mixed thoroughly until a slurry was formed. Then the slurry was stirred using a magnetic stirrer on a hot plate at 60 °C and 15% H_2SO_4 solution was slowly added . The process was continued until a clear solution was obtained.

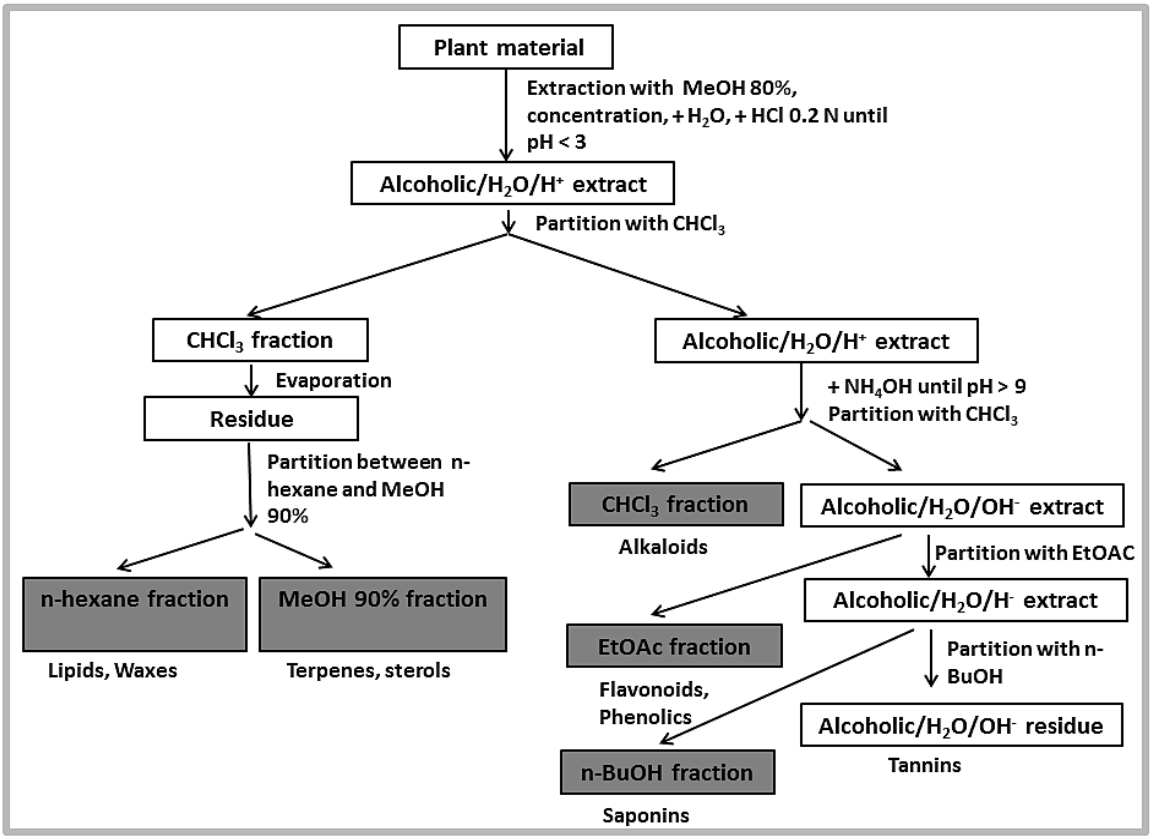


Figure 2.1 Schematic presentation of liquid-liquid fractionation

2.1.4. Flash Chromatography

Flash column chromatography was performed on a Reveleris iES system from Grace (Columbia, MD, USA) using the Reveleris® Navigator ™ software. The system is equipped with a binary pump with four solvent selection, an ultraviolet (UV) and evaporating light scattering detector (ELSD) and a fraction collector. The column used was a pre-packed Flash Grace Reveleris silica cartridge (80 g) with a particle size of 40 µm. The ELSD carrier solvent was isopropyl alcohol.

2.1.5. High performance liquid chromatography

HPLC analysis was carried out on an Agilent® 1200 series system with degasser, quaternary pump, automatic injection, thermostatic column compartment and a diode array detector (DAD) (Agilent® Technologies, Santa Clara, California, USA). A silica based Gracesmart C_{18} column (250 x 4.6 mm, 5 µm) (Grace Vydac, USA) and Phenomenex luna C_{18} (250 x 4.6 mm, 5 µm) (Phenomenex, Torrence, CA, USA) was used together with a suitable precolumn to endure the lifetime of columns.

2.1.6. Semi-preparative high performance liquid chromatography

The isolation of compounds was carried out on a semi-preparative HPLC-DAD-MS system (Waters®) using a Luna 5µm (C_{18}) 100A 250 X 10.0 mm column (Phenomenex™) and Masslynx® 4.1 software. The system was equipped with HPLC pump 515 (Waters™ 2767), make-up pump (Waters™ 511), system fluid organiser (SFO), diode array detector (DAD) (Waters™ 2998), triple quadrupole mass spectrometer (TQD-MS) and automatic fraction collector was used to isolate compounds.

2.2. SPECTROSCOPIC METHODS

2.2.1 Nuclear magnetic resonance spectroscopy

NMR spectra were recorded on a Bruker DRX-400 instrument (Rheinstetten, Germany), operating at 400 MHz for ^{1}H and at 100 MHz for ^{13}C, using a 3-mm broadband inverse (BBI) probe or a 5-mm dual $^{1}H/^{13}C$ probe using standard Bruker pulse sequences. "Distortionless Enhancement by Polarization Transfer" spectra (DEPT-135, DEPT-90) were also recorded. The chemical shifts are given in ppm and coupling constants (*J*) in Hz. The multiplicity is indicated as s for singlet, d for doublet, t for triplet, q for quartet

and m for multiplet. Additionally two dimensional NMR experiments (2D-NMR) were performed where necessary. The 2D-NMR experiments included correlation spectroscopy (COSY) for ^{1}H-^{1}H correlation, heteronuclear single quantum coherence (HSQC) for direct ^{1}H-^{13}C correlation and heteronuclear multiple bond coherence (HMBC) for long-range ^{1}H-^{13}C correlation. Structure elucidation was assisted by a ^{13}C-NMR database (NMR Predict version 4.8.57, Modgraph®). Deuterated solvents including $CDCl_3$ (99.8% D), DMSO-d_6 (99.9 % D) and CD_3OD (99.8% D) were purchased from Sigma-Aldrich.

2.2.2 Mass Spectrometry

High resolution mass spectra was obtained with an Agilent™ 6530 quadrupole-time-of-flight mass spectrometer (QTOF-MS) equipped with an Agilent™ Jetstream source that was used with the following parameters: gas temperature 325 °C, sheath gas flow 11L/min. Capillary, fragmentor and skimmer voltages were set to 3500 V, 150 V and 65 V, respectively, and OCT 1RF Vpp was set at 750 V. The mass spectrometer was operated in positive and negative ion mode at 20000 resolution. The instrument was calibrated and tuned with a tune mix (G1969-85000) and during acquisition the accuracy was monitored by using ES-TOF reference mass solution kit (G1969-85001) from Agilent™. Mass Hunter® (Agilent™ Technologies) software was used for acquisition and processing.

2.2.3 GC-MS analysis

GC experiments were performed on a TRACE® 2000 Ultra GC (Thermo Scientific) on a column AT-5MS (Grace): length 30 m; ID: 0.25 mm; film thickness: 0.25 µm. Derivatisation was performed on dried samples using 20 µL of BSTFA + TMCS, 99 : 1 (Supelco, USA) in pyridine (dry) (Seccosolv) in a molar ratio 2-1, by heating for 1h at 70°C.

2.2.4 Optical rotation

The specific optical rotation was determined on a Jasco P-2000 polarimeter. The samples were dissolved in methanol and optical rotation was recorded at 589 nm with a path length of 50 mm.

2.2.5 Others

UV-Vis (ultraviolet–visible light) absorbance was measured on a Genesys-10UV (Thermoscientific) spectrophotometer. Fluorescence (excitation 335 nm, emission 385 nm) was measured on a Tecan™ Infinite M200 spectrofluorometer.

2.3 PHYTOCHEMICAL SCREENING

Phytochemical screening for various compound classes was carried out on every fraction using published methods with slight modifications (Sofowora, 1993, Trease and Evans, 1989, Herborne, 1973).

2.3.1 Chemical test for alkaloids

About 0.2 g of the extract was added to 2 mL of 2% H_2S0_4 and the mixture was slightly heated on a water bath for 2-3 min. The mixture was filtered and 4-5 drops of Dragendorff's reagent were added. Orange-red precipitate indicated the presence of alkaloids.

2.3.2 Chemical test for tannins

About 0.2 g of extract was mixed with 10 mL distilled water in a test tube and heated on a water bath. The mixture was filtered and 4-5 drops 0.1% ferric chloride ($FeCl_3$) were added to the filtrate. A dark green or blue-green coloration indicated the presence of tannins.

2.3.3 Chemical test for saponins

About 0.2 g of the extract was shaken with 5 mL of distilled water and then heated to boiling. Appearance of foam indicated the presence of saponins.

2.3.4 Chemical test for flavonoids

About 0.2 g of extract was dissolved in 2 mL of 10% NaOH. A change from yellow to a colourless solution observed after addition of 0.5 mL HCl, indicated the presence of flavonoids.

2.3.5 Chemical test for steroids

About 0.2 g of the extract was mixed with 2 mL of acetic anhydride. To this mixture, 1 mL of H_2S0_4 conc. was added. The colour change from violet to blue or green indicated the presence of steroids.

2.3.6 Chemical test for terpenoids (salkowski test)

About 0.2 g of extract was mixed with 2mL of chloroform and 3mL H_2S0_4 conc. was carefully added to form a layer. A reddish-brown colouration of the interface indicated the presence of terpenoids.

2.3.7 Total phenolic contents (TPC)

The total phenolic content (TPC) of all fractions was estimated using Folin-Ciocalteu's reagent according to Bursal and Gulcin (2011) with slight modifications. Briefly, 10 µL of each sample (0.2 mg/mL) or reference compound (gallic acid 0.0156–0.5 mg/mL) was mixed with 100 µL of Folin-Ciocalteu's reagent, and incubated at room temperature and after 5 min, 90 µL of 10% Na_2CO_3 was added to the above mixture. These mixtures were then incubated at room temperature for 40 min. The absorbance was measured at 765 nm. TPC was expressed as gallic acid equivalents (GAE). Based on findings of Dudonne *et al*. (2009), TPC were categorized as very high (> 300 mg GAE/g), high (200 - 300 mg GAE/g), moderate (50-200 mg GAE/g), low (15-50 mg GAE/g), very low (<15 mg GAE/g).

2.3.8 Total flavonoid contents (TFC)

The total flavonoid content (TFC) of all fractions was assessed according to Sun *et al*. (2011). Briefly, 20 µL of each sample (1 mg/mL) or reference compound (rutin) was mixed with 30 µL of 5% sodium nitrite. After 6 min, 50 µL of a 10% $AlCl_3$ solution was added, and the mixture was kept untouched for 5 min. Next, 100 µL of a 10% NaOH solution was added, the mixture was incubated at 25°C for 15-20 min, and the absorbance was measured at 510 nm. TFC was expressed as rutin equivalents (RUE). TFC were categorized as very high (>300 mg RUE/g), high (200-300 mg RUE/g), moderate(50-200 mg RUE/g), low (15-50 mg RUE/g), very low (<15 mg RUE/g).

2.4. BIOLOGICAL ACTIVITIES

2.4.1 Antiglycation assay

Glucose-mediated protein glycation models are generally used to determine AGE inhibition. However, AGE-protein adducts can be formed both under oxidative and non-oxidative conditions, and therefore AGEs inhibitors should be differentiated from common antioxidants (Rahbar, 2007). Evaluation of the inhibition of AGEs formation starting from glucose and BSA involves all possible mechanisms. However, Reactive Carbonyl Substances (RCS) such as glyoxal and methyl glyoxal that are intermediate products of the Maillard reaction, have already undergone the oxidation processes starting from glucose. Inhibition of their reaction with BSA is representative of non-oxidative glycation reactions.

2.4.1.1 Antiglycation assay (BSA-Glucose assay)

The antiglycation assay was performed according to the method developed by Matsuura *et al.* (2002) with minor modifications. The reaction mixture (300 µL) contained bovine serum albumin (BSA) (10 mg/mL, 135 µL), D-glucose (500 mM, 135 µL) dissolved in phosphate buffer (50 mM, pH 7.4, containing sodium azide (0.02%, added to prevent bacterial growth) and test compounds (30 µL), at different final concentrations (1.5 – 0.023 mM for isolated constituents or 100–1.17 µg/mL for extracts) dissolved in 100 % DMSO. The mixtures were incubated at 60°C for 48 h. After incubation, the reaction mixture was allowed to cool down at room temperature. Then 100 µL reaction mixture was transferred to a new plastic tube (1.5 mL) and the reaction was stopped by adding 10 µL of 100% (w/v) trichloroacetic acid (TCA) and to precipitate proteins. The TCA-added mixture was kept at 4°C for 10 min, followed by centrifugation (14000 rpm, 4 °C, 4 min). The supernatant containing unreacted D-glucose, test sample and interfering substances was discarded, whereas the precipitate containing AGEs-BSA was redissolved with 0.8 mL alkaline PBS (137 mM NaCl, 8.1 mM Na_2HPO_4, 2.68 mM KCl, 1.47 mM KH_2PO_4, pH 10). The change in fluorescence intensity (excitation 335 nm, emission 385 nm; excitation 370, emission 440) due to formation of AGEs was monitored by spectrofluorometry (Tecan™ Infinite M200, Giessen, The Netherlands). In order to eliminate interference by autofluorescence of the test compounds, a parallel incubation of test substance with BSA at 60°C without D-glucose was performed for all

samples. Quercetin (0.5- 0.0312 mM final concentration) and aminoguanidine (3 - 0.046 mM final concentration) were used as reference compounds.

The AGEs inhibition was calculated as

$$\% \text{ inhibition} = \{1- (F_{BSA + glucose + test substance} - F_{BSA} + _{test substance}) / (F_{BSA + glucose} - F_{BSA})\} \times 100$$

where F is the fluorescence intensity. The concentration required for 50% inhibition (IC_{50}) was calculated using using Sigma plot® 13.0. The AGEs inhibition was categorized as mild (1-25%), moderate (26-50%), high (50-70%), and very high (70-100%).

2.4.1.2 Protein-glyoxal interaction (BSA-MGO assay)

The antiglycation assay using methylgloxyl (MGO) was performed according to the method developed by Peng *et al*. (2007) with slight modifications. Briefly, methylglyoxal (135 µL, 5.75 mM) was incubated with BSA (135 µL, 10 mg/mL) dissolved in phosphate buffer (50 mM, pH 7.4, containing sodium azide (0.02%), added to prevent bacterial growth) and test compounds (30 µL) at different final concentrations (1.5 – 0.023 mM) in 100% DMSO. The reaction mixture was incubated at 37 °C for one week. Control solutions only contained methylglyoxal (135 µL, 5.75 mM) BSA (135 µL, 10mg / mL) and DMSO (100%) only. The blank samples of similar composition were also prepared simultaneously and kept at 4 °C for one week. The change in fluorescence intensity (excitation 335 nm, emission 385 nm; excitation 370, emission 440) due to formation of AGEs was monitored by spectrofluorometry (Tecan® Infinite M200, Giessen, The Netherlands). Aminoguanidine (3-0.046 mM final concentration) was used as reference compound ,

The AGEs inhibition was calculated as

$$\% \text{ AGEs inhibition} = [1-(S-Sb)/(C-Cb)] \times 100$$

where S and C were fluorescence of test samples (in DMSO) and control (test mixtures containing only DMSO) incubated at 37 °C, and where Sb and Cb were fluorescence for

samples incubated at 4 °C. The concentration required for 50 % inhibition (IC_{50}) was calculated using using Sigma plot® 13.0.

2.4.2 Inhibition of α-glucosidase.

α-Glucosidase inhibitory activity was assayed according to method adopted by Choudhary *et al*. (2010) with some modifications. Briefly, 50 µL of a solution of α-glucosidase from *Saccharomyces cerevisiae* (0.2 U/mL in 0.1 M phosphate buffer at pH 6.8, Sigma-Aldrich, St. Louis, MO, USA) was incubated with 20 µL of test compounds or extracts at different concentrations (ranging from 6 mM – 0.372 mM in 25% DMSO for isolated constituents or from 5 mg to 0.039 mg/mL for extracts) at 37 °C for 10 min. Then 50 µl of the substrate, *p*-nitrophenyl-α-D-glucopyranoside (0.7 mM stock, final concentration 0.29 mM) was added to the reaction mixture and incubated again for 30 min at 37 °C. The final concentrations of test substances in the reaction mixture ranged from 1 – 0.062 mM for isolated constituents, and from 834 µg to 6.5 µg/mL for extracts. The reaction was stopped by adding Na_2CO_3 (100 µl, 200 mM stock) solution and the absorbance was measured at 400 nm. Final DMSO concentration remained below 7.5% during the assay. Acarbose (1 – 0.031 mM final concentration) was used as reference compound.

The % inhibition was calculated using the following formula:

$$\% \text{ Inhibition} = 100 - [OD_{(\text{test sample})} / OD_{(\text{control})}] \times 100$$

Alpha-glucosidase inhibition was categorised as mild (1-25%), moderate (26-50%), high (50-70%), and very high (70-100%).

2.4.3 DPPH radical scavenging activity.

The ability of the crude extract or fractions to scavenge DPPH radicals was evaluated according to Szabo *et al*. (2007) with some modifications. An aliquot (50 µL) of test sample (prepared in a concentration range (3.84 – 0.12 mg/mL in methanol) or quercetin (reference compound with stock concentration ranging from 1.08 – 0.0312 mg/mL in methanol) was mixed with PBS (450 µL, 10 mM, pH 7.4) and 1.0 mL of a methanolic solution of DPPH (0.1 mM), yielding a series of test samples in a final

concentration range of 128 – 4 µg/mL) and a series of quercetin samples in a final concentration of (1 – 36 µg/mL). After 30 min the absorbance was recorded at 517 nm and % inhibition was calculated using the following formula:

DPPH radical scavenging activity (%) $= [(OD_{control} - OD_{sample}) / OD_{control}] \times 100$

Based on findings of Dudonne *et al*. (2009), DPPH inhibition was categorized as very low (1-10%), low/week (10%-30%), moderate (30-60%), high (61-69%) and very high (70-100%).

2.4.4. Inhibition of 15-lipoxygenase

The soybean lipoxygenase (LOX) assay is used as an indication of anti-inflammatory activity (Muller, 1994). LOX is a key enzyme in the inflammatory cascade, whose inhibition is correlated to the ability of the inhibitors to reduce Fe^{3+} at the active site to the catalytically inactive Fe^{2+}. LOXs contain a "non-heme" iron per molecule in the enzyme active site as high-spin Fe^{+2} in the native state and the high-spin Fe^{+3} in the activated state. Several LOX inhibitors, such as phenolic derivatives, are excellent ligands for Fe^{3+}. The enzyme 15-lipoxygenase has consistently been reported as involved in oxidation of low-density lipoproteins (LDL) that is responsible for expansion of atherosclerosis (Steinberg, 1999).

The iridoids are more particularly known for their anti-inflammatory activities (Park *et al*., 2010; Gousiadou *et al*., 2013). As the major constituents of *Kickxia* were iridoids, it was therefore considered interesting to test the isolated iridoids for their potential to inhibit 15-lipoxygenase. Also flavonoids are reported to possess lipoxygenase inhibitory (Lyckander and Malterud, 1996; Malterud and Rydland, 2000), it was therefore decided also to investigate the isolated flavonoids in the 15-LOX assay.

15-LOX from soybean was used for peroxidation of linoleic acid, and inhibition was carried out as described previously with slight modifications (Malterud and Rydland 2000). Briefly, 12.5 µL (2 - 0.062 mM) of sample, dissolved in DMSO, was added to 487.5 µL of enzyme solution (200 U/mL). The mixture was incubated at room temperature for

5 min. Absorbance was recorded immediately after the addition of 500 µL of substrate (250 µM linoleic acid in 0.2 M borate buffer, pH 9) every min up to 5 min at 234 nm using a Genesys-10UV spectrophotometer.
% inhibition of enzyme activity was calculated as:

$$\% \text{ inhibition of enzyme activity} = ([\Delta A1/\Delta t] - [\Delta A2/\Delta t] / (\Delta A1/\Delta t) \times 100$$

where $\Delta A1/\Delta t$ and $\Delta A2/\Delta t$ are the increase rate in absorbance at 234 nm for sample without test substance and with test substance, respectively.

2.4.5 Inhibition of linoleic acid peroxidation

In general anti-inflammatory activity of compounds is believed to be the result of inhibition on the arachidonic acid (AA) pathway. AA is an unsaturated (C_{20}) fatty acid that is produced from membrane phospholipids. The derivatives of AA are potent mediators of inflammation. It is therefore considered that by inhibition of the biosynthesis of pro-inflammatory molecules, the inflammation can be reduced or ended by using antioxidants and anti-inflammatory drugs (Grantstrom 1984).

During the AAPH (2,2′-azobis(2-methylpropionamidine)-dihydrochloride) assay oxidative changes and the effect of the test compound are evaluated. AAPH (water soluble Azo compound) produces alkyl peroxyl free radicals through spontaneous thermal decomposition and induces linoleic acid peroxidation. Oxygen species are known to play a key role in various pathophysiological situations and also directly affect lipid peroxidation in membranes and cellular components.

The inhibition of linoleic acid lipid peroxidation was studied by a previously described method (Liegeois *et al*., 2000; Rajic *et al*., 2010). Briefly, 10 µL of a 16 mM linoleic acid dispersion and 10 µL of test compound (8-1 µM final concentration) was added to the UV cuvette containing 0.93 mL of 0.05 M phosphate buffer, pH 7.4. The oxidation reaction was initiated under air by the addition of 50 µL (40 mM) of AAPH solution. The rate of oxidation at 37 °C was monitored by recording the increase of absorption at 234 nm caused by conjugated diene hydroperoxides. The results were compared to the standard inhibitor (Trolox). The antioxidant power (AOP) is defined as the slope of the

curve representing the inhibition time of oxidation (T_{inh}) *vs.* the concentration of antioxidant and is expressed in min/µM.

2.4.6. Antimicrobial assays

The antimicrobial tests were performed in the Laboratory for Microbiology, Parasitology and Hygiene (LMPH) of the University of Antwerp. The primary *in vitro* evaluation of the samples was performed against a broad panel of pathogens to allow proper evaluation of selectivity. This approach involved integrated and robotic logistics to attain a reasonably high throughput. Compound stock solutions were prepared in DMSO (100%) at 20 mM (pure compounds) or 20 mg/mL (extracts or fractions). The compounds were then serially diluted (2 or 4 fold) in DMSO followed by a further (intermediate) dilution in demineralized water to ensure a final in-test DMSO concentration less than 1%. Test plates were identical for all screens and were produced as a single batch.

2.4.6.1 Antileishmanial activity

Leishmania infantum MHOM/MA(BE)/67 was maintained in Golden Hamster and spleen amastigotes were collected for preparing infected inocula. The primary peritoneal mouse macrophages were used as host cells and collected two days after peritoneal stimulation with a 2% potato starch suspension. The assay was performed in 96-microwell plates. Each well contained 10 µL of test compound, 190 µL of macrophage / parasite inoculum (3×10^5 cells and 3×10^6 parasite per well) and further incubated for 5 days at 37 °C and 5% CO_2. After five days the parasite burden (mean number of amastigotes/macrophage) was microscopically assessed after Giemsa staining, and expressed as a percentage of the blank controls without test compound. Fungizone was used as reference drug. (Cos *et al*., 2006; Balde' *et al*., 2010; Mesia *et al*., 2008).

2.4.6.2 Antitrypanosomal activity

Trypanosoma brucei Squib-427 strain (suramin-sensitive) was cultured at 37 °C and 5% CO_2 in Hirumi-9 medium (Hirumi and Hirumi,1989) supplemented with 10% fetal calf serum (FCS). The assay was performed in 96-microwell plates. Each well contains 10 microliter of test compound, 190 µL of trypomastogotes of an inoculation of 1.5×10^4

trypomastigotes/well . After an incubation of 72 h at 37°C under 5% CO_2. The parasite growth was assessed by adding 50 microliter/well resazurin (Raz *et al*., 1997). After 24h incubation at 37°C the fluorescence was measured (λ exi 550 nm, λ emi 590 nm). The results were expressed as % reduction in parasite growth compared to the blank controls. Suramine was used as reference drug.

For the Chagas disease, *Trypanosoma cruzi* Tulahuen CL2, β-galactosidase strain (nifurtimox- sensitive) was maintained on MRC-5 (human lung fibroblast) cells in minimum essential medium (MEM), supplemented with 200 mM L glutamine and 5% FCSi. All cultures and assays were conducted at 37 °C under 5% CO_2. The assays were performed using sterilized 96 micro well plates. Each well contained 10 of test substance, 190 µL MRC-5 cell/parasites inoculum (2 x 10^4/mL cell and 2 x 10^5 parasite/mL). The parasite growth was compared with untreated infected control (100% growth) and non-infected controls (0% growth) after seven days of incubation. The parasite burden was assessed by adding substrate chlorophenol red-β-D-galactopyranoside (CPRG): 50 µL/well of stock solution containing 15.2 mg of CPRG and 250µL Nonidet in 100 ml phosphate buffered saline (PBS) and the change in colour was measured spectrophotometrically at 540 nm after 4h of incubation at 37 °C. The results were expressed as % reduction in parasite burden compared to the control wells. The compounds were tested at five concentrations (ranging from 64 - 0.25 µM). Benznidazole was used as reference drug (Cos *et al*., 2006; Balde' *et al*., 2010; Mesia *et al*., 2008).

2.4.6.3 Antiplasmodial activity

The chloroquine-resistant *Plasmodium falciparum* strain K1 was used. Parasites were cultured in human erythrocytes at 37 °C under a low oxygen atmosphere (3% O_2, 4% CO_2 and 93% N_2) in a modular incubation chamber. The culture medium was RPMI-1640 supplemented with 0.5 m/v % albumax (Cos *et al*., 2006). The assay was performed in 96 micro well plates. Each well containing 10 µL of test compound, 190 µL of malaria parasite inoculum (1% parasitaemia, 2% haematocrit) incubated for 72 h at 37°C in modular chamber under low oxygen atmosphere (3% O_2, 4% CO_2 and 93% N_2). After incubation, test plates were frozen at -20 °C. Parasite multiplication was measured by the Malstat method. This method is based on the ability of the lactate dehydrogenase

(LDH) enzyme of *P. falciparum* and the coenzyme 3-acetyl pyridine (APAD) to transform the lactate into pyruvate. One hundred microliters of Malstat reagent was transferred in a new plate and mixed with 20 µL of the haemolysed parasite suspension for 15 min at room temperature. After addition of 20 µL nitro blue tetrazolium (NBT)/phenazine ethosulphate (PES) solution (2 mg/mL of NBT and 0.1 mg /mL of PES) and 2h incubation in the dark, the optical density was spectrophotometrically read at 655 nm. Percentage of growth inhibition was compared to the negative blanks. Chloroquine was used as reference drug (Cos *et al*., 2006; Balde' *et al*., 2010; Mesia *et al*., 2008).

2.4.6.4 Antibacterial activity

The bacterial strains *Staphylococcus aureus* representing gram-positive bacteria and *Escherichia coli* representing gram-negative bacteria were cultured in MHB (Mueller Hinton Broth) at 37 °C. Assays were performed in 96-well microtiter plates, each well containing 10 µL of test compound together with 190 µL of inoculum (5 x 10^3 cfu/mL). After 17 h incubation, bacterial viability was assessed fluorimetrically after addition of resazurin, as described above. The reference drugs used during analysis were erythromycin and trimethoprim (Cos *et al*., 2006; Balde' *et al*., 2010; Mesia *et al*., 2008)

2.4.6.5 Antifungal activity

The antifungal activity of extracts was tested against *Candida albicans*, *Aspergillus fumigatus* and *Microsporum canis.* The fungal strains were cultured in RPMI-1640 medium supplemented with Mops buffer and glucose at 37 °C. Assays were performed in 96-well microtiter plates, each well containing 10 µL of extract and 190 µL of fungal inoculum (5 x10^3 cfu/mL). After 24 h incubation, fungal viability was assessed fluorimetrically after addition of 10 µL resazurin per well (λ exi 550 nm, λ emi 590 nm). The results are expressed as % reduction in fungal growth/viability compared to control wells and an IC_{50} is determined. Reference drugs include miconazole and terbinafine (Cos *et al*., 2006; Balde' *et al*., 2010; Mesia *et al*., 2008)

2.4.6.6 Cytotoxicity

MRC-5SV2 (human fetal lung fibroblasts) cells were cultivated in MEM supplemented with L-glutamine (20 mM), 16.5 mM sodium hydrogen carbonate, and 5% FCS. For the assay, MRC-5 cells (3 x 10^4 cells / well) were seeded onto the test plates containing 10

μL the pre diluted test substance and incubated at 37 °C and 5% CO_2 for 72 h. Cell viability was assessed fluorimetrically 4 h after addition of 50 microliter/well resazurin. Fluorescence was measured (λ exi 550 nm, λ emi 590 nm) and the results are expressed as % reduction in cell viability compared to the blank controls. Tamoxifen was used as reference drug (Cos *et al*., 2006; Balde' *et al*., 2010; Mesia *et al*., 2008)

REFERENCES

Balde' ES, Megalizzi V, Traore' MS, Cos p, Meas L, Decaestecker C, Pieters L, Balde' AM . 2010. Antiprotozoal, antimicrobial and antitumor activities in vitro of Pavetta crassipes K. Schum leaves. *J Ethnopharmacol* **130**: 529–535.

Buckner FS, Verlinde CL, la Flamme AC, Van Voorhis WC. 1996. Efficient technique for beta-galactosidase. *Antimicrob Agents Chemother* **40**: 2592–2597.

Bursal E, Gülçin İ. 2011. Polyphenol contents and in vitro antioxidant activities of lyophilized aqueous extract of kiwifruit (Actinidia deliciosa). *Food Res Int* **44**:1482–1489

Choudhary MI, Shah SA, Atta-ur-Rahman, Khan SN, Khan MT, 2010. α- glucosidase and tyrosinase inhibitors from fungal hydroxylation of tibolone and hydroxytibolones. *Steroids* **75**: 956–966.

Cos P, Vlietinck AJ, Vanden Berghe D, Maes L, 2006. "Anti-infective potential of natural products: how to develop a stronger in vitro "proof-of-concept." *J Ethnopharmacol* **106**: 290–302.

Dudonne S, Vitrac X, Coutiere P, Woillez M, Merillon JM. 2009. Comparative Study of Antioxidant Properties and Total Phenolic Content of 30 Plant Extracts of Industrial Interest Using DPPH, ABTS, FRAP, SOD, and ORAC Assays. *J Agric Food Chem* **57:** 1768–1774.

Gousiadou C, Gotfredsen CH, Matsa M, Hadjipavlou-Litina D, Skaltsa H. 2013. Minor iridoids from *Scutellaria albida* ssp. *albid*a. Inhibitory potencies on lipoxygenase, linoleic acid lipid peroxidation and antioxidant activity of iridoids from *Scutellaria* sp. *J Enzyme Inhib Med Chem* **28**: 704–710.

Grantstrom E. 1984. The arachidonic acid cascade: the prostaglandins, thromboxanes and leukotrienes. *Inflammation* **8s**: 15–25.

Herborne JB. 1973. Phytochemical Methods 3rd Edn. Chapman and Hall Ltd., London, pp: 135-203.

Hirumi H, Hirumi K. 1989. Continuous cultivation of *Trypanosoma brucei* blood stream forms in a medium containing a low concentration of serum protein without feeder cell layers. *J Parasitol* **75**: 985–989.

Liegeois C, Lermusieau G, Collin S.2000. Measuring antioxidant efficiency of wort, malt, and hops against the 2,2'-azobis (2-amidinopropane) dihydrochloride-induced oxidation of an aqueous dispersion of linoleic acid. *J Agric Food Chem* **48**: 1129–1134.

Lyckander IM, Malterud KE. 1996. Lipophilic flavonoids from *Orthosiphon spicatus* inhibit oxidative inactivation of 15-lipoxygenase. *Prostagl Leukotr Essent Fatty Acids* **54:** 239-246.

Malterud KE, Rydland KM. 2000. Inhibitors of 15-lipoxygenase from orange peel. *J Agric Food Chem* **48**: 5576–80.

Matsuura N, Aradate T, Sasaki C, Kojima H, Ohara M, Hasegawa J. 2002. Screening system for the Maillard reaction inhibitor from natural product extracts. *J Health Sci* **48**: 520-526.

Mesia GK, Tona GL, Nanga TH, Apers S, Cos P, Meas L, Pieters L, Vlietink AJ. 2008. Antiprotozoal and cytotoxic screening of 45 plant extracts from Democratic Republic of Congo. *J Ethnopharmacol* **115**: 409–415.

Muller K. 1994. 5-Lipoxygenase and 12-lipoxygenase, Attractive targets for the development of novel antipsoriatic drugs. *Arch Pharm* (Weinheim) **327**:3–19.

Park KS, Kim BH, Chang IM. 2010. Inhibitory Potencies of Several Iridoids on Cyclooxygenase-1, Cyclooxygnase-2 Enzymes Activities, Tumor Necrosis factor-a and Nitric Oxide Production In Vitro. *Eid Based Complement Alternat Med*. **7:** 41-45.

Peng X, Zheng Z, Cheng KW, Shan F, Ren GX, Chen F, Wang M. 2007. Inhibitory effect of mungbean extract and its constituents vitexin and iso-vitexin on the formation of advanced glycation end products. *Food Chem* **106**: 475–481.

Rahbar S. 2007. Novel inhibitors of glycation and AGE formation. *Cell Biochem Biophys* **48:** 147–157.

Rajic Z, Hadjipavlou-Litina D, Pontiki E, Kralj M, Suman L, Zorc B. 2010. The novel ketoprofen amides–synthesis and biological evaluation as antioxidants, lipoxygenase inhibitors and cytostatic agents. *Chem Biol Drug Des* **75**: 641–652.

Raz B, Iten M, Grether-Buhler Y, Kaminsky R, Brun R.1997. The Alamar Blue asssay to determine drug sensitivity of African trypanosomes (*T.b.rhodesiense,T.b.gambiense*) in vitro. *Acta Trop* **68**: 139–147.

Sofowora A. 1993. Medicinal plants and traditional medicine in Africa. John Wiley and son Ltd., 150-153.

Steinberg D. 1999. At last, direct evidence that lipoxygenases play a role in athero-genesis. *J Clin Invest* **103:** 1487-1488.

Sun L, Zhang J, Lu X, Zhang L, Zhang Y. 2011. Evaluation to the antioxidant activity of total flavonoids extract from persimmon (Diospyros kaki L.) leaves. *Food Chem Toxicol* **49**: 2689–2696.

Szabo MR, Iditoiu C, Chambre D, Lupea AX. 2007. Improved DPPH determination for antioxidant activity spectrophotometric assay. *Chem Pap* **61**: 214-216.

Trease GE, Evans WC. 1989. Pharmacognosy.11th Edn. Brailliar Tiridel and Macmillian Publishers, London.

CHAPTER 3

PHYTOCHEMICAL AND BIOLOGICAL INVESTIGATIONS ON *NYMPHOIDES INDICA* LEAF EXTRACTS

Accepted for publication in Phytotherapy Research:

Phytochemical and pharmacological investigations on *Nymphoides indica* leaf extracts
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3.1 INTRODUCTION

Wetlands deliver several ecosystem services that afford a variety of human activities (Bostian *et al*., 2014) such as agriculture, food and water resources (Maya *et al*., 2003; Grossmann 2012). Furthermore the origin of the majority of human advancements bear their roots around wetlands (Swapna *et al*., 2011). Together with socioeconomic assistance wetlands also provide an imperative reservoir of medicinal plants worldwide (Cook, 1996; Marwat *et al*., 2011; Panda and Mishra, 2011) and numerous reports from literature can be consulted that provide clear evidence about synthesis of many secondary metabolites through their natural pathways (Jain *et al*., 2007, Das *et al*., 2006). Some promising bioactivities offered by aquatic plants include antibacterial (Abu Ziada *et al*., 2008; Fareed *et al*., 2008; Haroon *et al*., 2009; Sridevi *et al*.,2010), antifungal (Bhosale *et al*., 1999; Haroon, 2006) and antiviral (Verma *et al*., 2008; Shin *et al*., 2010) activities.

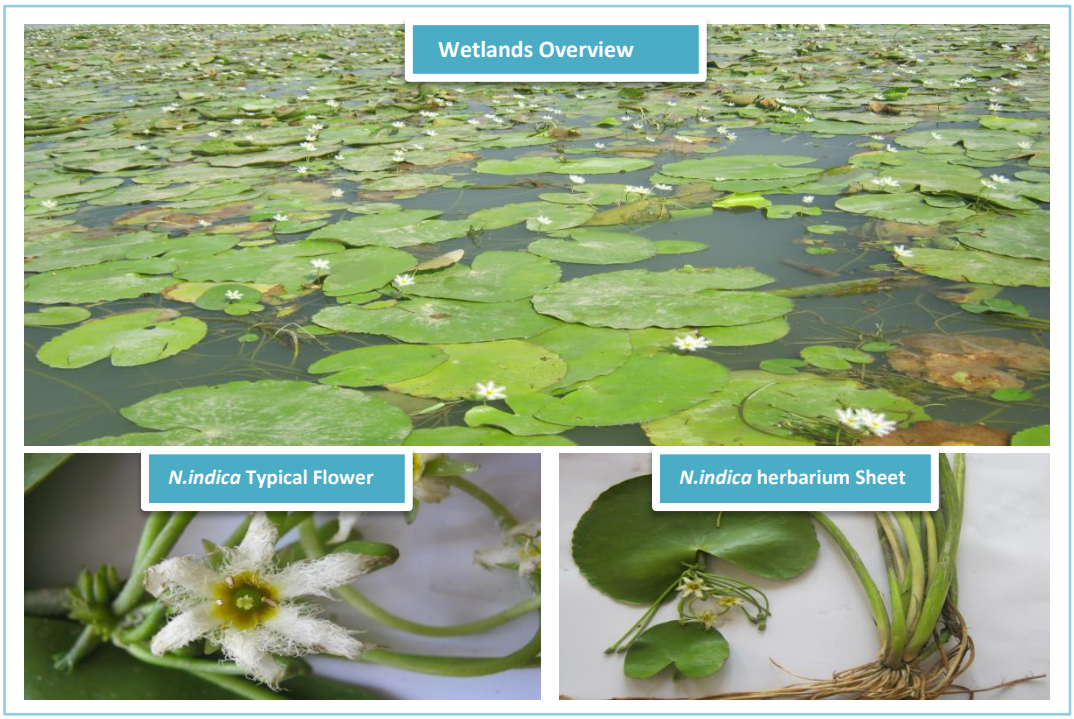

Figure 3.1 *Nymphoides indica* collection from natural habitat and herbarium sheet for identification.

Nymphoides indica (L.) Kuntze (Menyanthaceae) is a perennial rhizomatous free floating leaved aquatic herb (Schmidt, 2005). The genus *Nymphoides* is represented worldwide by about 50 species that are not only very diverse but also differ considerably in a number of biological features (Tippery *et al*., 2009). *Nymphoides indica* is native to tropical America (Iverson, 2010), however it is reported from various parts of world including Thailand (Ito and Barfod, 2014), India (Madhavan *et al*., 2009), Australia (Ohwi, 1984), Japan (Shibayama and Kadono, 2007). In Pakistan only three species occur, i.e *Nymphoides cristata* (Roxb.), *Nymphoides peltatum* (S.G. Gmel.) (Qaiser, 1977) and *Nymphoides indica* (Marwat *et al*., 2009). These species are primarily reported in the districts of Attock, Thatta, Kashmir and D. I. Khan.

N. indica is consumed as a vegetable (Cruz-Garcia and Price, 2011; Swapna *et al*., 2011) and has a consistent folklore usage in the Indian subcontinent for the management of numerous health indications by local healers (called hakeem or herbal doctors). A number of ethnomedicinal surveys can be accessed that principally emphasise on traditional use of this plant species for treatment of dysentery (Das *et al*., 2006; Kensa, 2011), scabies (Panda and Misra, 2011), snake bites (Coea and Anderson 2005), jaundice (Jain *et al*., 2007), anticonvulsant (Madhavan *et al*., 2009), aphrodisiac (Yumnam and Tripathi, 2012), and as antiproliferative (Kitdamrongtham *et al*., 2013).

Moreover it has been reported that it is a part of some important herbal formulations in India (Madhavan *et al*., 2009), Nepal (Siwakoti, 2006), Thailand (Manosroi *et al*., 2012) and Pakistan (Marwat *et al*., 2011) that are used for various therapeutic reasons locally. In spite of the multimedicinal usage of *Nymphoides indica*, no comprehensive published data is available to date concerning its phytochemical constituents and pharmacological properties. Therefore the aim of the present work was to evaluate various medicinal and pharmacological properties of *N. indica* and its constituents, as a part of running programmes on antimicrobial, antioxidant and antidiabetic (inhibition of α-glucosidase activity; inhibition of AGEs (Advanced Glycation Endproducts) properties of medicinal plants and natural products (Upadhyay *et al*., 2014_a, Upadhyay *et al.,* 2014_b; Mesia *et al*., 2008).

3.2 EXTRACTION AND ISOLATION

3.2.1 Plant material

N. indica leaves were collected in June and July 2012 from wetlands of the river Indus in Dera Ismail Khan (KPK) Pakistan. The collected plant leaves were identified by the Islamabad Herbarium at the Taxonomy Department, Quaid I Azam University, Islamabad, Pakistan, where the voucher specimen was deposited (5298-GA, accession No. ISL-120019). The leaves were shade dried, powdered and sieved through a 20 mesh filter, and stored below 20°C till further use (Fig 3.2).

Figure 3.2 *Nymphoides indica* dried leaf powder.

3.2.2. Extraction and fractionation

Powdered leaves (1.92 kg) were extracted with 90% (v/v) methanol by double cold maceration. The extract was instantaneously filtered through Whatman No.1 filter paper using a vacuum pump. The filtrates were combined together and evaporated on a rotary evaporator under reduced pressure below 40°C. The resultant semisolid material was lyophilised with a final yield of 88.61 g, and stored below 20°C. Further liquid–liquid partitioning of the crude extract was performed according to a standard extraction scheme (Chapter 2). After partitioning with different solvents as shown in the scheme, *n*-hexane (1.8 g), methanol 90% (11.2 g), chloroform (2.03 g), ethyl acetate

(1.2 g) and *n*-butanol (19.58 g) fractions were obtained. The collected fractions were dried under reduced pressure at 40°C and stored below 20°C.

3.2.3. Phytochemical analysis

Phytochemical screening for various compound classes was carried out on every fraction using published methods (Sofowora, 1993, Trease and Evans, 1989, Harborne, 1973; Egwaikhide and Gimba, 2007; Farnsworth, 1966) with slight modifications for sterols, triterpenes, carbohydrates and saponins flavonoids, alkaloids, and tannins (chapter 2). Phytochemical screening of the different extracts and fractions revealed that flavonoids, tannins, saponins and triterpenes are present with varying concentrations in the different fractions of the leaves extract (Table 3.1). No alkaloids were detected. Fractions of medium polarity contained flavonoids and terpenes. The residual aqueous fraction was positive for tannins and saponins. These finding are consistent with previous reports (Madhavan *et al*., 2009; Kitdamrongtham *et al*., 2013).

Table 3.1 Phytochemical analysis of the *N.indica* leaves

Extract fraction	**Phytochemicals**				
	Alkaloids	Flavonoids	Tannins	Steroids/ triterpenoids	Saponins
MeoH 90%	-	+++	-	+++	-
Chloroform	-	++	-	++	-
Ethyl acetate	-	+++	-	+	-
n-Hexane	-	-	-	-	-
n-butanol	-	+	-	++	++
Aqueous	-	-	+++	-	+++

profoundly present: high degree of precipitation (dark coloration), moderately present: less degree of precipitation (medium colouration), slightly present: very low precipitation (very little colouration), absent: no change in colour (Senguttuvan et al., 2014)

Phytochemicals: +++ profoundly present, ++ moderately present , + slightly present, - absent.

3.2.4. Thin layer chromatography

Each fraction was subjected to TLC (NP) using various solvent systems as mobile phase, including $CHCl_3$ / MeOH (70:30) with a few drops of NH_4OH for the methanolic fraction; $CHCl_3$ / MeOH (80:20) for the chloroform and ethyl acetate fractions; *n*-hexane / $CHCl_3$ (95:5) for the *n*-hexane fraction; and $CHCl_3$ / MeOH (25:75 or 37:63) the for *n*-butanol fraction. Developed TLC plates were examined under UV at 254 nm and 366 nm, after spraying with *p*-anisaldehyde reagent. For instance, the TLC profiling of the methanolic fraction (FR4) obtained after flash chromatography is shown below. In the highlighted area, four prominent spots are visible (Fig 3.3).

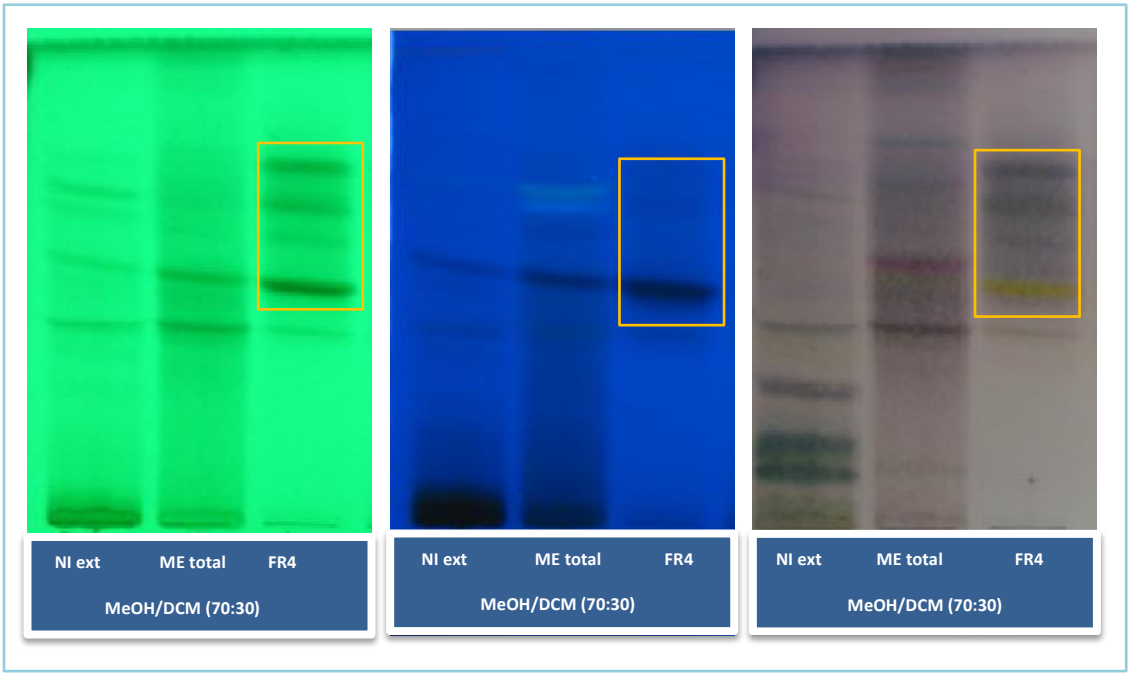

Figure 3.3 TLC profile of total plant extract (NI ext), methanolic extract (ME total) and fraction-4 (FR4) on silica TLC (NP) plate at 254 and 366 nm and after spraying anisaldehyde reagent.

3.2.5. Flash chromatography

An aliquot of 0.8 g from the *n*-hexane fraction was dissolved in 2 mL methanol, mixed with 1.1g silica and dried with nitrogen gas. The dried extract was subjected to flash chromatography with a gradient from *n*-hexane over methylene chloride to methanol. Based on UV and ELSD detection different subfractions were collected (Fig. 3.4). All fractions were analysed by TLC and similar fractions were combined. In this way **21** subfractions were obtained. Based on TLC profiling, subfractions NI**4** (190 mg) and NI**7** (150 mg) were selected for further HPLC profiling.

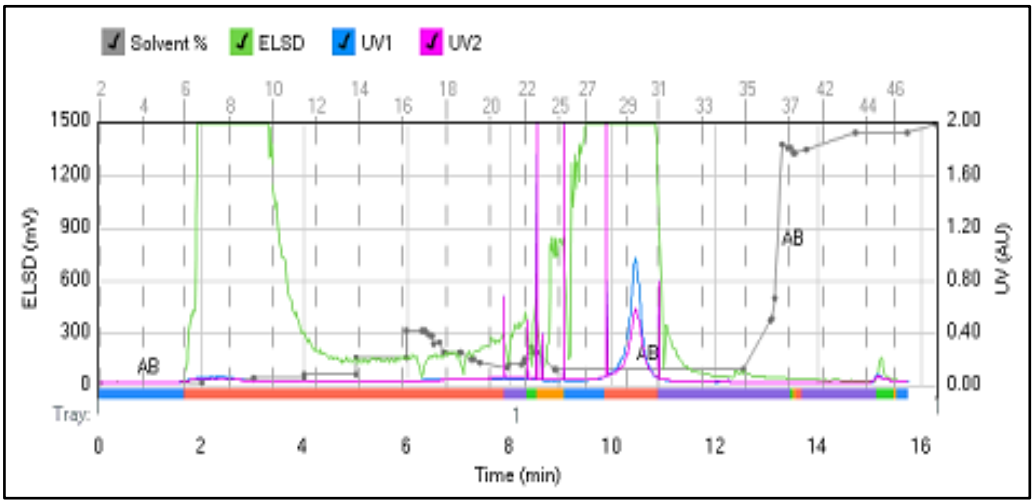

Figure 3.4 Flash chromatogram of *n*-hexane fraction.

An aliquot of 0.8 g from the methanolic fraction was subjected to flash chromatography with a gradient from ethyl acetate to methylene chloride over methanol. Based on UV and ELSD detection different subfractions were collected (Fig 3.5). All fractions were analysed by TLC and similar fractions were combined. A total of **19** subfractions were finally obtained. Finally subfractions NI**4** (250 mg), NI**6** (180 mg) and NI**7** (150 mg) were selected for further HPLC profiling.

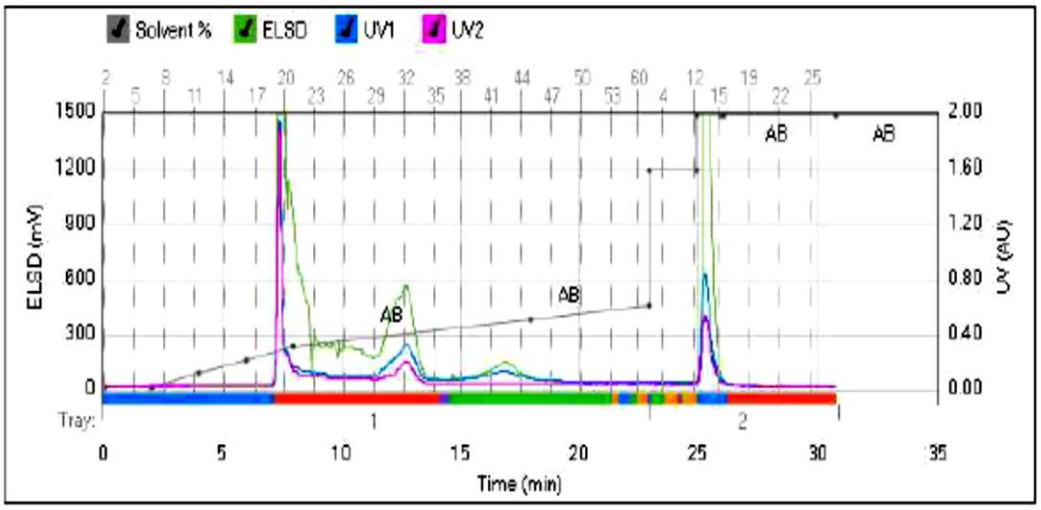

Figure 3.5 Flash chromatogram of methanolic fraction.

Similarly an aliquot of 0.9 g from the ethyl acetate fraction was subjected to flash chromatography with a gradient from ethyl acetate over methylene chloride to methanol. Based on UV and ELSD detection different subfractions were collected (Fig 3.6). All fractions were analysed by TLC and similar fractions were combined as described above; in this way **15** subfractions were obtained. Based on TLC profiling subfractions NI**4** (126 mg) and NI**7** (154 mg) were selected further HPLC analysis.

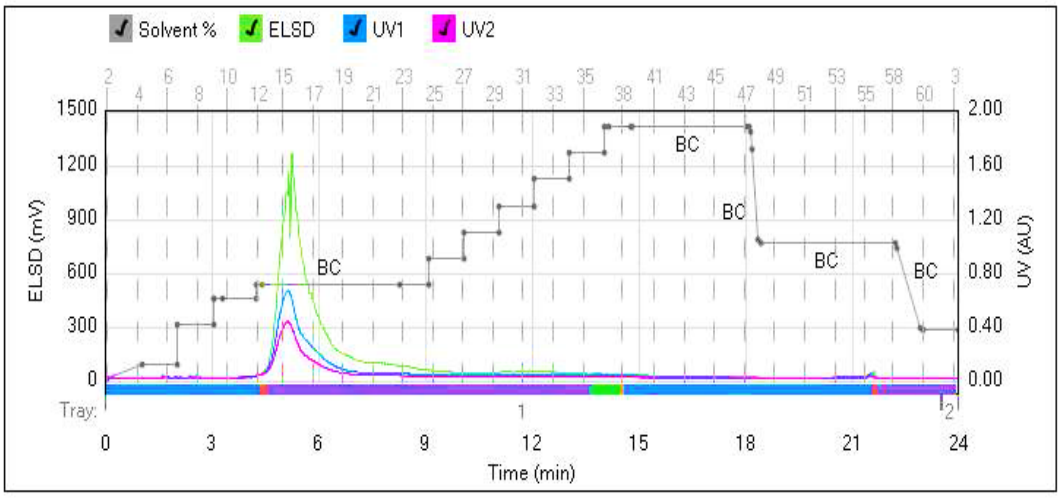

Figure 3.6 Flash chromatogram of ethyl acetate fraction.

Similarly an aliquot of 0.7 g from the chloroform fraction was subjected to flash chromatography with a gradient from ethyl acetate over methylene chloride to methanol as previously explained. Finally **10** subfractions were obtained (Fig 3.7). Based on TLC analysis subfraction NI**2** (130 mg) was selected for HPLC analysis.

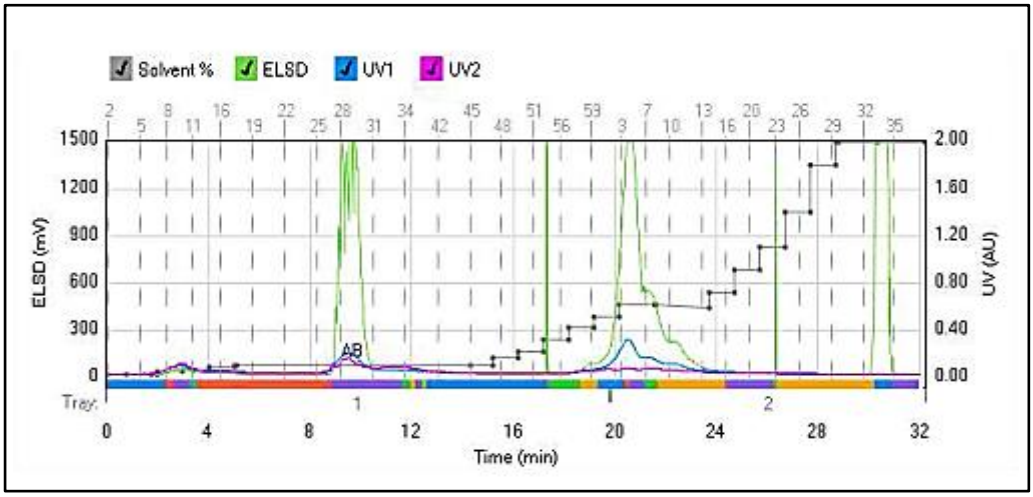

Figure 3.7 Flash chromatogram of chloroform fraction.

Finally an aliquot of 0.8 g from the *n*-butanol fraction was subjected to flash chromatography with a gradient from ethyl acetate over methylene chloride to methanol. Based on UV and ELSD detection different subfractions were collected (Fig 3.8). All fractions were analysed by TLC and similar fractions were combined. Finally **18** subfractions were obtained. Based on TLC analysis subfractions NI**2** (126 mg) and NI**5** (154 mg) were selected for further HPLC analysis.

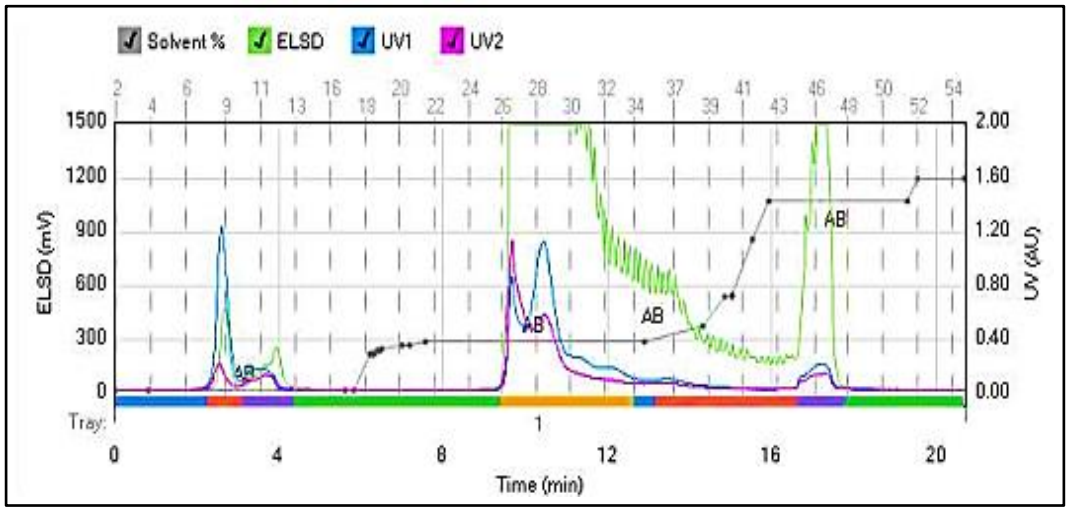

Figure 3.8 Flash chromatogram of *n*-butanol fraction.

3.2.6. HPLC analysis and isolation of compounds

The *n*-hexane fraction and all obtained subfractions NI**4** and NI**7** were analysed by HPLC using an optimized acetonitrile / H_2O + 0.1% formic acid gradient, ranging from 5% acetonitrile to 100% in 40 min at a flow rate of 1 mL/min. Samples were prepared in a concentration range from 1-10 mg/ mL in methanol (Fig 3.9). The isolation of pure compounds was performed by means of semi-preparative HPLC-DAD-MS using the same gradient at 3 mL/min, yielding compounds **1** (3.5 mg)**, 2** (4.2 mg)**, 3** (4.2 mg), **4** (3.6 mg) and **5** (5.2 mg).

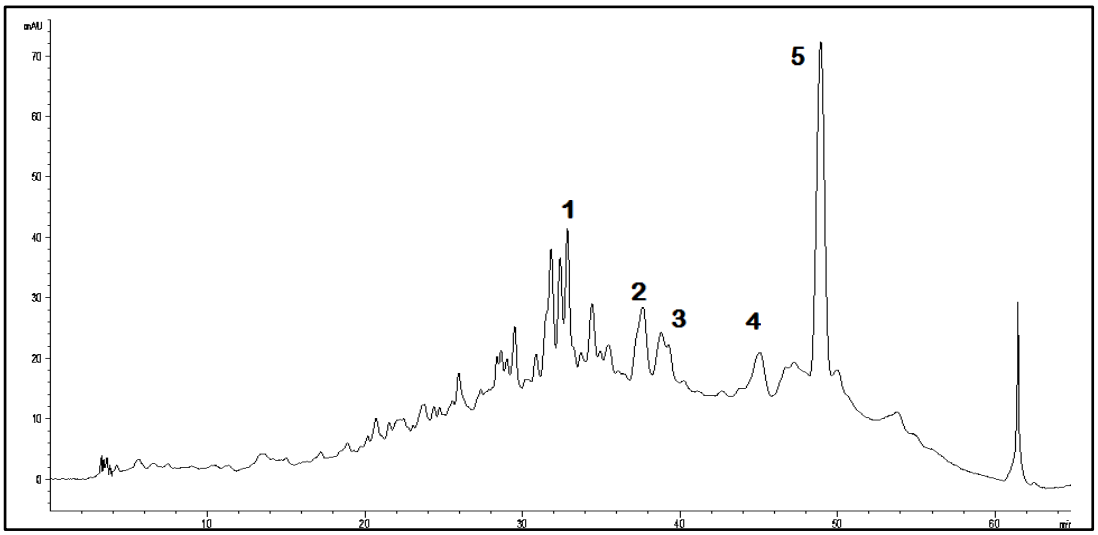

Figure 3.9 HPLC chromatogram of *n*-hexane fraction at 254 nm

The crude methanolic fraction and all subfractions NI**4** (250 mg), NI**6** (180 mg) and NI**7** (150 mg) were analysed by HPLC using an optimized acetonitrile / H_2O + 0.1% formic acid gradient ranging from 15% acetonitrile to 100% in 40 min at a flow rate of 1 mL/min Fig. 3.10). The isolation of pure compounds was performed by means of semi-preparative HPLC-DAD-MS using the same gradient at 3 mL/min Compounds **6** (5.2 mg), **7** (5.7 mg), **8** (7.5 mg), **9** (4.2 mg) and **12** (3.3 mg) were isolated.

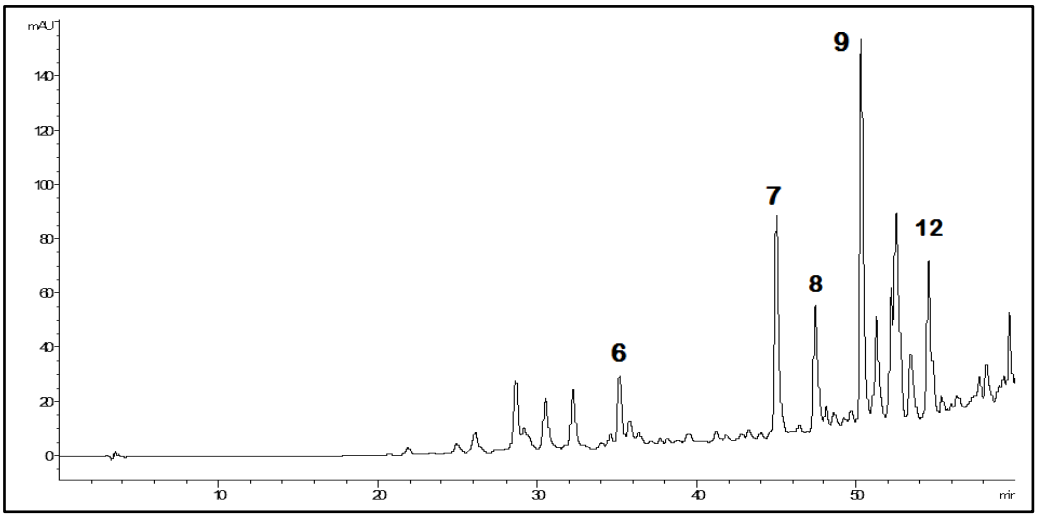

Figure 3.10 HPLC chromatogram of 90% methanol fraction at 254 nm

Similarly the crude ethyl acetate fraction and subfractions NI**4** (126 mg) and NI**7** (154 mg) were analysed by HPLC using an optimized acetonitrile/H_2O + 0.1% formic acid gradient ranging from 35% acetonitrile to 70% in 45 min at a flow rate of 1 mL/min (Fig. 3.11). The compounds were isolated by means of semi-preparative HPLC-DAD-MS system using same gradient at a flow rate of 3 mL/min. Finally the compounds **9** (4.5 mg), **10** (3.2 mg) and **13** (2.0 mg) were isolated.

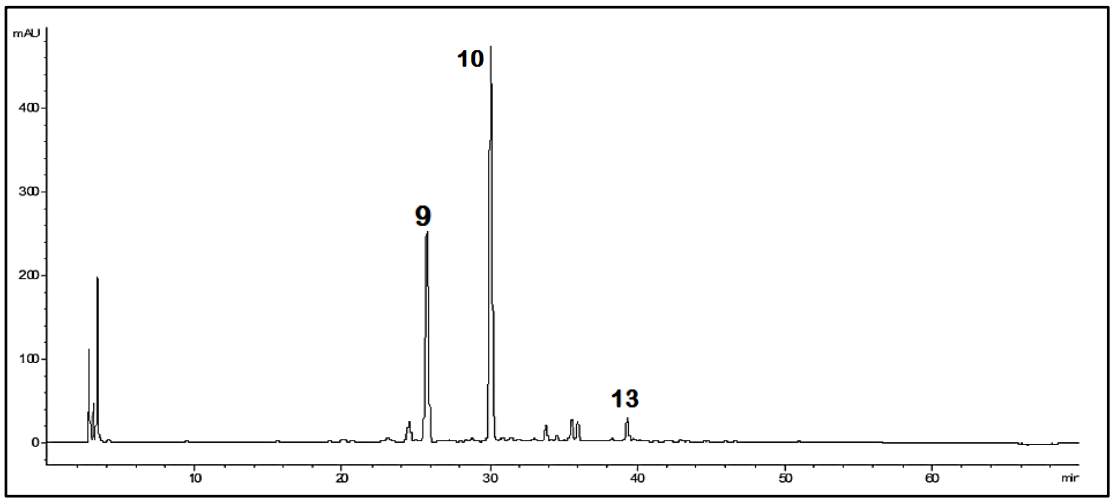

Figure 3.11 HPLC chromatogram of ethyl acetate fraction at 254 nm

The crude chloroform fraction and subfraction NI**2** was analysed by HPLC using an optimized acetonitrile/H_2O + 0.1% formic acid gradient ranging from 39% acetonitrile to 65% in 45 min at a flow rate of 1 mL/min (Fig. 3.12). Compound isolation was performed by means of semi-preparative HPLC-DAD-MS system using the same solvent gradient as HPLC at a flow rate of 3 mL/min. Finally compounds **10** (3.2 mg) and **11** (3.8 mg) were isolated.

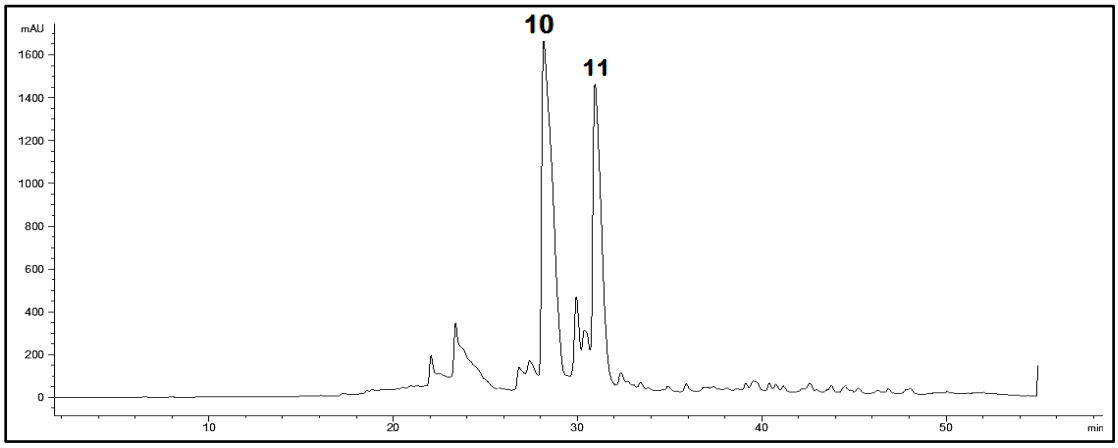

Figure 3.12 HPLC Chromatogram of ethyl acetate fraction at 254 nm

Finally the HPLC profiling of *n*-butanol fraction and subfractions NI**2** (126 mg) and NI**5** (154 mg) was performed using an optimized acetonitrile/H_2O + 0.1% formic acid gradient ranging from 30% acetonitrile to 65% in 50 min at a flow rate of 1 mL/min (Fig 3.13). The compounds **9** (7.3 mg), **10** (4.6 mg), **13** (2.3 mg) and **14**(2.8 mg), were finally isolated by means of semi-preparative HPLC-DAD-MS. The solvent gradient was same as HPLC at a flow rate of 3 mL/min.

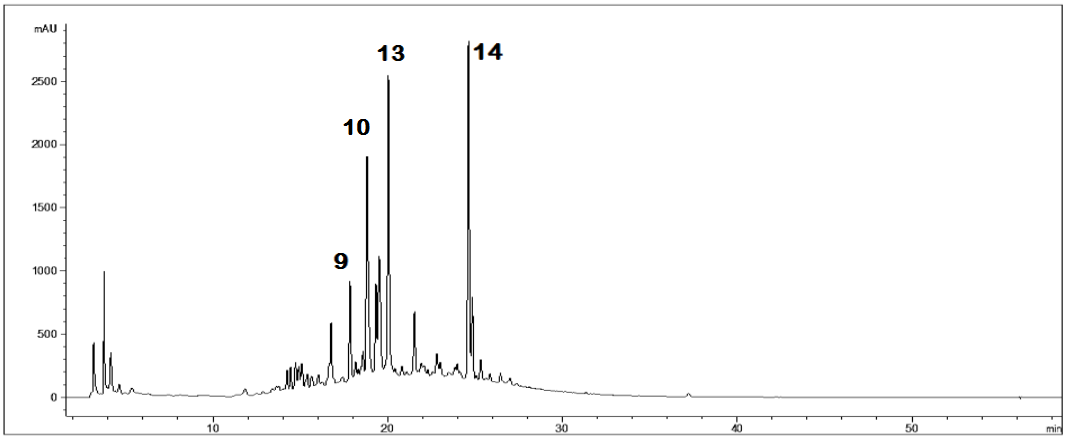

Figure 3.13 HPLC chromatogram of *n*-butanol fraction at 254 nm

3.3. STRUCTURE ELUCIDATION

3.3.1 Lipophilic compounds (1-5)

From the *n*-hexane fraction 5 lipophilic constituents were obtained. Their ^{1}H- and ^{13}C-NMR spectra showed the characteristic features of fatty acids and fatty alcohols, and after derivatisation and GC-MS analysis they were identified as the dicarboxylic acids azelaic acid (nonanedioic acid) (**1**) and 4-methyl-heptanedioic acid (**3**), the monocarboxylic acids hexadecanoic acid (**2**) and stearic acid (**5**) and the fatty alcohol hexadecanol (**4**) (Table 3.2)

Table 3.2 GC-MS profile of compounds isolated from *n*-hexane fraction.

Comp	RT(min)	MW	Prob.	Formula	Peak name
1	27.73	332	45.24	$C_{15}H_{32}O_4Si_2$	Azelaic acid-bis(trimethylsilyl)ester
2	35.57	328	72.86	$C_{19}H_{40}O_2Si$	Hexadecanoic acid trimethylsilyl ester
3	24.37	318	48.36	$C_{14}H_{30}O_4Si_2$	Heptanedioic acid, 4-methyl (bistrimethylsilylester)
4	39.10	314	17.38	$C_{19}H_{42}OSi$	1-trimethylsiloxyhexadecane
5	39.36	356	48.99	$C_{21}H_{44}O2Si$	Stearic acid trimethylsilylester

3.3.2 Seco iridoids (6-8)

3.3.2.1 7-Epiexaltoside (6):

The structure of compound **6** was elucidated by ^{1}H-NMR, ^{13}C-NMR (DEPT-135 and DEPT-90) as **7-epiexaltoside** (Fig. 3.14).

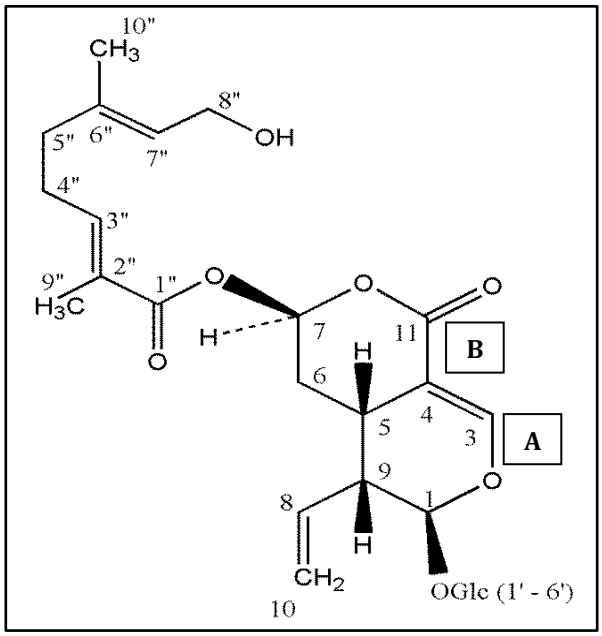

Figure 3.14 structure of 7-epiexaltoside (**6**)

Library search on the ^{13}C-NMR data (Table 3.3 and Fig. 3.15) suggested this compound was a seco-iridoid with a glucosyl moiety at C-1. Based on its ^{1}H-NMR, ^{13}C-NMR and mass spectra, and compared with data from literature (Junior, 1991) compound **6** was found to be identical to 7-epiexaltoside. The ^{1}H-NMR (Table 3.3 and Fig 3.16) and ^{13}C-NMR (Table 3.3 and Fig. 3.15) are in agreement with a geranyl-like side chain, with two double bonds having an *E*-configuration. The carbonyl group of the ester moiety showed a ^{13}C-NMR resonance at 167.1 ppm, which is typical for an α,β unsaturated ester.

The geranyl-like side chain has 2 tertiary carbons C-2"and C-6" showing signals at 128.3 ppm and 137.3 ppm, respectively. These tertiary carbons were substituted by a methyl group at C-9"and C-10" that showed signals at 12.4 ppm and 16.2 ppm, respectively.

Two secondary carbons in position C-4”and C-5” showed signals at 28.1 ppm and 39.1 ppm, respectively. The signal at 59.4 ppm was assigned to a primary carbon at position 8”.

The compound has a typical bicyclic ring system consisting of 2 rings A and B. In ring A C-1, showing a signal at 99.1 ppm, was attached to a β-D-glucopyranoside moiety. Carbon C-3 showed a CH-signal at 155.4 ppm that is characteristic to an iridoid. C-4 showed signal at 104.4 ppm. The signals at 23.1 ppm and 29.4 ppm were assigned to C-5 and C-6, respectively.

In ring B, a secondary carbon at C-7 showed a signal at 93.6 ppm attached to the geranyl ring through -O- bond with. The ring has a characteristic carbonyl group (C-11) of the ester moiety that resonates at 167.1 ppm. Finally, an unsaturated side chain is attached to ring A at C-9. The signals at 133.3 ppm and 121.5 ppm were assigned to C-8 and C-10, respectively.

The ^{1}H-NMR spectrum showed the characteristic H-3 iridoid signal at 7.67 ppm, as a doublet with a coupling constant of 2.5 Hz. The singlet at 5.55 ppm was assigned to H-1. Two multiplets at 3.35-3.48 ppm and 2.77 ppm were assigned to H-5 and H-9, respectively. The signal for H-8 (5.54 ppm) was attributed to the X part of an ABX system of a vinylidene group, coupling with the signals at 5.29-5.38 ppm (H-10a and H-10b), as the AB part. The methylene protons (H-6) of the ring B were assigned to signals at 1.86-1.98 ppm. The methylene protons showed a coupling with H-7. The absorption of H-7 occurred at 6.63 ppm with a typical small coupling constant (J=1.9 Hz) between H-7 and H-6a as well as H-6b, which is consistent with an axial arrangement (or β-orientation) of the ester at C-7, as demonstrated before for menthiafolin and dihydrofoliamenthin (Junior, 1989; 1991). The geranyl side chain consisted of 3 methylene protons, i.e. H-4”, H-5” and H-8”. The triplets at 2.38 ppm and 2.18 ppm were assigned to H-4”and H-5”, whereas a doublet at 4.09 ppm was assigned to H-8”.

With regard to the methine protons in the geranyl side chain,, the doublet at 7.67 ppm was attributed to H-3”, whereas the triplet at 7.67 ppm was assigned to H-7”. Finally, the spectrum was completed by the signals for a glucose moiety. Based on structural features compound 7-epiexaltoside can be classified as a secoiridoid ester glucoside.

Yellow powder; UV (acetonitrile / H_2O) λ_{max} 200 and 233 nm; ESI-MS (positive ion mode): *m/z* 563 $[M+Na]^+$ consistent with molecular formula $C_{26}H_{36}O_{12}$.

7-Epiexaltoside was originally obtained from *Villarsia exaltata* (Menyanthaceae) by Junior (1991), and interestingly *N. indica* is also a member of this small family of aquatic and wetland plants comprising five genera and fifty species worldwide.

Table 3.3 ^{1}H-NMR and ^{13}C-NMR assignments for 7-epiexaltoside (**6**) recorded in methanol-d_4

Position	**δ_H (ppm); multiplicity; *J* (Hz)**	**δ_C (ppm)**
1	5.55; s	99.1
3	7.67; d; *J*=2.5	155.4
4		104.5
5	3.35-3.48; m	23.1
6	1.86-1.98; m	29.4
7	6.63; t; *J*=1.9	93.6
8	5.54-5.60; m	133.3
9	2.77; m	43.5
10	5.29-5.38; m	121.5
11		167.1
1'	4.72; d; *J*=7.7	101.0
2'	3.20-3.89, m	74.8
3'	3.20-3.89, m	78.3
4'	3.20-3.89, m	71.5
5'	3.20-3.89, m	78.4
6'	3.20-3.89, m	62.6
1"		166.1
2"		128.3
3"	6.86; m	145.7
4"	2.38; m	28.1
5"	2.18; t; *J*=7.4	39.0
6"		137.3
7"	5.40; t partly overlapped by H-10	126.0
8"	4.09; d; *J*=6.7	59.4
9"	1.85; s	12.4
10"	1.70; s	16.2

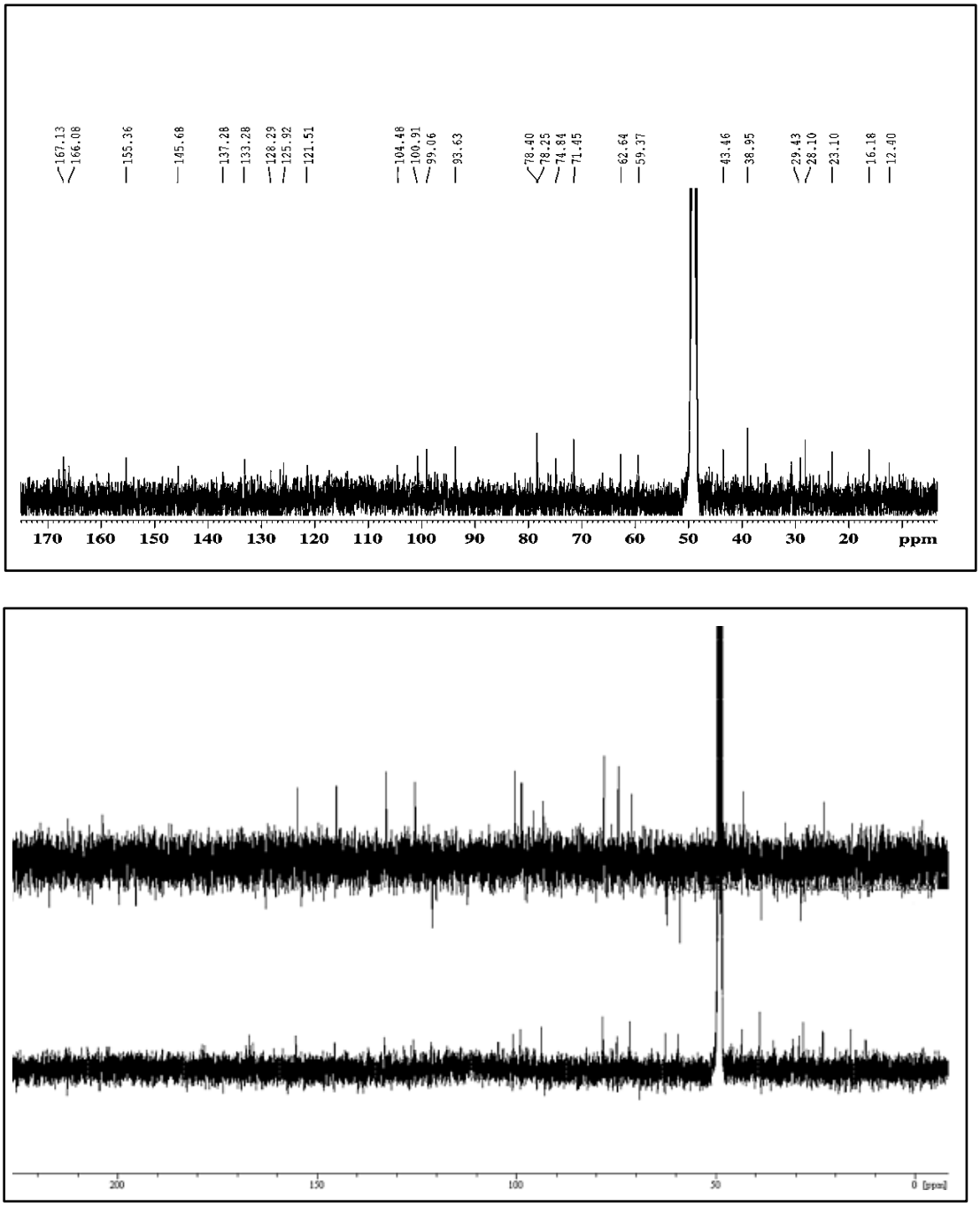

Figure 3.15 ^{13}C-NMR spectra including DEPT-135 of 7-epi exaltoside (**6**)

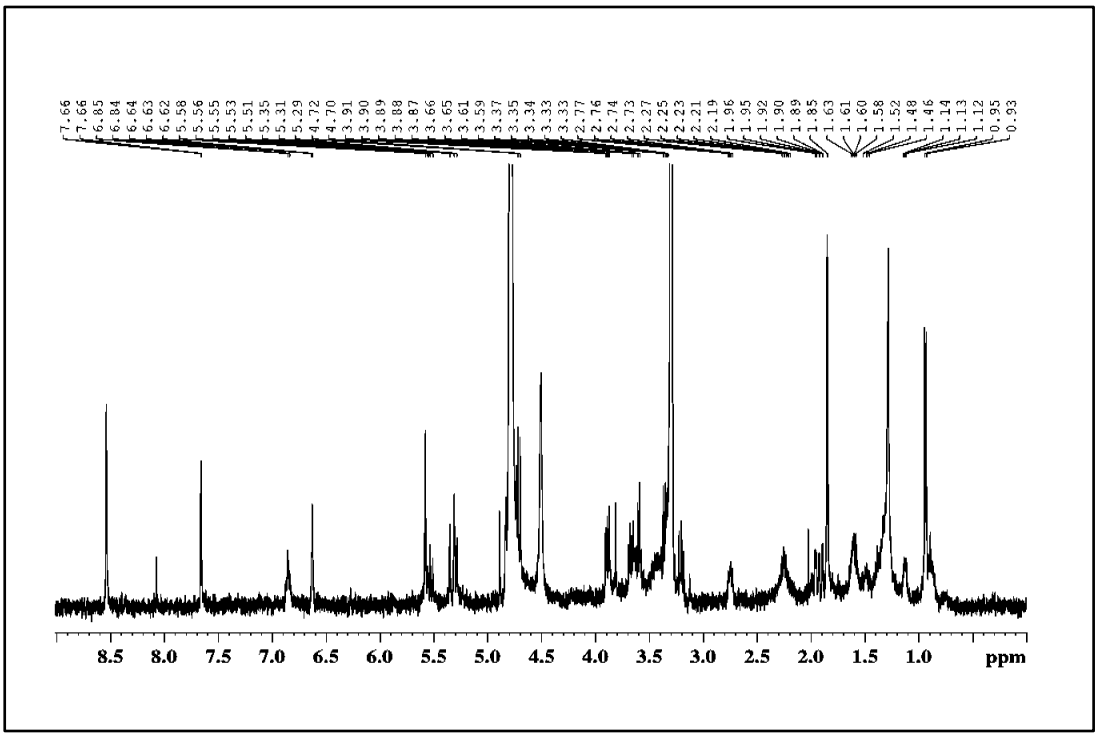

Figure 3.16 ^{1}H NMR spectrum of 7-epi exaltoside (**6**)

3.3.2.2 6”, 7”-Dihydro-7-epiexaltoside (7)

The structure of compound **7** was elucidated by ^{1}H-NMR and ^{13}C-NMR (DEPT-135 and DEPT-90) as **6”, 7”-dihydro-7-epiexaltoside** (Fig 3.17).

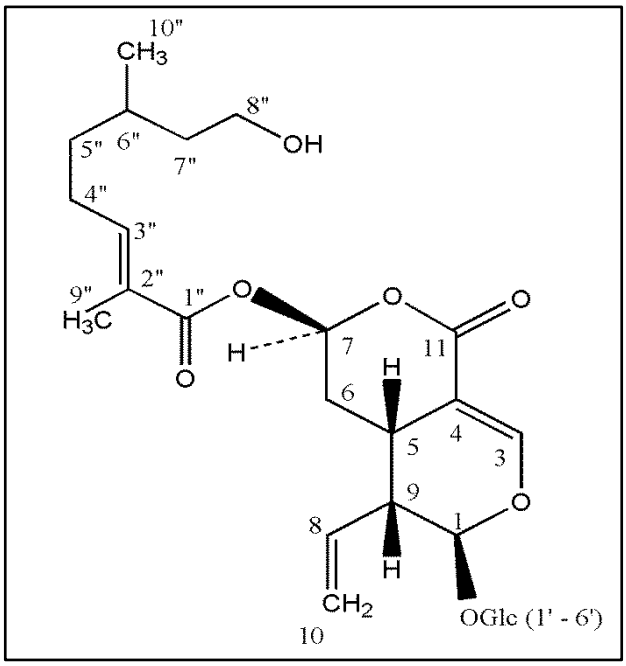

Figure 3.17 Structure of 6”,7”-dihydro-7-epiexaltoside (**7**)

Library search on the ^{13}C-NMR data (Table 3.4 and Fig. 3.18) suggested this compound is a seco-iridoid with a glucosyl moiety similar to compound **6** (Junior, 1991). Based on its ^{1}H-NMR (Table 3.4 and Fig. 3.19), ^{13}C-NMR (Table 3.4 and Fig. 3.20) and mass spectral data compound **7** was found to be 6”,7”-dihydro-7-epiexaltoside, containing a geranyl-like side chain, with only one double bond having an *E*-configuration. The carbonyl group of the ester moiety showed a ^{13}C-NMR resonance at 167.1 ppm, which is typical for an α,β-unsaturated ester as in compound **6**.

The NMR spectra of compound **7** were very similar to those observed for compound **6**, but it contained one double bond less, which was confirmed by the $[M+Na]^{+}$ signal observed in MS at *m/z* 565, rather than *m/z* 563 for compound **6**. Since the signals assigned to the seco-iridoid moiety in compound **6** were also observed in compound **7**, it was concluded that the saturation was located in the geranyl-like side chain. Because

the ester carbonyl was observed at δ 166.1, as expected in case of an α,β-unsaturation, it was concluded that, compared to compound **6**, the 6”,7”- double bond was saturated rather than the 2”,3”-double bond. A compound named dihydrofoliamenthin, containing a geranyl-like side chain saturated in position 2”,3” and a *Z*-configuration at the 6”,7”-double bond, has been reported before from *Villarsia exaltata* by Junior (1991). Hence, compound **7**, for which the name 6”,7”-dihydro-7-epiexaltoside was adopted, is reported here for the first time.

Yellow powder; UV (acetonitrile / H_2O) λ_{max} 200 and 233 nm; ESI-MS (positive ion mode): *m/z* 565 $[M+Na]^+$ consistent with a molecular formula $C_{26}H_{38}O_{12}$.

Table 3.4 ^{1}H-NMR and ^{13}C-NMR assignments for 6",7"-dihydro-7-epiexaltoside (**7**) recorded in methanol-d_4.

Position	δ_H (ppm); multiplicity; *J* (Hz)	δ_C (ppm)
1	5.57; d; *J*= 1.7	99.0
3	7.66; d; *J*= 2.3	155.3
4		104.6
5	3.35-3.45; m	27.4
6	1.88-1.98; m	30.6
7	6.62; t; *J*= 2.0	93.6
8	5.50; m	133.1
9	2.74; m	43.5
10	5.29-5.38; m	121.4
11		167.1
1'	4.70; d; *J*= 7.8	100.7
2'	3.32-3.90; m	74.8
3'	3.32-3.90; m	78.4
4'	3.32-3.90; m	71.5
5'	3.32-3.90; m	78.3
6'	3.32-3.90; m	62.7
1"		166.1
2"		127.9
3"	6.86; m	146.3
4"	2.24; m	27.4
5"	1.45-1.59; m	40.5
6"	1.40; m	23.1
7"	1.49-1.60; m	36.6
8"	3.60; m	61.0
9"	1.84; s	12.3
10"	0.93; d; *J*= 6.7	19.8

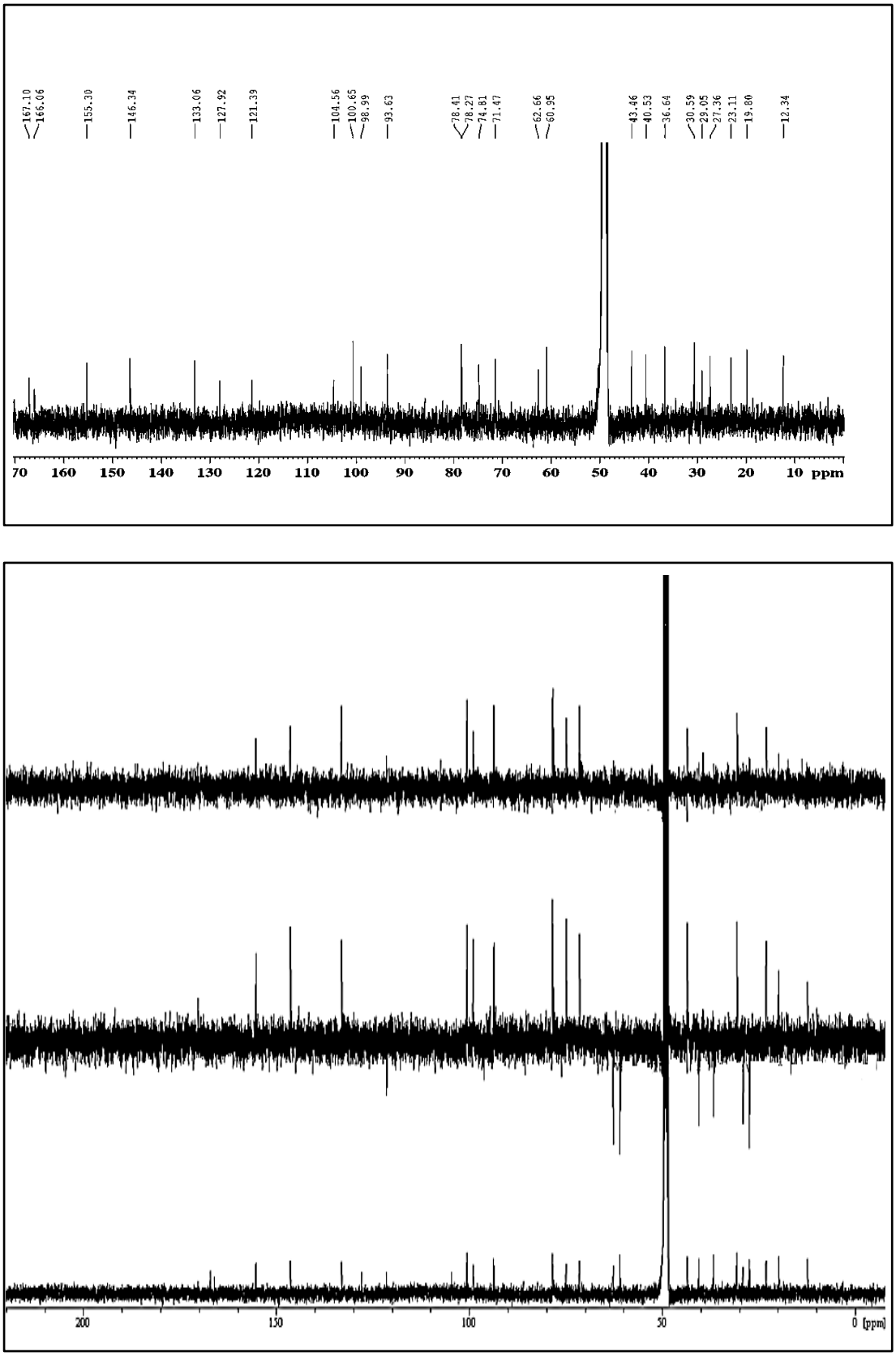

Figure 3.18 ^{13}C-NMR spectra including DEPT-135 and DEPT-90 of 6",7"-dihydro-7-epiexaltoside (**7**)

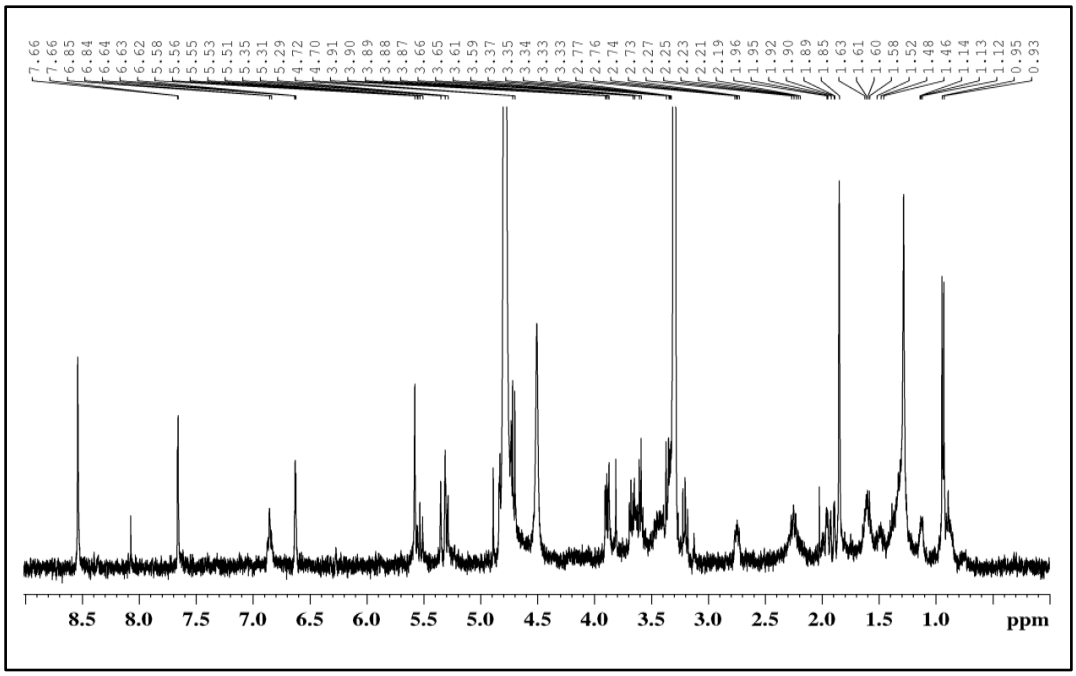

Figure.3.19 ^{1}H-NMR spectrum of 6”,7”-dihydro-7- epiexaltoside (**7**)

3.3.2.3 Menthiafolin (8)

The structure of compound **8** was elucidated by ^{1}H-NMR, ^{13}C-NMR (DEPT-135 and DEPT-90) as **menthiafolin (8)** (Fig. 3.20).

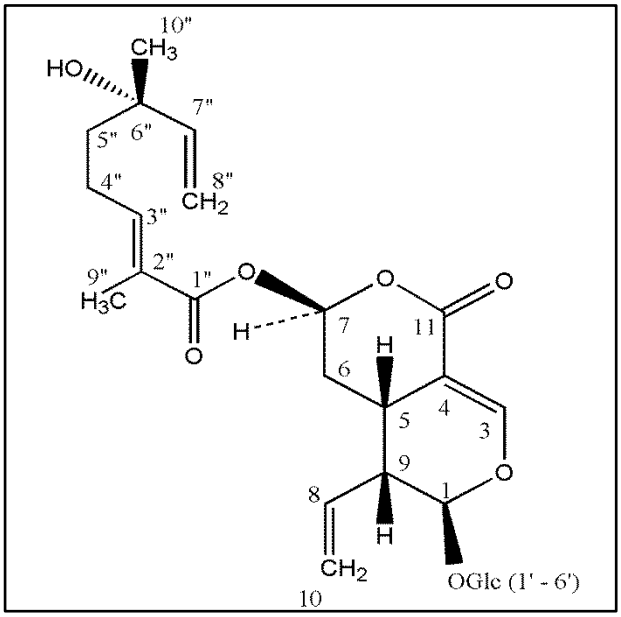

Figure 3.20 Structure of menthiafolin (**8**)

Library search on the ^{13}C-NMR data (Table 3.5 and Fig. 3.21) suggested this compound is a seco-iridoid with a glucosyl moiety. The ^{13}C-NMR spectrum of compound **8** revealed that it contained 26 carbon atoms, 16 of which belonging to the seco-iridoid core being in very good agreement with compounds **6** and **7**. However, a C10 side chain with a terminal double bond could be assigned as reported before by Junior (1989). Based on its ^{1}H-NMR (Table 3.5 and Fig. 3.22) and ^{13}C-NMR (Table 3.5 and Fig. 3.21) and mass spectral data compound **8** was found to be menthiafolin reported before from *Menyanthes trifoliata* ((Junior, 1989).

In fact the acyl substituent present in compound **8** is 6-hydroxy-2,6-dimethyl-2-(E),7-octa-dienoic acid, which was also isolated as a pure compound from the chloroform fraction (see below). The signals at 1.28 ppm (s) and 1.85 ppm (s) can be assigned to the methyl groups C-9”and C-10".

The vinyl protons at C-7 "and C-8" act as a typical ABX spectrum. The signal for H-7" (5.93; dd; *J*=17.4; 10.7 Hz) constitutes the X part of an ABX system, showing a coupling with signals at 5.07 ppm (dd; *J*=10.8; 1.5 Hz) and 5.24 ppm (dd; *J*=17.3; 1.5; Hz) (H- 8"a and H-8"b) as the AB part.

Yellow powder; UV (acetonitrile / H_2O) λ_{max} 200 and 233 nm; ESI-MS (positive ion mode): *m/z* 563 $[M+Na]^+$ consistent with a molecular formula $C_{26}H_{36}O_{12}$.

This is the first report on the occurrence of seco-iridoid in *N. indica*. Menthiafolin was previously isolated from *Menyanthes trifoliata* (Menynathaceae). *Nymphoides indica* also belongs to the Menynathaceae family that comprises mainly aquatic and wetland herbs.

Table 3.5 ^{1}H-NMR and ^{13}C-NMR assignments for menthiafolin (**8**) recorded in methanol-d_4.

Position	δ_H (ppm); multiplicity; *J* (Hz)	δ_C (ppm)
1	5.61; d; *J*= 1.8	96.6
3	7.64; d; *J*= 2.4	153.1
4		103.3
5	3.30-3.45 (1H, m, overlapped by sugar protons H-5	23.8
6	1.60-1.76; m	28.9
7	6.60; m	93.3
8	5.53; m	131.5
9	2.77; m	42.3
10	5.33-5.38; m, H-10a and H-10b	120.0
11		165.1
1'	4.70; d; *J*= 7.8	98.3
2'	3.18-3.95; m	73.4
3'	3.18-3.95; m	77.0
4'	3.18-3.95; m	70.2
5'	3.18-3.95; m	76.5
6'	3.18-3.95; m	61.3
1"		165.5
2"		126.3
3"	6.92; dt; *J*= 7.5;1.4	145.0
4"	2.23; m	23.3
5"	1.65; m	40.1
6"		73.30
7"	5.93; dd; *J*=17.4; 10.7	144.5
8a	5.07; dd; *J*=10.7;1.5	111.0
8b	5.24; dd; *J*=17.4;1.5	
9"	1.85; s	10.8
10"	1.28; s	26.4

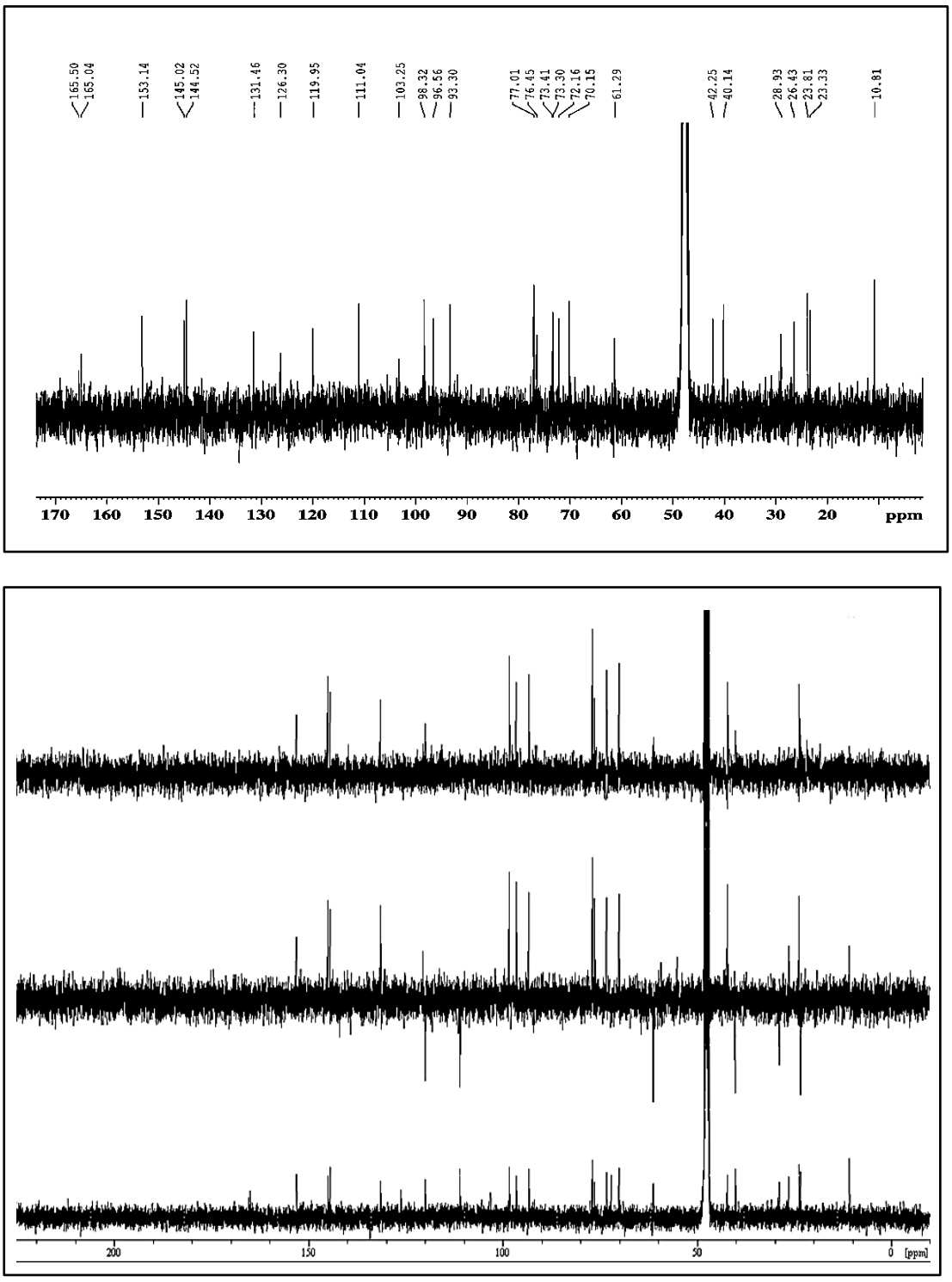

Figure 3.21 ^{13}C-NMR spectra including DEPT 135 and DEPT 90 of menthiafolin (**8**)

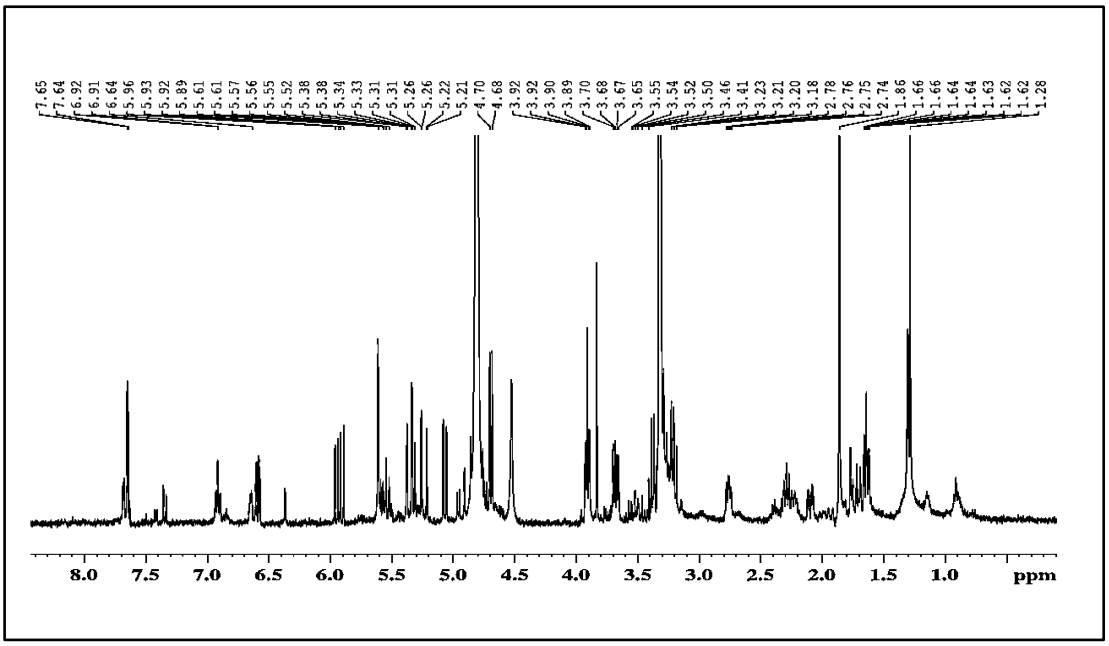

Figure 3.22 ^{1}H-NMR spectrum of menthiafolin (**8**)

3.3.3 Flavonoids (9-11)

3.3.3.1 3,7-Di-*O*-methylquercetin-4'-*O*-β-glucoside (9)

The structure of compound **9** was elucidated by ^{1}H-NMR, ^{13}C-NMR (DEPT-135 and DEPT-90) and 2D-NMR as **3,7-di-*O*-methylquercetin-4'-*O*-β-glucoside (9)** (Fig. 3.23).

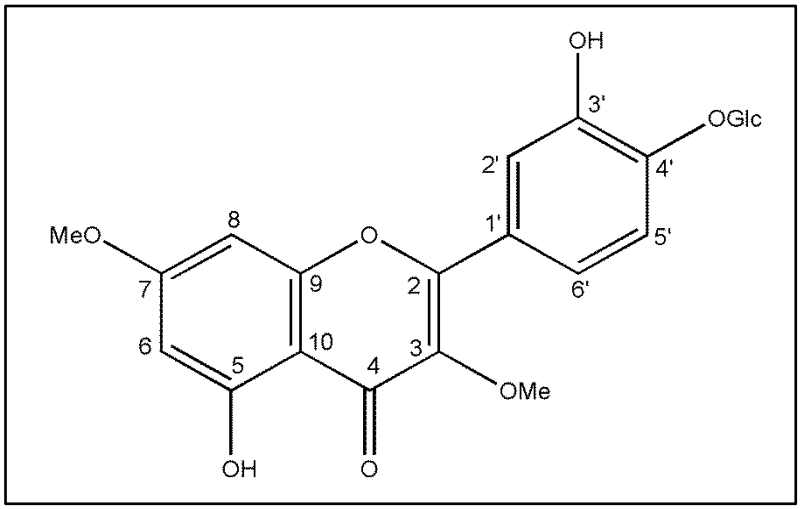

Figure 3.23. Structure of 3,7-di-*O*-methylquercetin-4'-*O*-β-glucoside (**9**)

Library search on the ^{13}C-NMR data (Table 3.6 and Fig. 3.24) suggested this compound is a flavonoid with glucosyl moiety. Its ^{1}H, ^{13}C-NMR and mass spectral data were compared with published data (Agarwal and Rastogi, 1981; Guvenalp and Demirezer, 2005), allowing its complete identification.

The ^{1}H-NMR (Table 3.6 and Fig. 3.25) and ^{13}C-NMR (Table 3.6 and Fig. 3.24) spectra of compound **9** exhibited resonances due to aromatic ring systems, typical for a quercetin moiety and a sugar residue. In the ^{1}H-NMR spectrum the aromatic region exhibited an ABX system at 7.66 ppm (1H, d, *J* = 2.2 Hz, H-2'), 7.63 ppm (1H, dd, *J*= 2.2 and 8.6 Hz, H-6'), and 7.33 ppm (1H, d, *J*= 8.6 Hz, H-5') due to a 3',4' substitution of ring B, and a typical meta-coupled pattern for H-6 (6.37 ppm, d, *J*=2.2Hz) and H-8 (6.56 ppm, d, *J*= 2.2 Hz). An anomeric proton signal appeared at 5.05 ppm (*d*, *J* = 7.3 Hz, H-1″) and the resonances in the region of 3.44 -3.96 ppm (6H, m, H-2″, H-3″, H-4″, H-5″, H-6″) together

with the corresponding carbon resonances inferred from the HSQC (Fig 3.26) spectrum suggested the presence of an *O*-glucopyranosyl unit. The signals at 3.82 ppm (s) and 3.91 ppm (s) are assigned to the methoxyl-groups at C-3 and C-7.

The ^{13}C-NMR spectrum of compound **9** showed the presence of 23 aromatic carbon signals. In the HMBC (Fig.3.27) spectrum, a cross-peak between C-4' and H-1" established the linkage point of quercetin and the sugar moiety. The ^{13}C-NMR signals at 55.1 and 59.3 ppm were assigned to the methoxyl groups at C-7 and C-3, respectively.

Yellow powder; UV (acetonitrile / H_2O) λ_{max} 200, 251 and 345 nm ESI-MS (positive ion mode): *m/z* 493 $[M+H]^+$ consistent with a molecular formula $C_{23}H_{24}O_{12}$.

3,7-Di-*O*-methylquercetin-4'-*O*-β-glucoside (**9**) was the major flavonoid isolated from leaves of *Nymphoides indica* during the current investigation. Previous records also suggested its occurrence in *Nymphoides indica* leaves (Bhom *et al*., 1986).

Table 3.6. ^{1}H-NMR and ^{13}C-NMR assignments for 3,7-di-*O* methylquercetin-4'-*O*-β-glucoside (**9**) recorded in methanol-d_4.

Position	δ ^{1}H ppm, multiplicity, *J* Hz,	δ ^{13}C ppm
1		
2		146.7
3		138.9
4		178.8
5		156.0
6	6.37; d; *J*= 2.2	97.7
7		166.0
8	6.56; d; *J*= 2.2	91.8
9		156.9
10		105.4
1'		
2'	7.66; d; *J*= 2.2	125.9
3'		116.2
4'		147.8
5'	7.33; d; *J*=8.6	115.8
6'	7.63; dd, *J*=8.6; 2.0	120.6
1"	5.05; d; *J*=7.3	101.8
2"	3.44-3.96; m	73.4
3"	3.44-3.96; m	69.9
4"	3.44-3.96; m	76.1
5"	3.44-3.96; m	61.1
6"	3.44-3.96; m	77.1
OMe (C-7)	3.91; s	55.1
OMe (C-3)	3.82; s,	59.3

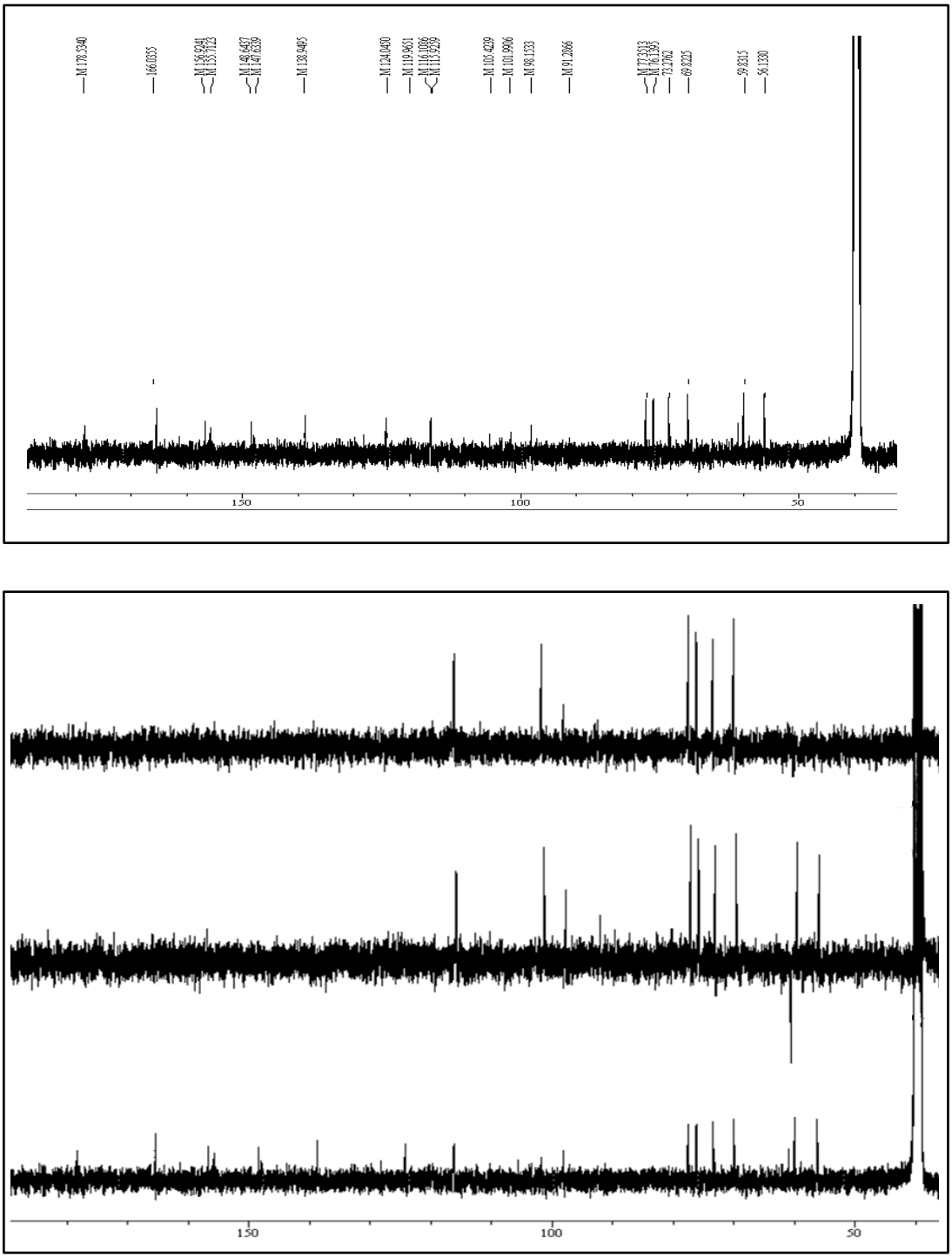

Figure. 3.24 ^{13}C-NMR spectra including DEPT-135 and DEPT-90 of 3,7-di-*O*-methylquercetin- 4'-*O*- β-glucoside (**9**)

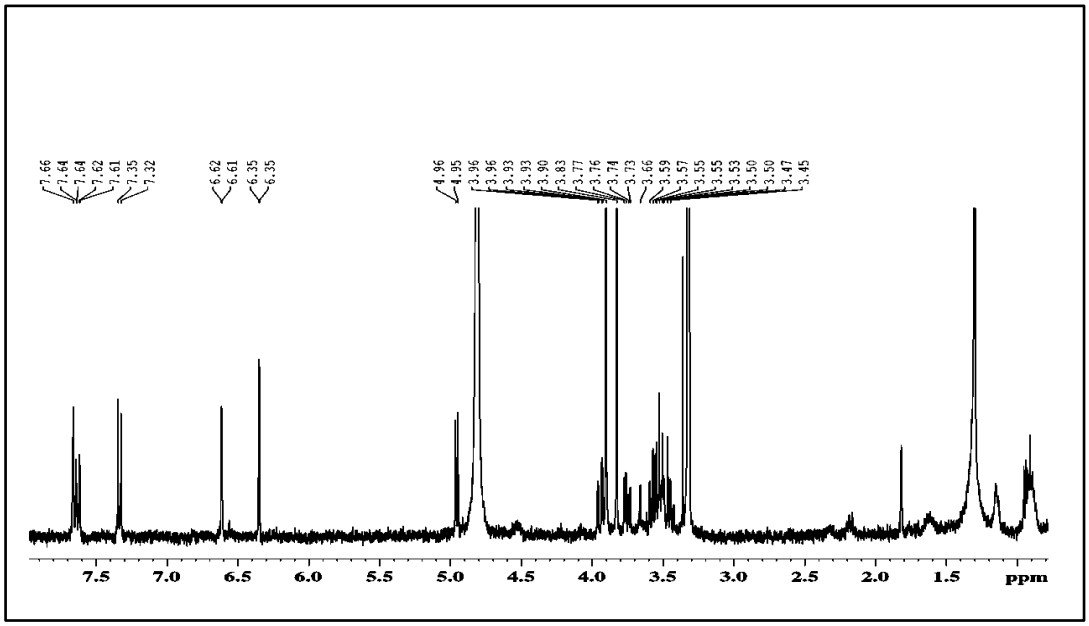

Figure.3.25 ^{1}H-NMR spectrum of 3,7-di-*O*-methylquercetin-4'- β-*O*-glucoside (**9**)

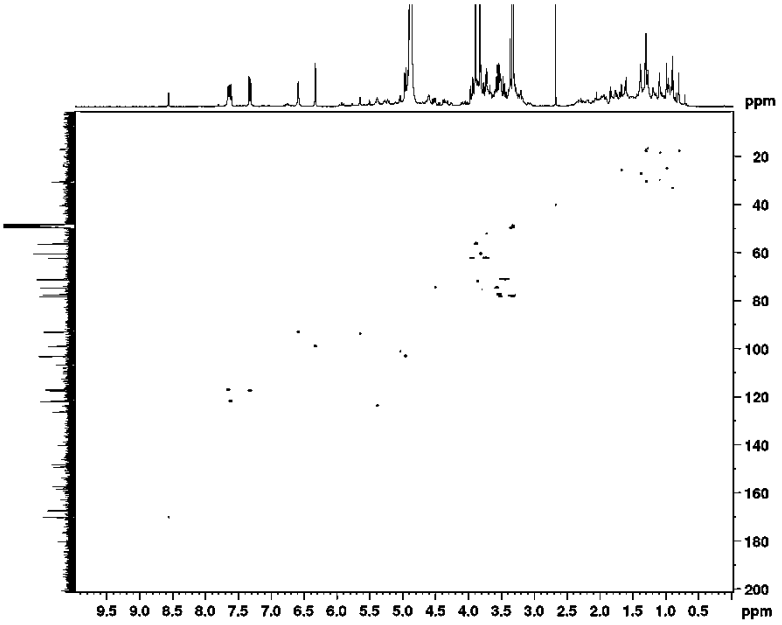

Figure.3.26 HSQC spectrum of 3,7-di-*O*-methylquercetin-4'-*O*- β-glucoside (**9**)

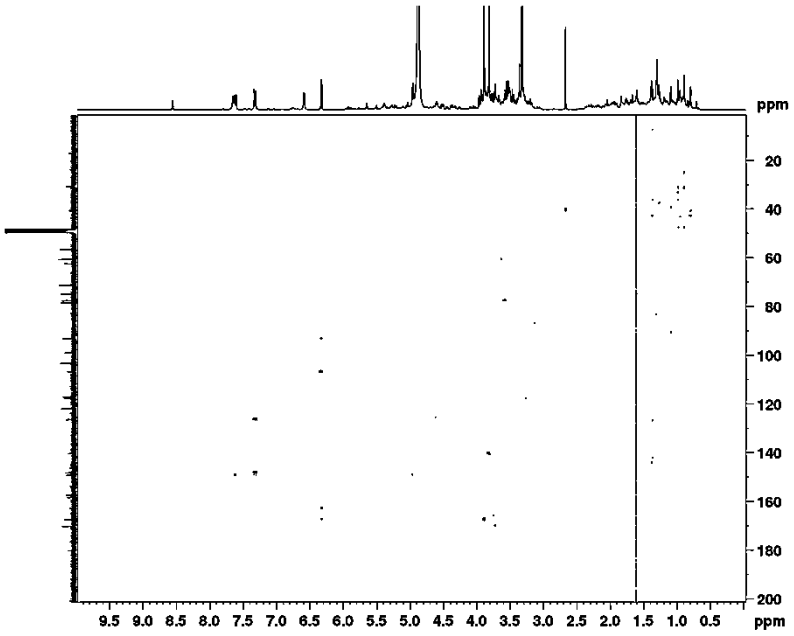

Figure.3.27 HMBC spectrum of 3,7-di-*O*-methylquercetin-4'-*O*- β-glucoside (**9**)

3.3.3.2 3-*O*-Methylquercetin-7-*O*- β-glucoside (10)

The structure of compound **10** was elucidated by ^{1}H-NMR, ^{13}C-NMR (DEPT-135 and DEPT-90) and 2D NMR (HSQC and HMBC) as **3-*O*-methylquercetin-7-*O*- β-glucoside** (**10**) (Fig. 3.28).

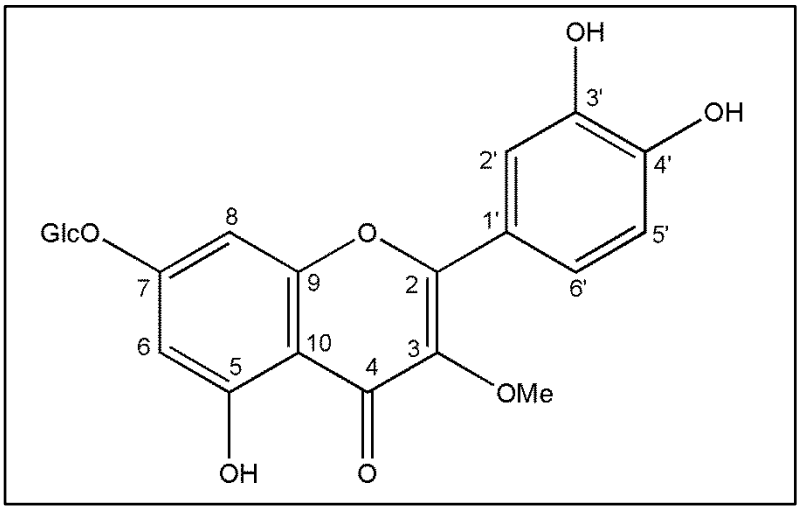

Figure 3.28 Structure of 3-*O*-methylquercetin-7-*O*- β-glucoside (**10**)

Library search on the ^{13}C-NMR data (Table 3.7 and Fig. 3.29) suggested this compound is a flavonoid with a glucosyl moiety. By comparison with published data (Krenn *et al*., 2003), compound **10** was identified as 3-*O*-methylquercetin-7-*O*-β-glucoside.

The ^{1}H-NMR (Table 3.7 and Fig. 3.30) and ^{13}C-NMR (Table 3.7 and Fig. 3.29) spectra of compound **10** exhibited resonances due to aromatic ring systems, typical for a quercetin moiety and a sugar residue as discussed earlier for compound **9**. Similar to the ^{1}H-NMR spectrum of **9**, the aromatic region exhibited a typical pattern (an ABX system) at 7.66 ppm (1H, d, *J* = 2.0 Hz, H-2'), 7.57 ppm (1H, dd, *J*= 2.0 and 8.4 Hz, H-6'), and 6.92 ppm (1H, d, *J*= 8.4 Hz, H-5') due to a 3', 4' substiution of ring B and a typical meta-coupled pattern for H-6 (6.50 ppm, d, *J*=2.0Hz) and H-8 (6.77 ppm, d, *J*= 2.0 Hz). An anomeric proton appeared at 5.07 ppm (*d*, *J* = 7.3 Hz, H-1″) and the resonances in the region of 3.41-3.96 ppm (6H, m, H-2″, H-3″, H-4″, H-5″, H-6″) suggested the presence of an O-

glucopyranosyl unit. The signals at 3.83 ppm (s) was assigned to a methoxyl group at C-3.

The ^{13}C-NMR spectrum of compound **10** showed the presence of 22 aromatic carbon signals. The chemical shift of C-7 (163.4 ppm) indicated substitution with a sugar moiety. The ^{13}C-NMR signal at 59.1 ppm was assigned to the methoxyl group at C-3. Based on its ^{1}H and ^{13}C-NMR assignments and literature (Krenn *et al*., 2003) the structure was confirmed as 3-*O*-methylquercetin-7-*O*-β-glucoside. The spectral assignments were confirmed by HSQC (Fig. 3.31) and HMBC (Fig. 3.32).

Yellow amorphous powder; UV (acetonitrile / H_2O) λ_{max} at 200, 251 and 345 nm; ESI-MS (positive ion mode): *m/z* 479 $[M+H]^+$ consistent with a molecular formula $C_{22}H_{22}O_{12}$.

This is a common flavonoid, previously isolated from a variety of medicinal plants, for instance *Achilllea nobilis* (Asteraceae) and *Leonotis leonurus* (Lamiaceaae).

Table 3.7 ^{1}H-NMR and ^{13}C-NMR assignments for 3-*O*-methylquercetin-7-*O*-β-glucoside (**9**) recorded in methanol-d_4.

Position	**δ ^{1}H ppm, multiplicity, J Hz,**	**δ ^{13}C ppm**
2		156.6
3		138.4
4		178.8
5		161.4
6	6.50; d; *J*=2.0	99.4
7		163.4
8	6.77; d; *J*=2.0	94.4
9		157.2
10		106.3
1'		121.1
2'	7.66; d; *J*=2.0	115.2
3'		145.1
4'		148.9
5'	6.92; d; *J*=8.4	115.1
6'	7.57; dd; *J*=8.4; 2.0	121.3
1"	5.07; d; *J*=7.3	100.3
2"	3.41-3.96; m	73.3
3"	3.41-3.96; m	76.5
4"	3.41-3.96; m	69.9
5"	3.41-3.96; m	77.0
6"	3.41-3.96; m	61.1
OMe (C-3)	3.83; s	59.1

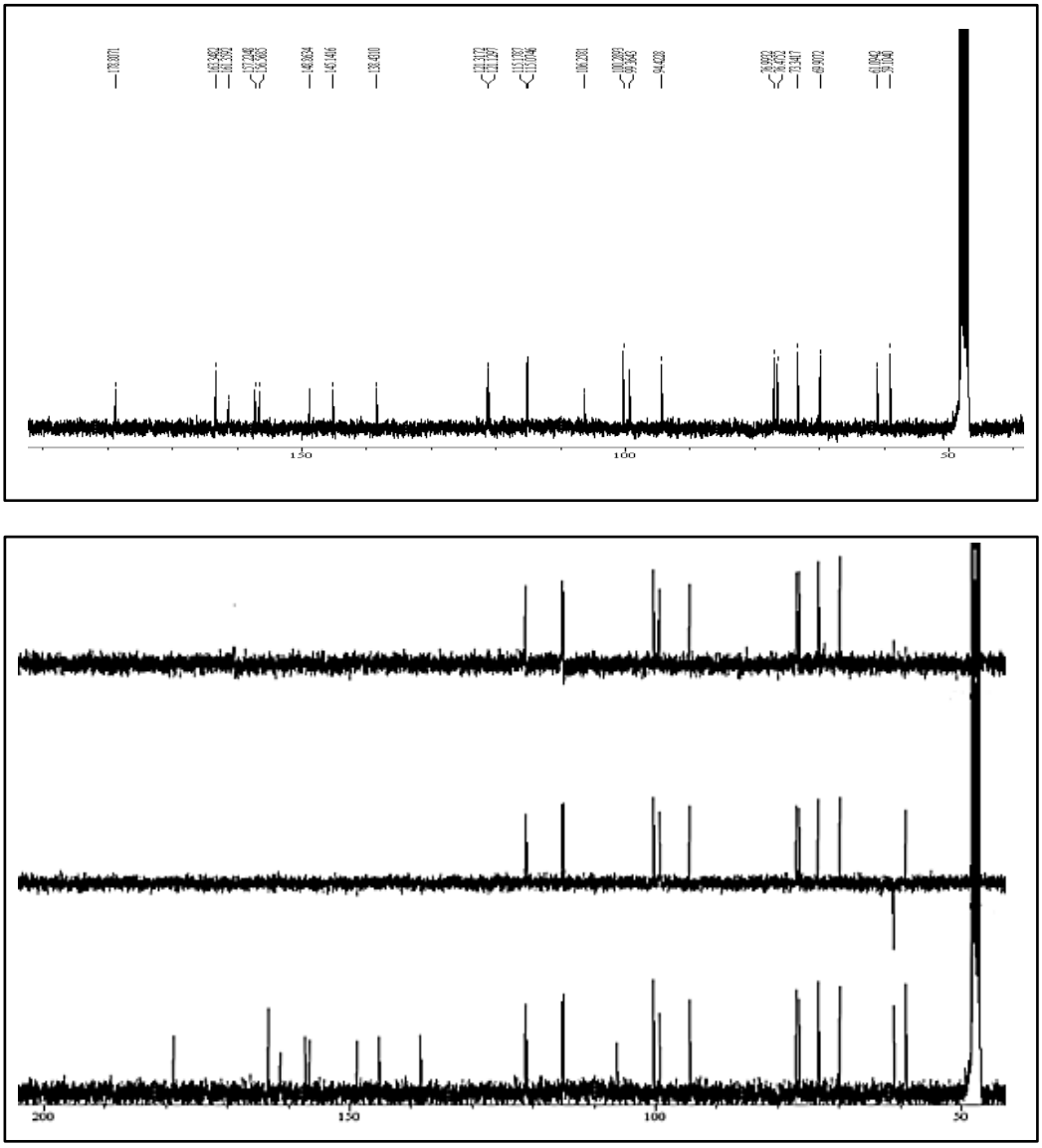

Figure.3.29 ^{13}C-NMR spectra including DEPT-135 and DEPT-90 of 3-*O*-β-methylquercetin-7-*O*-glucoside (**10**)

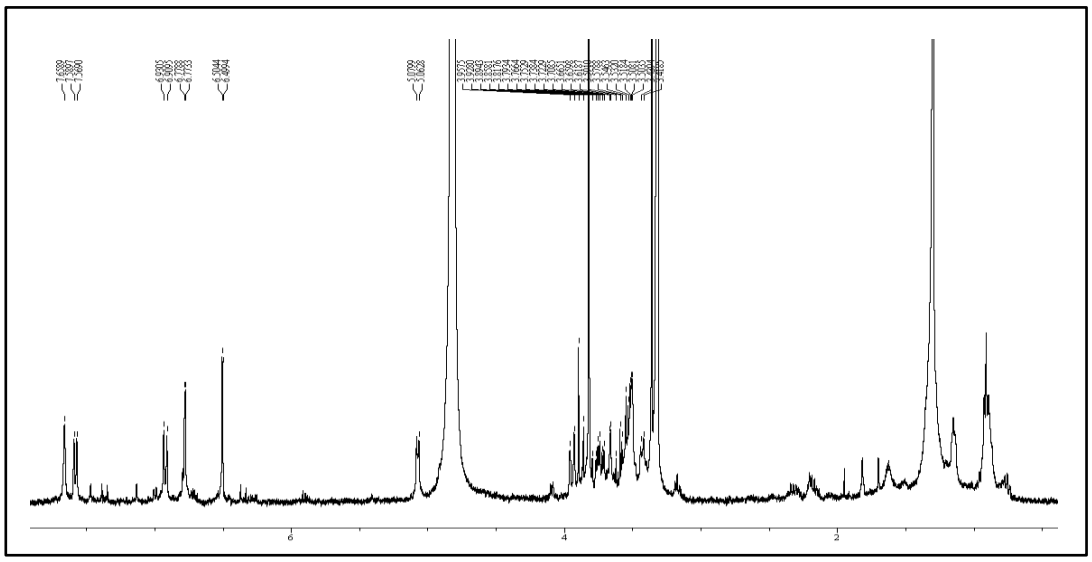

Figure.3.30 ^{1}H-NMR spectrum of 3,7-di-*O*-methylquercetin-4'-*O*- β-glucoside (**10**)

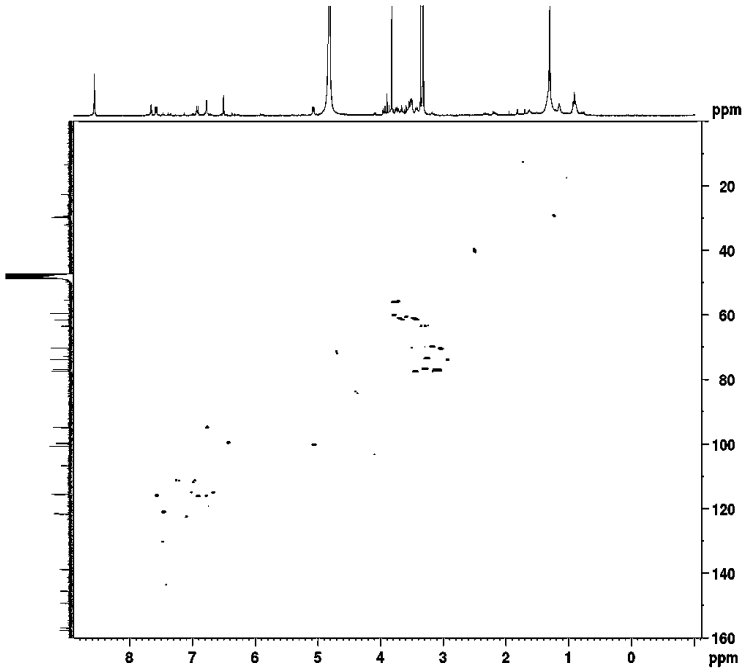

Figure.3.31 HSQC spectrum of 3,7-di-*O*-methylquercetin-4'-*O*- β-glucoside (**10**)

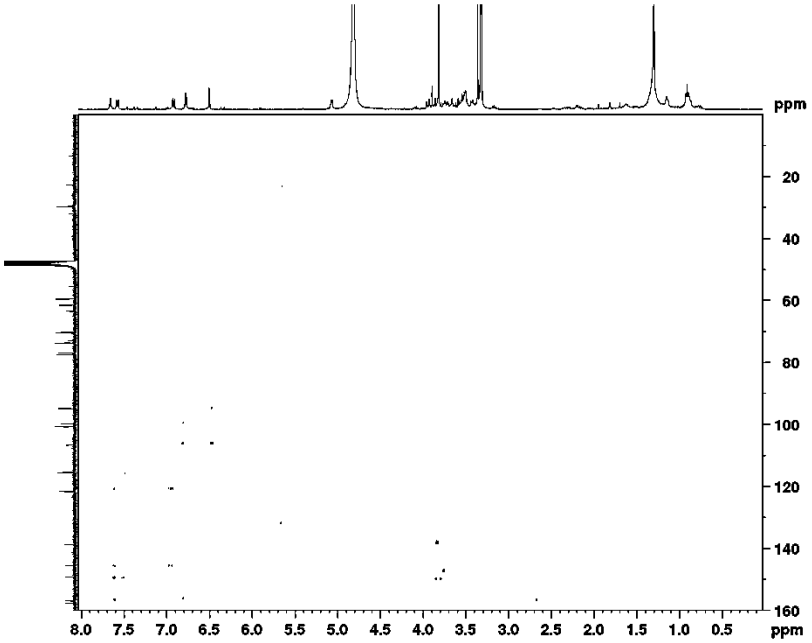

Figure 3.32 HMBC spectrum of 3,7-di-*O*-methylquercetin-4'-*O*- β-glucoside (**10**)

3.3.3.3 3,7-di-*O*-methylquercetin (**11**)

The structure of compound **10** was elucidated by ^{1}H-NMR, ^{13}C-NMR (DEPT-135 and DEPT-90) as **3,7-di-*O*-methylquercetin** (**11**) (Fig. 3.33).

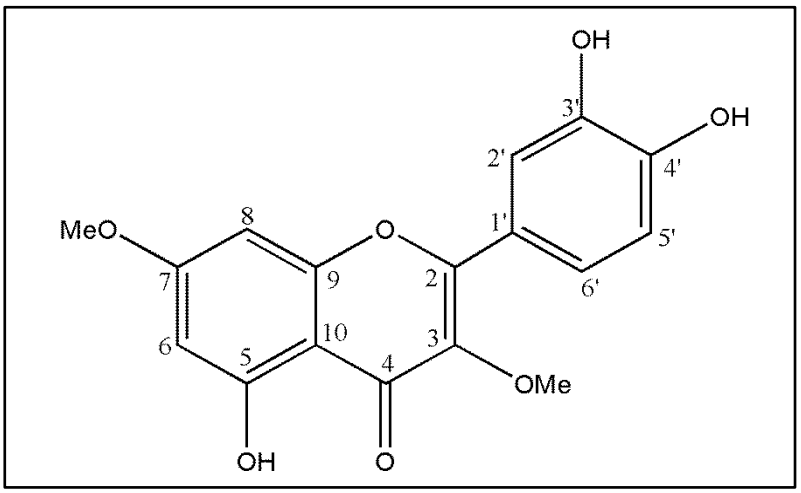

Figure 3.33 Structure of 3,7-di-*O*-methylquercetin (**11**)

Library search on the ^{13}C-NMR data (Table 3.7 and Fig. 3.34) suggested this compound is a flavonoid with a glucosyl moiety. By comparison with published data (Guerrero *et al*.,2002), compound **11** was identified as 3,7-di-*O*-methylquercetin.

The ^{1}H-NMR (Table 3.7 and Fig. 3.35) and ^{13}C-NMR (Table 3.7 and Fig. 3.34) spectra of compound **11** exhibited resonances due to aromatic ring systems, typical for a quercetin moiety. The ^{1}H-NMR spectrum exhibited a typical pattern (an ABX system) as discussed earlier (for compound **9** and **10**) at 7.65 ppm (1H, d, *J* = 2.0 Hz, H-2'), 7.56 ppm (1H, dd, *J*= 2.0 and 8.4 Hz, H-6'), and 6.92 ppm (1H, d, *J*= 8.4 Hz, H-5') due to a 3', 4' substiution of ring B and a typical meta-coupled pattern for H-6 (6.30 ppm, d, *J*=2.0Hz) and H-8 (6.62 ppm, d, *J*= 2.0 Hz). The signals at 3.83 ppm (s) and 3.90 ppm (s) was assigned to a methoxyl group at C-3 and C-7, respectively.

The ^{13}C-NMR spectrum of compound **11** showed the presence of 17 aromatic carbon signals. The chemical shift of C-4 (163.4 ppm) indicated the presence of a carbonyl moiety. The ^{13}C-NMR signals at 59.1 ppm and 55.1 were assigned to the methoxyl groups at C-3 and C-7, respectively. Based on its ^{1}H and ^{13}C-NMR assignments and literature (Guerrero *et al.*, 2002) the structure was confirmed as 3,7-di-*O*-methylquercetin. All correlations were confirmed by COSY (Fig. 3.36), HSQC (Fig. 3.37) and HMBC (Fig 3.38).

Yellow amorphous powder; UV (acetonitrile / H_2O) λ_{max} 203, 257 and 360 nm ESI-MS (positive ion mode): *m/z* 331 $[M+H]^+$ consistent with a molecular formula $C_{17}H_{14}O_7$.

This flavonoid was previously isolated from a variety of medicinal plants, for instance *Croton schiedeanus Schlecht* (Euphorbiaceae), *Saccopetalum horsfieldii* Benn (Annonaceae) and *Hypericum ternum* (*Hypericaceae*).

Table 3.8 ^{1}H-NMR and ^{13}C-NMR assignments for 3,7-di-*O*-methylquercetin (**11**) recorded in methanol-d_4.

Position	**δ ^{1}H ppm, multiplicity, J Hz,**	**δ ^{13}C ppm**
2		157.0
3		138.4
4		178.8
5		161.6
6	6.34; d; *J*= 2.0	97.5
7		166
8	6.62; d; *J*= 2.0	91.7
9		159.2
10		105.3
1'		121.4
2'	7.65; d; *J*=2.0	115.0
3'		145.1
4'		148.9
5'	6.92; d; *J*= 8.4	115.2
6'	7.56; dd; *J*= 8.4; 2.0	121.4
OMe (C-3)	3.81; s	55.1
OMe (C-7)	3.90; s	59.1

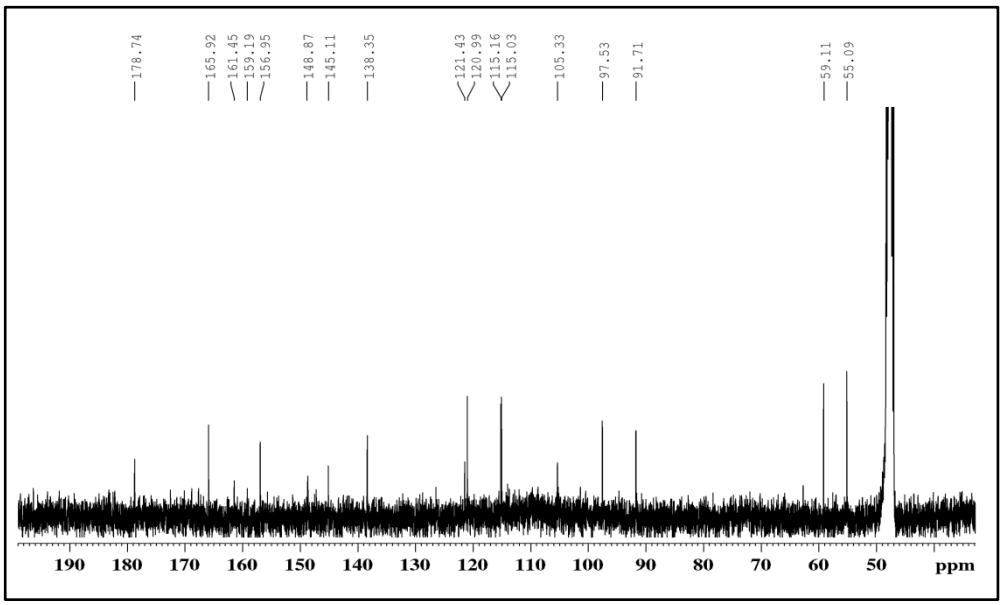

Figure.3.34 ^{13}C-NMR spectra of 3,7-di-*O*-methylquercetin (**11**)

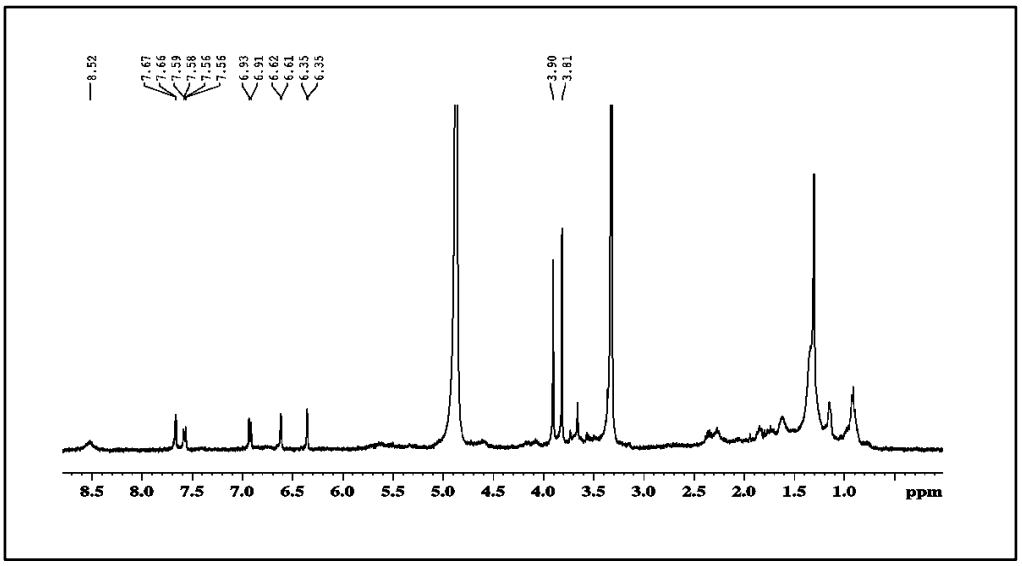

Figure.3.35 ^{1}H-NMR spectra of 3,7-di-*O*-methylquercetin (**11**)

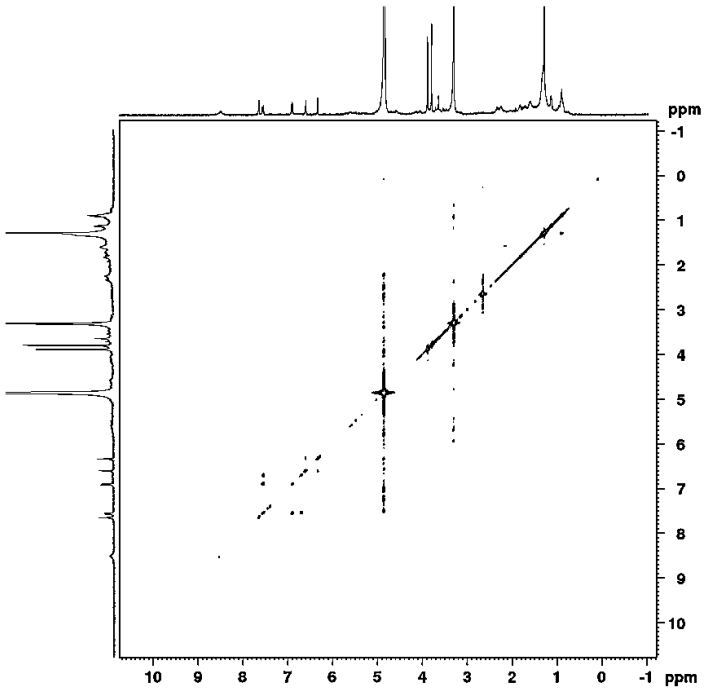

Figure. 3.36 COSY spectrum of 3,7-di-*O*-methylquercetin

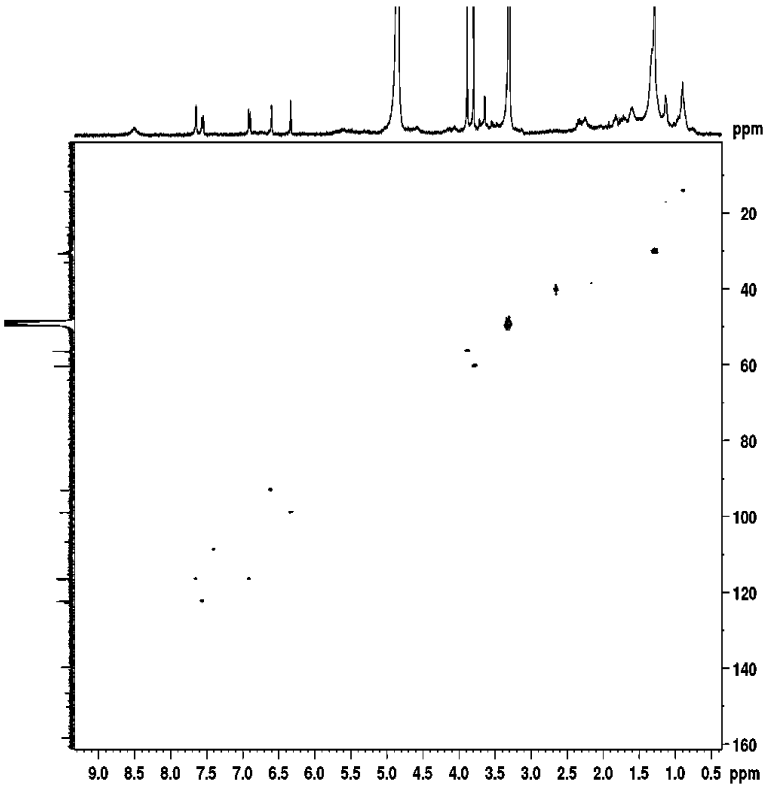

Figure 3.37 HSQC spectrum of 3,7-di-*O*-methylquercetin

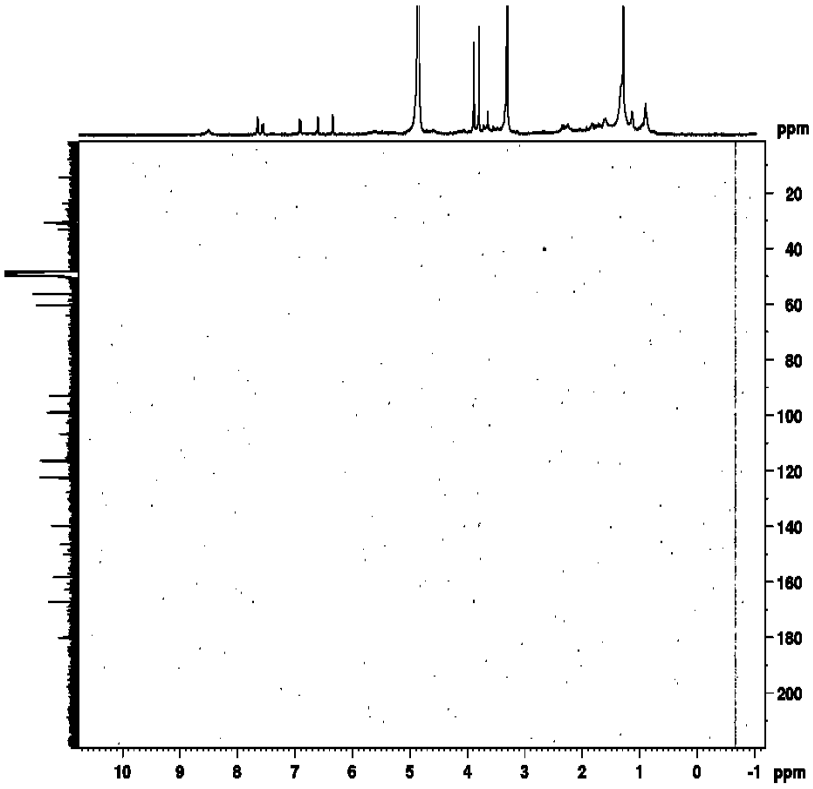

Figure 3.38 HMBC spectrum of 3,7-di-*O*-methylquercetin

3.3.4. Coumarins (12)

3.3.4.1 Scopoletin (12)

The structure of compound **12** was elucidated by ^{1}H- and ^{13}C-NMR as scopoletin (**12**) (Fig. 3.39).

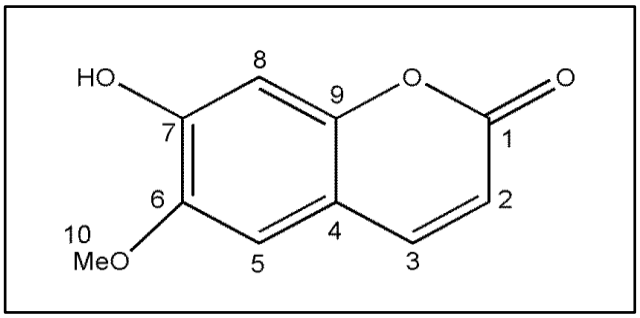

Figure 3.39 Structure of scopoletin (**12**)

Library search on the ^{13}C-NMR data (Table 3.8 and Fig. 3.40) suggested this compound is a methoxylated coumarin. Based on its ^{1}H, ^{13}C-NMR and mass spectral data compound **12** was found to be scopoletin (Darmawan *et al*., 2012).

The ^{1}H-NMR (Table 3.8 and Fig. 3.41) and ^{13}C-NMR (Table 3.8 and Fig. 3.40) spectra of compound **12** are indicative of an aromatic ring system. The ^{1}H-NMR spectrum revealed two coupled doublets at 6.21 ppm (J=9.4 Hz) and 7.87ppm (J=9.4 Hz) that were assigned to H-2 and H-3, respectively. Similarly two singlets appeared at 7.11 ppm and 6.77 ppm that were assigned to H-5 and H-8, respectively. Finally a methoxyl signal was observed at 3.92 ppm (s) that was assigned to H-10.

The ^{13}C-NMR spectrum indicated the presence of 11 carbons. The carbonyl group of the ester moiety presented a resonance at 168.8 ppm. In the same ring, the signal at 117.6 ppm was assigned to C-2 and the signal at 144.8 ppm to C-3. In the aromatic ring C-5 and C-8 were assigned 108.5 ppm and 102.6 ppm, respectively. Three quaternary aromatic carbons (C-4, C-6 and C-7) were assigned to the signals at 110.8 ppm, 145.9 ppm and 150.2 ppm, respectively. Similarly, C-9 was assigned to the signal at 152.6

ppm. The methoxylated carbon at 55.4 ppm was attributed to C-10. Further structure was confirmed by HSQC (Fig. 3.42) and HMBC (Fig 3.43).

Colourless needles; UV (acetonitrile / H_2O) λ_{max} 226, 287 and 338 nm ESI-MS (positive ion mode): *m/z* 193 $[M+H]^+$ consistent with a molecular formula $C_{10}H_8O_4$.

Scopoletin is a coumarin derivative that has been found in many medicinal plant species, for instance *Macaranga gigantifolia* Merr. (Darmawan *et al*., 2012), *Ipomoea digitata* (Khan and Hossain, 2015). This is however the first report of its presence in *Nymphoides indica*.

Table 3.8 ^{1}H-NMR and ^{13}C-NMR assignments for the scopoletin (**12**) recorded in methanol-d_4.

Position	**δ ^{1}H ppm; multiplicity; J Hz,**	**δ ^{13}C ppm**
1		162.8
2	6.21; d; *J*= 9.4	117.6
3	7.87; d; *J*=9.4	144.8
4		110.8
5	7.11; s	108.5
6		145.9
7		150.2
8	6.77; s	102.6
9		152.6
10	3.92; s	55.4

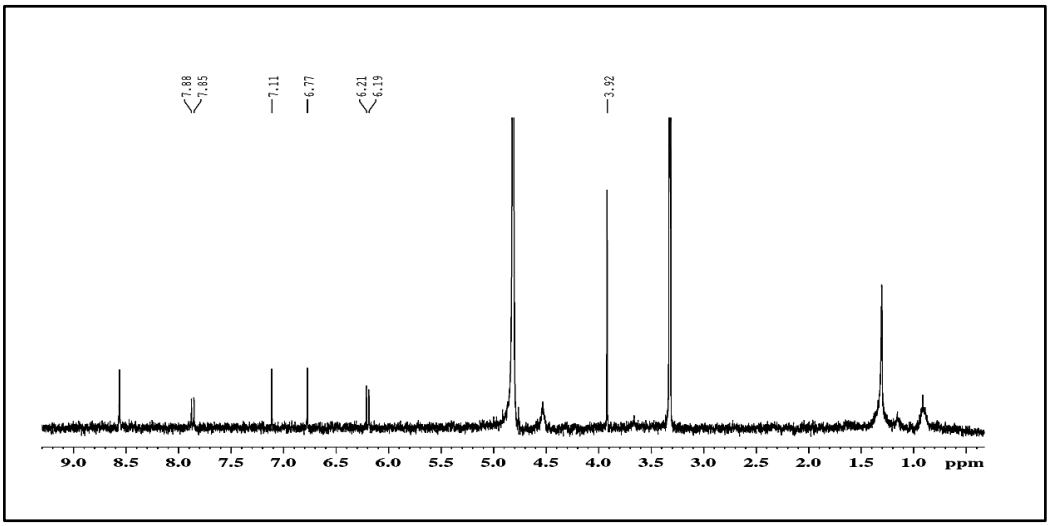

Figure.3.40 ^{1}H-NMR spectrum of scopoletin (**12**)

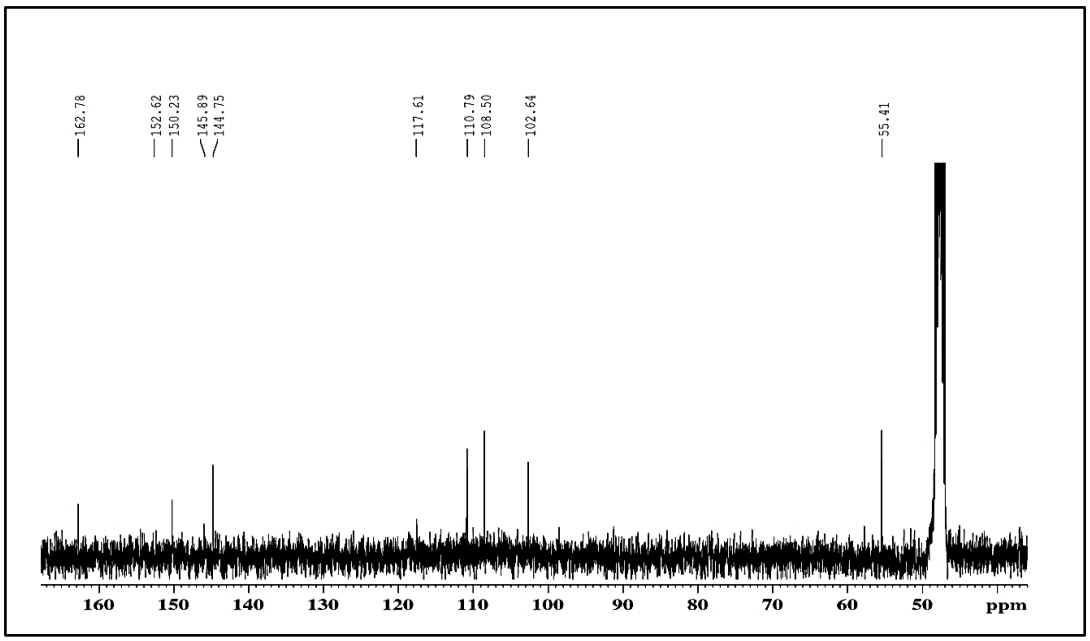

Figure.3.41 ^{13}C-NMR spectrum of scopoletin (**12**)

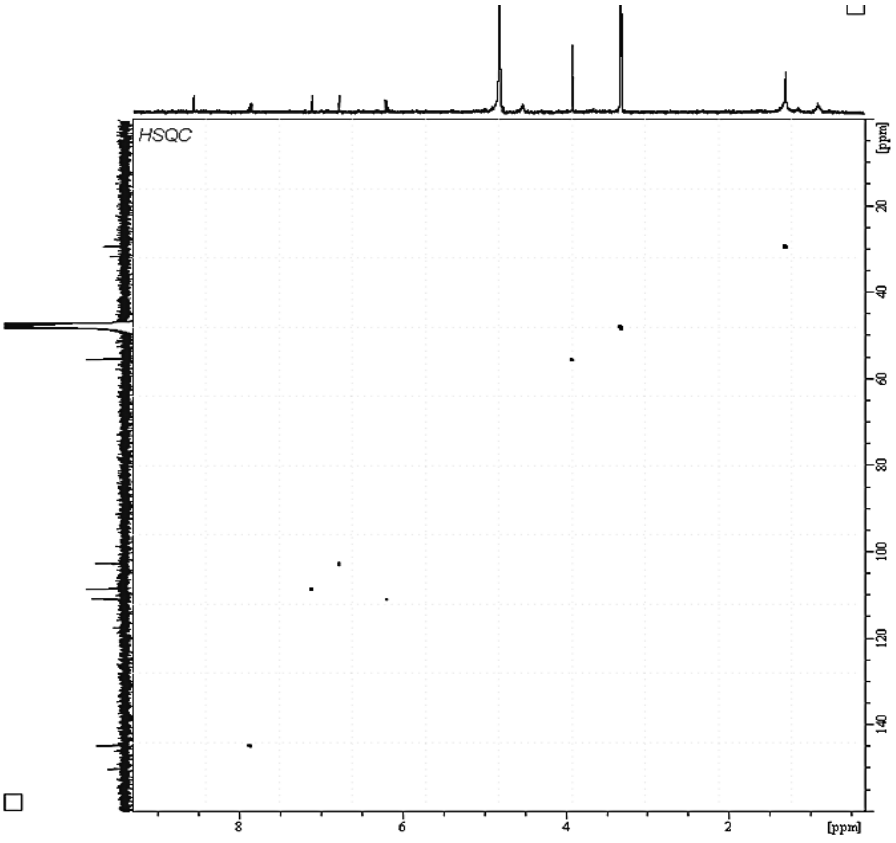

Figure 3.42 HSQC spectrum of scopoletin

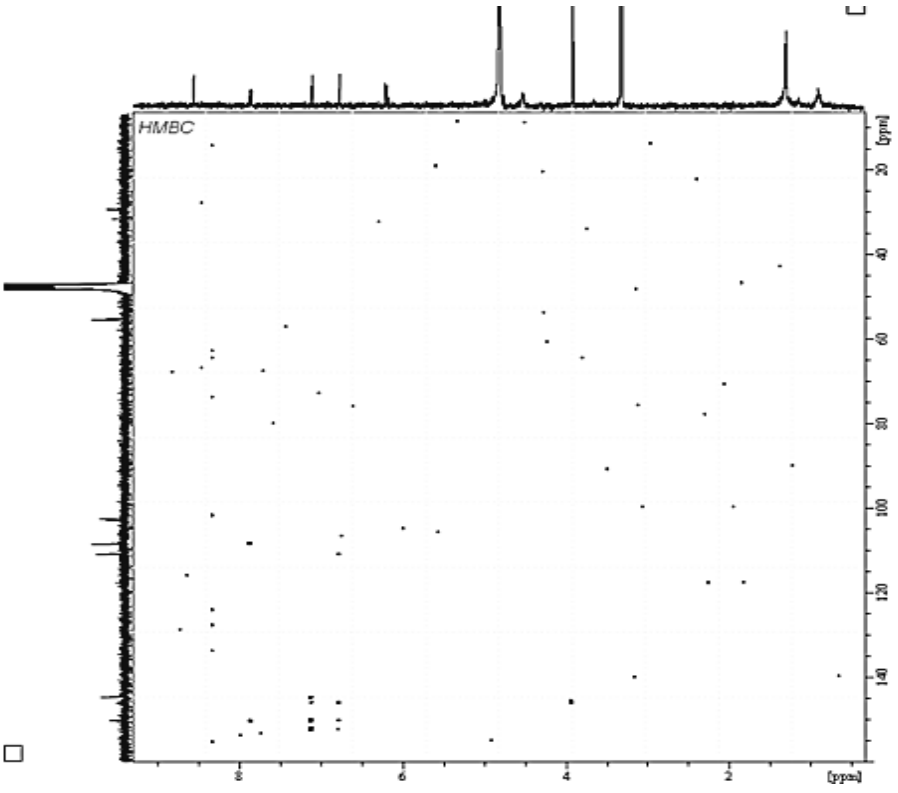

Figure 3.43 HMBC spectrum of scopoletin

3.3.5. Acyclic monoterpenes (13-14)

3.3.5.1 8-Hydroxy-2,6-dimethyl-(2*E*,6*E*)-octadienoic acid (13)

The structure of compound **13** was elucidated by ^{1}H- and ^{13}C-NMR (DEPT 135) as 8-hydroxy-2,6-dimethyl-(2*E*,6*E*)-octadienoic acid (**13**) (Fig 3.44).

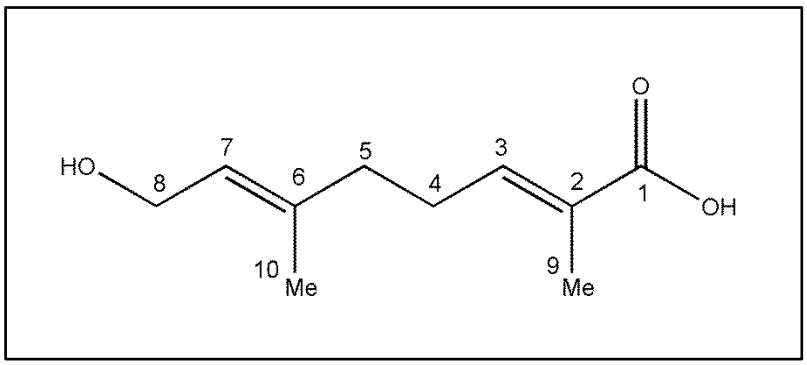

Figure 3.44 Structure of 8-hydroxy-2,6-dimethyl-(2*E*,6*E*)-octadienoic acid (**13**)

Library search on the ^{13}C-NMR data (Table 3.9 and Fig. 3.45) suggested this compound is an acyclic monoterpene. Based on its ^{1}H, ^{13}C-NMR and mass spectral data compound **13** was found to be 8-hydroxy-2,6-dimethyl-(2*E*,6*E*)-octadienoic acid (Iwagawa *et al*., 1990).

The ^{1}H-NMR spectrum (Table 3.9 and Fig. 3.46) revealed signals at 1.68 ppm (s, H-10) and 1.80 ppm (s, H-9) that were assigned to two olefenic methyl groups. Two methylene protons at 2.14 ppm (t, *J*=7.3Hz, H-5) were coupled to other methylene protons at 2.30 ppm (q, J=7.3Hz, H-4), which were in turn coupled with a proton at 6.67 ppm (m, H-3). A doublet at 4.09 ppm (H-8) was attributed to allylic methylene protons that were found adjacent to an hydroxyl group. These protons were coupled with an olefenic proton at 5.39 ppm (m, H-7). The olefenic protons were further coupled with methyl protons at 1.68 ppm. Comparison with related monoterpenes and the acyl side chains of the seco-iridoids **6 – 8** discussed above confirmed the E-configuration of both double bonds.

The ^{13}C-NMR spectrum (Table 3.9 and Fig 3.45) revealed 10 carbon signals. The carbonyl group of the ester moiety showed a signal at 171.1 ppm that was assigned to C-1. The signals at 11.2 ppm and 14.8 ppm were attributed to the methyl groups at C-9 and C-10, respectively. The methylene signals at 26.6 ppm, 37.8 ppm and 58.0 ppm were assigned to C-4, C-5 and C-8, respectively. Similarly two methine signals at 140.8 ppm and 124.2 ppm were attributed to C-3 and C-7, respectively. The signals at 128.6 ppm and 137.1 ppm were assigned to C-2 and C-6.

Yellowish powder; UV (acetonitrile / H_2O) λ_{max} 222 nm; ESI-MS (positive ion mode) *m/z* 207 $[M+Na]^+$ consistent with molecular formula $C_{10}H_{16}O_3$.

Compound **13** was previously reported from *Radermachia sinica* (Iwagawa *et al*., 1990) and was found as a substituent in some iridoids (Junior, 1983). Interestingly, during the current investigation, compound **13** was present as a side chain in compound **6**.

Table 3.9 ^{1}H-NMR and ^{13}C-NMR assignments for 8-hydroxy-2,6-dimethyl-(2*E*,6*E*)-octadienoic acid (**13**) recorded in methanol-d_4.

Position	**δ ^{1}H ppm; multiplicity; J Hz,**	**δ ^{13}C ppm**
1		171.1
2		128.6
3	6.67; m	140.8
4	2.30; q; 7.3	26.6
5	2.14; t; 7.3	37.8
6		137.1
7	5.38; m	124.2
8	4.04; d; *J*= 7.0	58.0
9	1.80; s	11.2
10	1.68; s	14.8

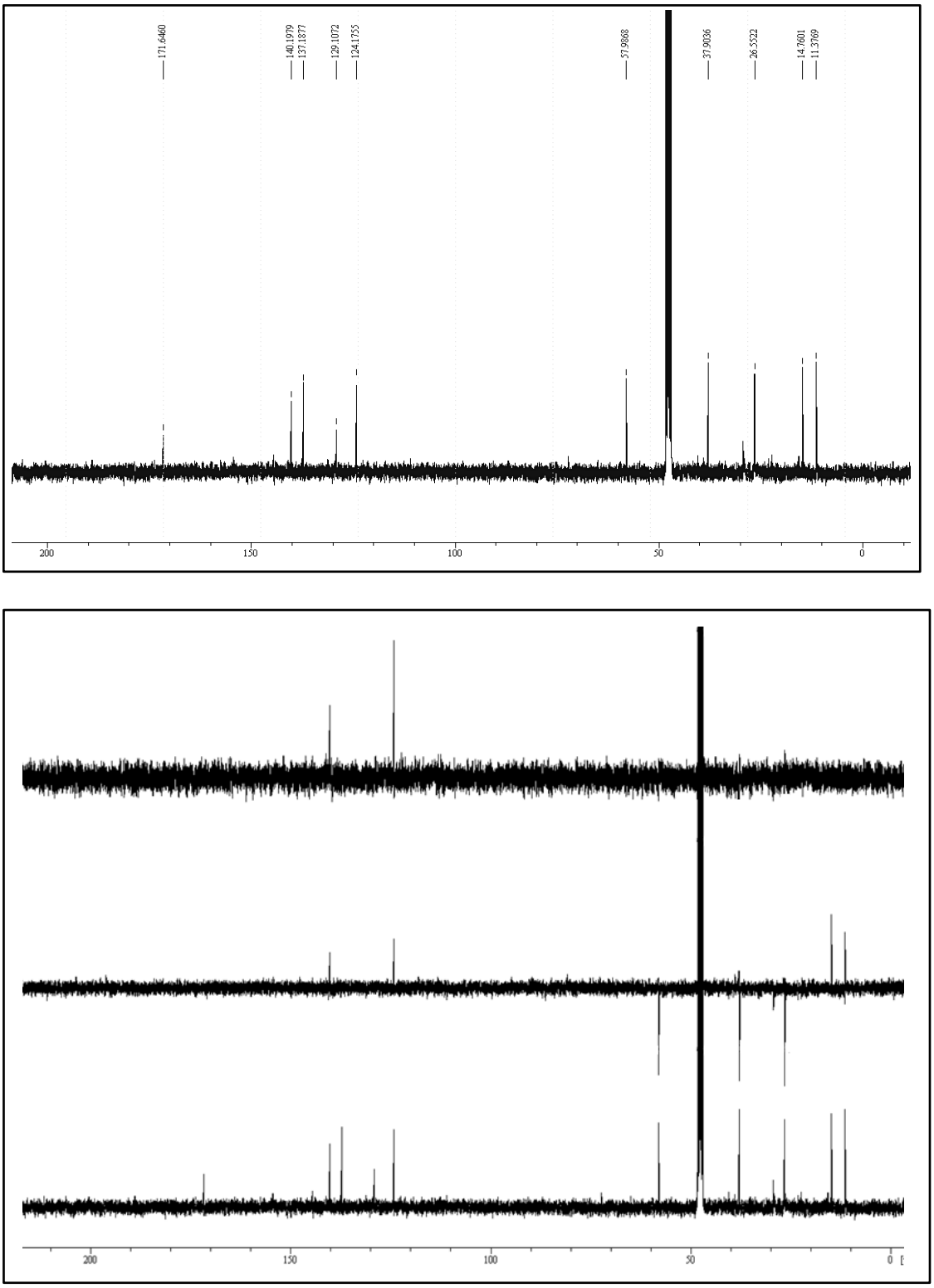

Figure 3.45 ^{13}C-NMR , DEPT-135 and DEPT-90 spectra of 8-hydroxy-2,6-dimethyl-(2*E*,6*E*)- octadienoic acid (**13**)

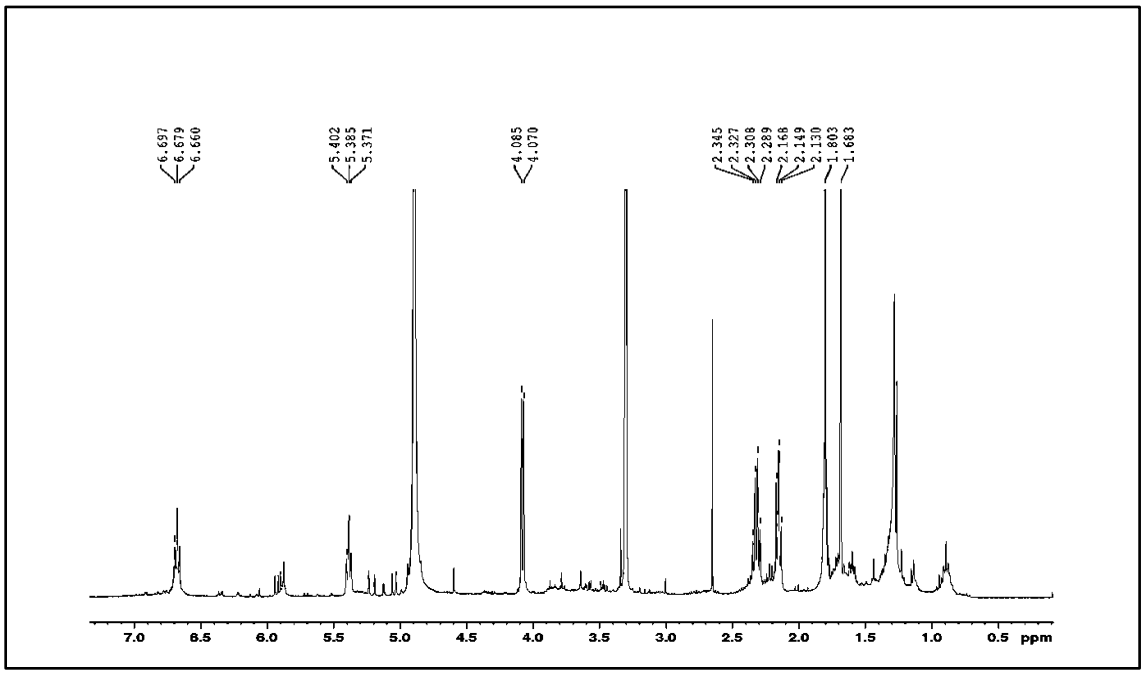

Figure 3.46 ^{1}H-NMR spectrum of 8-hydroxy-2,6-dimethyl-(2*E*,6*E*)-octadienoic acid (**13**)

3.3.5.2 8-Hydroxy-2,6-dimethyl-2-enoate (14)

The structure of compound **14** was elucidated by ^{1}H- and ^{13}C-NMR (DEPT-135) as 8-hydroxy-2,6-dimethyl-2-enoate (**14**) (Fig. 3.47).

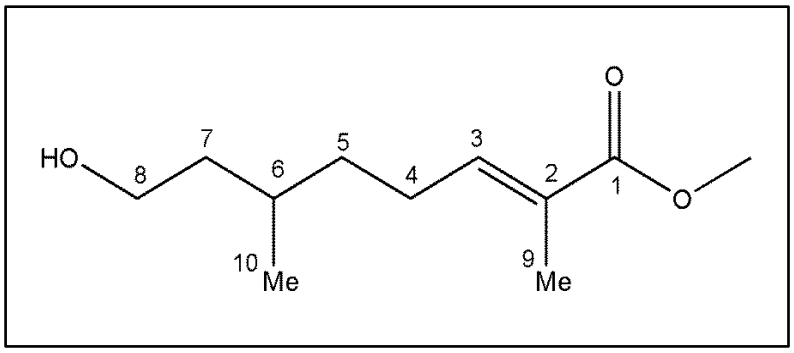

Figure3.47 Structure of 8-hydroxy-2,6-dimethyl-2-enoate (**14**)

Library search on the ^{13}C-NMR data (Table 3.10 and Fig 3.48) suggested this compound is an acyclic monoterpene. Based on its ^{1}H (Table 3.10 and Fig. 3.49) and ^{13}C-NMR spectra (Table 3.10 and Fig. 3.48), and its mass spectral data compound **14** was found to be 8-hydroxy-2,6-dimethyl-2-enoate (Otsuka *et al*., 1994).

In ^{1}H-NMR the signals at 0.94 ppm (d, *J*=7, H-10) and 1.84 ppm (d, *J*=1, H-9) were assigned to two olefenic methyl groups and the signal at 3.71 ppm (s) was attributed to a methoxyl group (-COOMe). A methylene group at 2.26 ppm (m, H-4) was coupled to a proton at 6.77 ppm (m, H-3). The multiplet signal at 1.25 - 1.65 ppm was assigned to H-5 (2H), H-6 (1H) and H-7 (2H), respectively. A multiplet at 3.60 ppm (H-8) was attributed to a methylene group adjacent to hydroxyl functionality.

The ^{13}C-NMR spectrum showed 11 carbon signals. The carbonyl group of the ester moiety was observed at 167.8 ppm. The signals at 11.0 ppm and 18.3 ppm were attributed to the methyl groups at C-9 and C-10, respectively, while the signal at 50.80 ppm was assigned to methoxylated protons adjacent to carbonyl moiety. The methylene signals of monoterpene at 25.7 ppm, 35.6 ppm, 39.2 ppm and 59.5 ppm were assigned to C-4, C-5, C-7 and C-8 respectively. Similarly two methane signals appeared

at 142.7 ppm and 29.1 ppm were attributed to C-3 and C-6 respectively. Finally the signal at 127.1 ppm was assigned to C-2. The *E* configuration of 2,3-double bond was defined from the ^{13}C-NMR chemical shift of the C-9 methyl group at 11.0 ppm, similar to compound **13**.

Yellowish powder; UV (acetonitrile / H_2O) λ_{max} 218 nm; ESI-MS (positive ion mode) *m/z* 201 $[M+H]^+$ consistent with a molecular formula $C_{11}H_{20}O_3$.

Compound **14** was previously reported from *Linaria japonica* (Otsuka *et al*., 1994) in its glycosylated form. This is first report of its occurrence in *N. indica*, although the possibility cannot be excluded that the methyl ester is formed as an artefact during extraction, and the free carboxylic acid is the genuine compound.

Table 3.10. ^{1}H-NMR and ^{13}C-NMR assignments for 8-hydroxy-2,6-dimethyl-2-enoate (**14**) recorded in methanol-d_4.

Position	**δ ^{1}H ppm; multiplicity; J Hz,**	**δ ^{13}C ppm**
1		168.8
2		127.1
3	6.77; m	142.7
4	2.26; m	25.7
5	1.25-1.65; m	35.6
6	1.25-1.65; m	29.1
7	1.25-1.65; m	39.2
8	3.60; m	59.5
9	1.84; d; *J*= 1.0	11.0
10	0.94; d; *J*=7.0	18.3
OMe	3.71; s	50.8

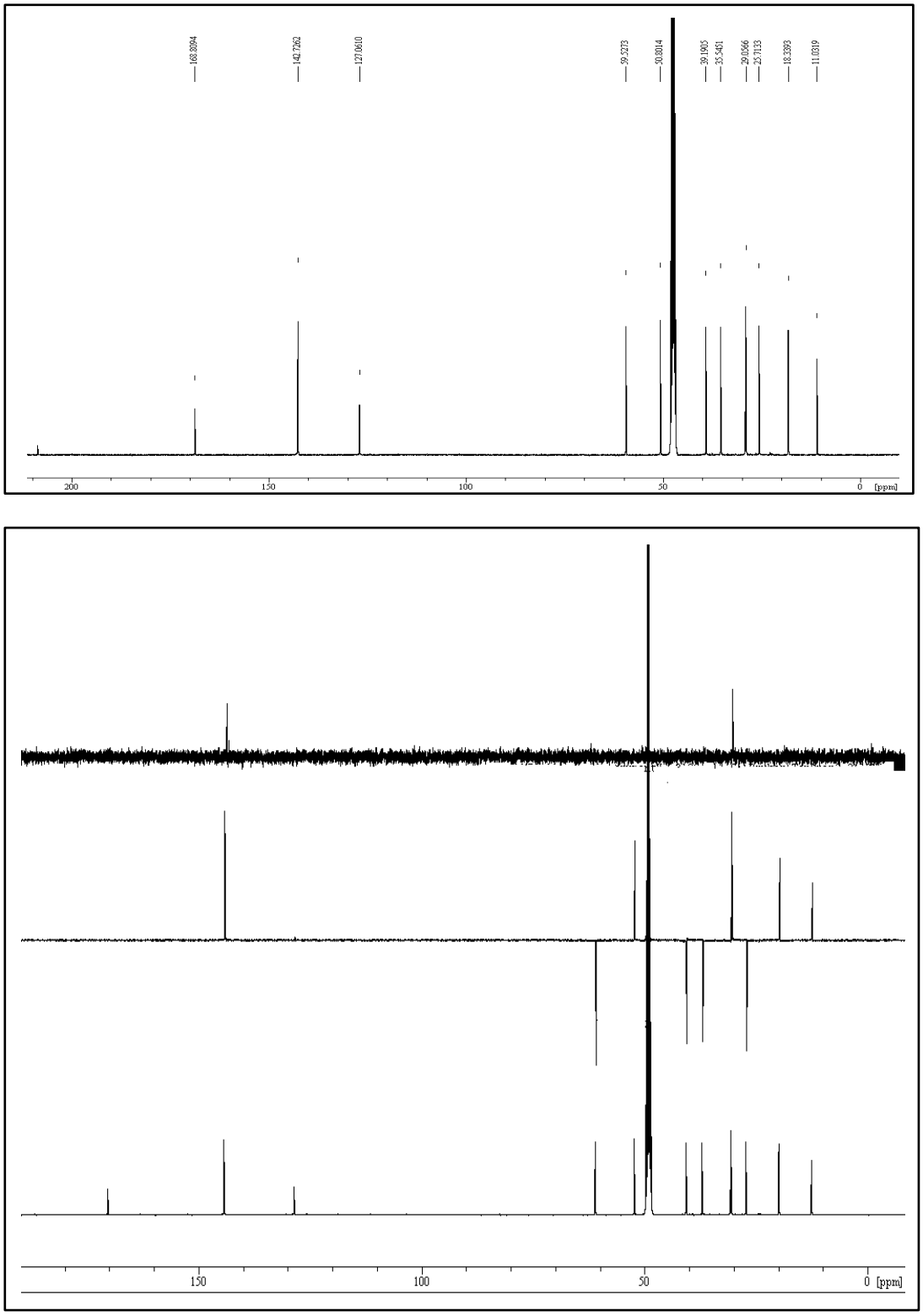

Figure 3.48 ^{13}C-NMR, DEPT-135 and DEPT-90 spectra of 8-hydroxy-2,6-dimethyl 2-enoate (**14**)

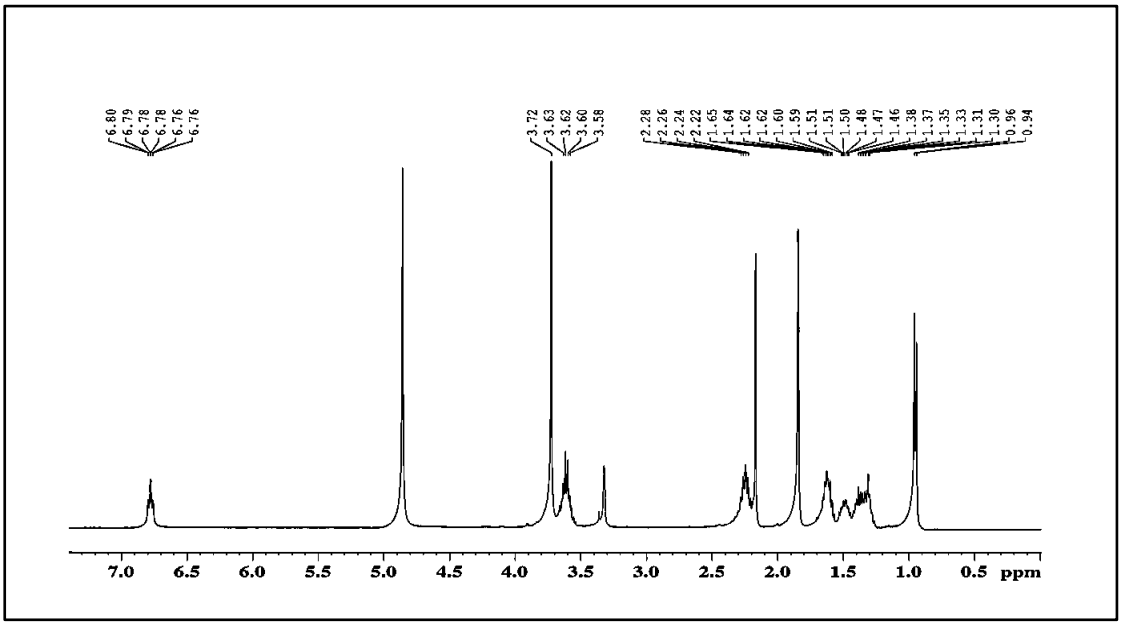

Figure 3.49 ^{13}C-NMR and DEPT-135 spectra of 8-hydroxy-2,6-dimethyl-2-enoate (**14**)

3.4. BIOLOGICAL ACTIVITIES

3.4.1. Total Phenolic Content (TPC), Total Flavonoid Content (TFC) and DPPH-scavenging activity

All fractions were analysed for their total phenolic, total flavonoid content (TPC and TFC, respectively) and DPPH scavenging activity (Table 3.11). The TFC of the crude extract was 70.28 mg RUE/g extract, as calculated from the rutin calibration curve**.** In the different fractions the highest flavonoid contents were observed in the ethyl acetate fraction (73.44 mg RUE/g) followed by the methanol fraction (62.34 mg RUE/g). The chloroform (17.52 mg RUE/g), *n*-butanol (10.57 mg RUE/g) had comparably low flavonoid contents. Similarly, the total phenolic content (TPC) of the total extract was 28.88 mg GAE/g as calculated from the gallic acid calibration curve, whereas the ethyl acetate (34.1 mg GAE/g) and methanol 90% (31.87 mg GAE/g) subfractions contained slightly higher amounts. This comparably high phenolic and flavonoid contents were indicative of possibly high antioxidant activities in the corresponding fractions. Indeed the ethyl acetate fraction presented moderate antioxidant potential (IC_{50} 81 µg/ml), followed by the methanol fraction (IC_{50} 97 µg/ml) and chloroform fractions (IC_{50} 119 µg/ml), respectively. These finding were in accordance with previous reports that correlate the antioxidant activities of plant extracts with high flavonoid and phenolic contents (Yoo *et al*., 2008; Pathirana and Shahidi, 2005; Goulasa *et al*., 2014). Furthermore, in an attempt to have an estimate of TPC activity, we compared our findings with a more broader investigation that involved a wide range of medicinal plants (30 in total) and categorised total phenolic contents into ranks (very low to very high phenolic contents). Upon comparison of our TPC findings, we could emphasise that total phenolic contents of *Nymphoides indica* lie in "very low"- "low" levels (Dudonne' *et al*., 2009). Likewise based on the findings of above cited work for antioxidant activity (DPPH scavenging), the *N.indica* leaves fractions was considered as "moderate" in inhibition for DPPH.

Table 3.11 Total phenolic (TPC), total flavonoid contents (TFC) and antioxidant activities of *N.indica* fractions.

Sample		TPC		TFC		DPPH	
	Extract Weight (g)	(mg GAE/g extract)[a]	amount of GAE (mg)	(mg RUE/g extract)[b]	amount of RUE (mg)	% inhibition [c]	IC_{50} µg/mL
Total Extract	88.61	28.88 ± 0.42	2561	70.28 ± 1.0	6221	53	121
MeOH 90%	11.2	31.87 ± 0.83	360	62.34 ± 0.9	698	55	97
Chloroform	2.03	9.76 ± 0.96	19	17.52 ± 1.22	35	52	119
Ethyl acetate	1.2	34.1 ± 0.54	41	73.44 ± 1.23	88	60	81
n-Butanol	19.58	16.38 ± 1.07	193	10.57 ± 0.39	818	48	-
Quercetin							6

(a) Total phenolic contents, gallic acid equivalents
(b) Total flavonoid contents, rutin equivalents
(c) at 128 µg/mL (final concentration).

3.4.2 Inhibition of AGEs formation and α-glucosidase inhibition by crude extracts

The different fractions were tested for potential antiglycation and α-glucosidase inhibitory activity (Table 3.12). A mild to moderate antiglycation activity was observed for the crude extract at 100μg/mL (33%). Likewise all tested fractions presented moderate (24-36%) inhibition of the formation of AGEs inhibition. Similarly mild α-glucosidase inhibitory activity was observed for the total extract at 834 μg/mL (13%), whereas the highest inhibition was shown by the methanolic (31%) and *n*-butanol fractions (25%) fractions at the same concentration. At higher test concentrations the inhibitory activity did not further increase, and the 50% inhibition level could not be reached.

Table 3.12. Anti-glycation and α-glucosidase inhibitory effects of *N. indica* fractions.

Sample	Antiglycation % Inhibition[a]	α-glucosidase inhibition % Inhibition[b]
Total Extract	33	13
MeOH 90%	36	31
Chloroform	23	12
Ethyl acetate	27	17
n-butanol	24	25
Standard inhibitor	78	67[b]

[a] at 100 μg/mL, Quercetin IC_{50} 0.23 mM
[b] at 834 μg/mL, Acarbose IC_{50} 0.26 mM

3.4.3 Antimicrobial activity of crude extracts

The crude extracts were evaluated for antibacterial and antifungal activities. The highest levels of antimicrobial and antifungal activities were observed for the *n*-hexane fraction, i.e. an $IC_{50\ of}$ 19.5 µg/mL against *S. aureus* and 32 µg/mL against *M. canis*. Similarly the methanol 90% fraction was active (IC_{50} 36.4 µg/mL) against *S. aureus* and cytotoxic (IC_{50} 38.9 µg /mL) against MRC-5 cells (Table 3.13).

Table 3.13 Antibacterial, antifungal and cytotoxic activities of *N. indica* fractions.

Sample	IC_{50} µg/mL				
	S. aureus	*E. coli*	*C. albicans*	*M. canis*	MRC-5
MeoH 90%	36.4	>64	>64	>64	38.9
Chloroform	>64	>64	>64	>64	>64
Ethyl acetate	>64	>64	>64	>64	>64
n-Hexane	19.5	>64	>64	32	>64
n-butanol	>64	>64	>64	>64	>64
Aqueous	>64	>64	>64	>64	>64

Reference: Tamoxifen(MRC-5) IC_{50} 11.4 µg/mL; *erythromycin (S.aureus)*IC_{50} 11.2 µg/mL, Trimethoprim (*E.coli*) IC_{50} 0.25 µg/mL; Miconazole(*C. albicans*) IC_{50} 5.99 µg/mL; Terbinafin (*M.canis*) IC_{50} 0.11 µg/mL.

MRC-5: Human fetal lung fibroblasts; *S. aureus: Staphylococcus aureus; E.coli: Escherichia coli; C. albicans: Candida albicans*

3.4.4. Biological activities of isolated constituents

In view of the antimicrobial activity of the *n*-hexane fraction, compounds **1** – **5** were evaluated in an integrated screening panel. Only azelaic acid (**1**) was active against *S. aureus* with an IC_{50} value of 55.1 µM (vs. 11.2 µM for erythromycin) (Table 3.14). It also showed weak antiplasmodial activities against *P. falciparum* K1, *T. brucei* and *T. cruzi*, but it was also cytotoxic against MRC-5 cells in the same concentration range (IC_{50} 32.2 µM). Compounds **2–5** showed comparable activity against some of the protozoal parasites. Lipophilic compounds such as medium and long chain fatty acids have been previously reported to contribute to antiprotozoal and antifungal activities (Krugliak *et al*., 1995; Wang & Johnson, 1992; Ozcelik *et al*., 2005a; Orhan *et al*., 2009). It was therefore concluded that the activity of the *n*-hexane fraction of *N. indica* was mainly due to presence of long chain mono and dicarboxylic fatty acids and fatty alcohols that may act synergistically.

Three seco-iridoid glucosides (**6–8**) were isolated from the methanolic fraction. In the antimicrobial screening panel, none of the secoiridoids presented activity in the set test ranges. Nevertheless, the possibility cannot be excluded that after oral intake the glycosidic moiety is hydrolysed, and that the resulting aglycones and their metabolites are active. However in the antidiabetic assays, compound **7** presented moderate antiglycation (54% inhibition at 0.67 mM, IC_{50} 0.36 mM) and mild α-glucosidase inhibitory (37% at 1 mM) activities, whereas only moderate antiglycation activities were observed for compound **6** (49% inhibition at 0.67 mM) and **8** (41% inhibition at 0.67 mM). The Compounds **6** and **8** did not present any α-glucosidase inhibitory activity (Table 3.15). Based on the observed antiglycation activities however, which are higher than the positive control aminoguanidine, a compound with proven clinical activity, it could be assumed that the isolated secoiridoids may have potential anti-AGEs activities. Also in this case the possibility cannot be excluded that after oral intake the glycosidic moiety is hydrolysed, and that the resulting aglycones and their metabolites are active.

In the antimicrobial screening panel, among the flavonoids, compound **9** showed weak antiprotozoal activities against *Trypanosoma brucei* (IC_{50} 8.4 µM)*, Trypanosoma cruzi*

(IC_{50} 30.0 µM) and *Leishmania infantum* (IC_{50} 32.5 µM), accompanied however by PMM (peritoneal murine macrophages) cytotoxicity.

Compound **9** is considered as the major flavonoid in *N. indica* (Bohm *et al*., 1986) with no previous evidence of pharmacological properties. Also during the current investigation, *it* was collected in comparatively large amount that shows its relative profusion in leaves and this may contribute to the antiprotozoal activities of *N. indica* extracts. A number of reports (Muzitano *et al*., 2006_a; Muzitano *et al*., 2006_b) covering the anti-leishmanial activities of flavonoids support these findings. Despite the fact that flavonoids are major contributors of plants to a wide array of activities including cytotoxicity and antimicrobial activities (Lovkova *et al*., 2001; Cushnie and Lamb 2005), the isolated flavonoids, however, presented rather moderate IC_{50} values and were therefore not considered for further antimicrobial evaluation.

In the antidiabetic assays the isolated flavonoids presented moderate activities. The highest antiglycation activity was shown by compound **10** (54% inhibition at 0.67 mM, IC_{50} 0.42 mM) followed by compound **9** (44% inhibition). These compounds also presented mild to moderate α-glucosidase inhibitory activities. Compound **10**, however, did not present any α-glucosidase inhibition (Table 3.15). Compound **11** was inactive in antimicrobial screening, but presented mild antiglycation (39% inhibition) and α-glucosidase inhibition (27% inhibition). Indeed it has been established that for antiglycation activity of flavonoids there are some essential features supporting the activity. Phenolic groups at C-3', C-4', C-5, and C-7 positions and methylation or glucosylation of the hydroxyl groups are considered important for the activity of flavones, flavonols and flavanones (Matsuda *et al*., 2003). In addition it has been established that oxidative injury associated with degenerative diseases can be inhibited by antioxidants and radical scavengers (Nakagawa *et al*., 2002). Compounds or plant extracts having antioxidative properties may also prevent AGEs formation (Yamaguchi *et al*., 2000, Kiho *et al*., 2004; Tung *et al*., 2007). It has been reported before that extracts containing compounds with different mechanisms of action may be more effective than isolated constituents (Xi *et al*., 2008, Yagi *et al*., 2013), as also observed during this investigation.

The coumarins are known for their cytotoxic and antimicrobial activities (De Souzaa *et al*., 2005; Smyth *et al*., 2009). The isolated coumarin (compound **12**) was identified as scopoletin. Antimicrobial activities have been reported for scopoletin (Ragasa and Hofilena 2011; More *et al*., 2012) but during current investigation this could not be confirmed for the test organisms (IC_{50} values >64 μM) (Table 3.13). However, as the crude methanolic fraction of *N. indica* showed significant cytotoxic and antimicrobial activities (Table 3), this could possibly due to the presence of flavonoids and coumarins. It could therefore be hypothesised that various constituents may contribute synergistically towards the antimicrobial activity of *N. indica* as indeed sometimes isolation of the components from the extract may cause a decrease in desired activity (Orhan *et al*., 2009).

Finally two acyclic monoterpenes (**13-14**) were isolated from the *n*-butanol fractions of *N. indica* leaves. These monoterpenes were not active in the antimicrobial screening panel. However, moderate antiglycation activity and α-glucosidase inhibition (Table 3.15) was observed. As the n-butanol fraction also showed antiglycation activity (24% inhibition) (Table 3.12), it can be hypothesised that the AGEs inhibitory potential of the n-butanol fraction is mainly contributed by flavonoids and monoterpenes (Al-Musayeib *et al*., 2011*;* Balasubramaniam and Anuradha, 2011; Joglekar *et al*., 2013).

Table. 3.14 Antimicrobial, antifungal, cytotoxic and antiprotozoal activities (IC_{50} µM)of isolated compounds from *N. indica* fractions.

Compound no.	IC_{50} µM									
	MRC5	*Pf-K1*	*T. bruc*	*T. cruz*	*L. inf*	*PMM*	*S. aureus*	*E. coli*	*C. albicans*	*A. fumigatus*
1	**32.2**	**62.2**	**32.6**	**34.6**	> 64	> 64	**55.1**	> 64	> 64	> 64
2	**28.5**	> 64	> 64	**33.5**	> 64	> 64	> 64	> 64	> 64	> 64
3	**32.2**	> 64	> 64	**32.8**	> 64	> 64	> 64	> 64	> 64	> 64
4	> 64	> 64	**32**	**31.5**	**50.8**	> 64	> 64	> 64	> 64	> 64
5	**32.6**	> 64	> 64	**33.6**	**32.4**	**32.0**	> 64	> 64	> 64	> 64
6	> 64	> 64	> 64	> 64	> 64	> 64	> 64	> 64	> 64	> 64
7	> 64	> 64	> 64	> 64	> 64	> 64	> 64	> 64	> 64	> 64
8	> 64	> 64	> 64	> 64	> 64	> 64	> 64	> 64	> 64	> 64
9	> 64	> 64	**8.4**	**30.0**	**32.5**	**32.0**	> 64	> 64	> 64	> 64
10	> 64	> 64	> 64	> 64	> 64	> 64	> 64	> 64	> 64	> 64
11	> 64	> 64	> 64	> 64	> 64	> 64	> 64	> 64	> 64	> 64
12	> 64	> 64	> 64	> 64	> 64	> 64	> 64	> 64	> 64	> 64
13	> 64	> 64	> 64	> 64	> 64	> 64	> 64	> 64	> 64	> 64
14	> 64	> 64	> 64	> 64	> 64	> 64	> 64	> 64	> 64	> 64

Reference: Tamoxifen (MRC-5), IC_{50} 11.35 µM; Suramine (*T. bruc*), IC_{50} 0.03 µM, Fungizone (*L. inf*), IC_{50} 1.15 µM; Chloroquine (*Pf-K1*), IC_{50} 0.16 µM, Benznidazole (*T. cruz*), IC_{50} 3.27 µM; Erythromycin (*S. aureus*), IC_{50} 11.31 µM; Chloramphenicol (*E. coli*), IC_{50} 4.88 µM; Miconazole (*C. albicans*), IC_{50} 10.5 µM; Terbinafine (*A. fumigatus*), IC_{50} 0.79 µM.

MRC-5: human fetal lung fibroblasts;, Pf-K1; *Plasmodium falciparum*; *T. bruc*: *Trypanosoma brucei; L.inf: Leishmania infantum*; PMM: peritoneal murine macrophages; *T. cruz*: *Trypanosoma cruzi; S. aureus: Staphylococcus aureus; E. coli: Escherichia coli; C. albicans: Candida albicans; A. fumigatus: Aspergillus fumigatus*

Table 3.15 Anti-glycation and α-glucosidase inhibitory effect of isolated compounds from *N. indica*

Compound no.	Protein glycation		α-glucosidase	
	% inhibition[a]	IC_{50} mM	% inhibition[b]	IC_{50} mM
6	49	-	na	-
7	54	0.36	37	-
8	41	-	na	-
9	44	-	45	-
10	54	0.42	na	-
11	39	-	27	-
12	nt	-	nt	-
13	47	-	38	-
14	51	0. 61	40	-
Standard Inhibitor		0.23[c] 1.75[d]		0.26[e]

na not active
nt not tested (low amount)
[a] (at 0.67 mM)
[b] (at 1 mM)
[c] Quercetin
[d] Aminoguanidine
[e] Acarbose

3.5. SUMMARY AND CONCLUSION

Nymphoides indica (L.) Kuntze (Menyanthaceae) is traditionally used in many ethnomedicinal formulations in the Indian subcontinent including Pakistan. However, scientific data reporting its constituents are poor. This study aimed at evaluating its traditional use and phytochemical constituents, as a part of running programmes on antimicrobial, antioxidant and antidiabetic properties (inhibition of alpha-glucosidase activity; inhibition of Advanced Glycation Endproducts - AGEs) of medicinal plants and natural products. Various extracts and fractions of different polarity of *N. indica* leaves showed moderate activities in these assays. Phytochemical investigations of the extracts and fractions resulted in the isolation and identification of 5 lipophilic compounds, i.e. the dicarboxylic acids azelaic acid (nonanedioic acid) (**1**) and 4-methyl-heptanedioic acid (**3**), the monocarboxylic acids hexadecanoic acid (**2**) and stearic acid (**5**) and the fatty alcohol hexadecanol (**4**); 3 seco-iridoids, i.e. 7-epiexaltoside (**6**), 6",7"-dihydro-7-epiexaltoside (**7**) (reported for the first time from nature) and menthiafolin (**8**); 3 flavonoids, i.e. 3,7-di-*O*-methylquercetin-4'-O-glucoside (**9**), 3-*O*-methylquercetin-7-*O*-glucoside (**10**) and 3,7-di-*O*-methylquercetin (**11**); scopoletin (**12**) and the monoterpenoids foliamenthoic acid (**13**) and 6,7-dihydrofoliamenthoic acid methyl ester (**14**). Compounds **1-5** showed moderate antimicrobial activities, whereas compound **9** presented mild antiprotozoal activities against *Trypanosoma brucei* (IC_{50} 8 µM), *Leishmania infantum* (IC_{50} 32 µM) *and Trypanosoma cruzi* (IC_{50} 30 µM). Likewise promising antiglycation activities were shown by compound **7** (IC_{50} 0.36 mM), **10** (IC_{50} 0.42 mM) and **14** (IC_{50} 0.61 mM). Finally mild to moderate α-glucosidase inhibition was shown by compounds **7**, **9**, **11** and **13** - **14**. It could be concluded that *N. indica* leaf extracts possess mild to moderate antimicrobial, antiprotozoal, antioxidant and antidiabetic activities. The traditional use of this plant species could be due to synergistic effects of its constituents, or in combinations with other plant species in traditional formulations.

REFERENCES

Abu Ziada E, Mashaly A, Abd El-Monem M, Torky M. 2008. Economic potentialities of some aquatic plants growing in north east Nile Delta. *Egypt J Appl Sci* **8**: 1395– 1405.

Agarwal PK, Rastogi RP. 1981. ^{13}C-NMR Spectroscopy of Flavonoids. *Heterocycles* **16**: 2181 - 2236.

Al-Musayeib N, Perveen S, Fatima I, Nasir M, Hussain A. 2011. Antioxidant, Anti Glycation and Anti-Inflammatory Activities of Phenolic Constituents from *Cordia sinensis. Molecules* **16**: 10214-10226.

Balasubramaniam D, Anuradha CV. 2011. Linalool, a plant derived monoterpene alcohol, rescues kidney from Diabetes induced Nephropathic changes via blood Glucose reduction. *Diabetol Croatic* **40**:121-127.

Bhosale SH, Jagtap TG, Naik CG.1999. Antifungal activity of some marine organisms from India, against food spoilage *Aspergillus strains*. *Mycopathologia* **147**: 133-138.

Bostian MB, Herlihy AT. 2014. Valuing trade-offs between agricultural production and wetland condition in the U.S. Mid-Atlantic region. *Ecol Econ* **105**: 284–291.

Bohm BA, Nicholls KW, Ornduff R. 1986. Flavonoids of the *Menyanthaceae*: Intra- and interfamilial relationships. *Am J Bot* **73**: 204-213.

Coea FG, Anderson GJ. 2005. Snakebite ethnophar-macopoeia of eastern Nicaragua*. J Ethnopharmacol* **96**: 303-323.

Connolly JD, Overton KH, Polonsky J. 1970. The chemistry and biochemistry of the linonoids and quassinoids. In: Reinhold, L., Liwashitz, Y. (Eds.), Programm Photochemistry. Wiley, London, p. 385.

Cook CDK. 1996. Aquatic and wetland plants of India. Oxford University press Inc., New York.

Cook CDK, Gut BJ, Rix EM, Schneller J, Seitz M. 1974. Water Plants of the World. A manual for the identification of the genera of fresh water Macrophytes. Dr. W. Junk b.v., Publishers The Hague. p. 329-330.

Cruz-Garcia GS, Price LL. 2011. Ethnobotanical investigation of 'wild' food plants used by rice farmers in Kalasin, Northeast Thailand. *J Ethnobiol Ethnomed* 7:33. **Doi**: 10.1186/1746-4269-7-33.

Cushnie TPT, Lamb AJ. 2005. Antimicrobial activity of flavonoids. *Int J Antimic Agents* **26**: 343–356.

Darmawan A, Kosela S, Leonardus BS, Kardono, Syah YM. 2012. Scopoletin, a coumarin derivative compound isolated from *Macaranga gigantifolia* Merr. *J Appl Pharm Sci* **2**: 175-177.

Das NJ, Saikia SP, Sarkar S, Devi K. 2006. Medicinal plants of district North-Kamrup district of Assam used in primary healthcare system. *Ind J Trad Knowl* **5**: 489- 493.

De Souzaa SM, Monacheb FD, Smania A. 2005. Antibacterial Activity of Coumarins. *Z Naturforsch* **60**: 693-700.

Dudonne' S, Vitrac X, Coutiere P, Woillez M, Merillon JM. 2009. Comparative Study of Antioxidant Properties and Total Phenolic Content of 30 Plant Extracts of Industrial Interest Using DPPH, ABTS, FRAP, SOD, and ORAC Assays. *J Agric Food Chem* **57:** 1768–1774.

Egwaikhide PA, Gimba CE. 2007. Analysis of the phytochemical content and antimicrobial activity of Plectranthus glandulosis whole part. Middl East J Sci Res 2: 135–138.

Fareed MF, Haroon AM, Rabh SA. 2008. Antimicrobial activity of some macrophytes from Lake Manzalah (Egypt). *Pak J Biol Sci* **11**: 2454–2463.

Farnsworth NR.1966. Biological and phytochemical screening of plants. *J Pharm Sci* **55**: 225–276.

Goulasa V, Exarchou V, Kanetisb L, Gerothanassis LP. 2014. Evaluation of the phytochemical content, antioxidant activity and antimicrobial properties of mountain tea (*Sideritis syriaca*) decoction. *J Funct Foods* **6**: 248 – 258.

Grossmann M. 2012. Economic value of the nutrient retention function of restored floodplain wetlands in the Elbe River basin. *Ecol Econ* **83**:108–117.

Guerrro MF. Puebla P, Carron R. Marthin ML, San Roman L. 2002. Quercetin 3, 7 dimethyl ether: a vasorelaxant flavonoid isolated from *Croton Schiedeanus schlecht*. *J Pharm Pharmacol* **54**: 1373-1378.

Haroon AM, Sharshar K, Fareed M. 2009. Investigation on *Vibrio sp.* isolated from diseased caryfish (*Procambarus calarkii*)with emphasis on biochemical characteristic and in vitro antibacterial effects of some plants extracts. *World Appl Sci J* **6**: 868–879.

Haroon AM. 2006. Effect of macrophytes extracts on growth of *Aspergillus parasitcus*. *Egypt J Aquat Res* **32**: 301-313.

Haung C, Tunon H, Bohlin L. 1995. Anti-inflammatory compounds isolated from *menynthes trifilata* L. *Acta Pharmacol Sin* **30**: 621-626.

Harborne JB. 1973. Phytochemical Methods 3rd Edn. Chapman and Hall Ltd., London, pp: 135-203

Ito MY, Barfod MAS. 2014. An updated checklist of aquatic plants of Myanmar and Thailand. *Biodivers Data J* **2**: e1019.

Iverson R. 2010. Risk assessments(RA) for *Nymphoides* crista, peltata and indica. Personal communication to A. Tasker on October 28, 2010, from Rick Iverson, North Carolina Department of Agriculture and Consumer Services, Weeds Specialist.

Iwagawa T, Asa H, Haso T, Sako S, Su R, Hagiwara N, Kim M.1990. Monoterpenes from *Radermachia sinica* . *Phytochemistry* **29**: 1913–1916

Jain A, Roshnibala S, Kanjilal BP, Singh RS, Singh HB. 2007. Aquatic/ semi aquatic plants used in herbal remedies in wetlands of Manipur, North-eastern India. *Indian J Tradit Know* **6**: 341-346.

Joglekar MM, Panaskar SN, Chougale AD, Kulkarni MJ, Arvindekar AU. 2013. A novel mechanism for antiglycative action of limonene through stabilization of protein conformation. *Mol Biosyst* **9**: 2463-72.

Junior P. 1983. Nemorosid und Nemorososid, zweineue Iridoidglucoside aus *Penstemon nemorosus. Planta Med* **47**: 67-70.

Junior P. 1989. Further investigation regrading distribution and structure of bitter principles from *Menyanthes trifoliate*. *Planta Med* **55**: 83-86.

Junior P. 1991. Exaltoside and 7-epiexaltoside, two novel acylated secoiridoid glucosides from *Villarsia exaltata*. *Planta Med* **57**: 181-183.

Kensa M. 2011. Floristic study in a Vembanur wetland, Kanyakumari District, Tamilnadu, South India. *Plant Sci Feed* **1**: 194–199.

Khan NMF, Hossain MD. 2015. Scopoletin and β-sitosterol glucoside from roots of Ipomoea digitate. *J Pharmacog Phytochem* **4**: 05-07

Kiho T, Usui S, Hirano K, Aizawa K, Inakuma T. 2004. Tomato paste fraction inhibiting the formation of advanced glycation end-products. *Biosci Biotechnol Biochem* **68**: 200-205.

Kitdamrongtham W, Manosroi A, Akazawa H, Gidado A, Stienrut P, Manosroi W, Warangkana L, Toshihiro A, Manosro J. 2013. Potent anti-cervical cancer activity: Synergistic effects of Thai medicinal plants in recipe N040 selected from the MANOSROI III database. *J Ethnopharmacol* **149**: 288–296.

Krenn L, Miron A, Pemp E, Petr Ursula, Kopp B. 2003. Flavonoids from *Achillea nobilis* L. *Z Naturforsch C* **58**: 11-16

Krugliak M, Deharo E, Shalmiev G, Sauvain M, Moretti C, Ginsburg H. 1995. Antimalarial effects of C18 fatty acids on *Plasmodium falciparum* in culture and on *Plasmodium vinckei petteri* and *Plasmodium yoelii nigeriensis* in vivo. *Exp Parasitol* **81**: 97–104.

Lovkova MY, Buzuk GN, Sokolova SM, Klimenteva NI. 2001. Chemical features of medicinal plants (Review). *Appl Biochem Microbiol* **37**: 229–237.

Madhavan V, Arora S, Murali A, Yoganarasimhan SN. 2009. Anti-convulsant activity of aqueous and alcohol extracts of roots and rhizomes of *Nymphoides indica* (L.) Kuntze in swiss albino mice. *J Nat Remedies* **9**: 68–73.

Madhavan V, Arora S, Yoganarasimhan SN, Gurudeva MR. 2011. Pharmacognostical studies on the rhizome and roots of *Nymphoides indica* (L.) Kuntze. alternate source for Tagara. *Asian J Trad Med* **6**: 14-125.

Manosroi J, Kitdamrongtham W, Manosroi A, Akazawa H, Gidado A, Stienrut P, Manosroi W, Lohcharoenkal W, Akihisa T. 2013. Potent anti-cervical cancer activity: Synergistic effects of Thai medicinal plants in recipe N040 selected from the MANOSROI III database. *J Ethnopharmacol* **149**: 288–296.

Manosroi J, Boonpisuttinant K, Manosroi W, Manosroi A. 2012. Anti-proliferative activities on HeLa cancer cell line of Thai medicinal plant recipes selected from MANOSROI II database. *J Ethnopharmacol* **142**: 422–431.

Marwat SK, Khan MA, Ahmad M, Zafar M. 2009. *Nymphoides indica*(L) Kuntze a new record for Pakistan. *Pak J Bot* **41**: 2657-2660.

Marwat SK, Khan MA, Rehman FU, Ahmad M, Zafar M. 2011. Biodiversity and Importance of floating weeds of Dera Ismail Khan District of KPK, Pakistan. *Afr J Trad Compl Alt Med* **8**: 97-107.

Matsuda H, Wang T, Managi H, Yoshikawa M. 2003. Structural requirements of flavonoids for inhibition of protein glycation and radical scavenging activities. *Bioorg Med Chem* **11**: 5317-23.

Maya S, Menon SV, Nair SG, 2003. Economic importance of river vegetation of Kerala, A case study. *J Econ Taxon Bot* **27**: 796-803.

Mesia GK, Tona GL, Nanga TH, Cimanga RK, Apers S, Cos P, Maes L, Pieters L, Vlietinck AJ. 2008. Antiprotozoal and cytotoxic screening of 45 plant extracts from Democratic Republic of Congo. *J Ethnopharmacol* **115**: 409–415

More G, Lall N, Hussein A, Tshikalange TE. 2012. Antimicrobial Constituents of *Artemisia afra* Jacq. Ex Willd. against Periodontal Pathogens. *Evid Based Complement Alternat Med* Pages. 7 (Article ID 252758) **do**i.org/10.1155/2012/252758

Muzitano MF, Cruz EA, Almeida AP, Silva SAG, Kaiser CR, Guette C, Rossi Bergmann B, Costa SS. 2006a. Quercitin from *Kalanchoe pinnata*: the first antileishmanial flavonoid glycoside. *Planta Med* **72**: 81–83.

Muzitano MF, Falcao CA, Cruz EA, Bergonzi MC, Bilia AR, Vincieri FF, Bergmann RB, Costa SS. 2009. Oral metabolism and efficacy of *Kalanchoe pinnata* flavonoids in a murine model of cutaneous Leishmaniasis. *Planta Med* **75:** 307–311.

Ohwi J. 1984. Flora of Japan (edited English version, reprint. Original 1954). National Science Museum, Tokyo, Japan. 1067 pp.

Orhan I, Deliorman OD, Ozcelik B. 2009. Antiviral activity and cytotoxicity of the lipophilic extracts of various edible plants and their fatty acids. *Food Chem* **115**: 701–705.

Otsuka H. Linarioside A-C and acyclic monoterpene diglucosides from *linaria Japonica.* 1994. *Phytochemistry* **37**: 461-465.

Ozcelik B, Aslan M, Orhan I, Karaoglu T. 2005a. Antibacterial, antifungal and antiviral activities of the lipophilic extracts of *Pistacia vera*. *Microbiol Res* **160**: 159–164.

Panda A, Misra MK. 2011. Ethnomedicinal survey of some wetland plants of South Orissa and their conservation. *Ind J Trad Knowl* **10**; 296–303.

Pathirana CL. Shahidi F. 2005. Optimization of extraction of phenolic compounds from wheat using response surface methodology. *Food Chem* **93**: 47-56.

Qaiser M. 1977. *Menyanthaceae* in flora of Pakistan, Karachi. 111: 4. (Eds.): E. Nasi & S.I. Ali.

Ragasa CY, Hofilena JG. 2011. Antimicrobiol caumarine derivative from *Delonix regia*. *Manil J Sci* **7**: 7-11.

Schmidt MU. 2005. Notes on the genus Nymphoides (Menynthaceeae) of Columbia. **27**: 127–130.

Senguttuvan J, Paulsamy, S., Karthika K. 2014. Phytochemical analysis and evaluation of leaf and root parts of the medicinal herb, *Hypochaeris radicata* L. for in vitro antioxidant activities. *Asian Pac J Trop Biomed* **4**(Suppl 1)**:** 359-367.

Shibayama Y, Kadono Y. 2007. The effect of water-level fluctuations on seedling recruitment in an aquatic macrophyte *Nymphoides indica* (L.) Kuntze (Menyanthaceae). Aquatic Bot 87 **4**:320-324.

Shin WJ, Lee KH, Park MH, Park, Seong BL. 2010. Broad-spectrum antiviral effects of *Agrimonia palosa* extract on influenza viruses. *Microbiol Immunol* **54**: 11–19.

Siwakoti M. 2006. An overview of floral diversity in wetlands of Terai region of Nepal. *Our Natur* **4**: 83-90.

Smyth T, Ramachandran VN, Smyth WF. 2009. A study of the antimicrobial activity of selected naturally occurring and synthetic coumarins. *Int J Antimicrobiol Agent* **33:** 421–426.

Sofowora A. 1993. Medicinal plants and traditional medicine in Africa. John Wiley and son Ltd., 150-153.

Swapna MM, Prakashkumar R, Anoop KP, Manju CN, Rajith NP. 2011. A review on the medicinal and edible aspects of aquatic and wetland plants of India. *J Med Plant Res* **5**: 7163-7176.

Tippery NP, Donald H, Les JC, Regalado J, Averyanov LV, Long VN, Raven PH. 2009. Transfer of *Villarsia cambodiana* to *Nymphoides* (Menyanthaceae). *Systemat Bot* **34**: 818-823.

Trease GE, Evans WC. 1989. Pharmacognosy.11th Edn. Brailliar Tiridel and Macmillian Publishers, London.

Tung Y, Wu J, Kuo Y, Chang S. 2007. Antioxidant activities of natural phenolic compounds from *Acacia confusa* bark. *Bioresour Technol* **98**: 1120-1123.

Upadhyay A, Tuenter E, Rizwan Ahmad Amin A, Exarchou V, Apers S, Hermans N Pieters L. 2014a. Kavalactones, A Novel Class of Protein Glycation and Lipid Peroxidation Inhibitors. *Planta Med* **80**: 1001–1008.

Upadhyay A, Tuenter E, Amin A, Exarchou V, Hermans N, Apers S, Pieters L. 2014b. 5-O-Demethylnobiletin, a polymethoxylated flavonoid, from *Citrus depressa Hayata* peel prevents protein glycation. *J Funct Food* **11**: 243–249.

Verma H, Patil PR, Kolhapure RM, Gopalkrishna V. 2008. Antiviral activity of the Indian medicinal plant extract *Swertia chirata* against herpes simplex viruses: a study by *in vitro* and molecular approach. *Ind J Med Microbiol* **26**: 322–326.

Wang LL, Johnson EA. 1992. Inhibition of Listeria monocytogenes by fatty acids and monoglycerids. *Appl Environ Microbiol* **58**: 624–629.

Wolf HH, Swinyard EA, Goodman LS. 1962. Anticonvulsant properties of some N-substituted hydanoins. *J Pharm Sci* **51**: 74–76.

Xi M, Hai C, Tang H, Chen M, Fang K, Liang X. 2008. Antioxidant and antiglycation properties of total saponins extracted from traditional Chinese medicine used to treat diabetes mellitus. *Phytother Res* **22**: 228-237.

Yagi S, Drouart N, Bourgaud F, Henry M, Chapleur Y , Laurain-Mattar D. 2013. Antioxidant and antiglycation properties of *Hydnora johannis* roots. *S Afr J Bot* **84**:124-127.

Yamaguchi F, Ariga T, Yoshimura Y, Nakazawa H. 2000. Antioxidative and antiglycation activity of garcinol from *Garcinia indica* fruit rind. *J Agri Food Chem* **48**: 180-185.

Yoo KM, Lee CH, Lee H, Moon B, Lee CY. 2008. Relative antioxidant and cytoprotective activities of common herbs. *Food Chem* **106**: 929–936.

Yumnam JY, Tripathi OP. 2012. Traditional knowledge of eating raw plants by the Meitei of Manipur as medicine/neutrient supplement in their diet. *Ind J Trad Knowl* **11**: 45-50.

CHAPTER 4

PHYTOCHEMICAL AND BIOLOGICAL INVESTIGATIONS ON *KICKXIA RAMOSISSIMA*

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Phytochemical and biological investigations of *Kickxia ramosissima:* Adnan Amin, Emmy Tuenter, Ken Foubert, Atul Uphadaya, Paul Cos, Louis Maes, Vassiliki Exarchou, Sandra Apers and Luc Pieters

4.1 INTRODUCTION

Although *Nanorrhinum ramosissimum* (Wall.) Betsche is the accepted name of the species in the genus *Nanorrhinum* (family Plantaginaceae) (Adopted in 1984) (The Plant List, 2016), common synonyms are *Linaria ramosissima* Wall. (family Scrophulariaceae)(1831); *Linaria somalensis* Vatke (family Scrophulariaceae)(1882); *Kickxia ramosissima* (Wall.) Janchen (family Scrophulariaceae) (1933); *Kickxia somalensis* (Vatke) Cuf. (family Scrophulariaceae)(1963) and *Pogonorrhinum somalense* (Vatke) Betsche (family Scrophulariaceae)(1984) (Plants Jstore, 2016). Due to the fact that synonym *Kickxia ramosissima* is more commonly used in literature, it was adopted during current project. *Kickxia* (Scrophulariaceae) is a small genus of herbs geographically distributed from West-Africa to India (Khan and Aqil, 1993). The genus *Kickxia* is comprised of 47 species worldwide (Mabberley, 1997). *Kickxia ramosissima* (Wall.) Janch. is a perennial herb with numerous filiform branches, membranous leaves and yellow flowers (Pandya *et al*., 2012). It is mainly found in rocky areas with shady places (Kirtikar and Basu 2005) and high salt contents (Mathur *et al*., 1980) (Fig. 4.1). In Pakistan it has been reported from various places including the district Attock (Ahmad *et al*., 2009), the district Karak (Khan *et al.,* 2011), and the Nara desert (Qureshi *et al.,* 2010).

Figure 4.1 *Kickxia ramosissima* natural habitat and herbarium sheet

Kickxia ramosissima has been used in the indigenous system of treatment of the Indian subcontinent (Vardhana, 2008). In Pakistan it is known as "Wal", "Shin beeta" or "Khunger booti". It is used for a number of ailments, for instance as diuretic, against kidney stones (Pandya et al., 2012), fever and rheumatism (Jain et al., 2008) and during management of snake and scorpion bites (Bole and Pathak, 1988). Traditionally in the Indian subcontinent including Pakistan this species has been reported as an effective remedy for diabetes mellitus (Qureshi and Bhatti, 2008; Ahmad et al., 2009; Qureshi et al., 2010; Patel and Sachdeva, 2014).

Despite its ethnomedicinal importance only a few *Kickxia* species worldwide were chemically investigated. This has resulted in the isolation of mainly flavonoids and iridoid glycosides (Yuldashev *et al*., 1996; Khan and Aqil 1993; Khan *et al*., 2001; Al-Rehaily *et al*., 2006; Ahmad *et al*., 2006; Ferhat *et al*., 2010), fatty acids (Morteza-Semnani *et al*., 2008) and a hexitol, D-mannitol (Khan and Aqil, 1993) (Fig. 4.2). *Kickxia ramosissima*, however, is one of the least explored species.

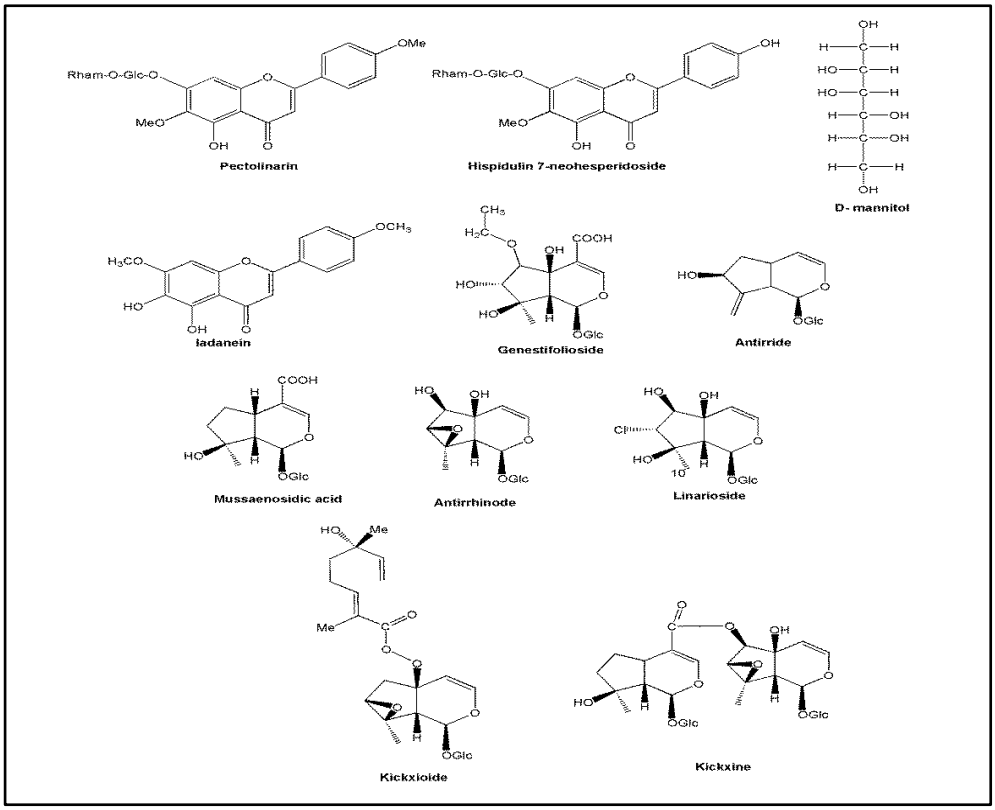


Figure 4.2. Compounds isolated from *Kickxia* sp.

4.2 EXTRACTION AND ISOLATION

4.2.1 Plant material

Kickxia ramosissima whole plant was collected in October 2012 from Takht-e-Nusrati, district Karak (KPK), Pakistan. Afterwards the herbarium sheets of the collected whole plant were submitted for identification at the Islamabad Herbarium of the Taxonomy Department, Quaid-I-Azam University, Islamabad, Pakistan, where the voucher specimen was deposited (voucher No. 48 CJ, accession no. ISL-44586). The whole plant was dried under shade followed by powdering and sieving through a 20 mesh filter (Fig. 4.3). All powdered material was stored below 20 °C till further use.

Figure 4.3 *Kickxia ramosissima* dried whole plant powder.

4.2.2. Extraction and fractionation

Powdered plant material (0.955 kg) was extracted with 90% (v/v) methanol by double cold maceration. The extract was filtered through Whatman No.1 filter paper using a vacuum pump. The collected filtrate was dried using a rotary evaporator under reduced pressure below 40 °C. The resultant semisolid material was lyophilised with a final yield of 83.54 g, and stored below 20 °C. The liquid–liquid partitioning was performed on the crude extract according to a standard extraction scheme (see chapter 2). After partitioning with different solvents as shown in the scheme, *n*-hexane (1.0 g), methanol

90% (3 g), chloroform (1.69 g), ethyl acetate (1.22 g), *n*-butanol (9.63 g) and aqueous fractions (67.0 g) were obtained. The collected fractions were dried under reduced pressure at 40 °C, lyophilized and stored below 20 °C.

4.2.3 Phytochemical analysis

Phytochemical screening of extracts and fractions for various compound classes was carried out using published methods (Sofowora, 1993, Trease and Evans, 1989, Harborne, 1973; Egwaikhide and Gimba, 2007; Farnsworth, 1966) with slight modifications for sterols, triterpenes, carbohydrates and saponins flavonoids, alkaloids, and tannins (chapter 2). Phytochemical screening of extracts and fractions indicated that flavonoids, tannins, saponins and triterpenes were present at different concentrations according to the fraction (Table 4.1). No alkaloids were detected. Fractions of medium polarity contained flavonoids and terpenes. The residual aqueous fraction was positive for tannins and saponins.

Table 4.1 Phytochemical analysis of *Kickxia ramosissima* fractions

Extract/ fraction	**Phytochemicals**				
	Alkaloids	Flavonoids	Tannins	Steroids/ triterpenoids	Saponins
MeoH 90%	-	+++	-	+++	-
Chloroform	++	+++	-	+++	-
Ethyl acetate	-	+++	-	+	-
***n*-Hexane**	-	-	-	-	-
***n*-butanol**	-	++	-	++	+++
Aqueous	-	-	+++	-	+++

profoundly present: high degree of precipitation (dark coloration), moderately present: less degree of precipitation (medium colouration), slightly present: very low precipitation (very little colouration), absent: no change in colour (Senguttuvan et al., 2014)

Phytochemicals: ++++ profoundly present; ++ moderately present; + slightly present; - absent.

4.2.4. Thin layer chromatography

The NP (normal phase) TLC for all obtained fractions was performed using various solvent systems as mobile phase, including CH_2Cl_2/MeOH (70:30) with a few drops of NH_4OH and CH_2Cl_2/MeOH (75:25) for the methanolic fraction; CH_2Cl_2 / MeOH (78 : 22) for the chloroform and ethyl acetate fractions; *n*-hexane/$CHCl_3$ (93:7) for the *n*-hexane fraction; CH_2Cl_2/MeOH (25:75 or 36:64) the for *n*-butanol fraction and MeOH/CH_2Cl_2 (85:15) for the aqueous fraction, respectively. Developed TLC plates were examined under UV at 254 nm and 366 nm and after spraying with *p*-anisaldehyde. For instance, TLCs for the methanolic and ethyl acetate fractions are shown in Fig 4.4.

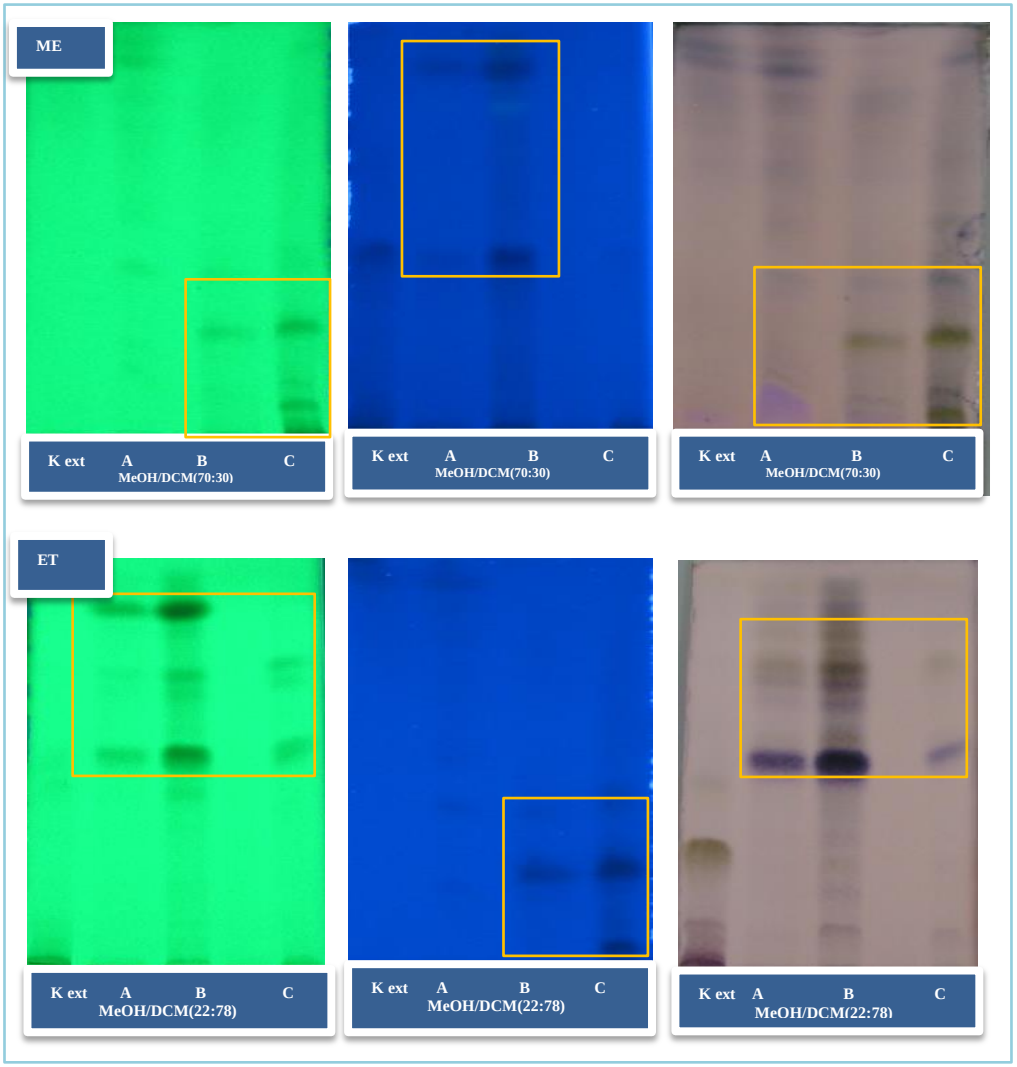


Figure 4.4. TLC profile of the total plant extract (K ext), methanolic(ME) and ethyl acetate(ET) fractions A,B,C on silica TLC (NP) plate at 254 and 366 nm after spraying with *p*-anisaldehyde reagent.

4.2.5. Flash chromatography

An aliquot of 0.8 g from the methanol 90% fraction was dissolved in 2 mL methanol and mixed with 1.1g silica; the mixture was dried with nitrogen gas. The dried extract was loaded on a prepacked Flash Grace Reveleris® silica cartridge of 80 g. The compounds were eluted using a gradient from ethyl acetate over methanol to methylene chloride. Based on UV and ELSD detection, multiple subfractions were collected (Fig. 4.5). All fractions were further analysed by TLC and similar fractions were combined. In this way 14 subfractions were obtained. Flash chromatography with similar conditions was repeated when necessary. Finally, based on TLC profiling, subfractions **KRM2** (200 mg), **KRM3**(150 mg), **KRM5** (210 mg), **KRM7** (200 mg) and **KRM14** (110 mg) were selected for further HPLC profiling.

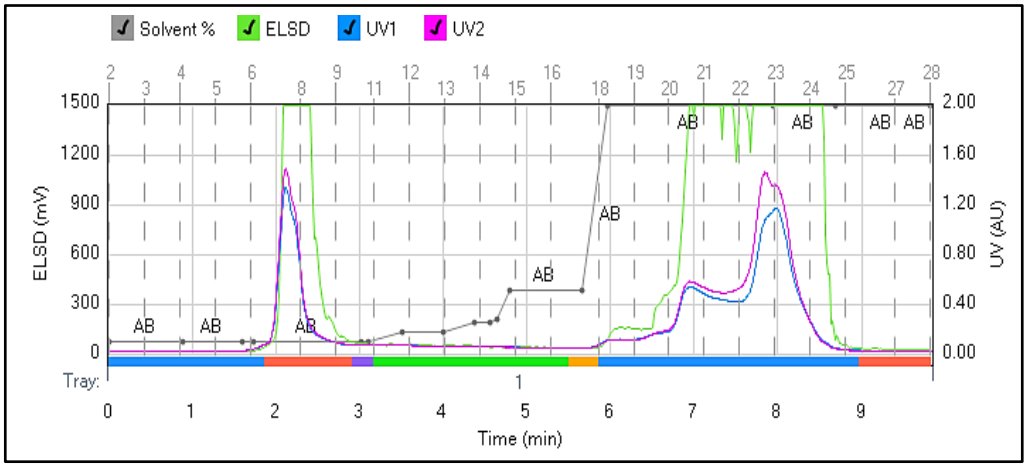


Figure 4.5 Flash chromatogram of the methanolic fraction.

Similarly an aliquot of 0.8 g from the ethyl acetate fraction was subjected to flash chromatography as above. The gradient used was from ethyl acetate over methanol to methylene chloride. Based on UV and ELSD detection different subfractions (8 in total) were collected (Fig 4.6). Finally a total of 4 subfractions was obtained and subfractions **KRET2** (210 mg), **KRET3** (160 mg), **KRET4** (100mg) and **KRET5** (270 mg) were selected for further HPLC profiling.

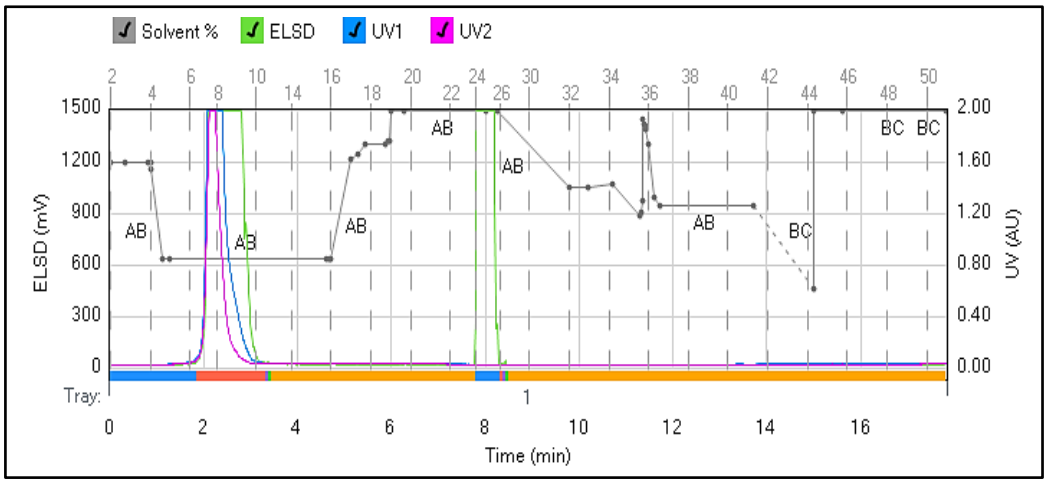


Figure 4.6. Flash chromatogram of the ethyl acetate fraction.

Likewise an aliquot of 0.8 g from the chloroform fraction was loaded on a flash column as discussed above. The compounds were eluted using a gradient from ethyl acetate over methylene chloride to methanol. Based on UV and ELSD detection different subfractions were collected (Fig 4.7). All fractions were analysed by TLC and similar fractions were combined as described above; in this way 8 subfractions were obtained. Based on TLC profiling subfractions **KRCL1** (135 mg), **KRCL2** (125 mg), **KRCL4** (160 mg), **KRCL7** (150 mg) and **KRCL8** (120 mg) were selected further HPLC analysis.

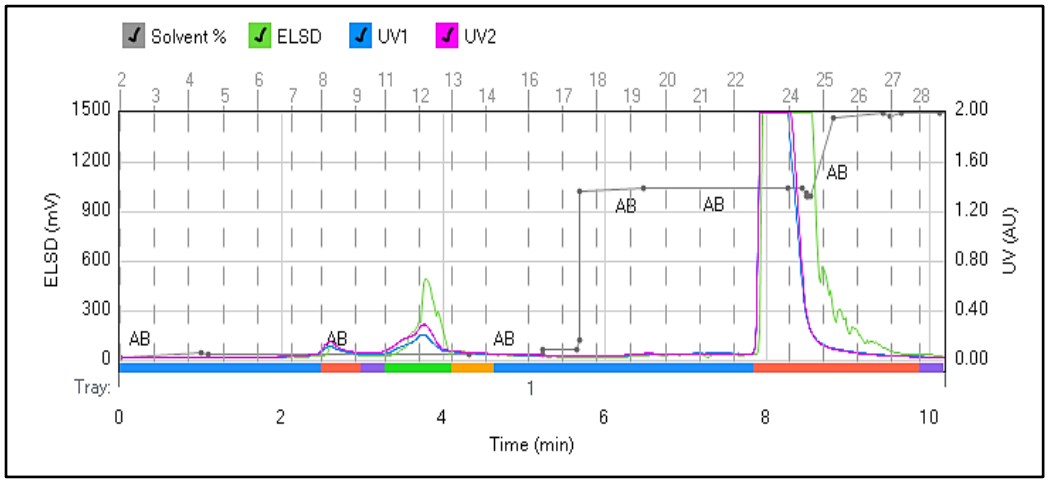


Figure 4.7 Flash chromatogram of the chloroform fraction.

Similarly an aliquot of 0.8 g from the *n*-butanol fraction was subjected to flash chromatography with a gradient from ethyl acetate over methylene chloride to methanol as previously explained. Finally 10 subfractions were obtained (Fig 4.8). Based on TLC analysis, subfractions **KRB2** (110 mg), **KRB4** (127 mg), **KRB5** (131 mg), **KRB6** (120 mg) and **KRB7** (126 mg) was selected for HPLC analysis.

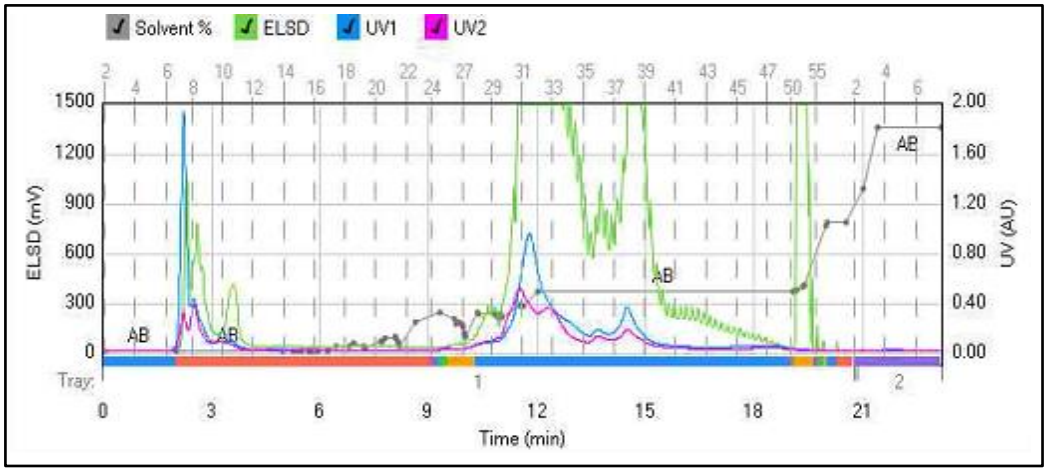


Figure 4.8 Flash chromatogram of the *n*-butanol fraction.

4.2.6. HPLC analysis and isolation of compounds

The chloroform fraction and all obtained subfractions **KRCL1**, **KRCL2**, **KRCL4**, **KRCL7** and **KRCL8** were analysed by HPLC using an optimized methanol/H_2O + 0.1% formic acid gradient, ranging from 15% acetonitrile to 80% in 50 min at a flow rate of 1 mL/min. Samples were prepared in a concentration range from 1-10 mg/ mL in methanol (Fig 4.9). The isolation of pure compounds was performed by means of semi-preparative HPLC-DAD-MS using the same gradient at 3 mL/min, yielding compounds **1** (7.5 mg)**, 2** (5.2 mg) and **3** (5.2 mg)**.**

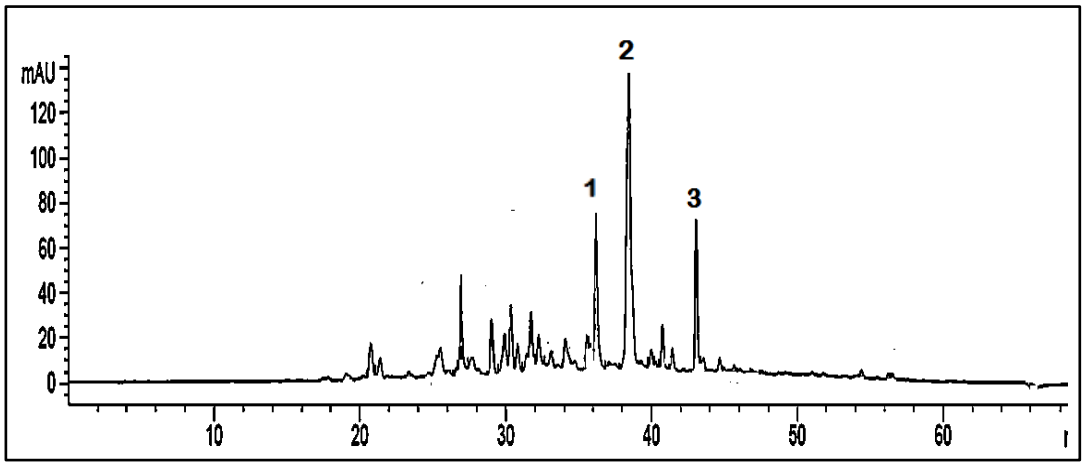


Figure 4.9 HPLC chromatogram of chloroform fraction **KRCL2** at 254 nm.

The crude ethyl acetate fraction and subfractions **KRET3** (250 mg), **KRET4** (180 mg) and **KRET5** (150 mg) were analysed by HPLC using an optimized acetonitrile/H_2O + 0.1% formic acid gradient ranging from 15% acetonitrile to 100% in 60 min at a flow rate of 1 mL/min (Fig. 4.10). The isolation of pure compounds was performed by means of semi-preparative HPLC-DAD-MS using the same gradient at 3 mL/min. Compounds **2** (2.2 mg), **3** (5.2 mg) and **4** (5.7 mg) were finally obtained.

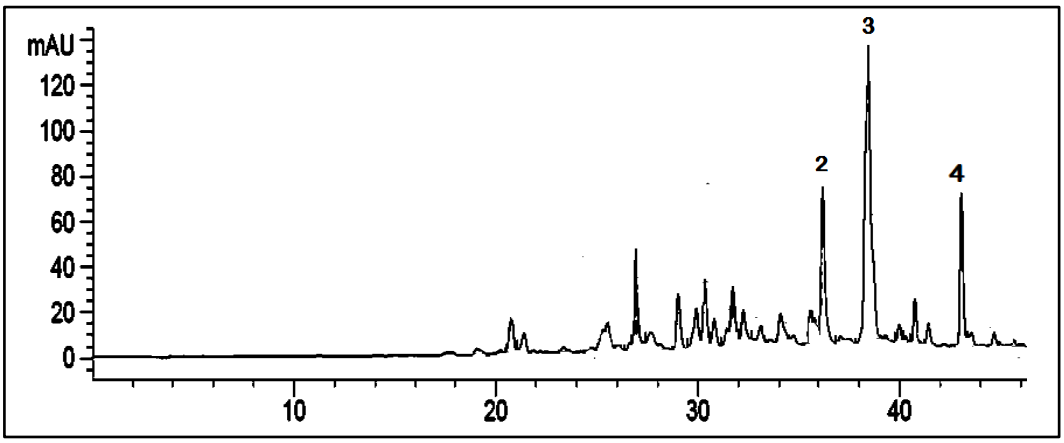


Figure 4.10 HPLC chromatogram of ethyl acetate fraction **KRET3** at 254 nm.

Similarly the crude methanol fraction and subfractions **KRM2, KRM3, KRM5, KRM7** and **KRM14** were analysed by HPLC using an optimized acetonitrile/H_2O + 0.1% formic acid gradient ranging from 35% acetonitrile to 70% in 50 min at a flow rate of 1 mL/min (Fig. 4.11). The compounds were isolated by means of semi-preparative HPLC-DAD-MS system using the same gradient at a flow rate of 3 mL/min. Finally compounds **2** (3.3mg), **5** (4.5 mg) and, **6** (3.2 mg) were isolated.

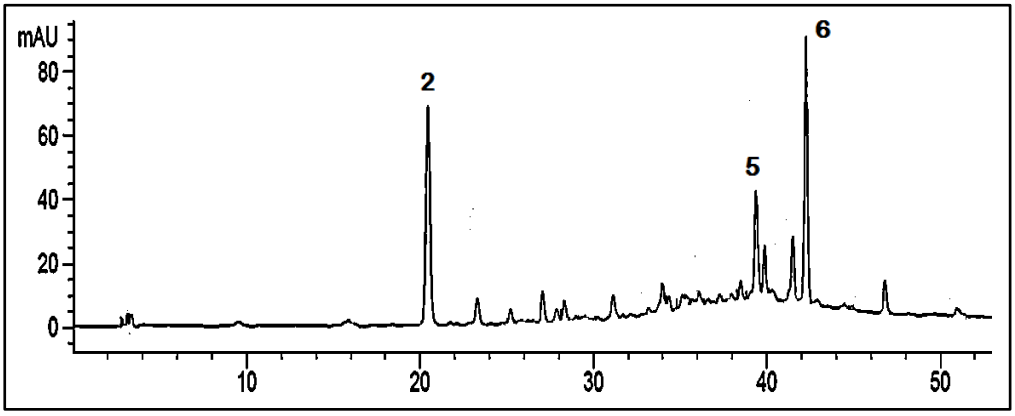


Figure 4.11 HPLC chromatogram of methanol fraction **KRM2** at 254 nm

The crude *n*-butanol and subfraction **KRB2, KRB4, KRB6 and KRB7** was analysed by HPLC using an optimized acetonitrile/H_2O + 0.1% formic acid gradient ranging from 39% acetonitrile to 65% in 45 min at a flow rate of 1 mL/min (Fig. 4.12). Compound isolation was performed by means of semi-preparative HPLC-DAD-MS system using the same solvent gradient as HPLC at a flow rate of 3 mL/min. Compounds **3** (3.2 mg), **5** (3.2 mg) and **7** (3.8 mg) were finally isolated.

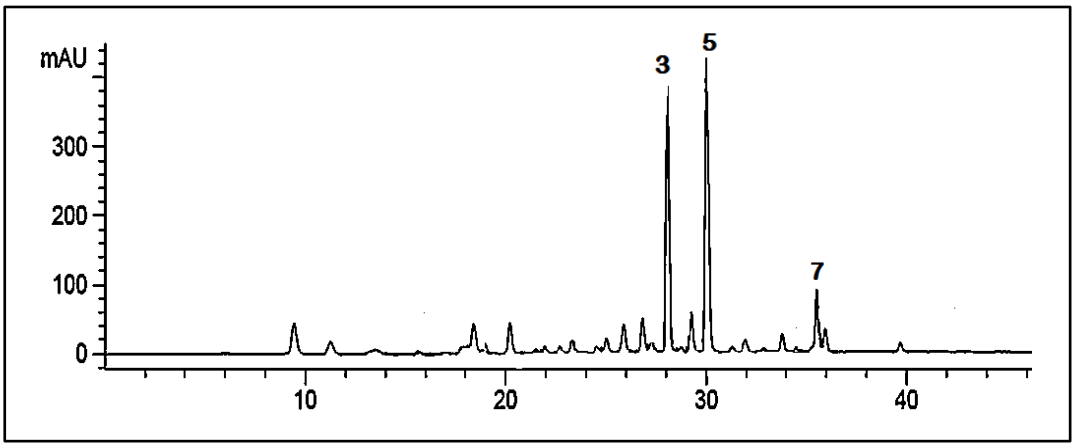


Figure 4.12 HPLC chromatogram of *n*-butanol fraction **KRB2** at 254 nm

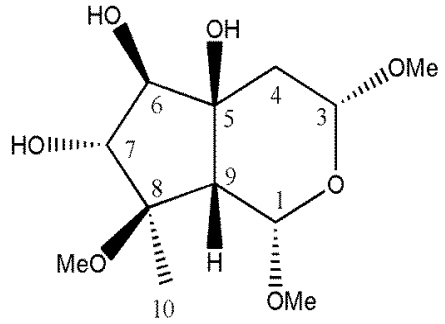
4.3. STRUCTURE ELUCIDATION

Structures of the isolated compounds from *Kickxia ramosissima* were elucidated using ^{1}H- and ^{13}C- NMR (including DEPT-135 and DEPT-90) as well as 2D-NMR (COSY, HSQC and HMBC) spectroscopy. The molecular ion was derived from the high resolution mass spectra obtained with an Agilent™ 6530 quadrupole-time-of-flight mass spectrometer (QTOF-MS) and from the semi-preparative HPLC-DAD-MS system.

4.3.1 Iridoids (1-4)

4.3.1.1 Kickxiasine (1)

The structure of compound **1** was elucidated by ^{1}H-NMR, ^{13}C-NMR (including DEPT-135 and DEPT-90) and 2D-NMR (COSY, HSQC and HMBC). Since this compound had not been reported before the name **kickxiasine** was adopted (Fig. 4.13).



(1*S*,3*R*,4a*S*,5*R*,6*R*,7*R*,7a*S*)-1,3,7-trimethoxy-7-methylhexahydrocyclopenta[*c*]pyran-4a,5,6(1*H*)-triol

Figure 4.13 Structure of kickxiasine (**1**)

Library search on the ^{13}C-NMR data (Table 4.2 and Fig 4.14) using the NMR Predict database and ^{1}H-NMR (Table 4.2, Fig 4.15 and Fig 4.16) suggested that this compound has a similarity with 5-deoxy-8-deacetyl-3-*O*-methyl-6-*O*-*p*-hydroxybenzoyl-1,3-di-*epi*-clandonensine (Awale *et al.*, 2005) with some structural modifications on the cyclopentanopyran ring system. By the analysis of COSY (Fig 4.17), HSQC (Fig 4.18) and HMBC (Fig 4.19) correlations the structure was elucidated as shown.

The ^{1}H-NMR spectrum (Fig 4.15 and Fig 4.16) showed signals due to one methyl, one methylene, six methines and three methoxyls. The methylene signals at 1.51 ppm (dd) and 2.13 ppm (m) were assigned to H-4. A methine at 4.88 ppm (dd, *J*=5.3, 8.4 Hz) was attributed to H-3. A methine signal at 2.05 ppm (d, H-9) was coupled with another methine at 4.94 ppm (d, H-1), an upfield signal that was indicative of substitution with oxygen. Finally two methine protons showed coupled doublets at 3.76 ppm (*J*=4.3 Hz) and 3.92 ppm (*J*= 4.3 Hz), assigned to H-7 and H-6, respectively. The methyl group at 1.35 ppm (s, H-10) was attached to an oxygen-bearing quaternary carbon as determined by HMBC correlations.

The ^{13}C-NMR spectrum showed 12 carbon signals. The signals at 98.7 ppm and 98.8 ppm were assigned to carbons C-1 and C-3, respectively. In the cyclopentane ring the signals at 75.9 ppm and 77.3 ppm were attributed to C-6 and C-7, respectively. In the same ring the signals at 83.2 ppm, 77.4 ppm and 60.3 ppm were assigned to C-8, C-5 and C-9, respectively. The methyl group (C-10) showed a resonance signal at 17.1 ppm. Three methoxyl groups in position 1, 3 and 8 showed signals at 50.3, 55.6, and 56.3 ppm, respectively. The protons of these methoxyl groups were correlated in the HMBC spectrum with C-1, C-3 and C-8. The methyl group (C-10) at 1.35 ppm was attached to C-8, which was also established by HMBC correlations of the methyl protons with C-8 and C-9.

HMBC correlations from C-1 to the methoxyl group attached to C-8 and to H-9, and from C-6 to H-10 confirmed that the cyclopentane ring was substituted by a methyl group (C-10) and a methoxyl group. Similarly in the second ring, the signals at 97.3 ppm, 97.4 ppm and 39.5 ppm were assigned to C-1, C-3 and C-4, respectively. Also the HMBC correlations from H-1 to the methoxyl group attached to C-3 and from C-8 to the methoxyl group attached to C-1 confirmed the position of these methoxyl groups at C-1 and C-3.

The relative configuration was assigned on the basis of chemical shifts and coupling constants reported for related compounds (Morota *et al*., 2010; Ahmad *et al*., 2006; Awale *et al*., 2005), and by analogy with linarioside (compound **4**, see below). The ^{13}C-

NMR chemical shifts and 2D NMR experiments support the structure of compound **1** as a new compound for which the name kickxiasine was adopted.

Yellow powder; $[\alpha]^{20}_{D}$ -19.072 (c = 0.0011, MeOH); UV (acetonitrile / H_2O) λ_{max} 202, 209, 216 and 225 nm; HR-MS (positive ion mode): *m/z* 301.1274 $[M+Na]^+$ (calculated 301.1258) consistent with a molecular formula $C_{12}H_{22}O_7Na$, or $C_{12}H_{22}O_7$ for kickxiasine.

Compound **1** (kickxiasine) is reported here for the first time from nature. Although a number of polymethoxylated iridoids have been reported in nature from different medicinal plants, for instance *Tabebuia avellanedae* (Awale *et al*., 2005), *Rehmannia glutinosa* (Morota *et al*., 2010; Liu *et al*., 2014) and *Gonocaryum calleryanum* (Kaneko *et al*., 1995), however, the possibility that compound **1** is an artefact formed during extraction and isolation cannot be excluded.

Table 4.2 ^{1}H and ^{13}C NMR assignments for kickxiasine (**1**) recorded in MeOH-d_4

Position	**δ_H (ppm); multiplicity; *J* (Hz)**	**δ_C (ppm)**
1	4.94; d; *J*=7.3	98.7
3	4.88; dd; 8.4, 5.3	98.8
4_a	2.13; m	40.9
4_b	1.51; dd; *J*= 14.0, 8.4*	
5		77.4
6	3.93 ; d; *J*=4.3	75.9
7	3.77; d; *J*=4.3	77.3
8		83.2
9	2.05; d, *J*= 7.3	60.3
10	1.35; s	17.1
OMe (C-1)	3.47; d; *J*=6.7	50.3
OMe (C-3)	3.47; s	55.6
OMe (C-8)	3.36; s	56.3

* Multiplicity and coupling constants were calculated on a spectrum recorded in acetone-d_6 because of overlapping with the HDO peak in the spectrum recorded in MeOH-d_4.

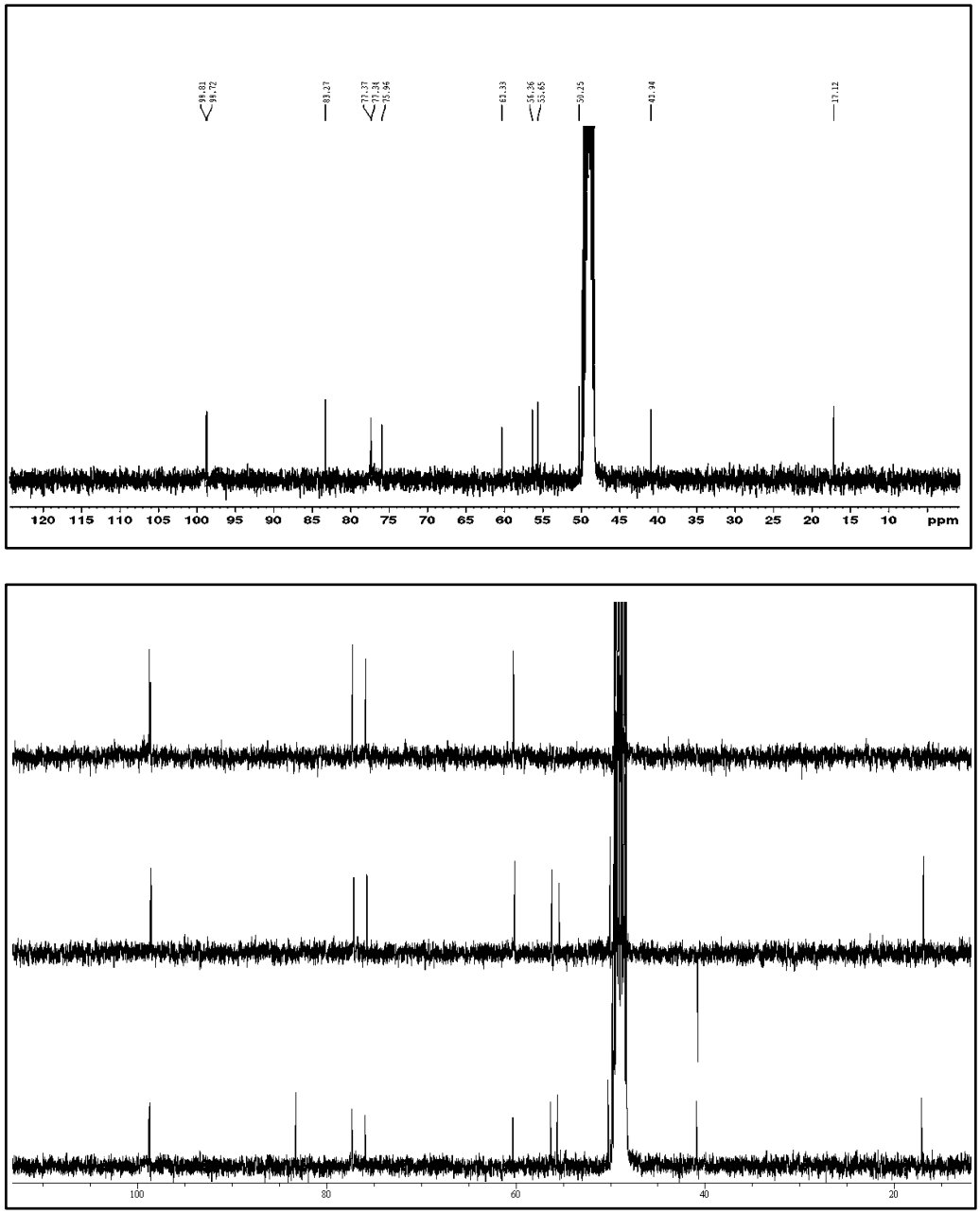


Figure 4.14 ^{13}C-NMR, DEPT-135 and DEPT-90 spectra of kickxiasine **1** in methanol-d_4.

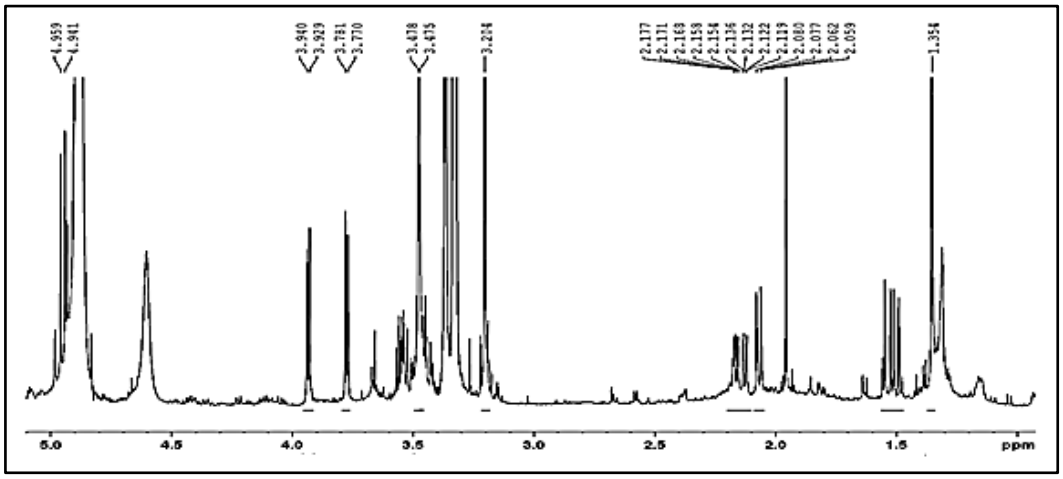


Figure 4.15 ^{1}H-NMR spectrum of kickxiasine **1** in methanol-d_4

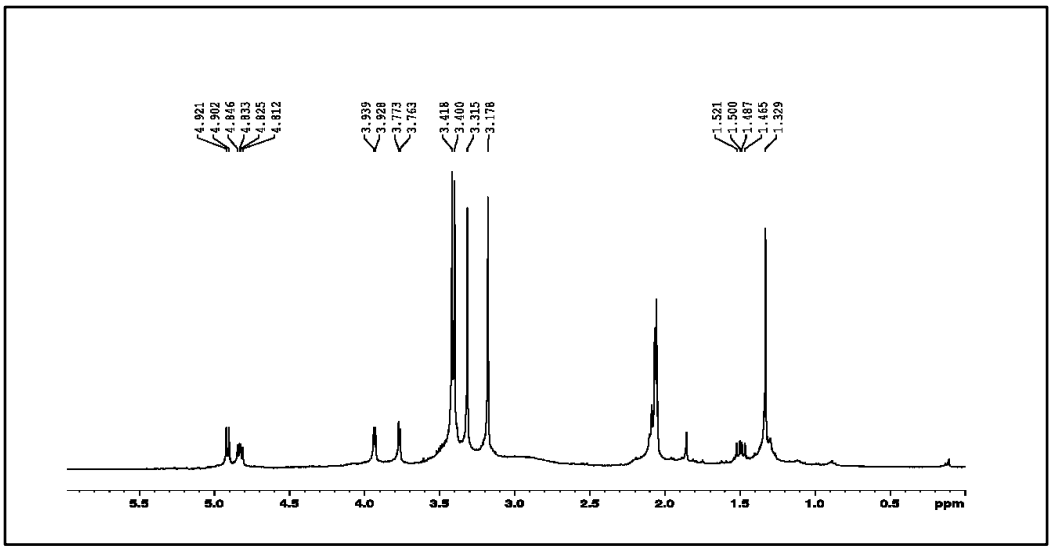


Figure 4.16 ^{1}H-NMR spectrum of kickxiasine **1** in acetone-d_6

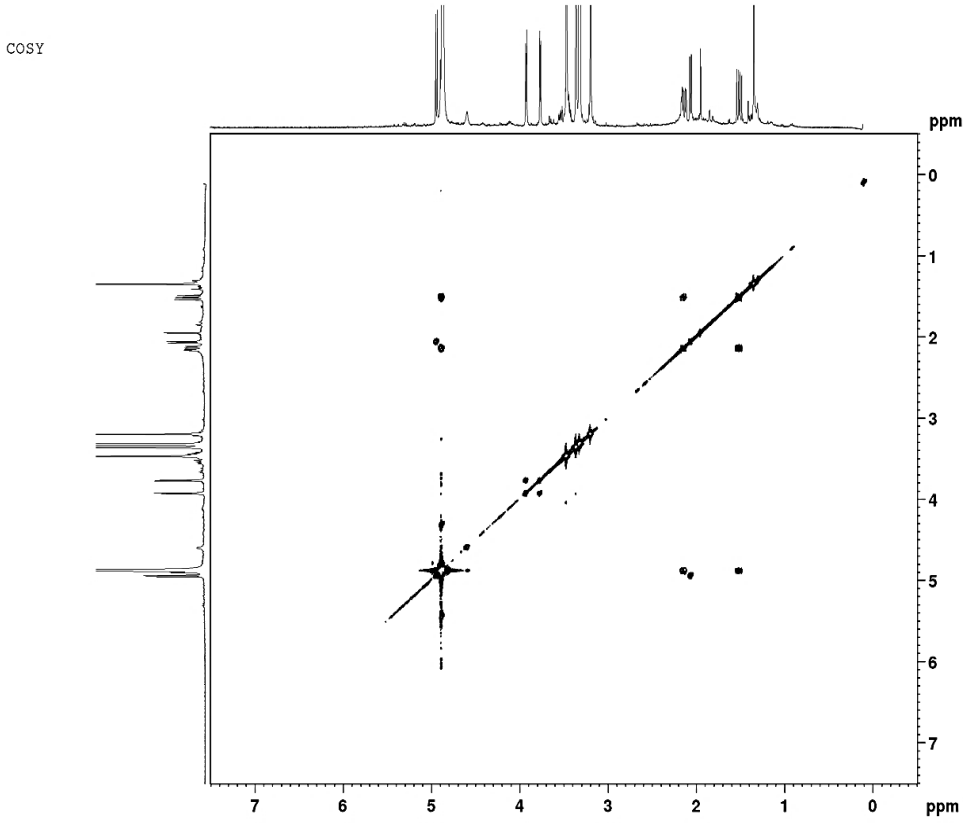


Figure 4.17 COSY spectrum of kickxiasine **1**

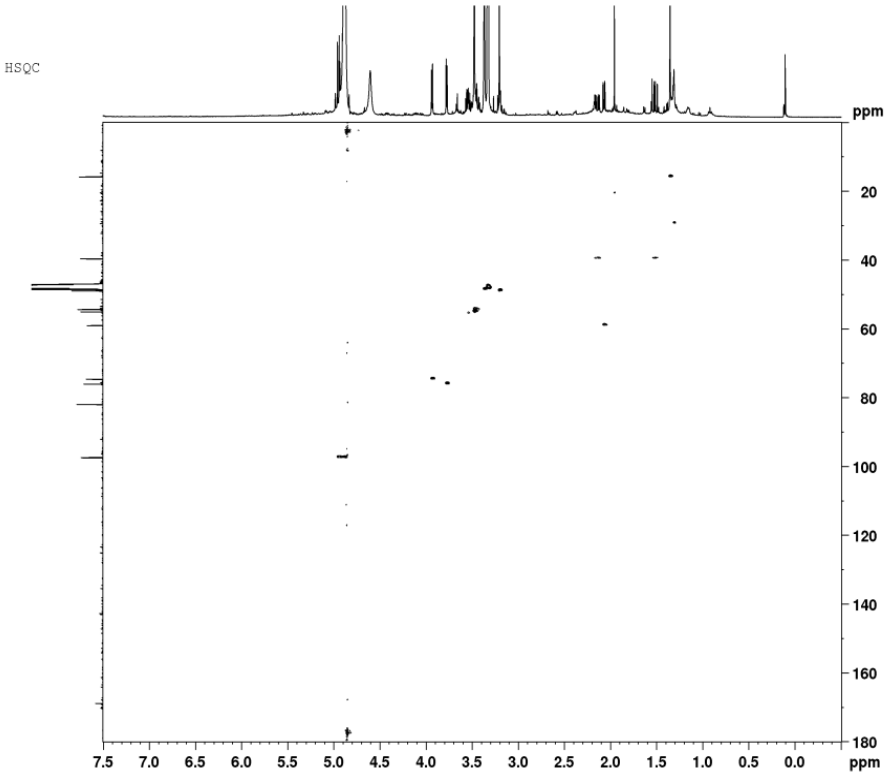


Figure 4.18 HSQC spectrum of kickxiasine **1**

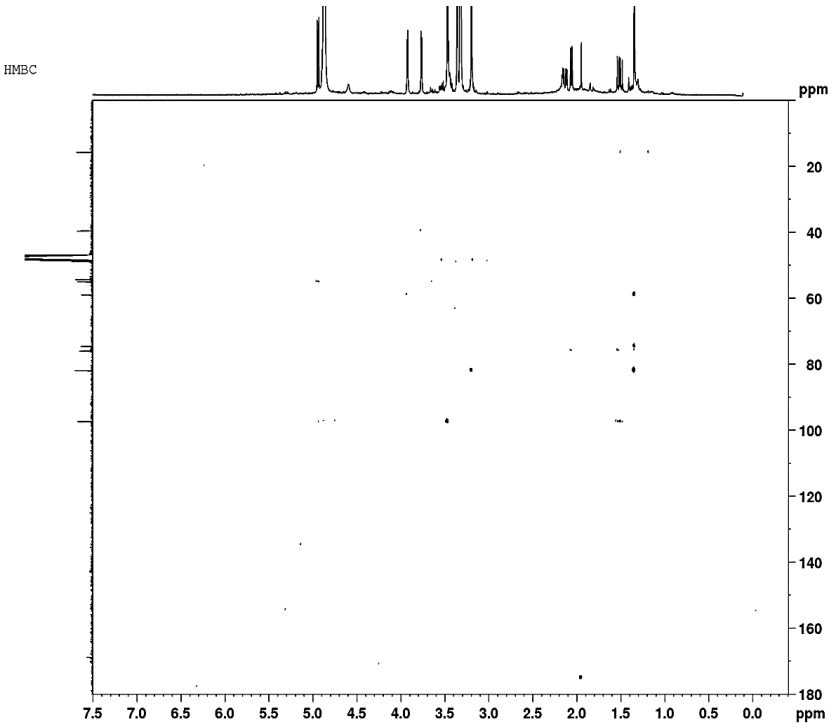


Figure 4.19 HMBC spectrum of kickxiasine **1**

4.3.1.2 Mussaenosidic acid (2)

The structure of compound **2** was elucidated by ^{1}H-NMR, ^{13}C-NMR, DEPT-135 and DEPT-90 as mussaenosidic acid (Fig. 4.20).

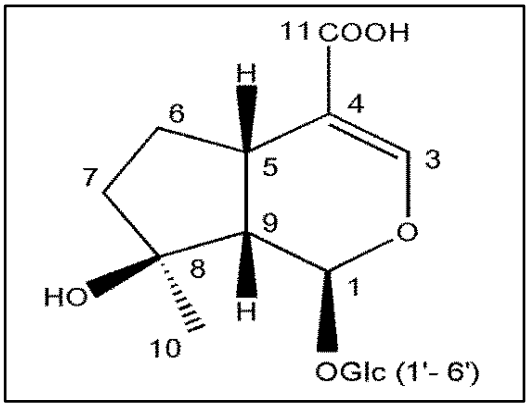


Figure 4.20 Structure of mussaenosidic acid (**2**)

Library search on the ^{13}C-NMR data (Table 4.3 and Figure 4.21) using the NMR Predict database and ^{1}H-NMR (Fig 4.22) suggested that this compound was an iridoid glucoside. Comparison with NMR data from literature (Venditti *et al.*, 2016) suggested that compound **2** was mussaenosidic acid.

The ^{1}H-NMR spectrum (Fig. 4.22) of compound **2** displayed two methylene signals (multiplets) at 2.23 ppm (assigned to H-6) and at 1.74 / 1.52 ppm assigned to H-7a and H-7_b, respectively. Another multiplet at 3.10 ppm was attributed to H-5. A singlet at 1.30 ppm was assignable to a methyl group attached to a hydroxyl bearing carbon. Similarly a multiplet appearing at 2.36 ppm was assigned to H-9 whereas a doublet at 5.60 ppm (*J*=2.8 Hz) was attributed to H-1. The anomeric proton H-1'resonated at 4.80 ppm (partly overlapping with the HDO signal). Finally the sugar protons were observed between 3.0-3.52 ppm (H-2'; H-3'; H-4'; H-5'). The methylene protons of the sugar moiety (H-6') resonated at 3.92 ppm (dd; *J*=2.0, 12.0 Hz, H-6_a') and 3.72 ppm (dd; *J*=6.0, 12.0 Hz, H-6_b').

The ^{13}C-NMR spectrum showed signals of 16 carbons and a β-glucopyranose moiety. The signals at 94.3 ppm and 29.5 ppm were assigned to C-1 and C-5, respectively. The

^{13}C-NMR spectrum showed the presence of a double bond with the olefinic carbons at 151.0 ppm and 112.6 ppm that were assigned to C-3 and C-4, respectively. The resonance at 169.4 ppm was assigned to a carbonyl group (C-11). Two methylene signals were recorded at 28.7 ppm and 39.6 ppm, which were attributed to C-6 and C-7, respectively. Finally the signals at 79.6 ppm and 50.6 ppm were attributed to C-8 and C-9, respectively. The methyl signal appearing at 24.6 ppm was assigned to C-10.

Yellow powder; UV (acetonitrile / H_2O) λ_{max} 237 nm; ESI-MS (positive ion mode): *m/z* 399 $[M+Na]^+$ consistent with a molecular formula $C_{16}H_{24}O_{10}$.

Mussaenosidic acid has been reported from various medicinal plants including *Pedicularis kerneri* (Venditti *et al*., 2016), *Kickxia elatine* (L.) Dum., *Kickxia spuria* (L.) Dum. (Handjieva *et al*., 1995), *Lagochilus ilicifolius* (Guangzhou *et al*., 2012) and *Vitex negundo* (Sehgal *et al.,* 1982*)*. Mussaenosidic acid (**2**) is reported here for the first time in *Kickxia ramosissima*.

Table 4.3 ^{1}H-NMR and ^{13}C-NMR assignments for mussaenosidic acid (**2**) recorded in D_2O.

Position	**δ_H (ppm), multiplicity, *J* (Hz)**	**δ_C (ppm)**
1	5.6; d; *J*= 2.8	94.3
3	7.47; s	151.0
4		112.6
5	3.10; m	29.5
6	2.23 (2H; m)	28.7
7	1.74 (1H, m):1.52 (1H, m)	39.6
8		79.6
9	2.36; m	50.6
10	1.30; s	22.9
11		171.2
1'	4.80 (1H, overlapped with solvent)	98.2
2'	3.07-3.52; m	72.6
3'	3.07-3.52; m	77.2
4'	3.07-3.52; m	69.5
5'	3.07-3.52; m	76.2
6a'	3.92; dd; *J*=12.0; 2.0	60.6
6b'	3.72; dd; *J*=12.0; 6.0	

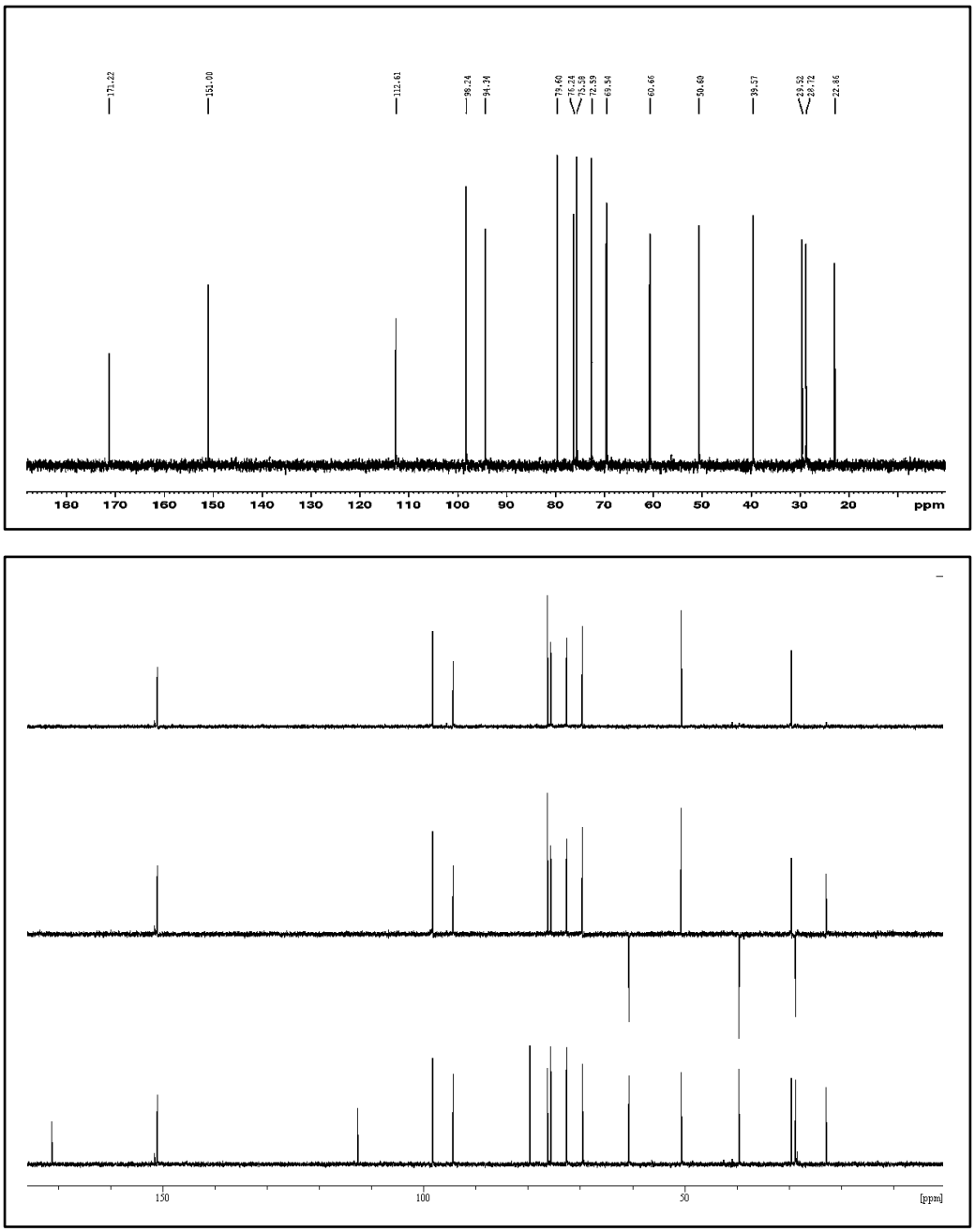


Figure 4.21 ^{13}C-NMR spectra including DEPT-135 and DEPT-90 of mussaenosidic acid (**2**) in D_2O.

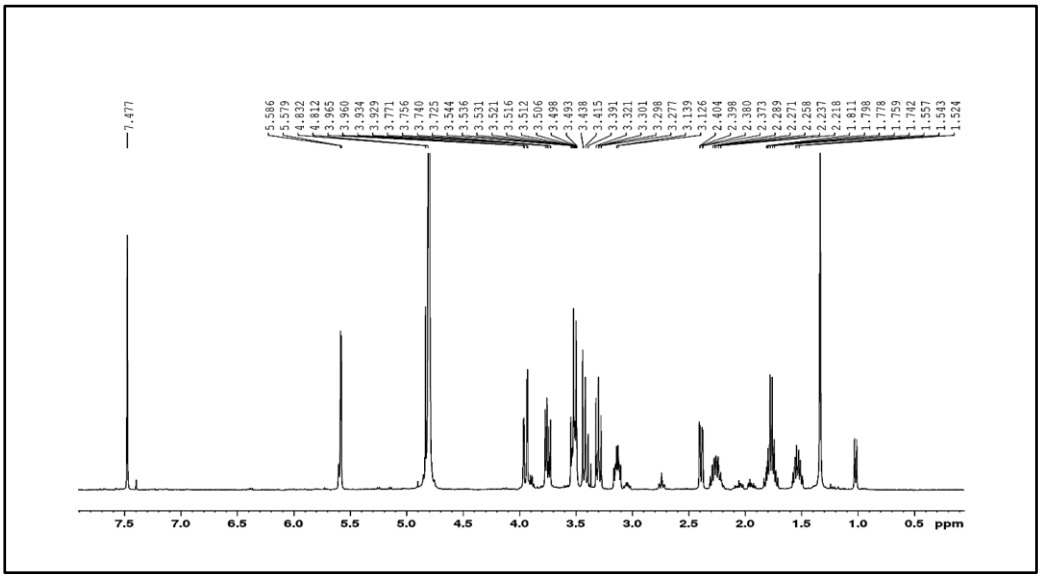


Figure 4.22 ^{1}H-NMR spectrum of mussaenosidic acid (**3**) in D_2O.

4.3.1.3 **Mussaenoside** (**3**):

The structure of compound **3** was elucidated by ^{1}H-NMR, ^{13}C-NMR, DEPT-135 and DEPT-90 as mussaenoside (Fig. 4.23).

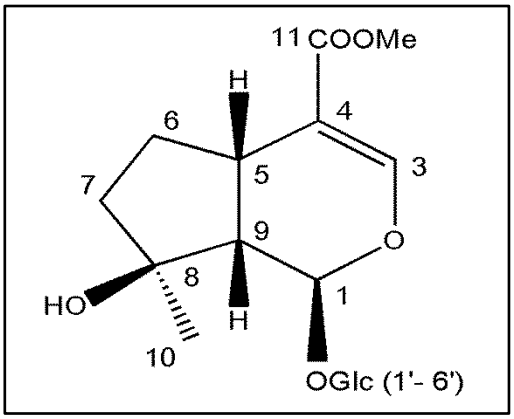


Figure 4.23 Structure of mussaenoside (**3**)

Library search on the ^{13}C-NMR data (Table 4.4 and Fig 4.24) using the NMR Predict database and ^{1}H-NMR (Fig 4.25) suggested that this compound was an iridoid glucoside. Comparison with NMR data from literature (Ersoz *et al*., 1998; Haznagy-Radni *et al*., 2014) suggested that compound **2** was mussaenoside.

The ^{1}H-NMR (Fig 4.25) spectrum of compound **3** displayed two methylene signals (multiplets) at 2.30 ppm and 1.45 ppm assigned to H-6$_a$ and H-6$_b$, respectively, and 1.75 ppm assigned to H-7a and H-7$_b$. Another multiplet at 3.20 ppm was attributed to H-5. A singlet at 1.33 ppm was assignable to a methyl group attached to a hydroxyl bearing carbon, whereas a methoxyl group appeared at 3.71 ppm. Similarly a doublet of doublet appearing at 2.24 ppm (*J*=4.2, 9.3 Hz) was assigned to H-9 whereas a doublet at 5.48 ppm (*J*=4.2 Hz) was attributed to H-1. The anomeric proton H-1' resonated at 4.25 ppm (d; *J*=7.9 Hz). Finally sugar protons were observed at 3.0-3.72 ppm (H-2'; H-3'; H-4'; H-5'). The methylene protons of the glucose moiety (H-6'$_a$ and H-6'$_b$.) were observed at 3.92 ppm (dd; *J*= 2.0, 11.8 Hz) and 3.65 ppm(dd; *J*= 6.2, 11.8).

The ^{13}C-NMR spectrum showed signals of 17 carbons and a β-glucopyranose moiety. Two olefinic carbons were observed at 152.0 ppm and 113.3 ppm that were assigned to C-3 and C-4, respectively. The carbon resonances at 169.4 ppm (C-11) and 51.6 ppm confirmed the presence of the $COOCH_3$ ester group. The signals at 95.3 ppm and 32.0 ppm were assigned to carbons C-1 and C-5, respectively. The methylene signals at 30.7 ppm and 40.7 ppm were assigned to C-6 and C-7, respectively. Finally signals at 80.5 ppm and 52.3 ppm were attributed to C-8 and C-9, respectively. The methyl signal (C-10) appeared at 24.6 ppm.

Yellow amorphous solid; UV (acetonitrile / H_2O) λ_{max} 237 nm; ESI-MS (positive ion mode): *m/z* 413 $[M+Na]^+$ consistent with a molecular formula $C_{17}H_{26}O_{10}$.

Mussaenoside is a common iridoid and has been isolated from a number of plants species, for instance *Melampyrum* sp. (Damtoft *et al*., 1984), *Bellardia trixago* (Ersoz *et al.,* 1998) and *Mussaenda incana* (Dinda *et al*., 2005). This is the first report of its occurrence in *Kickxia ramosissima*. Since mussaenoside is the methyl ester of mussaenosidic acid (**2**), the possibility that it is an artefact formed during extraction procedures in which methanol is used cannot be excluded.

Table 4.4 ^{1}H-NMR and ^{13}C-NMR assignments for mussaenoside (**3**) recorded in methanol-d_4.

Position	**δ_H (ppm); multiplicity; *J* (Hz)**	**δ_C (ppm)**
1	5.48; d; *J*=4.2	95.3
3	7.4; s	152.0
4		113.3
5	3.20; m	32.0
6	2.30(1H, m) / 1.45(1H, m)	30.7
7	1.75 (2H, m)	40.7
8		80.5
9	2.24; dd; *J*=9.3; 4.2	52.3
10	1.33; s	24.6
11		169.4
1'	4.25; d; *J*=7.8	99.8
2'	3.0-3.72; m	74.7
3'	3.0-3.72; m	78.4
4'	3.0-3.72; m	71.7
5'	3.0-3.72; m	78.0
6_a'	3.92; dd; *J*= 11.8; 2.0	62.9
$6'_b$	3.65; dd; *J*= 11.8; 6.2	
OMe	3.71; s	51.6

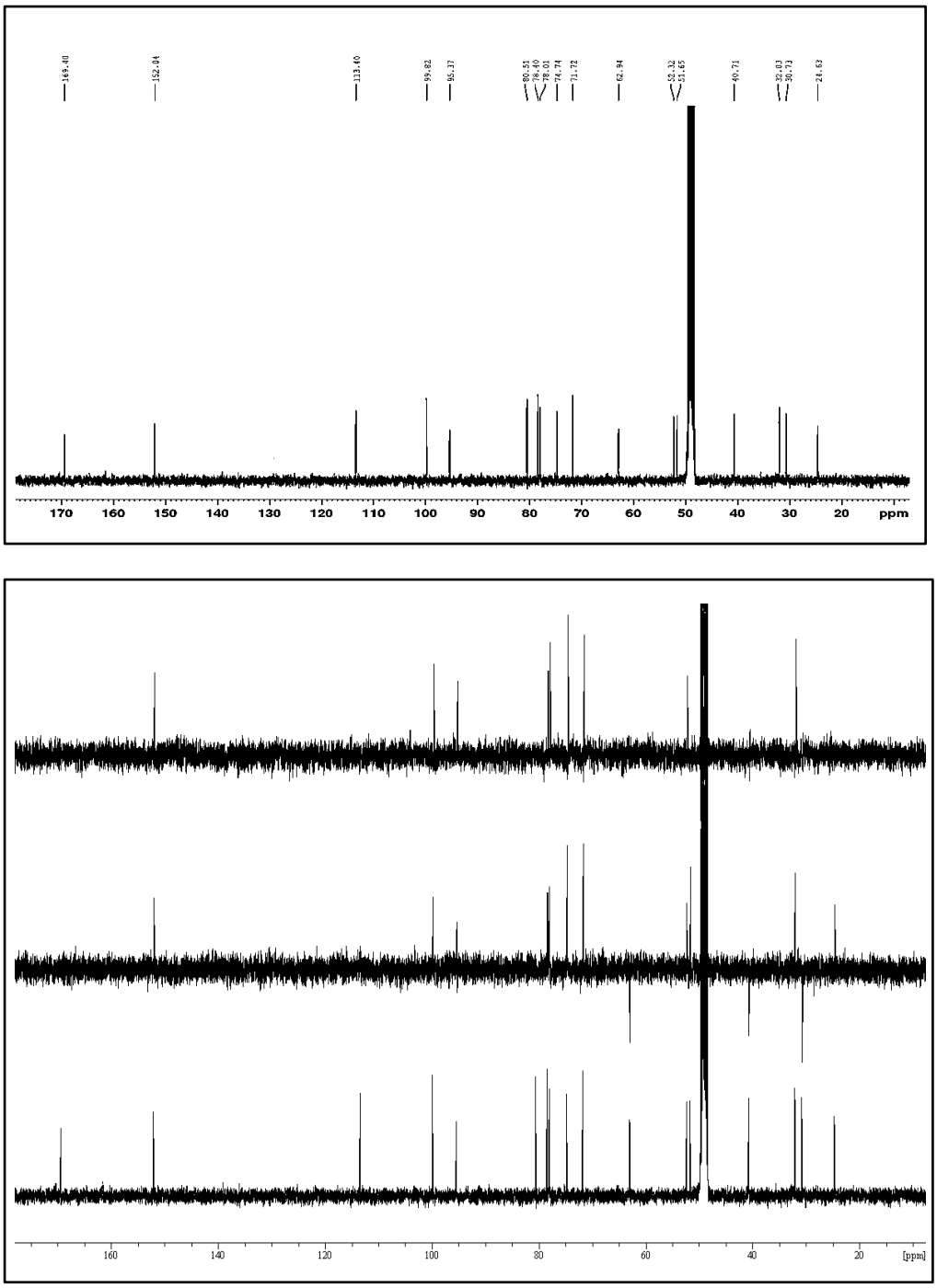


Figure 4.24 ^{13}C-NMR spectra including DEPT-135 and DEPT-90 of mussaenoside(**3**) in methanol-d_4.

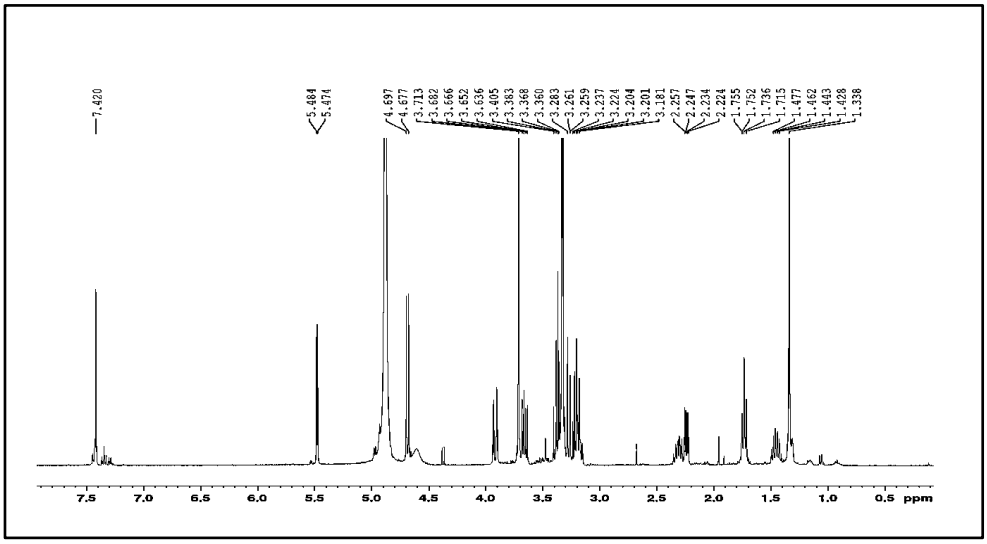


Figure 4.25 ^{1}H-NMR spectrum of mussaenoside(**3**) in methanol-d_4.

4.3.1.4 Linarioside (4):

The structure of compound **4** was elucidated by ^{1}H-NMR, ^{13}C-NMR, DEPT-135 and DEPT-90 as linarioside (Fig. 4.26).

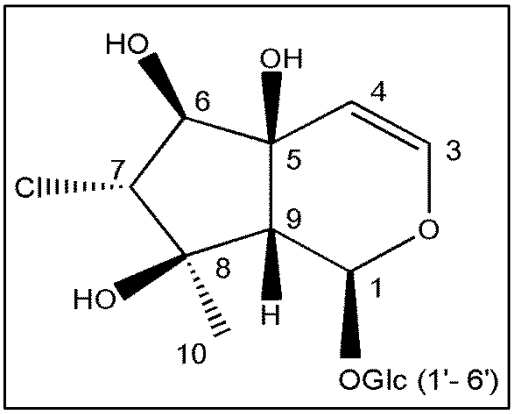


Figure 4.26 Structure of linarioside (**4**)

Library search on the ^{13}C-NMR data (Table 4.5 and Fig 4.27) using the NMR Predict database and ^{1}H-NMR (Fig 4.28) suggested that this compound was an iridoid glucoside. Comparison with spectral data obtained from literature (Kitagawa *et al*.,1973; Otsuka, 1993) suggested compound **4** was linarioside.

The signals at 6.42 ppm and 5.30 ppm were assigned to the olephinic protons H-3 and H-4 that were coupled with each other. The singlets appearing at 5.80 ppm and 2.57 ppm were attributed to H-1 and H-9, respectively. These singlets were indicative of the absence of coupling (i.e. very small J) between H-1 and H-9. Multiplets at 3.42-4.2 ppm represented H-6 and H-7, respectively. The methyl signal was recorded at 1.30 ppm and assigned to H-10. The anomeric proton (H-1') showed a resonance at 4.20 ppm (d; *J*= 7.8 Hz). Finally the sugar protons were observed at 3.42-4.2 ppm (H-2'; H-3'; H-4'; H-5' and H-6').

The ^{13}C-NMR spectrum showed signals of 15 carbons and a β-glucopyranose moiety. The signals at 91.0 ppm, 139.2 ppm and 108.1ppm were assigned to C-1, C-3 and C-4, respectively. Similarly C-5 and C-6 were assigned to the signals at 64.6 ppm and 79.2

ppm, respectively. The methyl group appearing at 17.1 ppm was attributed to C-10. Finally the signals at 72.5 ppm and 74.0 ppm were assigned to C-7 and C-8, respectively.

Yellow powder; UV (acetonitrile / H_2O) λ_{max} 210 nm; ESI-MS (positive ion mode): *m/z* 422 $[M+Na]^+$ consistent with a molecular formula $C_{15}H_{23}O_{10}Cl$.

Linarioside was the first example of a chlorine containing iridoid glucoside in nature isolated from *Linaria japonica* that was reported by Kitagawa *et al*. (1973). A detailed investigation proved that the compound linarioside is a naturally occurring compound and not formed as an artefact during the extraction process. Afterwards a number of other medicinal plants were reported as having linarioside as one of their constituents. These include *Cymbalaria muralis* (Kapoor and Reisch, 1974), *Asystasia Bella* (Demuth *et al*., 1989), *Linaria aegyptiaca* (Ferhat *et al*., 2010) and *Linaria genistifolia* (Ilieva *et al*., 1992). We are reporting linarioside for the first time in *Kickxia ramosissima*.

Table 4.5 ^{1}H-NMR and ^{13}C-NMR assignments for linarioside(**4**) recorded in D_2O.

Position	**δ_H (ppm); multiplicity; *J* (Hz)**	**δ_C (ppm)**
1	5.80; s	91.0
3	6.42; d; *J*=6.5	139.2
4	5.30; dd; *J*=1.2; 6.5	108.1
5		64.6
6	3.42-4.2; m	79.2
7	3.42-4.2; m	72.5
8		74.0
9	2.57; s	56.2
10	1.30; s	17.1
1'	4.20; d; *J*= 7.8	97.8
2'	3.42-4.2; m	75.2
3'	3.42-4.2; m	79.2
4'	3.42-4.2; m	69.7
5'	3.42-4.2; m	76.2
6'	3.42-4.2; m	61.6

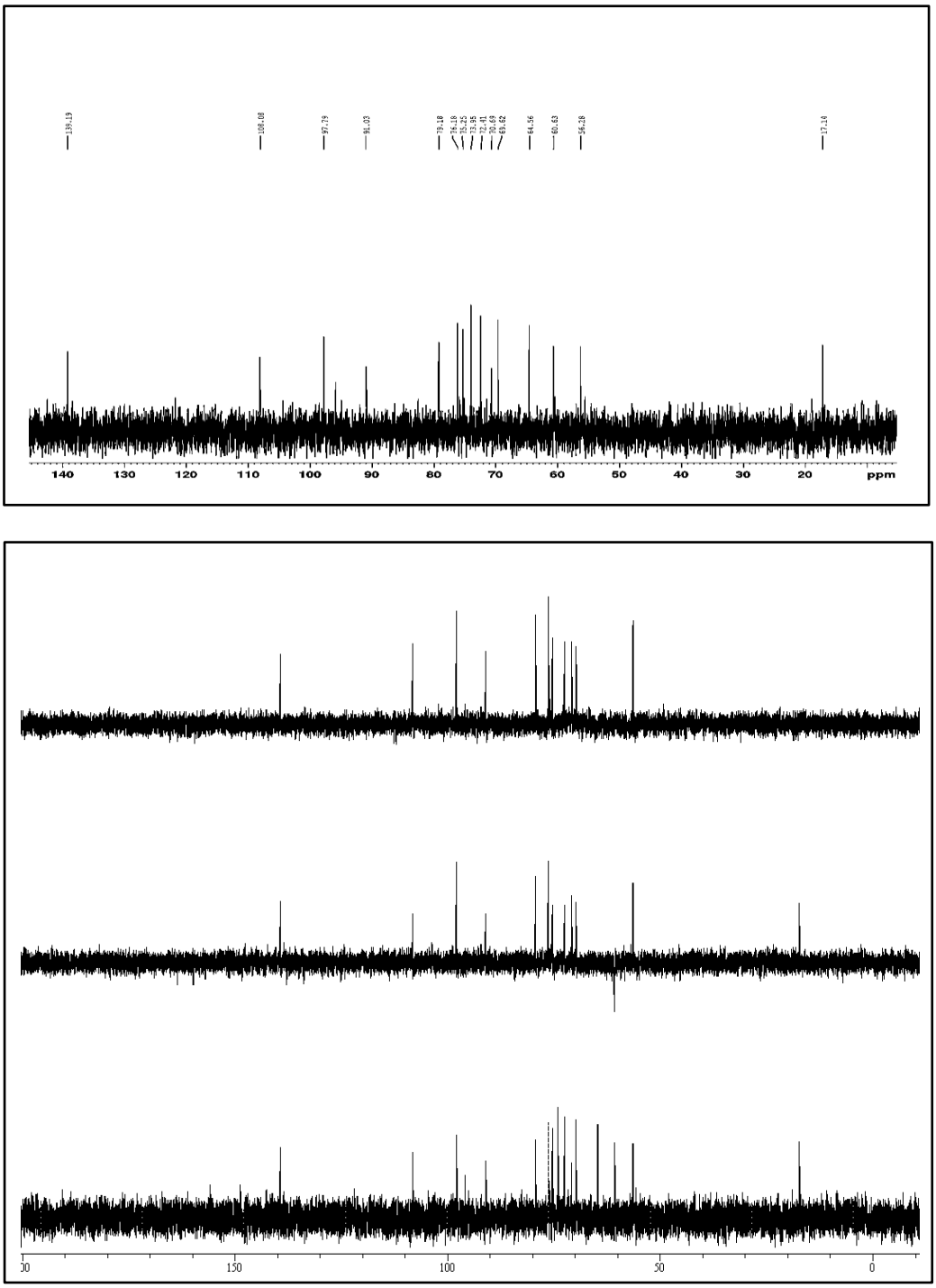


Figure 4.27 ^{13}C-NMR spectra including DEPT-135 and DEPT-90 of linarioside(**4**) in D_2O.

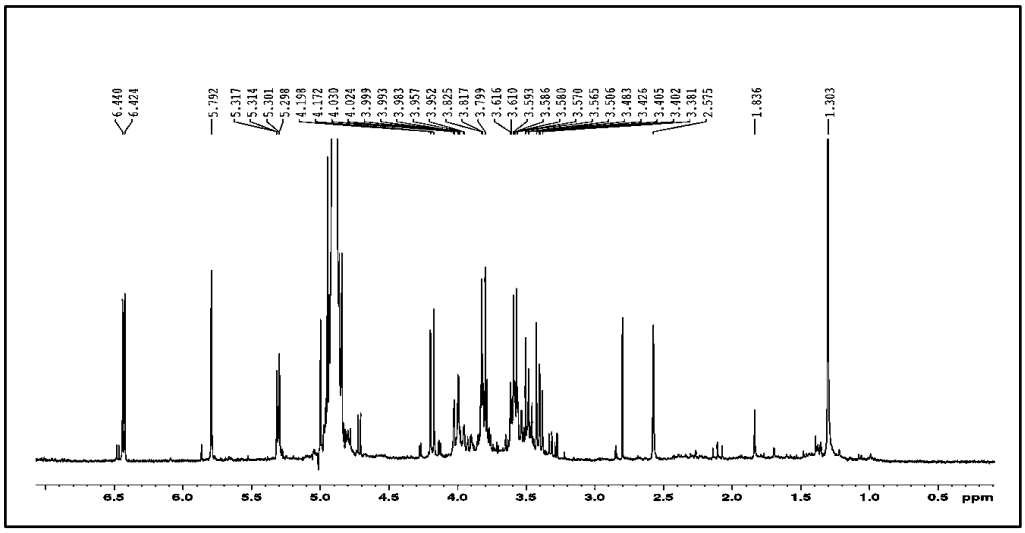


Figure 4.28 ^{1}H-NMR spectrum of linarioside(**4**) in D_2O.

4.3.2 Flavonoids

4.3.2.1 Pectolinarigenin (**5**):

The structure of compound **5** was elucidated by ^{1}H-NMR, ^{13}C-NMR, DEPT-135 and DEPT-90 as pectolinarigenin (Fig. 4.29).

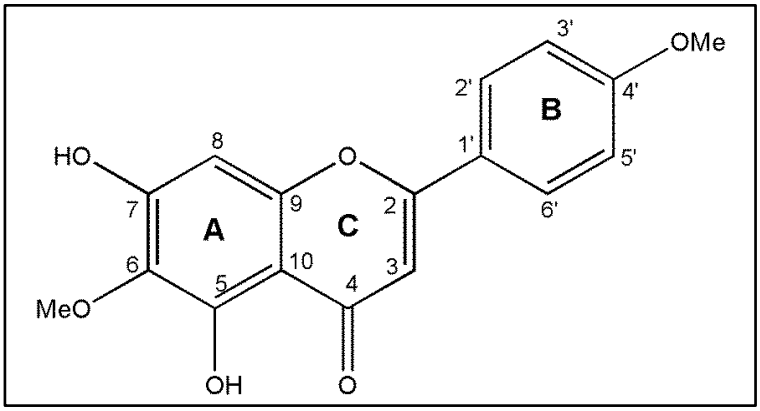


Figure 4.29 Structure of pectolinarigenin (**5**)

Library search on the ^{13}C-NMR data (Table 4.6 and Fig 4.30) suggested this compound is a flavonoid with a glucosyl moiety. By comparison with published data (Lim *et al*., 2008), compound **5** was identified as pectolinarigenin.

The ^{1}H-NMR (Table 4.6 and Fig 4.31) and ^{13}C-NMR (Table 4.6 and Fig 3.30) spectra of compound **5** exhibited resonances due to aromatic ring systems, typical for a flavonoid. In the ^{1}H-NMR spectrum typically 4 protons (H-2', H-3', H-5'and H-6') appearing as 2 doublets (J=9.0 Hz each) at 7.77 ppm and 6.97 ppm suggested an A_2B_2 pattern of ring B. Two singlets at 6.53 ppm and 6.51 ppm were assigned to H-3 and H-8, respectively. The typical pattern of singlets suggested the presence of a 5, 6, 7-oxygenated flavone ring. Similarly the proton spectrum exhibited 2 methoxyl signals at 3.84 ppm and 3.94 ppm that were assigned to the substituents at C-6 and C-4', respectively.

The ^{13}C-NMR spectrum of compound **5** showed the presence of 17 unsaturated carbon signals. The signals at 61.0 ppm and 55.6 were assigned to the methoxyl groups at C-6 and C-4', respectively. The carbonyl group (C-4) showed a resonance at 183.0 ppm, indicating the absence of a hydroxyl group at C-3 and a free hydroxyl group at C-5. A

hydrogen bond between the latter hydroxyl and the carbonyl group leads to relative deshielding of C-4. In ring C the chemical shifts at 164.4 ppm, 103.9 ppm and 152.2 were assigned to C-2, C-3 and C-5 respectively. The signals at 130.4 ppm and 155.0 ppm were attributed to C-6 and C-7, respectively, whereas in ring A the resonances at 93.4 ppm, 153.1 ppm and 105.8 ppm were assigned to C-8, C-9 and C-10, respectively. Finally in ring B the signals at 123.6 ppm, 128.2 ppm and 114.5 ppm were attributed to C-1', C-2'/C-6' and C-3'/C-5', respectively, and the signal at 162.8 ppm to C-4'.

Yellow powder; UV (acetonitrile / H_2O) λ_{max} 275, 335 nm; ESI-MS (positive ion mode): *m/z* 315 $[M+H]^+$ consistent with a molecular formula $C_{17}H_{14}O_6$.

Pectolinarigenin is a common flavonoid that has been reported from various medicinal plants including *Linaria reflexa* (Cheriet *et al*., 2014) *Circium chanroenicum* (Lim *et al*., 2008), and it has also been reported before from *Kickxia ramosissima* (Singh and Parakash, 1987).

Table 4.6 ^{1}H-NMR and ^{13}C-NMR assignments for pectolinarin (**5**) recorded in $CDCl_3$.

Position	**δ_H (ppm), multiplicity, *J* (Hz)**	**δ_C (ppm)**
1		
2		164.4
3	6.53; s	103.9
4		183.0
5		152.2
6		130.4
7		155.0
8	6.51; s	93.4
9		153.1
10		105.8
1'		123.6
2'	7.77; d; *J*=9.0	128.2
3'	6.97; d; *J*=9.0	114.5
4'		162.8
5'	6.97; d; *J*=9.0	114.5
6'	7.77; d; *J*=9.0	128.2
OMe (C-6)	3.98; s	61.0
OMe (C-4')	3.84; s	55.6

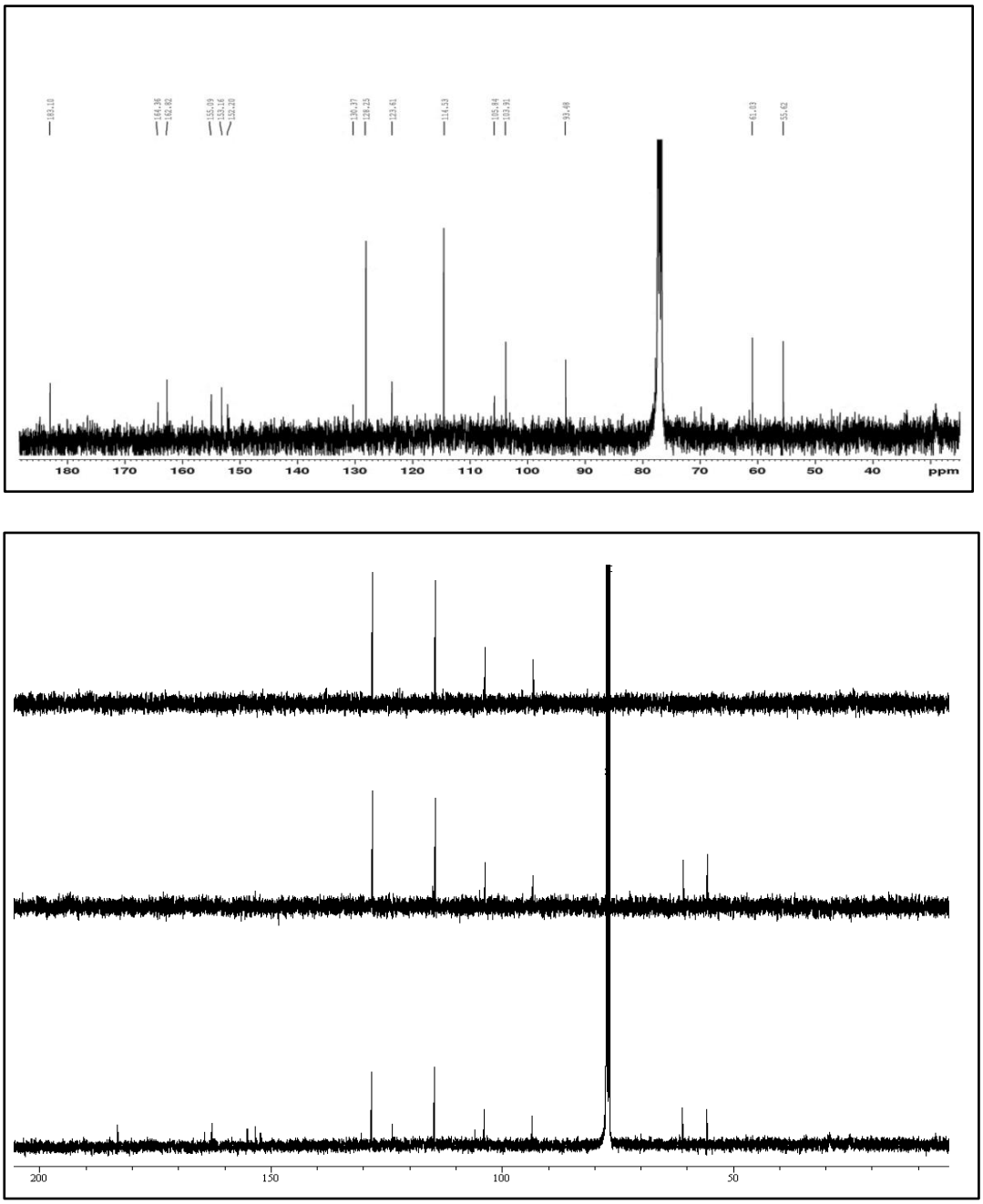


Figure 4.30 ^{13}C-NMR spectra including DEPT-135 and DEPT-90 of pectolinarigenin (**5**) in $CDCl_3$.

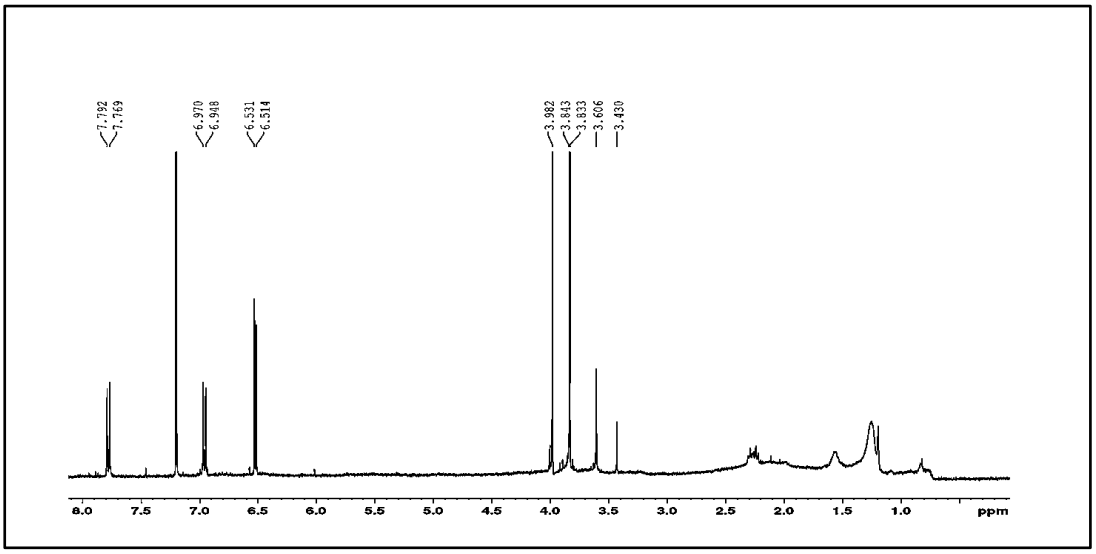


Figure 4.31 ^{1}H-NMR spectrum of pectolinarigenin (**5**) in $CDCl_3$.

4.3.2.2 Pectolinarin (6):

The structure of compound **6** was elucidated by ^{1}H-NMR, ^{13}C-NMR, DEPT-135 and DEPT-90 as pectolinarin (Fig. 4.32).

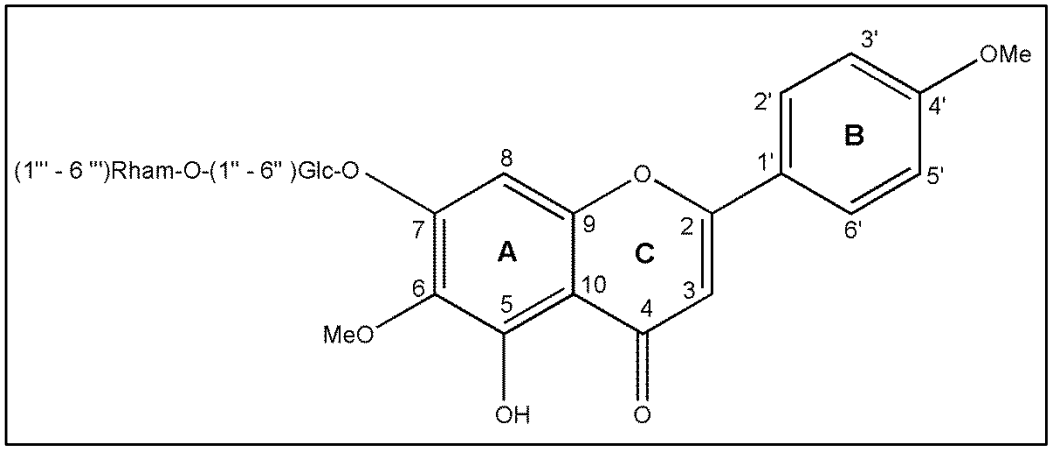


Figure 4.32 Structure of pectolinarin(**6**)

Library search on the ^{13}C-NMR data (Table 4.7 and Fig 4.33) suggested this compound is a flavonoid with a rutinosyl moiety. Its ^{1}H, ^{13}C-NMR and mass spectral data were compared with published data (Lim *et al*., 2008), allowing its complete identification.

The ^{1}H-NMR (Table 4.6 and Fig 4.34) and ^{13}C-NMR (Table 4.6 and Fig 4.33) spectra of compound **9** exhibited resonances due to aromatic ring systems, typical for a flavonoid moiety and sugar residues. In the ^{1}H-NMR spectrum the aromatic region exhibited an AA'XX' system of spins at 8.04 ppm (2H each; d; *J*= 8.9 Hz) attributed to H-2' and H-6', and 7.16 ppm (2H each, d; *J*=8.9 Hz) assigned to H-3'and H-5', respectively, indicating a 4′-oxygenated B ring.

The doublets at 4.56 ppm (*J* = 1.1 Hz, H-1'") and 5.12 ppm (*J* =7.0 Hz, H-1") are typical of anomeric hydrogens. According to the coupling constants, the configurations of the D-glucosyl- and L-rhamnosyl moieties were determined as β (*J*= 7.0 Hz) and α (*J*= 1.1 Hz), respectively. The resonances in the region of 3.12 -3.75 ppm (m, H-2", H-3", H-4", H-5", H-6" and H-2"', H-3"', H-4"', H-5"') can be assigned to the remaining sugar protons. The doublet at 1.06 ppm (3H, *J*=6.0 Hz, H-6"') !s related to the rhamnose methyl group. The singlets at 6.92 ppm and 6.94 ppm were assigned to H-8 and H-3, respectively. Two

methoxyl signals at 3.85 ppm (s) and 3.76 ppm(s) were assigned to C-4' and C-6, respectively.

The ^{13}C-NMR spectrum of compound **6** showed the presence of 29 carbon signals. Nine signals were observed in the range of 66.4 - 76.9 ppm, corresponding to two sugar units i.e glucose and rhamnose. The ^{13}C-NMR signals at 56.0 ppm and 60.8 ppm were assigned to the methoxyl groups at C-4' and C-6, respectively. The methyl group of rhamnose was observed at 18.2 ppm.

The chemical shifts of the carbon signals for D-glucose and L-rhamnose, indicated L-rhamnose as the terminal sugar, linked to C-6" of glucose (66.0 ppm), i.e. rutinose. The DEPT experiment confirmed that this signal was a methylene group.

Yellow powder; . UV (acetonitrile / H_2O) λ_{max} 275, 330 nm; ESI-MS (positive ion mode): *m/z* 623 $[M+Na]^+$ consistent with a molecular formula $C_{29}H_{34}O_{15}$.

Pectolinarine has already been isolated from a number of plants species including *Cirsium japonicum* (Liu *et al*., 2007), *Circium chanroenicum* (Lim *et al*., 2008) and *Kickxia abhaica* (Al-Rehaily *et al*., 2006). It has also been reported from *Kickxia ramosissima* before (Ahmad *et al*., 2006).

Table 4.7 ^{1}H-NMR and ^{13}C-NMR assignments for pectolinarin (**6**) recorded in DMSO-d_6.

Position	δ_H (ppm); multiplicity; *J* (Hz)	δ_C (ppm)
1		
2		164.6
3	6.92; s	103.9
4		182.8
5		152.6
6		133.3
7		157.0
8	6.94; s	94.8
9		152.7
10		106.3
1'		123.2
2'	8.04; d; *J*=8.9	129.0
3'	7.16; d; *J*= 8.9	115.3
4'		163.0
5'	7.16; d; *J*= 8.9	115.2
6'	8.04; d; *J*=8.9	128.9
1"	5.12; d; *J*=7.0	100.9
2"	3.12-3.75; m	73.6
3"	3.12-3.75; m	76.9
4"	3.12-3.75; m	69.9
5"	3.12-3.75; m	76.2
6"	3.12-3.75; m	66.4
1'''	4.56; d;1.1	100.7
2'''	3.12-3.75; m	70.9
3'''	3.12-3.75; m	71.5
4'''	3.12-3.75; m	72.4
5'''	3.12-3.75; m	68.2
6'''	1.06; d; 6.0	18.2
OMe(C-6)	3.76; s	60.8
OMe(C-4')	3.85; s	56.0

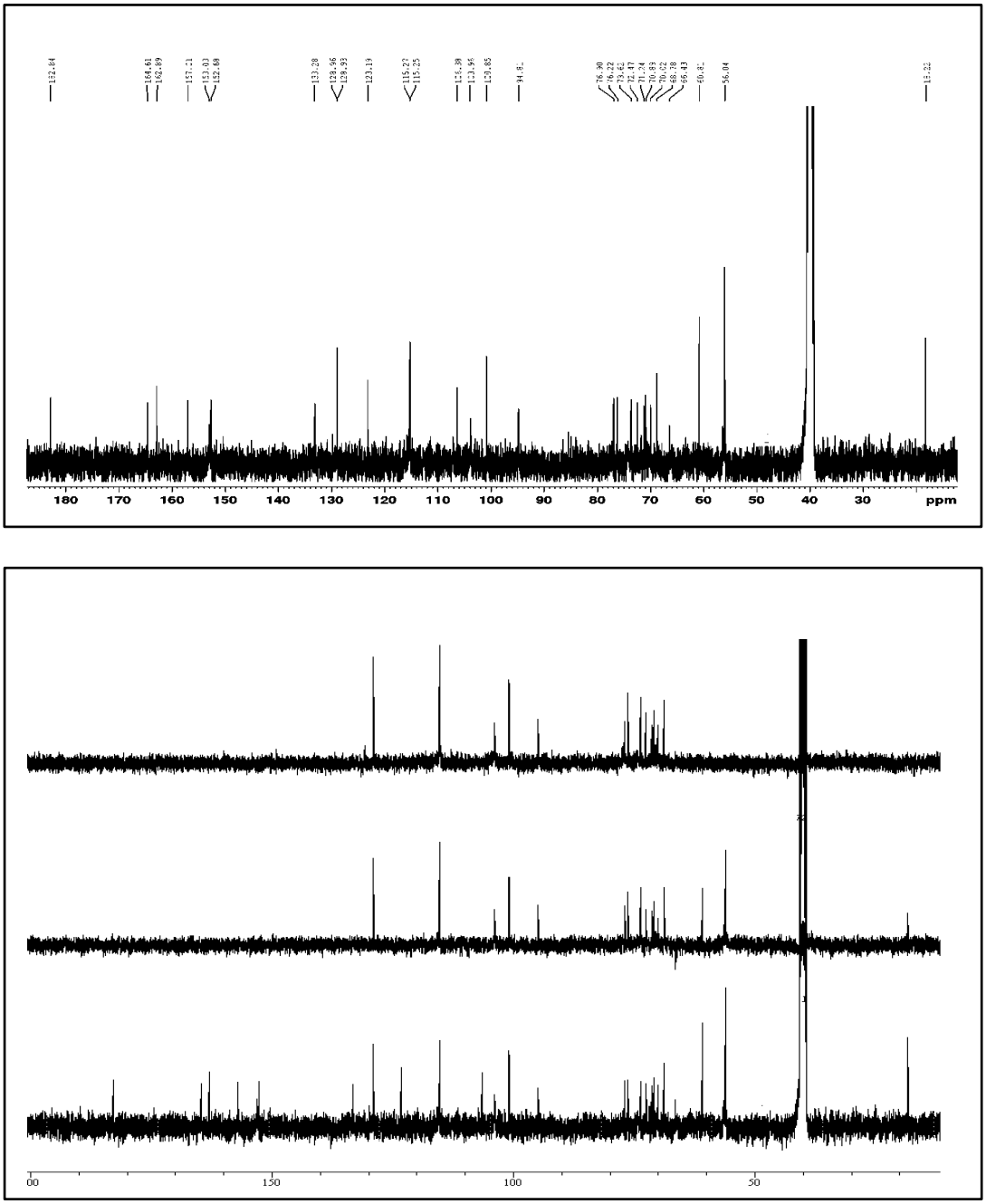


Figure 4.33 ^{13}C-NMR spectra including DEPT-135 and DEPT-90 of pectolinarin (**6**) in DMSO-d_6.

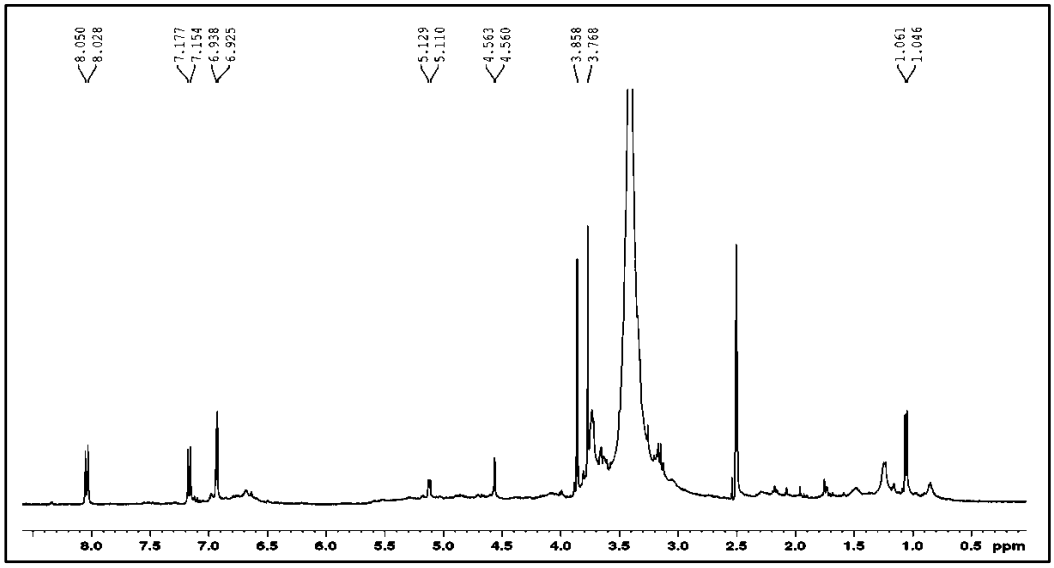


Figure 4.34 ^{1}H-NMR spectrum of pectolinarin (**6**) in DMSO-d_6.

4.3.3 Benzoic acid derivatives

4.3.3.1 4-Hydroxy benzoic acid methyl ester (**7**):

The structure of compound **7** was elucidated by ^{1}H-, ^{13}C-NMR, DEPT-135 and DEPT-90 as 4-hydroxybenzoic acid methyl ester (**7**) (Fig 4.35).

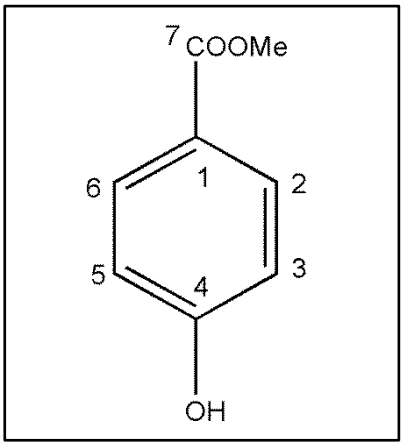


Figure 4.35. 4-hydroxy benzoic acid methyl ester (**7**)

Library search on the ^{13}C-NMR data (Table 4.8 and Fig 4.36) suggested this compound is a benzoic acid derivative. Based on its ^{1}H, ^{13}C-NMR and mass spectral data compound 7 was found to be 4-hydroxy benzoic acid methyl ester (Yoshioka *et al*., 2004).

The ^{1}H-NMR (Table 4.8 and Fig 4.37) and ^{13}C-NMR (Table 4.8 and Fig 4.36) spectra of compound **7** are indicative of an aromatic ring system. The ^{1}H-NMR spectrum revealed a doublet at 7.88 ppm (*J*=8.9 Hz) that were assigned to H-2 and H-6, respectively. Similarly a doublet observed at 6.84 ppm (*J*= 8.9) was assigned to H-3 and H-5. Finally a methoxyl signal was observed at 3.92 ppm (s).

The ^{13}C-NMR spectrum indicated the presence of 8 carbons. The carbonyl group of the ester moiety presented a resonance at 168.6 ppm. The signal at 121.8 ppm and 132.5 ppm were assigned to C-1 and C-2/C-6, whereas the signals 116.0 and 163.6 ppm were attributed to C-3/C-5 and C-4, respectively. The methoxyl group appeared at 52.0 ppm.

Yellow powder; UV (acetonitrile / H_2O) λ_{max} 226, 287, 338 nm; ESI-MS (positive ion mode): *m/z* 153 $[M+H]^+$ consistent with a molecular formula $C_8H_8O_3$.

4-Hydroxy-benzoic acid methyl ester has been found in many medicinal plant species for instance *Vitex rotundifolia* (Yoshioka *et al*., 2004) and *Houttuynia cordata* (Jong and Jean, 1993). This is, however, the first report of its presence in *Kickxia ramosissima*.

Table 4.8 ^{1}H-NMR and ^{13}C-NMR assignments for 4- hydroxy benzoic acid methyl ester (7) recorded in methanol-d_4.

Position	δ_H (ppm); multiplicity; *J* (Hz)	δ_C (ppm)
1		121.8
2	7.88; d; *J*= 8.9	132.5
3	6.84; d; *J* = 8.9	116.0
4		163.6
5	6.84; d; *J*= 8.9	116.0
6	7.88; d; *J*= 8.9	132.5
7		168.6
OMe	3.86; s	52.0

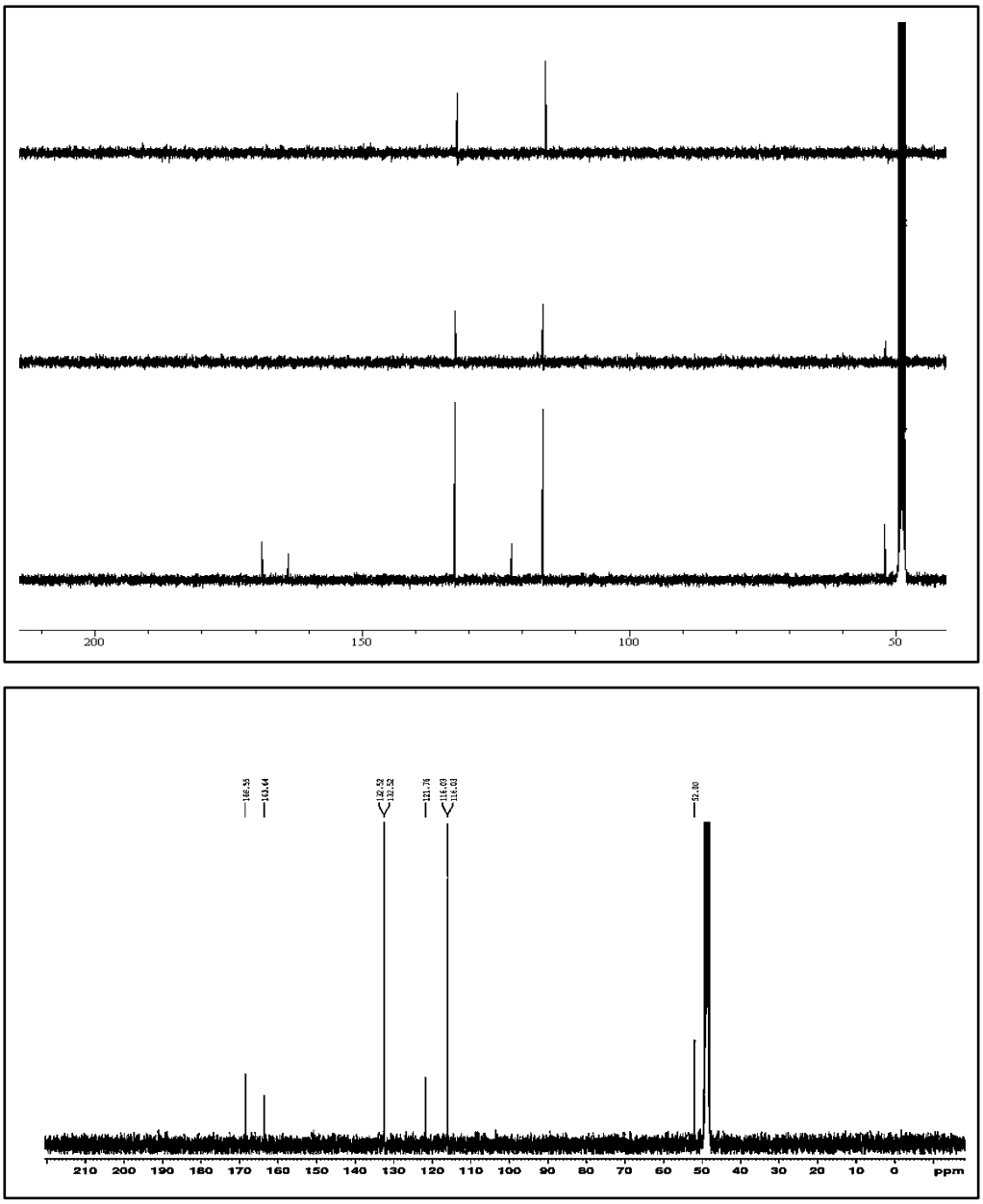


Figure 4.36 ^{13}C-NMR spectra including DEPT-135 and DEPT-90 of 4-hydroxy-benzoic acid methyl ester (**7**) in methanol-d_4.

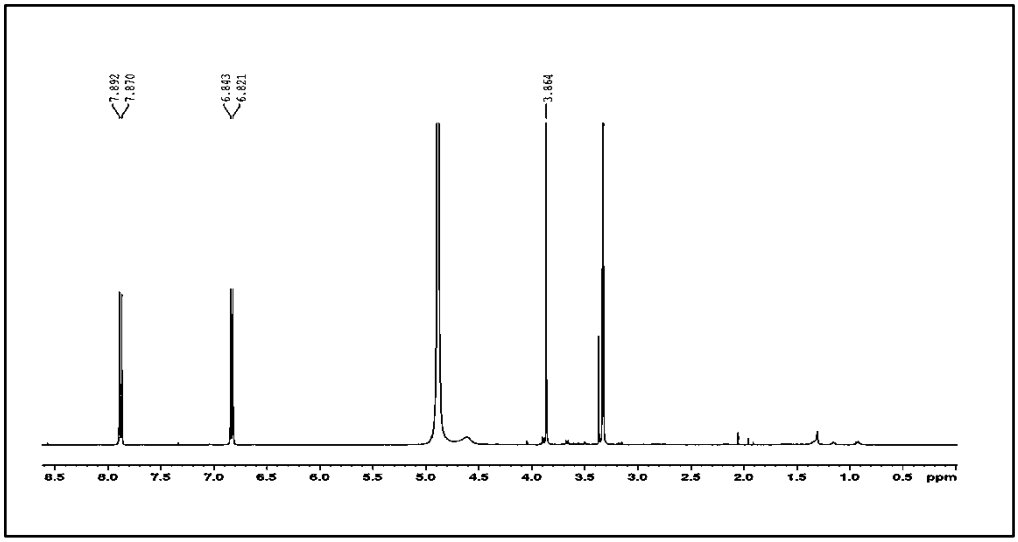


Figure 4.37 ^{1}H-NMR spectrum of 4-hydroxy-benzoic acid methyl ester (**7**) in methanol-d_4.

4.4. BIOLOGICAL ACTIVITIES

4.4.1 Inhibition of AGEs formation by crude extracts

The different fractions were tested for potential antiglycation activity (Table 4.9). The ethyl acetate fraction was highly active (IC_{50} 88 μg/mL), whereas a moderate antiglycation activity was observed for the *n*-butanol (36% inhibition at 100 μg/mL) and methanol (32% inhibition) fractions. The chloroform fraction presented a mild inhibition of protein glycation (20% inhibition).

Table 4.9. Anti-glycation effects of *K. ramosissima* fractions.

Sample	Antiglycation	
	% Inhibition[a]	IC_{50} (μg/mL)
Total Extract	5	-
MeOH 90%	32	-
Chloroform	20	-
Ethyl acetate	64	88
n-Butanol	36	-
Aminoguanidine		19.9

[a] at 100 μg/mL,

4.4.2 Inhibition of AGEs formation by isolated constituents

In the BSA-glucose assay the prevention of AGE formation was investigated by determining the fluorescence of the complex formed after incubation of a sugar (glucose) and protein (BSA) in the presence or absence of test compounds. In the case of iridoids (compounds **1**-**4**) only mild inhibition of protein glycation was noticed (Table 4.8). In particular compound **2** was most active (35% inhibition at the highest test concentration) followed by compound **3** (28% inhibition) and compound **1** (26% inhibition). Compound **4** did not present any activity. A nearly similar moderate trend was seen in the BSA-MGO assay. There has only been one previous report on weak inhibition of protein glycation by iridoids (West *et al*., 2014).

Contrary to iridoids, the isolated flavonoids compound **5** (IC_{50} 0.79 mM) and **6** (IC_{50} 2.29 mM) were more active. The fact that the glycosylated compound **6** was less active than the aglycone **5** was indicative of the fact that glycosylation of flavonoids mainly decreases the activity (Matsuda *et al*., 2003). This inhibition was mainly due to the non-oxidative mode of inhibition as obvious in the BAS- MGO assay, since the IC_{50} values for compounds **5** (IC_{50} 0.19 mM) and **6** (IC_{50} 0.13 mM) were lower than in the glucose-BSA assay. Finally the benzoic acid derivative **7** did not present any inhibition in both models.

Table 4.10. Antiglycation (AGEs) potential of isolated compounds from *Kickxia ramossisima*

Compound	Protein glycation			
	BSA-Glucose		BSA-MGO	
	% inhibition[a]	IC_{50} mM	% inhibition[b]	IC_{50} mM
1	26		31	
2	35		23	
3	28		24	
4	no activity		9	
5		0.79		0.19
6		2.29		0.13
7	no activity		no activity	
Aminoguanidine		1.75		0.15
Quercetin		0.23		-

[a] at 3 mM final concentration
[b] at 1.3 mM final concentration

4.4.3 Inhibition of α-glucosidase by isolated constituents

In a sequel of testing the isolated compounds for their antidiabetic potential, the assay on inhibition of α-glucosidase was performed. These agents are able to prevent the fast breakdown of sugars by competitively inhibiting α-glycosidase activity and thus controlling the blood sugar levels. This category of oral hypoglycaemic agents is important in cases of post-prandial blood glucose elevation in diabetic patients. There are quite a few commercially available α-glucosidase inhibitors, but gastrointestinal side effect in patients mainly limit their use. It was therefore considered important to test the isolated compounds for α-glucosidase inhibition.

It was noticed that iridoids presented only mild levels of inhibition (Table 4.9). The highest inhibition (29% inhibition at the highest test concentration) was seen for compound **4** followed by compound **2** (15% inhibition) and compound **3** (9% inhibition). The newly isolated compound **1** did not present any activity. In general a number of reports suggested the potential of iridoids as α-glucosidase inhibitors, but mainly moderate inhibition was noticed (Hua *et al*., 2014; Lin *et al*., 2015). Keeping in mind the results of this investigation and the literature, we can conclude that as *K. ramosissima* contains a number of iridoids, the traditional claims about antidiabetic potential could be due in part to the combined effect of all iridoids of the plant.

The flavonoids however presented better activity compared to tested iridoids. Compound **5** showed the highest activity (IC_{50} 0.23 mM), whereas its glycosylated counterpart compound **6** presented moderate α-glucosidase inhibition (48% inhibition at the highest test concentration). The flavonoids are generally known for their multiple therapeutic effects including α-glucosidase inhibitory activity (Tadera *et al*., 2006; Hong *et al*., 2013). Also based on our findings, we could conclude that these flavonoids may contribute to the traditional use of *K. ramosissima* in diabetic conditions.

Finally moderate α-glucosidase inhibition (47% inhibition) was observed for the benzoic acid derivative **7.** In conclusion we could emphasise that the combined effect of all isolated compounds i.e iridoids, flavonoids and the benzoic acid derivative are contributing towards the α-glucosidase inhibitory potential of *Kickxia ramosissima*.

Table 4.11 α-Glucosidase inhibitory effect of isolated compounds from *Kickxia ramosissima*.

Compound	% inhibition [a]	IC_{50} (mM)
1	no activity	-
2	15	-
3	9	-
4	29	-
5	-	0.23
6	48	-
7	47	-
Acarbose		0.26

[a] at 2 mM final concentration

4.4.4 Inhibition of 15-lipoxygenase by isolated constituents

During the current investigation some interesting results were observed for the iridoids as well as the flavonoids. The highest inhibition was seen for compound **2** (IC_{50} 0.22 mM) followed by compound **3** (37% inhibition at the highest test concentration) and **1** (30% inhibition) (Table 4.12). Likewise in the case of flavonoids compound **6** (IC_{50} 0.25 mM) presented the highest inhibition, whereas only a moderate activity (46% inhibition) was seen for compound **5.** Finally the benzoic acid derivative **7** presented only a moderate level of inhibition (30% inhibition). Based on these findings we could confirm the possible role of iridoids and flavonoids as anti-inflammatory agents in *Kickxia ramosissima*.

4.4.5 Inhibition of linoleic acid lipid peroxidation - AAPH Assay

During our investigation the isolated iridoids (**1-4**) were evaluated for inhibition of AAPH induced linoleic acid peroxidation. All iridoids exhibited weak or no activity (Table 4.10) compared to standard inhibitor Trolox (Fig 4.36 and Fig 4.37). Our findings were in agreement with earlier reports covering mussaenoside and mussaenosidic acid that also reported week inhibition (Gousiadou *et al*., 2013). Also for the flavonoids (**5-6**) a similar trend was observed as previously reported for a different type of flavonoids (Peyrat- Maillard *et al*., 2003). Finally compound **7** did not present any activity. In conclusion we could say that the isolated compounds presented a weak inhibition of linoleic acid peroxidation that could play a limited role in inhibition of lipid peroxidation.

Table 4.12. 15-lipoxygenase inhibition and inhibition of linoleic acid (AAPH assay) by isolated compounds from *Kickxia ramosissima*.

Compound	**15-LOX assay**		**AAPH assay**
	% inhibition[a]	IC_{50} (mM)	AOP (min/μM)[c]
1	30	-	0.3
2	-	0.22	0.2
3	37	-	0.1
4	8	-	Not tested
5	46	-	0.2
6	-	0.25	0.3
7	30	-	Not active
Quercetin	-	0.14	-
Trolox	-	-	14.12[d]

[a] at 0.312 mM final concentration
[b] at 0.06 mM final concentration
[c] at 100 μM
[d] at 8 μM

AOP antioxidant power

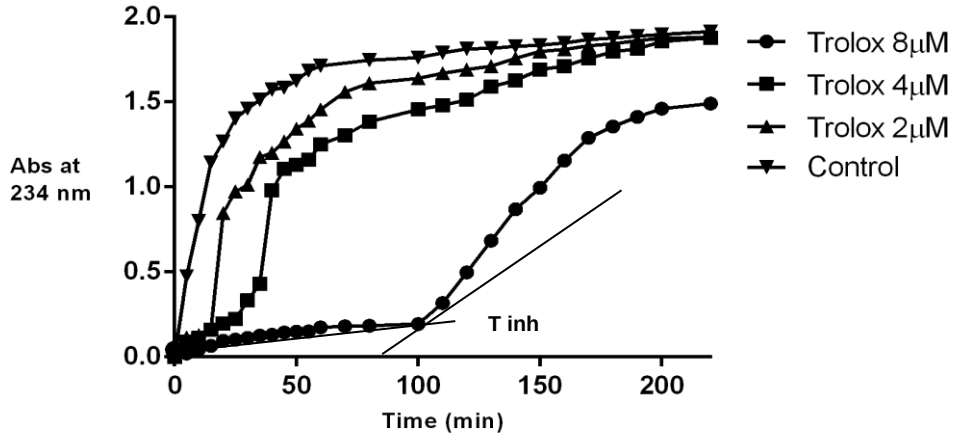


Figure 4.38 Effect of Trolox on AAPH induced linoleic acid oxidation

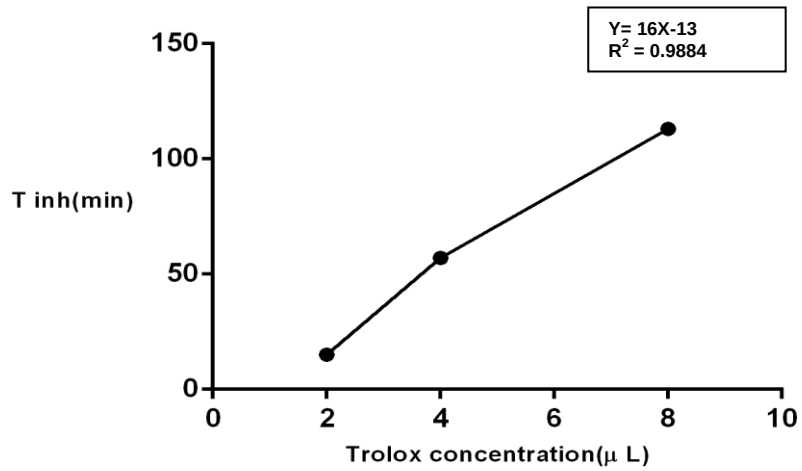


Figure 4.39 Time of inhibition(T_{inh}) as a measure of time.

4.5. Antimicrobial activity

4.5.1 Antimicrobial activity of fractions

The crude extracts were evaluated for antibacterial and antifungal activities. The highest levels of antimicrobial and antifungal activities were observed for n the *n*-hexane fraction, i.e. an IC_{50} of 8.0 µg/mL against *S. aureus* and 24.4 µg/mL against *M. canis*. None of the other fractions were found active in the tested concentration range (Table 4.13).

Table 4.13 Antibacterial, antifungal and cytotoxic activities of *K. ramosissima* fractions.

Sample	IC_{50} µg/mL				
	S. aureus	*E. coli*	*C. albicans*	*M. canis*	MRC-5
MeOH 90%	>64	>64	>64	>64	>64
Chloroform	>64	>64	>64	>64	>64
Ethyl acetate	>64	>64	>64	>64	>64
n-Hexane	8.0	>64	>64	24.4	>64
n-Butanol	>64	>64	>64	>64	>64
Aqueous	>64	>64	>64	>64	>64

Reference: Tamoxifen(MRC-5) IC_{50} 11.4 µg/mL; erythromycin *(S.aureus)*IC_{50} 11.2 µg/mL, Trimethoprim (*E.coli*) IC_{50} 0.25 µg/mL; Miconazole(*C. albicans*) IC_{50} 5.99 µg/mL; Terbinafin (*M.canis*) IC_{50} 0.11 µg/mL.

MRC-5: Human fetal lung fibroblasts; *S. aureus: Staphylococcus aureus; E. coli: Escherichia coli; C. albicans: Candida albicans.*

4.5.2 Antimicrobial activity of isolated constituents

Compounds **2**–**7** were evaluated in an integrated screening panel for antimicrobial activity. Compound **1** was not tested due to the limited amount available. None of the isolated iridoids including the chlorinated iridoid (**4**) presented antimicrobial activity in the test range. Although few iridoids are well characterized for their antimicrobial activities (Ishiguro *et al*., 1983; Davini *et al*., 1986), such activity is surely related to certain structural variations. A number of investigations have reported antimicrobial activity of iridoid rich fractions of medicinal plants, for instance *Morinda citrifolia* (noni) fruits (West *et al*., 2012). As stated before for *Nymphoides indica*, iridoids can be considered as prodrugs that may be deglycosylated and activated after oral administration.

The flavonoids, however, are well known for their antimicrobial activities (Xie *et al*., 2015; Mohanty *et al*., 2015, da Rocha *et al*., 2015). Also during the current investigation, pectolinarigenin (**5**) was found active against *Staphylococcus aureus* (IC_{50} 49.8 µM). This compound also showed weak antiprotozoal activities against *Plasmodium falciparum* K1 (IC_{50} 41.8 µM) and *Trypanosoma cruzi* (IC_{50} 32.0 µM), accompanied however by PMM cytotoxicity. Moreover it was not cytotoxic against MRC-5 cells (Table 4.11). However, the glucoside of compound **5**, i.e pectolinarine (**6**) did not present any activity in the antimicrobial screening panel.

Table 4.14. Antimicrobial, antifungal cytotoxic and antiprotozoal activities of isolated compounds from *Kickxia ramosissima* (IC_{50}, µM)

Compound	**IC_{50} (µM)**									
	MRC5	*Pf-K1*	*T. bruc*	*T. cruz*	*L. inf*	*PMM*	*S. aureus*	*E. coli*	*C. albicans*	*A. fumigatus*
1	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT
2	> 64	> 64	> 64	> 64	> 64	> 64	> 64	> 64	> 64	> 64
3	> 64	> 64	> 64	> 64	> 64	> 64	> 64	> 64	> 64	> 64
4	> 64	> 64	> 64	> 64	> 64	> 64	> 64	> 64	> 64	> 64
5	> 64	**41.8**	> 64	**32.0**	> 64	> 64	**49.8**	> 64	> 64	> 64
6	> 64	> 64	> 64	> 64	> 64	> 64	> 64	> 64	> 64	> 64
7	> 64	> 64	> 64	> 64	> 64	> 64	> 64	> 64	> 64	> 64

Reference: Tamoxifen (MRC-5), IC_{50} 10.48 µM; Suramine (*T. bruc*), IC_{50} 0.03 µM, Chloroquine (*Pf-K1*), IC_{50} 0.08 µM, Miltefosine (*L. inf*), IC_{50} 9.02 µM; Benznidazol (*T.cruz*), IC_{50} 2.13 µM; Erythromycin (*S. aureus*) IC_{50} 11.30 µM; Chloramphenicol (*E.coli*), IC_{50} 2.42 µM; Miconazole (*C. albicans*), IC_{50} 4.70 µM; Terbinafine (*A. fumigatus*), IC_{50} 1.38 µM.

MRC-5: human fetal lung fibroblasts;, Pf-K1; *Plasmodium falciparum*; *T.bruc*: *Trypanosoma brucei; T. cruz*: *Trypanosoma cruzi; L.inf: Leishmania infantum*; PMM: peritoneal murine macrophages; *S.aureus: Staphylococcus aureus; E.coli: Escherichia coli; C. albicans: Candida albicans; A. fumigatus: Aspergillus fumigatus*

4.6. SUMMARY AND CONCLUSION

Kickxia ramosissima (Wall.) Janch is a well-known medicinal plant in Pakistan that is traditionally used to treat diabetes mellitus (DM). Indeed quite a few studies have been carried out and some flavonoids, iridoids and sugars were isolated, but the detailed phytochemical and biological analysis of isolated constituents still needed to be performed. Therefore we evaluated various fractions of whole plant material and a series of isolated constituents. The compounds were further analysed for antidiabetic properties (inhibition of α-glucosidase activity; inhibition of Advanced Glycation Endproducts-AGEs), antioxidant activity (AAPH induced linoleic acid peroxidation), inhibition of 15-LOX (anti-inflammatory activity) and antimicrobial activities, as part of running programmes in the host laboratory. Phytochemical analysis of the extracts and fractions led to isolation and identification of **7** compounds, including iridoids (**1-4**) with a new compound, kickxiasine (**1**), mussaenosidic acid (**2**), mussaenoside (**3**), linarioside (**4**); the flavonoids, pectolinarigenin (**5**) and pectolinarin (**6**); and the benzoic acid derivative 4-hydroxy-benzoic acid methyl ester (**7**) respectively.

In the antidiabetic assays, the ethyl acetate fraction presented highest activity (IC_{50} 88.0 µg/mL), whereas all other fractions were only moderately active. The isolated iridoids (**1-4**) presented week inhibition in the BSA-glucose assay, and comparable results in the BSA-MGO assay. The flavonoids, however, showed interesting results as pectolinarigenin (**5**) was highly active (IC_{50} 0.79 mM) followed by pectolinarigenin (**6**) with IC_{50} 2.29 mM. Also in the BSA-MGO assay both flavonoids show high levels of inhibition i.e pectolinarigenin (**5**) IC_{50} 0.19 mM and pectolinarigenin (**6**) IC_{50} 0.13 mM. This could clearly indicate the inhibition through non-oxidative pathways. Compound 7 was inactive in all AGEs experiments. In the α-glucosidase inhibition assay, moderate inhibition was recorded for the iridoids. However, with regard to the flavonoids, pectolinarigenin showed interesting activity (IC_{50} 0.23 mM), followed by pectolinarin (**6**) which showed moderate inhibition (48% inhibition at a concentration of 2 mM). Finally the benzoic acid derivative (**7**) presented moderate inhibition (47% at a concentration of 2 mM).

In the 15-LOX experiment, moderate inhibition was recorded for most compounds, and pectolinarigenin (**5**) showed the highest inhibition (46% inhibition at a concentration of 0.312 mM) followed by mussaenoside (**3**) (37% inhibition) and compound **1** (30% inhibition). Compounds **2** and **6** were found inactive. In the AAPH assay, weak or no inhibition was recorded for all compounds. Finally in the antimicrobial screening panel among the fractions, only the *n*-hexane fraction presented moderate activity against *Staphylococcus aureus* (IC_{50} 8.0 µg/mL), *Micrococcus canis* (IC_{50} 24.0 µg/mL). With regard to the isolated constituents, only pectolinarigenin (**5**) showed moderate activity against *Plasmodium falciparum* K1 (IC_{50} 41.8 µM), *Staphylococcus aureus* (IC_{50} 49.8 µM) and *Trypanosoma cruzi* (IC_{50} 30.0 µM) with no cytotoxicity. Based on our findings we could demonstrate the moderate antidiabetic, anti-inflammatory and antimicrobial activity of some constituents of *Kickxia ramosissima*.

REFERENCES

Ahmad M, Qureshi R, Arshad M, Khan MA, Zafar M. 2009. Traditional Herbal remedies used for the treatment of diabetes from District Attock. *Pak J Bot* **41**: 2777-2782

Ahmad VA, Kousar F, Zubair M, Khan A, Ali MS, Choudhary MI, Sener B. 2006. A new iridoid glycoside from *Linaria genestifolia*. *Fitoterapia* **77:** 12–14.

Al-Rehaily A J, Abdel-Kader MS., Ahmad MS, Mossa JS. 2006. Iridoid glucosides from *Kickxia abhaica* D.A. Sutton from Scrophulariaceae. *Phytochemistry* 67: 429–432.

Awale S, Kawakami T, Tezuka Y., Ueda JY., Tanaka K., Kadota S. 2005. Nitric Oxide (NO) Production Inhibitory Constituents of *Tabebuia avellanedae* from Brazil. *Chem Pharm Bull* **53**: 710-713.

Bole PV, Pathak JM. 1988. Flora of Saurashtra. The Director Botanical Survey of India, New Delhi: Deep Printer;

Cheriet T, Aouabdia S, Mancini I, Defant A, Seghiri R, Boumaza O, Mekkiou R, Sarri D, Leon F, Brouard I, Benayache F, Benayache S. 2014. Chemical constituents of *Linaria reflexa* Desf. (Scrophulariaceae). *Der Pharmacia Lettere* **6**: 54-57.

Cornicelli JA, Trivedi BK. 1999. 15-Lipoxygenase and its inhibition: A novel therapeutic target for vascular disease. *Curr Pharm Des* **5:** 11-20.

da Rocha CQ, Queiroz EF, Meira CS, Moreira DR, Soares MB, Marcourt L, Vilegas W, Wolfender JL. 2014. Dimeric flavonoids from *Arrabidaea brachypoda* and assessment of their anti-*Trypanosoma cruzi* activity. *J Nat Prod* **77**: 1345-50.

Demuth H, Jensen SR, Nielsen BJ. 1989. Iridoid glucosides from *Asystasia bella. Phytochemistry* **28**: 3361-3364.

Davini E, Javarone C, Trogolo C, Aureli P, Pasolini.1986. The quantitative isolation and antimicrobial activity of the aglycone of aucubin. *Phytochemistry* **25**: 2420–2422

Dinda B, Debnath S, Majumder S, Arima S, Sato N, Harigaya Y. 2005. chemical contstituetnts of Morinda incana. *Ind J Chem* **44:** 2362-2366.

Egwaikhide PA, Gimba CE. 2007. Analysis of the phytochemical content and antimicrobial activity of Plectranthus glandulosis whole part. Middl East J Sci Res 2: 135–138.

Ersoz T, Yalcini FN, Tasdemir D, Sticher O, Calis I. 1998. Iridoid and Lignan Glucosides from *Bellardia trixago* (L.) All. *Tr J of Med Sci* **28:** 397-400.

Farnsworth NR.1966. Biological and phytochemical screening of plants. *J Pharm Sci* **55**: 225–276.

Ferhat M, Harkat H, Lavaud C, Haba Hamada H, Long C, Benkhaled M. 2010. Iridoids and flavonoid from *Linaria aegyptiaca* (L.) Dum. subsp. *fruticosa*. *Biochem Sys Ecol* **38:** 833–835.

Gousiadou C, Gotfredsen CH, Matsa M, Hadjipavlou-Litina D, Skaltsa H. 2013. Minor iridoids from *Scutellaria albida* ssp. *albid*a. Inhibitory potencies on lipoxygenase, linoleic acid lipid peroxidation and antioxidant activity of iridoids from *Scutellaria* sp. *J Enzyme Inhib Med Chem* **28**: 704–710.

Grantstrom E. 1984. The arachidonic acid cascade: the prostaglandins, thromboxanes and leukotrienes. *Inflammation* **8s**: 15–25.

Guangzhou Li, Dumaa M, Xiang PU, Jinhai Y, Guolin G, Yinggang LOU. 2012. Chemical Components of Aerial Parts of *Lagochilus ilicifolius*. *Chin J App Environ Biol* **18:** 924-927 doi: 10.3724/SP.J.1145.2012.00924

Handjieva N, Tersieva L, Popov S, Evstatieva L. 1995. Two iridoid 5-O-menthia foloyl kickxioside and kickxin, from *Kickxia Dum*. species. *Phytochemistry* 39: 925–927.

Herborne JB. 1973. Phytochemical Methods 3rd Edn. Chapman and Hall Ltd., London, pp: 135-203.

Hong HC, Li SL, Zhang XQ, Ye WC, Zhang QW. 2013. Flavonoids with α-glucosidase inhibitory activities and their contents in the leaves of *Morus atropurpurea*. *Chin Med* **8**: 19 doi: 10.1186/1749-8546-8-19

Hua J, Qi J, Yu BY. 2014. Iridoid and phenylpropanoid glycosides from *Scrophularia ningpoensis* Hemsl. and their α-glucosidase inhibitory activities. *Fitoterapia* **93:** 67–73.

Ilieva E, Handjieva N, Popov S. 1992. Genistifolin and other Iridoid Glucosides from *Linaria genistifolia* (L.) Mill. *Z Naturforsch* **47c**: 791-793.

Ishiguro K, Yamaki, Takagi S.1983. Studies on Iridoid-Related Compounds, II. The structure and antimicrobial activity of aglucones of Galioside and Gardenoside. *J Nat Prod* **46:** 532–536

Jain A, Katewa SS, Galave P, Nag A. 2008. Some therapeutic uses of biodiversity among tribes of Rajhistan. *Ind J Trad Med* **7:** 256-62.

Jong TT, Jean MY. 1993. Constituents of *Houttuyniae Cordata* and the Crystal Structure of Vomifoliol. *J Chin Chem Soc* **40:** 399–402.

Kapoor SK, Reisch J. 1974. Iridoids of *Cymbralaria Muralis*. *Phytochemistry* **13:** 1018-1019.

Kaneko T, Sakamoto M, Ohtani K, Ito A, Kasai R, Yamasaki K, Padorina WG. 1995. Secoiridoid and flavonoid glycosides from *Gonocaryum calleryanum. Phytochemistry* **39:** 115–120

Khan IZ, Aqil M. 1993. Isolation and identification of Pectolinarine and Mannitol from *Kickxia ramosissima*(wall). *Chem Environ Res* **2:** 287-289.

Khan M, Hussain F, Musharaf S. 2011. Preliminary floristic range of Tehsil Takht-e-Nasrati Pakistan. *Int J Bio sci* **1:** 88-99, 2011.

Khan IZ, Aqil M, Kolo BG. 2001. A new flavone glycoside from *Kickxia ramosissima* (Wall). *Ultra-Physical Sci* **13:** 112–115.

Kirtikar KR, Basu BD. Indian medicinal Plants. 2nd ed. Dehra Dunb: International book distributors; 2005.

Kitagawa I, Tani T, Akita K, Yosioka I. 1973. On the constituents of *Linaria Japonica Miq*. I. the structure of Linarioside, a new chlorinated Iridoid glucoside and identification of two related glucosides. *Chem Pharm Bull* **21:** 1978-1987.

Liegeois C, Lermusieau G, Collin S. 2000. Measuring antioxidant efficiency of wort, malt, and hops against the 2, 2'-azobis (2- amidinopropane) dihydrochloride-induced oxidation of an aqueous dispersion of linoleic acid. *J Agric Food Chem* **48:**1129–1134.

Lim H, Son KH, Chang HW, Bae K, Kang SS, Kim HP. 2008. Anti-inflammatory Activity of Pectolinarigenin and Pectolinarin Isolated from *Cirsium chanroenicum. Biol Pharm Bull* **31:** 2063-2067.

Lin Y, Xu W, Huang M, Xu W, Li H, Ye M, Zhang X, Chu K.2015. Qualitative and Quantitative Analysis of Phenolic Acids, Flavonoids and Iridoid Glycosides in Yinhua Kanggan Tablet by UPLC-QqQ-MS/MS. *Molecules* **20**: 12209-12228.

Liu YF, Liang D, Luo H, Hao ZY, Wang Y, Zhang CL, Ni G, Chen RY, Yu DQ. 2014. Ionone glycosides from the roots of *Rehmannia glutinosa*. *J Asian Nat Prod Res* **16**:11-9.
doi: 10.1080/10286020.2013.875011

Liu S, Zhang J, Li D, Liu W, Luo X, Zhang R, Li L, Zhao J. 2007. Anticancer activity and quantitative analysis of flavone of *Cirsium japonicum* DC. *Nat Prod Res* **21:** 915-22.

Lyckander IM, Malterud KE. 1996. Lipophilic flavonoids from *Orthosiphon spicatus* inhibit oxidative inactivation of 15-lipoxygenase. *Prostagl Leukotr Essent Fatty Acids* **54:** 239-246.

Mabberley AJ. 1997. The Plant-book. Cambridge University Press, Cambridge.

Malterud KE, Rydland KM. 2000. Inhibitors of 15-lipoxygenase from orange peel. *J Agric Food Chem* **48:** 5576–5580.

Mathur AK, Ganapathy PS, Johri, BM. 1980. Isolation of sodium chloride-tolerant plantlets of Kickxia ramosissima under in vitro conditions. *Zeitschrift fuer Pflanzenphysiologie* **99:** 287-94.

Mohanty S, Maurya AK, Jyotshna, Saxena A, Shanker K, Pal A, Bawankule DU. 2015. Flavonoids rich fraction of *Citrus limetta* fruit peels reduces pro-inflammatory cytokine production and attenuates malaria pathogenesis. *Curr Pharm Biotechnol* **16:** 544-52.

Morota T, Sasaki H, Sugama KG, Nishimura H, Chen M, Mitsuhashi H. 1990. Two non glycosidic Iridoids from *Rehmani glutinosa*. . Phytochemistry **29**: 523-526.

Morteza-Semnani K, Saeedi M, Akbarzadeh M. 2008. The Essential Oil Composition of *Kickxia spuria* (L.) Dum .*J Essen Oil Res* **20:** 24-25.

Muller K. 1994.5-Lipoxygenase and 12-lipoxygenase: attractive targets for the development of novel antipsoriatic drugs. *Arch Pharm* (Weinheim) **327**:3–19.

Otsuka H. 1993. Iridoid glucosides from *Linaria Japonica. Phytochemistry* 33: 617-622.

Pandya PN, Aghera HB, Ashok BK. 2012. Diuretic activity of *Linaria ramosissima* (Wall.) Janch leaves in albino rats. *J AYU* **33:**576-578

Park KS, Kim BH, Chang IM. 2010. Inhibitory Potencies of Several Iridoids on Cyclooxygenase-1, Cyclooxygnase-2 Enzymes Activities, Tumor Necrosis factor-a and Nitric Oxide Production In Vitro. *Eid Based Complement Alternat Med*. **7:** 41-45.

Patel BI, Sachdeva PD. 2014. Antidiabetic activity of *linaria ramosissima* (wall) Janch in Streptozotocin induced Diabetic Rats. *Int J Pharm Pharm Sci* **6:** 166-171.

Plants J store, 2016. (http://plants.jstor.org/stable/10.5555/al.ap.flora.ftea009671; data accessed on 2/06/2016).

Qureshi R, Bhatti GR. 2008. Ethnobotany of plants used by the Thari people of Nara Desert, Pakistan. *Fitoterapia* **79**: 468–473

Qureshi R, Bhatti GR, Memon RA. 2010. Ethanomedicinal uses of Herbs from Northern Part of Nara desert, Pakistan. *Pak J Bot* **42:** 839-851.

Rajic Z, Hadjipavlou-Litina D, Pontiki E, Kralj M, Suman L, Zorc B. 2010. The novel ketoprofen amides–synthesis and biological evaluation as antioxidants, lipoxygenase inhibitors and cytostatic agents. *Chem Biol Drug Des* **75:** 641–652.

Senguttuvan J, Paulsamy, S., Karthika K. 2014. Phytochemical analysis and evaluation of leaf and root parts of the medicinal herb, *Hypochaeris radicata* L. for in vitro antioxidant activities. *Asian Pac J Trop Biomed* **4**(Suppl 1)**:** 359-367.

Singh M, Prakash L. 1987. A new flavone glycoside and other chemical constituents from *Kickxia ramosissima* Wall. (Scrophulariaceae). *Pharmazie* **42:** 490-491.

Sehgal CK, Taneja SC, Dhar KL, Atal CK. 1982. '2'-p-hydroxybenzoyl mussaenosidic acid a new iridoid glucoside from *Vitex negundo*. *Phytochemistry* **21:**363–366.

Sofowora A. 1993. Medicinal plants and traditional medicine in Africa. John Wiley and son Ltd., 150-153.

Steinberg D. 1999. At last, direct evidence that lipoxygenases play a role in atherogenesis. *J Clin Invest* **103:** 1487-1488.

Tadera K, Minami Y, Takamatsu K, Matsauka T. 2016. Inhibition of α-glucosidase and α-amylase by flavonoids. *J Nutri Sci Vitaminaol* **52**: 149-153.

The plant list, 2016. http://www.theplantlist.org/tpl1.1/record/kew-2382576; data accessed on 2/06/2016).

Trease GE, Evans WC. 1989. Pharmacognosy.11th Edn. Brailliar Tiridel and Macmillian Publishers, London.

Vardhana R. 2008. Direct Uses Of Medicinal Plants And Their Identification 1st edition. Sarup & Sons; New Delhi, India. pp198.

Venditti A, Frezza C, Serafini M, Bianco A.2016. Iridoids and phenylethanoid from *Pedicularis kerneri* Dalla Torre growing in Dolomites, Italy. *Nat Prod Res* **30**: 327-31. doi: 10.1080/14786419.2015.1060230.

West BJ, Palmer SK, Deng S, Pal AK. 2012.Antimicrobial Activity of an Iridoid Rich Extract from *Morinda citrifolia* Fruit. *Curr Res J Biol Sci* **4**: 52-54.

West BJ, Uwaya, Isami, Deng, Nakajima, Jensen CJ. 2014. Antiglycation Activity of Iridoids and Their Food Sources. *Int J Food Sci* Article ID 276950. 8 pages doi.org/10.1155/2014/276950.

Xie Y, Yang W, Tang F, Chen X, Ren L.2015. Antibacterial activities of flavonoids: structure-activity relationship and mechanism. *Curr Med Chem* **22:**132-49.

Yoshiokaa T, Inokuchib T, Fujiokac S, Kimurab Y. 2004. Phenolic Compounds and Flavonoids as Plant Growth Regulators from Fruit and Leaf of *Vitex rotundifolia. Z Naturforsch* **59c**: 509-514.

Yuldashev MP, Batirov EK, Malikov VM. 1996. Flavonoids from aerial parts of *Kickxia elatine*. *Khim Prir Soedin* **1**: 38–41.

CHAPTER 5

PHYTOCHEMICAL AND BIOLOGICAL INVESTIGATIONS ON *FERULA NARTHEX* EXUDATE

Submitted in part for publication in Food Chemistry:

Terpenyl Coumarins from *Ferula narthex* exudate with antiglycation and antimicrobial properties. Adnan Amin, Emmy Tuenter, Paul Cos, Louis Maes, Vassiliki Exarchou, Sandra Apers and Luc Pieters.

5.1 INTRODUCTION

The genus *Ferula* (Apiaceae) is quite diverse and includes around 180 species worldwide. It is commonly found at high altitudes in central Asia and the Middle East (Pimenov and Leonov, 2004). *Ferula narthex* Boiss. is a perennial herb growing at high altitudes (Fig. 5.1). In general its milky exudate is used as spice and folk medicine. Just before flowering in March/ April cuts are made in the upper part of the rhizome root and the milk is collected and dried. The genus *Ferula* is distributed throughout the world, especially in Afghanistan, Iran, India and Pakistan. (Indrayan, 2009). In Pakistan *F. narthex* has been reported mainly from Gilgit, Chitral, Kashmir and Balochistan (Shinwari and Gilani, 2003; Khan *et al*., 2011; Kakari *et al*., 2013, Alam *et al*., 2016). In the Indian subcontinent several species, such as *Ferula gummosa*, *Ferula asafoetida, Ferula narthex*, *Ferula costata* and *Ferula latisecta* have been reported (Kapoor, 1990; Eigner and Scholz, 1999; Khan *et al*., 2011; Kakari *et al*., 2013).

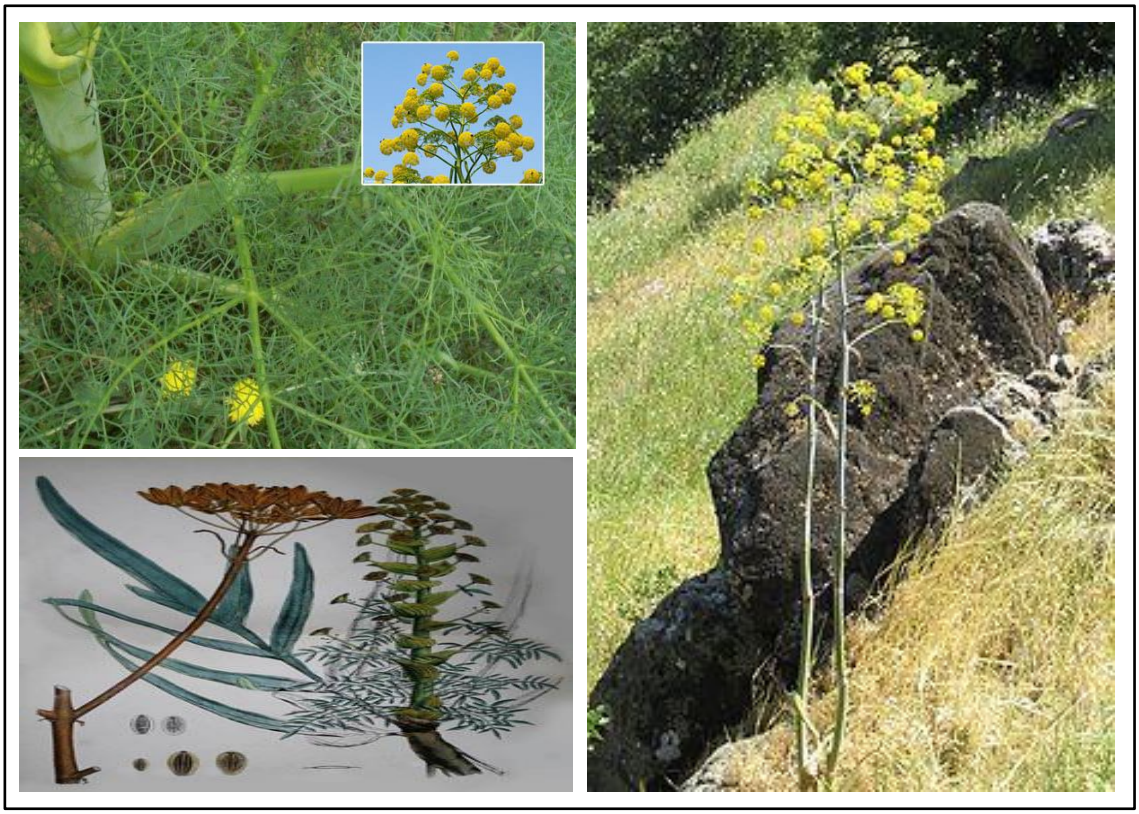


Figure 5.1 *Ferula narthex* in its natural habitat

Ferula narthex Boiss. is commonly called “Heeng”, “Hilteet” or “Raw”. It is used for treatment of a number of ailments including cough, asthma, toothache, gastric problems and also against constipation and angina pectoris (Shinwari and Gilani, 2003, Khan *et al*., 2011, Srinivasan, 2005, Mahendra and Bisht, 2012). Most recently extracts of *Ferula narthex* have been reported as analgesic (Bashir *et al*., 2013), insecticidal (Bashir *et al*., 2013), antimicrobial (Bashir *et al*., 2014a) and antidiabetic (Iranshahy and Iranshahi, 2011). It is also an important constituent of some traditional formulations (Achliya *et al*., 2004)

Indeed the genus *Ferula* is mainly known for the occurrence of coumarins, sesquiterpenes and sesquiterpene coumarins (Fig. 5.2) (Iranshahi *et al*., 2010a; Appendino *et al*., 1994; Bandyopadhyay *et al*., 2006,). Compounds isolated from members of the genus *Ferula* have been reported to possess a wide array of activities including antileishmanial, anti-inflammatory, cytotoxic, anticancer, antibacterial and antiviral (Lee *et al*., 2009; Nazari and Iranshahi, 2011; Bashir *et al*., 2014b). The extensive traditional usage of the exudate of *Ferula narthex* provoked us to investigate into detail its phytochemical and biological properties.

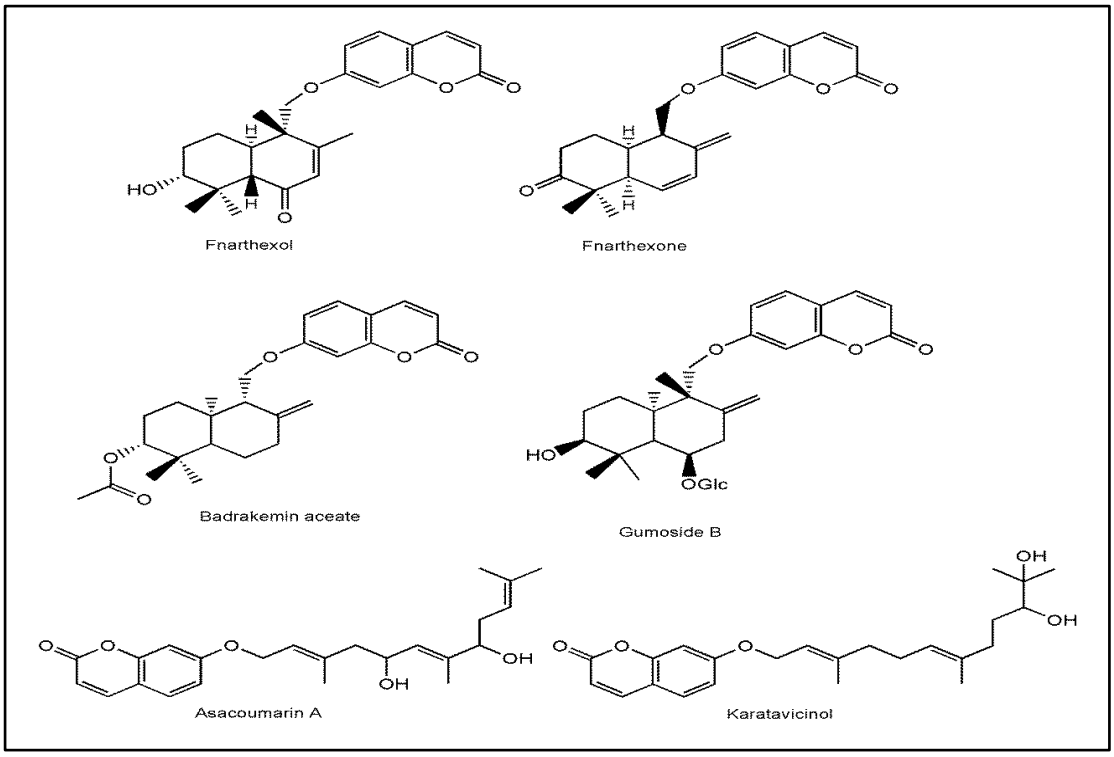


Figure 5.2 Compounds isolated from *Ferula* sp.

5.2 EXTRACTION AND ISOLATION

5.2.1 Plant material

Ferula narthex exudate was collected in September 2012 from the district Chitral (KPK), Pakistan. The samples were identified at the Islamabad Herbarium in the Taxonomy Department, Quaid-I-Azam University, Islamabad, Pakistan, where the voucher specimen was deposited (voucher No. 568 BC, accession no. ISL-72568). The semisolid exudate was stored below 20 °C till further use (Fig. 5.3).

Figure 5.3 *Ferula narthex* dried exudate.

5.2.2. Extraction and fractionation

Dried plant exudate (1 kg) was extracted with 90% (v/v) methanol by double cold maceration. The extract was filtered through Whatman No.1 filter paper using a vacuum pump. The collected filtrate was dried using a rotary evaporator under reduced pressure below 40 °C. The resultant semisolid material was lyophilised with a final yield of 87.1 g, and stored below 20 °C. The liquid–liquid partitioning was performed on the crude extract according to a standard extraction scheme (see chapter 2). After partitioning with different solvents as shown in the scheme, *n*-hexane (1.21g), methanol 90% (25.0 g), chloroform (1.96 g), ethyl acetate (0.2 g), *n*-butanol (4.87 g) and aqueous fractions (53.86 g) were obtained. All collected fractions were dried under reduced pressure at 40 °C, lyophilized and stored below 20 °C.

5.2.3 Phytochemical analysis

Phytochemical screening of extracts and fractions for various compound classes was carried out using published methods (Sofowora, 1993, Trease and Evans, 1989, Harborne, 1973; Egwaikhide and Gimba, 2007; Farnsworth, 1966) with slight modifications for sterols, triterpenes, carbohydrates and saponins flavonoids, alkaloids, and tannins (chapter 2). Phytochemical screening of extracts and fractions indicated that terpenes, alkaloids, tannins and saponins were present at different concentrations depending on their polarity (Table 5.1).

Table 5.1 Phytochemical analysis of *Ferula narthex* extracts and fractions

Extract / fraction	**Phytochemicals**				
	Alkaloids	Flavonoids	Tannins	Steroids/ triterpenoids	Saponins
MeOH 90%	+	++	-	+++	-
Chloroform	++	++	-	+++	-
Ethyl acetate	+	++	-	+	-
***n*-Hexane**	-	-	-	-	-
***n*-Butanol**	+	+++	+	++	-
Aqueous	-	-	+++	-	-

profoundly present: high degree of precipitation (dark coloration), moderately present: less degree of precipitation (medium colouration), slightly present: very low precipitation (very little colouration), absent: no change in colour (Senguttuvan et al., 2014)

Phytochemicals: ++++ profoundly present; ++ moderately present; + slightly present; - absent.

5.2.4. Thin layer chromatography

The NP (normal phase) TLC for all obtained fractions was executed using various solvent systems as mobile phase. The solvent systems used included CH_2Cl_2 / MeOH (65:35) with a few drops of NH_4OH for the methanolic fraction; CH_2Cl_2 / MeOH (72:28) for the chloroform fraction; *n*-hexane / CH_2Cl_2 (93:7) for the *n*-hexane fraction; CH_2Cl_2 / MeOH (40:60) for the *n*-butanol fraction and CH_2Cl_2 / MeOH (15:85) for the aqueous fraction. Developed TLC plates were examined under UV at 254 nm and 366 nm and after spraying with *p*-anisaldehyde reagent. For instance, TLCs for the methanolic and ethyl acetate fractions are shown in Fig 5.4.

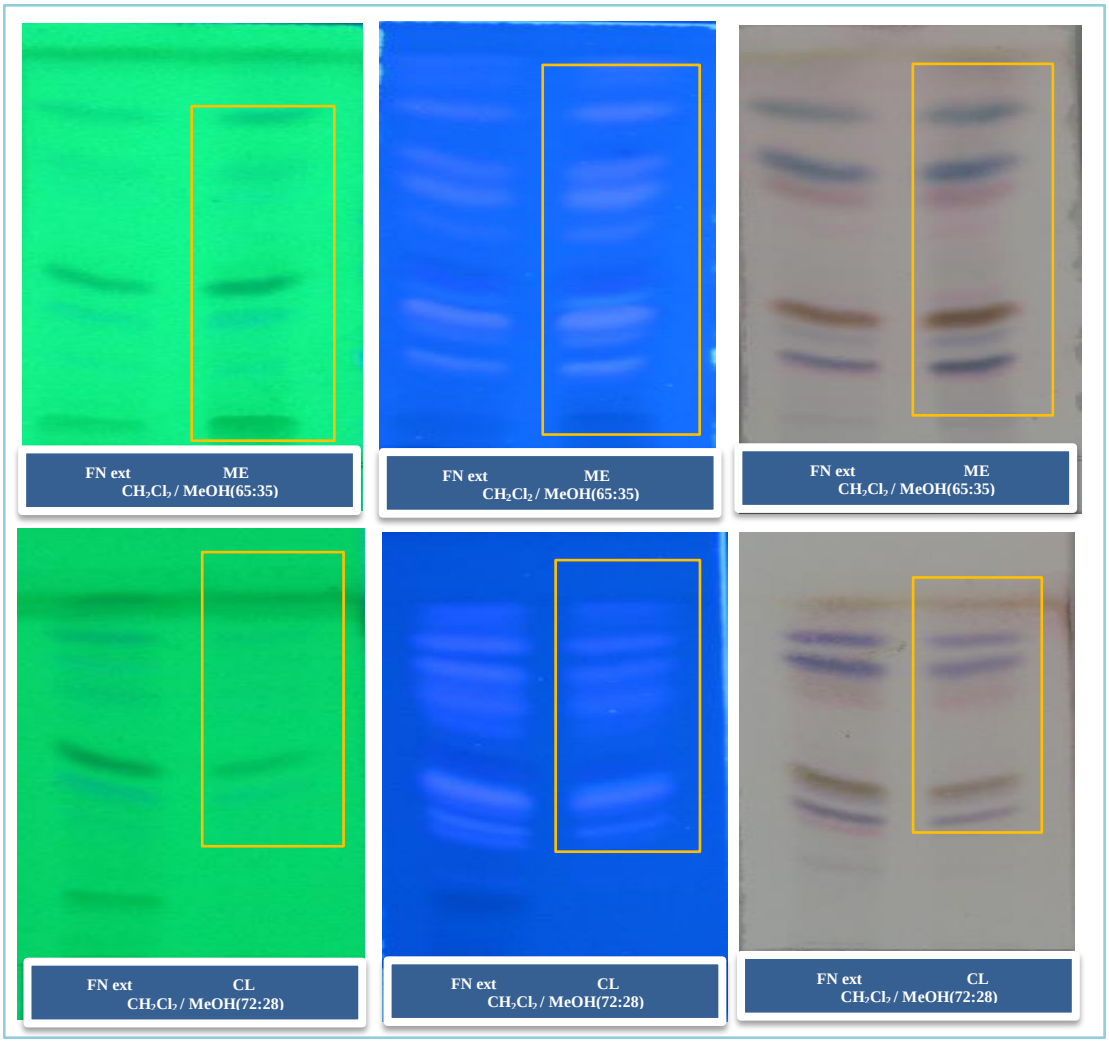


Figure 5.4. TLC profile of total extract (FN ext), methanolic (ME) and chloroform(CL) fractions on silica TLC (NP) plates at 254 and 366 nm after spraying with *p*-anisaldehyde reagent.

5.2.5. Flash chromatography

An aliquot of 0.8 g from the methanol 90% fraction was dissolved in 2 mL methanol and mixed with 1.1g silica; the mixture was dried with nitrogen gas. The dried extract was loaded on a prepacked Flash Grace Reveleris® silica cartridge of 80 g. The compounds were eluted using a gradient from methylene chloride to methanol. Based on UV and ELSD detection, multiple subfractions were collected (Fig. 5.5). All fractions were further analysed by TLC and similar fractions were combined. In this way 16 subfractions were obtained. Flash chromatography with similar conditions was repeated when necessary. Finally, based on TLC profiling, subfractions **FME1** (200 mg), **FME2** (150 mg), **FME4** (220 mg) and **FME5** (210 mg) were selected for further HPLC profiling.

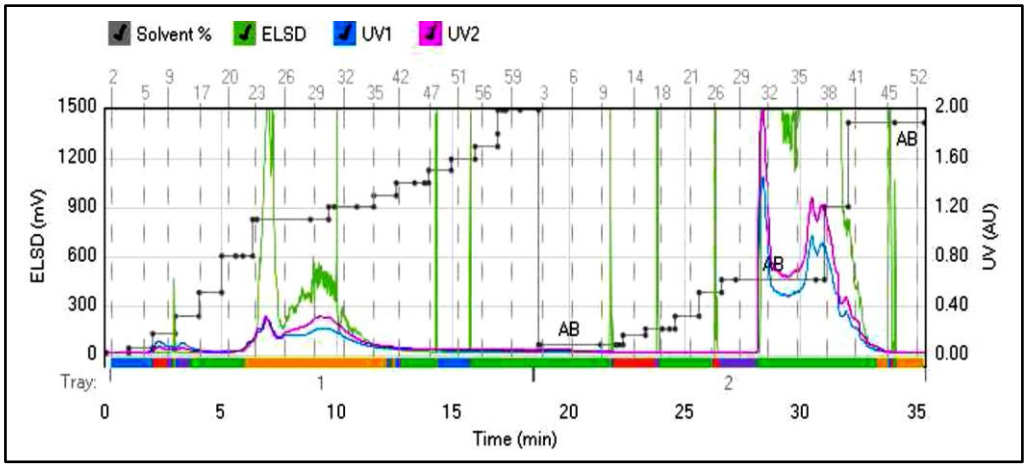


Figure 5.5 Flash chromatogram of the methanolic fraction.

Likewise an aliquot of 0.8 g from the chloroform fraction was loaded on a flash column as discussed above. The compounds were eluted using a gradient from methylene chloride to methanol. Based on UV and ELSD detection different subfractions were collected (Fig 5.6). All fractions were analysed by TLC and similar fractions were combined as described above; in this way 5 subfractions were obtained. In general the TLC of collected fractions revealed similarity with the methanolic fraction as obvious from Fig 5.4. Based on TLC profiling subfractions **FCL1** (120 mg), **FCL2** (140 mg), **FCL3** (180 mg), **FCL4** (100 mg) and **FCL5** (140 mg) were selected further HPLC analysis.

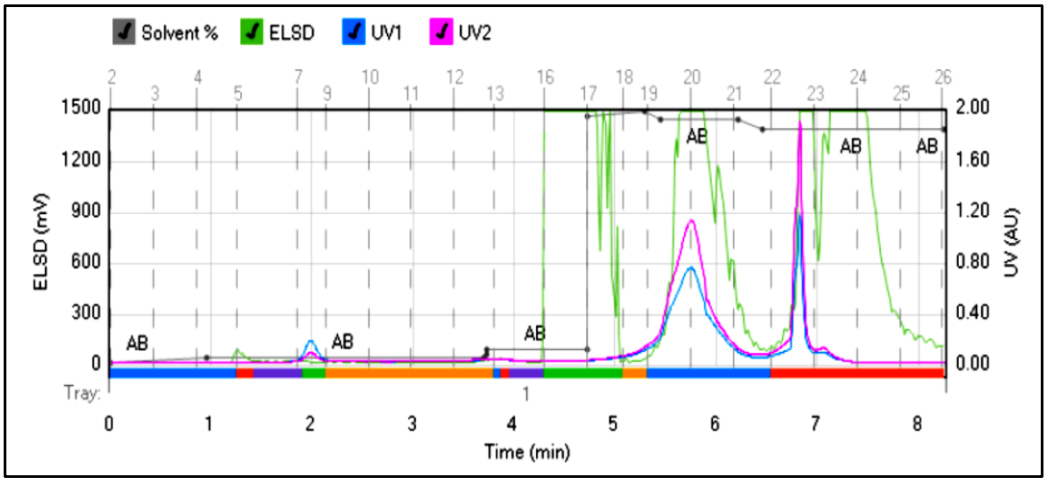


Figure 5.6 Flash chromatogram of the chloroform fraction.

Similarly an aliquot of 0.8 g from the *n*-butanol fraction was subjected to flash chromatography with a gradient from methylene chloride to methanol as previously explained. Finally 10 subfractions were obtained (Fig 5.7). As noticed for the chloroform fraction, the TLC profiling of the *n*-butanol fraction shared some common bands with the methanolic and chloroform fractions. Based on TLC analysis, subfractions **FB2** (140 mg), **FB4** (125 mg), **FB5** (154 mg), **FB6** (200 mg) and **FB7** (116 mg) were selected for HPLC analysis.

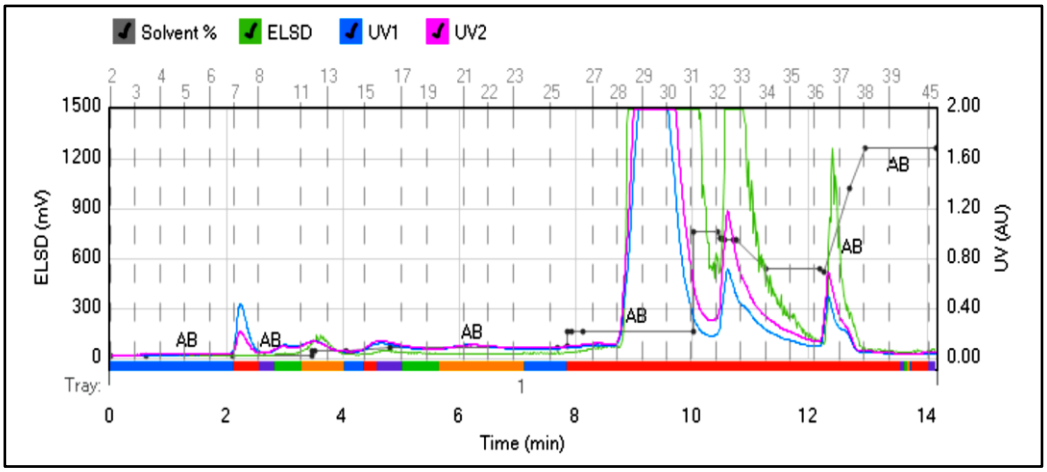


Figure 5.7 Flash chromatogram of the *n*-butanol fraction.

5.2.6. HPLC analysis and isolation of compounds

The methanolic fraction and all obtained subfractions **FME1**, **FME2**, **FME4** and **FME5** were analysed by HPLC using an optimized methanol/H_2O + 0.1% formic acid gradient, ranging from 20% acetonitrile to 80% in 60 min at a flow rate of 1 mL/min. Samples were prepared in a concentration range from 1-10 mg / mL in methanol (Fig 5.8). The isolation of pure compounds was performed by semi-preparative HPLC-DAD-MS using the same gradient at 3 mL/min, yielding compounds **1** (5.5 mg)**, 2** (4.6 mg) and **3** (6.2 mg)**.**

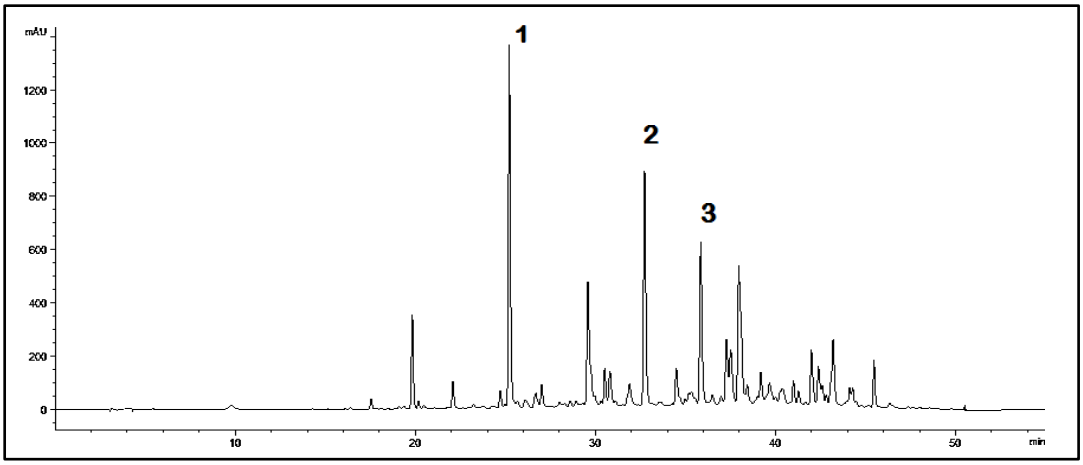


Figure 5.8 HPLC chromatogram of the chloroform fraction **FME2** at 254 nm.

Similarly the crude chloroform fraction and subfractions **FCL1**, **FCL2, FCL3, FCL4** and **FCL5** were analysed by HPLC using an optimized acetonitrile / H_2O + 0.1% formic acid gradient ranging from 35% acetonitrile to 80% in 45 min at a flow rate of 1 mL/min (Fig. 5.9). The compounds were isolated by semi-preparative HPLC-DAD-MS using the same gradient at a flow rate of 3 mL/min. Finally compounds **2** (2.5 mg), **3** (4.2 mg) and **5** (4.3 mg) were isolated.

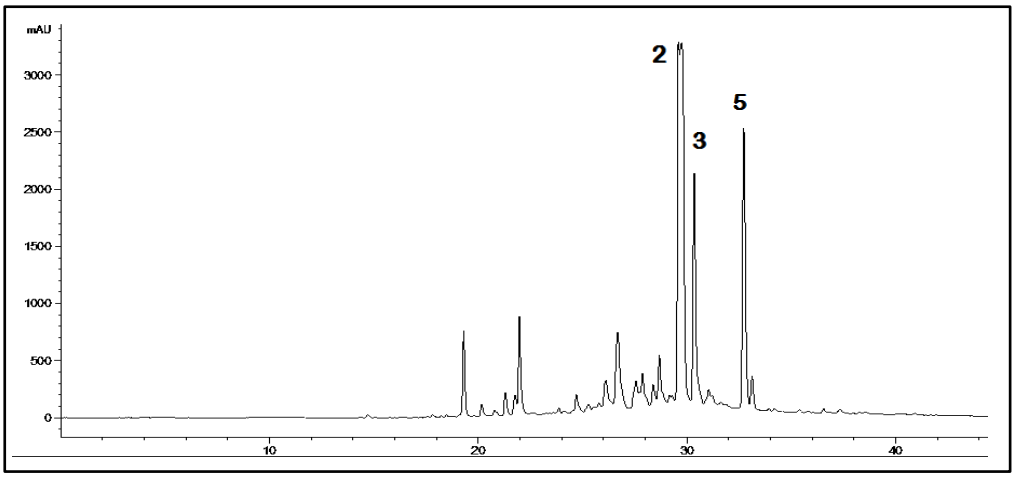


Figure 5.9 HPLC chromatogram of the methanol fraction **FCL 5** at 254 nm

The crude *n*-butanol fraction and subfractions **FB2**, **FB4, FB5, FB6** and **FB7** were analysed by HPLC using an optimized acetonitrile / H_2O + 0.1% formic acid gradient ranging from 40% acetonitrile to 65% in 50 min at a flow rate of 1 mL/min (Fig. 5.10). Compound isolation was performed by semi-preparative HPLC-DAD-MS using the same solvent gradient as HPLC at a flow rate of 3 mL/min. Compounds **2** (3.6 mg), **5** (4.2 mg) and **6** (4.8 mg) were finally isolated.

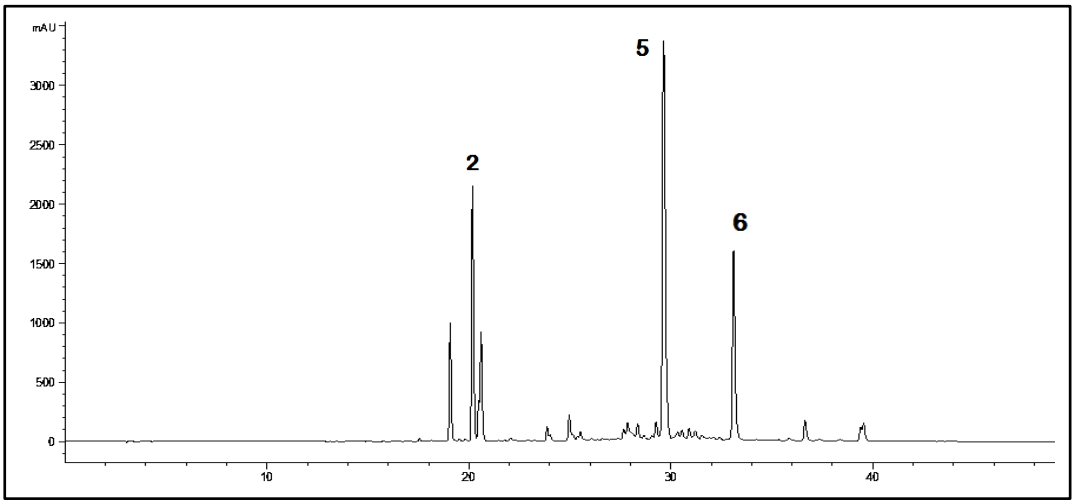


Figure 5.10 HPLC chromatogram of the *n*-butanol fraction **FB4** at 254 nm

5.3. STRUCTURE ELUCIDATION

Structures of the isolated compounds from *Ferula narthex* were elucidated using ^{1}H- and ^{13}C- NMR (including DEPT-135 and DEPT-90) as well as 2D-NMR (COSY, HSQC and HMBC) spectroscopy. The molecular ion was derived from the mass spectra obtained with the semi-preparative HPLC-DAD-MS system.

5.3.1 Fnarthexol (1)

The structure of compound **1** was elucidated by ^{1}H-NMR, ^{13}C-NMR (DEPT-135 and DEPT-90) and 2D-NMR (COSY and HSQC) as fnarthexol (Fig. 5.10).

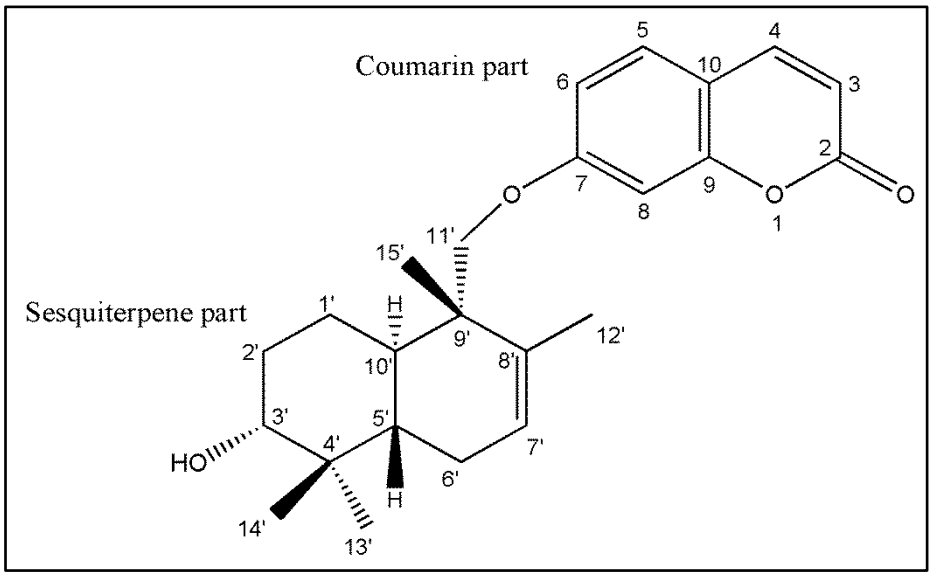


Figure 5.10 Structure of fnarthexol (**1**)

Library search on the ^{13}C-NMR data (Table 5.2 and Fig. 5.11) using the NMR Predict database and ^{1}H-NMR (Table 4.2 and Fig. 5.12) suggested that this compound was a sesquiterpene-coumarin. The spectral data were compared with NMR data from literature (Bashir *et al.*, 2014$_b$), which indicated that compound **1** was fnarthexol. Analysis of the COSY (Fig. 5.13) and HSQC (Fig. 5.14) correlations confirmed the structure as shown.

In ^{13}C-NMR (Table 5.2, Fig. 5.14) the sesquiterpene ring carbons C-1' and C-2' showed resonance signals at 24.3 ppm and 28.0 ppm, respectively. The signals at 79.6 ppm (indicative of oxygen substitution), 39.9 ppm and 50.9 ppm were assignable to C-3', C-4' and C-5', respectively. Similarly C-6' was attributed to 39.0 ppm. In the same ring C-7', C-8' and C-9' were assigned to the signals at 124.5 ppm, 133.9 ppm and 37.0 ppm respectively. The resonance at C-10 was attributed to the signal at 55.4 ppm whereas the peak at 68.2 ppm was assignable to C-11', i.e. a methylene group substituted by oxygen. Four methyl signal appearing at 21.8 ppm, 15.3 ppm, 28.7 ppm and 15.9 ppm were assigned to C-12', C-13', C-14'and C-15', respectively.

In the coumarin ring the carbonyl group (C-2) appeared at 163.4 ppm. The resonance signals at 113.6 ppm and 145.9 ppm were assigned to the double bond carbons C-3 and C-4. Similarly the CH-groups C-5, 6 and C-8 were attributed to the peaks at 130.5 ppm, 114.4 ppm and 102.2 ppm, respectively. Finally the signals at 163.7 ppm, 157.2 ppm and 114.1 ppm were assigned to the quaternary carbons C-7, C-9 and C-10, respectively.

In the ^{1}H-NMR spectrum (Fig 4.15 and Fig 4.16) of the sesquiterpene part, methylene signals at 2.07 ppm (m) and 1.63 / 0.92 ppm (m) and 2.0 ppm (m) were assigned to H-1', H-2' and H-6', respectively. An oxygenated methylene signal at 3.70 ppm (d, J=8.5 Hz) and 3.99 ppm (d, J= 8.5 Hz) was assigned to H-11'. The oxygenated methane signal observed at 3.22 ppm (dd) was assignable to H-3'. A multiplet appearing at 1.98 ppm was assigned to H-5', whereas the signal at 2.20 ppm was assigned to H-10'. A broad singlet appearing at 5.53 ppm was assigned to the olefinic proton H-7'. Finally four methyl signals at 1.69 ppm, 0.87 ppm, 0.98 ppm and 0.93 ppm were assigned to H-12', H-13', H-14' and H-15', respectively.

In the coumarin ring protons H-3 and H-4 (i.e. the double bond) corresponded to signals at 6.23 ppm and 7.90 ppm, respectively, whereas protons H-5 and H-6 resonated 4.55 ppm (d) and 6.95 ppm (dd), respectively. Finally a broad singlet appearing at 6.93 ppm was assigned to H-8.

Yellow powder; UV (acetonitrile / H_2O) λ_{max} 243, 325 nm; ESI-MS (positive ion mode): *m/z* 405 $[M+Na]^+$ consistent with a molecular formula $C_{24}H_{30}O_4$.

Fnarthexol has only been isolated recently from *Ferula narthex* (Bashir *et al*., 2014_b).

Table 5.2 ^{1}H and ^{13}C NMR assignments for compound (**1**) recorded in MeOH-d_4

Position	δ_H (ppm); multiplicity; *J* (Hz)	δ_C (ppm)
1		
2		163.4
3	6.23; d; *J*= 9.5	113.6
4	7.90; d; *J*= 9.5	145.9
5	7.54; d; *J*= 8.5	130.5
6	6.93; br d; *J*= 8.5	114.4
7		163.7
8	6.93; br s	102.2
9		157.2
10		114.1
1'	2.07; m	24.3
2'	1.63; 0.92; m	28.0
3'	3.23; dd; *J*=10.9; 4.5	79.6
4'		39.9
5'	1.26; m	50.9
6'	2.0; m	39.0
7'	5.53,br s	124.5
8'		133.9
9'		37.0
10'	2.20; br s	55.4
11'	3.70; d; *J*= 8.5 3.99; d; *J*= 8.5	68.2
12'	1.69; s	21.8
13'	0.87; s	15.3
14'	0.98; s	28.7
15'	0.93; s	15.9

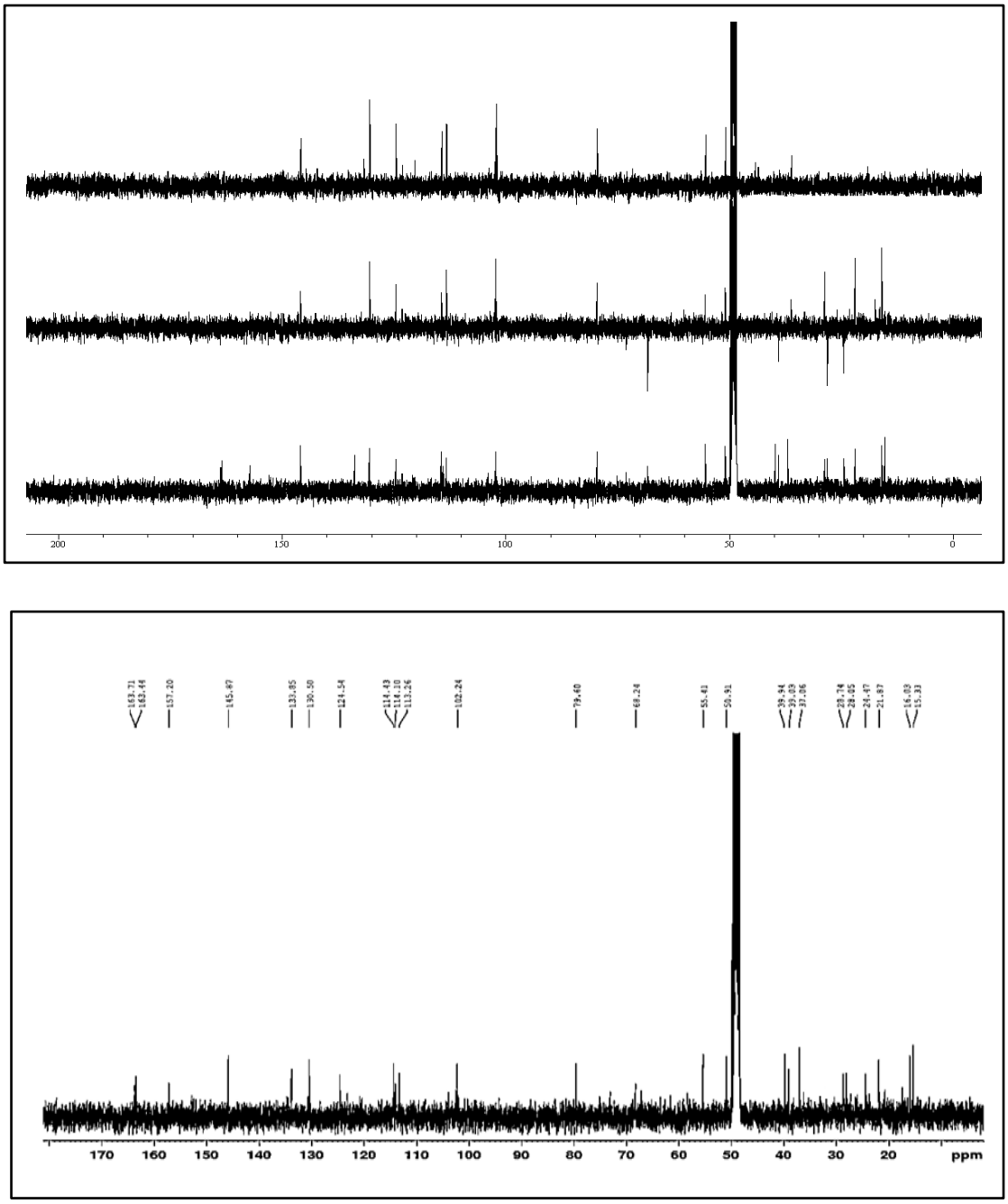


Figure 5.11 ^{13}C-NMR, DEPT-135 and DEPT-90 spectra of compound **1** in methanol-d_4.

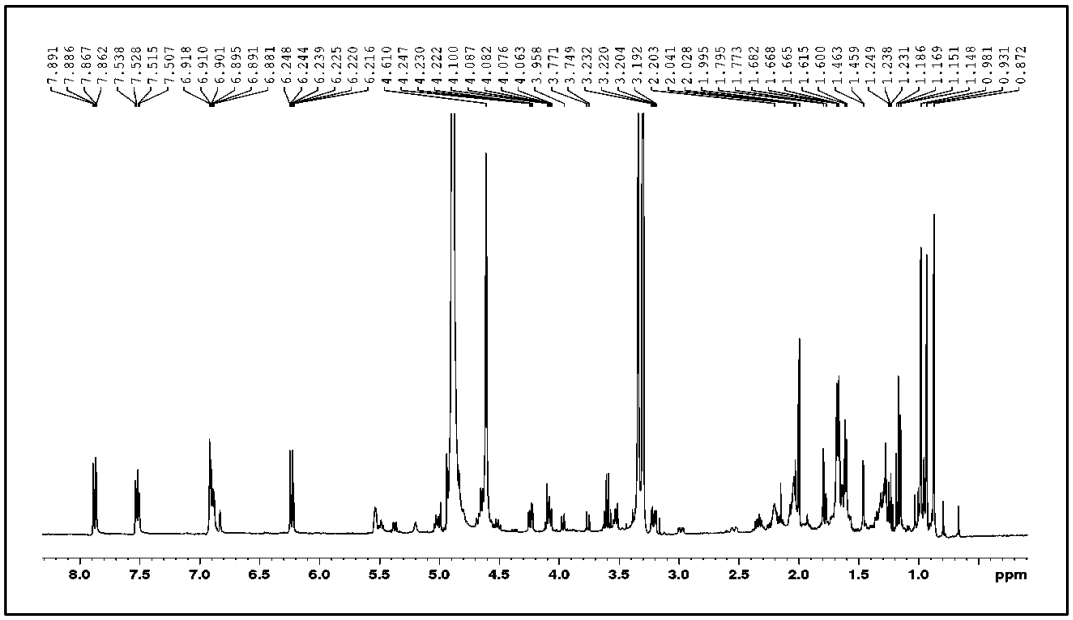


Figure 5.12 ^{1}H-NMR spectrum of compound **1** in methanol-d_4

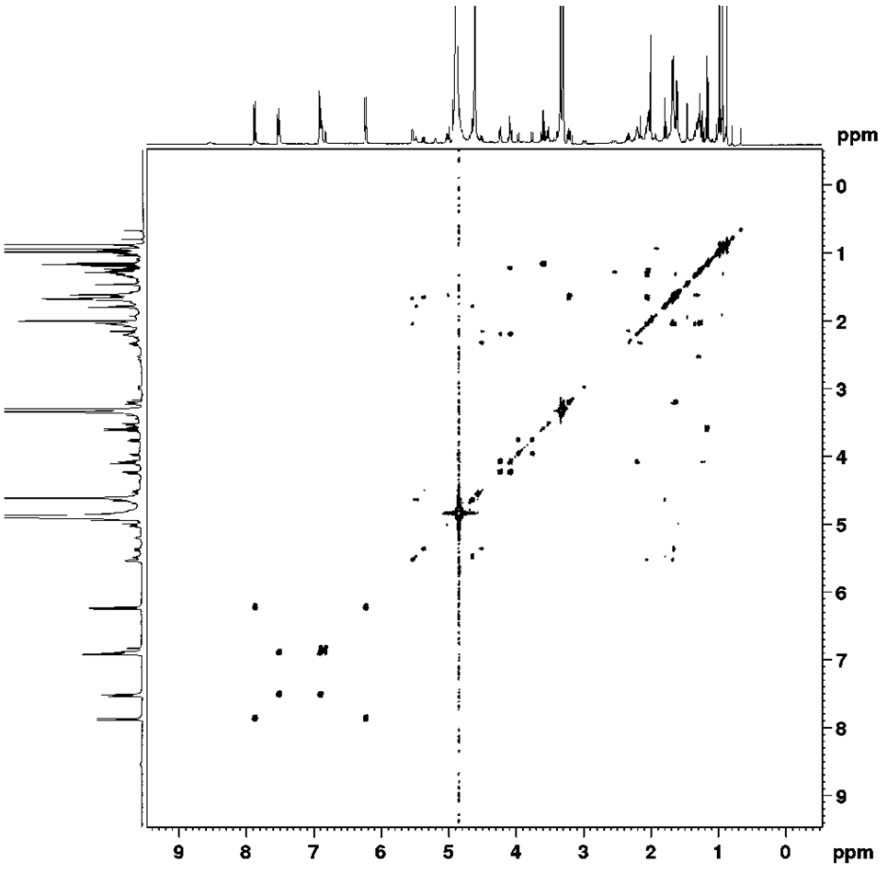


Figure 5.13 COSY spectrum of compound **1**

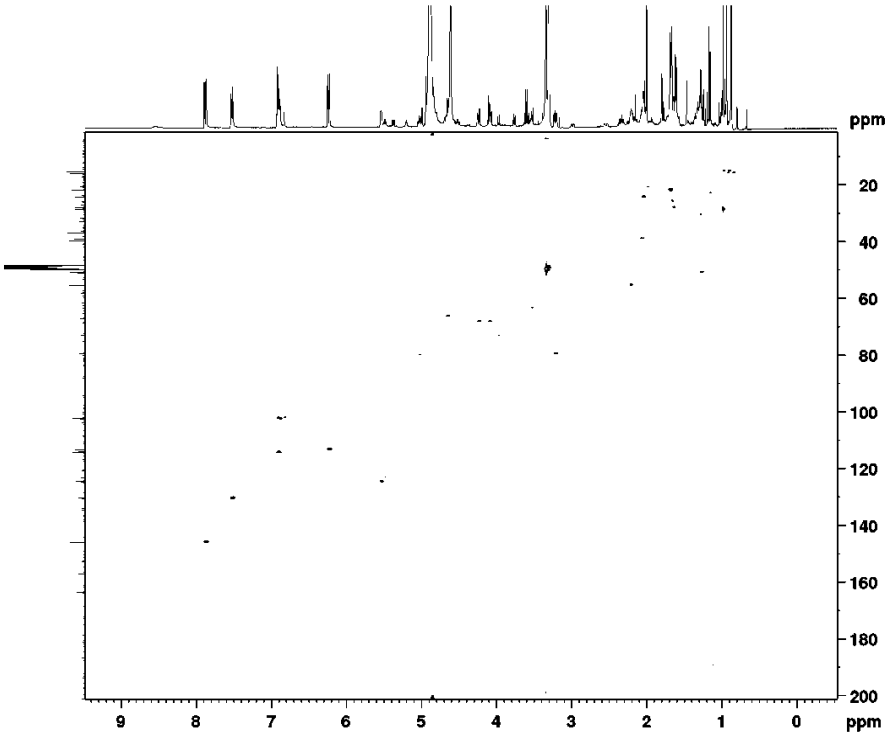


Figure 5.14 HSQC spectrum of compound **1**

5.3.2 Ligupersin A (2)

The structure of compound **2** was elucidated by ^{1}H-NMR, ^{13}C-NMR (DEPT-135 and DEPT-90) and 2D-NMR (COSY, HSQC and HMBC) as **ligupersin A** (Fig. 5.15).

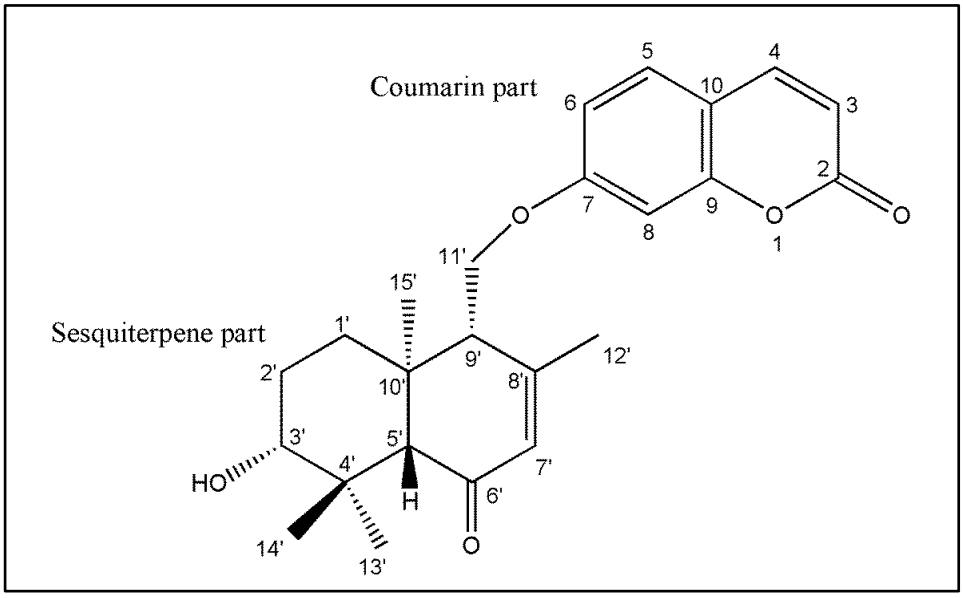


Figure 5.15 Structure of ligupersin A (**2**)

Library search on the ^{13}C-NMR data (Table 5.3 and Fig 5.16) using the NMR Predict database and ^{1}H-NMR (Table 5.3 and Fig. 5.17) suggested that this compound was a sesquiterpene-coumarin. The spectral data were compared with NMR data from literature (Marco *et al*., 1991) which suggested that the compound was ligupersin A. Analysis of the COSY (Fig. 5.18), HSQC (Fig. 5.19) and HMBC (Fig. 5.20) correlations confirmed the structure as shown.

In ^{13}C NMR (Table 5.3, Fig. 5.18) the sesquiterpene ring carbons C-1' and C-2' (both methylene groups) showed resonance signals at 38.5 ppm and 27.6 ppm, respectively. The signals at 79.4 ppm (indicative of oxygen substitution), 39.1 ppm and 63.7 were assignable to C-3', C-4' and C-5', respectively. The carbonyl group at C-6 was attributed to the peak at 201.9 ppm. In the same ring C-7' and C-8' (double bond) were assigned to the signals at 130.7 ppm and 157.4 ppm, respectively. In the same ring C-9' and C-10' were observed at 56.5 ppm and 43.6 ppm, respectively. The resonance at 67.1 ppm was assignable to the oxygen-substituted methylene group C-11'. Finally four methyl signals appearing at 22.0 ppm, 16.0 ppm, 29.1 ppm and 16.7 ppm were assigned to C-12', C-13', C-14'and C-15', respectively.

In the coumarin ring the carbonyl group (C-2) appeared at 163.2 ppm. The resonance signals at 114.0 ppm and 145.9 ppm (double bond) were assigned to C-3 and C-4, respectively. C-5, 6 and C-8 were attributed to signals at 130.7 ppm, 114.5 ppm and 102.6ppm, respectively. Finally signals at 163.5 ppm, 159.6 ppm and 114.4 ppm were assigned to C-7, C-9 and C-10, respectively.

In the ^{1}H-NMR spectrum (Table 5.3, and Fig. 5.19) of the sesquiterpene part, multiplets appearing at 1.67 ppm and 2.06 ppm were assigned to coupled protons at H-1', whereas multiplets at 1.65 ppm and 1.72 ppm were attributed to H-2'. The resonance at 3.20 ppm (dd, *J*= 4.3, 11.0 Hz) was assignable to H-3'. Indeed, the chemical shift indicated the presence of a hydroxyl group with α-orientation, as the *J* values were in agreement with published data (Marco *et al*., 1991). A singlet appearing at 2.33 ppm was assigned to H-5'. The resonance signals appearing at 5.84 ppm (s) and 2.81 ppm (m) were assigned to H-7' and H-9', respectively. Likewise the resonances at 4.32 ppm (dd) and 4.40 ppm (dd) were attributed to H-11'. Four methyl signals were recorded at 2.0 ppm, 1.16 ppm, 1.26 ppm and 1.06 ppm were assigned to H-12', H-13', H-14' and H-15', respectively. This typical pattern of methyl groups suggested a drimane skeleton in the sesquiterpene part of the compound.
In the coumarin ring, protons H-3 and H-4 (double bond) corresponded to signals at 6.24 ppm and 7.88 ppm, respectively, whereas H-5 and H-6 were observed at 5.84 ppm (d) and 6.95 ppm (dd), respectively. Finally a doublet (*J*=2.5) appearing at 6.98 ppm was assigned to H-8.

Yellow powder; UV (acetonitrile / H_2O) λ_{max} 218, 236, 296, 325 nm; ESI-MS (positive ion mode): *m/z* 419 $[M+Na]^+$ consistent with a molecular formula $C_{24}H_{28}O_5$.

Ligupersin A has been isolated from various medicinal plants including *Ligularia persica* (Marco *et al*., 1991), *Ferula gummosa* (Iranshahi *et al*., 2010$_a$), *Ferula flabelliloba* (Iranshahi *et al*., 2010$_b$). However this is the first report in *Ferula narthex.*

Table 5.3 ^{1}H and ^{13}C NMR assignments for compound (**2**) recorded in MeOH-d_4

Position	δ_H (ppm); multiplicity; *J* (Hz)	δ_C(ppm)
2		163.3
3	6.24; dd; *J*= 9.5	114.0
4	7.88; d; *J*= 9.5	145.9
5	7.84; d; *J*= 8.5	130.7
6	6.95; *J*= 8.5; 2.5	114.5
7		163.5
8	6.98; d; *J*= 2.5	102.6
9		159.6
10		114.4
1'	1.67; 2.06; m	38.5
2'	1.65;1.72; m	27.6
3'	3.20; *J*= dd; 4.3; 11.0	79.4
4'		39.1
5'	2.33; s	63.7
6'		201.9
7'	5.84; br s	130.1
8'		157.4
9'	2.81; m	56.5
10'		43.6
11'	4.32; dd; *J*= 10.5; 5.2 4.40; dd; *J*= 10.5; 3.0	67.1
12'	2.0; s	22.0
13'	1.16; s	16.0
14'	1.26; s	29.1
15'	1.06; s	16.7

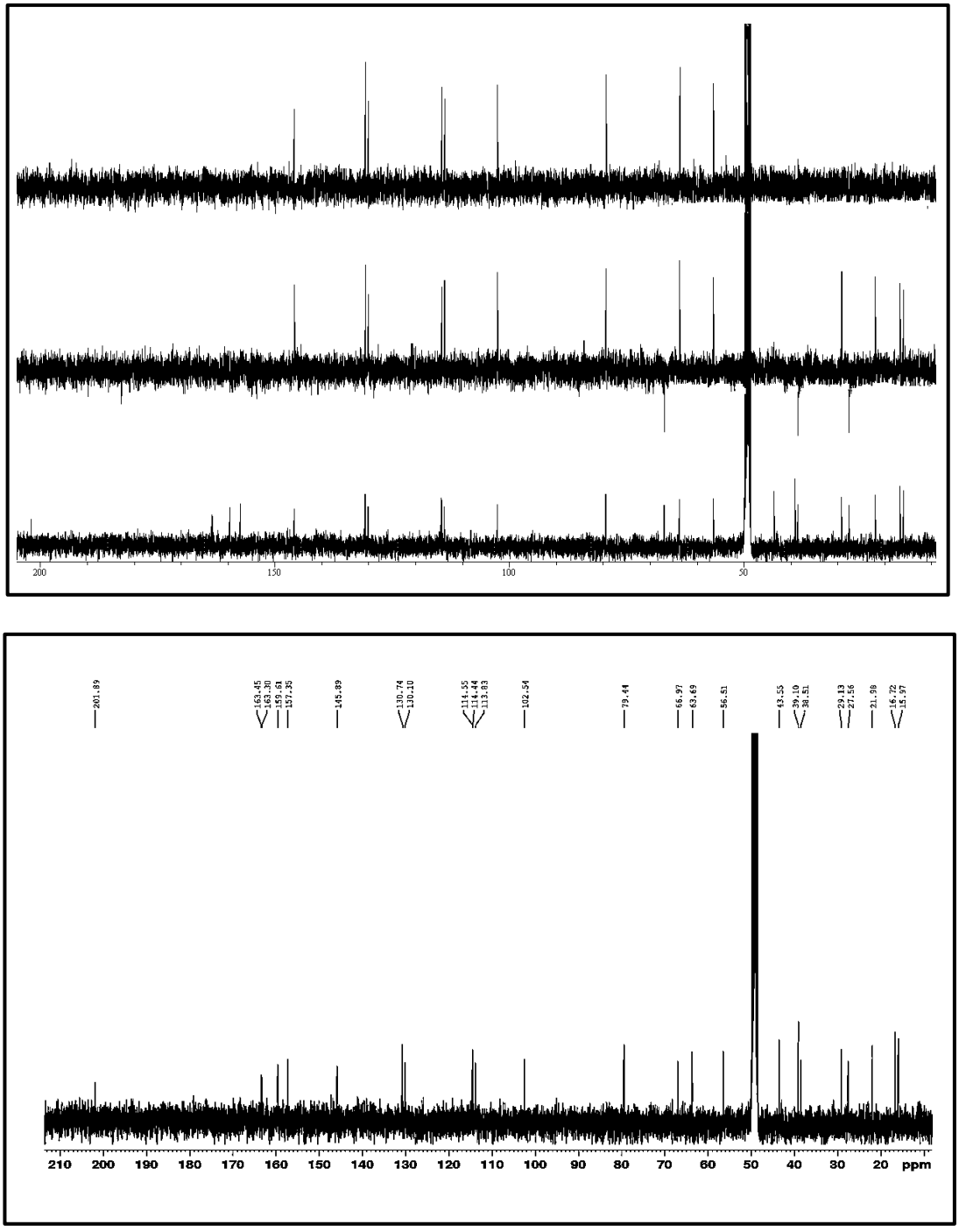


Figure 5.16 ^{13}C-NMR, DEPT-135 and DEPT-90 spectra of compound **2** in methanol-d_4.

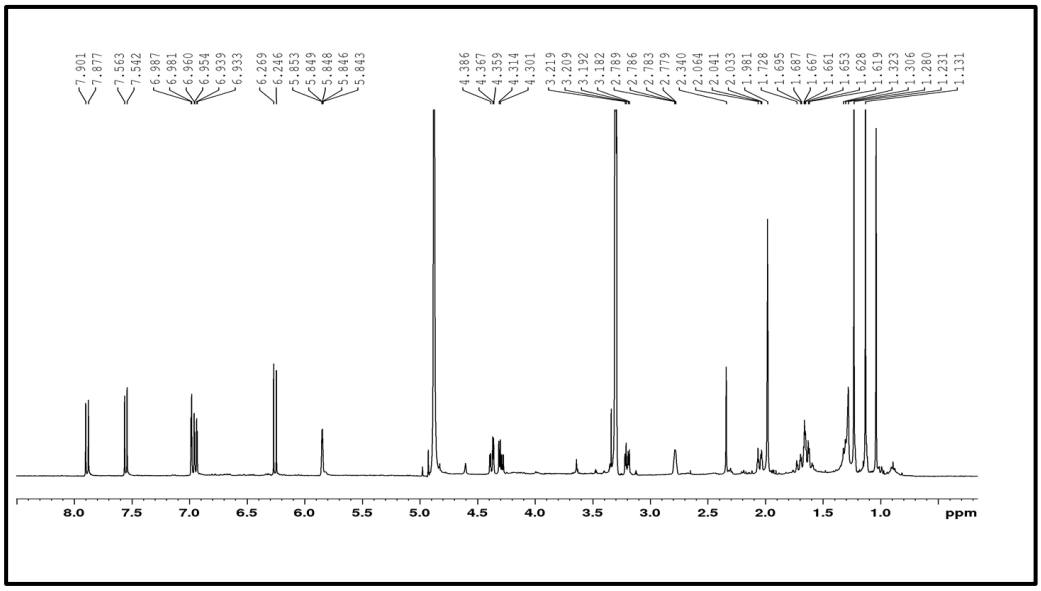


Figure 5.17 ^{1}H-NMR spectrum of compound **2** in methanol-d$_4$

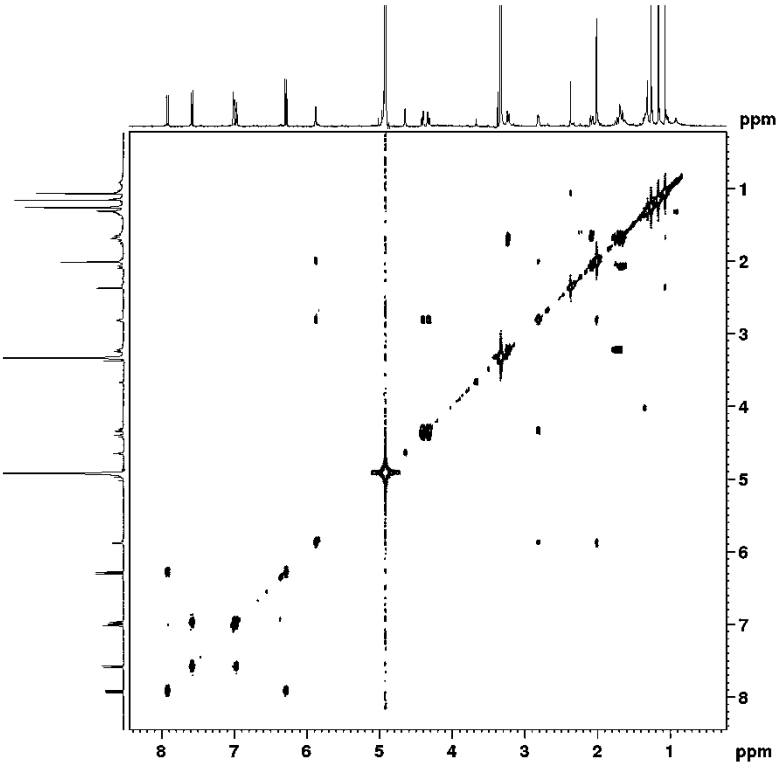


Figure 5.18 COSY spectrum of compound **2**

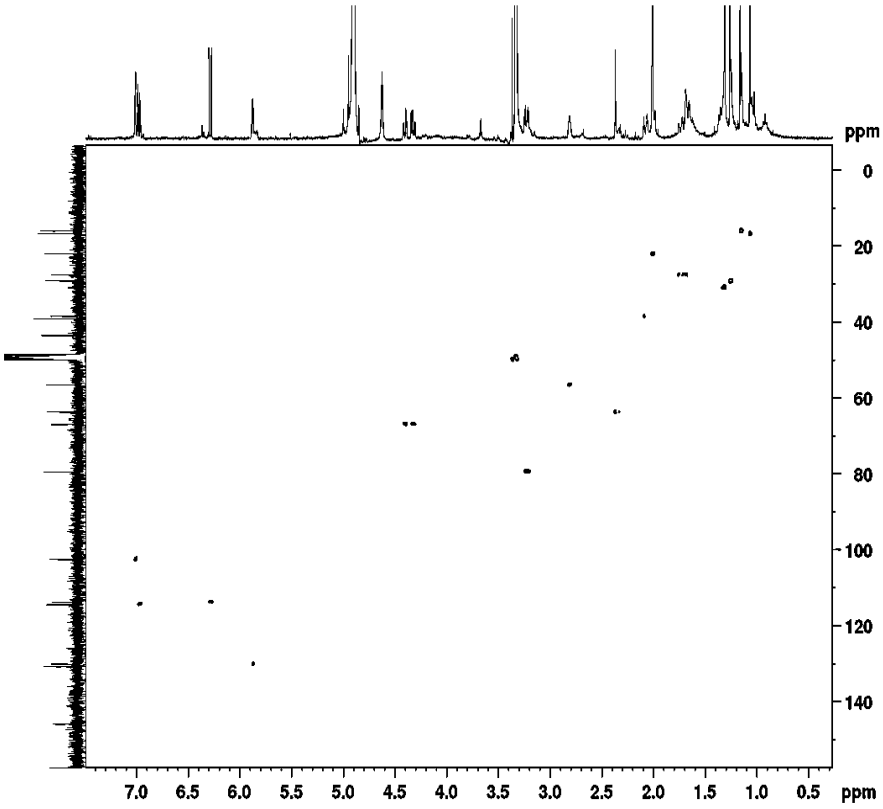


Figure 5.19 HSQC spectrum of compound **2**

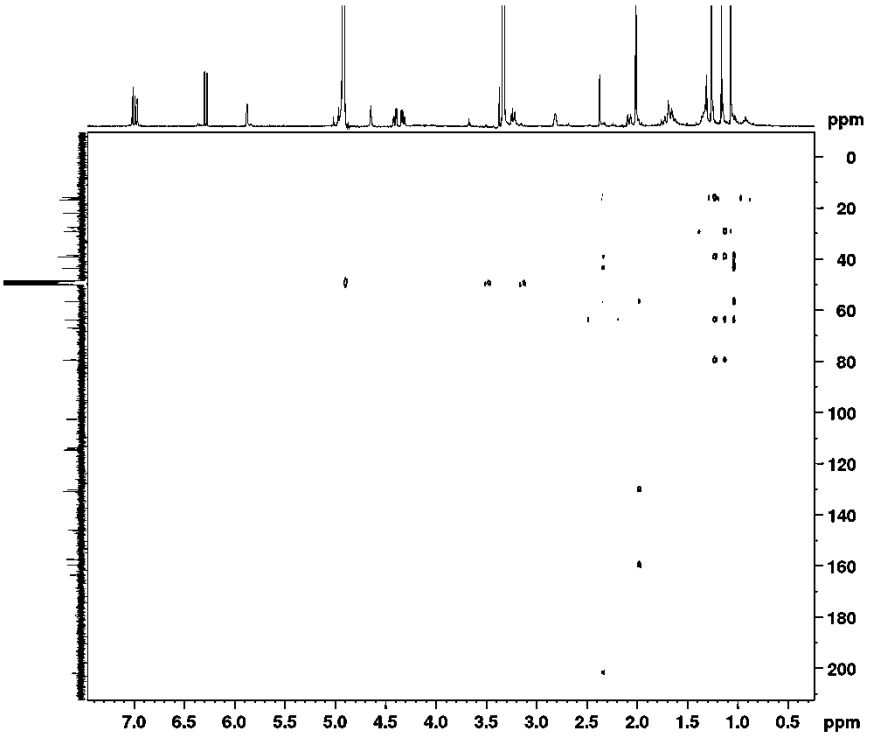


Figure 5.20 HMBC spectrum of compound **2**

5.3.3 8'- Acetoxy-5'-hydroxyumbelliprenin (3)

The structure of compound **3** was elucidated by ^{1}H-NMR, ^{13}C-NMR (DEPT-135 and DEPT-90) as **8'-acetoxy-5'-hydroxyumbelliprenin** (Fig. 5.21).

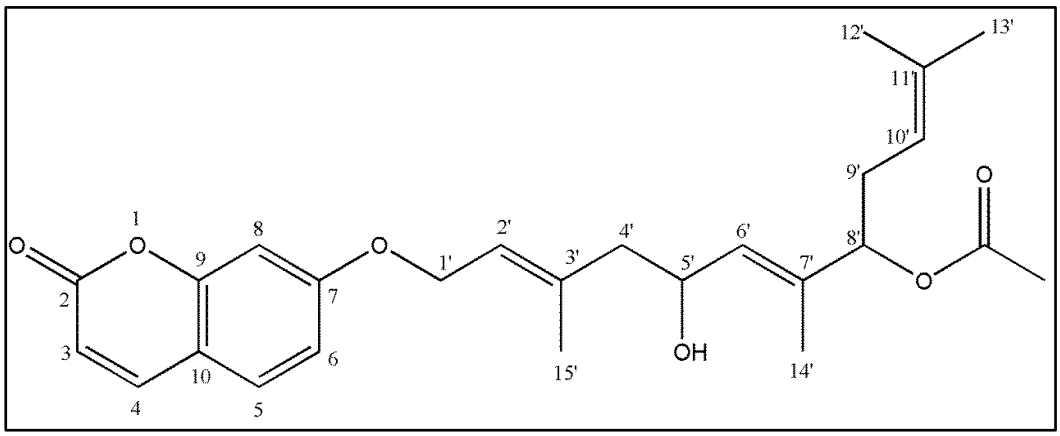


Figure 5.21 Structure of 8'-acetoxy-5'-hydroxyumbelliprenin **(3)**

Library search on the ^{13}C-NMR data (Table 5.4 and Fig 5.22) using the NMR Predict database and ^{1}H-NMR (Table 5.4 and Fig. 5.23) suggested that this compound was a substituted coumarin with a terpene side chain. The spectral data were compared with NMR data from literature (Appendino *et al*., 1994), which indicated that the compound was 8'-acetoxy-5'-hydroxy umbelliprenin.

In ^{13}C-NMR the coumarin ring carbonyl group (C-2) showed a resonance at 163.4 ppm. The signals at 113.4 ppm and 145.8 ppm (double bond) were assigned to C-3 and C-4, respectively. C-5, 6 and C-8 were attributed to peaks at 130.5 ppm, 114.4 ppm and 102.6 ppm, respectively, and signals at 163.8 ppm, 157.1 ppm and 114.4 ppm to C-7, C-9 and C-10, respectively. In the aliphatic chain three methylene signals appeared at 66.7 ppm, 48.3 ppm and 32.9 ppm, and were assigned to C-1', C-4' and C-9', respectively. Resonance signals at 123.2 ppm, 139.3 ppm and 67.3 ppm were attributed to C-2', C-3' and C-5'. Carbon signals appearing at 131.9 ppm, 136.4 ppm and 80.0 were assignable to C-6', C-7' and C-8'. Finally C-10' and C-11' were assigned to the peaks at 120.3 ppm and 135.4 ppm.

Four methyl signals at 12.8 ppm, 18.0 ppm, 17.4 ppm and 26.0 ppm were attributed to C-12', C-13', C-14' and C-15', respectively. The acetoxy group appeared at 172.0 ppm (carbonyl) and 21.0 ppm (methyl).

In the ^{1}H-NMR spectrum (Fig 4.15 and Fig 4.16) of the coumarin part H-3 and H-4 (double bond) corresponded to signals at 6.21 ppm and 7.90 ppm, respectively, whereas H-5 and H-6 resonated at 7.51 ppm (d) and 6.90 ppm (dd), respectively. Finally a doublet (*J*=2.5 Hz) appearing at 6.84 ppm was assigned to H-8.

In the aliphatic chain signals appearing at 4.64 ppm and 5.50 ppm were attributed to H-1' and H-2', whereas multiplets at 2.34 ppm and 2.16 ppm were assigned to H-4'. The upfield chemical shift of the signal at 4.51 ppm, showed the presence of a hydroxyl group and this signal was assigned to H-5'. A broad doublet (*J*=8.2 Hz) at 5.40 ppm corresponded to H-6', whereas a triplet appearing 5.02 ppm was assigned to H-8'. The methylene protons at H-9' were assigned to signals at 2.33 ppm and 2.24 ppm (m), whereas H-10' was attributed to the signal at 4.83 ppm. The methyl groups H-12', H-13', H-14' and H-15' were assigned to signals at 1.60 ppm, 1.70 ppm, 1.80 pp and 1.80 ppm, respectively. The singlet appearing at 2.0 ppm was assigned to the acetoxy group.

Yellow powder; UV (acetonitrile / H_2O) λ_{max} 205, 325 nm; ESI-MS (positive ion mode): *m/z* 463 consistent with a molecular formula $C_{26}H_{32}O_6$.

8'-Acetoxy-5'-hydroxyumbelliprenin has been reported previously been reported from asafetida, the gum resin obtained by incision of the roots of various plants from the genus *Ferula* (Appendino *et al.,* 1994; Lee *et al.,* 2009).

Table 5.4 ^{1}H and ^{13}C NMR assignments for compound (**3**) recorded in MeOH-d_4

Position	δ_H (ppm); multiplicity; *J* (Hz)	δ_C (ppm)
2		163.4
3	6.21; d; *J*= 8.5	113.4
4	7.90; d; *J*= 9.5	145.8
5	7.51; d; *J*= 8.5	130.5
6	6.90; dd; *J*= 8.0; 2.5	114.4
7		163.8
8	6.84; d; *J*= 2.5	102.6
9		157.1
10		114.0
1'	4.64; br d; *J*= 6.2	66.7
2'	5.50; br t; *J*=6.2	123.2
3'		139.3
4'	2.34; m 2.16; m	48.3
5'	4.51; m;	67.3
6'	5.40; br d; *J*= 8.2	131.9
7'		136.4
8'	5.02; t; *J*= 7.0	80.0
9'	2.33; m 2.24; m	32.8
10'	4.83; overlapped with solvent peak	120.3
11'		135.4
12'	1.60; br s	12.8
13'	1.70; s	18.0
14'	1.68; s	17.4
15'	1.80; br s	26.0
Ac	2.0; s	21.0 172.0

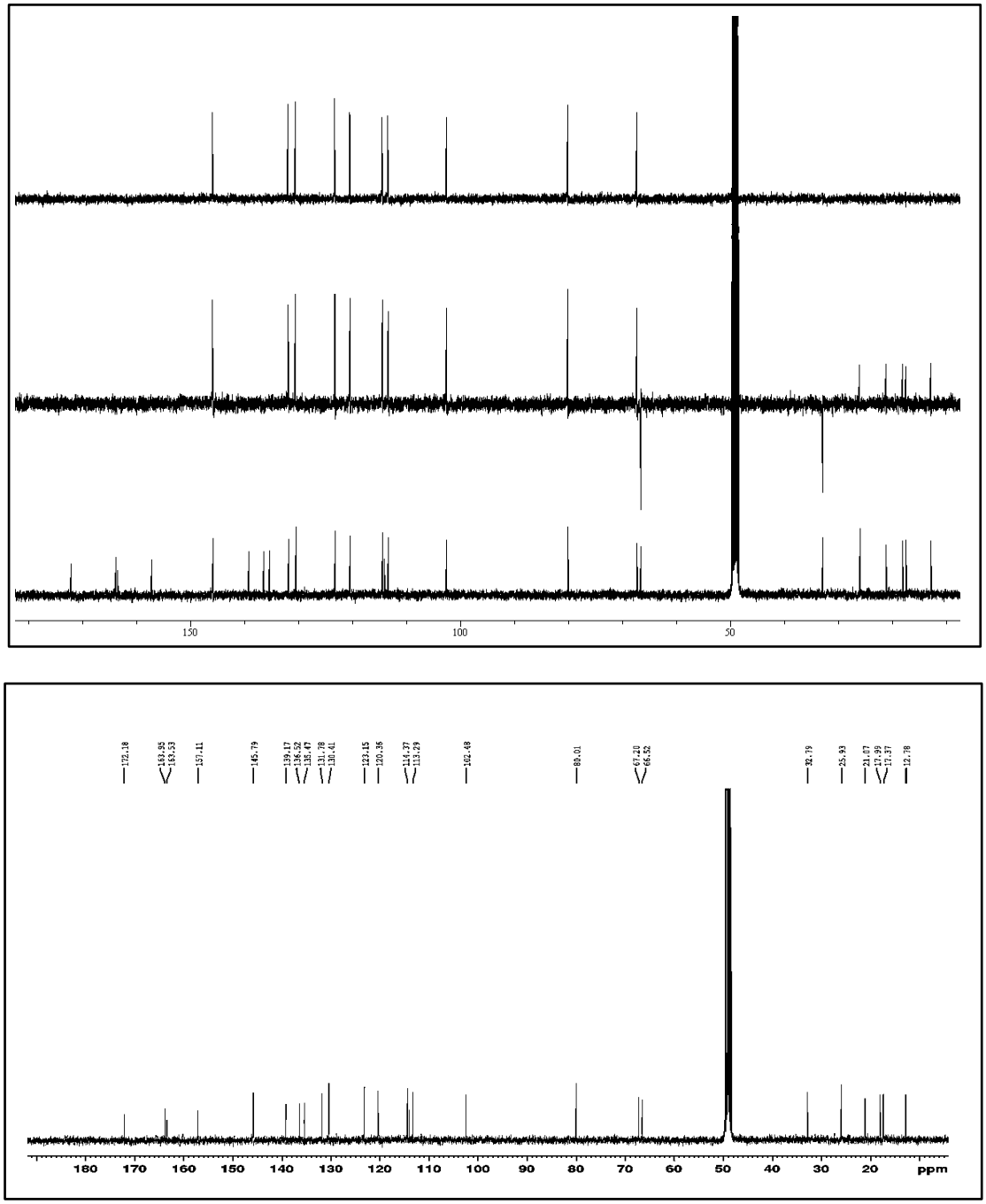


Figure 5.22 ^{13}C-NMR, DEPT-135 and DEPT-90 spectra of compound **3** in methanol-d$_4$.

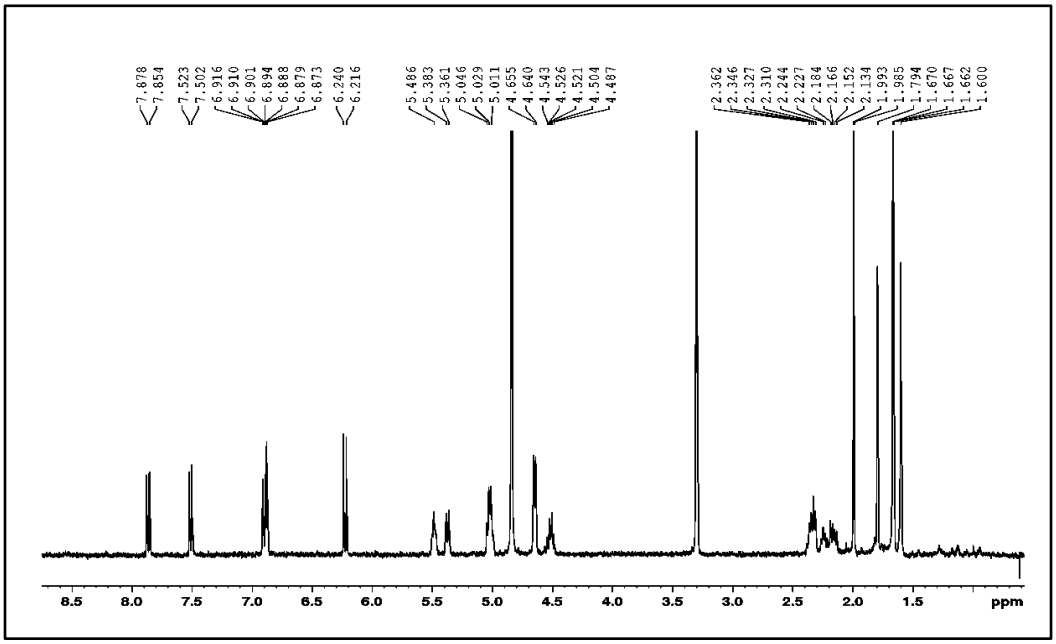


Figure 5.23 ^{1}H-NMR spectrum of compound **3** in methanol-d_4

5.3.4 Asacoumarin A (4)

The structure of compound **4** was elucidated by ^{1}H-NMR, ^{13}C-NMR (DEPT-135 and DEPT-90) and 2D-NMR (COSY, HSQC, and HMBC) as **asacoumarin A** (Fig. 5.24).

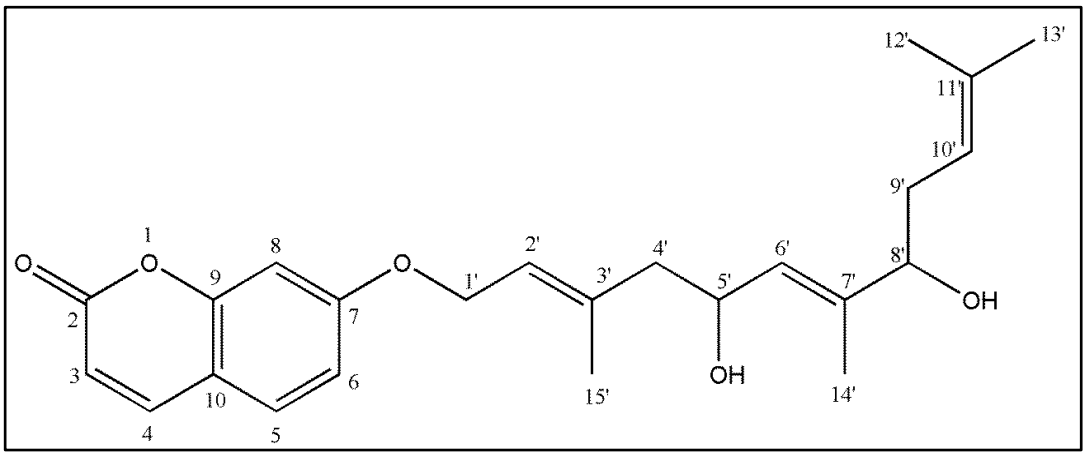


Figure 5.24 Structure of asacoumarin A (**4**)

Library search on the ^{13}C-NMR data (Table 4.5 and Fig 5.25) using the NMR Predict database and ^{1}H-NMR (Table 4.5 and Fig 5.26) suggested that this compound was a substituted coumarin. The spectral data were compared with NMR data from the literature (Abd El-Razek *et al*., 2007) and analysis of the COSY (Fig. 5.27), HSQC (Fig. 5.28) and HMBC (Fig. 5.29) correlations confirmed the structure as asacoumarin A.

In ^{13}C-NMR 24 signals were recorded. The coumarin carbonyl (C-2) show a resonance at 163.4 ppm. The signals at 113.2 ppm and 145.8 ppm were assigned to C-3 and C-4. Similarly, the CH-groups C-5, 6 and C-8 were attributed to the signals at 130.3 ppm, 114.4 ppm and 102.2 ppm, respectively, whereas the at163.8 ppm, , 157.1 ppm and 113.9 ppm were assigned to the quaternary carbons C-7, C-9 and C-10, respectively.

In the aliphatic chain three methylene signals appeared at 66.5 ppm, 48.3 ppm and 34.9 ppm, assigned to C-1', C-4' and C-9', respectively. Likewise the signals at 123.0 ppm, 140.1 ppm and 67.4 ppm were attributed to C-2', C-3' and C-5'. Carbon signals appearing at 130.4 ppm, 139.3 ppm and 78.5 were assignable to C-6', C-7' and C-8'. Finally C-10' and C-11' were assigned to 121.8 ppm and 133.9 ppm. Four methyl signals

at 18.0 ppm, 26.0 ppm, 17.3 ppm and 11.6 ppm were attributed to C-12', C-13', C-14' and C-15', respectively.

In the ^{1}H-NMR spectrum (Table 4.5 and Fig. 5.28) coumarin and terpene signals were recorded. In the coumarin part, protons H-3 and H-4 (double bond) corresponded to signals at 6.22 ppm and 7.88 ppm, respectively, whereas H-5 and H-6 resonated 7.50 ppm (d) and 6.88 ppm (dd), respectively. A doublet appearing at 6.85 ppm was assigned to H-8.

In the terpene part (aliphatic chain) signals appearing at 4.64 ppm and 5.50 ppm were attributed to H-1' and H-2', whereas multiplets observed at 2.34 ppm and 2.14 ppm were assigned to H-4'. The upfield chemical shift of the signal at 4.51 ppm, due to the presence of a hydroxyl group, was assigned to H-5'. Similarly a broad doublet at 5.30 ppm corresponded to H-6', whereas a triplet appearing at 3.89 ppm was assigned to H-8'. The methylene protons at H-9' were assigned to a multiplet at 2.11 ppm, whereas H-10' was attributed to the peak at 5.05 ppm. The methyl signals at H-12', H-13', H-14' and H-15' were assigned to signals at1.60 ppm, 1.66 ppm, 1.80 ppm and 1.60 ppm, respectively.

Yellow powder; UV (acetonitrile / H_2O) λ_{max} 203, 325 nm; ESI-MS (positive ion mode): *m/z* 421 $[M+Na]^+$ consistent with a molecular formula $C_{24}H_{30}O_5$.

Asacoumarine A has been isolated before from asafoetida (Appendino *et al.,* 1994; Kajimoto *et al*., 1989; Abd El-Razek *et al*., 2007), However, this is the first report in *Ferula narthex.*

Table 5.4 ^{1}H and ^{13}C NMR assignments for compound (**4**) recorded in MeOH-d_4

Position	δ_H (ppm); multiplicity; *J* (Hz)	δ_C (ppm)
2		163.4
3	6.22; d; *J*= 9.5	113.2
4	7.88; d; *J*= 9.5	145.8
5	7.50; d; *J*= 8.5	130.3
6	6.88; dd; *J*= 8.0; 2.5	114.4
7		163.8
8	6.85; d; *J*= 2.5	102.2
9		157.1
10		113.9
1'	4.64;d; *J*= 6.4	66.5
2'	5.50; br t; *J*=6.2	123.0
3'		140.1
4'	2.34; m 2.14; m	48.3
5'	4.51;dd; *J*=15.0; 6.4	67.4
6'	5.3; d; *J*= 8.2	130.4
7'		139.3
8'	3.89; t; *J*= 6.7	78.5
9'	2.11; m	34.9
10'	5.05; t; *J*=7.0	121.8
11'		133.9
12'	1.60; br s	18.0
13'	1.66; s	26.0
14'	1.8; s	17.3
15'	1.60; br s	11.6

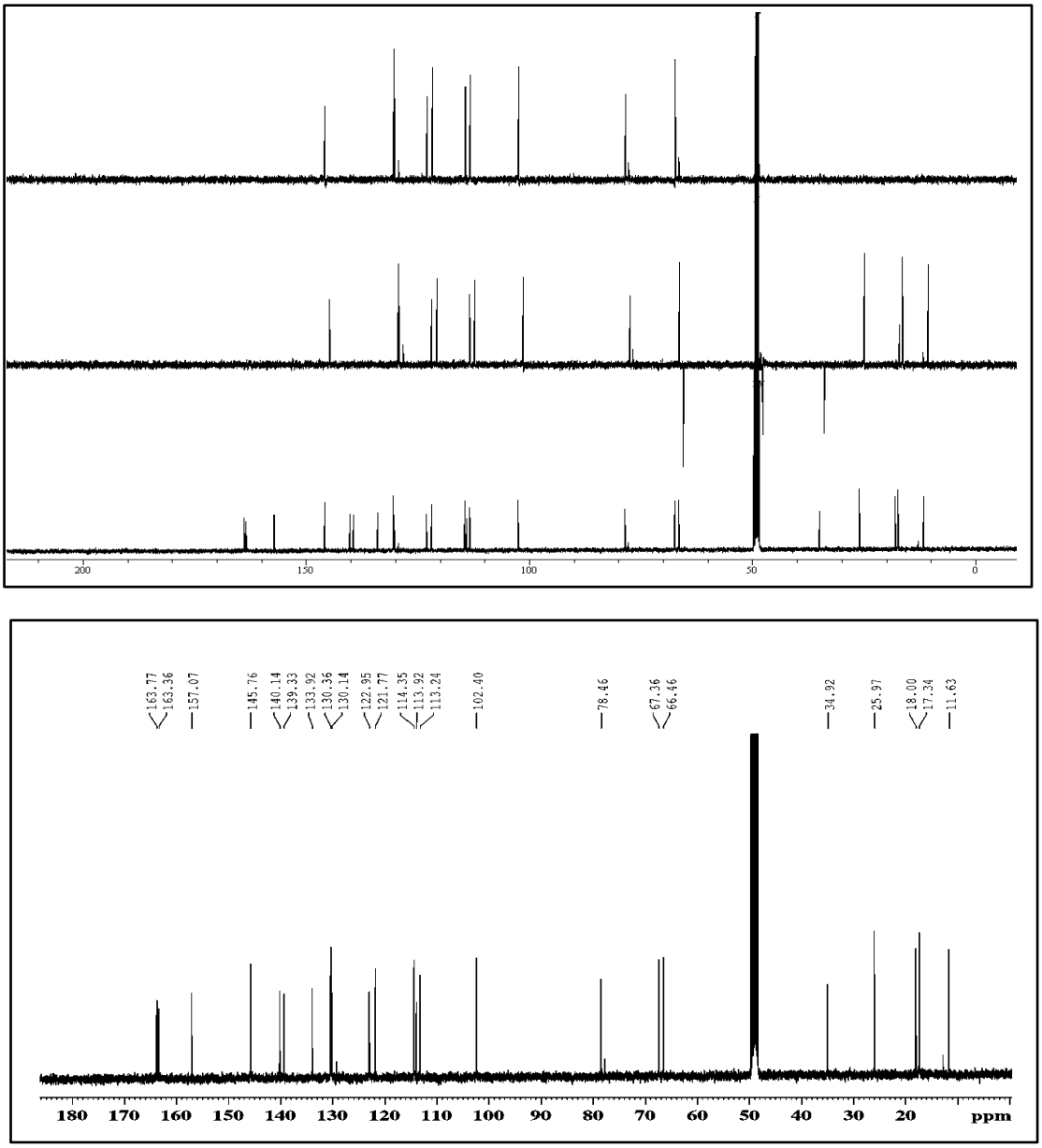


Figure 5.25 ^{13}C-NMR, DEPT-135 and DEPT-90 spectra of compound **4** in methanol-d$_4$.

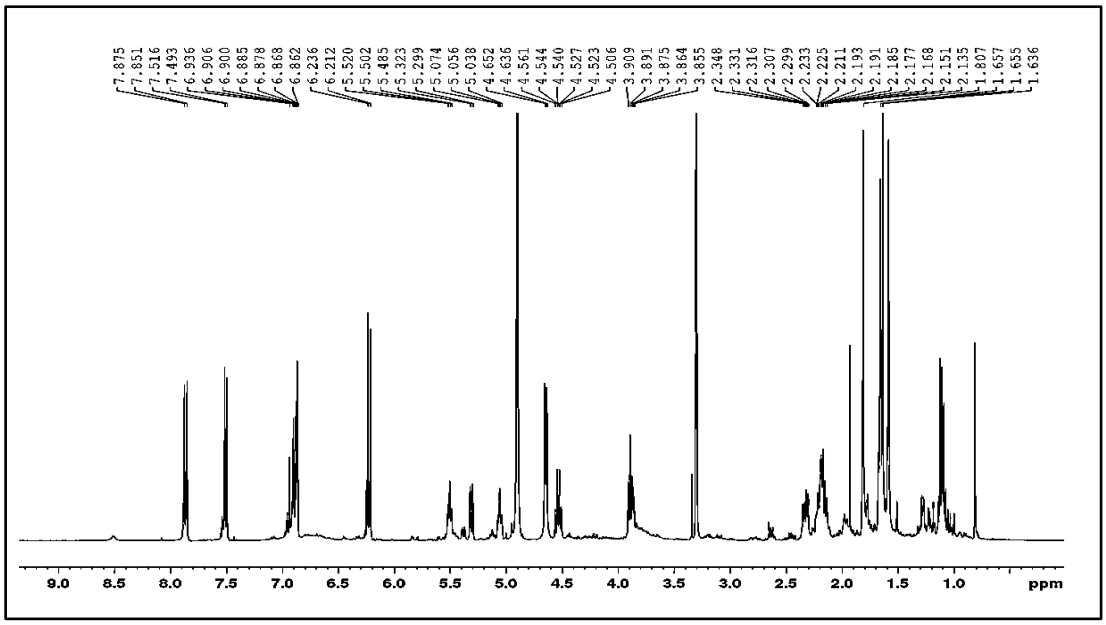


Figure 5.26 ^{1}H-NMR spectrum of compound **4** in methanol-d$_4$

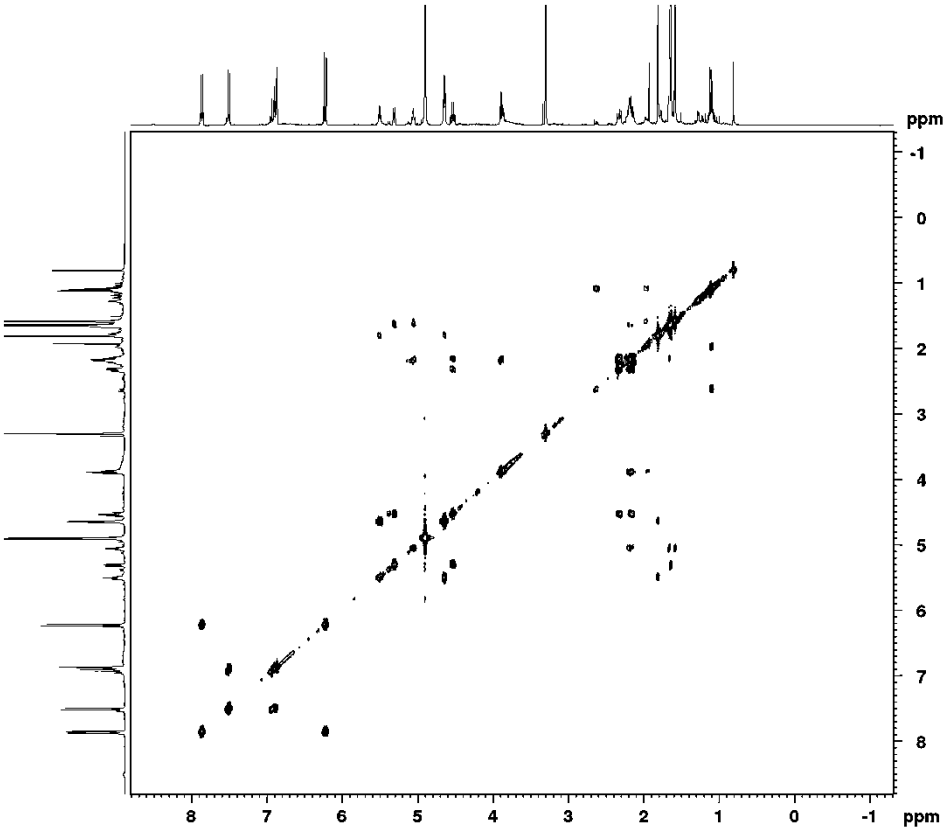


Figure 5.27 COSY spectrum of compound **4**

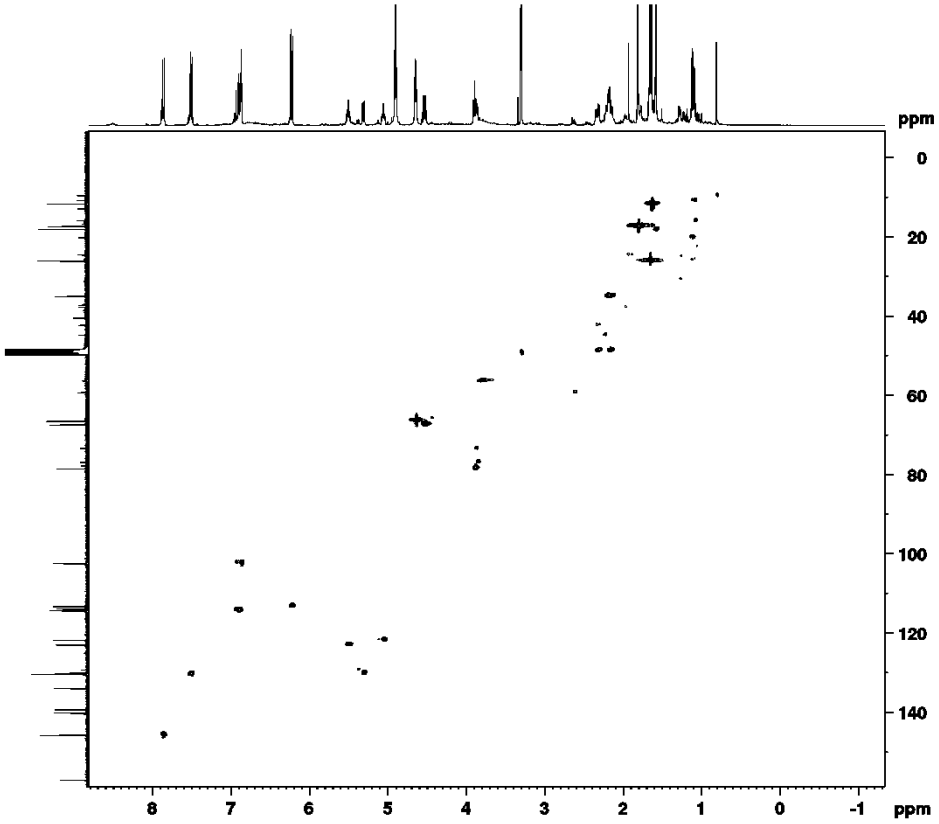


Figure 5.28 HSQC spectrum of compound **4**

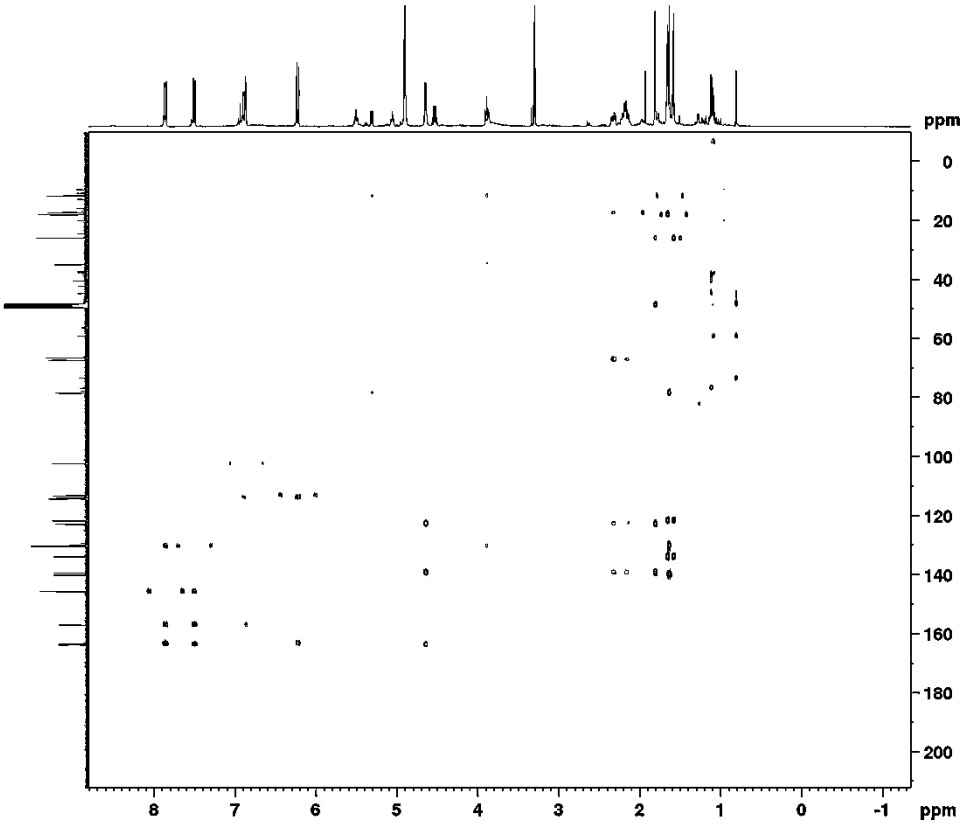


Figure 5.29 HMBC spectrum of compound **4**

5.3.5 10'*R*-Karatavacinol (5)

The structure of compound **5** was elucidated by ^{1}H-NMR, ^{13}C-NMR (DEPT-135 and DEPT-90) as 10'*R*-**karatavacinol** (Fig. 5.30).

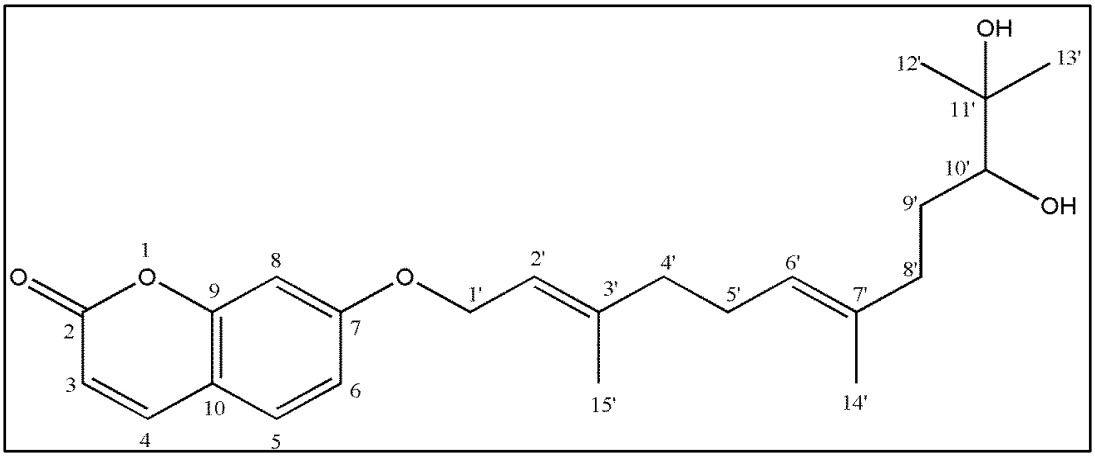


Figure 5.30 Structure of 10'*R*-karatavicinol (**5**)

Library search on the ^{13}C-NMR data (Table 5.6 and Fig. 5.31) using the NMR Predict database and ^{1}H-NMR (Table 5.6 and Fig. 5.32) suggested that this compound was a substituted coumarin with a terpene side chain. The spectral data were compared with NMR data from literature (Abd El-Razek *et al*., 2007; Ahmad, 1999), which indicated that compound **5** was 10'*R*-karatavacinol.

In ^{13}C-NMR 24 signals were recorded. Indeed karatavicinol shared structural similarities with compounds **3** and **4**. However, five methylene signals were recorded in case of karatavicinol at C-1', C-5', C-6', C-8'and C-9' that corresponded to signals at 66.6 ppm, 30.8 ppm, 37.8 ppm, 40.5 ppm and 27.2 ppm, respectively. The carbonyl group appearing at 163.8 ppm was assigned to C-2 (carbonyl) of the coumarin moiety.

Similarly in the ^{1}H-NMR spectrum (Table 5.6 and Fig. 5.35) methylene signal appearing at 4.65 ppm (d; *J*= 6.0) was assigned to H-1'; whereas the multiplets appearing at 2.17 ppm, 1.20 ppm, 1.32 ppm and 1.70 ppm were assigned to H-4', H-5, H-8', H-9' in the

aliphatic chain, respectively. The upfield chemical shift of the signal at 3.21 ppm was indicative of an oxygenated CH at position 10'.

Yellow powder; UV (acetonitrile / H_2O) λ_{max} 211, 318 nm; ESI-MS (positive ion mode): *m/z* 423 $[M+Na]^+$ consistent with a molecular formula $C_{24}H_{32}O_5$.

Karatavicinol has been isolated before from a number of *Ferula* species including *Ferula karatavica* (Kiryalov *et al.,* 1969*), Ferula sinica* (Ahmad, 1999), *Ferula foetida* (Abd EL-Razek *et al*., 2007) and *Ferula sinkiangensis* (Teng *et al*., 2013). This is however, the first time it is reported in *Ferula narthex.*

Table 5.6 ^{1}H and ^{13}C NMR assignments for compound (**5**) recorded in MeOH-d_4

Position	δ_H (ppm); multiplicity; *J* (Hz)	δ_C (ppm)
2		163.8
3	6.22; d; *J*= 9.5	113.8
4	7.90; d; *J*= 9.5	145.8
5	7.51; d; *J*= 8.5	130.3
6	6.91; dd; *J*= 8.0; 2.5	114.4
7		163.4
8	6.90; d; *J*= 2.5	102.5
9		157.0
10		113.2
1'	4.65; d; *J*= 6.0	66.6
2'	5.46; t; *J*=6.0	120.3
3'		142.8
4'	2.17; m	37.8
5'	1.20; m	30.8
6'	5.16; br t; *J*=6.0	125.0
7'		136.5
8'	1.32; m	40.5
9'	1.70; m	27.2
10'	3.21; dd; *J*=10.0; 2.0	77.7
11'		73.7
12'	1.10; s	25.0
13'	1.13; s	16.7
14'	1.61; s	25.6
15'	1.77; s	16.2

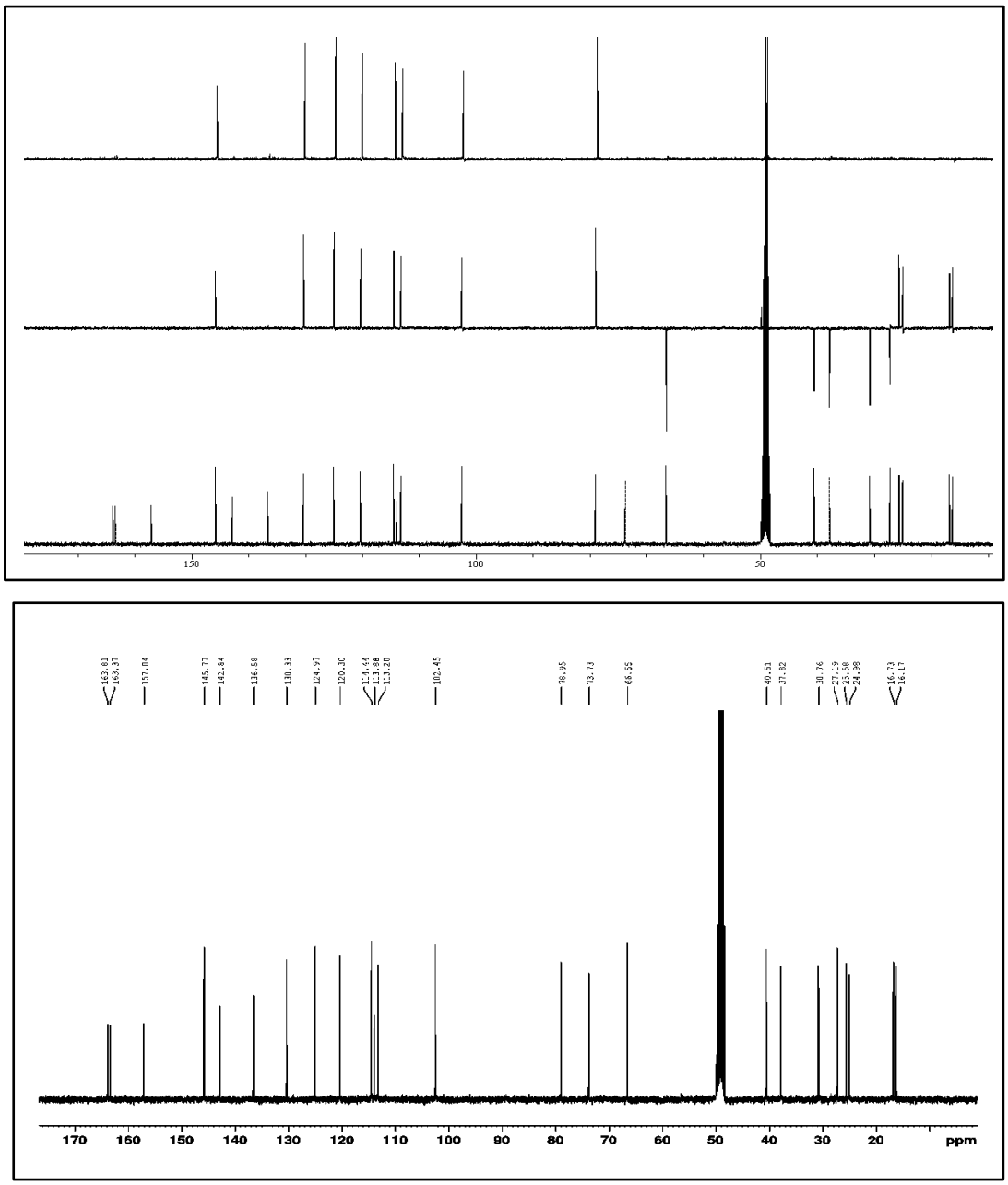


Figure 5.31 ^{13}C-NMR, DEPT-135 and DEPT-90 spectra of compound **5** in methanol-d_4.

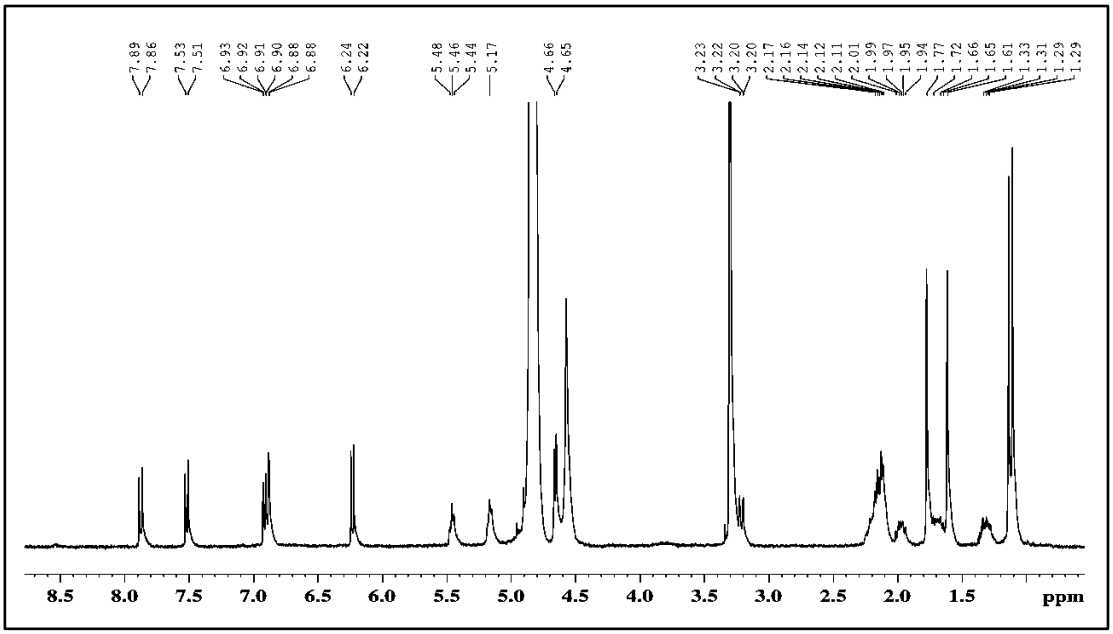


Figure 5.32 ^{1}H-NMR spectrum of compound **5** in methanol-d_4

5.3.6 10′*R*-Acetoxy-11′-hydroxyumbelliprenin (6)

The structure of compound **6** was elucidated by ^{1}H-NMR, ^{13}C-NMR (DEPT-135 and DEPT-90) as **10′*R*-acetoxy-11′-hydroxyumbelliprenin** (Fig. 5.33).

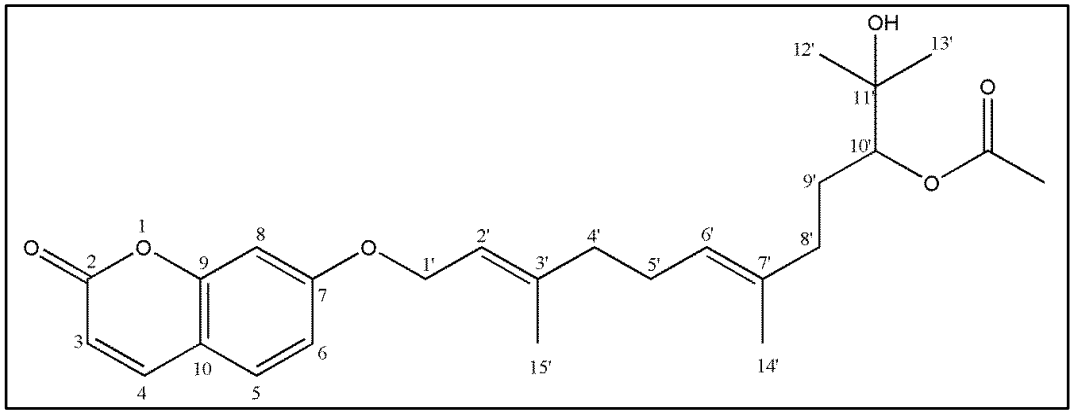


Figure 5.33 Structure of 10′*R*-acetoxy-11′-hydroxyumbelliprenin (**6**)

Library search on the ^{13}C-NMR data (Table 5.7 and Fig. 5.34) using the NMR Predict database and ^{1}H-NMR (Table 5.7 and Fig. 5.35) suggested that this compound was a terpene-substituted coumarin. The spectral data were compared with NMR data from literature (Lee *et al*., 2009), which indicated that compound **6** was 10′*R*-acetoxy-11′ hydroxy-umbelliprenin.

In ^{13}C-NMR 24 signals were recorded. Compound **6** showed structural resemblance with karatavacinol (**5**). Five methylene signals were recorded for C-1', C-4', C-5', C-8' and C-9' that could be assigned to the signals at 66.6 ppm, 40.4 ppm, 27.2 ppm, 37.4 ppm and 29.0 ppm, respectively. The carbonyl group appearing at 164.0 ppm was assigned to C-2 of the coumarin moeity. The signal at 172.8 ppm was assigned to the acetoxy group (carbonyl). Compound **6** is in fact the acetylated analogue of 10'*R*-karatavacinol, i.e 10′*R*-acetoxy-11′ hydroxy-umbelliprenin.

In the ^{1}H-NMR spectrum, signals for the two main moieties i.e a coumarin and a sesquiterpene as in compounds **4** and **5**, were recorded. The sesquiterpene moiety showed signals for four methyl groups at 1.12 ppm, 1.13 ppm, 1.60 ppm and 1.77 ppm,

assigned to H-12', H-13', H-14'and H-15', respectively. The methylene signals observed at 2.05-2.16 (m) were assigned to H-4'and H-5', whereas multiplet signals at t 1.92 ppm and 1.67 ppm were assigned to H-8' and H-9', respectively. The oxygenated methylene signal at 4.99 ppm was attributed to H-1'. The olefenic proton at H-2' was assignable to the signal at 5.50 ppm.

Yellow powder; UV (acetonitrile / H_2O) λ_{max} 213, 321 nm; ESI-MS (positive ion mode): *m/z* 465 $[M+Na]^+$ consistent with a molecular formula $C_{26}H_{34}O_6$.

10′-Acetoxy-11′-hydroxyumbelliprenin has been previously isolated from *asafoetida* (Lee *et al*., 2009). This is, however, the first time it is reported in *Ferula narthex.*

Table 5.7 ^{1}H- and ^{13}C-NMR assignments for compound **6** recorded in MeOH-d_4

Position	**δ_H (ppm); multiplicity; *J* (Hz)**	**δ_C (ppm)**
2		164.0
3	6.23; d; *J*= 9.5	113.2
4	7.90; d; *J*= 9.5	145.9
5	7.51; d; *J*= 8.5	130.4
6	6.91; dd; *J*= 8.0; 2.5	114.5
7		163.5
8	6.90; d; *J*= 2.5	102.5
9		157.1
10		113.9
1'	4.66; d; *J*= 6.0	66.6
2'	5.50; t; *J*=6.0	120.5
3'		143.0
4'	2.16-2.05; m	40.4
5'	2.16-2.05; m	27.2
6'	5.16; br t; *J*=6.0	125.5
7'		135.7
8'	1.92; m	37.4
9'	1.67; m	29.0
10'	4.75; dd; *J*=10.5; 2.0	80.6
11'		72.8
12'	1.12; s	25.7
13'	1.13; s	25.9
14'	1.60; s	16.0
15'	1.77; s	16.7
OAc (C-10')	2.0; s	21.1
		172.8

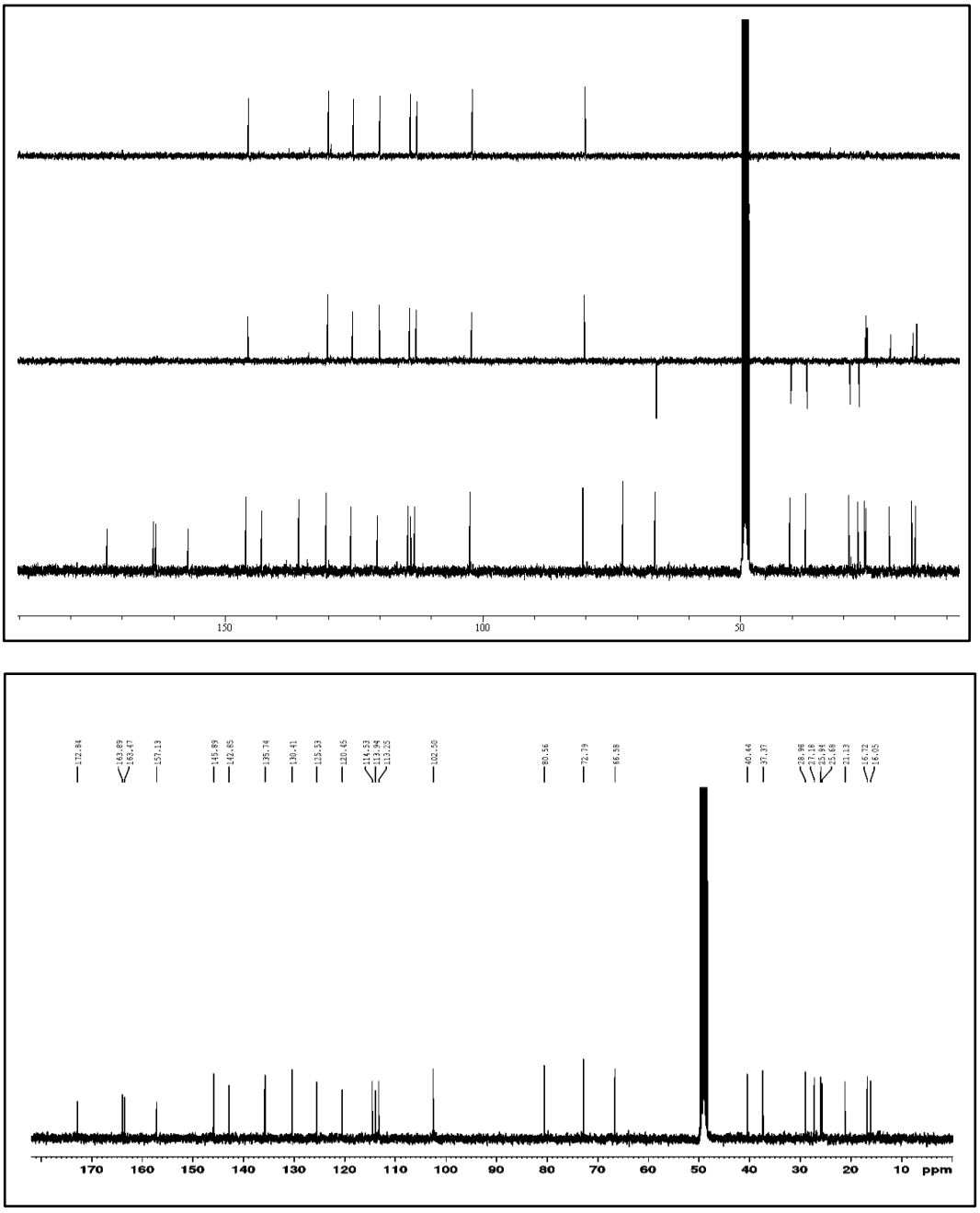


Figure 5.34 ^{13}C-NMR, DEPT-135 and DEPT-90 spectra of compound **6** in methanol-d_4.

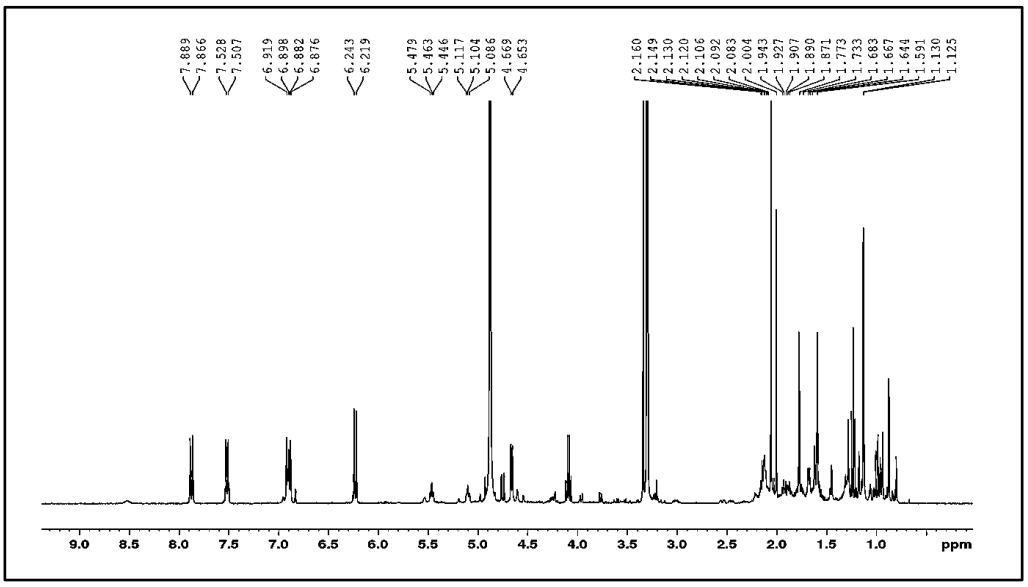


Figure 5.35 ^{1}H-NMR spectrum of compound **6** in methanol-d_4

5.4. BIOLOGICAL ACTIVITIES

5.4.1. Inhibition of AGEs formation

All fractions and crude extracts of *Ferula narthex* were evaluated for AGEs inhibition; however, due to practical issues of solubility and autofluorescence, it was not possible to obtain conclusive data.

All isolated compounds from *Ferula narthex* (**1**-**6**) were tested for their antiglycation potential in the BSA-Glucose and BSA-MGO models. Primarily in the BSA-glucose test, compound **2** displayed the highest activity (IC_{50} 0.414 mM), being more active than the positive control aminoguanidine, followed by compound **4** (IC_{50} 1.83 mM), whereas compounds **1** (47 % inhibition at the highest test concentration of 2 mM), **3** (44 % inhibition), **5** (32 % inhibition) and **6** (36% inhibition) showed moderate inhibition (Table 5.9). On the other hand, in the BSA-MGO assay, the highest activity was shown by compound **2** (IC_{50} 1.03 mM), followed by **1** (IC_{50} 1.71 mM) and **3** (IC_{50} 1.03 mM), whereas moderate activities were observed for compounds **2** (40% inhibition at the highest test concentration of 2 mM), **4** (35% inhibition) and **6** (20% inhibition). Therefore it could be concluded that the antiglycation potential of the isolated constituents was due to both oxidative and non-oxidative modes of inhibition.

Table 5.9. Antiglycation (inhibition of AGEs) potential of isolated compounds from *Ferula narthex*

Compound	**Protein glycation**			
	BSA-Glucose		BSA-MGO	
	% inhibition[a]	IC_{50} mM	% inhibition[b]	IC_{50} mM
1	47			1.71
2	-	0.414	40	-
3	44			1.03
4		1.83	35	-
5	32		-	1.86
6	36		20	
Aminoguanidine		1.75		0.15
Quercetin		0.23		-

[a] at 0.25 mM final concentration
[b] at 2 mM final concentration

5.4.2 Antimicrobial activity

The crude extract and fractions were evaluated for antibacterial and antifungal activities. Highest levels of antimicrobial and antifungal activities were observed for the methanolic fraction, i.e. an IC_{50} of 19.9 µg/mL against *S. aureus* and 22.0 µg/mL against *M. canis*, whereas this fraction was also found to be cytotoxic towards MRC-5 cells (IC_{50} 36.5 µg/mL). Similarly, the *n*-hexane fraction was active (IC_{50} 8.3 µg/mL) against *M. canis,* whereas the chloroform fraction was cytotoxic (IC_{50} 49.8 µg /mL) against MRC-5 cells (Table 5.8).

Table 5.8 Antibacterial, antifungal and cytotoxic activities of *Ferula narthex* fractions.

Sample	IC_{50} µg/mL				
	S. aureus	*E. coli*	*C. albicans*	*M. canis*	MRC-5
MeOH 90%	**19.9**	>64	>64	**22.0**	**36.5**
Chloroform	>64	>64	>64	>64	**49.8**
Ethyl acetate	>64	>64	>64	>64	>64
n-Hexane	>64	>64	>64	8.3	>64
n-Butanol	>64	>64	>64	>64	>64
Aqueous	>64	>64	>64	>64	>64

Reference: Tamoxifen(MRC-5) IC_{50} 11.4 µg/mL; *erythromycin (S. aureus)*IC_{50} 11.2 µg/mL, Trimethoprim (*E. coli*) IC_{50} 0.25 µg/mL; Miconazole(*C. albicans*) IC_{50} 5.99 µg/mL; Terbinafin (*M. canis*) IC_{50} 0.11 µg/mL.

MRC-5: Human fetal lung fibroblasts; *S. aureus: Staphylococcus aureus; E. coli: Escherichia coli; C. albicans: Candida albicans; M. canis: Microsporom canis.*

The isolated compounds **1**, **3**, **4** and **6** were evaluated in an integrated screening panel for antimicrobial activity (Table 5.10). Compounds **2** and **6** were not tested due to the limited amount available. Although the methanolic fraction showed activity in the preliminary screening (Table 5.8), however none of the tested compounds presented anti-bacterial (*Staphylococcus aureus*, *Escherichia coli*) or antifungal activities (*Candida albicans*, *Aspergillus fumigatus*) in the tested range. It could be assumed that the individual compounds may act synergistically in the crude fractions that were found active. A few investigations have reported anti-bacterial properties of sesquiterpenes (Al-Yahya *et al*., 1998; Iric *et al*., 2012; Dastan *et al*.,2016); however, such activity is

surely related to certain structural variations that may not be met here. Also, it could be emphasized that such compounds may act as prodrugs and therefore could get activated in gastrointestinal tract and/or after absorption.

Compound **1** (fnarthexol) was moderately active against *Plasmodium falciparum* K1 (IC_{50} 22.4 μM), whereas equal levels of inhibition were observed for *Trypanosoma brucei* (IC_{50} 8.1 μM), *Trypanosoma cruzi* (IC_{50} 8.6 μM) and *Leishmania infantum* (IC_{50} 6.8.0 μM), accompanied however by PMM cytotoxicity (IC_{50} 8 μM).

Structurally similar compounds (**3**, **4**, and **5**) also were active in the antiprotozoal assays. Compound **4** was found most active against *Plasmodium falciparum* K1 (IC_{50} 1.3 μM), while a moderate activity against *Trypanosoma brucei* (IC_{50} 32.6 μM), *Trypanosoma cruzi* (IC_{50} 10.5 μM) and *Leishmania infantum* (IC_{50} 12.7 μM) that was accompanied however by PMM cytotoxicity (IC_{50} 32.0 μM). Compound **3** also displayed interesting activity against *Plasmodium falciparum* K1 (IC_{50} 7.4 μM), with a moderate activity against *Trypanosoma brucei* (IC_{50} 32.4 μM), *Trypanosoma cruzi* (IC_{50} 19.1 μM) and *Leishmania infantum* (IC_{50} 12.7 μM) that was accompanied by PMM cytotoxicity (IC_{50} 32.0 μM).

Similarly compound **5** presented activity against *Plasmodium falciparum* K1 (IC_{50} 16.0 μM), while a moderate activity was observed against *Trypanosoma brucei* (IC_{50} 32.4 μM), *Trypanosoma cruzi* (IC_{50} 9.4 μM) and *Leishmania infantum* (IC_{50} 32.4 μM) that was accompanied by PMM cytotoxicity (IC_{50} 32.0 μM). All tested compounds demonstrated mild cytotoxicity against MRC-5 cells.

Table 5.10. Antimicrobial, antifungal cytotoxic and antiprotozoal activities of isolated compounds from *Ferula narthex* (IC_{50}, µM)

Compound	**IC_{50} (µM)**									
	MRC5	*Pf-K1*	*T. bruc*	*T. cruz*	*L. inf*	*PMM*	*S. aureus*	*E. coli*	*C. albicans*	*A. fumigatus*
1	8.0	22.4	8.1	8.6	6.8	8	> 64	> 64	> 64	> 64
2	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT
3	31.7	7.4	32.4	19.1	12.7	32	> 64	> 64	> 64	> 64
4	11.7	1.3	32.6	10.5	12.7	32	> 64	> 64	> 64	> 64
5	20.4	16.0	32.4	9.4	32.4	32	> 64	> 64	> 64	> 64
6	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT

Reference: Tamoxifen (MRC-5), IC_{50} 10.48 µM; Suramine (*T.bruc*), IC_{50} 0.03 µM, Chloroquine (*Pf-K1*), IC_{50} 0.08 µM, Miltefosine (*L. inf*), IC_{50} 9.02 µM; Benznidazol (*T. cruz*), IC_{50} 2.13 µM; Erythromycin (*S. aureus*) IC_{50} 11.30 µM; Chloramphenicol (*E. coli*), IC_{50} 2.42 µM; Miconazole (*C. albicans*), IC_{50} 4.70 µM; Terbinafine (*A. fumigatus*), IC_{50} 1.38 µM.

MRC-5: human fetal lung fibroblasts;, Pf-K1; *Plasmodium falciparum*; *T. bruc*: *Trypanosoma brucei; T. cruz*: *Trypanosoma cruzi; L. inf: Leishmania infantum*; PMM: peritoneal murine macrophages; *S. aureus: Staphylococcus aureus; E. coli: Escherichia coli; C. albicans: Candida albicans; A. fumigatus: Aspergillus fumigatus*

5.5. SUMMARY AND CONCLUSION

Ferula narthex Boiss. is a well-recognised medicinal plant in Pakistan. Its milky exudate obtained from bark is commonly used as a spice in everyday food and also as part of certain folk medicines in the subcontinent, such as "Unmadnashak Ghrita". Traditionally it is used to treat a number of ailments including antimicrobial infections and diabetes mellitus. Therefore we performed a detailed phytochemical and biological investigation on the exudate of *Ferula narthex* and have isolated a series of compounds. The isolated compounds were analysed for their antiglycation properties (inhibition of Advanced Glycation Endproducts-AGEs), and antimicrobial activities, as part of running programmes in the host laboratory.

A total of 6 compounds were isolated (sesquiterpene- substituted coumarins) including fnarthexol (**1**), ligupersin A (**2**), 8-acetoxy-5-hydroxyumbelliprenin (**3**), asacoumarin A (**4**), 10' R- karatavicinol (**5**) and 10′-acetoxy-11′-hydroxyumbelliprenin (**6**).

In the antiglycation assays (inhibition of AGEs formation) compound **2** displayed the highest activity (IC_{50} 0.414 mM) in the BSA-glucose assay, followed by compound **4** (IC_{50} 1.83 mM). Compounds **2-6** presented moderate inhibition in the BSA-glucose assay. In the BSA-MGO assay, compound **3** presented the highest activity (IC_{50} 1.03 mM), followed by compounds **1** (IC_{50} 1.71 mM) and **5** (IC_{50} 1.86 mM), indicating their potential to inhibit non-oxidative pathways of AGEs formation. Compounds **2**, **3** and **6** presented moderate inhibition in the same assay. It was evident that both oxidative and non-oxidative pathways contributed towards the antiglycation activity of isolated compounds.

In the antimicrobial assay for the crude extracts and fractions the methanolic fraction was considered as highly active, i.e. an IC_{50} 19.9 µg/mL against *Staphylococcus aureus* and 22.0 µg/mL against *Microsporum canis*. However, this fraction was found to be cytotoxic towards MRC-5 cells (IC_{50} 36.5 µg/mL). Similarly, the *n*-hexane fraction was active (IC_{50} 8.3 µg/mL) against *Microsporum canis,* whereas the chloroform fraction was cytotoxic (IC_{50} 49.8 µg/mL) against MRC-5 cells.

Nevertheless in the antimicrobial screening of isolated constituents, none of the tested compounds demonstrated anti-bacterial (*Staphylococcus aureus*, *Escherichia coli*) or antifungal activities (*Candida albicans*, *Aspergillus fumigatus*) in the test range. However, all tested compounds (**1**, **3**, **4** and **5**) were found active in the antiprotozoal and antileishmanial assays. Compound **1** was active against *Plasmodium falciparum* K1 (IC_{50} 22.4 μM), whereas equivalent inhibitions were recorded for *Trypanosoma brucei* (IC_{50} 8.1 μM), *Trypanosoma cruzi* (IC_{50} 8.6 μM) and *Leishmania infantum* (IC_{50} 8.0 μM). Among compounds **3**, **4** and **6** that are structurally similar, compound **4** was the most active one against *Plasmodium falciparum* K1 (IC_{50} 1.3 μM), while moderate activities were observed against other protozoal organisms.

Based on findings of the current investigation, it could be hypothesized that the isolated constituents may contribute in part to the traditional use of *Ferula narthex* in diabetic conditions and for antimicrobial applications.

REFERENCES

Abd EL-Razek MH, Wu YC, Chang FR.2007.Sesquiterpen coumarins from *Ferula foetida*. *J Chin Chem Soc* **54:** 235-238.

Achliya GS, Wadodkar SG, Dorle AK. 2004. Evaluation of sedative and anticonvulsant activities of Unmadnashak Ghrita. *J Ethnopharmacol* **94**:77–83

Ahmad AA. 1999. Sesquiterpene coumarin and sesquiterpene from *Ferula sinaica. Phytochemistry* **50**: 109-112.

Alam M, Khan A, Wadood A, Khan A, Bashir S, Aman A, Jan AK, Rauf A, Ahmad B, Khan AR, Farooq U. 2016. Bioassay-Guided Isolation of Sesquiterpene Coumarins from *Ferula narthex* Bioss: A New Anticancer Agent. *Front Pharmacol* **7:** 26.

Al-Yahya MA, Muhammad I, Mirza HH, El-Feraly FS. 1998. Antibacterial Constituents from the Rhizomes of *Ferula communis*. *Phytother Res* **12**: 335–339.

Appendino, G., Tagliapietra, S., Nano, G.M., Jakupovic, J., 1994. Sesquiterpene coumarin ethers from asafetida. *Phytochemistry* 35, 183–186.

Bandyopadhyay D, Basak B, Chatterjee A, Lai TK, Banerji A, Banerji J, Neuman A, Prange T. 2006. Saradaferin, a new sesquiterpenoid coumarin from *Ferula assafoetida*. *Nat Prod Res* **20**: 961–965.

Bashir S, Alam M, Ahmad B, Aman A, Ali J. 2013. Screening of *Ferula narthex* Boiss Crude Methanolic Extract for Analgesic, Gastrointestinal Motility and Insecticidal Activity. *Middle-East J Sci Res* **14:** 471-475.

Bashir S, Alam M, Ahmad B, Aman A. 2014a. Antibacterial, Anti-fungal and Phytotoxic activities of *Ferula narthex* Boiss. *Pak J Pharm Sci* **27:** 1819-1825

Bashir S, Alam M, Adhikari A, Shrestha RM, Yousaf S, Ahmad B, Parveen S, Aman A, Choudhary MI. 2014b. New antileishmanial sesquiterpene coumarins from *Ferula narthex* Boiss. *Phytochem Lett* **9**: 46–50.

Dastan D, Salehi P, Aliahmadi A, Gohari AR, Maroofi H, Ardalan A. 2016. New coumarin derivatives from *Ferula pseudalliacea* with antibacterial activity. *Nat Prod Res* 1-7 **doi:**10.1080/14786419.2016.1149705

Egwaikhide PA, Gimba CE. 2007. Analysis of the phytochemical content and antimicrobial activity of Plectranthus glandulosis whole part. Middl East J Sci Res 2: 135–138.

Eigner D, Scholz D. 1999. *Ferula asa-foetida* and *Curcuma longa* in traditional medical treatment and diet in Nepal. *J Ethnopharmacol* **67**: 1–6

Farnsworth NR.1966. Biological and phytochemical screening of plants. *J Pharm Sci* **55**: 225–276.

Herborne JB. 1973. Phytochemical Methods 3rd Edn. Chapman and Hall Ltd., London, pp: 135-203.

Indrayan A, Agrawal P, Rathi AK, Shatru A, Agrawal NK and Tyagi DK 2009. Nutritive value of some indigenous plant rhizomes resembling Ginger. *Nat Prod Rad* **8**: 507-513.

Iranshahi M, Masullo M, Asili A, Hamedzadeh A, Jahanbin B, Festa M Piacente S. 2010a. Sesquiterpene coumarins from *Ferula gumosa*. *J Nat Prod* **73**:1958-62.

Iranshahi M, Kalategi F, Shebkar A, Sardashti, Schneider B. 2010b. New sesquiterpene coumarins from roots of *Ferula flabelliloba. Pharm Biol 48:* 217-220.

Iranshahy, M., Iranshahi, M., 2011. Traditional uses, phytochemistry and pharmacology of asafoetida (*Ferula assafoetida* oleo-gum-resin) – a review. *J Ethnopharmacol* **134**: 1–10.

Iranshahi M, Barthomuef C, Bayet-Robert M, Chollet P, Davoodi D, Piacente, Rezaee R, Sahekbar A. 2014. Drimane-Type sesquiterpene coumarins from ferula gummosa fruits enhance Doxorubincin uptake in Doxorubicin-resistant human breast cancer cell lines. *J Trad Med* **4**:118-125.

Iric AC, Kariotia A, Koukoulitsaa C, Sokovic M, Skaltsa H. 2012. Sesquiterpene Lactones from *Centaurea zuccariniana* and Their Antimicrobial Activity. *Chem Biodivers* **9**: 2843-285.

Kakari SA, Tareen RB, Sandhu ZU, Kakar MA, Kakar SR, Iqbal Z, Jabeen H. 2013. In vitro and in vivo anthelminthis activity of *Ferula costata* (KOR.) against gastrointestinal nematodes of sheep. *Pak J Bot* **45**(SI): 263-268.

Kapoor LD. 1990. Handbook of Ayurvedic medicinal plants. Boca Raton: CRC Press: 184

Khan N, Ahmed N, Ahmed A, Shaukat SS, Wahab M, Ajaib M, Siddiqui MF, Nasir M. 2011. Important medicinal plants of Chitral Gol National Park (CGNP) Pakistan. *Pak J Bot* **43**: 797-809.

Kiryalov NP, Bagirov VY. 1969. The structure of Karatavicinol**.** *Chem Nat Comp* **15**: 191-192.

Kajimoto T, Yahiro K, Nohara T. 1989. Sesquiterpene and disulphide derivative from ferula Assaofoetida. *Phytochemistry* **28:** 1761-1763.

Lee CL, Chiang LC, Cheng LH, Liaw CC, Abd El-Razak MH, Chang FR, Wu YC. 2009. Influenza A (H_1N_1) Antiviral and Cytotoxic Agents from *Ferula assa-foetida. J Nat Prod* **72 :** 1568–1572

Lierman JC, Thines E, Opatz T, Anke H. 2012. Drimane Sesquiterpenoids from *Marasmius* sp. Inhibiting the Conidial Germination of Plant-Pathogenic Fungi. *J Nat Prod* **75** : 1983–1986

Mahendra P, Bisht S. 2012. *Ferula asafoetida*: Traditional uses and pharmacological activity. *Pharmacogn Rev* **6**: 141.

Marco JA, Sanz JF, Yuste A, Rustaiyan. 1991. New Umbelliferon sesquiterpene ethers from roots of *Ligulari persica*. *Liebigs Ann Chem* **1991**: 929-931.

Nazari ZE, Iranshahi M. 2011. Biologically active sesquiterpene coumarins from *Ferula* species. *Phytother Res* **25**:315-23. doi: 10.1002/ptr.3311. Epub 2010 Oct 28.

Pimenov MG, Leonov MV. 2004. The Asian Umbelliferae biodiversity database (ASIUM) with particular reference to South-West Asian taxa. *Turk J Bot* **28**: 139–145

Senguttuvan J, Paulsamy, S., Karthika K. 2014. Phytochemical analysis and evaluation of leaf and root parts of the medicinal herb, *Hypochaeris radicata* L. for in vitro antioxidant activities. *Asian Pac J Trop Biomed* **4**(Suppl 1)**:** 359-367.

Shinwari ZK, Gilani SS. 2003. Sustainable harvest of medicinal plants at Bulashbar Nullah, Astore (Northern Pakistan). *J Ethnopharmacol* **84**(2-3):289-98.

Sofowora A. 1993. Medicinal plants and traditional medicine in Africa. John Wiley and son Ltd., 150-153.

Srinivasan K. 2005. Spices as influencers of body metabolism: An overview of three decades of research. *Food Res Inter* **38**: 77-86.

Teng L, Ma GZ, Li L, Ma LY, Xu XQ. 2013.Karatavicinol a, a new anti-ulcer sesquiterpene coumarin from *Ferula sinkiangensis. Chem Nat Prod 49: 60-66*

Trease GE, Evans WC. 1989. Pharmacognosy.11th Edn. Brailliar Tiridel and Macmillian Publishers, London.

Zhou P, Takaishi Y, Duan H, Chen B, Honda G, Itoh M, Takeda Y, Kodzhimatov OK, Lee KH. 2000. Coumarins and bicoumarin from *Ferula sumbul*: anti-HIV activity and inhibition of cytokine release. *Phytochemistry* **53**: 689-97.

CHAPTER 6
GENERAL CONCLUSIONS AND FUTURE PERSPECTIVES

During the current project three medicinal plants from Pakistan were investigated for their phytochemical constituents and biological activities. These medicinal plants were selected based on the fact that they were used traditionally for the treatment of diabetes mellitus and microbial infections by local people. Also the traditional healers use them in formulations which are based on the "Ayurveda", "Unani" or "Tib" medicinal systems of the Indian subcontinent.

From *Nymphoides indica* leaves extracts a series of seco-iridoids and flavonoids was isolated. All crude extracts, fractions and compounds were tested for a number of biological activities. We were able to conclude that the extracts possessed moderate antimicrobial and antidiabetic activities. It was emphasised that the phenomenon of synergism and of metabolic conversions of constituents after oral intake may explain its traditional medicinal use, in spite of rather moderate *in vitro* activities.

For *Kickxia ramosissima*, we were able to isolate some iridoids and flavonoids, which were tested for various biological activities. It could be concluded that the crude extracts, fractions and isolated compounds presented some promising activities including antidiabetic effects, for which the plant is known. Similarly to *N. indica*, synergistic effects and the occurrence of many glycosylated compounds that are metabolised after oral intake could possibly play a key role in the pharmacological activity of *Kickxia ramosissima.*

Finally some coumarin-sesquiterpenes were isolated from the *Ferula narthex* exudate. Based on our findings, it was hypothesized that the isolated constituents may contribute in part to the traditional use of *Ferula narthex* in diabetic conditions and for antimicrobial applications. As we were able to present some antiprotozoal and anti-AGEs compounds, further detailed investigations are suggested for *in vivo* blood glucose lowering effects and a broader screening for antimicrobial analysis.

Although no highly active constituents have been identified, this study has contributed to the phytochemical and biological knowledge of three medicinal plants widely used in Pakistan. The results of the present investigation may in part support their traditional use against microbial infections and/or in diabetic conditions. Especially for

Nymphoides indica and Kickxia ramosissima, the possibility that prodrugs such as iridoid- and flavonoid glycosides, showing weak in vitro activities, are deglycosylated and metabolically activated after oral administration, cannot be excluded, and should be investigated in more detail. As indeed *in vitro* assays cannot really mimic *in vivo* situations, we suggest further *in vivo* trials of isolated constituents and the crude extracts. Based on the constituents isolated in the course of this project, these extracts can now fully be characterised, which is a necessary condition for performing reliable and reproducible *in vivo* assays.

As the traditional medicinal therapy is mainly based on observations obtained on human beings by traditional healers, it would be very interesting if the selected plants extracts were further evaluated in controlled clinical trials.

As traditionally these plant species are mostly used in formulations, it would also be very interesting to explore the other plants of these formulations individually. The comparison of the *in vivo* evaluation of the individual plants of such formulation and that of the total formulation would give us a better understanding of the therapy and more comprehensive conclusions could be made.

SUMMARY

Traditional medicine (TM) is an essential part of the health care system in the Indian subcontinent, including Pakistan. The roots of this system are as old as human civilization. Various forms of TM are practiced in this region including Ayurveda, Rigveda, Siddha, Tib and Yunani. These systems are either based on religious traditions or believes of super natural “healing powers” that are associated with the “earth's natural systems” that include medicinal plants and animal species, ambient healthy air and spring water. Even modern scientific techniques have confirmed the concept of personalised medicine, as it has been used for centuries in the traditional medicinal system of India. Irrespective of its scientific basis, religious believes, easy availability and effectiveness are the major reasons for acceptance of TM in this region.

This project focused on some medicinal plants traditionally used in diabetic conditions, and for antimicrobial applications, as part of running research programmes in the host laboratory.

Diabetes mellitus is a common chronic disorder that has become a challenging disease these days because of its continuously increasing prevalence world-wide, especially in developing nations. This growing prevalence is primarily due to bad life style, progressive urbanization, health status and social set up of the population. The chronic nature of the disease could lead to increased risk of severe complications, for instance kidney failure, blindness, cardiovascular diseases and foot ulcers. Diabetes mellitus is a multifarious metabolic disorder involving carbohydrate, fat and protein metabolism, that results from either insufficiency or dysfunction of insulin, ending up in elevated fasting and postprandial blood sugar levels.

Microbial infections are considered as major health issues in developing countries including Pakistan that constitute a major part of total annual death count. Poor hygiene, lack of proper health facilities and lack of knowledge are the major reasons for this widespread health concern in developing countries. Moreover, excessive and over-usage of antibiotics, leading to development of resistance has become critical.

Likewise, protozoal infections also contribute significantly to the total health burden in Pakistan. Geographical location, poor economic conditions, lack of health facilities and

heavy uncontrolled migration of Afghan refugees are major reasons for the widespread prevalence of protozoal infections in Pakistan. Cutaneous leishmaniasis (*Leishmania major, Leishmania tropica*) and malaria (*Plasmodium vivax* for 64% and *Plasmodium falciparum* for 26%) are main protozoal infection in Pakistan. Also, malaria accounts for 16% of the total disease burden of the country.

Although a range of drugs are available for the treatment of all such diseases, the cost of therapy, side effects and development of resistance in the cases of antimicrobial drugs, generally limit their use. Local people look towards traditional medicine for treatment of diabetes mellitus, microbial and protozoal infections. Keeping in mind the effectiveness and acceptance of TM, we selected some traditional medicinal plants and investigated their potential against some aspects of diabetes mellitus, and microbial infections.

Phytochemical investigations of ***Nymphoides indica*** extracts and fractions led to the isolation and identification of 15 compounds including 5 lipophilic compounds, i.e. the dicarboxylic acids azelaic acid (nonanedioic acid) and 4-methyl-heptanedioic acid, the monocarboxylic acids hexadecanoic acid and stearic acid and the fatty alcohol hexadecanol; 3 seco-iridoids, i.e. 7-epiexaltoside, 6",7"-dihydro-7-epiexaltoside (reported for the first time from nature) and menthiafolin; 3 flavonoids, i.e. 3,7-di-*O*-methylquercetin-4'-O-β-glucoside, 3-*O*-methylquercetin-7-*O*-β-glucoside and 3,7-di-*O* methylquercetin; scopoletin and ferulic acid; and the monoterpenoids foliamenthoic acid and 6,7-dihydrofoliamenthoic acid methyl ester.

In the antidiabetic *in vitro* assays various fractions were assayed for potential antiglycation and α-glucosidase inhibitory activity. All tested fractions presented moderate inhibition (24-36% at a concentration of 100 µg/mL) of the formation of AGEs. Similarly mild α-glucosidase inhibitory activity was observed for the total extract at 834 µg/mL (13% inhibition), whereas the highest inhibition was shown by the methanolic (31% inhibition) and *n*-butanol fractions (25% inhibition) at the same concentration. At higher test concentrations the inhibitory activity did not further increase, and the 50% inhibition level could not be reached. Among isolated constituents interesting antiglycation activities were shown by 6",7"-dihydro-7-

epiexaltoside (IC_{50} 0.36 mM), 3-*O*-methylquercetin-7-*O*-β-glucoside (IC_{50} 0.42 mM) *and* 6,7-dihydrofoliamenthoic acid methyl ester (IC_{50} 0.61 mM). Finally mild to moderate α-glucosidase inhibition was shown by 3,7-di-*O*-methylquercetin-4'-O-β-glucoside, 3-*O*-methylquercetin-7-*O*-β-glucoside and 3,7-di-*O* methylquercetin; scopoletin, ferulic acid, foliamenthoic acid and 6,7-dihydrofoliamenthoic acid methyl ester.

In the antimicrobial assays of the different fractions, the highest levels of inhibition were observed for the *n*-hexane fraction, i.e. an IC_{50} of 19.5 μg/mL against *S. aureus* and 32.0 μg/mL against *M. canis*. Similarly the methanol 90% fraction was active (IC_{50} 36.4 μg/ml) against *S. aureus* and cytotoxic (IC_{50} 38.9 μg /mL) against MRC-5 cells. Among the isolated constituents the lipophilic compounds showed moderate antimicrobial activities, whereas 3,7-di-*O*-methylquercetin-4'-O-β-glucoside presented mild antiprotozoal activities against *Trypanosoma brucei* (IC_{50} 8.0 μM), *Leishmania infantum* (IC_{50} 32.0 μM) and *Trypanosoma cruzi* (IC_{50} 30.0 μM). It was concluded that *N. indica* leaf extracts possess mild to moderate antimicrobial, antiprotozoal, antioxidant and antidiabetic activities. The traditional use of this plant species can possibly be due to synergism between its constituents, and to metabolic conversions (deglycosylations) of iridoids and flavonoid glycosides after oral intake.

The study on ***Kickxia ramosissima*** involved phytochemical and biological investigation of crude fractions and isolated constituents, including assessment of *in vitro* antidiabetic properties (inhibition of α-glucosidase activity; inhibition of Advanced Glycation Endproducts-AGEs), antioxidant activity (AAPH induced linoleic acid peroxidation), inhibition of 15-LOX (anti-inflammatory activity) and antimicrobial activities.

A total of 7 compounds were isolated including iridoids, i.e a new compound kickxiasine, mussaenosidic acid, mussaenoside, linarioside, the flavonoids pectolinarigenin and pectolinarin, and the benzoic acid derivative 4-hydroxy-benzoic acid methyl ester.

In the antidiabetic assays of crude extracts and fractions, the highest inhibition was recorded for the ethyl acetate fraction (IC_{50} 88.0 μg/mL) whereas moderate levels of

antiglycation effects were observed for all other fractions. Also the isolated iridoids showed weak inhibition in the BSA-glucose assay, and comparable results in the BSA-MGO assay. The flavonoids, however, showed interesting results as pectolinarigenin was highly active (IC_{50} 0.79 mM) followed by pectolinarigenin with IC_{50} 2.29 mM. Also in the BSA-MGO assay both flavonoids showed high levels of inhibition i.e pectolinarigenin IC_{50} 0.19 mM and pectolinarigenin IC_{50} 0.13 mM. This trend clearly indicated the inhibition through non-oxidative pathways. The benzoic acid derivative did not present any activity in all AGEs experiments. In the α-glucosidase inhibition assay the iridoids presented a weak inhibition. However, with regard to the flavonoids, pectolinarigenin showed an interesting activity (IC_{50} 0.23 mM), whereas moderate inhibition was observed for pectolinarin and the benzoic acid derivative.

In the 15-LOX experiment, moderate inhibition was recorded for pectolinarigenin, mussaenoside and kickxiasine. In the AAPH assay, weak or no inhibition was recorded for all compounds.

In the antimicrobial screening panel the *n*-hexane fraction presented activity against *Staphylococcus aureus* (IC_{50} 8.0 μg/mL) and *Microsporum canis* (IC_{50} 24.4 μg/mL), whereas none of the other fractions presented any activity in the test range. Among isolated constituents, only pectolinarigenin showed moderate activity against *Plasmodium falciparum K1* (IC_{50} 41.8 μM), *Staphylococcus aureus* (IC_{50} 49.8 μM) and *Trypanosoma cruzi* (IC_{50} 30.0 μM) with no cytotoxicity. Based on our findings we could demonstrate the moderate antidiabetic, anti-inflammatory and antimicrobial activity of some constituents of *Kickxia ramosissima.*

As mentioned before for *N. indica*, the traditional use of this plant species can possibly be due to synergism between its constituents, and to metabolic conversions (deglycosylations) of iridoids and flavonoid glycosides after oral intake.

During the phytochemical analysis of crude extracts of ***Ferula narthex*** exudate**,** 6 compounds (sesquiterpene-substituted coumarins) were isolated, including fnarthexol, ligupersin A, 8'-acetoxy-5'-hydroxyumbelliprenin, asacoumarin A, 10'*R*-karatavicinol and 10′*R*-acetoxy-11′-hydroxy umbelliprenin.

In the antidiabetic assays ligupersin A displayed the highest activity (IC_{50} 0.414 mM) in the BSA-Glucose assay, followed by asacoumarin A (IC_{50} 1.83 mM). In the BSA-MGO assay, 8'-acetoxy-5'-hydroxyumbelliprenin presented the highest activity (IC_{50} 1.03 mM), followed by feselol (IC_{50} 1.71 mM) and 10'*R*-karatavicinol (IC_{50} 1.86 mM). It was evident that both oxidative and non-oxidative pathways contributed towards the antiglycation activity of the isolated compounds.

In the antimicrobial assays the methanolic fraction was considered as highly active, i.e. an IC_{50} 19.9 µg/mL against *Staphylococcus aureus* and IC_{50} 22.0 µg/mL against *Microsporum canis*. However, this fraction was found cytotoxic towards MRC-5 cells (IC_{50} 36.5 µg/mL). Similarly the *n*-hexane fraction was active (IC_{50} 8.3 µg/mL) against *Microsporum canis,* whereas the chloroform fraction was found cytotoxic (IC_{50} 49.8 µg/mL) against MRC-5 cells.

In the antimicrobial screening of the isolated constituents, none of the isolated compounds demonstrated anti-bacterial or antifungal activities. However, moderate antiprotozoal and antileishmanial activities were noticed for all tested compounds.

Fnarthexol was moderately active against *Plasmodium falciparum* K1 (IC_{50} 22.4 µM), whereas equivalent inhibitions were recorded for *Trypanosoma brucei* (IC_{50} 8.1 µM), *Trypanosoma cruzi* (IC_{50} 8.6 µM) and *Leishmania infantum* (IC_{50} 8.0 µM). Among the other compounds that are structurally similar, asacoumarin A was the most active one against *Plasmodium falciparum* K1 (IC_{50} 1.3 µM), while moderate activities were observed against other protozoal organisms. Also mild cytotoxicity against MRC-5 cells was observed for all tested compounds.

Based on findings of the current investigation, it could be hypothesized that the isolated constituents may contribute in part to the traditional use of *Ferula narthex* in diabetic conditions and for antimicrobial applications.

SAMENVATTING

Traditionele geneeskunde is zo oud als de menselijke beschaving. De traditionele geneeskunde maakt een essentieel deel uit van de gezondheidszorg in het subcontinent Indië, waaronder Pakistan. In deze regio worden verschillende vormen van traditionele geneeskunde beoefend: de Ayurveda, Rigveda, Siddha, Tib en Yunani. Deze systemen zijn gebaseerd op religieuze tradities en geloof in bovennatuurlijke krachten, die geassocieerd zijn met aardse natuurlijke systemen, zoals medicinale planten en dieren, gezonde lucht en bronwater. Het concept van gepersonaliseerde geneeskunde, dat nu ook ingang vindt in de Westerse geneeskunde, wordt reeds vele eeuwen in de traditionale geneeskunde van Indië toegepast. Los van de wetenschappelijke basis, zijn religieuze opvattingen, beschikbaarheid en efficaciteit de voornaamste redenen voor de populariteit van traditionele geneeskunde.

In dit project staan enkele traditionele medicinale planten centraal, gebruikt bij diabetes en infectieziekten, als onderdeel van lopende onderzoeksprogramma's in deze domeinen in het gastlaboratorium. Diabetes mellitus is een veel voorkomende chronische ziekte, waarvan de prevalentie wereldwijd toeneemt, ook in ontwikkelingslanden. Deze toename is in de eerste plaats te wijten aan slechte voedingsgewoonten, voortschrijdende verstedelijking, de algemene gezondheidsstatus en sociale structuur van de bevolking. De chronische aard van deze aandoening kan leiden tot een verhoogd risico op complicaties, zoals nierfalen, blindheid, cardiovasculaire problemen en voetzweren. Ook infectieziekten anderzijds worden beschouwd als grote gezondheidsproblemen in ontwikkelingslanden, ook in Pakistan, die verantwoordelijk zijn voor een hoge jaarlijkse dodentol. Dit is voornamelijk te wijten aan slechte hygiëne, gebrek aan goede faciliteiten in de gezondheidszorg, en gebrek aan kennis over deze ziekten.

Ook protozoale infecties dragen aanzienlijk bij aan de totale ziektelast in Pakistan. Geografische ligging, de slechte economische omstandigheden, gebrek aan medische voorzieningen en zware ongecontroleerde migratie van Afghaanse vluchtelingen zijn belangrijke redenen voor de wijdverbreide prevalentie van protozoaire infecties in Pakistan. Cutane leishmaniasis (*Leishmania major*, *Leishmania tropica*) en malaria (*Plasmodium vivax* voor 64% en *Plasmodium falciparum* voor 26%) zijn de belangrijkste protozoaire infecties in Pakistan. Maartdaarme 16% van de totale ziektelast van het land uit.

Alhoewel heel wat geneesmiddelen beschikbaar zijn, vormen de kostprijs, de beschikbaarheid, de neveneffecten en in het geval van infectieziekten het ontstaan van resistentie een aantal beperkende factoren, waardoor de plaatselijke bevolking zijn toevlucht neemt tot de traditionele geneeskunde. Om deze reden werden enkele traditionele medicinale planten geselecteerd, met als doel hun potentiële bruikbaarheid na te gaan bij diabetes en bij infectieziekten.

Fytochemisch onderzoek van extracten van de bladeren van ***Nymphoides indica*** leidde tot de isolatie en identificatie van 15 inhoudsstoffen, waaronder 5 lipofiele producten (de dicarbonzuren azelainezuur (nonaandicarbonzuur) en 4-methyl-heptaandicarbon-zuur; de monocarbonzuren palmitinezuur en stearinezuur; en het vetalcohol hexadecanol); 3 seco-iridoiden (7-epiexaltoside; 6",7"-dihydro-7-epiexaltoside, voor de eerste keer gerapporteerd; en menthiafoline); 3 flavonoiden (3,7-di-*O*-methylquercetin-4'-*O*-β-glucoside; 3-*O*-methylquercetin-7-*O*-β-glucoside; en 3,7-di-*O* methylquercetin); scopoletine en ferulazuur; en de monoterpenen foliamenthoine zuur en 6,7-dihydrofoliamenthoine zuur methyl ester.

In de antidiabetische *in vitro* testen vertoonden alle geëvalueerde subfracties een matige activiteit tegen proteïn-glycatie (24-36% inhibitie van de vorming van Advanced Glycation Endproducts (AGEs) bij een concentratie van 100 µg/mL), en als inhibitor van α-glucosidase activiteit (tot 31% inhibitie bij een concentratie van 834 µg/mL voor het methanolisch extract). Wat betreft de geïsoleerde producten werden voor de anti-glycatie werking, de hoogste activiteiten gevonden voor 6",7"-dihydro-7-epiexaltoside (IC_{50} 0.*36* mM), 3-*O*-methylquercetin-7-*O*-glucoside (IC_{50} 0.*42* mM) *en* 6,7-dihydro-foliamenthoine zuur methyl ester (IC_{50} 0.61 mM); terwijl 3,7-di-*O*-methylquercetin-4'-O-β-glucoside; 3-*O*-methylquercetin-7-*O*-β-glucoside; 3,7-di-*O* methylquercetin; scopoletine; ferulazuur; foliamenthoine zuur en 6,7-dihydrofoliamenthoine zuur methyl ester meest actief waren als inhibitor van α-glucosidase.

In de antimicrobiële testen was de n-hexaan fractie het meest actief, met een IC_{50} van 19.5 µg/mL tegen *Staphylococcus aureus* en 32.0 µg/mL tegen *Microsporum canis*. De methanol 90% fractie was actief (IC_{50} 36.4 µg/ml) tegen *S. aureus* en cytotoxisch (IC_{50} 38.9 µg /mL) op MRC-5 cellen. Wat betreft de geïsoleerde producten, vertoonden de lipofiele verbindingen een matige activiteit. 3,7-Di-*O*-methylquercetin-4'-*O*-β-

glucoside was matig actief tegen *Trypanosoma brucei* (IC_{50} 8.0 µM)*, Leishmania infantum* (IC_{50} 32.0 µM) en *Trypanosoma cruzi* (IC_{50} 30.0 µM)*.*

Extracten van de bladeren van *N. indica* bezitten dus matige antimicrobiële, anti-glycatie en α-glucosidase inhiberende werking. Het traditioneel gebruik van deze plant kan mogelijk verklaard worden door synergisme tussen de inhoudsstoffen, of door metabole omzettingen (deglycosylaties) en activatie van de seco-iridoiden en flavonoiden na orale inname.

Uit ***Kickxia ramosissima*** werden 7 inhoudsstoffen geïsoleerd, meer bepaald de iridoiden kickxiasine (een nieuw product), mussaenosidine zuur, mussaenoside en linarioside; de flavonoiden pectolinarigenine and pectolinarine; en 4-hydroxy-methylbenzoaat.

De hoogste anti-glycatie activiteit werd vastgesteld voor de ethyl acetaat fractie (IC_{50} 88.0 µg/mL); ook de geïsoleerde iridoiden vertoonden een matige activiteit. De flavonoiden pectolinarigenine (IC_{50} 0.79 mM) en pectolinarigenine (IC_{50} 2.29 mM) waren actiever. Ook in BSA-MGO test waren deze flavonoiden zeer actief (IC_{50} 0.19 mM en 0.13 mM, respectievelijk), wat aangeeft dat ook inhibitie van de niet-oxidatieve wegen betrokken is bij de werking. In de α-glucosidase inhibitie test waren de iridoiden slechts zwak actief, in tegenstelling tot de flavonoiden, met een IC_{50} waarde van 0.23 mM voor pectolinarigenine.

In de antimicrobiële screening was de *n-hexaan fractie actief tegen Staphylococcus aureus* (IC_{50} 8.0 µg/mL) en *Microsporum canis* (IC_{50} 24.4 µg/mL). Enkel *pectolinarigenine was matig actief tegen Trypanosoma cruzi* (IC_{50} 30.0 µM*), Plasmodium falciparum* K1 (IC_{50} 41.8 µM) en *Staphylococcus aureus* (IC_{50} 49.8 µM), in de afwezigheid van cytotoxiciteit.

Zoals reeds vermeld voor *N. indica*, kan het traditioneel gebruik van *K. ramosissima* mogelijk verklaard worden door synergisme tussen de inhoudsstoffen, of door metabole omzettingen (deglycosylaties) en activatie van de iridoiden en flavonoiden na orale inname.

Uit het exudaat van ***Ferula narthex*** tenslotte werden 6 bestanddelen verkregen, behorend tot de sesquiterpeen-gesubstitueerde coumarines, namelijk fnarthexol, ligupersine A, 8'-acetoxy-5'-hydroxyumbelliprenine, asacoumarine A, 10'*R*-karatavicinol

en 10′*R*-acetoxy-11′-hydroxy-umbelliprenine. Ligupersine A vertoonde de hoogste anti-glycatie activiteit (IC_{50} 0.414 mM), gevolgd door asacoumarine A (IC_{50} 1.83 mM). In de BSA-MGO test was 8'-acetoxy-5'-hydroxyumbelliprenine het meest actief (IC_{50} 1.03 mM), gevolgd door fnarthexol (IC_{50} 1.71 mM) en 10'*R*-karatavicinol (IC_{50} 1.86 mM). Zowel inhibitie van de oxidatieve als de niet-oxidatieve weg droeg dus bij tot de activiteit. De methanolische fractie vertoonde een hoge antimicrobiële activiteit (IC_{50} 19.9 µg/mL tegen *Staphylococcus aureus* en IC_{50} 22.0 µg/mL tegen *Microsporum canis*), maar was ook cytotoxisch tegen MRC-5 cellen (IC_{50} 36.5 µg/mL). The *n*-hexane fractie was actief (IC_{50} 8.3 µg/mL) tegen *Microsporum canis*. *Fnarthexol* was matig actief tegen *Plasmodium falciparum* K1 (IC_{50} 22.4 µM), *Trypanosoma brucei* (IC_{50} 8.1 µM), *Trypanosoma cruzi* (IC_{50} 8.6 µM) en *Leishmania infantum* (IC_{50} 8.0 µM). Asacoumarine A echter was zeer actief tegen *Plasmodium falciparum* K1 (IC_{50} 1.3 µM). De geïsoleerde producten kunnen dus ten minste voor een deel het traditioneel gebruik verklaren.

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EDUCATION

November 2012-2016 Ph D research fellow Department of Pharmaceutical Sciences University of Antwerp, Belgium.

March 2005-2007 M.Phil (Biotechnology) Department of Biotechnology Quaid I Azam University, Islamabad Pakistan.

May 1999-2003 Bachelors in Pharmacy, Faculty of Pharmacy, Gomal University D.I.Khan, Pakistan.

REASERACH EXPERIENCE

2012-2106 Ph D Research fellow Department of Pharmaceutical Sciences, University of Antwerp, Belgium.

Project aim: "Phychemical and biological investigations on medicinal plant from Pakistan."

Techniques used: Flash chromatography, HLPC-DAD, LC-MS-DAD, GC-MS, NMR.

2008-2011 Antimicrobial, antifungal bioactivity testing of crude plant extracts.

Techniques used: Extraction, Agar diffusion assay, broth macrodilution assay.

2005-2007 M.Phil Quaid I Azam University Islamabad Pakistan.

Project aim: "Studies on resistivity pattern of clinical isolates of *Klebsiella pneumonia".*

Techniques used : API 20E, Culture sensitivity, Agar dilution, Broth dilution methods.

SCIENTIFIC TECHNIQUES

Flash chromatography, HLPC DAD, LC-DAD-MS, GC-MS, NMR.

PROJECT SKILLS

- Experimental protocol: designing, planning and budget management
- Effective presentations
- Training and management of master students
- Computing: Word, Power point, Excel, Access, Chemdraw, NMR predict

MEMBERSHIPS

2014-2016 Society for Medicinal Plants and Natural Product Research (GA).

2013 -2015 Belgian Society of Pharmaceutical Sciences (BSPS).

2009-2016 National Academy of Young Scientists (NAYS), Pakistan.

2006-2007 Pakistan Society for Microbiology (PSM).

2003-2005 Pakistan Pharmacist Association (PPA).

PROFESSIONAL EXPERIENCE

2015-continued Assistant Professor Gomal University, D.I.Khan Pakistan.
2007-2014 Lecturer Gomal University, D.I.Khan Pakistan.
2006-2007. Hospital Pharmacist (DHQT Hospital D.I.Khan KPK Pakistan).
2005-2006 Provincial Inspector of Drugs (KPK) Pakistan.
2004-2005 Medical Information Officer, Servier Research and Pharmaceuticals (Pvt) Ltd. Pakistan.

SCIENTIFIC PUBLICATIONS

Papers in peer-reviewed journals

2016 Adnan Amin, Emmy Tuenter, Vassiliki Exarchou, Atul Upadahay, Paul Cos, Louis Maes, Sandra Apers and Luc Pieters. 2016. Phytochemical and pharmacological investigations on *Nymphoides indica* leaf extracts. Accepted for publication in Phytotherapy Research.

Adnan Amin, Emmy Tuenter, Atul Uphadaya, Paul Cos, Louis Maes, Vassiliki Exarchou, Ken Foubert, Sandra Apers and Luc Pieters. 2016. Phytochemical and biological investigations of *Kickxia ramosissima.* Submitted for publication in Journal of Ethnopharmacology.

Adnan Amin, Emmy Tuenter, Paul Cos, Louis Maes, Vassiliki Exarchou, Sandra Apers and Luc Pieters. Terpenyl Coumarins from *Ferula narthex* exudate with antiglycation and antimicrobial properties. Submitted for publication in Food Chemistry.

2014 Atul Upadhyay, Emmy Tuenter, Adnan Amin, Vasiliki Exarchou, Nina Hermans, Sandra Apers, Luc Pieters. 2014. 5-*O*-Demethylnobiletin, a polymethoxylated flavonoid, from *Citrus depressa* Hayata peel prevents protein glycation. Journal of Functional Foods, 11 243–249.

Atul Upadhyay, Emmy Tuenter, Rizwan Ahmad, Adnan Amin, Vasiliki Exarchou, Sandra Apers, Nina Hermans, Luc Pieters. 2014. Kavalactones, A Novel Class of Protein Glycation and Lipid Peroxidation Inhibitors. Planta Med 80:1001–1008.

2013 Muhammad Iqbal, Imam Bakhsh Baloch, Musa Kaleem Baloch, Muhammad Ayaz Khan, Adnan Amin and Muhammad Akram. 2013. Bio-active Compounds from *Coriandrum sativum* L. Seeds. Wulfenia, 20 (1)105-120.

Adnan Amin, Muhammad Ayaz Khan, Swahid Shah, Mushatq Ahmad, Muhammad Zafar and Abdul Hameed. 2013. Inhibitory effects of *Olea ferruginea* crude leaves extract against some bacterial and fungal pathogen. Pak J Pharm Sci 26(2) 251-254.

Oral / Poster Presentations

2015 Adnan Amin, Paul Cos ,Louis Maes, Vasiliki Exarchou, Sandra Apers and Luc Pieters. Phytochemical and Pharmacological investigations on *Kickxia ramossisima*. Planta Med 2015; 81 – YRW-4 DOI: 10.1055/s-0035-1565362.

2014 Adnan Amin, Atul Upadhyay, Muhammad Zafar, Paul Cos, Louis Maes, Sandra Apers, Vasiliki Exarchou, Luc Pieters. Antibacterial, antifungal, cytotoxic, antioxidant and antidiabetic compounds from *Nymphoides indica*; the first comprehensive phytochemical and pharmacological study. Planta Med 2014; 80 - P1L115 DOI: 10.1055/s-0034-1394772.

2013 Adnan Amin, Paul Cos, Louis Maes, Sandra Apers and Luc Pieters. Phytochemical and pharmacological investigations on medicinal plants from Pakistan. BJPS 17th Forum of Pharmaceutical Sciences. pp 2. (Congress Abstract and Oral Presentation).