EÜ FEN BİLİMLERİ ENSTİTÜSÜ

EGE UNIVERSITY

DOCTOR of PHILOSOPHY THESIS

ISOLATION and STRUCTURAL DETERMINATION of BIOLOGICALLY ACTIVE NATURAL PRODUCTS from THREE *Cephalaria* SPECIES

Veysel Umut ÇELENK

Supervisor: Prof. Dr. Süheyla KIRMIZIGÜL

Chemistry Department

Presentation Date: 05.07.2018

Bornova-İZMİR

2018

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Sayın **Veysel UMUT ÇELENK** tarafından DOKTORA tezi olarak sunulan "**Isolation and Structural Determination of Biologically Active Natural Products from Three *Cephalaria* Species**" başlıklı bu çalışma E.Ü. Lisansüstü Eğitim ve Öğretim Yönetmeliği ile E.Ü. Fen Bilimler Enstitüsü Eğitim ve Öğretim Yönergesi'nin ilgili hükümleri uyarınca tarafımızdan değerlendirilerek savunmaya değer bulunmuş ve **05.07.2018** tarihinde yapılan tez savunma sınavında aday oybirliği/oyçokluğu ile başarılı bulunmuştur.

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EÜ Lisansüstü Eğitim ve Öğretim Yönetmeliğinin ilgili hükümleri uyarınca Doktora Tezi olarak sunduğum "**Isolation and Structural Determination of Biologically Active Natural Products from Three *Cephalaria* Species**" başlıklı bu tezin kendi çalışmam olduğunu, sunduğum tüm sonuç, doküman, bilgi ve belgeleri bizzat ve bu tez çalışması kapsamında elde ettiğimi, bu tez çalışmasıyla elde edilmeyen bütün bilgi ve yorumlara atıf yaptığımı ve bunları kaynaklar listesinde usulüne uygun olarak verdiğimi, tez çalışması ve yazımı sırasında patent ve telif haklarını ihlal edici bir davranışımın olmadığını, bu tezin herhangi bir bölümünü bu üniversite veya diğer bir üniversitede başka bir tez çalışması içinde sunmadığımı, bu tezin planlanmasından yazımına kadar bütün safhalarda bilimsel etik kurallarına uygun olarak davrandığımı ve aksinin ortaya çıkması durumunda her türlü yasal sonucu kabul edeceğimi beyan ederim.

05 / 07 / 2018

Veysel UMUT ÇELENK

ÖZET

ÜÇ *Cephalaria* TÜRÜNDEN BİYOLOJİK AKTİF DOĞAL BİLEŞİKLERİN İZOLASYONU ve YAPI TAYİNİ

UMUT ÇELENK, Veysel

Doktora Tezi, Kimya Bölümü

Tez Yöneticisi: Prof. Dr. Süheyla KIRMIZIGÜL

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Cephalaria hirsuta, Cephalaria procera ve *Cephalaria elazigensis* var. *elazigensis* bitki türlerinden biyolojik aktif doğal bileşiklerin izolasyonu ve elde edilen bileşiklerin molekül yapılarının belirlenmesinin hedeflendiği bu çalışmada, kurutulmuş ve öğütülmüş bitkilerin sırasıyla MeOH, *n*-BuOH:H_2O (1:1) ve *n*-hekzan ile ektraksiyonları yapıldı. Glikozidik bileşiklerce zengin olan *n*-BuOH fazı üzerinde izolasyon ve saflandırma çalışmaları gerçekleştirildi. Doğal bileşiklerin elde edilmesi sırasında çeşitli kromatografik (TLC, VLC, MPLC, HPLC, CC) yöntemler kullanıldı.

İzolasyon ve saflandırma işlemleri sonrası; *C. hirsuta* bitkisinden **13** triterpen saponin ile **1** iridoit glikozit, *C. procera* bitkisinden **8** triterpen saponin, *C. elazigensis* var. *elazigensis* bitkisinden ise **1** iridoit aglikon elde edildi. Toplam **23** doğal bileşiğin yapıları 1D- ve 2D- NMR teknikleri kullanılarak belirlendi. Yapısı belirlenen bileşiklerden **4**'ü Caprifoliaceae familyasında, **1**'i ise *Cephalaria* cinsinde ilk kez tanımlandı.

Çalışılan bitkilerin içerdiği glikozitlerin kalitatif ve kantitatif analizleri UPLC-ESI-MS/MS cihazı ile ilk kez gerçekleştirildi. Uygulanan yöntemin geçerliliği analitik validasyon metodu ile ispatlandı. *n*-BuOH fazı üzerinde yapılan analizler sonucunda *C.hirsuta* bitkisinde **28**, *C. procera* bitkisinde **22** ve *C. elazigensis* var. *elazigensis* bitkisinde ise **4** adet glikozidik bileşiğin varlığı tespit edildi.

Anahtar sözcükler: Caprifoliaceae, *C. hirsuta, C. procera, C. elazigensis* var. *elazigensis,* Triterpen Glikozit, İridoit Glikozit, İridoit Aglikon, UPLC-ESI-MS/MS, Validasyon

ABSTRACT

ISOLATION and STRUCTURAL DETERMINATION of BIOLOGICALLY ACTIVE NATURAL PRODUCTS from THREE *Cephalaria* SPECIES

UMUT ÇELENK, Veysel

Doctor of Philosophy, Thesis in Chemistry

Supervisor: Prof. Dr. Süheyla KIRMIZIGÜL

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This study was aimed to isolation and structural determination of biologically active natural products from *Cephalaria hirsuta*, *Cephalaria procera* ve *Cephalaria elazigensis* var. *elazigensis* species. Dried and milled plants were extracted with MeOH, *n*-BuOH:H_2O (1:1) and *n*-hexane. Isolation and purification studies were performed on glycosidic compound-rich *n*-BuOH phase. Various chromatographic methods (TLC, VLC, MPLC, HPLC, CC) were employed to obtain natural products.

After isolation and purification studies; **13** triterpene saponins and **1** iridoid glycoside from *C. hirsuta*, **8** triterpene saponins from *C. procera* and **1** iridoid aglycone from *C. elazigensis* var. *elazigensis* were obtained. Structures of **23** compounds were determined by 1D- and 2D- NMR techniques. Among the structural designated compounds, **4** compounds in Caprifoliaceae family and **1** compound in *Cephalaria* genus were identified for the first time.

Qualitative and quantitative analysis of glycosidic compounds in studied plants were performed UPLC-ESI-MS/MS instrument for the first time. Validity of applied method was proven by analytical method validation. As a result of analysis on *n*-BuOH phase, **28** glycosidic compounds in *C.hirsuta,* **22** glycosidic compounds in *C. procera* and **4** glycosidic compounds in *C. elazigensis* var. *elazigensis* were detected.

Keywords: Caprifoliaceae, *C. hirsuta, C. procera, C. elazigensis* var. *elazigensis,* Triterpene Glycoside, Iridoid Glycoside, Iridoid Aglycone, UPLC-ESI-MS/MS, Validation

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SYMBOLS and ABBREVIATIONS

Symbols	**Explanations**
Hz	: Hertz
J	: Coupling Constant
m/z	: Mass/Charge
ppm	: Parts per million
δ	: Chemical Shift

Abbreviations	**Explanations**
UV-Vis	: Ultraviolet-Visible
FT	: Fourier-Transform
IR	: Infrared
NMR	: Nuclear Magnetic Resonance
1D	: One Dimensional
2D	: Two Dimensional
^{13}C-NMR	: Carbon-13 Nuclear Magnetic Resonance
^{1}H-NMR	: Proton Nuclear Magnetic Resonance
COSY	: Correlation Spectrum
TOCSY	: Total Correlation Spectrum
HSQC	: Heteronuclear Single Quantum Correlation

Abbreviations	**Explanations (Continue)**
HMBC	: Heteronuclear Multiple Bond Correlation
APT	: Attached Proton Test
RF	: Radiofrequency
Ara	: Arabinopyranosyl
Gal	: Galactopyranosyl
Glc	: Glucopyranosyl
Rha	: Rhamnopyranosyl
Xyl	: Xylopyranosyl
n-BuOH	: *n*- Butanol
GC	: Gas Chromatography
UPLC	: Ultra pressure liquid chromatography
ESI	: Electrospray ionization
UPLC-ESI-MS/MS	: Ultra pressure liquid chromatography coupled with electrospray ionization tandem mass spectrometry
HR-ESI/MS	: High Resolution Electron Spray Ionization Mass spectrometry
TLC	: Thin Layer Chromatograpy
RP	: Reverse Phase
VLC	: Vacuum-Liquid Chromatograpy

Abbreviations	**Explanations (Continue)**
CC	: Column Chromatography
Comp.	: Compound
MeOH	: Methanol
Fr.	: Fraction

1. INTRODUCTION

Natural products are chemical substances obtained from plants, microorganisms and animals: in brief various natural sources. Naturally originated compounds have been used since ancient times for medicinal purposes and daily consumptions such as wood and wood derivatives, fibers, clothings, dyeing materials, gum, resin, rubber, cleaning and personal care products. Nowadays, natural products are used lots of industrial areas especially food, textile, cosmetic, agriculture, cleaning and pharmaceutical (Bohlin et al., 2010; Dias et al., 2012).

As mentioned above nature has been a great source of therapeutic compounds for thousands of years. One of the main areas is medicine. So, the effective number of modern drugs have been isolated or derived from natural sources. Some of the top-selling drugs (approximately 40%) have been enhanced from natural products. Between 1983 and 1994, 39% of 520 new approved drugs were developed from natural resources (Sarker et. al., 2006). Most of the antibacterial and anticancer drugs (60-80%) were isolated from natural products used as origins in those years. The multiplicity of cancers were tried to be cured by natural originated drugs in clinical trials in 2000. In 2001, eight of the 30 top-selling medicines (paclitaxel, pravastatin, clavulanic acid, amoxycillin, azithromycin, ceftriaxone, cyclosporin, and simvastatin) were natural products and market share of these eight drugs reached the US $16 billion. Contribution to the exploration of new drugs was provided by natural products in three ways; new drug in an unmodified state, for synthesizing more complex molecules and by indicating new pharmacological action. Due to their “drug-like” properties and incomparable structural diversity, natural products will be major sources of new drugs in the time to come (Sarker et. al., 2006; Sarker and Nahar, 2007).

Animals, microorganisms, marine organisms and especially plants include major and most important natural products. For that reason plants have been constituted a large part of traditional medicines than other areas (Cragg et al., 1997; Newman and

Cragg, 2007). All essential organic compounds which are originated from nature (nucleotides, amino acids, phytosterols, acyl lipids and organic acids) named as "primary metabolites". Furthermore some natural compounds which have not part in vital activities like growth, development and reproduction known as "secondary metabolites" (Bennett and Wallsgrove, 1994; Bunchanan et al., 2000). Secondary metabolites are biosynthesized by an organism as a result of the organism adapting to its surrounding environment. Secondary metabolites are derived from glycolysis, photosynthesis and Krebs cycle (Dias et al., 2012).

Characterization and identification of natural originated compounds were simplified and accelerated due to spectroscopic techniques (nuclear magnetic resonance-NMR and mass spectrometry-MS), chromatographic methods (high-performance liquid chromatography-HPLC, gas chromatography-GC and medium-pressure liquid chromatography-MPLC) and highly specific *in vitro* bioassay techniques.

1.1 History of Therapeutic Plants

There is a time-honored history of the plants use as therapeutic purposes. Natural remedies for various illnesses have been recorded on a Sumerian clay tablet at 2000 BC as the earliest known document. Over 250 species were used to prepare drug recipe (Kelly, 2009).

The Chinese book *Pen T`Sao* and the Indian holy books *Vedas* includes many treatments for various illnesses and many of them are used even this century such as nutmeg, clove, pepper, camphor, ginseng, cinnamon bark, *Rhei rhizoma*, *Podophyllum* and jimson weed (Bottcher, 1965; Tucakov, 1971; Wiart, 2006).

The Ebers papyrus (Papyrus Ebers), written circa 1550 BC, has many remedies and definition for various illnesses eg. asthma, belly, cancer *etc.* This historical document describes the usage of 700 plant species as therapy such as senna, aloe,

garlic, fig, willow, castor oil plant, juniper, pomegranate etc. (Glesinger, 1954; Tucakov, 1964).

Sixty three plant species from Mycenaean, Minoan, and Egyptian Assyrian pharmacotherapy were mentioned in Homer`s epics, The Iliad and The Odysseys, circa 800 BC. Some of these species were termed after mythological characters from these epics; for instance, the name of Elecampane (*Inula helenium* L. Asteraceae) was created in memoriam Elana, who was the personage of the Trojan War. The name of the genus *Artemisia* which was believed to protect health was derived from Greek word *Artemis*. Hippocrates (459-370 BC) has classified 300 various medicinal plants by physiological action (Bojadzievski, 1992; Gorunovic et al., 2001).

Theophrast (371-287 BC) classified more than 500 medicinal plants in his books "De Causis Planetarium" and "De Historia Planetarium. " The father of botany referred to cinnamon, mint, pomegranate, cardamom, fragrant hellebore, monkshood and so on (Katic, 1958; Pelagic, 1970).

Dioscorides, "the father of pharmacognosy" was the most prominent researcher on plant drugs and wrote "De Materia Medica". Description of 944 drugs and 657 plant origins with their specifications appearance, locality, and therapeutic effects was constituted in the book. Most aforementioned domestic plants are as follows: ivy, willow, garlic, marshmallow, nettle, sage, common centaury, garlic, camomile and false helle bore. Camomile (*Matricaria recutita* L.), known as Chamaeleon is used to cure wounds, burns, and ulcers as an antiphlogistic (Nikolovski, 1961; Katic, 1980).

Slavic people used *Ocimum basilicum, Rosmarinus officinalis, Mentha Viridis,* and *Iris germanic* in cosmetic mixtures. *Veratrum album, Cucumis sativus, Urtica dioica, Achillea millefolium, Artemisia maritime* L., *Lavandula officinalis, Sambuci flos* were used against several insects (Bojadzievski, 1992).

The founder of the esteemed medical school in Salerno, Charles the Great (742 AC–814), has quoted approximately 100 plants which have been used till today. Especially sage (*Salvia officinalis L.*), which called a salvation plant (*salvare* meaning "*save, cure*"), was appreciated by the emperor Charles the Great (Tucakov, 1990; Jancic, 2002).

Beyond any doubt, many medicinal plants became widespread under favour of Marco Polo's journeys (1254-1324) and Vasco De Gama's journeys to India (1498). After these discoveries, contents of the book Materia-Medica were enriched with new medicinal plants such as Ratanhia, Jalapa, Ipecacuanha, Cacao, Lobelia, Senega, Vanilla, red pepper, tobacco and so on (Bojadzievski, 1992; Toplak, 2005).

Linnaeus (1707-1788), who is the main researcher in botanical area, was provided many plant species as their classification and brief description of in the 18th century by in his work *Species Planetarium* (1753). A polynomial system was applied as follows; the genus was denoted by the first name while remaining polynomial phrase explained other characteristics of the plant (Bojadzievski, 1992; Toplak, 2005).

Early 19th century was a milestone for usage of medicinal plants. The discovery, isolation and purification of alkaloids from poppy, ipecacuanha, strychnos, quinine and pomegranate, then the isolation of glycosides, was accepted as the beginning of pharmacy. The initial point of the scientific pharmacy was accepted as the isolation of glycosides was employed successfully (Dervendzi, 1992).

In late 19th and early 20th centuries, glycosides, alkaloids and other therapeutics isolated in pharmaceutical grade and many stabilization methods were enhanced for production processes (Lukic, 1985; Kovacevic, 2000).

Nowadays, venerable pharmacopoeias prohibited plant drugs of medicines for their usage purposes. But, unofficial drugs have many usage areas in social life. Applications of these unofficial drugs based on the traditional medicine and

conventional medicine. Most of the medicinal drugs are used in combination with synthetic drugs as a complementary medicine. Professional applications and standardization of plant drug extracts of medicinal plants have met the requirements for pharmaceutical quality of drugs.

1.2 Drug Discovery from Natural Products

Medicinal plants have mega diversity and a long history of attending humankind all over the earth. Approximately 350 000 plant species identified and investigated so far, but only 10 percent of these species are utilized as medicinal goals. World Health Organization (WHO) confirms that herbal medicines have a huge area of utilization in upstate areas of third world countries. Besides, consumers in developed countries have realized importance and efficiency of therapeutic plants as a result of alternative pursuits.

The essence of matter, some medicine companies have produced several chemotherapy drugs from natural sources which have cytotoxic activities *e.g.* teniposide (Vumon®), etoposide (Toposar®), irinotecan (Camptosar®), topotecan (Hycamtin®), paclitaxel (Taxol®), vinblastine (Alkaban-AQ®) and vincristine (Oncovin®) (Cragg et al., 1997; Chemocare, 2018). Because of bioactive compounds from natural resources have a great market share; modern drug producers have increased the allowance of research & development for drug discovery from natural resources. The results of efficient and decisive investigations, seven plants derived anticancer drugs have approved by Food and Drug Administration (FDA) for commercial production (Kong et al., 2003).

Paclitaxel (Taxol®): Primer election for several tumorous cancer and isolated from Pacific Yew tree (*Taxus brevifolia*).

Vinblastine (Alkaban-AQ®): It is the first drug of choice in leukemia and discovered Madagascar periwinkle in the 1950s. This chemical has a respectable success rate (%80) of childhood leukemia.

Vincristine (Oncovin®): Another plant drug from Madagascar periwinkle which is used for the treatment of leukemia.

***Topotecan (Hycamtin®):** A plant alkaloid from *Camptotheca acuminata* (from China) is used for the treatment of ovarian and small cell lung cancers.

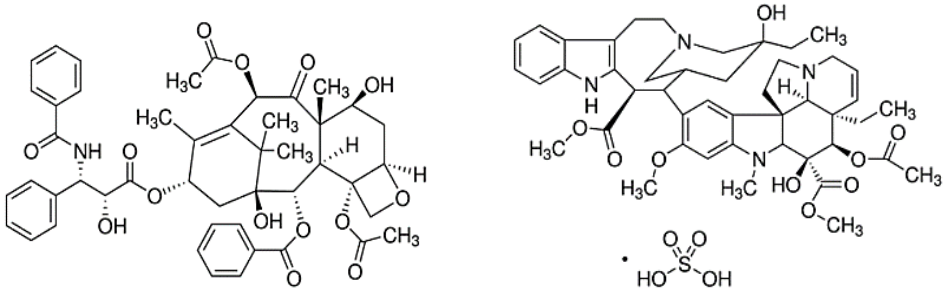
Irinotecan (Camptosar®): An analogue compound (synthesized chemical) which has been modified from plant (*Camptotheca acuminata*) alkaloid for the treatment of metastatic colorectal cancer.

Etoposide (Toposar®): It is a semisynthetic form of epipodophyllotoxin which has been discovered in the (*Podophyllum peltatum*).

Teniposide (Vumon®): Another semisynthetic compound from *Podophyllum peltatum.*

Taxus brevifolia *Camptotheca acuminata* *Podophyllum peltatum*

Figure 1.1 Some plants used for anticancer drugs



Paclitaxel Vinblastine

Figure 1.2 Some structures of plant-derived anticancer drugs (Sarker and Nahar, 2007)

After investigation of more than 40 000 plants, only five chemicals discovered as an active compound against AIDS. Plants listed below are still under research for AIDS and cancer.

(+)-Calanolide A and (-)-Calanolide B: The chemicals are isolated from *Calophyllum lanigerum* and *Calophyllum teysmanii*, respectively (Sarawak, Malaysia).

Conocurovone: Isolated from *Conospermum incurvu,* located in Western Australia.

Michellamine B: The compound from the leaves of *Ancistrocladus korupensis* is under preclinical studies. According to the clinical reports, it is considered too toxic.

Prostratin: It has been isolated from the wood of *Homolanthus nutans*, located Western Samoa.

9-Aminocamptothecin: Like many other anticancer drugs, this compound was isolated from *Camptotheca acuminata.* It has been investigated in clinical trials for ovarian and stomach cancers and T-cell lymphoma (Lee et al., 1995; Keung et al., 1995).

As a complementary point of view, herbal medication and therapeutic plants cannot be reduced to the isolation and purification of major constituents or active ingredients. So, much traditional medicine is prepared using various parts of plants to interact together. A list of chemicals and drugs from plants was given in Table 1.1 (Kong et al., 2003).

Table 1.1 Drugs and chemicals from plant sources and their usage areas

Drug/Chemical	Clinical Use	Plant Species
Acetyldigoxin	Cardiogenic	*Digitalis lanata*
Adoniside	Cardiogenic	*Adonis vernalis*
Aesin	Anti-inflammatory	*Aesculus hippocastanum*
Atropine	Antichlolinergic	*Atropa belladonna*
Berberine	Bacillary dysentery	*Berberis vulgaris*
Betulinic acid	Anticancerous	*Betula alba*
Borneol	Antipyretic, analgesic	*Several plants*
Caffeine	CNS stimulant	*Camellia sinensis*
Camphor	Rubafacient	*Cinnamomum camphora*
Camptothecin	Anticancerous	*Camptotheca acuminata*
Cissampeline	Skeletal muscle relaxant	*Cissampelos pareira*
Cocaine	Local anaesthetic	*Erythroxylum coca*
Codeine	Analgesic, antitussive	*Papaver somniferum*
Curcumin	Choleretic	*Curcuma longa*
Cynarin	Choleretic	*Cynara scolymus*
Danthron	Laxative	*Cassia species*
Demecolcine	Antitumor agent	*Colchicum autumnale*
L-Dopa	Anti-parkinsonism	*Mucuna sp*
Digitoxin	Cardiotonic	*Digitalis purpurea*
Digoxin	Cardiotonic	*Digitalis purpurea*
Emetine	Amoebicide, emetic	*Cephae lisipecacuanha*
Ephedrine	Sympathomimetic	*Ephedra sinica*
Etoposide	Antitumor agents	*Podophyllum peltatum*
Gitalin	Cardiotonic	*Digitalis purpurea*
Glaucarubin	Amoebicide	*Simaroub aglauca*
Glaucine	Antitussive	*Glaucium flavum*
Glasiovine	Antidepressant	*Octea glaziovii*
Kawain	Tranquillizer	*Piper methysticum*
Menthol	Rubefacient	*Mentha species*
Morphine	Analgesic	*Papaver somniferum*
Nicotine	Insecticide	*Nicotiana tabacum*
Papaverine	Smooth muscle relaxant	*Papaver somniferum*
Phyllodulcin	Sweetner	*Hydrangea macrophylla*
Pseudoephredrine	Sympathomimetic	*Ephedra sinica*
Reserpine	Tranquillizer	*Rauvolfia serpentina*
Salicin	Analgesic	*Salix alba*
Silymarin	Antihepatotoxic	*Silybum marianum*
Sparteine	Oxytocic	*Cytisus scoparius*
Stevioside	Sweetner	*Stevia rebaudiana*
Strychnine	CNS stimulant	*Strychnos nux-vomica*
Teniposide	Antitumor agent	*Podophyllum peltatum*
α-Tetrahydrocannabinol	Antiemetic	*Cannabis sativa*
Tetrahydro palmatine	Analgesic	*Corydalis ambigua*
Theobromine	Diuretic	*Theobroma cacao*
Theophylline	Diuretic	*Theobroma cacao*
Thymol	Antifungal	*Thymus vulgaris*
Topotecan	Antitumor	*Camptotheca acuminata*
Vasicine	Cerebral stimulant	*Vinca minor*
Vinblastine	Antitumor, Antileukemic agent	*Catharanthus roseus*
Vincristine	Antitumor, Antileukemic agent	*Catharanthus roseus*

1.3 Isolation Methods for Natural Products

Discovery of bioactive natural products is closely associated with isolation and purification process. These processes are not really suited to practical methods due to nature of natural products. Natural products include; an entire organism, part of an organism, an extract of an organism, pure compounds (e.g. sugars, terpenoids, flavonoids, alkaloids, steroids and glycosides, etc.) obtained from plants, animals or microorganisms (Xiao, 1988; Sarker et al., 2006). Over the last decades, methods and strategies in the natural product research area have evolved into two categories as old and modern (Sarker et al., 2006).

Older Strategies:

a) Chemotaxonomic research
b) Selection of material based on traditional uses and ethnopharmacological information.
c) After *in vivo* biological activity testing, isolation and identification of compounds.
d) Chemistry of compounds was more important than the activity of compounds.

Modern Strategies:

a) Identification and isolation of bioactive compounds based on bioassay-guided applications (mainly *in vitro*).
b) Constitution of libraries for natural products.
c) Generation of bioactive molecules in cell or tissue culture, natural combinatorial chemistry, genetic manipulation, etc.
d) Importance of bioactivity increased.
e) Chemical fingerprinting, metabolomics and chemical fingerprinting.
f) Selection of materials based on folkloric reputations, ethnopharmacological information and traditional uses.

1.3.1 Extraction procedure

The extraction method is directly related to the compounds and the source material. The target of extraction must be resolved prior to choosing method. A typical extraction process for plant resources was listed below (Sarker et al., 2006);

1) Homogeneity must be provided before application of extraction process. Drying and grinding operations or maceration of plant materials with a suitable solvent must be put in the top of the operation sequence.
2) Solvent system must be decided considering the polarity (Polar, Medium and Nonpolar).
3) One of (or the mixture) the extraction methods must be chosen, for example; maceration, boiling, soxhlet, supercritical fluid extraction, sublimation, steam distillation, and so on.
4) Operations for extraction must not affect the structure of willed compounds or nature of the material.

1.3.2 Fractionation of extracts

Single separation technique is inadequate to isolate requested compounds individually from the crude plant. Initially, the crude extract is separated into various sub-fractions based on molecular size and polarity. Physically discrete divisions, e.g., liquid-liquid extraction or elution from a chromatography column such as solid-phase extraction (SPE), size-exclusion chromatography (SEC), column chromatography (CC), vacuum liquid chromatography (VLC), medium-pressure liquid chromatography (MPLC), etc. may be employed to isolate compounds from fractions. For better fractionation, on-line detection such as ultraviolet (UV) or refractive index (RID) for traceability and preparative high-performance liquid chromatography (HPLC) or medium-pressure liquid chromatography (MPLC) can be employed. In the absence of detector, thin layer chromatography may be used to identify compounds (Sarker et al., 2006).

1.3.3 Isolation of compounds

Isolation protocol has to be prepared heedfully before starting purification and separation processes. Physicochemical characteristic of the molecule that is helpful to find out isolation process includes charge, stability, acid-base properties, solubility (hydrophobicity and hydrophilicity) and molecular size. Isolation of a known compound is easy because of the information on the chromatographic behavior. But, design an isolation process for a crude extract which includes unknown compounds is more difficult. So, qualitative or quantitative tests for the presence of compounds are helpful and accelerator operations for the isolation process. The feature of extracts can give an idea to choose the appropriate technique. For example, polar compounds in MeOH extracts or fractions are more feasible to separating by reversed phase HPLC (RP-HPLC). Physicochemical properties of the extracts and the fractions can be determined in a series of experiments. Some of them are listed below;

1) Hydrophobicity or hydrophilicity: Dried extract must dissolve in various solvents or solvent systems which involves the range of polarities e.g. water, ethyl acetate, methanol, dichloromethane, acetonitrile, petroleum ether, chloroform, *n*-hexane, etc.
2) Acid-base properties: The acid-base properties of compounds in extracts can be determined by creating pH range typically 3, 7 and 10 in aqueous solutions. Hereby stability of compounds at various pH values can be tested.
3) Charge: Charge properties of the compounds can be found out by adding various ion exchangers to the mixture.
4) Heat stability: A general test for heat stability experiment includes incubation of the sample at ~90 ºC for 10 min in a water bath followed by an analysis of undisturbed compounds.

Many chromatographic techniques are present for isolation and purification processes of various types of natural products. These techniques can be listed under two main categories:

Classical chromatographic techniques:

1) Flash chromatography (FC)
2) Thin-layer chromatography (TLC)
3) Preparative thin-layer chromatography (PTLC)
4) Open-column chromatography (CC)

Modern chromatographic techniques:

1) High-performance thin-layer chromatography (HPTLC)
2) Vacuum liquid chromatography (VLC)
3) Droplet countercurrent chromatography (DCCC)
4) High-performance liquid chromatography (HPLC)
5) Combined techniques (HPLC-UV, HPLC-RID, LC-MS, LC-NMR, LC-MS-NMR)

1.4 Identification of the Structures

The conclusive structure determination of isolated pure compounds is the final phase of the process; however, identification of novel or known compounds isolated from plants, fungi or bacteria is the most crucial part of the whole study. Many spectroscopic methods can be used to obtain information about the structures of compounds. But, remarking of spectra requires the specialist who has a good grasp of natural product chemistry and detailed spectroscopic knowledge; on the other hand, there is a number of computer software for structure elucidation available to resolve the structure of compounds (Blinov et al., 2003; Steinbeck, 2004). Structures of the known compounds are compared with spectroscopic data in literature or primary standard compounds. However, if the compound is unknown and has a complex structure, a systematic approach must be employed including chemical, physical and spectroscopic techniques. Identification and structural determination of complex structures obtained from natural compounds can be elucidated by various spectroscopic techniques such as UV, IR, NMR and MS.

1.4.1 Ultraviolet-visible spectroscopy (UV-Vis)

Ultraviolet-visible spectroscopy is a confirmed analytical technique with advanced methods, software and hardware equipment. It is commonly used in classic analytical applications and environmental analyses. UV-Vis spectrometers are available almost all laboratories such as in chemistry, physics, biochemistry and bioscience fields. Amount of light absorbed or transmitted by the compound is measured as a function of the wavelength. Besides, the amount of light absorbed or transmitted by a molecule depends on the path length of cells.

The compounds must have chromophore groups which have electronic bands with energy differences comparable to the energies. Chromophores, for example, nitriles, diens, and carbonyl groups often contain π bonds or transition metal complexes and their ions. Many compounds such as triterpenes don't absorb the light in the UV part. Alkanes, water or alcohols are ideal solvents for ultraviolet-visible spectroscopy because of they do not include any chromophores. This group of chemicals gives characteristic absorbance bands. Changes in molecule cause alterations in their energy levels, so the wavelength and the intensity of absorbance are affected. Color is a function of absorbance and emittance of light and it refers to daylight as an illuminant. Eyes of humankind are capable to detect light in a range from 380 to 780 nm, the visible part of a UV-Visible spectrum (Agilent Tech., 2000).

1.4.2 Infrared spectroscopy (IR)

Infrared spectroscopy is simple and quick spectroscopic technique that can determine the functional groups such as –C=O, -OH, $-NH_2$, aromaticity and so on. The IR spectrum is divided into, Near Infrared [NIRS (0.7 μm to 2.5 μm)], Mid Infrared [MIRS (2.5 μm to 25 μm)] and Far Infrared [FIRS (25 μm to 300 μm)]. In terms of wavenumbers the three regions in cm^{-1} are NIRS: 14000-4000 cm^{-1}, MIRS: 4000-400 cm^{-1} and FIRS: 400-10 cm^{-1}. NIRS, the first region, allows the work on overtones and

harmonic vibrations. MIRS region is for the fundamental vibrations and the rotation-vibration structure of relatively small molecules. The third region, FIRS, is for the low heavy atom vibrations (metal-ligand vibrations). There are many research about IR spectroscopy was employed on complex molecules in the field of biosciences such as proteins, membranes and DNA (Herzberg, 1969; Maas, 1972; Fowles, 1975; Colthup, 1990; Theophanides, 1990; Anastasopoulou et al., 1997).

1.4.3 Mass spectrometry (MS)

Mass spectroscopy is the prevalent, accurate, robust and sensitive technique for identification and quantification of various compounds at ppq (Parts Per Quadrillion) levels. This method is based on the motion of an ion, in a magnetic field. The mass to charge ratio (*m/z*) of the ion leads the motion. Vacuum system to provide the low pressure, is available in all mass systems. Operation under high vacuum minimizes neutralization, ion-molecule reactions and scattering of the ions. A block diagram for mass spectrometer was shown below as illustrated Figure 1.3 (Bramer, 1998).

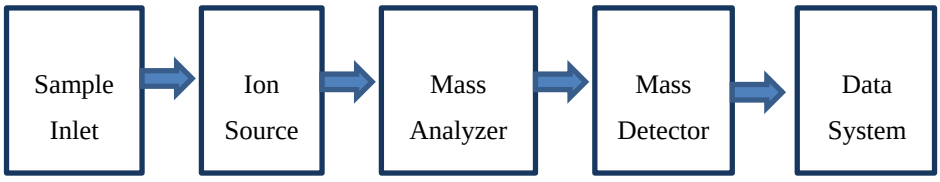


Figure 1.3 Basic diagram for a mass spectrometer

Sample inlet transfers the molecule that is analyzed into the mass spectrometer. In the ion source, neutral molecules are ionized and sent into the mass analyzer. Molecules might break into charged fragments during the ionization process. In the mass analyzer, ions are separated according to their mass-to-charge ratio (*m/z*). Ions are detected by a mechanism capable of detecting charged particles. Results are displayed as spectra of the relative abundance as a function of *m/z* ratio. Identification is done by correlating known masses to the identified masses or through a characteristic fragmentation pattern (Hoffman and Stroobant, 2007).

1.4.3.1 Ionization techniques for mass spectrometry

The analyzed compounds are ionized prior to analysis in the ion sources. Various ionization techniques are employed for mass spectrometry. The neutral molecule ejects an electron to form a radical cation (M^+) and is the remaining unpaired electron of the radical in almost all ionization techniques. But, ion-molecule reactions that reveal adduct ions (MH^+) in the other techniques. Gas phase ionization is provided only by electron ionization and chemical ionization. On the other hand, ionization condensed phase samples are performed by secondary ion mass spectrometry, fast atom bombardment, and matrix-assisted laser desorption. Some ionization techniques produce only molecular ions, so these techniques are named soft ionization techniques. But, other techniques which are very energetic cause fragmentation of ions (Bramer, 1998).

Direct ion sources can be explained under two titles as solid-state ion sources and liquid phase ion sources. In liquid phase ion sources, analyte solution is introduced as droplets into ion source at atmospheric pressure by a nebulizer. Electrospray ionization (ESI), atmospheric pressure chemical ionization (APCI) and atmospheric pressure photoionization (APPI) sources are interior this type. In solid state ion sources, involatile compounds are treated as solid or a viscous fluid. These compounds are irradiated by photons or energetic particles and then ions can be extracted by an electric field. This strategy was used by various techniques to produce ions such as secondary ion mass spectrometry, matrix-assisted laser desorption (MALDI), plasma desorption and field desorption. Besides, the involatile liquid matrix can be ionized by fast atom bombardment technique (Hoffman and Stroobant, 2007).

Electron Ionization (EI): Electron ionization technique has extensive usage for MS systems and it works very well for many gas phase molecules. Electron ionization technique is very reproducible and causes fragmentations that provide structural information for unknown molecules. The electrons are ionized by current through a filament (Figure 1.4).

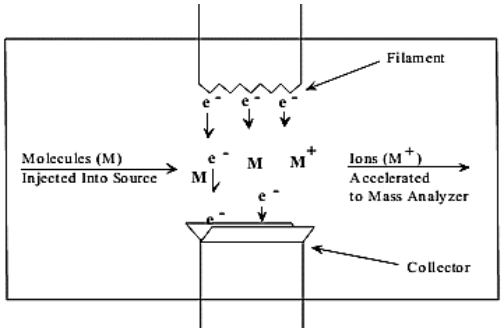


Figure 1.4 Electron ionization source (Bramer, 1998)

The quantity of electrons emitted by filament depends on the amount of current. These electrons are accelerated by an electric field across to the source to produce a beam of high energy electrons. When molecules pass through this electron beam, a valence shell electron can be removed from the molecule to produce a positive ion or a radical cation. Generally, the molecular ion is not observed for many analytes due to extensive fragmentation (Bramer, 1998).

Chemical Ionization (CI): This "soft" ionization technique produces ions with the low energy and consequently less fragmentation occurs in the spectra. So, CI technique is used to verify the molecular mass of unknown molecules. Chemical ionization technique is based on generating the ions through a collision of the molecule. So, the pressure of the source has to be optimized for frequent collisions. Ions occur by the loss of a proton or a hydride or the addition of a proton. The molecular ions (pseudomolecular ions) refers to M^+ or M^- (Munson and Field, 1966; Munson, 1977).

Fast Atom Bombardment (FAB) and Secondary Ion Mass Spectrometry (SIMS): Ionization of the molecules in a single step is performed by Fast Atom Bombardment (FAB) and Secondary Ion Mass Spectrometry (SIMS) techniques. A beam of rare gas neutrals or ions is focused on the sample and it causes the analyte molecules transition into the gas phase and ionizes. High molecular weight (up to a few thousand daltons) and thermally labile molecules can be analyzed by these techniques (Barber et al., 1982; Fenselau, 1982; Biemann, 1986).

Atmospheric Pressure Ionization (API) and Electrospray Ionization (ESI): Atmospheric Pressure Ionization (API) sources ionize thermally labile molecules (peptides, proteins and polymers) at atmospheric pressure before the transfer the ions to the mass spectrometer. Introducing the sample through pumped stages maintains the pressure difference between the ion source and the mass spectrometer. Electrospray Ionization (ESI) is used for LC-MS systems as the most common API application to ionize thermally labile and high weighted molecules. The large potential between inlet needle and the skimmer is applied in ESI technique (Figure 1.5).

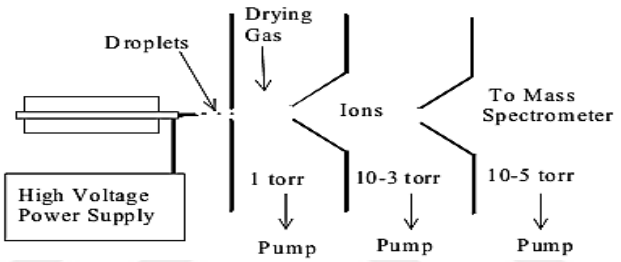


Figure 1.5 Electrospray ionization source (Bramer, 1998)

Inductively Coupled Plasma Mass Spectrometry (ICP-MS) involves API sources. Plasma temperature is high enough (6000 to 10000 K) to ionize most elements and destroy any chemical bonds. This sensitive and selective technique is used for elemental analysis at very low levels (Houk, 1986; Vela et al., 1993).

Matrix Assisted Laser Desorption/Ionization (MALDI): Extremely large molecules can be analyzed Matrix Assisted Laser Desorption/Ionization (MALDI) systems. The condensed phase of analytes (peptides, proteins and polymers) are ionized and vaporized by this technique. Molecules weight up to 200000 daltons is analyzed by MALDI. Single laser pulse provides ionization and desorption of the analyte in an appropriate matrix. Laser energy subtracts analyte ions from the surface to generate the molecular ion, charged ions and fragment ions (Figure 1.6) (Karaz and Hillenkamp, 1988; Fenselau, 1997).

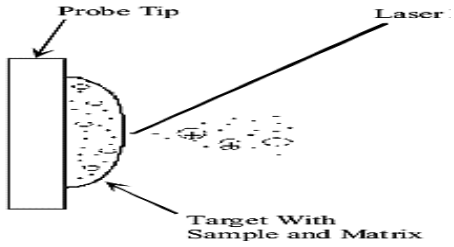


Figure 1.6 Matrix assisted laser desorption/ionization (MALDI) (Bramer, 1998)

1.4.3.2 Mass analyzers

The ions formed in the source are transferred into the mass analyzer and separated according to *m/z* values. Selectivity of ions depends on resolution, mass range, scan rate and limit of detections. There is various mass analyzer have different characteristic. Generally, analyzers are separated into two types; continuous and pulsed. Continuous analyzers are similar to a monochromator and include magnetic sectors and quadrupole filters. These analyzers transfer selected *m/z* to the detector and ions at unselected *m/z* ratios are lost.

Quadrupole: Quadrupole, the most common mass analyzer has some features (modest vacuum requirements, fast scan rate, compact size, and high transmission efficiency) ideal for small benchtop instruments. The selected ions are accelerated by an electric field in the quadrupole analyzer which consists of four electrodes or rods (Figure 1.7). The ions are filtered according to *m/z* values and only selected *m/z* can reach the detector. Radio Frequency (RF) and Direct Current (DC) voltages determine the *m/z* value transmitted by the quadrupole (Steel and Henchman, 1998).

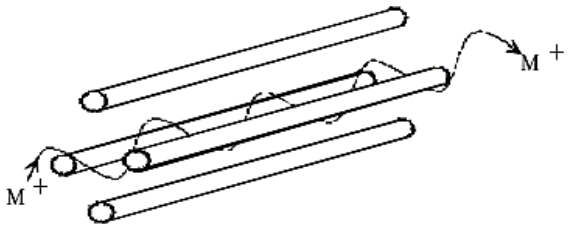


Figure 1.7 Quadrupole mass analyzer and ion trajectory through the quadrupole (Bramer, 1998)

Magnetic Sector: Magnetic sector instruments are more selective and sensitive than quadrupole instruments, but larger vacuum pumps and lower scan rate are required. These instruments are often used for tandem mass spectrometry experiments and mass range of these instruments is between *m/z* 5000 and *m/z* 30,000. Separation of ions in a magnetic field is based on momentum and charge of the ion. Ions are transmitted into the magnetic sector by a 1 to 10 kV electric field (Figure 1.8). As the ions move through the analyzer, the ion beam is bent in an arc by the magnetic field.

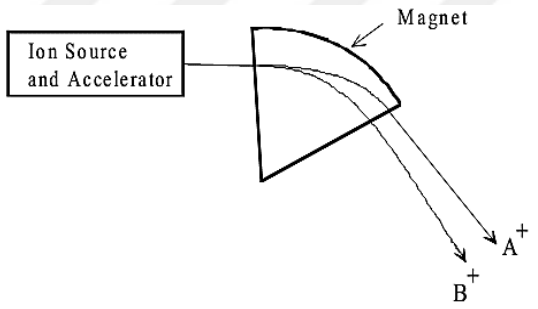


Figure 1.8 Magnetic sector mass spectrometer (Bramer, 1998)

Time-of-Flight (TOF): Ions are separated as their time of flight in a flight tube. The time of flight mass analyzer have the poor mass resolution (usually < 500 *m/z*) and uses constant voltages but does not need a magnetic field. Fast scan rate, high transmission efficiency and no upper *m/z* limit are the advantages of this technique, but the very low detection limit is a disadvantage. Ionisation pulse generates a packet of ions in the source of the analyzer. The electric field accelerates the ions into the flight tube. Under the same kinetic energy mass, lighter ions will transfer faster (Cotter, 1992).

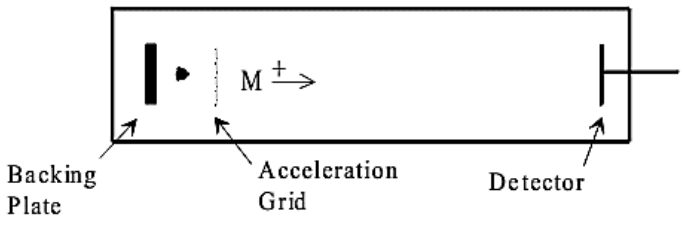


Figure 1.9 Time-of-flight mass spectrometer (Bramer, 1998)

1.4.3.3 Detectors

Ions are detected according to charge and momentum. The ion signal is amplified by a collector as a photomultiplier tube. Besides, detectors include channeltrons, multichannel plates and electron multipliers. Changing the voltage of the detector provides the gain control (Bramer, 1998). There are several types of detectors. The choice of a detector depends on the experimental application and the instrument type. Ions charge, mass and velocity change the detection technique. There are various detectors in use such as Photographic Plate, Faraday Cup, Electron Multipliers and Electro-Optical Ion Detectors (Hoffman and Stroobant, 2007).

Photographic Plate: Photographic plate is the first mass detector for analytical purposes. Ions make spot after reaching the plate and the tone of spots gives an average value of abundance.

Faraday Cup: A Faraday cup includes a metal cup with the small orifice. Ions are accelerated to the cylinder and neutralized as they reach the walls. This process generates a current through the resistor. The discharge current is detected and measured.

Electron Multipliers (EM): After 2006, electron multipliers become most widely used detector in mass spectrometry. Acceleration of ions from the analyzer enhances the detection efficiency and capability. An electrode called a conversion dynode archives high potential from ±3 to ±30 kV. Ions (positive or negative) striking the dynode causes the emission of secondary particles which includes positive ions, negative ions, neutrals and electrons. Positive ions generate negative ions and electrons after the strike the negative high voltage conversion dynode. Negative ions generate positive ions after the strike the negative high voltage conversion dynode. A current is produced after amplified by the electron multiplier. A schematic diagram of electron multiplier was shown in Figure 1.10.

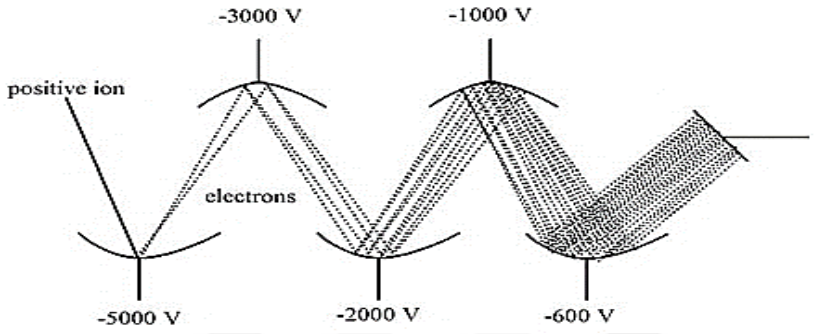


Figure 1.10 Schematic diagram of the electron multiplier (Bramer, 1998)

Electro-Optical Ion Detectors (EOID): Electro-optical ion detector includes ion and photon detection tools. Converting ions to electrons and photons were operated by EOID. Daly detector is the most common electro-optical ion detector. This detector can detect both positive and negative ions.

1.4.4 Nuclear magnetic resonance (NMR)

Nuclear magnetic resonance is the most extensive technique for structural elucidation of organic compounds. This instrumental technique not only identifies the numbers and types of nuclei present in a compound but also characterize their individual chemical environments. In 1952, F.Bloch and E. Purcell shared a Nobel Prize in physics for combining the Nuclear Zeeman Effect to practical use by performing the first NMR spectrometer. After this defining moment, NMR has become most widely used application for identification of molecular structure in the field of chemistry and biochemistry year by year. The acronym "NMR" is often reputed to ^{1}H-NMR (Proton Nuclear Magnetic Resonance). Other techniques such as ^{13}C-NMR, ^{15}N-NMR, ^{19}F-NMR, ^{31}P-NMR and other magnetic nuclei were developed and widely used later. In contemporary instrumental techniques, pulsed Fourier Transform (FT) spectrometers are widely used. The advantage of these modern instruments is magnetic field and rf pulse properties. Computer software converts the intensity-versus-time data into intensity-versus-frequency information in a mathematical process which is known as Fourier Transform. So, this spectrum called a Fourier Transform NMR (FT-NMR) (Macomber, 1998; Bruice, 1998).

In ^{13}C-NMR, the number of signals indicates how many different kinds of carbons a compound has. The working principles of ^{1}H-NMR and ^{13}C-NMR spectroscopy are essentially identical. Because of the signals in ^{13}C-NMR spectroscopy obtained from a weak single scan; this method requires Fourier Transform techniques. A lot of scans can be recorded whereby ^{13}C-NMR scans can be repeated quickly. In case of absence Fourier Transform, analysis takes days to achieve the number of scans required.

Distinguishing among CH_3, CH_2 and CH groups is provided by APT^{13}C-NMR (APT: Attached Proton Test) technique that is widely used to determine the number of

hydrogens attached to a carbon. But, the signal cannot be obtained for a carbon that is not attached to a hydrogen atom.

Two dimensional (2D) NMR is used for identification of complex molecules (saponins, proteins, nucleic acids and high molecular weighted natural products). This technique allows identifying the structure in a solvent. In recent years, 3D and 4D NMR spectroscopy techniques are enhanced to identify structures of highly complex compounds. Prevalent 2D spectra involve ^{1}H - ^{1}H shift correlations that are called ^{1}H-^{1}H shift correlated spectroscopy (COSY). The obtained pulse batches are more complex than calculated ones. Phase cycling technique is used in many spectrometers to change the RF (Radio frequency) pulse in a regular manner. The phase cycles are performed to remove artefacts and peculiarities. Adding a third $\pi/2$ pulse after the second $\pi/2$ pulse in COSY pulse a very popular technique is employed called Double Quantum Filtered ^{1}H-^{1}H COSY (DQF-COSY). So, the double quantum filter will select only two spins (at least) systems. Correlation of ^{13}C nuclei with attached protons are applied by ^{13}C - ^{1}H COSY (HETCOR- HETeronuclear CORrelation) technique. HMQC (Heteronuclear Multiple Quantum Correlation) techniques correlate ^{13}C nuclei with directly attached protons (one band couplings). Long range (two and three bonds) proton-carbon coupling is determined *via* their coupling constants by HMBC (Heterocycles Multiple Bond Correlation) techniques (Bruice, 1998; Silverstein et al., 2005).

1.4.5 Other techniques

1.4.5.1 X-ray crystallography technique

X-ray crystallography is a widespread scientific technique for determining the 3D structure of molecules such as viruses, immune complexes, proteins and nucleic acids. After crystallization of an isolated or purified sample, the crystals are exposed to an X-ray beam. The structure of molecules is then refined to fit the conformation (Smyth and Martin, 2000; Picknett and Brenner, 2001).

1.4.5.1.1 Polarimetry

Optical activity of biological, inorganic and organic compounds is measured by polarimetry which is a sensitive and nondestructive technique. A polarimeter includes a LED source, a fixed polarizer and a rotating polarizer (analyzer) to detect changes in rotation of plane-polarized light in the presence of an optically active compound. An optically active compound can rotate the linearly polarized light to clockwise (+) or counterclockwise (-) when passing through it. The rotation depends on the structure of molecule and concentration of chiral molecules (Gross et al., 2012).

1.5 Chemical, Biological and Physical Assays

Identification and characterization of natural compounds are guided by chemical, biological, or physical assays with developing the technology. Target natural compounds may have chemical classes, biological activities, or physical properties. Therefore, the results of the appropriate assays should be evaluated before isolation and purification process. Before performing an assay, insoluble matters must be defecated. Acidified or basified solutions of the compound should be readjusted to original pH. The assay must have some validation parameters such as linearity, reproducibility, repeatability, and robustness. Chromatographic and spectroscopic behaviors of natural complex products can be analyzed by the various modern techniques such as TLC, HPLC, GC, LC-MS, GC-MS, LC-NMR. Bioassays can be explained as the use of the biological system to identify properties (antifungal, anti-HIV, anticancer, antibacterial, antidiabetic, etc.) of fractions, extracts, pure compounds, or a mixture. Bioassays are performed *in vivo* (clinical trials, animal experiments), *in vitro* (cultured cells) and *ex vivo* (isolated tissues) systems (Sarker et al., 2006).

1.5.1 Chromatographic analyses

The most widely used separation technique is chromatography in the determination of compounds. Nevertheless, this technique is used for preparative purposes, to isolate small amounts of compounds that have high intrinsic. A mixture can be separated into individual components in a single step operation by chromatography. Besides, it provides qualitative and quantitative analysis for each constituent. Chromatography has been explained as follows; A separation procedure that is provided by distributing the components of a mixture between two phases, a stationary phase and a mobile phase. The compounds held in the stationary phase as preferentially are detained longer than that are distributed in the mobile phase selectively (Scott, 2003).

1.5.1.1 Gas chromatography

In 1941, Martin and Synge developed Gas Chromatography (GC) as an analytical technique that uses gas as a mobile phase instead of liquid. Since the development of gas chromatography instruments, GC techniques are performed in the food industry, pharmaceutical chemistry, environmental chemistry, biotechnology, petrochemistry and natural product chemistry fields. Volatile compounds could be separated and identified by this efficient technique. GC instruments transport volatile compounds through packed columns that contain polymeric liquid stationary phase by various gaseous mobile phases. Required gases for the process are; Carrier Gas: H_2, He, N_2; Makeup Gas: H_2, He, N_2; Detector Gas: H_2 & Air, Ar or Ar & CH_4, N_2. Elution time of compounds depends on interactions between stationary and mobile phase. The interactions can be categorized into three headings such as dispersive, dipole and hydrogen bonding. The most important part of a GC is the chromatographic column that varies in length and ID (internal diameter) depending on analyte or application. Besides, detectors for GC are variable. The detector detects the analysts according to physical properties of the compound. There are various detectors in GC applications such as Flame Ionization (FID), Electron Capture (ECD), Flame Photometric (FPD),

Nitrogen Phosphorous (NPD), Thermal Conductivity (TCD) and Mass Spectrometer (MS) (Jennings et al., 1997; Crawford Sci., 2018a). Gas chromatography is an excellent instrument for qualitative or quantitative determination of volatile or volatilizable compounds, non-polar or middle polar molecules, thermally stable analytes and compound that molecular weight under 500 Da.

1.5.1.2 Liquid chromatography

Russian botanist Mikhail Tswett (1872-1919) first used the term "Chromatography" in 1906, to explain the separation of pigments in an alumina filled column using petroleum ether. Today, just as Tswett, modern science uses a liquid mobile phase to separate the compounds through a stationary phase packed column. In 1941, it was reported that “the most efficient columns should have very small particles and high-pressure differences". A few decades later, high-pressure liquid chromatography (HPLC) was developed for specific applications (Meyer, 2004; Snyder et al., 2010; Crawford Sci., 2018b).

Table 1.2 Comparison of gas chromatography and liquid chromatography

Gas Chromatography	**Liquid Chromatography**
Volatile substances are separated by gas chromatography	Unvolatile substances are not separated by gas chromatography
Low molecule weighted compounds is suitable for GC analysis	Middle and high molecule weighted compounds is suitable for LC analysis
Not suitable for thermally unstable and nonvolatile compounds	Suitable for thermally unstable and nonvolatile compounds
Not Suitable for preparative purposes	Suitable for preparative purposes
It requires little quantities of high purity gas (He, ArCH_4, H_2, N_2 etc.)	It requires large quantities of expensive organic solvents

In contrast gas chromatography, HPLC uses a liquid mobile phase to resolve the analytes through the chromatographic column. The non-volatile analytes, molecules have various polarities and molecular weight can be detected by HPLC applications. HPLC applications involve many parameters need to be optimized to perform a suitable qualitative and quantitative analysis. Each of the listed parameters below needs to be optimised for satisfactory separation;

- Mobile phase composition and flow rate
- Column and packing dimensions
- Sample pre-treatment and concentration
- Bonded phase chemistry
- Column temperature
- Injection volume
- Detector parameters

Liquid chromatography separation modes are explained by the combination of mobile phase and stationary phase. The most important modes of liquid chromatography are Reversed Phase, Ion Pair, Normal Phase, Ion Exchange and Size Exclusion modes (Crawford Sci., 2018b).

Reversed Phase Chromatography: Non-polar compounds are eluted later than polar compounds by a relatively polar (water to THF) mobile phase through a non-polar stationary phase (C18, C8, C4, cyano and amino).

Normal Phase Chromatography: Polar compounds are eluted later than apolar compounds by a non-polar mobile phase through a polar stationary phase (silica, diol).

Ion Pair Chromatography: Separation of ionic compounds (secondly, acids, bases and neutral products) is performed by ion pair chromatography. Ionic compounds are “masked” by a counter ion in mostly reversed phased (C18, C8) system.

Ion Exchange Chromatography: The ionic groups of the sample molecules interact with the ionic groups (e.g. NR_3^+or SO_3^-) in the stationary phase (Anion or Cation Exchange Resin) to separate ionic metabolic products, amino acids and organic ions.

Size Exclusion Chromatography: Size exclusion chromatography can be divided into two main subtitles; gel filtration chromatography (GFC) (with aqueous solutions) and gel permeation chromatography (GPC) (with organic solvents). This technique can be used when a mixture includes molecules with the molecular mass difference of at least 10%. Generally, high molecular weight compounds and polymers are separated through polystyrene silica.

1.5.2 Analysis of glycosides by combined techniques

There are many reports for glycosides analyzed by combined chromatographic techniques, such as liquid chromatography-electrospray ionization mass spectrometry (ESI-LC-MS), high performance liquid chromatography-evaporative light scattering detection (HPLC-ELSD), liquid chromatography-photodiode array electrospray ionization tandem mass spectrometry (LC-PDA-ESI-MS/MS), liquid chromatography-ultraviolet nuclear magnetic resonance (LC-UV-NMR) and ultra-performance liquid chromatography- electrospray ionization tandem mass spectrometry (Han et al., 2011; Yang et al., 2015; Mikolajczyk-Bator et al., 2016). Especially, electrospray ionization multi-stage tandem mass spectrometry (ESI-MS^n) and nano-HPLC (nLC) presented a new way for quantitative determination of natural products (Madl et al., 2006; Celenk et al., 2018).

1.6 Glycosides

Glycosides are the most special seconder metabolites in all natural sources, especially in plants which have at least one sugar moiety in their structures. After the acidic hydrolyses proses, a glycoside splits up two portions, sugar portion (glycone) and a non-sugar portion (aglycone). Many different aglycones of glycosides exist in the plants. Most of these glycosides are composed of phenols, steroidal alcohols and polyphenols through glyosidic attachment to sugars. Natural glycosides include D-glucose sugar form more common than L-rhamnose, D- and L-fructose and L-arabinose forms. If the sugar moiety of a glycoside bonded to the aglycone via an oxygen atom, it is named *O*-glycoside. On the other hand, a chemical bond can also be a carbon (C-glycoside), a nitrogen (N-glycoside) or a sulphur atom (S-glycoside). Glycosides are usually named as the anomeric prefix (α- or β-) and the configurational prefix (D- or L-) before the base sugar name. The chemical name of the aglycone leads the name of the sugar (Sarker and Nahar, 2007).

1.6.1 Formation of glycosides

The cyclic hemiacetal formed by a monosaccharide can react with a hydroxide part of another molecule to form an acetal. The acetal form of a sugar is called a glycoside, and the bond between the anomeric carbon and the alkoxy oxygen is called glycosidic bond. The reaction of a single anomer with an alcohol leads to the formation of both the α- and β-glycosides. The mechanism of the reaction shows why both glycosides are formed. The -OH group bonded to the anomeric carbon becomes in the acidic solution, and a lone pair on the ring oxygen helps expel a molecule of water. When the alcohol comes in from the top of the plane, the β-glycoside is formed; when the alcohol comes in from the bottom of the plane, the α-glycoside is formed (Bruice, 2007; Solomons et al., 2014).

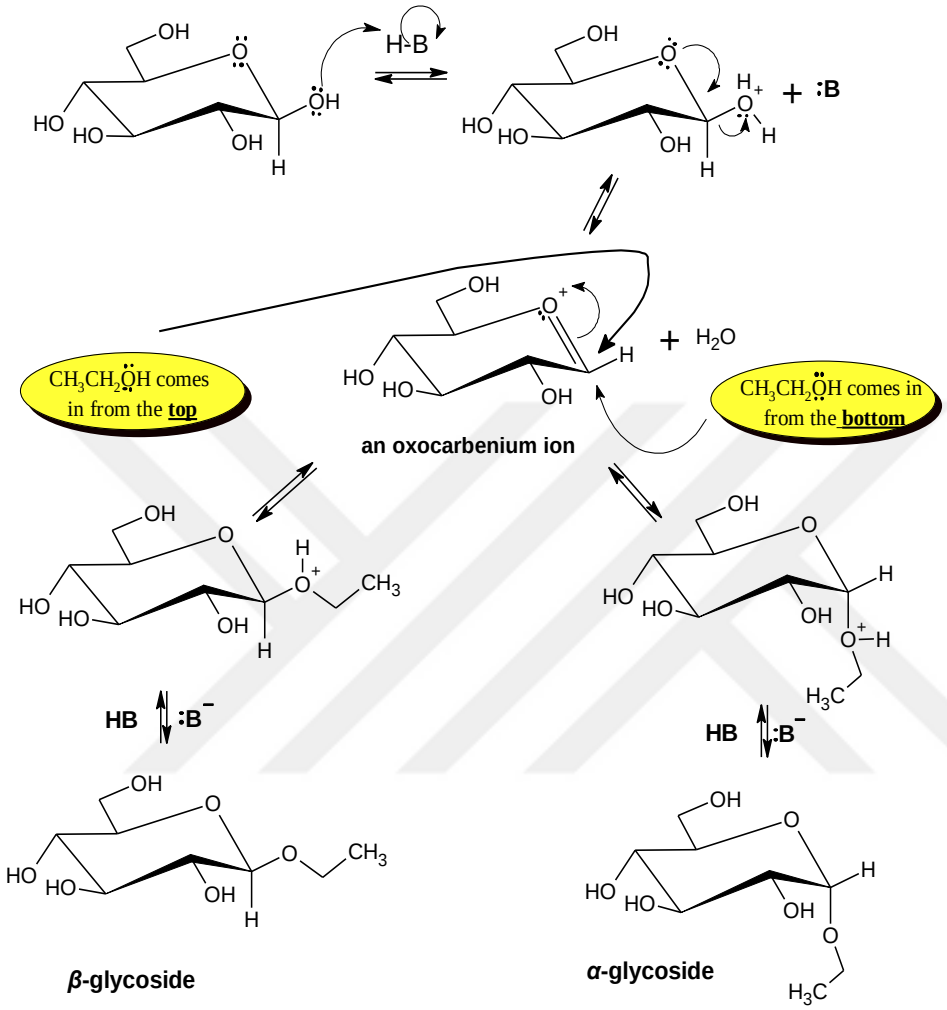


Figure 1.11 Mechanism for glycoside formation

1.6.2 Classification of glycosides

The main classification of the glycosides can be categorized based on the structural types of aglycones. For example, cyanogenic, flavonoid, anthracene, antraquinone, iridoid, steroid and triterpene glycosides. Some important glycosides are described in the next sections.

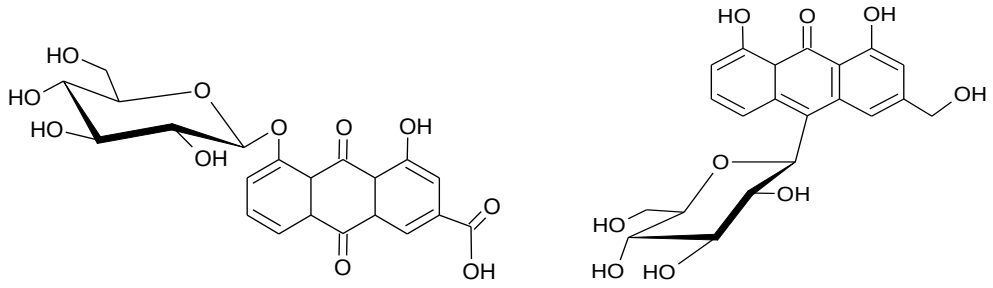
1.6.2.1 Cyanogenic glycosides

Prunasin, amygdalin and variously related glycosides liberate hydrocyanic acid upon hydrolysis and this type glycosides are classified as cyanogenic glycosides. The aglycones of cyanogenic glycosides are biosynthesized from L-amino acids. Linamarin and amygdalin are the major cyanogenic glycosides from *Linum usitatissimum* and *Prunus amygdalus*, respectively.

Cyanogenic glycosides can be determined by the indirect chemical assay based on the effect of HCN on a sodium picrate paper. When the paper color turns yellow to red, it means sodium iso-purpurate is formed by contact with HCN.

1.6.2.2 Flavanoid and anthracene/anthraquinone glycosides

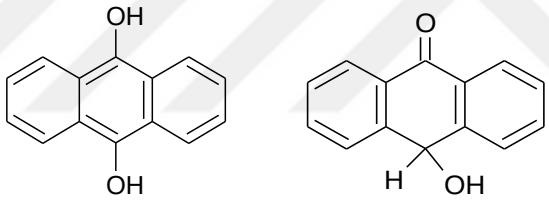
Anthracene derivate glycosides possess an anthraquinone skeleton, e.g. aloin (*C*-glucoside) and rhein 8-*O*-glucoside. Glucose and rhamnose are most common sugars in anthracene derivate glycosides. Due to their laxative and purgative properties, anthraquinone glycosides are included in various drugs. In spite of causing intestinal tumours in long-term usage, laxative preparations contain anthraquinone glycosides.



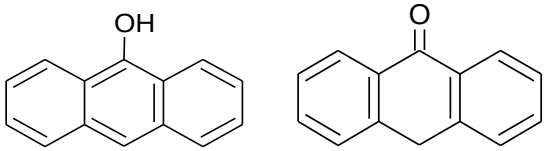
a) Rhein 8-*O*-glucoside b) Aloin

Figure 1.12 Molecular structures of a) Rhein 8-*O*-glucoside and b) Aloin

Anthraquinones are present in various plant species such as Polygonaceae, Liliaceae, Rhamnaceae, Fabaceae and Rubiaceae.



Anthrahydroquinone Oxanthrone



Anthranol Anthrone

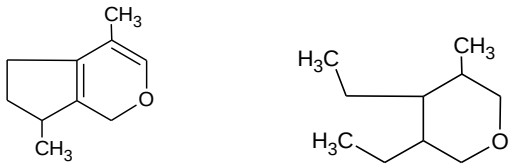
Figure 1.13 Structures of most common anthraquinone aglycones in nature

Determination of the free anthraquinones can be performed by a visual examination. A powdered plant substance is dissolved in an organic solvent and then filtered and a base solution added to an organic phase. A violet or pink colour in aqueous phase indicates the presence of anthraquinones in the plant material.

Cardiac Glycosides; Cardiac glycosides exert a prominent effect on heart muscle e.g. digitoxin (from *Digitalis purpurea*). Atrioventricular conduction and myocardial contraction are affected by cardiac glycosides. The aglycone moieties of cardiac glycosides are steroids that include unsaturated lactone ring (γ-lactone or δ-lactone). Digitoxose, digitals, cymarose, sarmentose and rhamnose are the main cardiac glycosides (Sarker and Nahar, 2007).

1.6.2.3 Iridoid and secoiridoid glycosides

The iridoids and secoiridoids are usually found as glycosides in a large group of plants e.g. harpagoside is an active iridoid glycoside of *Harpagophytum procumbens*. Most of natural iridoids and secoiridoids include an additional oxygenation (hydroxy) at C-1, which is usually involved in the glycoside formation. Besides, there is a double bond between C-3 and C-4, and a carboxylation at C-11 (Sarker and Nahar, 2007).



a) Iridoid b) Secoiridoid

Figure 1.14 Molecular structures of a) Iridoid and b) Secoiridoid

1.6.2.4 Steroid and triterpene glycosides

The aglycone of isoprenoid glycosides is derived from isoprene units by biosynthetically. Isoprenoid glycosides can be separated two major groups as saponins and cardiac glycosides.

Saponins; Saponins are widely distributed glycosides that shows "soap-like" properties in water. Saponin glycosides are characterized by their molecular structure containing a triterpenoid or steroidal aglycone and number of sugar chains (Sarker and Nahar, 2007). Saponins produce an aglycone after hydrolysis that is called sapogenin. Physicochemical and biological properties of saponins are considered in traditional and industrial applications (Oakenfull, 1981; Price et al., 1987; Oakenfull and Sidhu, 1989; Fenwick et al., 1991; Hostettmann and Marston, 1995). In industrial and food processes they used as surface active and foaming reagents. But, saponins have been considered as anti-nutritional properties in the food industry, due to their bitter taste (Ridout et al., 1991; Thompson, 1993; San Martin and Briones, 1999). In recent years there is an increasing demand for saponins in food and pharmaceutical industries due to their health benefits such as anticancer, cholesterol lowering, antifungal, antimicrobial, antitumour, anti-inflammatory, anti-ulcerogenic, spermicidal, analgesic and diuretic properties (Hostettmann and Marston, 1995; Gurfinkel and Rao, 2003; Kim et al., 2003). Saponins are classified according to the number of sugar chains as monodesmosidic, bidesmosidic or tridesmosidic. Single sugar chain attached at C-3 are called monodesmosidic saponins. Two sugar chains attached through an ether linkage at C-3 and one attached through an ester linkage at C-28 (triterpene saponins) or an ether linkage at C-26 (furastanol saponins) are called bidesmosidic saponins. The most common bonded monosaccharides in saponins are D-glucose (Glc), D-fucose (Fuc), D-xylose (Xyl), D-galacturonic acid (GalA), L-arabinose (Ara), L-rhamnose (Rha), D-glucuronic acid (GlcA) and D-galactose (Gal). A various chemical, physical and biological properties of saponins depends on the structural complexity. For example, amphiphilic nature of saponins based on a lipid-soluble aglycone and water-soluble

sugar chains (Mitra and Dungan, 1997; Ibanoglu and Ibanoglu, 2000; Sarnthein-Graf and La Mesa, 2004; Wang et al., 2005). The solubility of saponins is depended on solvent properties, temperature and pH. Most common solvents are water, methanol and ethanol or aqueous alcohols, but ether, chloroform, benzene and ethyl acetate have also been reported (Hostettmann and Marston, 1995).

The biological activities of the pure natural compounds and extracts are investigated many times to define of structure and bioactivity relationships. One of the most experienced features of saponins is *in vitro* haemolytic activity that is swelling and rupturing of erythrocytes. Types of aglycones and sugar chains are related to the activity, but there is no apparent relation between components of this diverse family (Oda et al., 2000).

Adjuvant activity of saponins can affect the immune system due to their ability to improve the effectiveness of orally administered vaccines by facilitating the absorption of large molecules, and their immunostimulatory effects. The cholesterol-lowering activity of saponins has been investigated in human and animal trials. As a result, inhibition of the absorption of cholesterol from small intestine has been attributed by saponins (Bingham et al., 1978; Matsuura, 2001; Kim et al., 2003). Anticancer activity has been reported for a number of triterpene and steroid saponins (Rao and Sung, 1995; Plewa et al., 1998; Berhow et al., 2000; Kerwin, 2004). Reported biological activities of saponins are listed in Table 1.3 (Hostettmann and Marston, 1995; Milgate and Roberts, 1995; Lacaille-Dubois and Wagner, 1996; Francis et al., 2002).

Table 1.3 Some reported biological activities of saponins (Sarker and Nahar, 2007)

Adjuvant	Chemopreventive
Analgesic activity	Cytotoxic
Antiallergic	Diuretic
Antiedematousx	Effect on absorption of minerals and vitamins
Antiexudative	Effect on animal growth
Antifeedant	Effect on cognitive behaviour
Antifungal	Effect on ethanol-induced amnesia
Antigenotoxic	Effect on morphine/nicotine
Antihepatotoxic inhibitory effect	Effects on ruminal fermentation
Anti-inflammatory	Expectorant
Antimicrobial	Haemolytic
Antimutagenic	Hepaprotective
Antiobesity	Hypocholesterolemic
Antioxidant	Hypoglycemic
Antiparasitic	Immunostimulatory effects
Antiphlogistic	Increase permeability of intestinal mucosa cells
Antiprotozoal	Inhibit active nutrient transport
Antipsoriatic	Molluscicidal
Antipyretic	Neuroprotective
Antispasmodic	Reduction in fat absorption
Antithrombotic	Reduction in ruminal ammonia concentrations
Antitussive	Reductions in stillbirths in swine
Antiulcer	Ruminant bloat
Antiviral	Sedative

1.7 Hydrolysis of Glycosidic Compounds

Chemical or enzymatic agents degrade the glycosidic bonds due to the susceptibility to acidic and basic conditions. While chemical hydrolysis is nonspecific, the enzymatic hydrolysis is regiospecific and stereospecific.

Acetolysis reaction is performed by subjecting a glycoside to acid conditions. Especially *O*-glycoside bonds can be broken even in weak acid conditions. On the other hand, S-glycosides are more resistant than *O*-glycosides in acidic conditions. Polysaccharides disintegrate to oligosaccharides, disaccharides, and monomers depending on the acid concentration, branching, and solubility.

Glycosides even have naturally high acid sensitivity can be partially sensitive to basic conditions. Three classes of *O*-glycosides; phenolic glycosides, enolic glycosides and *β*-substituted alcohol glycosides might be subject to basic hydrolysis.

The rule of most glycosidases is not totally well understood, but the correlation between feeding and detoxification processes was related. Some of the best-studied hydrolyses are the *β*-glycosidases and among them *β*-glucosidases, *β*-glucuronidases, *β*-glucanases, *β*-chitinases, all of them with important biological and economic implications (Brito-Arias, 2016).

Glycosidic compounds are seconder metabolites found in plants, one of the most important natural compound sources. The *Cephalaria* genus from plants containing such compounds is an important natural resource that is distributed in our country.

1.8 Introduction to the Genus *Cephalaria* (Caprifoliaceae)

The name of *Cephalaria* Schrad. ex Roem. & Schult. originates from the word kephale that means head. The genus *Cephalaria* is distributed particularly in South Africa and in the Holarctic Kingdom (Mediterranean area, West China, Caucasia, South Ukraine and the Middle East) (Szabó, 1940). The total number of *Cephalaria* species is 94 in the world. The number of species in Turkey is 40 and the total number of taxa of Cephalaria is also 42 in Turkey (Gokturk and Sumbul, 2014, 2016). The genus *Cephalaria* has belonged to the Dipsacaceae for a long time, but according to APG III (Angiosperm Phylogeny Group III) the genus is included Caprifoliaceae (Reveal and Chase, 2011). There is lots of research based on chemical constituents and biological activities of various species of *Cephalaria*. *Cephalaria* taxa growing in Turkey is listed below (Table 1.4).

Table 1.4 *Cephalaria* species growing in Turkey (Gokturk and Sumbul, 2014)

1. *Cephalaria aristata* K.Koch	21. *Cephalaria tuteliana* Kuş & Göktürk
2. *Cephalaria joppensis* (Rchb.) Coult. ex DC.	22. *Cephalaria isaurica* V.A.Matthews
3. *Cephalaria transylvanica* (L.) Schrad.	23. *Cephalaria media* Litv.
4. *Cephalaria cilicica* Boiss. & Kotschy	24. *Cephalaria aytachii* Göktürk & Sümbül
5. *Cephalaria setosa* Boiss. & Hohen.	25. *Cephalaria balansae* Raus
6. *Cephalaria syriaca* Schrad.	26. *Cephalaria gigantea* (Ledeb.) Bobrov
7. *Cephalaria stapfii* Hausskn.	27. *Cephalaria procera* Fisch. & Avé-Lall.
8. *Cephalaria speciosa* Boiss. & Kotschy	28. *Cephalaria tchihatchewii* Boiss.
9. *Cephalaria duzceënsis* Aksoy & Göktürk	29. *Cephalaria kotschyi* Boiss. & Hohen.
10. *Cephalaria davisiana* Göktürk & Sümbül	30. *Cephalaria sparsipilosa* V.A.Matthews
11. *Cephalaria sumbuliana* Göktürk	31. *Cephalaria microcephala* Boiss.
12. *Cephalaria demirizii* Göktürk & Sümbül	32. *Cephalaria hakkiarica* V.A.Matthews
13. *Cephalaria stellipilis* Boiss.	33. *Cephalaria lycica* V.A.Matthews
14. *Cephalaria elazigensis* Göktürk & Sümbül var.*elazigensis* *Cephalaria elazigensis* Göktürk & Sümbül var. *purpurea* Göktürk & Sümbül	34. *Cephalaria scoparia* Contandr. & Quézel 35. *Cephalaria dirmilensis* Hub.-Mor.
15. *Cephalaria elmaliensis* Hub.-Mor. & V.A. Matthews	36. *Cephalaria gazipashensis* Sümbül subsp. *gazipashensis*
16. *Cephalaria hirsuta* Stapf	*Cephalaria gazipashensis* Sümbül subsp. *pilifera* Parolly, Kürschner & Eren
17. *Cephalaria peshmenii* Sümbül	37. *Cephalaria ekimiana* Göktürk & Sümbül
18. *Cephalaria szaboi* Hayek	38. *Cephalaria salicifolia* Post
19. *Cephalaria paphlagonica* Bobrov	39. *Cephalaria taurica* Szabó
20. *Cephalaria anatolica* Shkhiyan	40. *Cephalaria anamurensis* Göktürk & Sümbül

Cephalaria hirsuta Stapf (Figure 1.15) is stout and erect perennial herb, that stem up 1 m tall and striate. Coriaceous leaves, simple or lyrate lower leaves, densely hirsute hairy at the margin are identified. Capitula globose, 1–2.5 cm in diameter in flower, 1–1.7 cm in diameter in fruit. It has corolla, 7–12 mm long and yellow or cream. *Cephalaria hirsuta* occurs on high mountain steppe and rocky places (2210–2450 m). It is distributed Turkey (East Anatolia) and West Iran at the Irano-Turanian element. Type: [W. Iran] in agro Ecbatanensi (Media), Pichlers.n.(holo. WU; iso. K) (Gokturk and Sumbul, 2014).

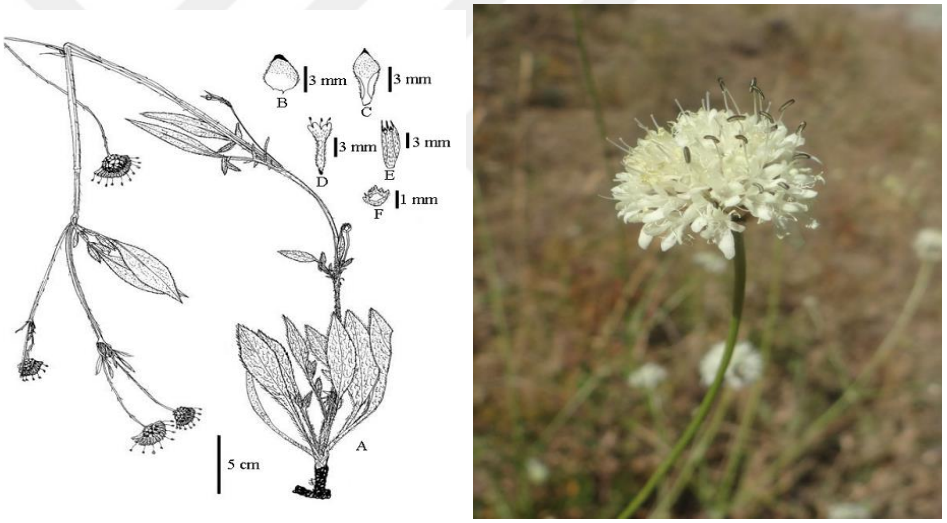


Figure 1.15 *C. hirsuta* Stapf (Gokturk and Sumbul, 2014)

Cephalaria procera Fisch. & Avé-Lall. (Figure 1.16) is stout and erect perennial herb, that stem up 2 m tall, striate, hollow, glabrous or sparsely pilose. Herbaceous leaves and lyrate-pinnatisect lower leaves are identified. Capitula globose, 2–4 cm in diameter in flower, 1.5 – 3 cm in diameter in fruit. It has corolla, 13–15 mm long and sulphur yellow or cream. *Cephalaria procera* occurs on Rocky slopes, meadows, steppe and roadsides (900–2600 m). It is distributed Turkey (Black Sea, Central Anatolia, East Anatolia, and northeast of Mediterranean Region), Armenia, North Iran, Caucasia, and Transcaucasia at the Irano-Turanian element. Lectotype: Anatolia. Juldis Dagh, Wiedemann s.n.(LE) (Gokturk and Sumbul, 2014).

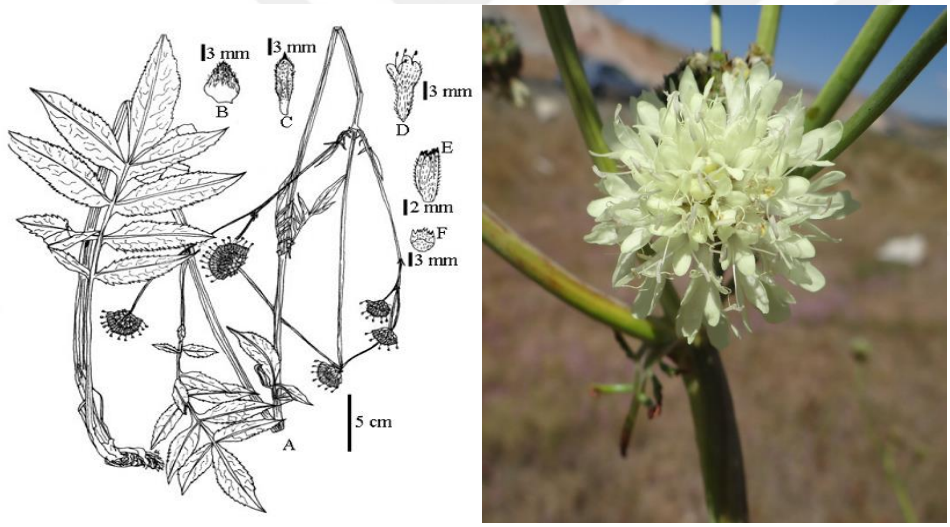


Figure 1.16 *C. procera* Fisch. & Avé-Lall (Gokturk and Sumbul, 2014)

Cephalaria elazigensis* var. *elazigensis Göktürk & Sümbül (Figure 1.17) slender and erect perennial herb, that stem up 1 m tall. Coriaceous leaves and minute stellate hairs on both surfaces are identified. Capitula ovoid or ovoid-subglobose, 1–1.75 cm in diameter in flower, 1 – 1.5 cm in diameter in fruit. It has corolla, 8–10 mm long and yellow or purple. *C. elazigensis* var. *elazigensis* occurs on dry slopes (900 m). It is distributed Turkey (East Anatolia) at the Irano-Turanian element. Type: Turkey. B7 Elazığ: Maden, 8 km from Maden to Ergani, dry slopes, 900 m, 30.7.2001, R.S.Göktürk4698 & M.Göktürk (holo. Akdeniz Univ. Herb.; iso. ANK, HUB, GAZI) (Gokturk and Sumbul, 2014).

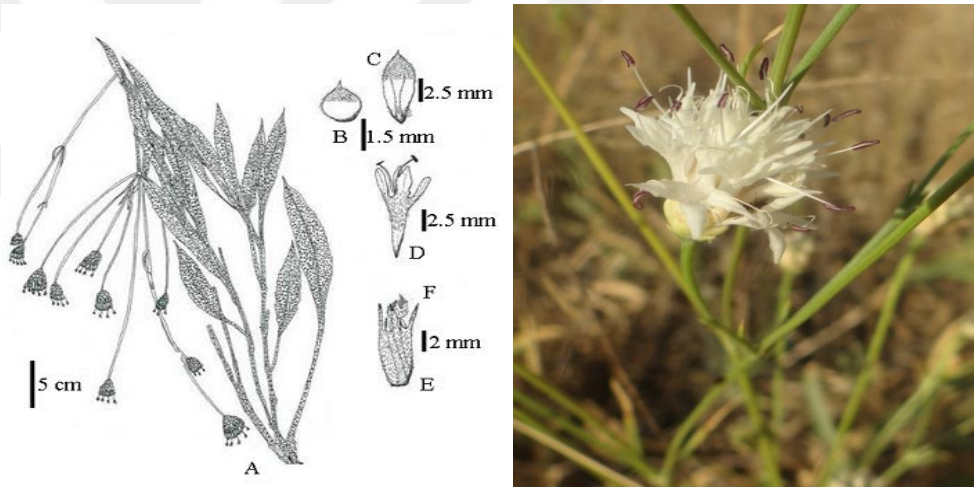


Figure 1.17 *C. elazigensis* var. *elazigensis* Göktürk & Sümbül (Gokturk and Sumbul, 2014)

1.8.1 Scientific reports for *Cephalaria* species

A number of flavonoids, triterpene saponins, iridoids, lignans, alkaloids and their glycosidic compounds have been reported as results of phytochemical investigations on *Cephalaria* species. There are many studies on isolation and bioactivity of triterpene saponins from *Cephalaria* species. The first recorded investigation on *Cephalaria* species was performed in 1929 and two glucosides (*β*-methyl glycoside and an amorphous glycoside) were found in *C. alpina* Schrod. (Wattiez, 1929). Biochemical and chemical studies on other *Cephalaria* species have followed up this study.

Approximately seventy investigations on various species have been reported since 1929. There are three reports for *C. syriaca* on biochemical and seed oil properties (Lys, 1951; Yazicioglu et al., 1978; Ali et al., 2012). Chemical investigations, biochemical, isolation and characterisation studies were performed on *C. gigantea* and reported (Zemtsova et al., 1970; Zviadadze et al., 1976, 1980, 1981, 1983; Tabatzade et al., 2002, 2003, 2005, 2007; Movsumov et al., 2006; Gogitidze et al., 2017). *C. leucantha, C. kotschyi* and *C. nachiczevanica* species were researched and mostly focused on triterpenoid and alkaloid compounds (Bouillant et al., 1972; Aliev et al., 1974, 1975a; 1975b, 1976, 1981; Movsumov et al., 1975a; 1975b; Godevac et al., 2006a; Mustafaeva et al., 2008, 2011). Many triterpenoid saponins were isolated and identified from *C. transsylvanica* and biological activities were investigated (Tagiev et al., 1976; Kirmizigul et al., 1994a; 1994b, 1995, 1996a; 1996b, 1997, 2002). Novel compounds and various biological data were reported from *C. uralensis, C. procera, C. ambrosioides, C. pastricensis, C. grossheimii* and *C. velutina* between 1977 and 2016 (Zemtsova et al., 1977; Ulubelen et al., 1978; Pasi et al., 2002a; 2002b, 2009; Movsumov et al., 2009a; 2009b, 2010, 2013; Godevac et al., 2004, 2006b, 2010; Sarikahya et al., 2015b; Vukicevic et al., 2016). Recently, more investigation was performed on *Cephalaria* species. Phytochemical analysis, immunomodulatory, hemolytic, cytotoxic activity potentials, fatty acid profile, structural elucidations, isolation and characterization experiments were reported various *Cephalaria* species. For example *C. joppica, C. isaurica, C. dipsacoides, C. lycica, C. davisiana, C. elazigensis* var. *purpurea, C. cilicica, C. stellipilis, C. media, C. paphlagonica, C. balansae, C. aristata, C. scoparia, C. gazipashensis, C. elmaliensis, C. aytachii, C. anatolica, C. taurica, C. tuteliana, C. speciosa, C. tchihatchewii, C. hirsuta, C. elazigensis* var. *elazigensis* (Kirmizigul et al., 2007, 2012; Capanlar et al., 2010; Kayce et al., 2010, 2014, 2017a; 2017b; Halay et al., 2010; Sarikahya et al., 2010, 2011,2012a; 2012b, 2013, 2014, 2015a; 2015b, 2018; Garaev et al., 2014; Top et al., 2017; Ozer et al., 2018).

2. MATERIALS and METHODS

2.1 General

Medium pressure liquid chromatography (MPLC) applications were performed on Buchi C-605 instrument with various appropriate Buchi glass columns (15–49 mm x 100–920 mm). MPLC and CC (Open column chromatography) studies were carried out on silica gel 60 (0.063–0.200 mm, Merck 7734). LiChroprep RP-18 (25–40 mm, Merck 9303) was used as stationary phases for VLC (Vacuum liquid chromatography) studies. All Thin Layer Chromatography (TLC) analyses were performed on silica gel F254 (Merck 5554) pre-coated aluminum plates. Eluted compounds were detected by spraying with H_2SO_4:H_2O (1:5, v/v) followed by heating at 120 ºC. 1D- and 2D- NMR results were obtained from Varian ASP 600 *MHz* and 400 *MHz* in DMSO-d_6. Chemical shifts were given in ppm under the guidance of tetramethylsilane (TMS) as reference standard. The coupling constants (*J*) were recorded in *Hz*. HRESIMS analyses were performed by a Bruker LC micro-Q-TOF mass spectrometer. GC-MS analyses were applied by Shimadzu GCMS QP 2010 plus instrument with Rtx-CLPestisides2 (20 m x 0.18 mm ID x 0.14 μm df) capillary column.

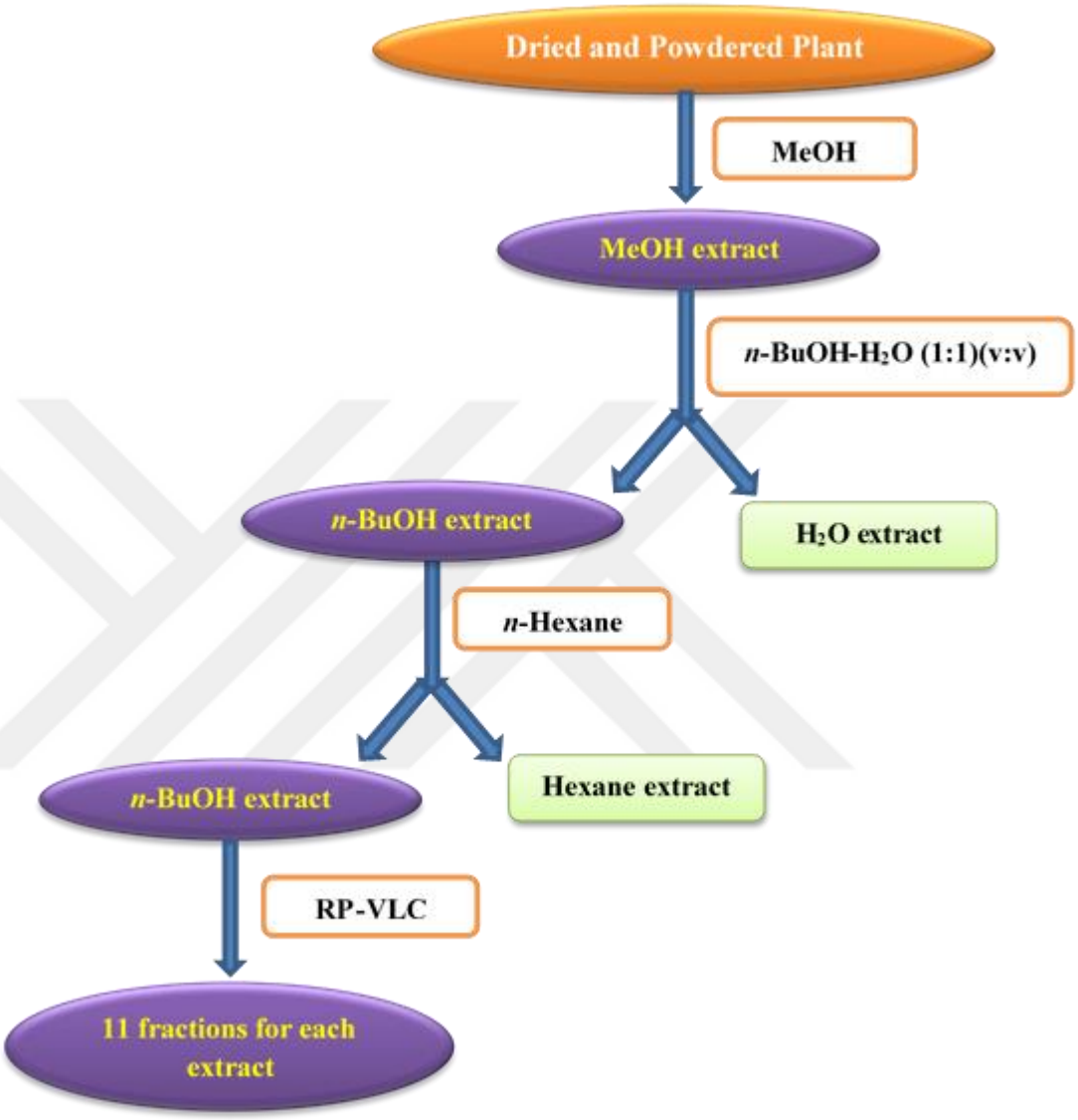
Qualitative and quantitative determination of saponin contents in 3 different plants' extracts were performed comparing with standard saponins by an ultra-high-performance liquid chromatography combined with electrospray ionization tandem mass spectrometer (UPLC-ESI-MS/MS) (Waters, Milford, MA). The mass spectrometer was controlled by MassLynx 4.1 software. The samples were separated on a BEH C18 column (50 mm x 2.1 mm, 1.7 μm particle size; Waters).

2.2 Plant Material

The aerial parts of *Cephalaria hirsuta* Stapf. (Caprifoliacea), *Cephalaria procera* Fisch. & Avé-Lall (Caprifoliacea) and *Cephalaria elazigensis* var. *elazigensis* Göktürk & Sümbül (Caprifoliacea) were harvested in vegetative season of 2013 and identified by Prof. Dr. RS. Göktürk (Department of Biology, Faculty of Arts and Science, Akdeniz University). *C. hirsuta* was collected from Erzurum, Erzurum-Cat road 5 km (AKDU 4142, RS Göktürk 7676), *C. procera* was collected from Sivas-Zara, Zara-Imranli road (AKDU 3535, RS Göktürk 7672) and *C. elazigensis* var. *elazigensis* was collected from Elazig-Maden, Maden-Ergani road 8. km (AKDU 1962, RS Göktürk 7679). Plant materials were deposited in Herbarium Research and Application Centre of Akdeniz University.

2.3 Extraction of Plant Materials

Air-dried and milled *C. hirsuta* (2.0 kg), *C. procera* (2.0 kg) and *C. elazigensis* var. *elazigensis* (1.6 kg) were extracted with methanol (95% purity) (4 x 5 L) by a laboratory mixer (Silverson, L5M-A, USA) for 12 h at room temperature individually. The extracts were concentrated by rotary evaporator under reduced pressure till dryness at 40 ºC. MeOH residues (*C. hirsuta*: 291.1 g, *C. procera*: 275.0 g and *C. elazigensis* var. *elazigensis*: 213.4 g) were extracted with *n*-BuOH:H_2O (1:1, 5×200 mL) solvent system. After the separation and evaporation of *n*-BuOH and H_2O portions, the *n*-BuOH fractions (*C. hirsuta*: 124.4 g, *C. procera*: 81.8 g and *C. elazigensis* var. *elazigensis*: 111.0) were defatted with *n*-hexane (100 mL x 10) to remove chlorophylls and oily partitions. After that, the saponin-rich *n*-BuOH fractions were concentrated under reduced pressure (20 mbar). The *n*-BuOH residues (*C. hirsuta*: 71.0 g, *C. procera*: 48.0 g and *C. elazigensis* var. *elazigensis*: 72.6 g) were subjected to VLC using Lichroprep RP-18 as an adsorbent by MeOH:H_2O solvent system with a gradient from 0% to 100% MeOH to give 11 fractions for each species.



Scheme 2.1 Flow chart for extraction of plant materials

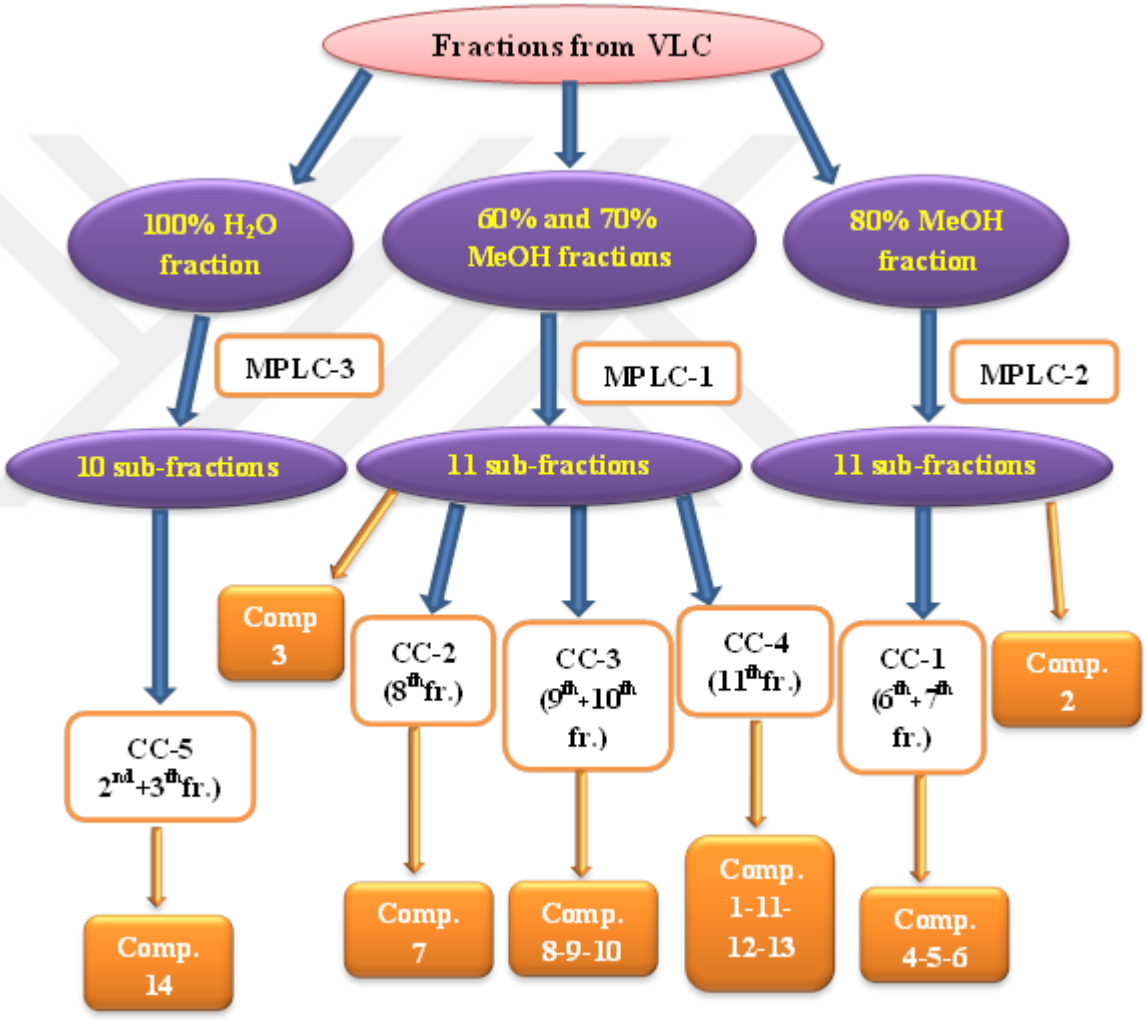
2.3.1 Isolation and purification of *C. hirsuta*

The 60% and 70% MeOH fractions (19.0 g) of RP-VLC were combined and applied to MPLC-1 over silica gel using a suitable column (26 x 920 mm) and programme [max. pressure: 40 bar, flow rate: 23 mL/min, solvent system [61:32:7 (1000 mL)]. Eleven sub-fractions were obtained and analyzed by TLC. Compound **3** (380 mg) was obtained from the 6th fraction as a pure compound. Compound **7** (280 mg) was purified from 8th fraction (2.3 g) of MPLC-1 by a silica gel CC-2 performing $CHCl_3$:MeOH:H_2O solvent system in order of 90:10:0 (500 mL) and 80:20:2 (3000 mL). Compounds **8** (155 mg), **9** (1120 mg) and **10** (600 mg) were obtained from the combined 9th and 10th fractions (7.5 g) of MPLC-1 by a silica gel CC-3 performing $CHCl_3$:MeOH:H_2O solvent system in order of 90:10:0 (1000 mL), 80:20:2 (1000 mL), 85:25:2.5 (500 mL), 70:30:3 (1000 mL), 65:35:4 (1000 mL) and 61:32:7 (1000 mL). Compound **1** (50 mg), **11** (330 mg), **12** (430 mg), and **13** (170 mg) were purified from 11th fraction (2.5 g) of MPLC-1 by a silica gel CC-4 and the solvent systems ($CHCl_3$:MeOH:H_2O) were used in order of 90:10:0 (1000 mL), 80:20:2 (1000 mL), 85:25:2.5 (500 mL), 70:30:3 (1000 mL), and 61:32:7 (1000 mL).

The 80% MeOH fraction (6.2 g) of RP-VLC was exposed to MPLC-2 over silica gel using a suitable column (26 x 920 mm) and programme [max. pressure: 40 bar, flow rate: 23 mL/min, solvent system $CHCl_3$:MeOH:H_2O / 90:10:0.5 (1500 mL), 80:20:2 (1000 mL), 75:25:2.5 (1000 mL), 70:30:3 (1000 mL)]. Nine sub-fractions were obtained from this application. Compound **2** (406 mg) was obtained from 4th fraction as a pure compound. The combined fractions 6th and 7th (1.082 g) of MPLC-2 were applied to another silica gel CC-1 and eluted with $CHCl_3$:MeOH:H_2O in order of 90:10:0 (1500 mL), 80:20:2 (2000 mL) and 75:25:2.5 (1000 mL) to give the compounds **4** (120 mg), **5** (120 mg), and **6** (300 mg).

The 100% H_2O fraction (5.4 g) of RP-VLC was subjected to MPLC-3 over silica gel using a suitable column (26 x 920 mm) and programme [max. pressure: 40 bar,

flow rate: 23 mL/min, solvent system $CHCl_3$:MeOH:H_2O / 90:10:0.5 (1000 mL), 80:20:2 (1000 mL), 70:30:3 (1000 mL)]. Ten sub-fractions were obtained from this application. MPLC-3 fractions 2^{nd}+3^{rd} combined (336.0 mg). Lastly they eluted with CC-5 to give Compound **14** (70 mg).



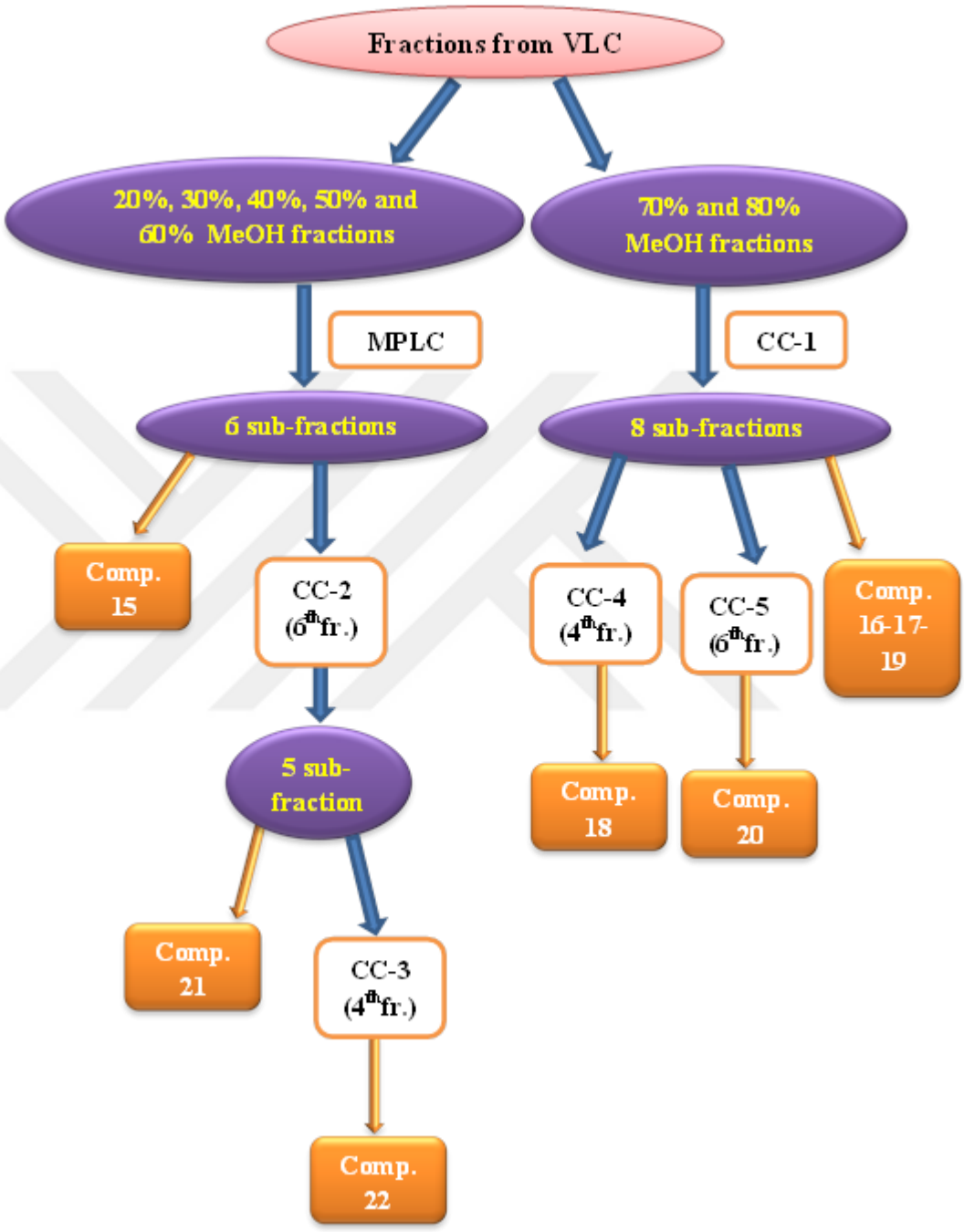
Scheme 2.2 Flow chart for extraction of *C. hirsuta*

2.3.2 Isolation and purification of *C. procera*

The 20%, 30%, 40%, 50% and 60% MeOH fractions of RP-VLC were combined (9.0 g) and applied to MPLC over silica gel using a suitable column (26 x 920 mm) and programme [max. pressure: 40 bar, flow rate: 23 mL/min, solvent system $CHCl_3$:MeOH:H_2O / 90:10:0 (1000 mL), 80:20:2 (1500 mL), 70:30:3 (1100 mL) and 61:32:7 (1500 mL)]. Six sub-fractions were obtained and analyzed by TLC. Compound **15** (120 mg) was obtained from 4th fraction directly.

Sixth fraction (300 mg) was applied to CC-2 and five sub-fractions were resulted. Compound **21** (10 mg) was obtained from the 3th fraction of CC-2 directly. Compound **22** (25 mg) were obtained from the 4^{h} fraction (40 mg) by a silica gel CC-3 performing $CHCl_3$:MeOH:H_2O solvent system in order of 80:20:2 (50 mL), 70:30:3 (50 mL) and 61:32:7 (300 mL).

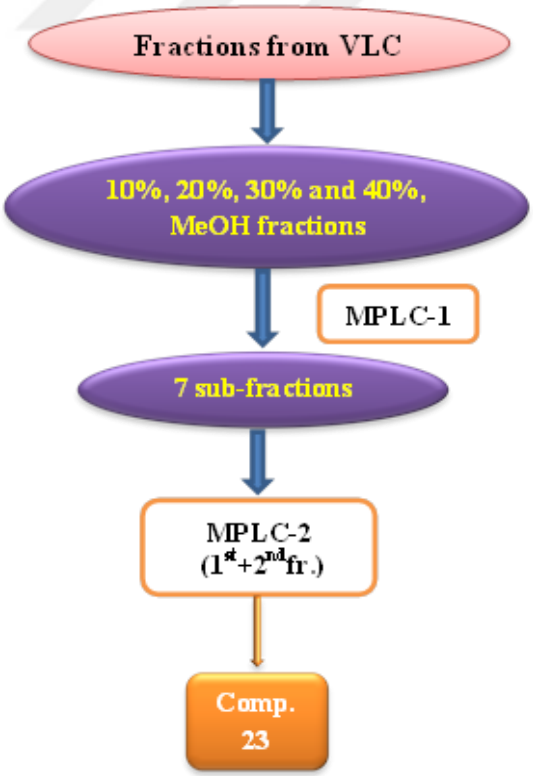
Fractions 70% and 80% of MeOH (4.0 g) of RP-VLC were exposed to CC-1 over silica gel using a suitable column, solvent system [$CHCl_3$:MeOH:H_2O / 90:10:0.5 (1000 mL), 80:20:2 (1000 mL), 70:30:3 (1000 mL) and 61:32:7 (1200 mL)]. Eight sub-fractions were obtained. Compound **16** (250 mg) and **17** (90 mg) were directly obtained from 2nd and 3rd fractions, respectively. Compound **19** (250 mg) was obtained from 5th fraction directly. Compound **18** (30 mg) was purified from 4th fraction (180 mg) by a silica gel CC-4 performing $CHCl_3$:MeOH:H_2O solvent system in order of 80:20:2 (100 mL), 70:30:3 (50 mL) and 61:32:7 (200 mL). Compound **20** (9 mg) was purified from the 6th fraction (40 mg) by a silica gel of CC-5 performing $CHCl_3$:MeOH:H_2O solvent system in order of 80:20:2 (50 mL), 70:30:3 (50 mL) and 61:32:7 (100 mL).



Scheme 2.3 Flow chart for extraction of *C. procera*

2.3.3 Isolation and purification of *C. elazigensis* var. *elazigensis*

The RP-VLC fractionation of *n*-BuOH extract was given eleven fractions using H_2O:MeOH beginning from 100% H_2O to 100% MeOH by gradiating these solvents. Fractions 10%, 20%, 30% and 40% of RP-VLC were combined (13.4 g) because of their TLC similarities. For that reason the combined fractions was applied to MPLC-1 over silica gel using a suitable column (26 x 920 mm) and programme [max. pressure: 40 bar, flow rate: 23 mL/min, solvent system $CHCl_3$:MeOH:H_2O / 90:10:0 (500 mL), 80:20:2 (1000 mL), 70:30:3 (1250 mL) and 61:32:7 (700 mL)]. Seven sub-fractions were obtained from this MPLC-1 application. Fractions 1 and 2 were combined and this combination applied to MPLC-2 application using silicagel and EtOAc : *n*-hexane 1:2 (900 mL), 1:1 (500 mL) and 2:1 (300 mL). Compound **23** (308 mg) was obtained from the 3th fraction.



Scheme 2.4 Flow chart for extraction of *C. elazigensis* var. *elazigensis*

2.4 Determination of Triterpene Saponins by UPLC-ESI-MS/MS

2.4.1 Preparation of standard and sample solutions

The saponin-rich *n*-BuOH fractions (Section 2.3) each of three *Cephalaria* species were 1.0 mg, and they seperately dissolved in 10.0 mL MS grade of methanol and filtered through a 0.22 μm PTFE syringe for the UPLC-ESI-MS/MS analysis. Dilution factor (D.F.) was $1x10^4$ for this application. Before validation studies, a trial analyse was performed to estimate the concentrations of saponins in the *n*-BuOH fractions. The highest concentration was approximately 10000 mg/kg for Scoposide G in the *n*-BuOH fraction of *C. hirsuta*. Due to high concentration of Scoposide G, highest level of calibration range was selected as 1.0 mg/L. So, stock solution of forty triterpenoid compounds were prepared by dissolving 1.0 mg of each compounds in 10 mL volumetric flask. Six levels (0.025, 0.050, 0.100, 0.250, 0.500, and 1.00 mg/L) of calibration standards were prepared in 5 mL volumetric flask with MeOH. The results were calculated considering dilution factor as $1x10^4$.

2.4.2 Chromatographic conditions

The mobile phases were 0.1 mM ammonium formate solution in H_2O (A) and 0.1 mM ammonium formate solution in methanol (B). Gradient programme was adjusted to 0.4 mL/min flow rate at 40 ^oC. After 5 μL injection, (B) linearly increased from 5% to 50% in 2 min, linearly increased to 75% in 1 min, linearly increased to 95% in 1.5 min and stayed at this concentration for 0.5 min. Then, the system was returned to the initial conditions in 1 min.

2.4.3 Mass spectrometry conditions

Tandem mass spectrometer with an ESI system operated in positive-ion mode. The desolvation gas (N_2) flow and cone gas (Ar) flow were set at 500 L/h and 100

L/h, respectively. Capillary energy was set at 3.0 kV. Cone energies, collision energies, precursor and transition ions were set up individually considering their response and peak shapes. Ion source and desolvation gas (N_2) were heated at 120 ^oC and 350 ^oC, respectively. The scan range was *m/z* 50–2000, and the dwell time was 0.100 second.

2.4.4 Method validation

Linearity parameter for each compound was determined by analyzing standard solutions. The linearity range was determined from 0.025 to 1.0 mg/L for all compounds. External calibrations were applied to calculate the concentration of triterpenoid compounds in the *n*-BuOH fractions of three plant extracts. The recovery experiments were evaluated at two fortification levels (500.0 mg/kg and 1000.0 mg/kg) and three times injection. Dilution factor (D.F.= $1x10^4$) was used to calculate the results. The precision (repeatability) of the reported method was evaluated by repeating the measurements 6 times at 500.0 mg/kg and 1000.0 mg/kg spiked the concentrations in the same day. The LOD and LOQ values of triterpenoid compounds were calculated *via* signal to noise (S/N) ratio.

3. RESULTS and DISCUSSION

Isolation, purification and determination of natural products especially glycosidic ones in *Cephalaria hirsuta, Cephalaria procera* and *Cephalaria elazigensis* var. *elazigensis* was aimed in this study. As it is mentioned in Second Part, all plant materials have been dried in the shade and then powdered. The plant materials were extracted with MeOH, *n*-BuOH:H_2O and *n*-hexane individually. Isolation and determination process were applied on *n*-BuOH which were the saponin rich extracts of three plants.

3.1 Spectroscopic Determinations

3.1.1 *Cephalaria hirsuta*

After isolation and purification process on *Cephalaria hirsuta* Stapf. (Caprifoliacea), 14 pure glycosides were obtained. These compounds were structurally identified as Aristatoside B (**1**), *α*-Hederin (**2**), 3-*O*-*α*-L-rhamnopyranosyl-(1→2)-*α*-L-arabinopyranosyl hederagenin 28-*O*-*β*-D-glucopyranosyl ester (**3**), Sapindoside B (**4**), Macranthoside A (**5**), Scoposide D (**6**), 3-*O*-*β*-D-glucopyranosyl (1→3)-*α*-L-rhamnopyranosyl-(1→2)-*α*-L-arabinopyranosyl hederagenin 28-*O*-*β*-D glycopyranosyl ester (**7**), Decaisoside D (**8**), Dipsacoside B (**9**), Elmalienoside C (**10**), Elmalienoside A (**11**), Macranthoidin A (**12**), Cephoside B (**13**) and 7*β*-7-*O*-methyl morroniside (**14**).

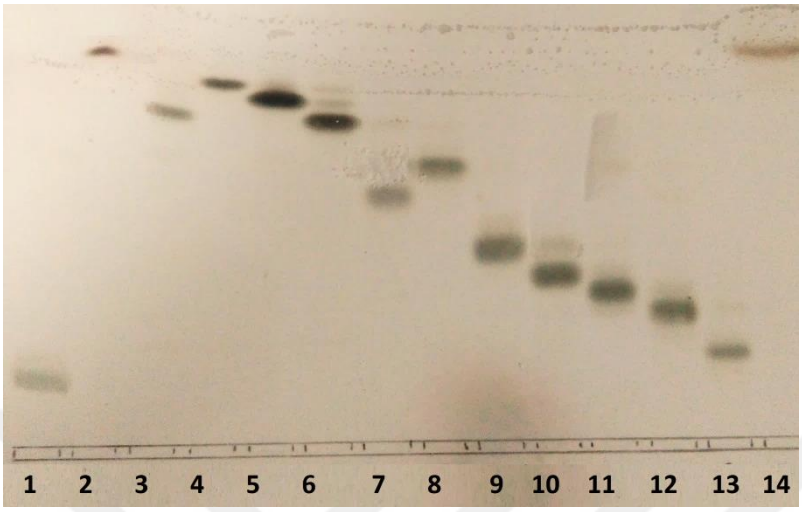


Figure 3.1 TLC spots of isolated pure compounds from *C. hirsuta*

Compound 1 (**Aristatoside B**): A white, amorphous powder (50.0 mg), bisdesmosidic triterpenoid glycoside, ^{1}H-NMR (DMSO-d_6, 600 *MHz*) and ^{13}C-NMR (DMSO-d_6, 150 *MHz*) and, see Figure 3.16 and 3.17 (Sarikahya, 2014).

According to the report, compound **1** (**Aristatoside B**) did not show any anti-bacterial activity against *S. aureus* and *B. subtilis* whereas activity against *E. coli* at range of MIC value 0.62–2.5 mg/mL. Also this was found most active at the concentration of 1.25 mg/mL for *E. coli*, *E. faecalis*, *P. aeruginosa*, *S. typhimurium* and *C. albicans*. In addition, it showed antifungal activity against *C. albicans* with the MIC value 1.25–2.5 mg/mL. Gentamycin and Clotrimazole were used as referance control in this experiment (Sarikahya, 2014). Another report showed that compound **1** demonstrated moderate immunomodulatory activity (Sarikahya et al., 2018). The molecular structure of the compound was presented as in Figure 3.2.

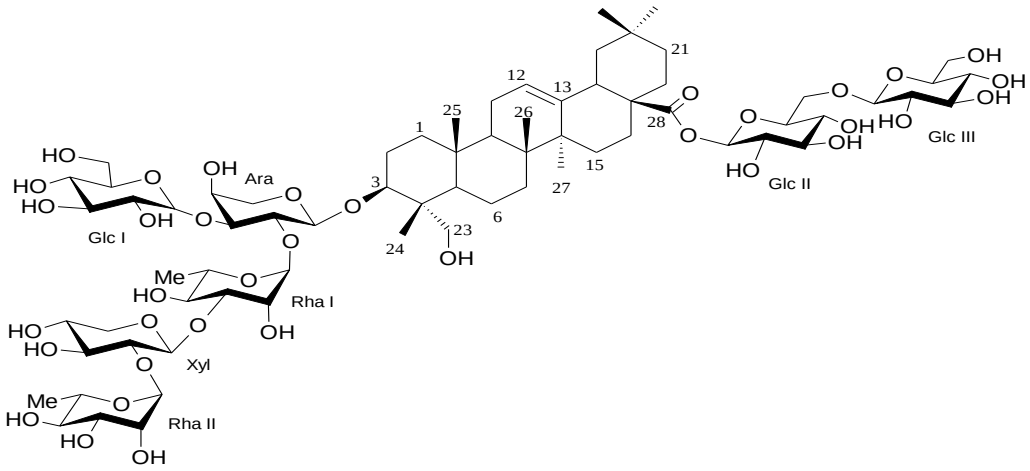


Figure 3.2 Structure of compound **1**

Compound 2 (***α*-Hederin**): A white, amorphous powder (400.0 mg), monodesmosidic triterpenoid glycoside; ^{1}H-NMR (DMSO-d_6, 400 *MHz*) and ^{13}C-NMR (DMSO-d_6, 100 *MHz*), see Figure 3.29 and 3.30 (Aliev and Movsumov, 1976).

According to report, compound **2** (***α*-Hederin**) inhibits the growth of breast cancer cells anticancer activity. MTT assay was performed to determine the inhibitory rate of growth on breast cancer cell lines, MCF-7 and MDA-MB-231 (Cheng et al., 2014). Another experiment on antitumor activities of *α*-Hederin was presented in 2008. In this study, antitumor activity of *α*-Hederin was tested by MTT assay on HT-29 cell line with 5-fluorouracil (5-FU) (Bun et al., 2008). Besides, hemolytic, antifungal (Takechi et al., 1990) and anticancer (Cheng et al., 2014) activities were reported in various studies. The molecular structure of the compound was presented as in Figure 3.3.

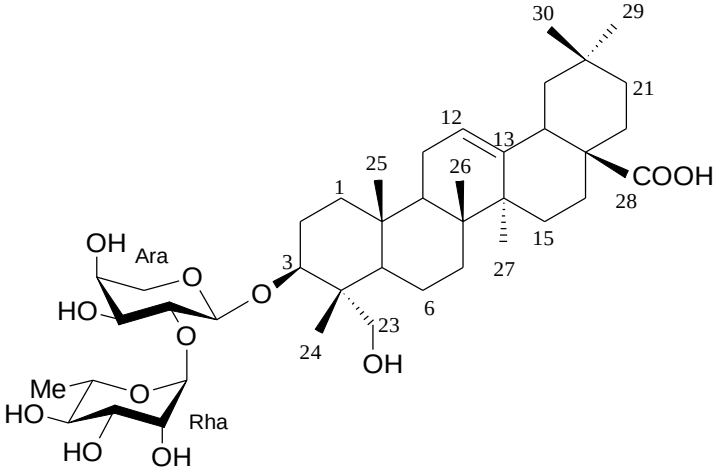


Figure 3.3 Structure of compound **2**

Compound 3 (3-*O*-*α*-L-rhamnopyranosyl-(1→2)-*α*-L-arabinopyranosyl hederagenin 28-*O*-*β*-D-glucopyranosyl ester): A white, amorphous powder (380.0 mg), bisdesmosidic triterpenoid saponin; ^{1}H-NMR (DMSO-d_6, 400 *MHz*) and ^{13}C-NMR (DMSO-d_6, 100 *MHz*), see Figure 3.31 and 3.32 (Kawai et al., 1988).

According to the report, compound **3** showed hemolytic activity in the ratio of 47% in sheep red blood cells (Kawai et al., 1988). The molecular structure of the compound was presented as in Figure 3.4.

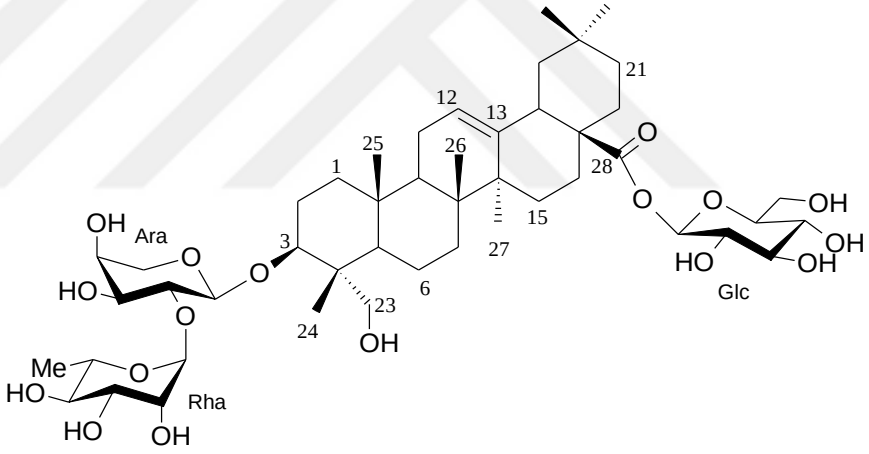


Figure 3.4 Structure of compound **3**

Compound 4 (**Sapindoside B**): A white, amorphous powder (120.0 mg), monodesmosidic triterpenoid saponin; ^{1}H-NMR (DMSO-d_6, 600 *MHz*) and ^{13}C-NMR (DMSO-d_6, 150 *MHz*), see Figure 3.33 and 3.34 (Chirva et al., 1969).

According to reports, compound **4** (**Sapindoside B**) inhibited mycelial growth of *V. inaequalis* and *B. cinerea* by 45 and 43%, respectively (Porsche et al., 2008). Hemolytic activity of Sapindoside B was tested in Sheep erythrocytes and 100% haemolytic dose was determined as the saponin concentration which is 22.7 mg/mL (Voutquenne et al., 2008). This compound showed strong antimicrobial activity (MIC values 0.90-6.50 μg/mL) (Pasi et al., 2009). The molecular structure of the compound was presented as in Figure 3.5.

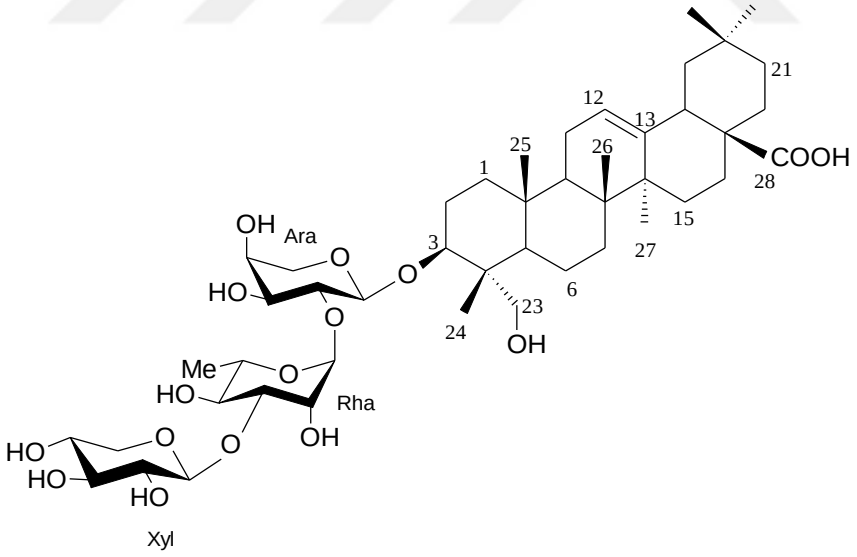


Figure 3.5 Structure of compound **4**

Compound 5 (**Macranthoside A**): A white, amorphous powder (120.0 mg), monodesmosidic triterpenoid saponin; ^{1}H-NMR (DMSO-d_6, 600 *MHz*) and ^{13}C-NMR (DMSO-d_6, 150 *MHz*), see Figure 3.35 and 3.36 (Saito et al., 1990).

According to reports, compound **5** (**Macranthoside A**) was metabolized to kalopanaxsaponin I and hederagenin by human intestinal microflora. Among Macranthoside A metabolites, hederagenin showed the most potent antidiabetic activity (Kim et al., 1998). The molecular structure of the compound was presented as in Figure 3.6.

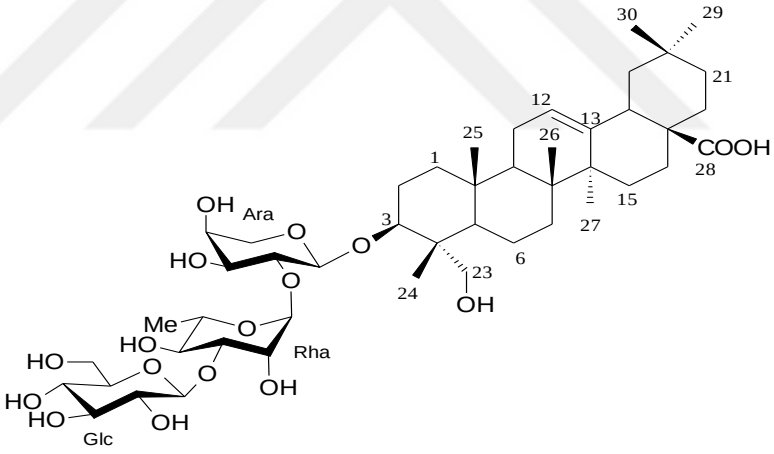


Figure 3.6 Structure of compound **5**

Compound 6 (**Scoposide D**): A white, amorphous powder (300.0 mg), oleanane-type triterpenoid saponin; ^{1}H-NMR (DMSO-d_6, 600 *MHz*) and ^{13}C-NMR (DMSO-d_6, 150 *MHz*), see Figure 3.37 and 3.38 (Sarikahya and Kirmizigul, 2010).

According to report, compound **6** (**Scoposide D**) showed antimicrobial activity. The antimicrobial effect of Scoposide D were examined by MIC method and found to be moderately active for both Gram-positive and Gram-negative bacteria (Sarikahya and Kirmizigul, 2010). Another report showed that compound **6** did not demonstrate any immunomodulatory activity (Sarikahya et al., 2018). The molecular structure of this compound was presented as in Figure 3.7.

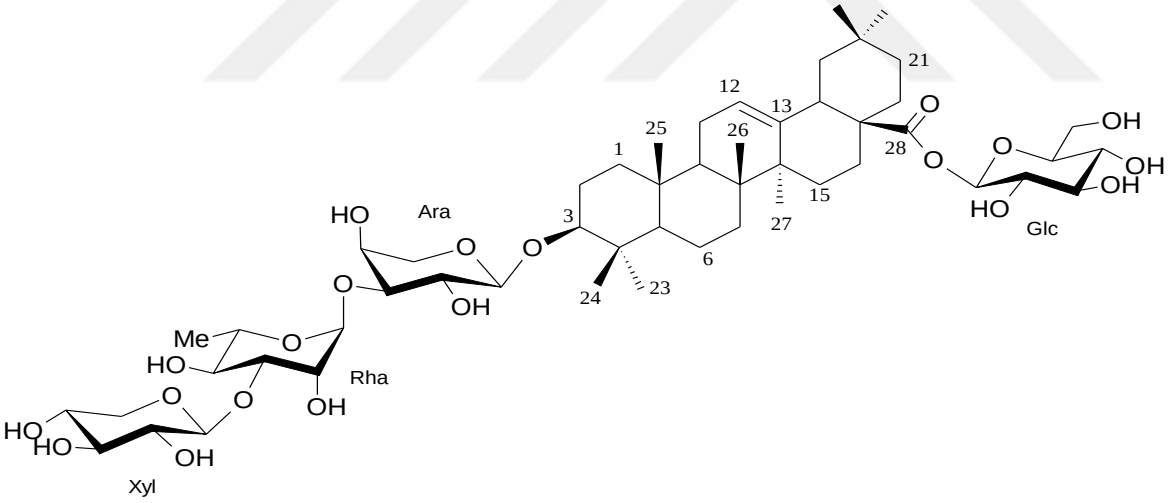


Figure 3.7 Structure of compound **6**

Compound 7 (3-*O*-*β*-D-glucopyranosyl-(1→3)-*α*-L-rhamnopyranosyl-(1→2)-*α*-L-arabinopyranosyl hederagenin 28-*O*-*β*-D-glycopyranosyl ester): A white, amorphous powder (280.0 mg), bisdesmosidic triterpenoid saponin, ^{1}H-NMR (DMSO-d_6, 600 *MHz*) and ^{13}C-NMR (DMSO-d_6, 150 *MHz*), see Figure 3.39 and 3.40 (Braca et. al., 2004).

According to report, compound **7** showed anti-proliferative activity in J774.A1, HEK 293 and WEHI-164 cell lines. The IC_{50} values were presented 0.51 μM for j774.A1, 1.74 μM for HEK 293 and 1.8 μM for WEHI-164 (Braca et al., 2004). The molecular structure of this compound was presented as in Figure 3.8.

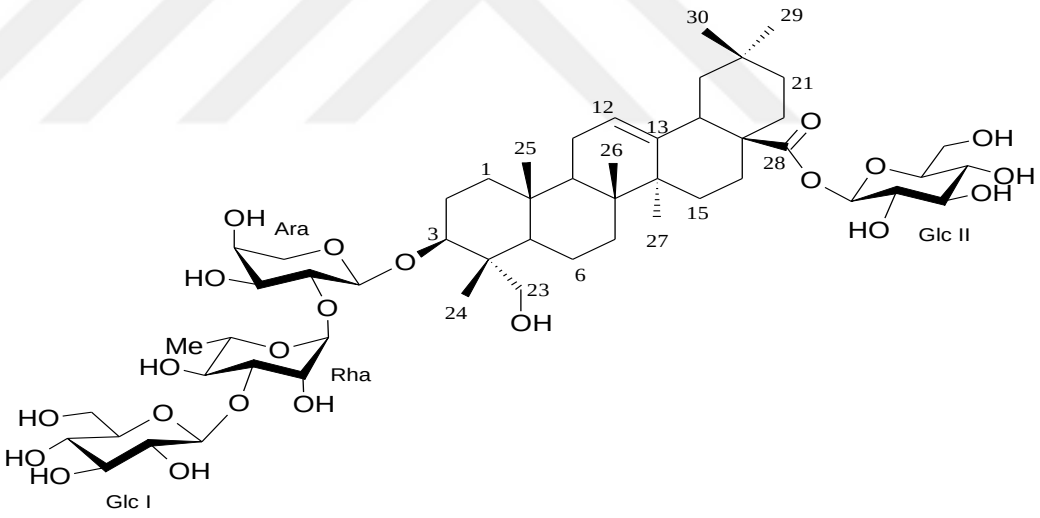


Figure 3.8 Structure of compound **7**

Compound 8 (**Decaisoside D**): A white, amorphous powder (155.0 mg), bisdesmosidic triterpenoid saponin, ^{1}H-NMR (DMSO-d_6, 600 *MHz*) and ^{13}C-NMR (DMSO-d_6, 150 *MHz*), see Figure 3.41 and 3.42 (Kong et al., 1993).

According to report, compound **8** (**Decaisoside D**) was tested on bovine erythrocytes, as well as their cytotoxic activity using brine shrimps (*Artemia salina*) bioassay. Compound **8** showed 58% of hemolysis at concentration of 1.0 mg/mL. It did not show cytotoxic activity on brine shrimps at the same concentrations (Godevac et al., 2006b). The molecular structure of this compound was presented as in Figure 3.9.

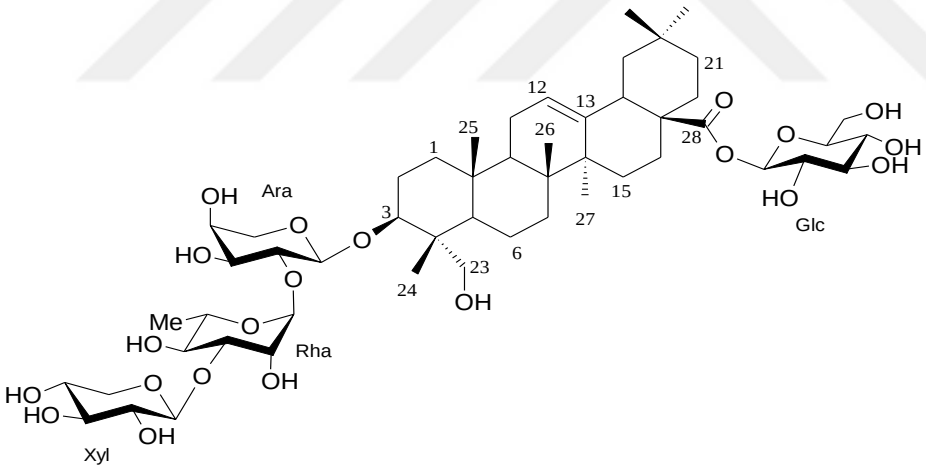


Figure 3.9 Structure of compound **8**

Compound 9 (**Dipsacoside B**): A white, amorphous powder (1120.0 mg), bisdesmosidic triterpenoid saponin, ^{1}H-NMR (DMSO-d_6, 600 *MHz*) and APT (DMSO-d_6, 150 *MHz*), see Figure 3.43 and 3.44 (Mukhamedziev et al., 1971).

According to report, compound **9** (**Dipsacoside B**) tested for antimicrobial properties and it showed a strong inhibitory activity. MIC values were 0.70 μg/mL for *C. glabrata*, 0.90 μg/mL for *C. C. tropicalis*, 1.90 μg/mL for *C. albicans*, 1.80 μg/mL for *K. pneumoniae*, 2.90 μg/mL for *E. cloacae*, 2.50 μg/mL for *E. coli*, 3.50 μg/mL for *P. aeruginosa*, 1.40 μg/mL for *C. S. epidermidis* and 2.10 μg/mL for *S. aureus*. The antimicrobial activities of Dipsacoside B comparable to those of standard antibiotics used in the tests, and in some cases were even better (Pasi et al., 2009). The molecular structure of this compound was presented as in Figure 3.10.

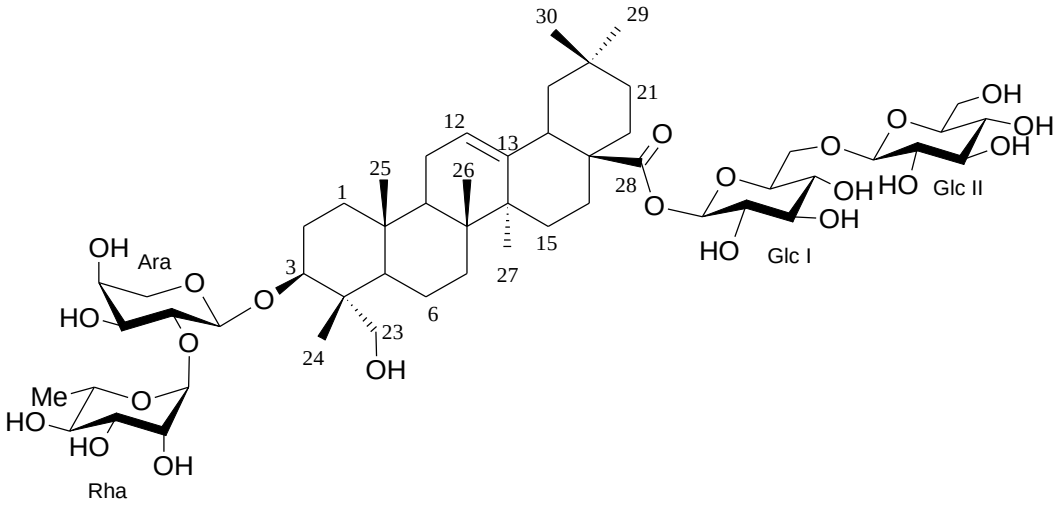


Figure 3.10 Structure of compound **9**

Compound 10 (**Elmalienoside C**): A white, amorphous powder (600.0 mg), bisdesmosidic triterpenoid saponin, ^{1}H-NMR (DMSO-d_6, 600 *MHz*) and ^{13}C-NMR (DMSO-d_6, 150 *MHz*), see Figure 3.45 and 3.46 (Sarikahya and Kirmizigul, 2012a).

According to the report, compound **10** (**Elmalienoside C**) showed anti-bacterial activity. MIC results were found as 64 μg/mL for *S. aureus*, 32 μg/mL for *S. epidermidis*, 32 μg/mL for *S. typhimurium*, 32 μg/mL for *E. coli*, 32 μg/mL for *B. cereus*, 32 μg/mL for *K. pneumoniae*, 4 μg/mL for *E. faecalis* and 32 μg/mL for *P. aeruginosa*. Gentamycin was used as a standard in this study. (Sarikahya and Kirmizigul, 2012a). The molecular structure of this compound was presented as in Figure 3.11.

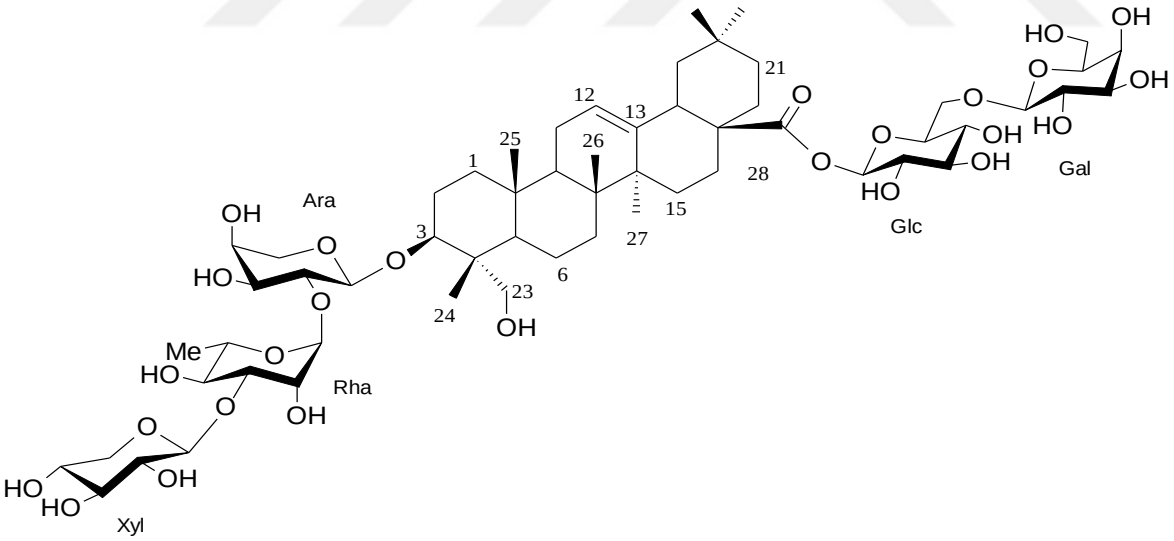


Figure 3.11 Structure of compound **10**

Compound 11 (**Elmalienoside A**): A white, amorphous powder (330.0 mg), bisdesmosidic triterpenoid saponin, ^{1}H-NMR (DMSO-d_6, 600 *MHz*) and ^{13}C-NMR (DMSO-d_6, 150 *MHz*), see Figure 3.47 and 3.48 (Sarikahya and Kirmizigul, 2012a).

According to the report, compound **11** (**Elmalienoside A**) showed anti-bacterial activity. MIC results were found as 32 μg/mL for S. aureus, 16 μg/mL for *S. epidermidis*, 16 μg/mL for *S. typhimurium*, 16 μg/mL for *E. coli*, 8 μg/mL for *B. cereus*, 32 μg/mL for *K. pneumoniae*, 2 μg/mL for *E. faecalis* and 16 μg/mL for *P. aeruginosa*. Gentamycin was used as a standard in this study. (Sarikahya and Kirmizigul, 2012a). Another report showed that compound **11** demonstrated moderate immunomodulatory activity (Sarikahya et al., 2018). The molecular structure of this compound was presented as in Figure 3.12.

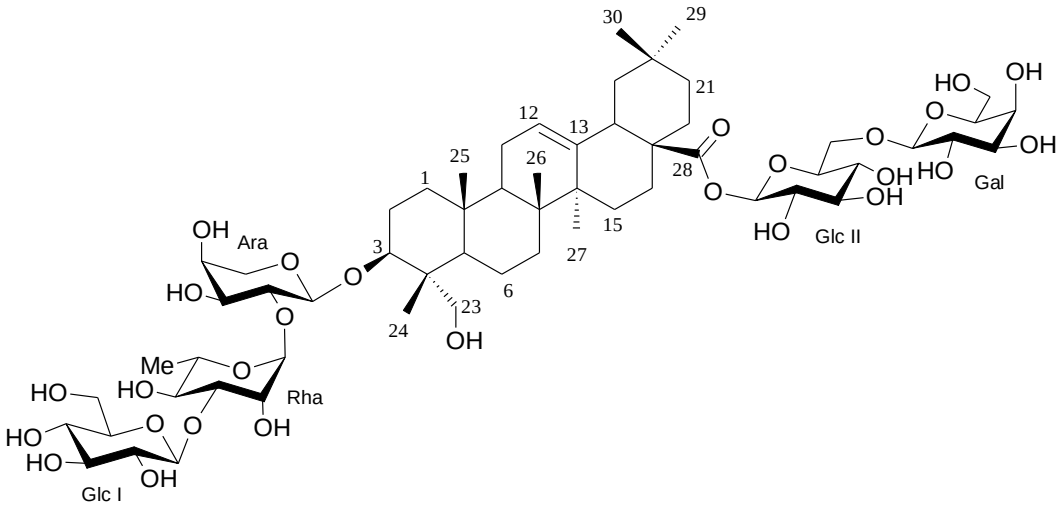


Figure 3.12 Structure of compound **11**

Compound 12 (**Macranthoidin A**): A white, amorphous powder (430.0 mg), bisdesmosidic triterpenoid saponin, ^{1}H-NMR (DMSO-d_6, 600 *MHz*) and ^{13}C-NMR (DMSO-d_6, 150 *MHz*), see Figure 3.49 and 3.50 (Mao et. al., 1993).

According to the report, compound **12** (**Macranthoidin A**) exhibited antitumor activity against human mammary adenocarcinoma MCF-7 (Liu et al., 2018). The molecular structure of this compound was presented as in Figure 3.13.

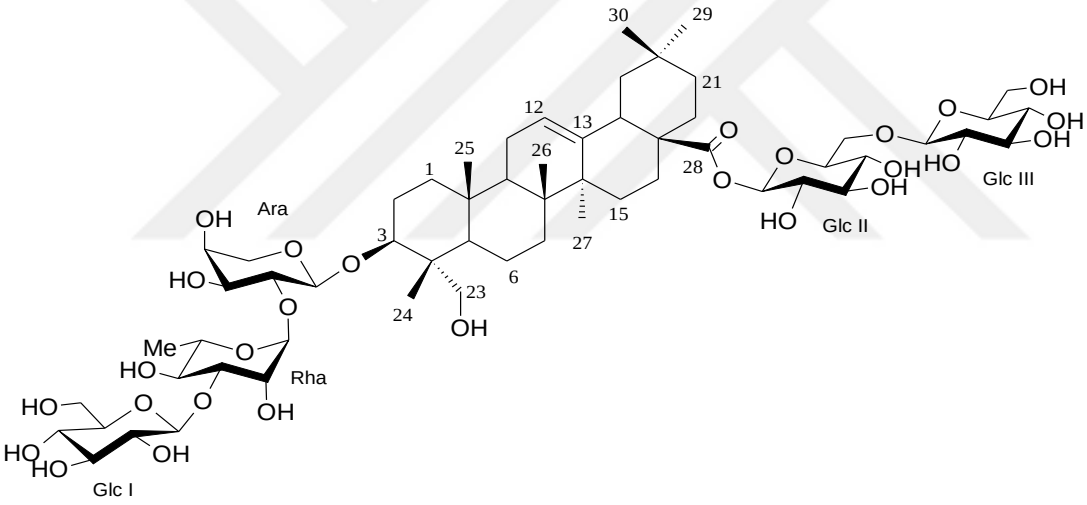


Figure 3.13 Structure of compound **12**

Compound 13 (**Cephoside B**): A white, amorphous powder (170.0 mg), bisdesmosidic triterpenoid saponin, ^{1}H-NMR (DMSO-d_6, 600 *MHz*) and ^{13}C-NMR (DMSO-d_6, 150 *MHz*), see Figure 3.51 and 3.52 (Sumer et al., 2017).

According to the report, compound **13** (**Cephoside B**) did not exhibit a significant hemolytic activity in human red blood cells (Sumer et al., 2017). The molecular structure of this compound was presented as in Figure 3.14.

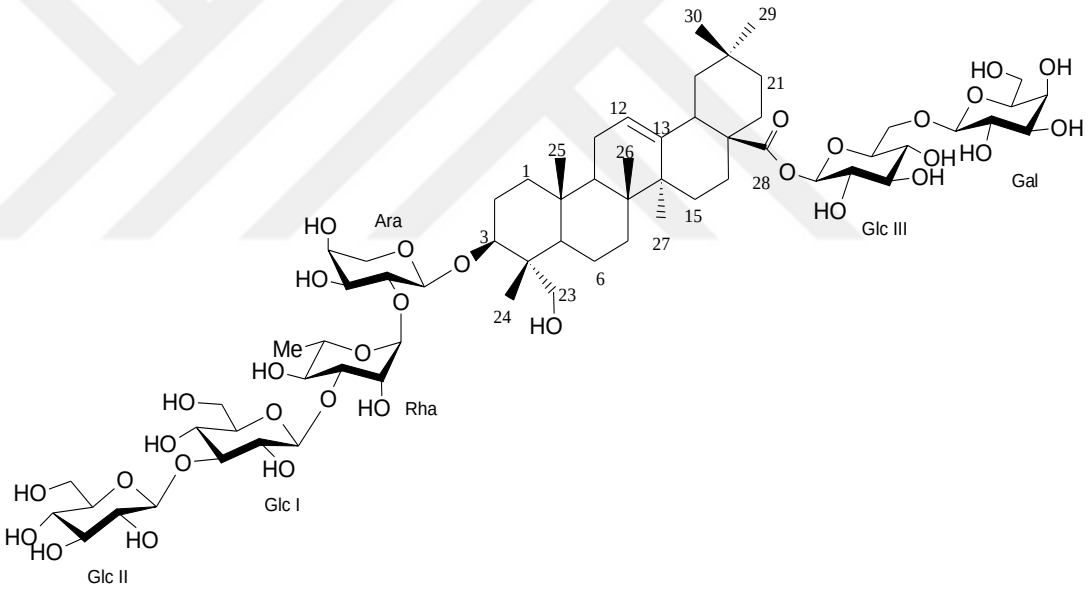


Figure 3.14 Structure of compound **13**

Compound 14 (7*β*-7-*O*-methyl morroniside): A white, amorphous powder (70.0 mg), iridoid glycoside, ^{1}H-NMR (DMSO-d_6, 400 *MHz*) and ^{13}C-NMR (DMSO-d_6, 100 *MHz*), see Table 3.1 and Figure 3.53 and 3.54 (Ye et. al., 1993). This compound was identified in Caprifoliceae family for the first time.

Anti-inflammatory effect of compound **14** (**7*β*-7-*O*-methyl morroniside**) was tested based on the evaluation of the inhibitory effect on NO production in lipopolysaccharide (LPS)-induced RAW 264.7 cells by the MTT method. Compound **14** did not display any significant activity (Effects of inhibiting NO production IC_{50} >100μM and α-Glucosidase inhibitory activity IC_{50} > 20 mM) (Liu et al., 2015). Another study showed that compound **14** did not showed antitrypanosomal and opioid receptor binding activity (Mohamed et al., 2015). The molecular structure of this compound was presented as in Figure 3.15.

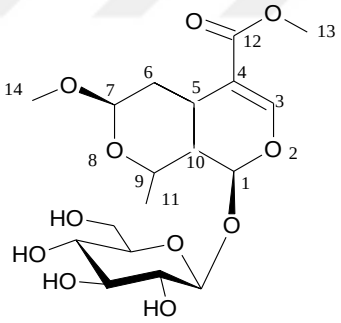


Figure 3.15 Structure of compound **14**

Table 3.1 ^{1}H and ^{13}C-NMR data for compound **14**[a-d]

Position	^{13}C-NMR	^{1}H-NMR
1	94.2	5.75,d (9.6)
2	-	-
3	153.2	7.44, m
4	110.7	-
5	26.5	2.87, m
6	32.5	1.38, 1.71, m
7	97.4	4.64, d (3.2)
8	-	-
9	64.4	4.10, m
10	39.0	1.71, m
11	19.5	1.19, d (6.4)
12	166.6	-
13	51.5	3.60, s
14	54.2	3.17, s
	Glc	
1	98.7	4.60,d (7.6)
2	73.9	2.98, m
3	77.0	3.20, m
4	70.6	3.04, m
5	77.8	3.15, m
6	61.6	3.44 ; 3.70, m

[a] ^{1}H and ^{13}C-NMR data (δ) were measured in DMSO-d_6 at 400 and 100 *MHz*, respectively.
[b] The assignments are based on COSY, HMQC and HMBC experiments.
[c] Coupling constants (J) in *Hz* are given in parentheses.
[d] nd: not detected.

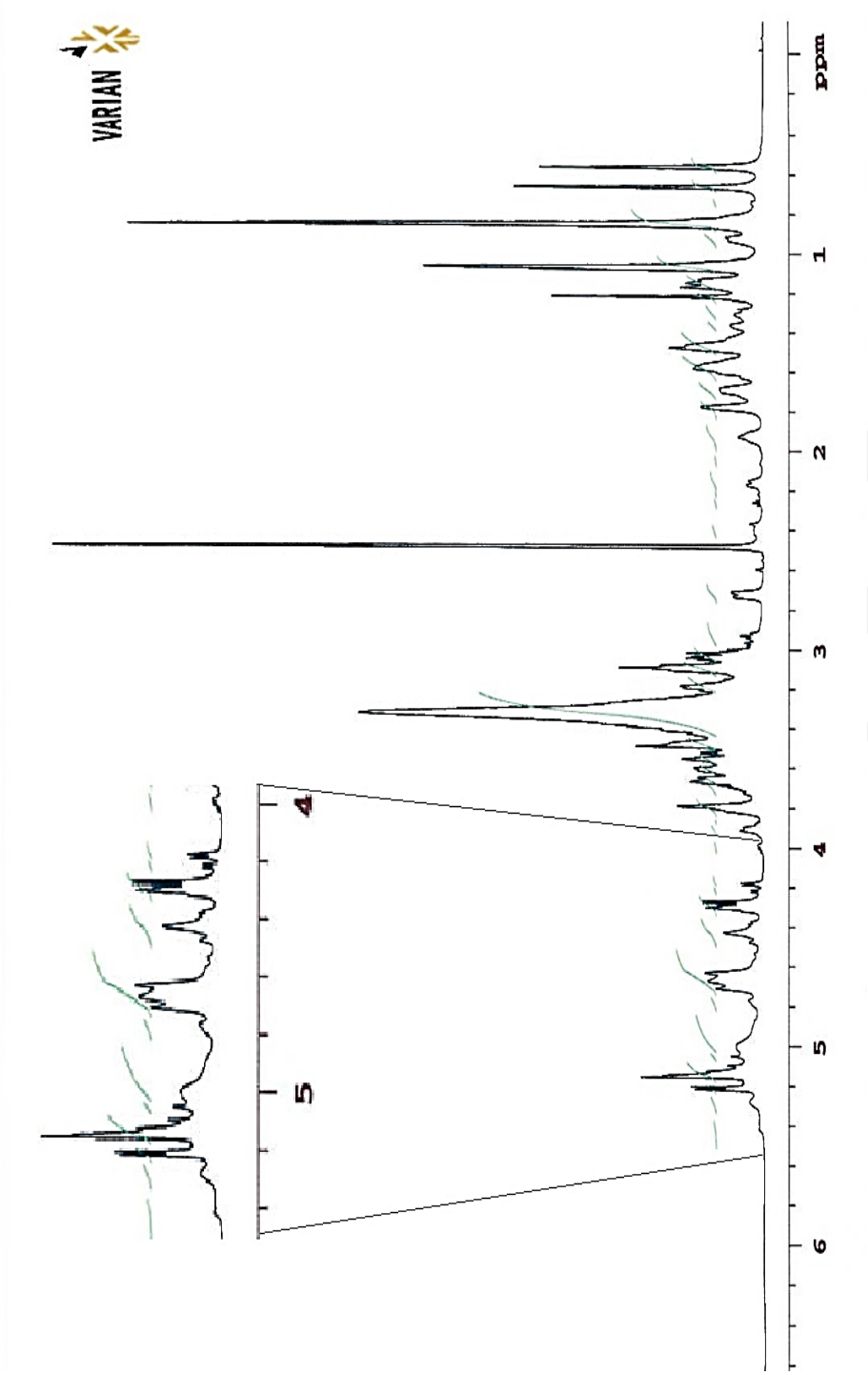


Figure 3.16 ^{1}H-NMR (600 *MHz*, DMSO-d_6) spectrum of compound **1**

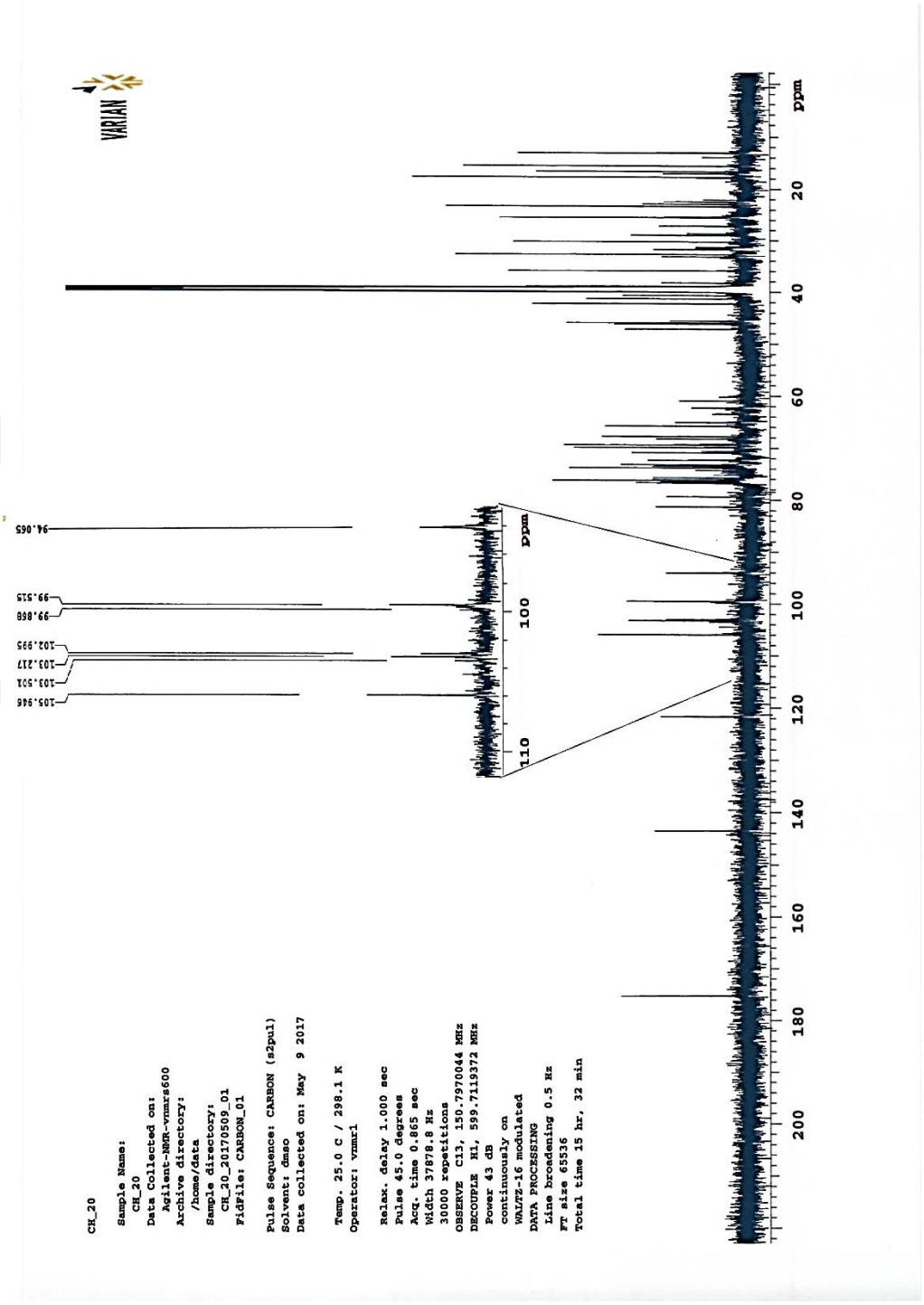


Figure 3.17 ^{13}C-NMR (150 *MHz*, DMSO-d_6) spectrum of compound **1**

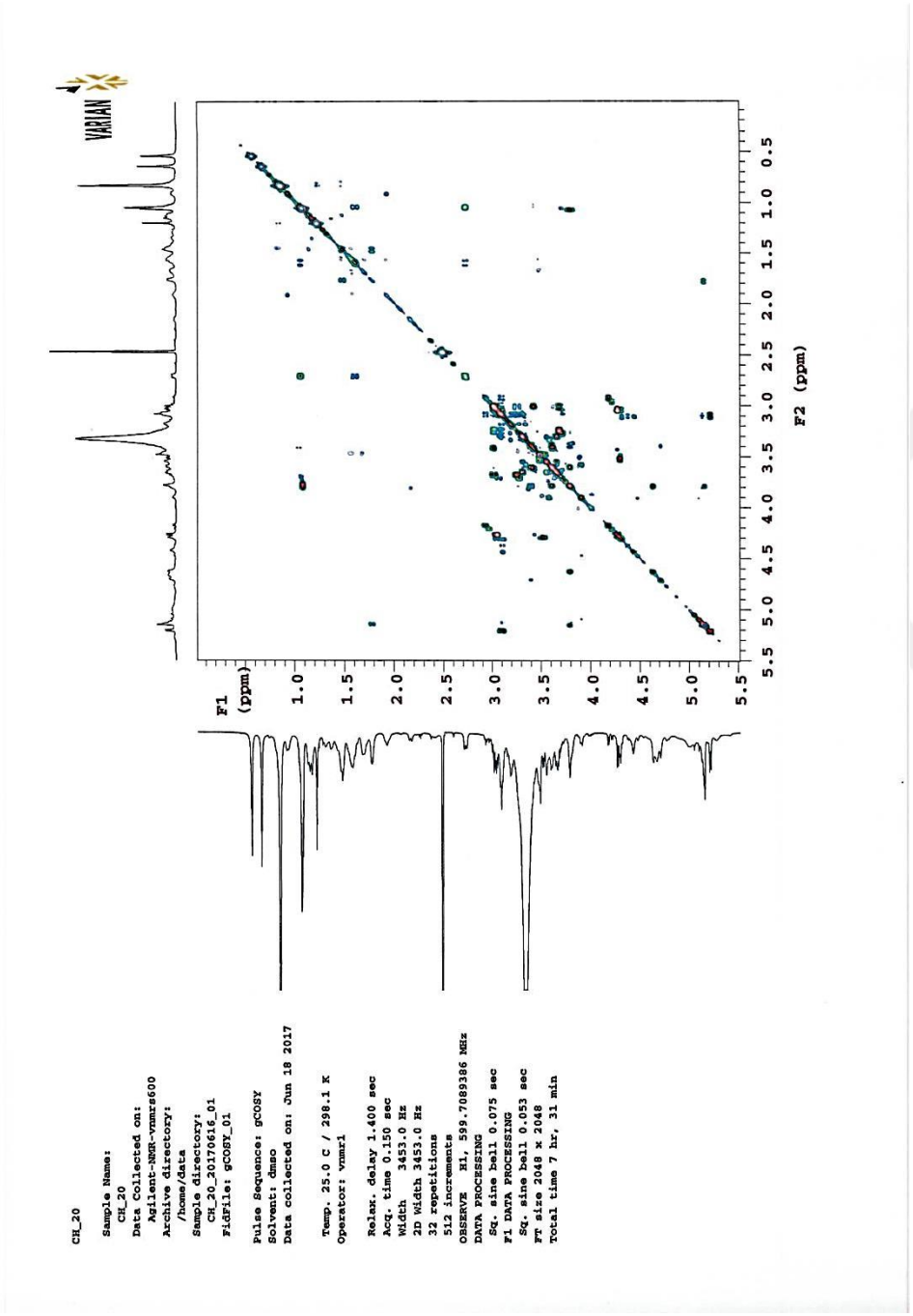


Figure 3.18 COSY (600 *MHz*) spectrum of compound **1**

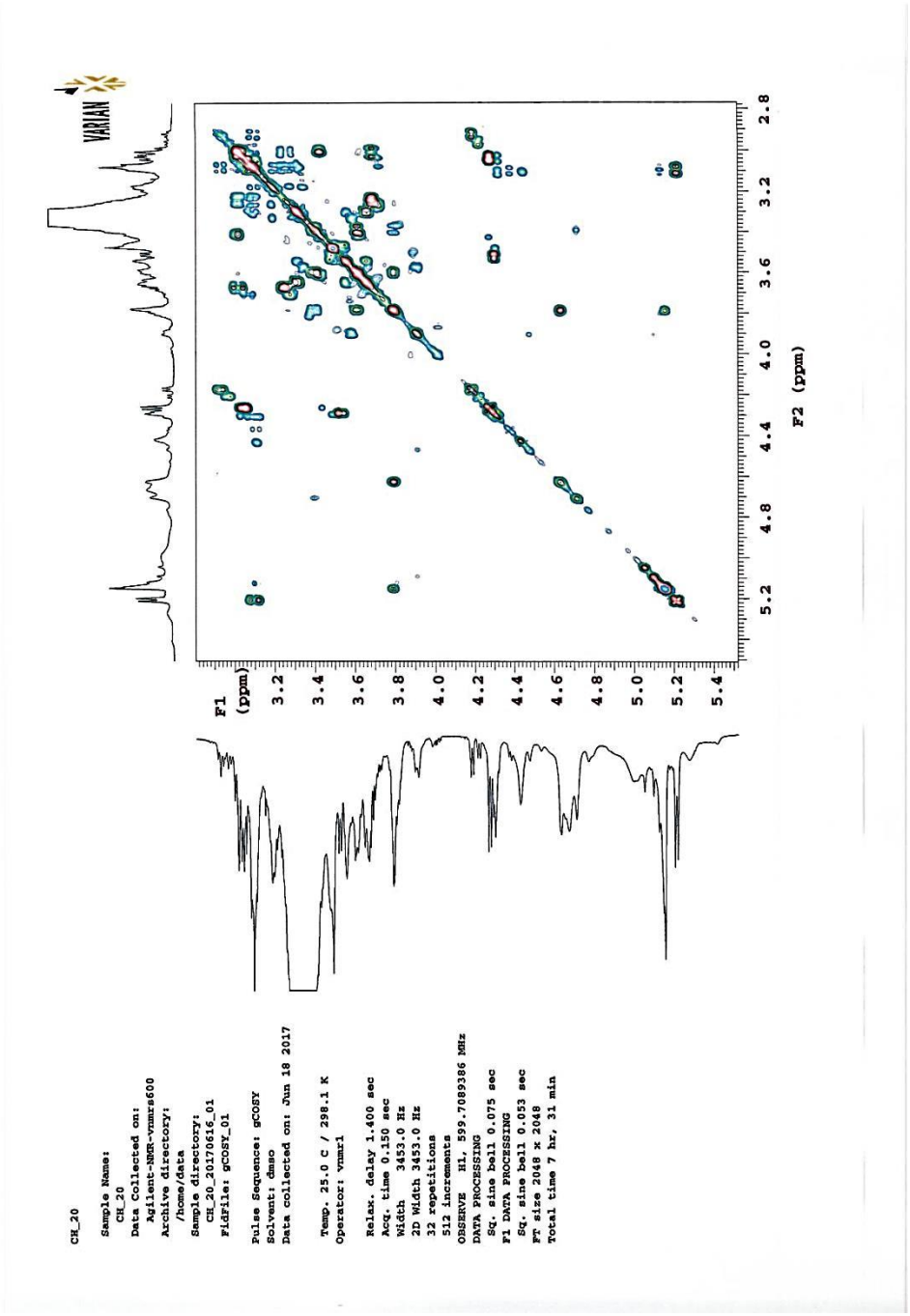


Figure 3.19 Expansion of COSY (600 *MHz*) spectrum of compound **1**

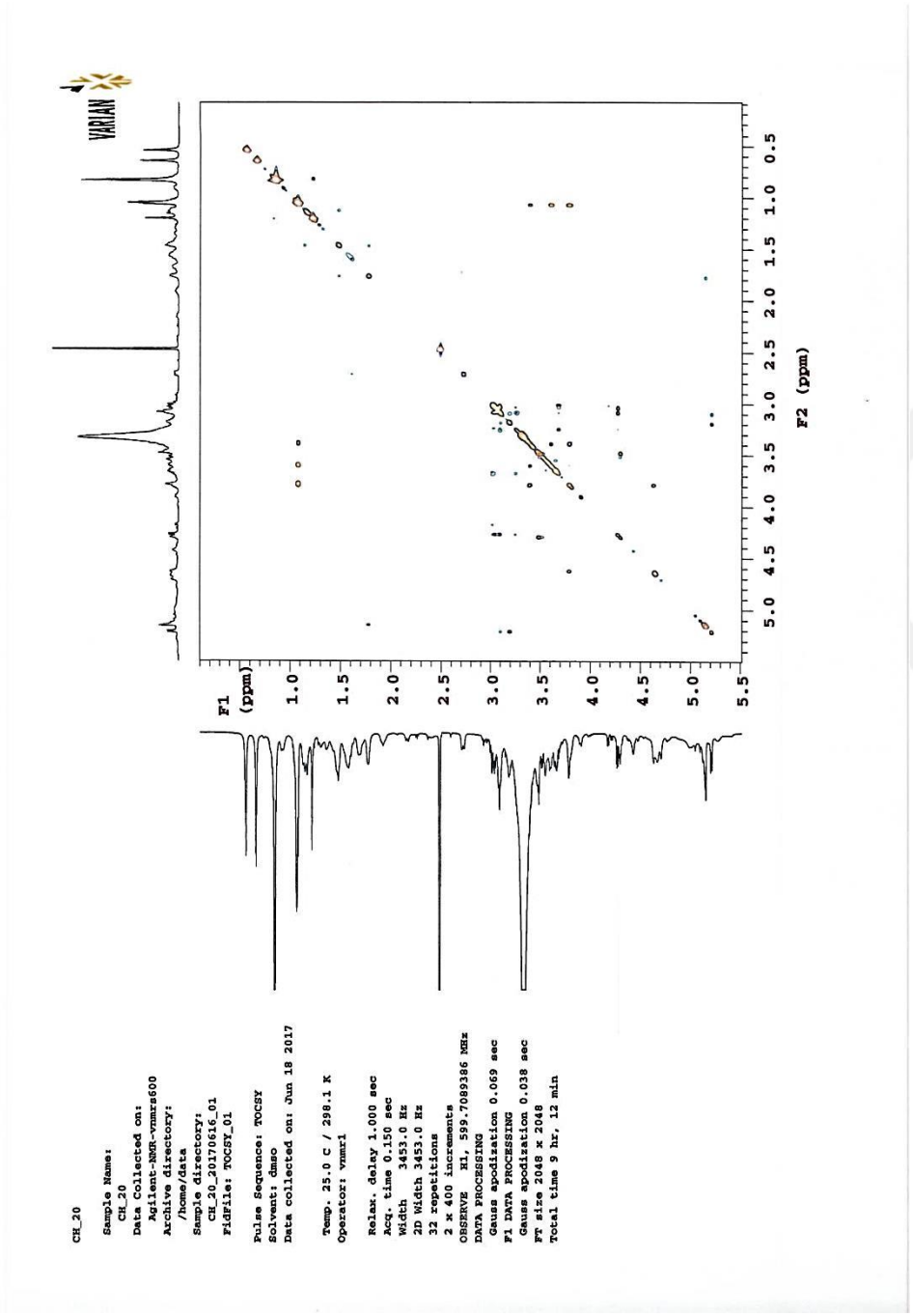


Figure 3.20 TOCSY (600 *MHz*) spectrum of compound **1**

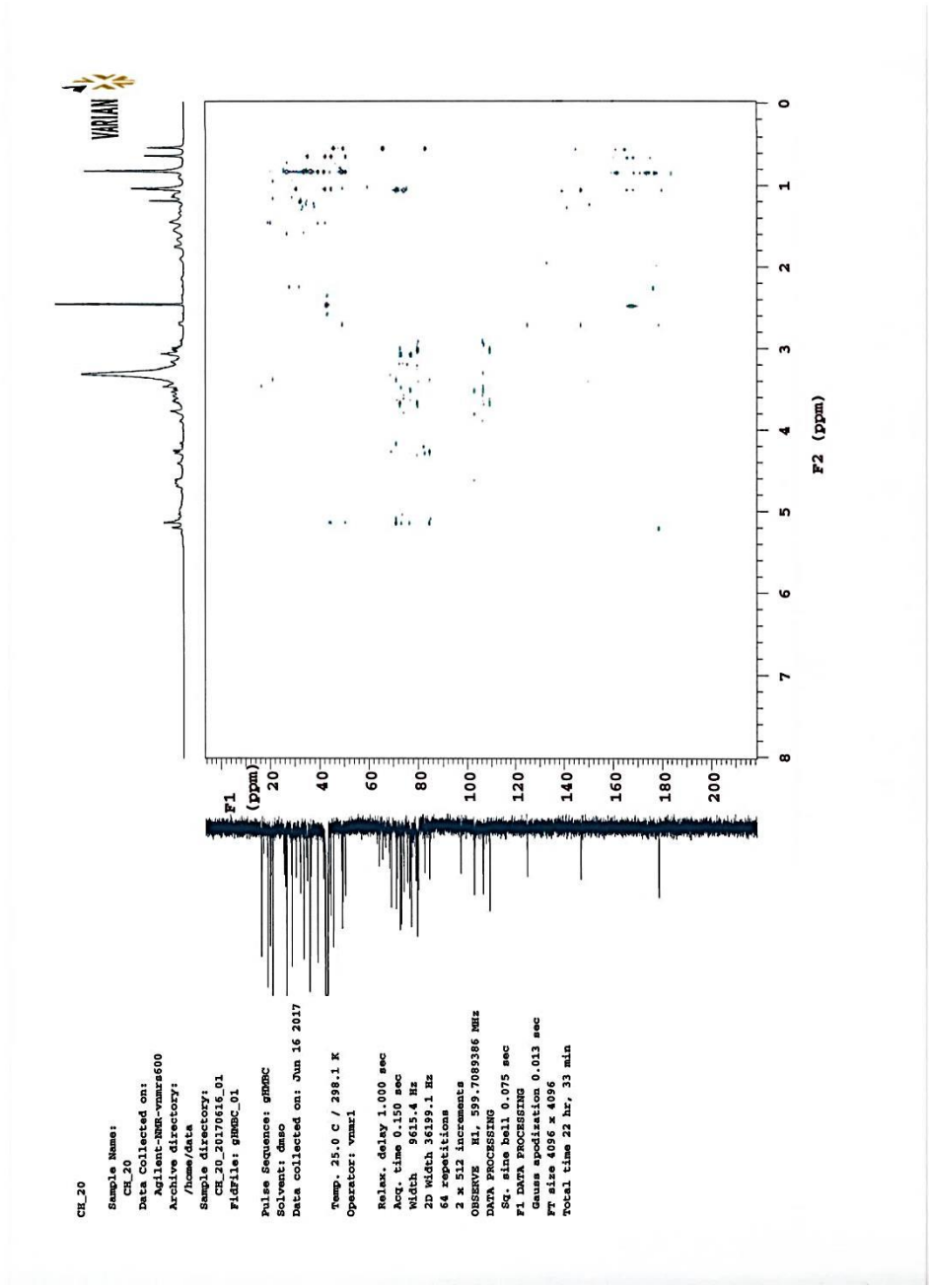


Figure 3.21 HMBC (600 *MHz*) spectrum of compound **1**

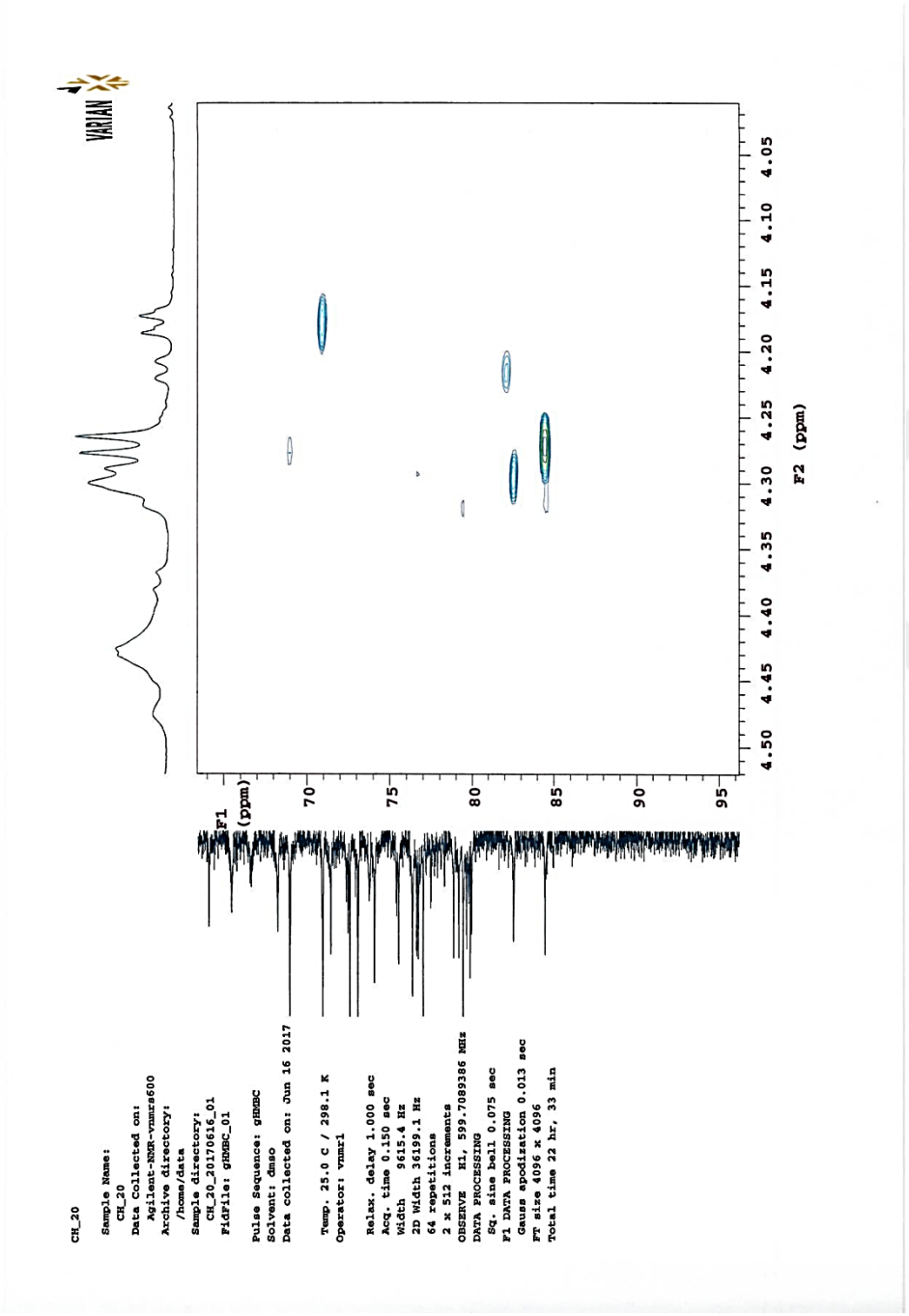


Figure 3.22 Expansion of HMBC (600 *MHz*) spectrum of compound **1**

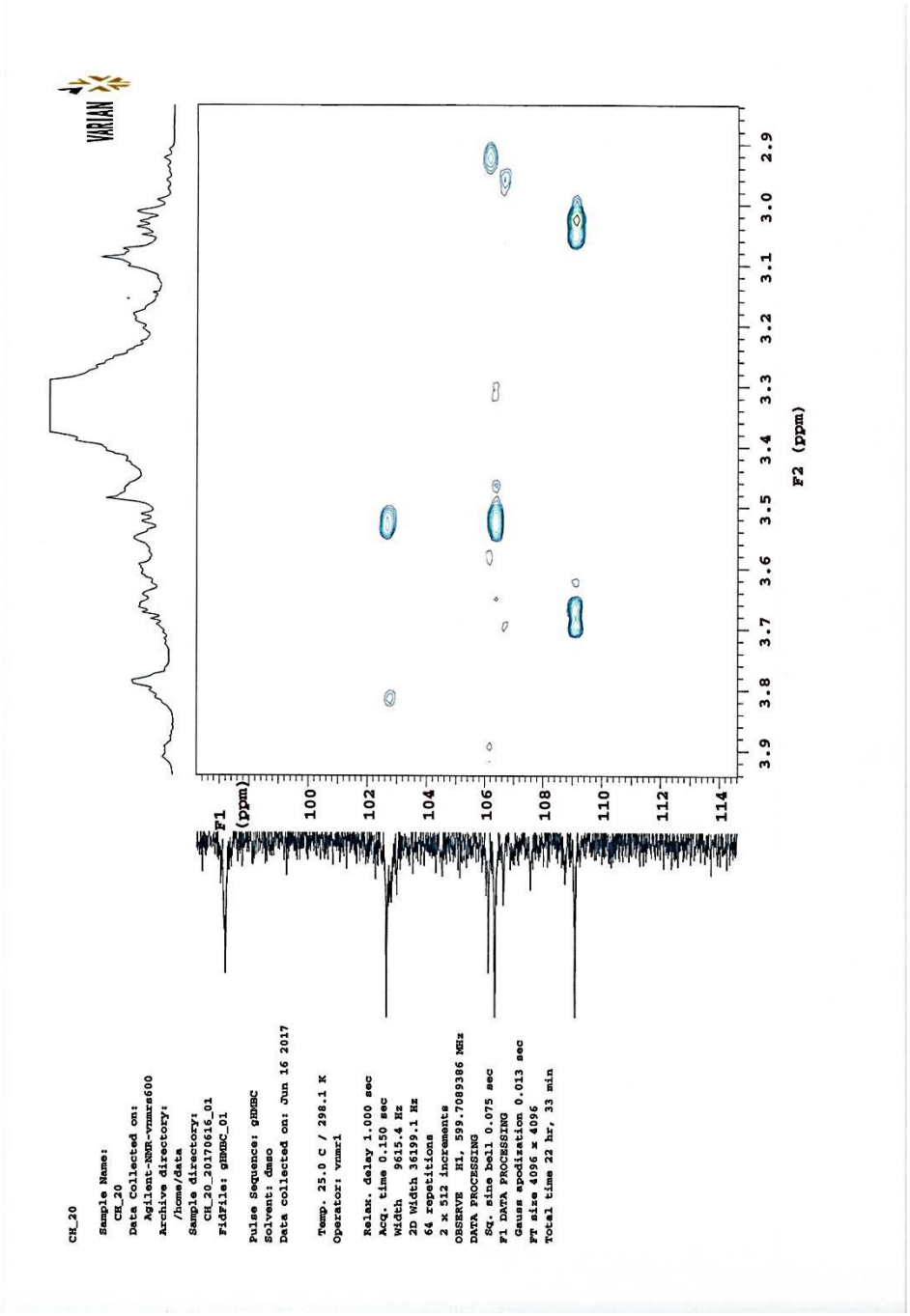


Figure 3.23 Expansion of HMBC (600 *MHz*) spectrum of compound **1**

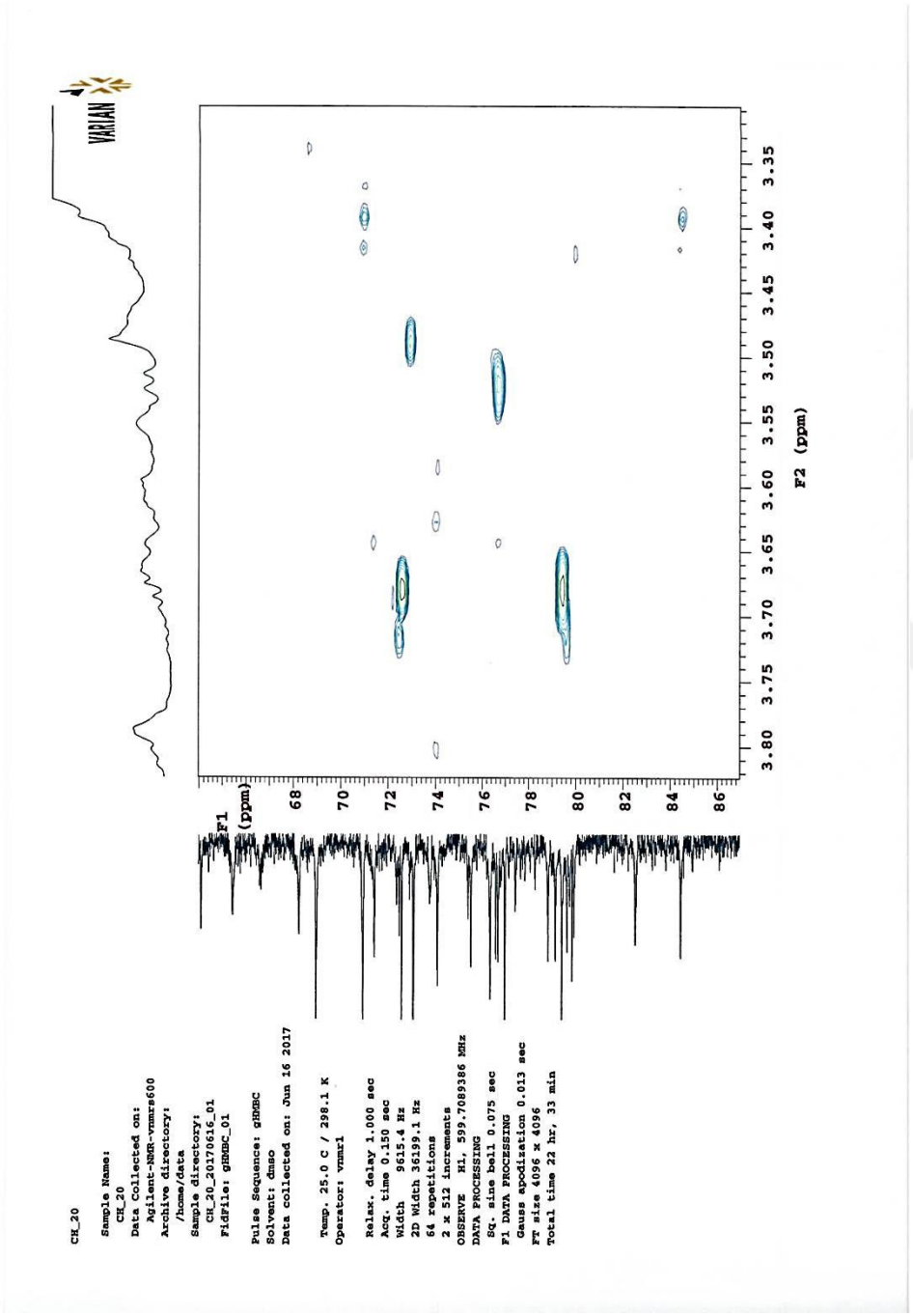


Figure 3.24 Expansion of HMBC (600 *MHz*) spectrum of compound **1**

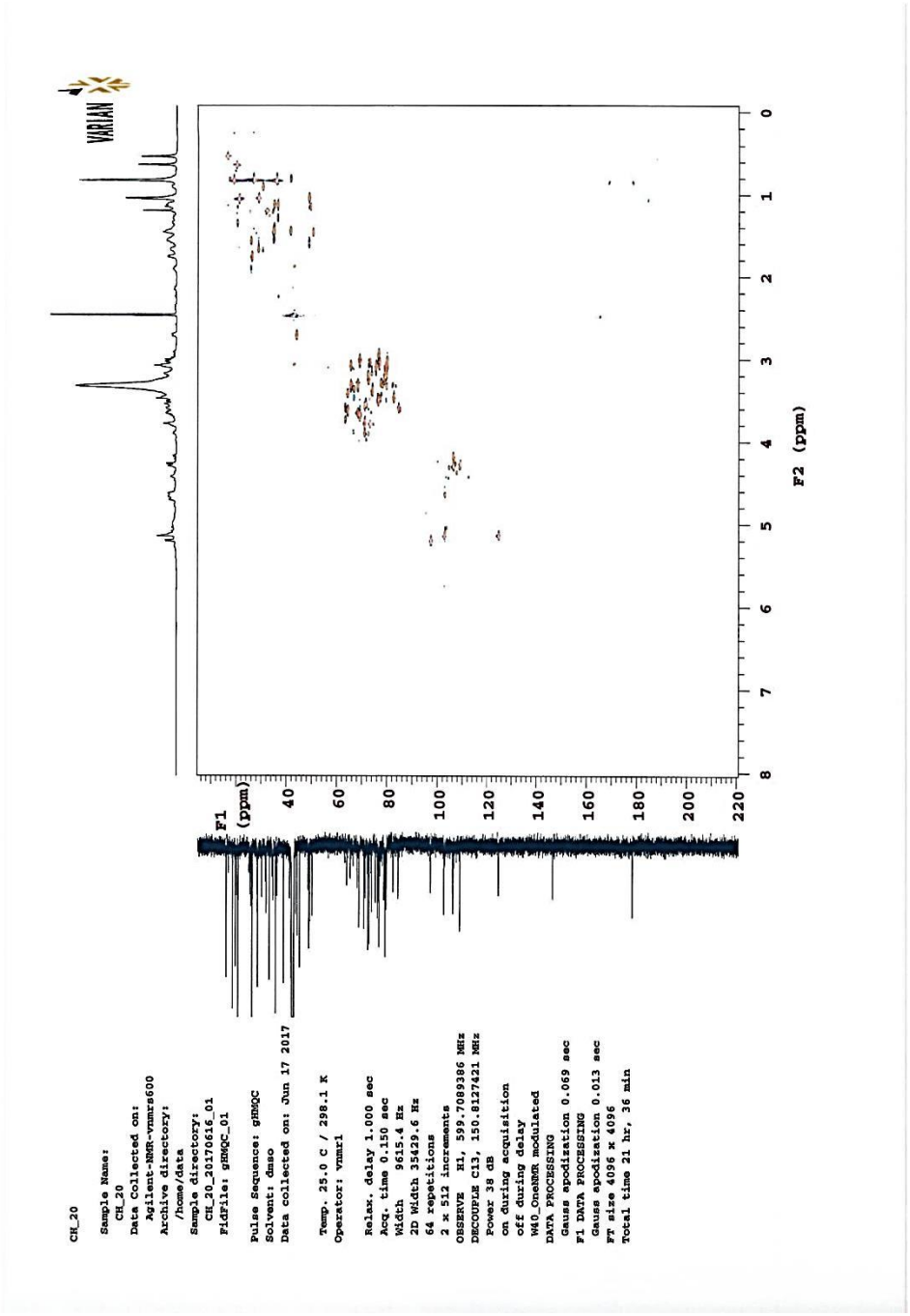


Figure 3.25 HMQC (600 *MHz*) spectrum of compound **1**

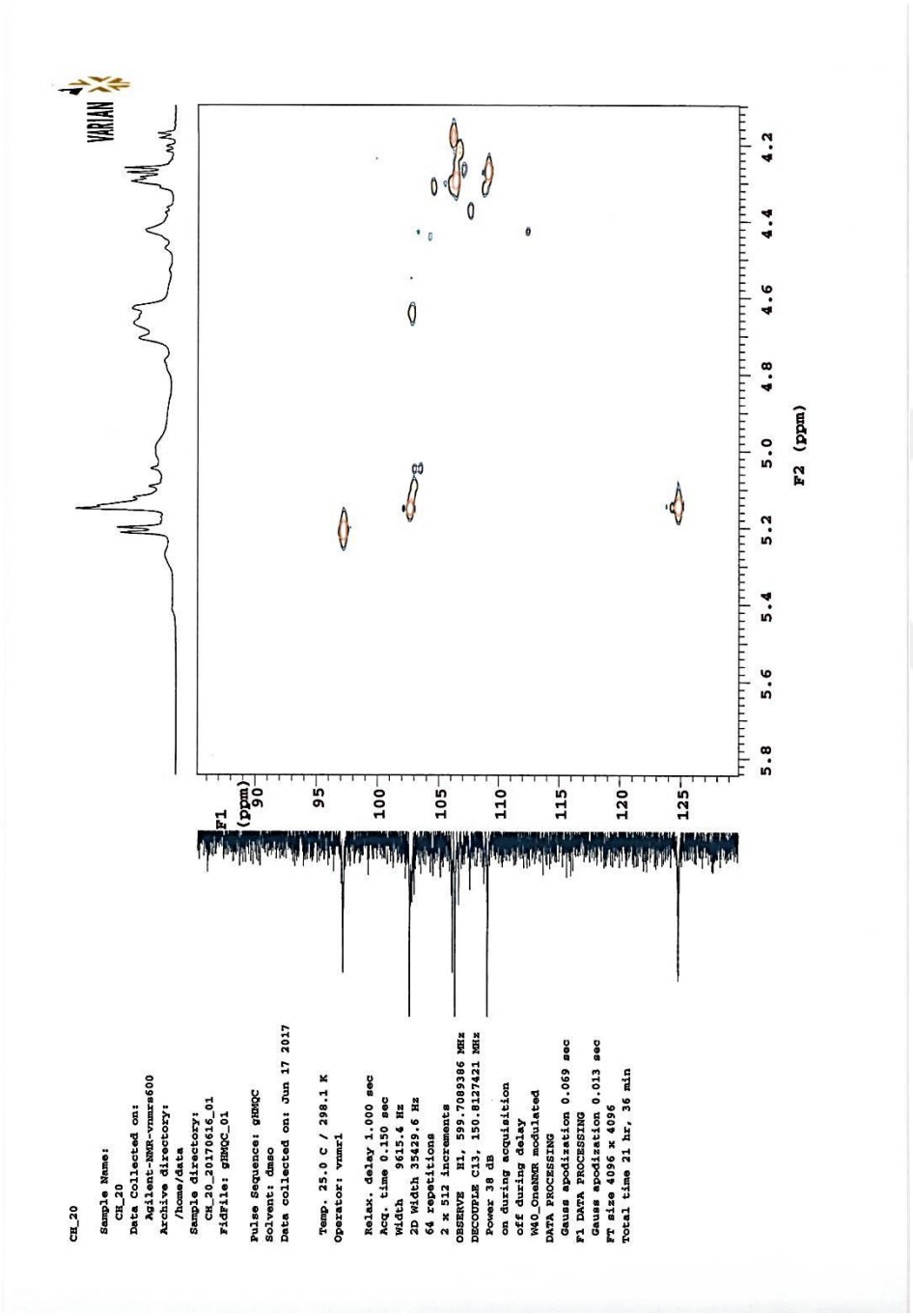


Figure 3.26 Expansion of HMQC (600 *MHz*) spectrum of compound **1**

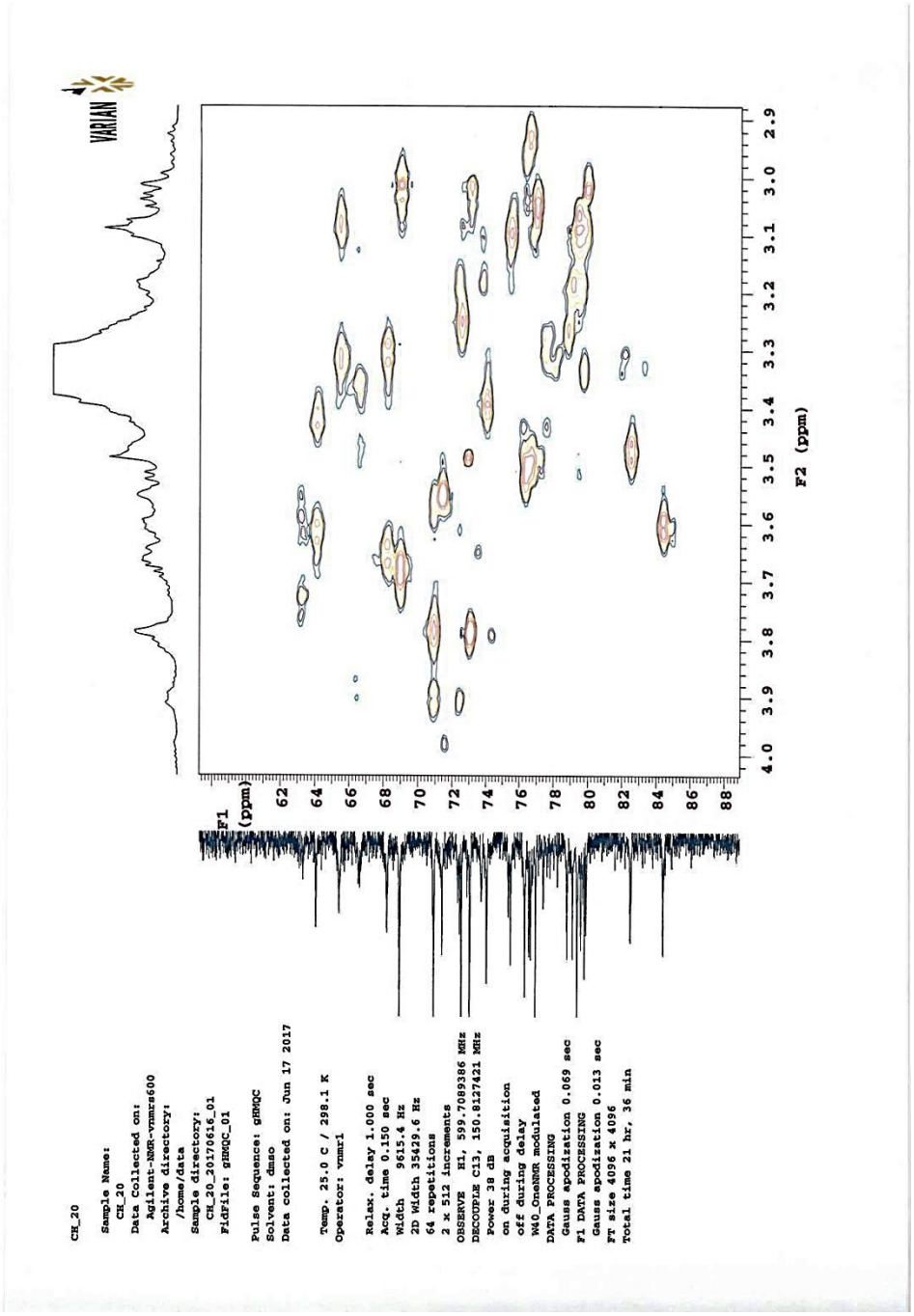


Figure 3.27 Expansion of HMQC (600 *MHz*) spectrum of compound **1**

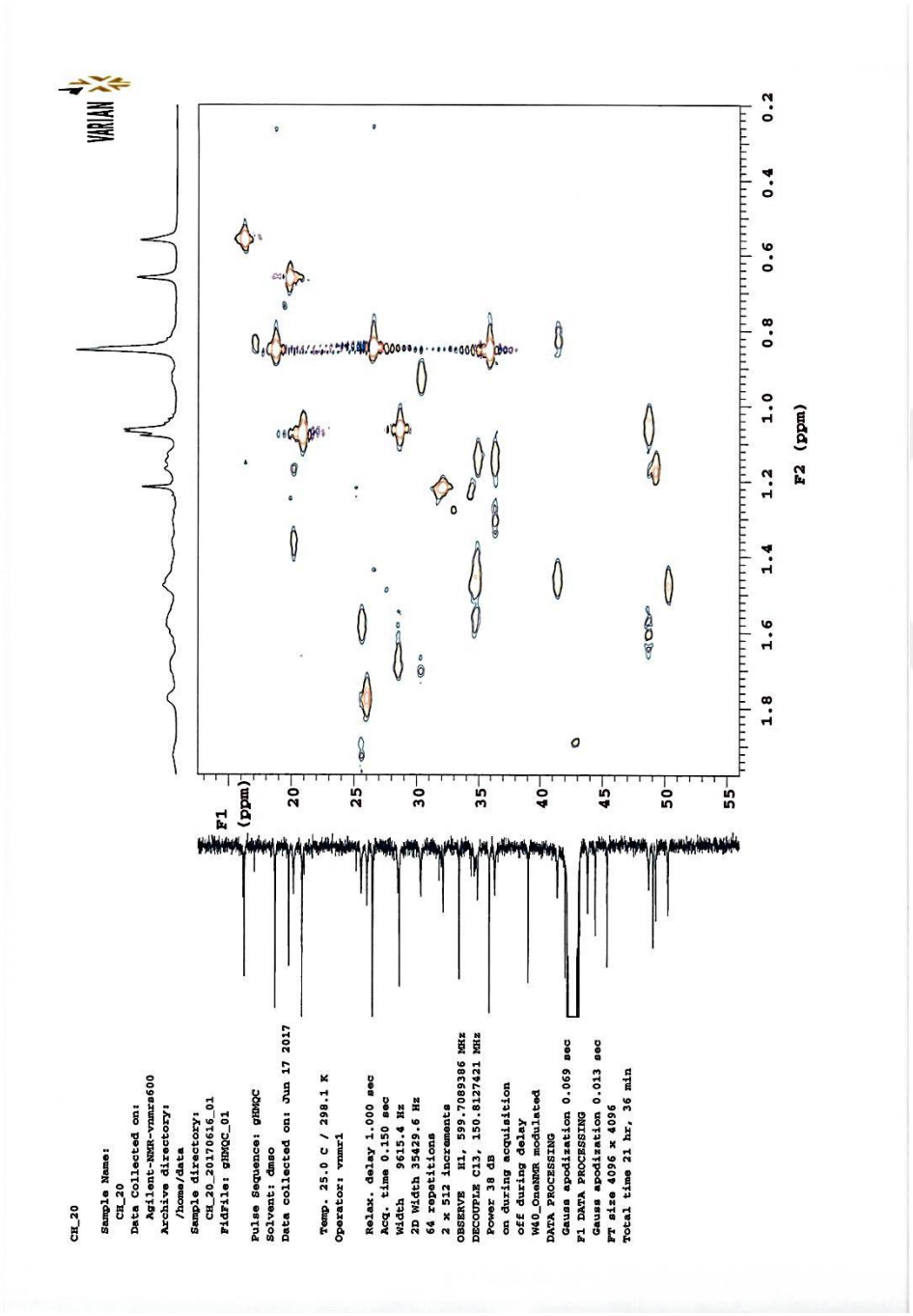


Figure 3.28 Expansion of HMQC (600 *MHz*) spectrum of compound **1**

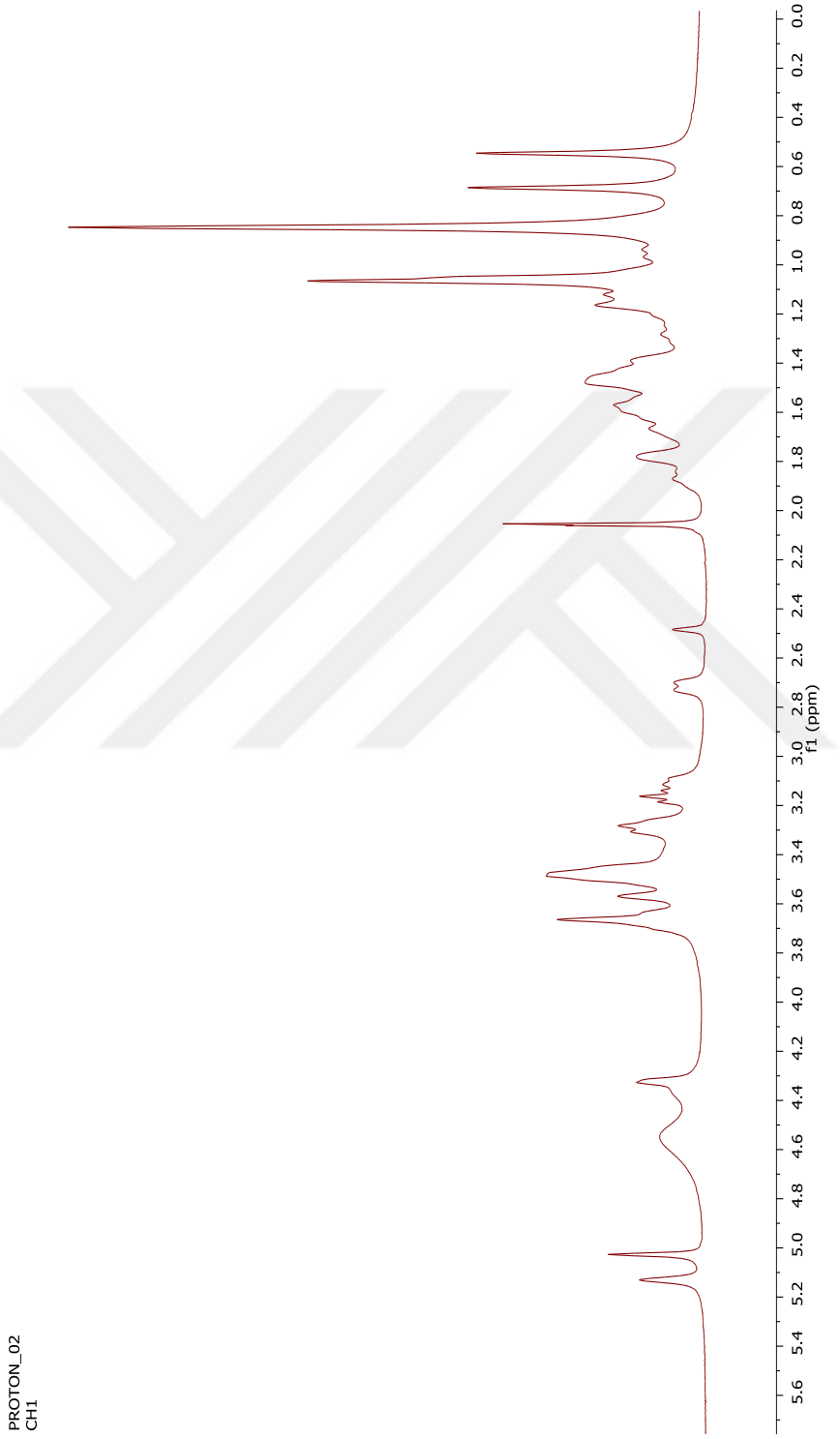


Figure 3.29 ^{1}H-NMR (400 *MHz*, DMSO-d_6) spectrum of compound **2**

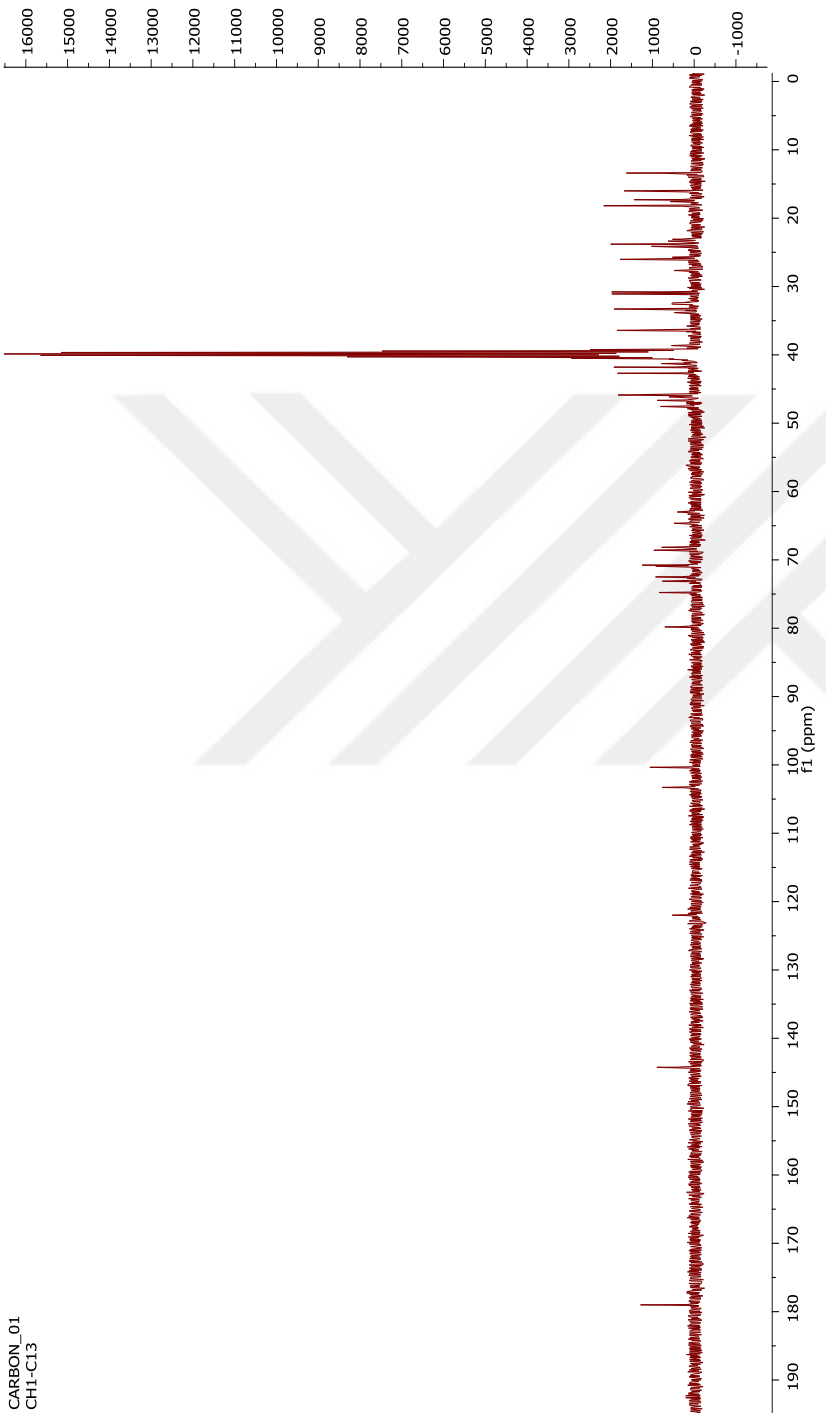


Figure 3.30 ^{13}C-NMR (100 *MHz*, DMSO-d_6) spectrum of compound **2**

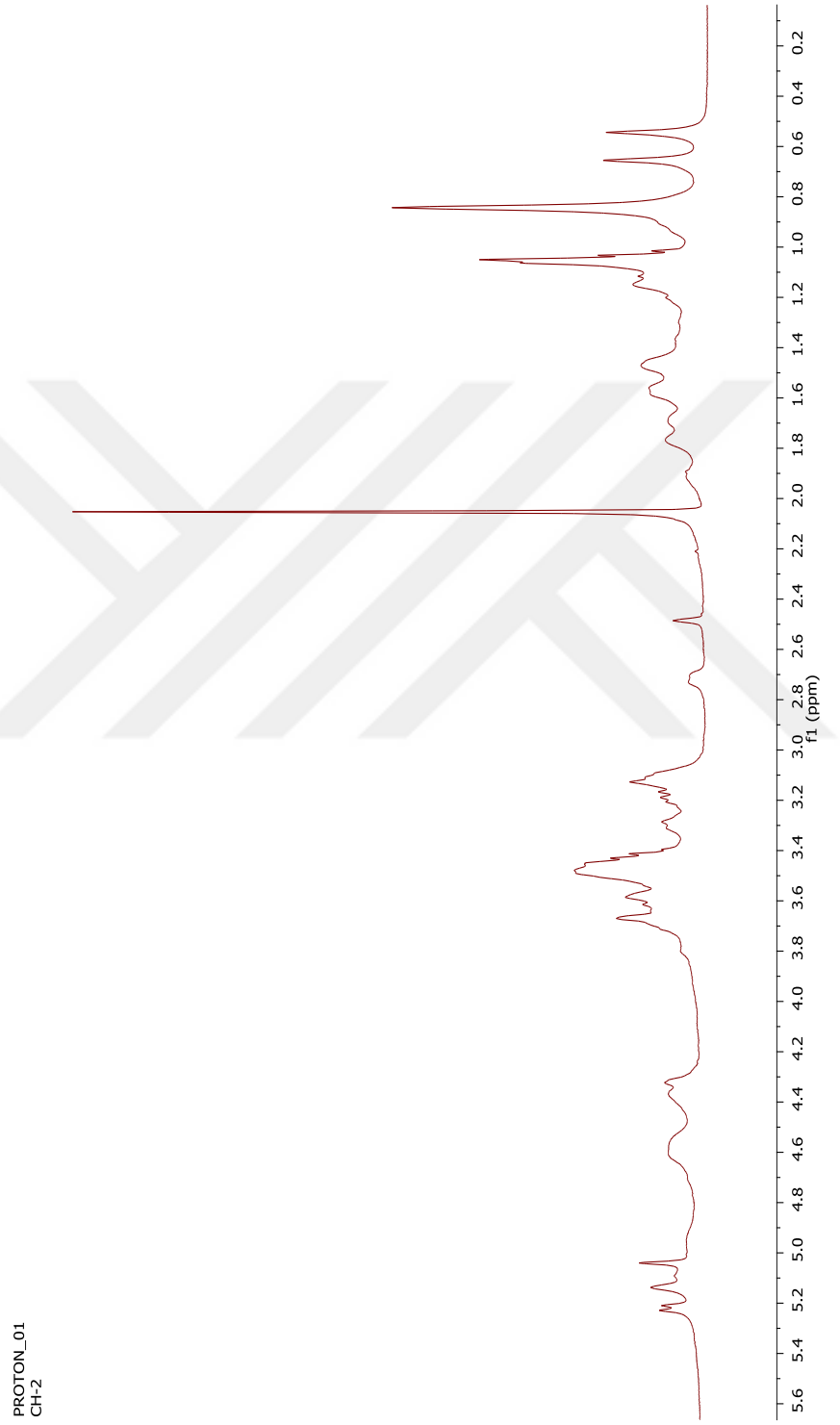


Figure 3.31 ^{1}H-NMR (400 *MHz*, DMSO-d_6) spectrum of compound **3**

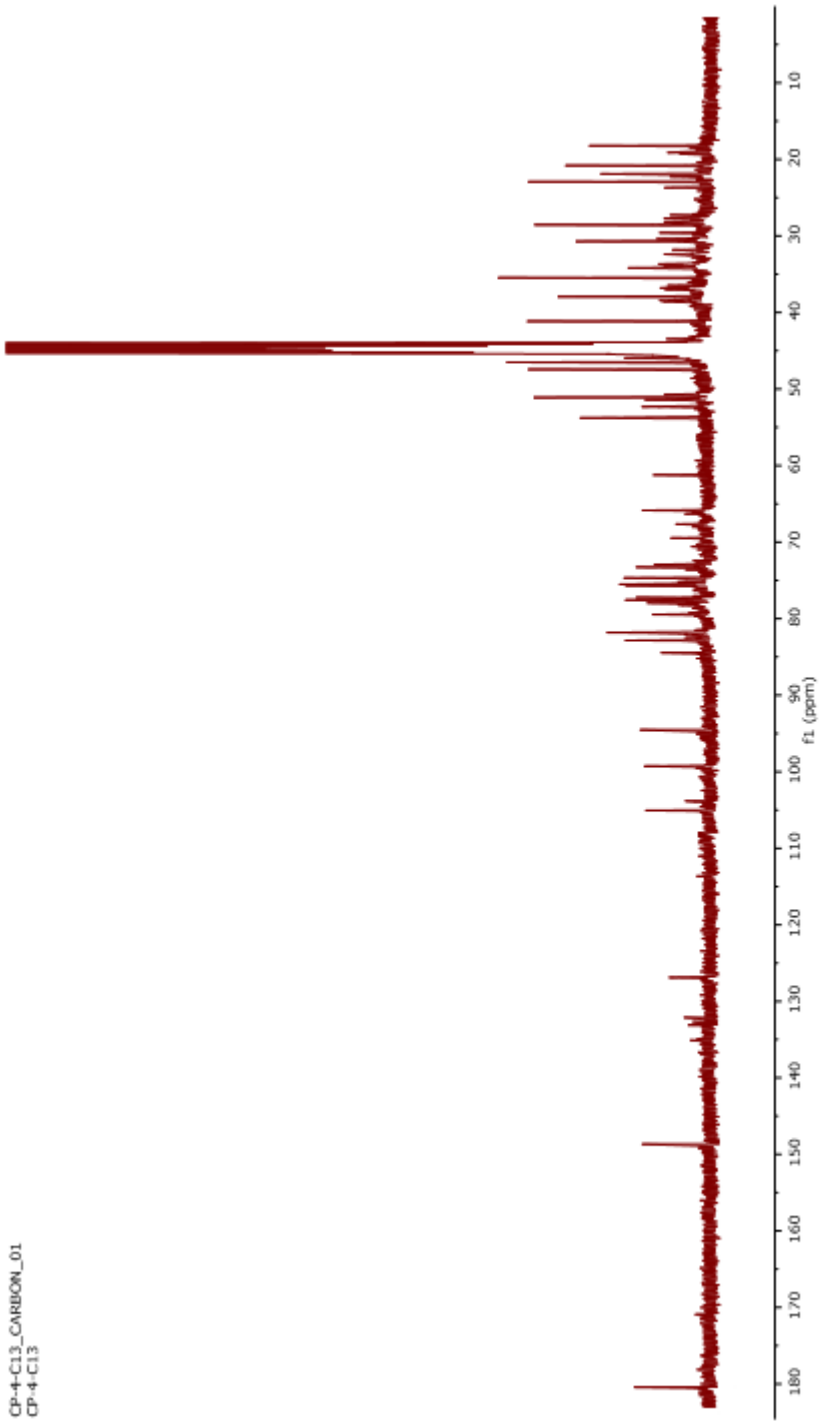


Figure 3.32 ^{13}C-NMR (100 *MHz*, DMSO-d_6) spectrum of compound **3**

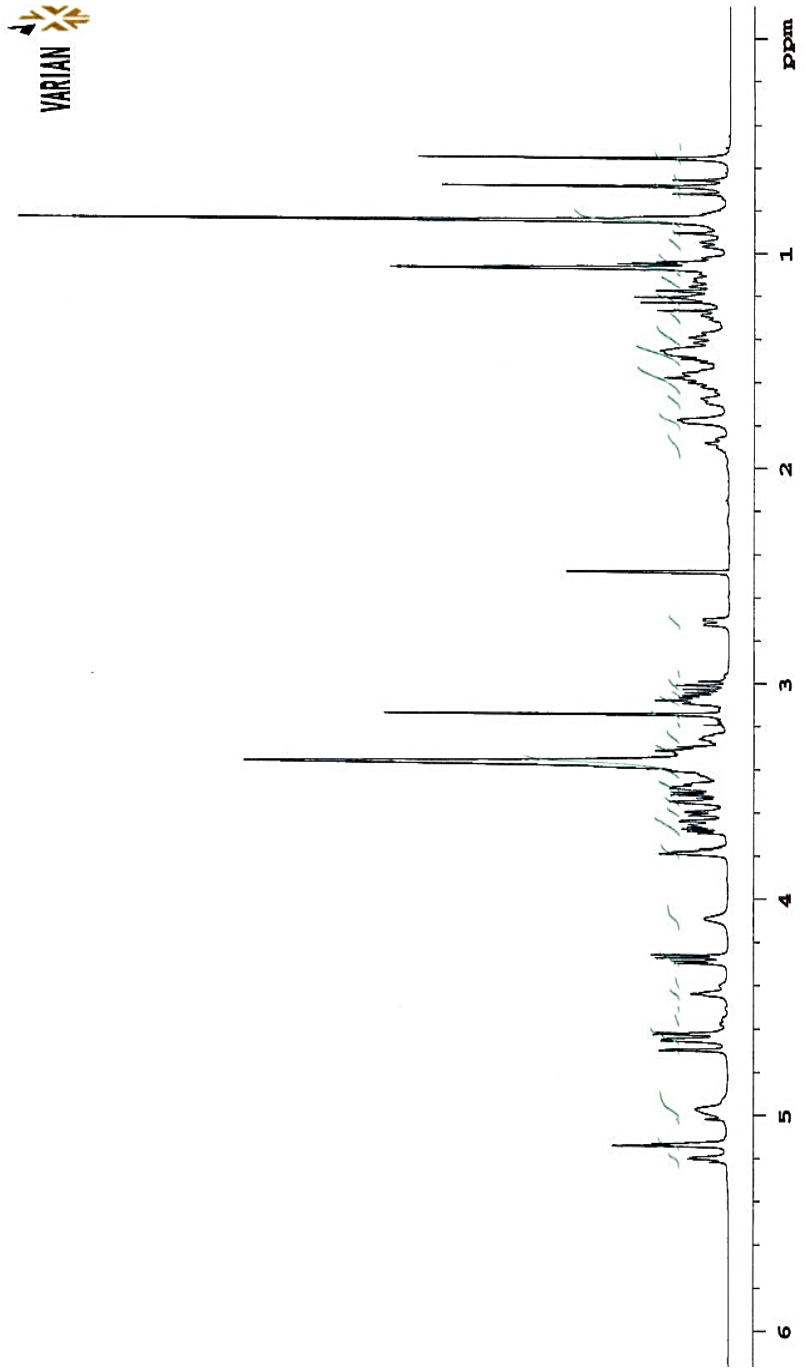


Figure 3.33 ^{1}H-NMR (600 *MHz*, DMSO-d_6) spectrum of compound **4**

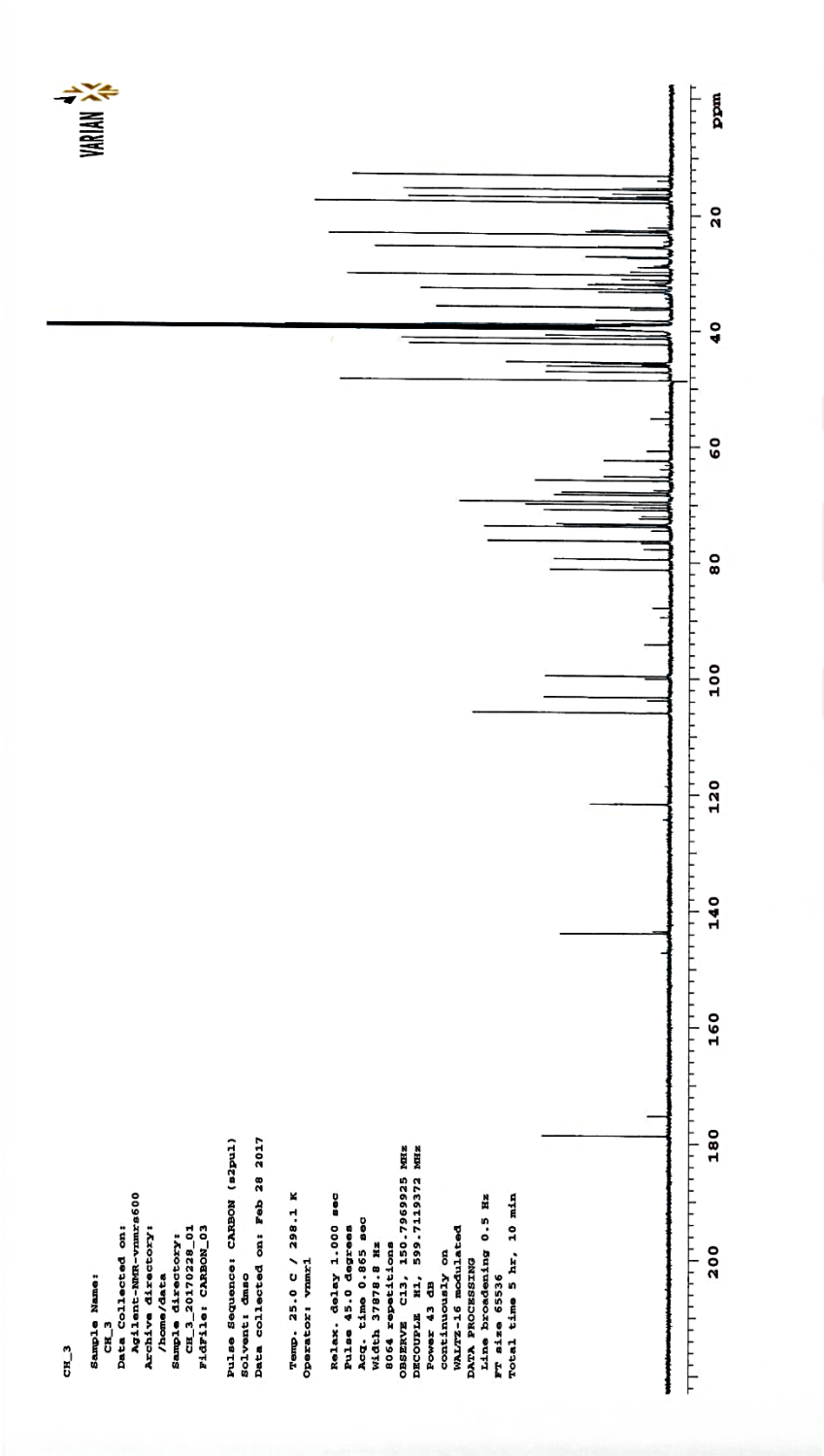


Figure 3.34 ^{13}C-NMR (150 *MHz*, DMSO-d_6) spectrum of compound **4**

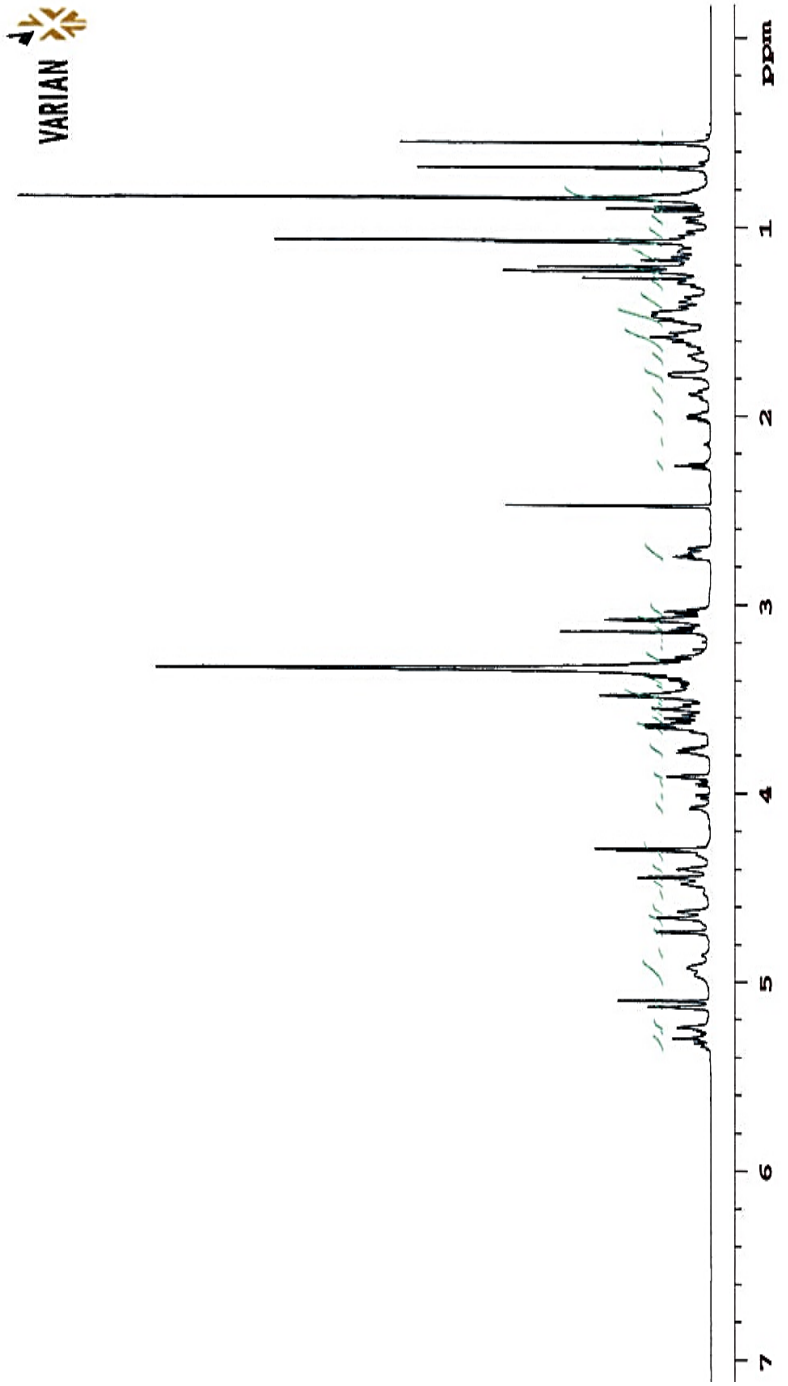


Figure 3.35 ^{1}H-NMR (600 *MHz*, DMSO-d_6) spectrum of compound **5**

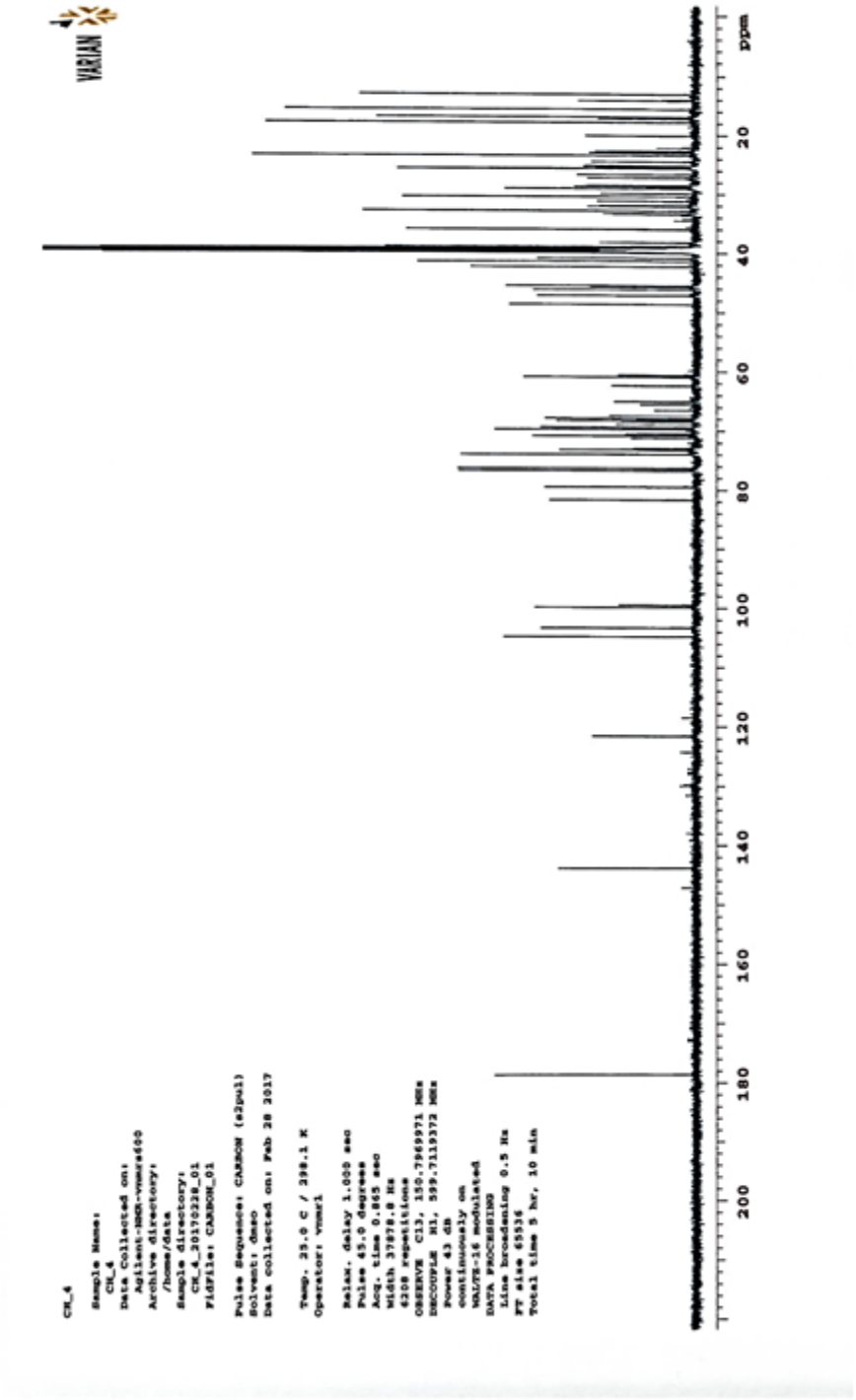


Figure 3.36 ^{13}C-NMR (150 *MHz*, DMSO-d_6) spectrum of compound **5**

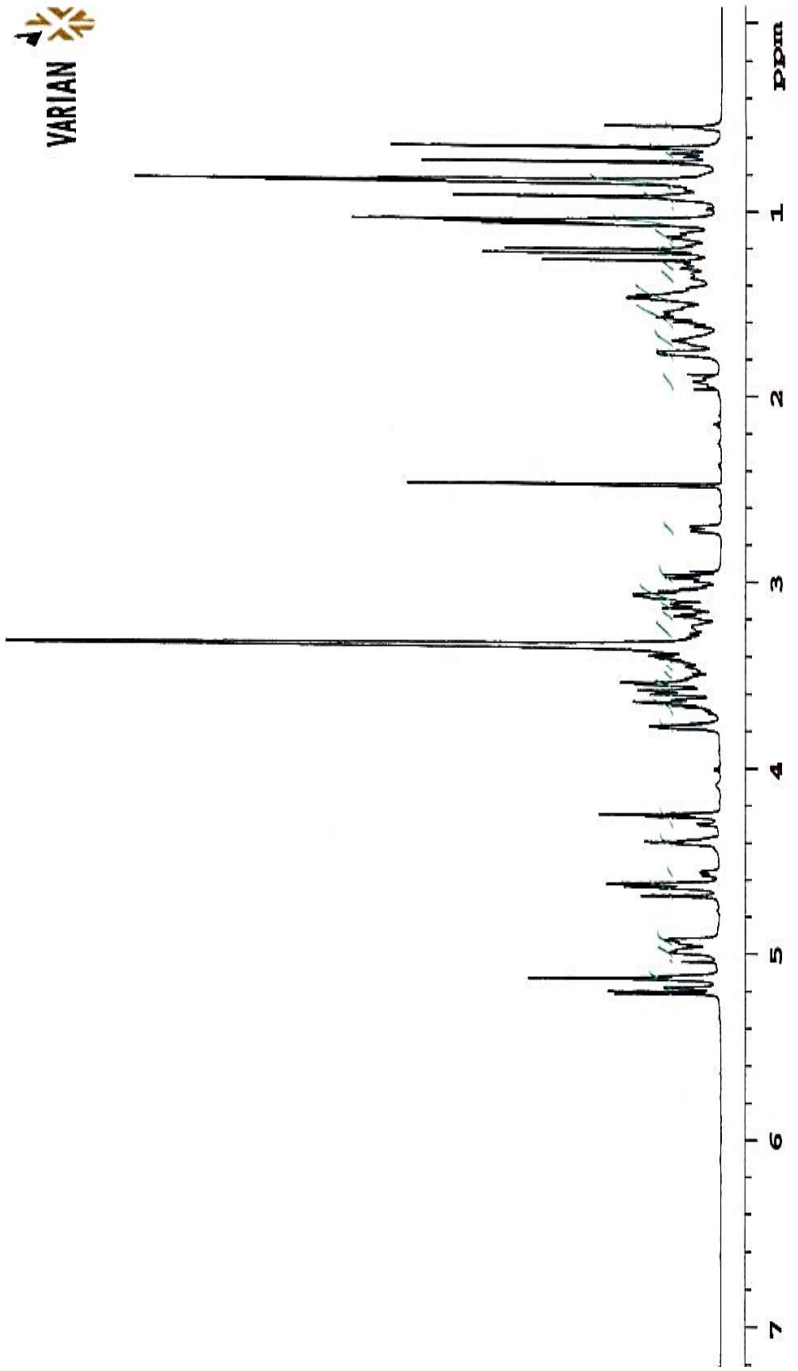


Figure 3.37 ^{1}H-NMR (600 *MHz*, DMSO-d_6) spectrum of compound **6**

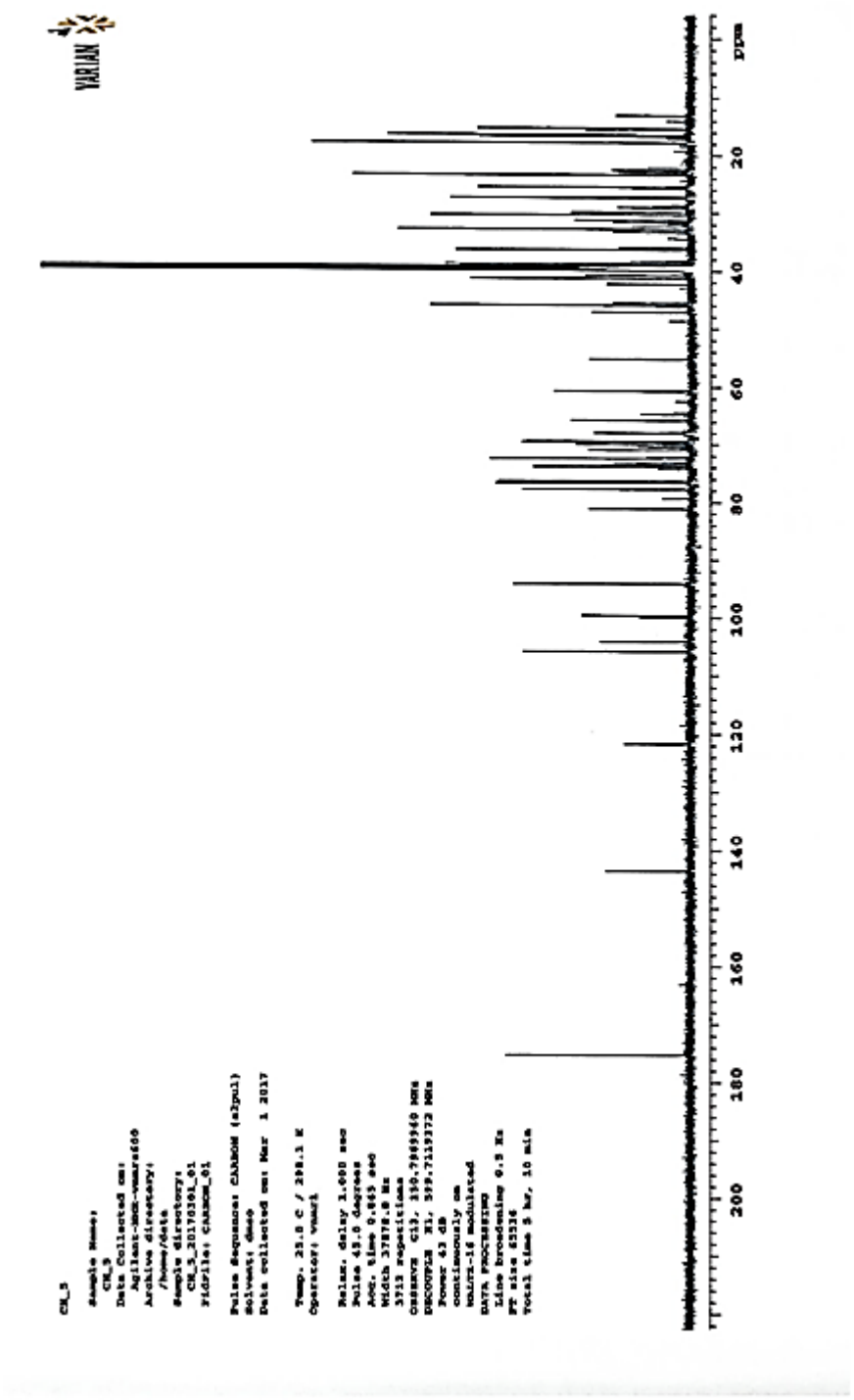


Figure 3.38 ^{13}C-NMR (150 *MHz*, DMSO-d_6) spectrum of compound **6**

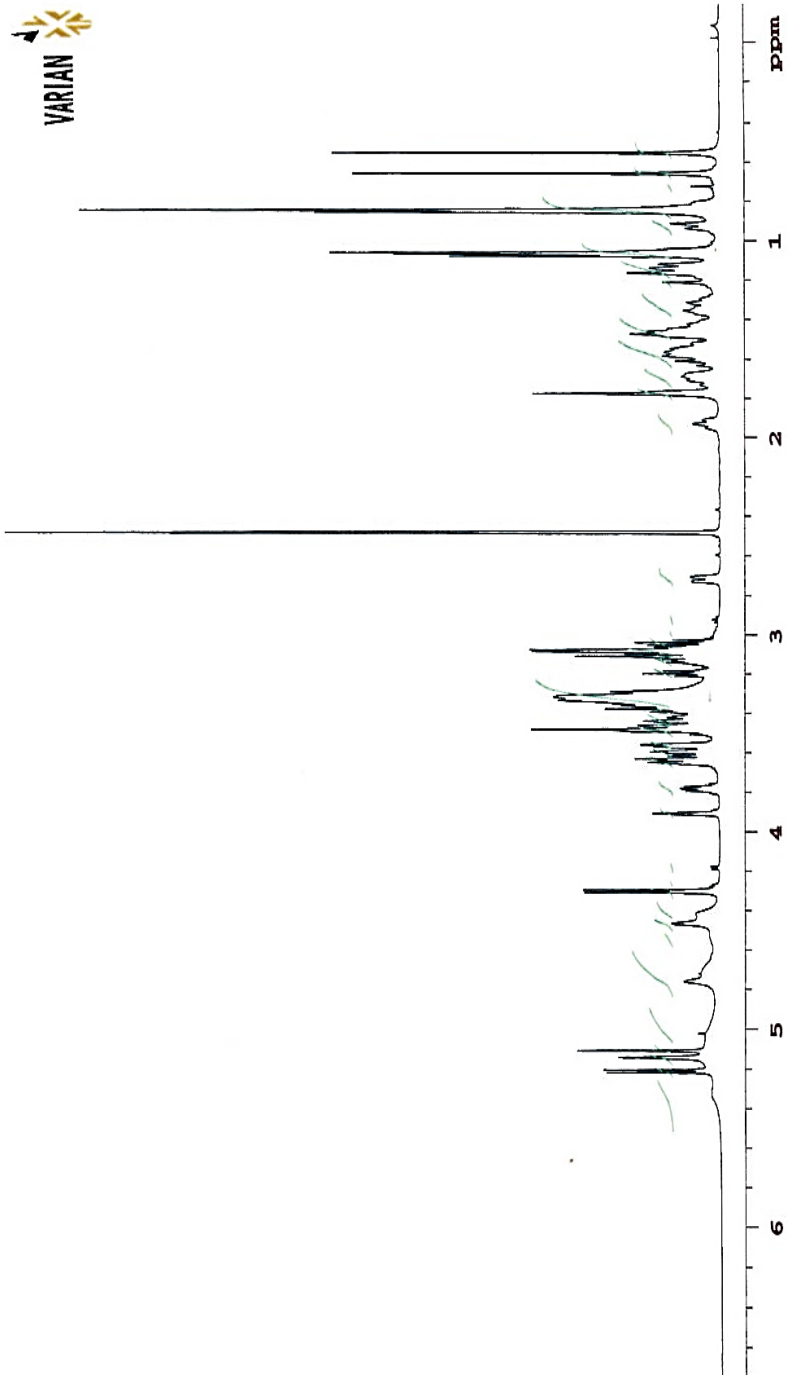


Figure 3.39 ^{1}H-NMR (600 *MHz*, DMSO-d_6) spectrum of compound **7**

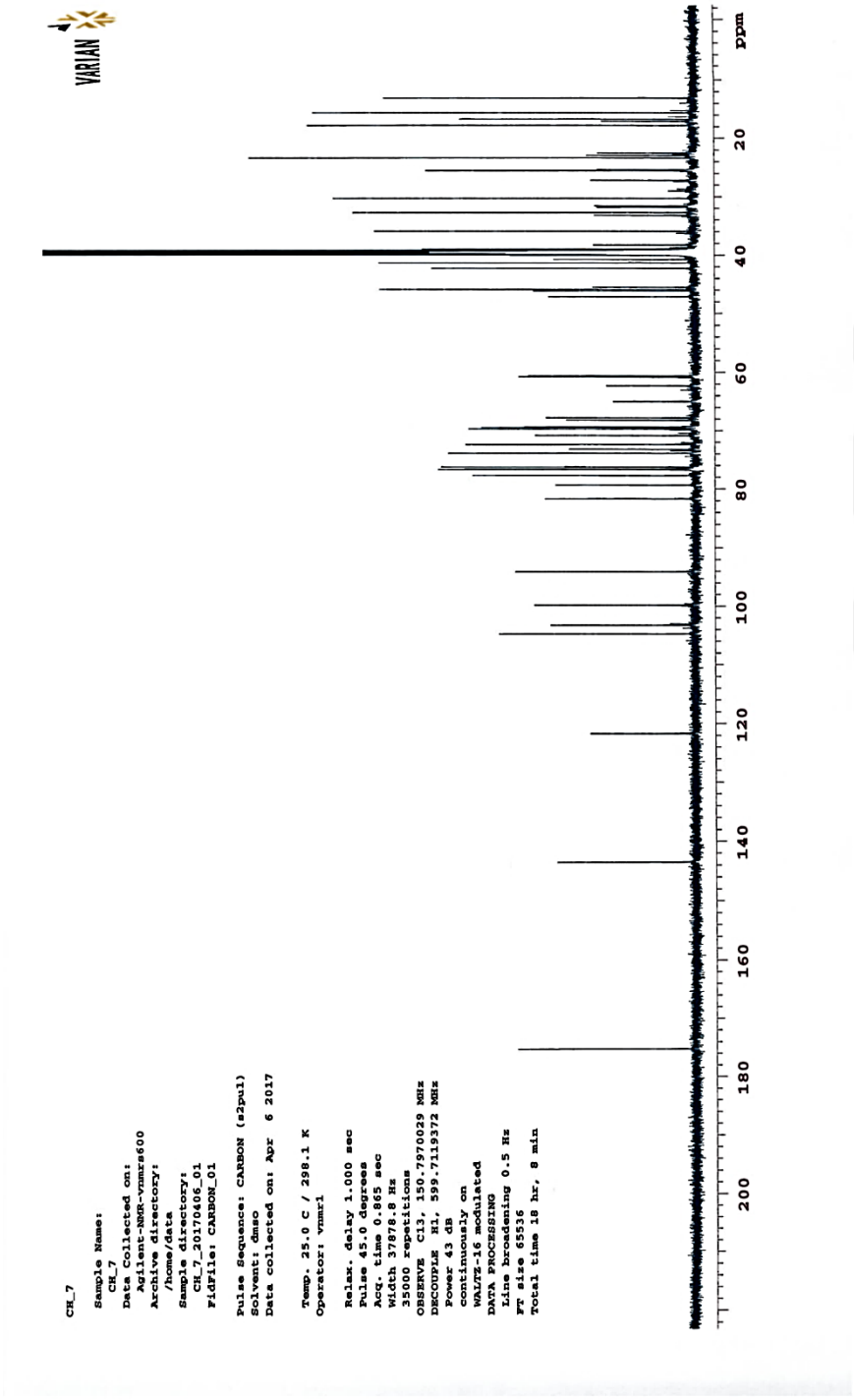


Figure 3.40 ^{13}C-NMR (150 *MHz*, DMSO-d_6) spectrum of compound **7**

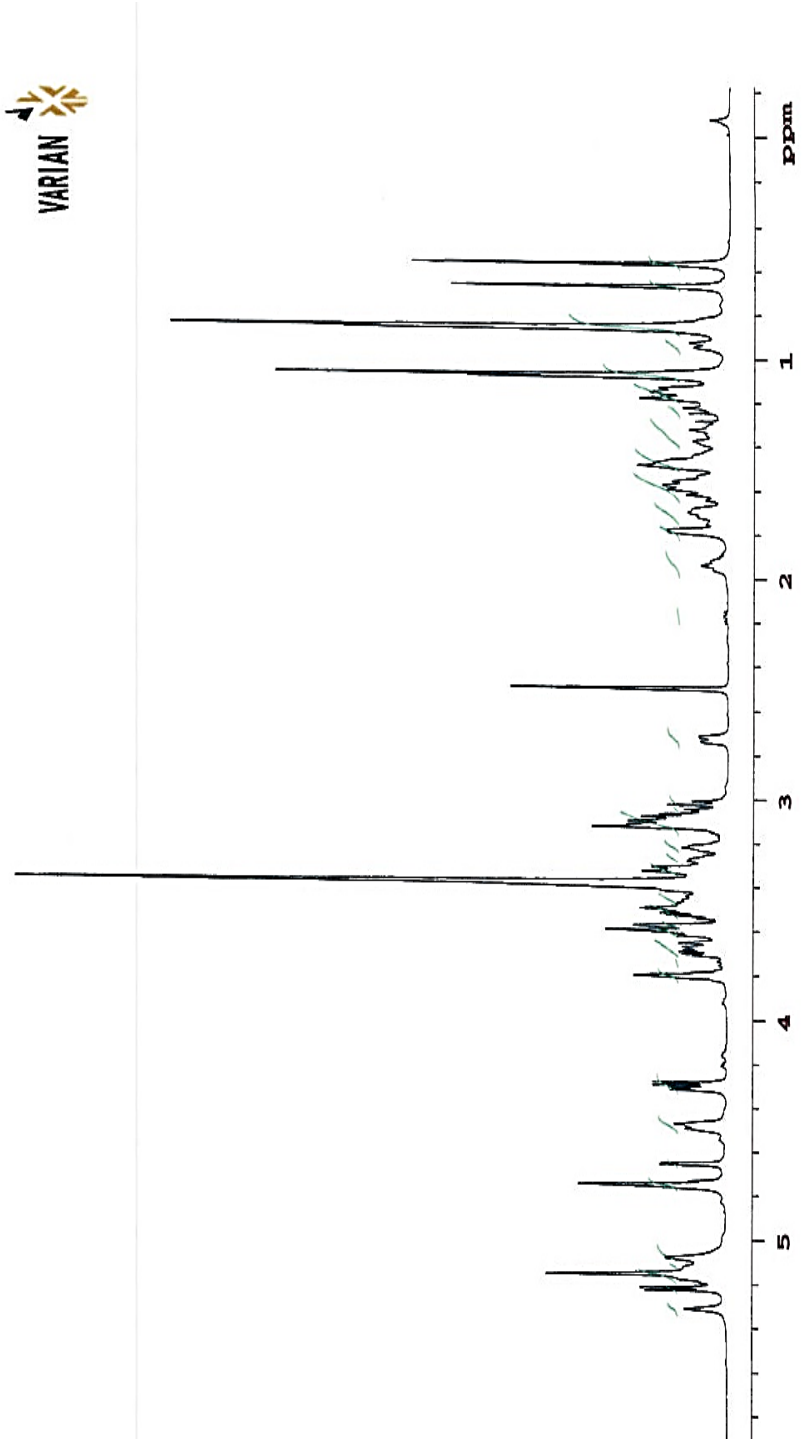


Figure 3.41 ^{1}H-NMR (600 *MHz*, DMSO-d_6) spectrum of compound **8**

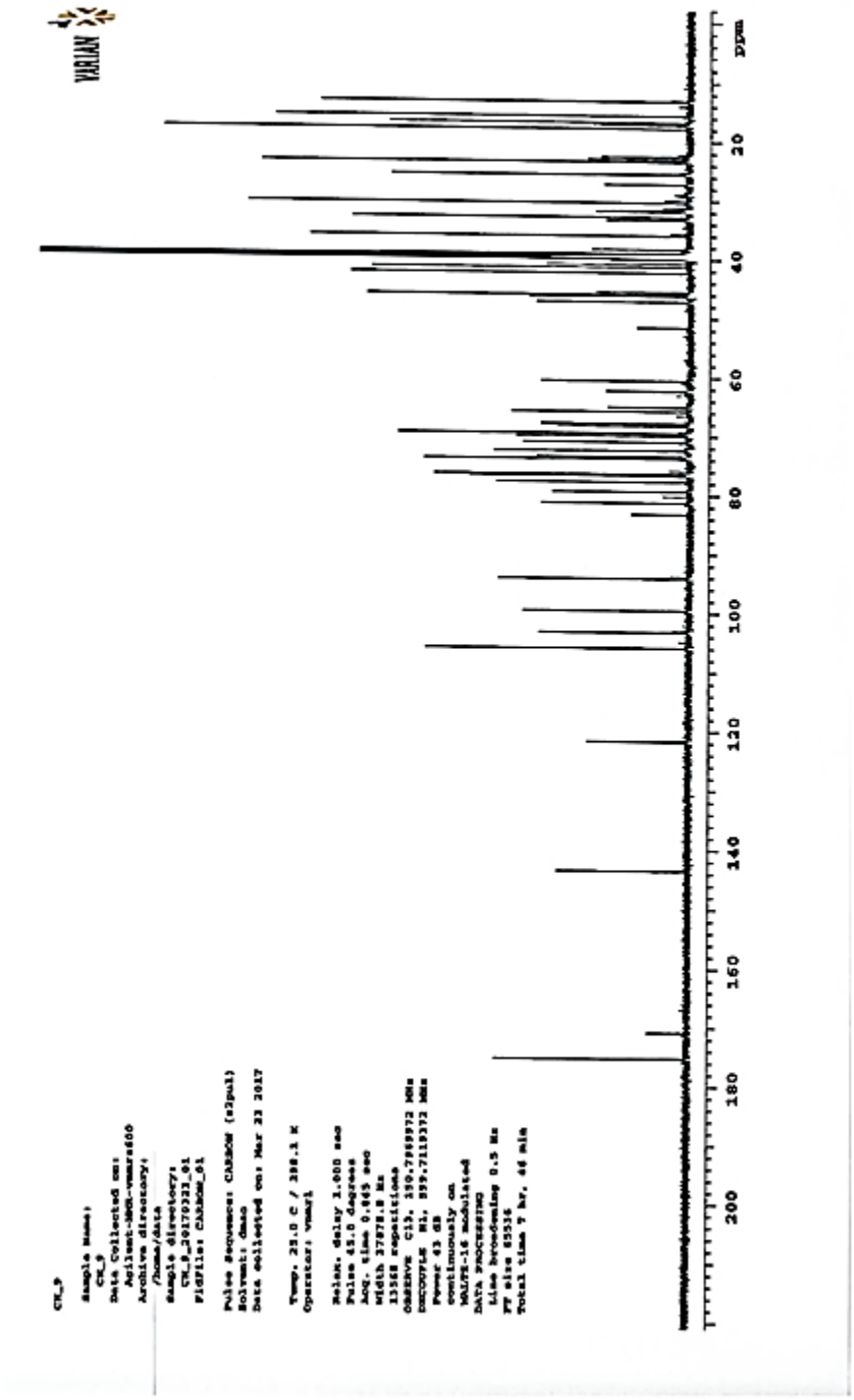


Figure 3.42 ^{13}C-NMR (150 *MHz*, DMSO-d_6) spectrum of compound **8**

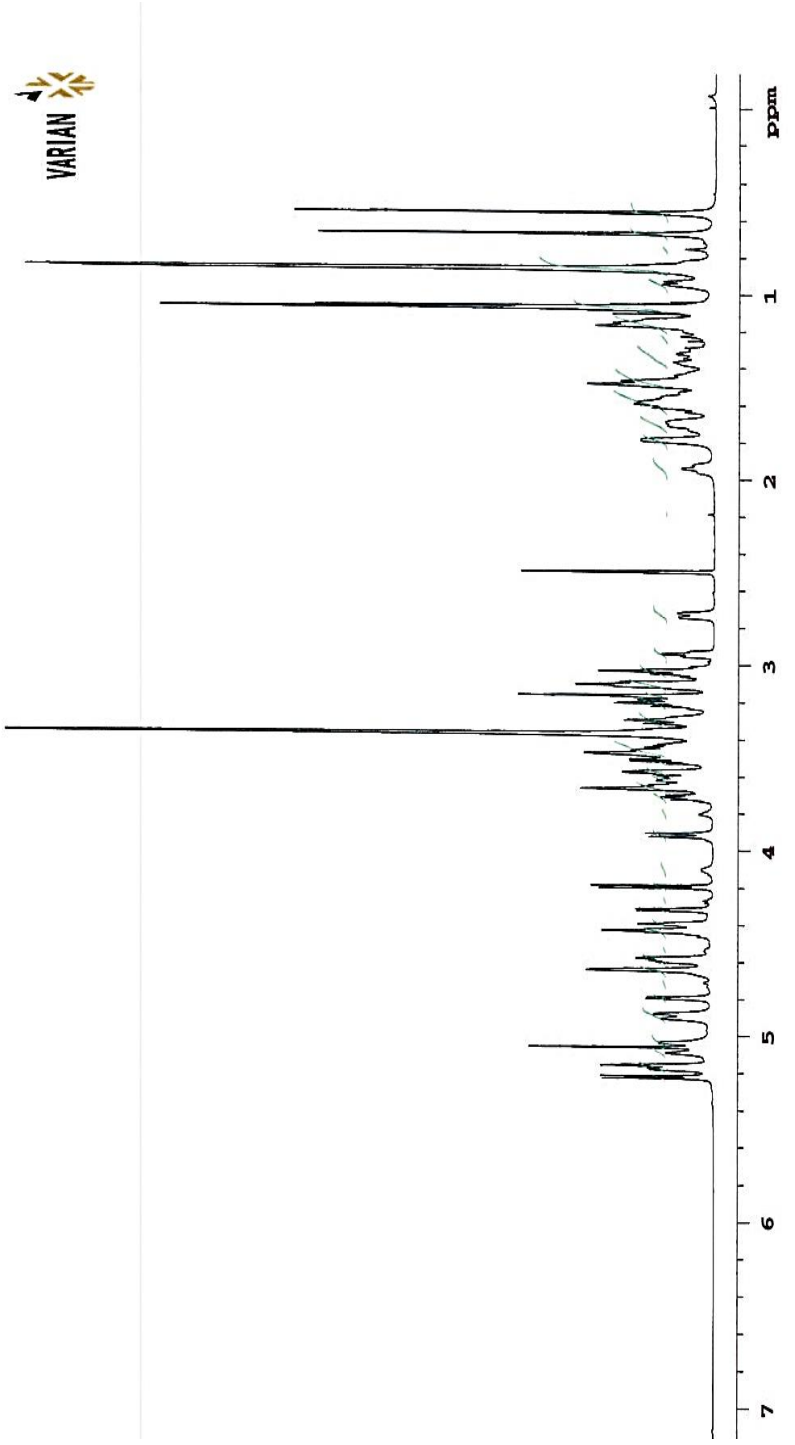


Figure 3.43 ^{1}H-NMR (600 *MHz*, DMSO-d_6) spectrum of compound **9**

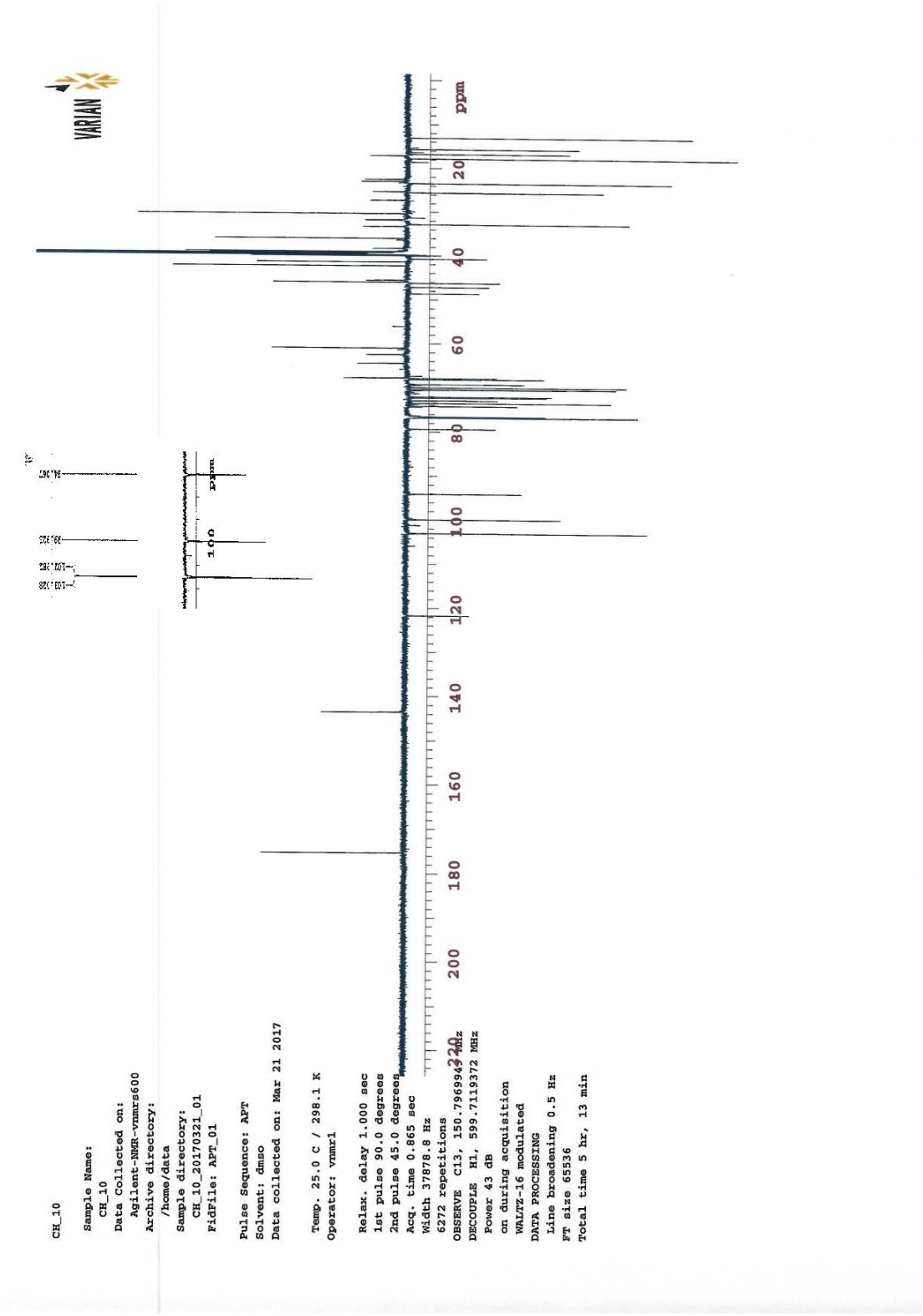


Figure 3.44 APT (150 *MHz*, DMSO-d_6) spectrum of compound **9**

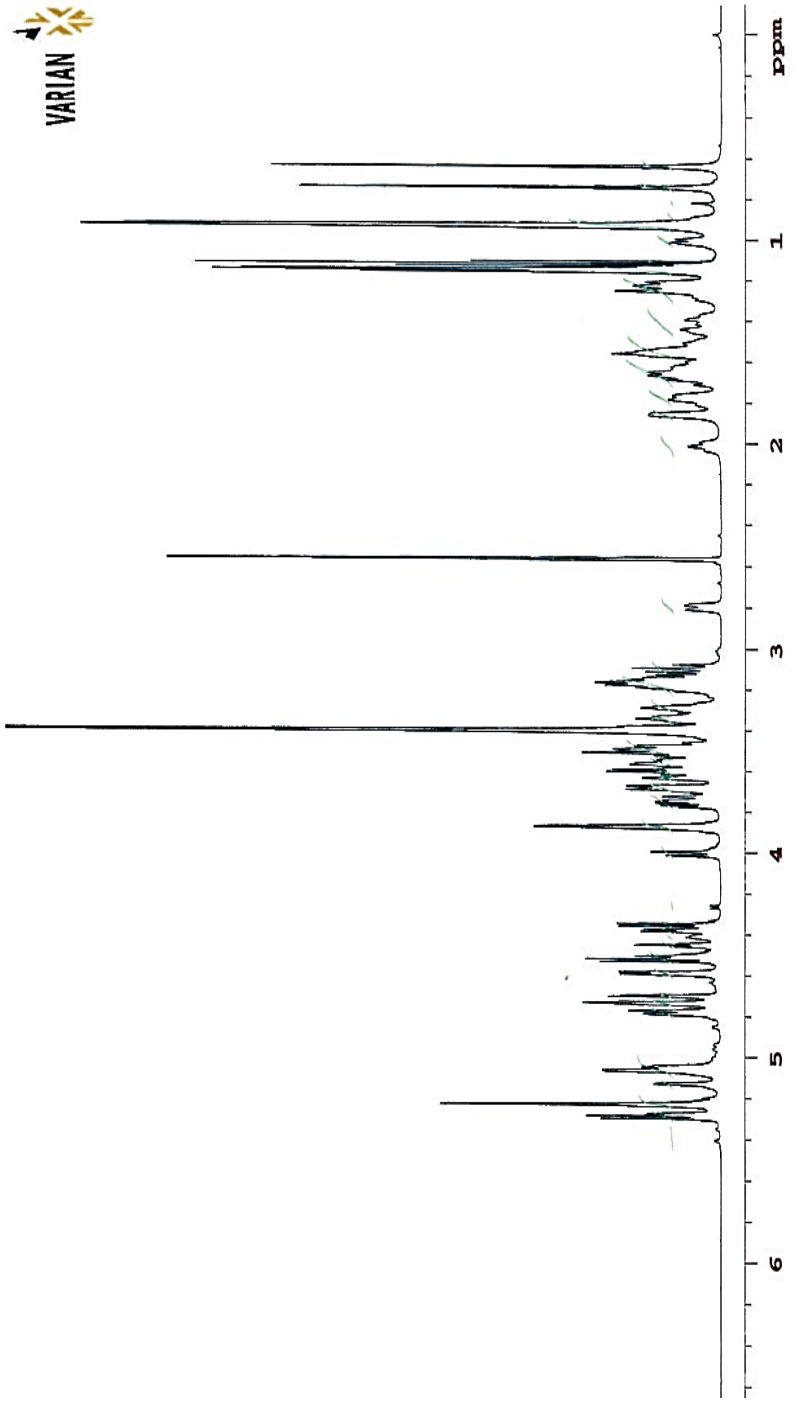


Figure 3.45 ^{1}H-NMR (600 *MHz*, DMSO-d_6) spectrum of compound **10**

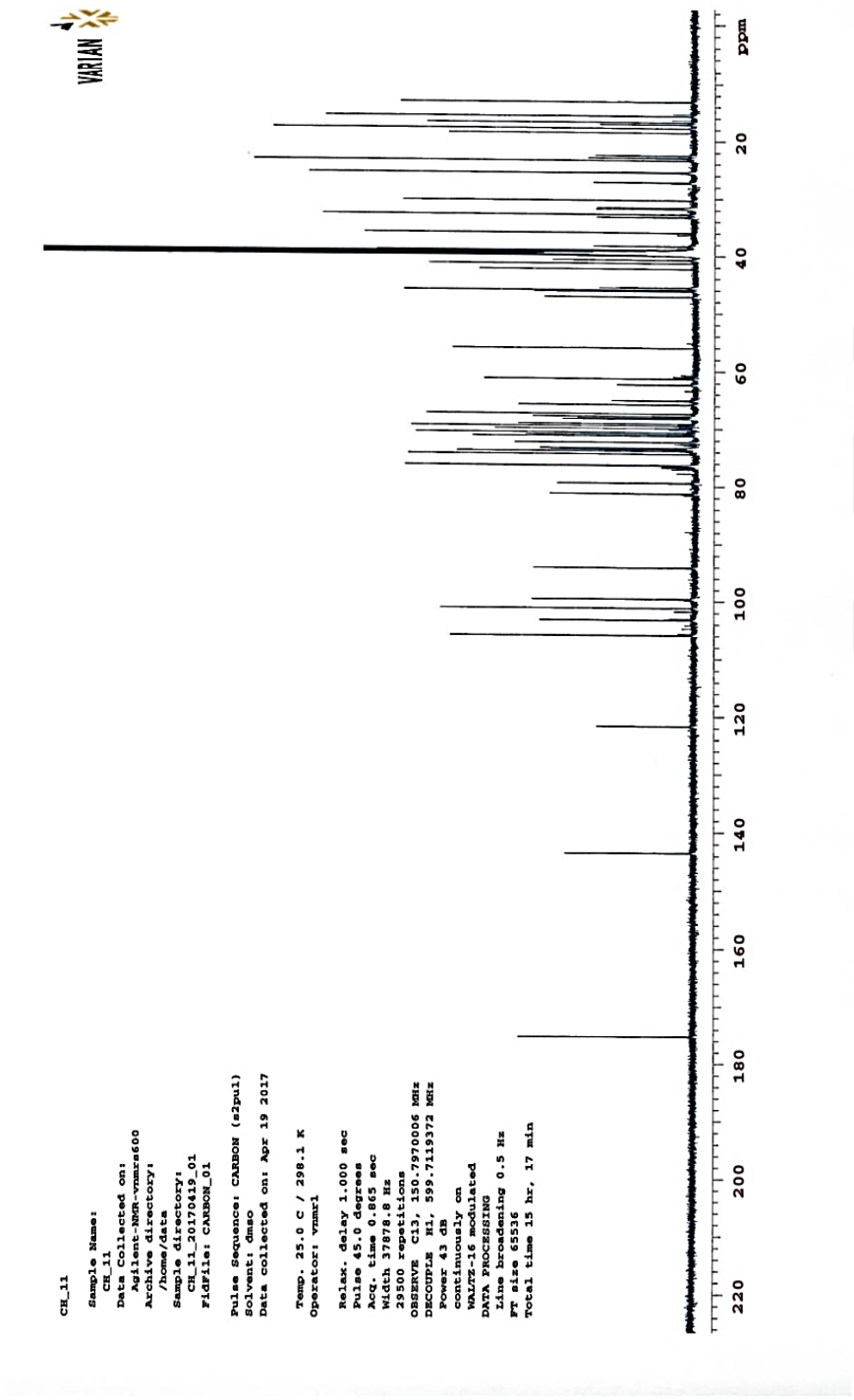


Figure 3.46 ^{13}C-NMR (150 *MHz*, DMSO-d_6) spectrum of compound **10**

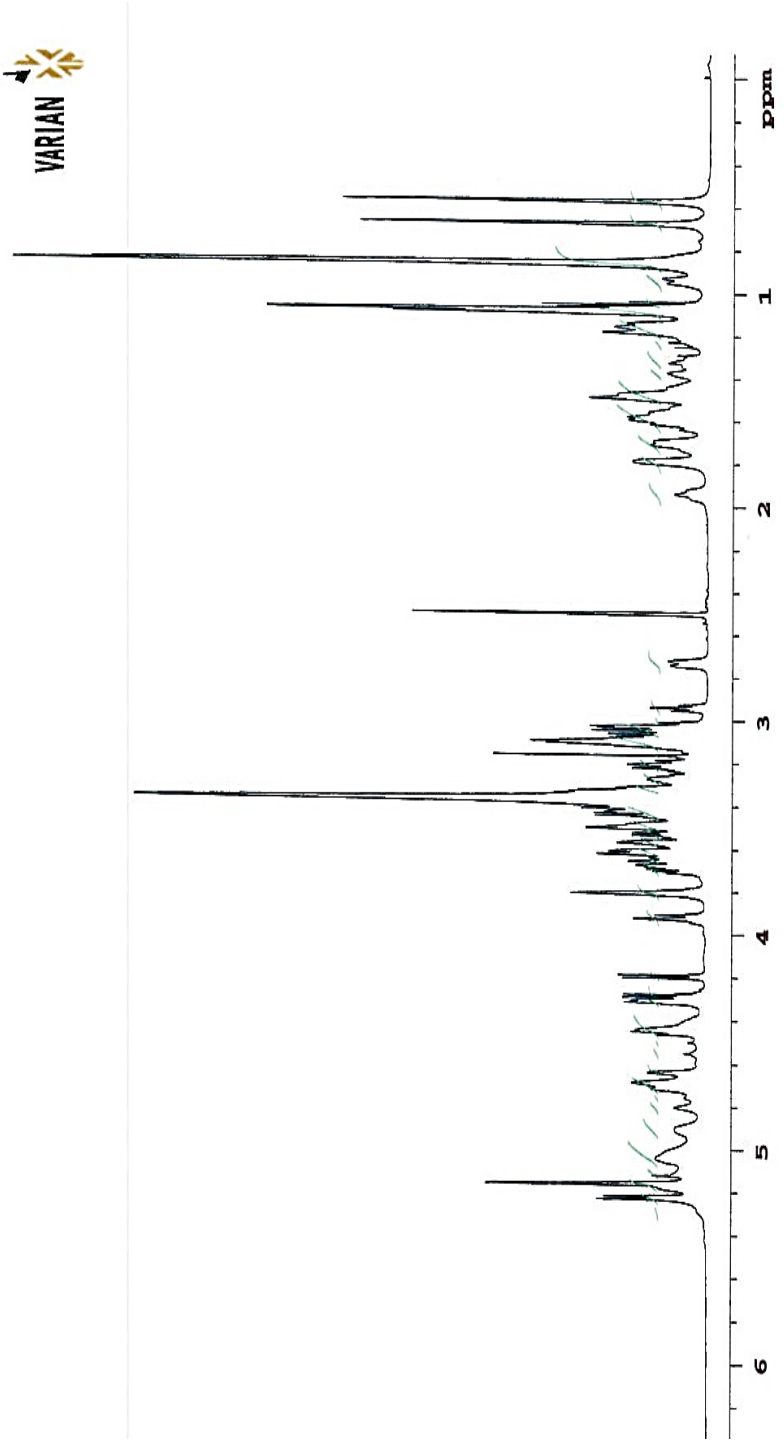


Figure 3.47 ^{1}H-NMR (600 *MHz*, DMSO-d_6) spectrum of compound **11**

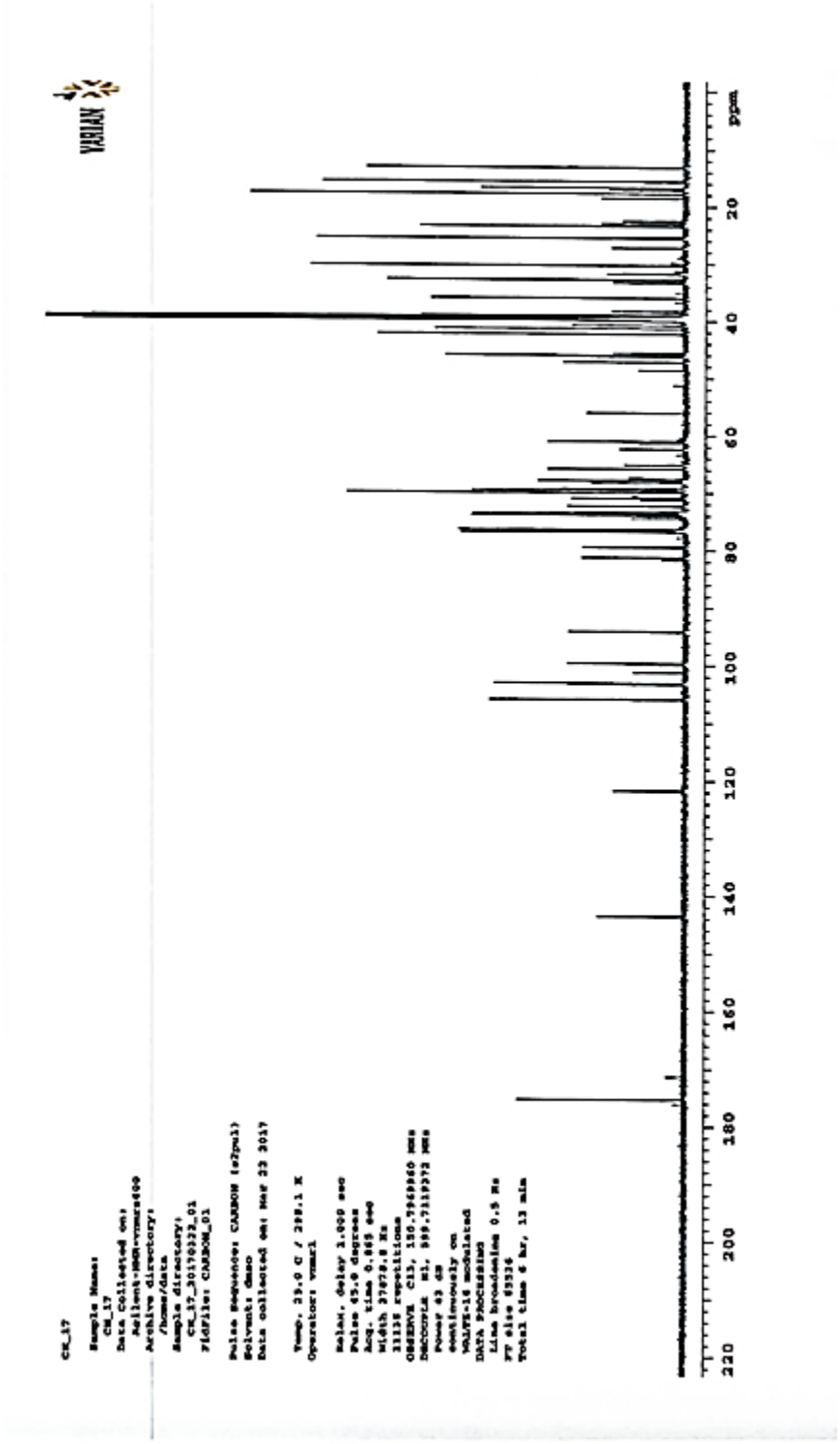


Figure 3.48 ^{13}C-NMR (150 *MHz*, DMSO-d_6) spectrum of compound **11**

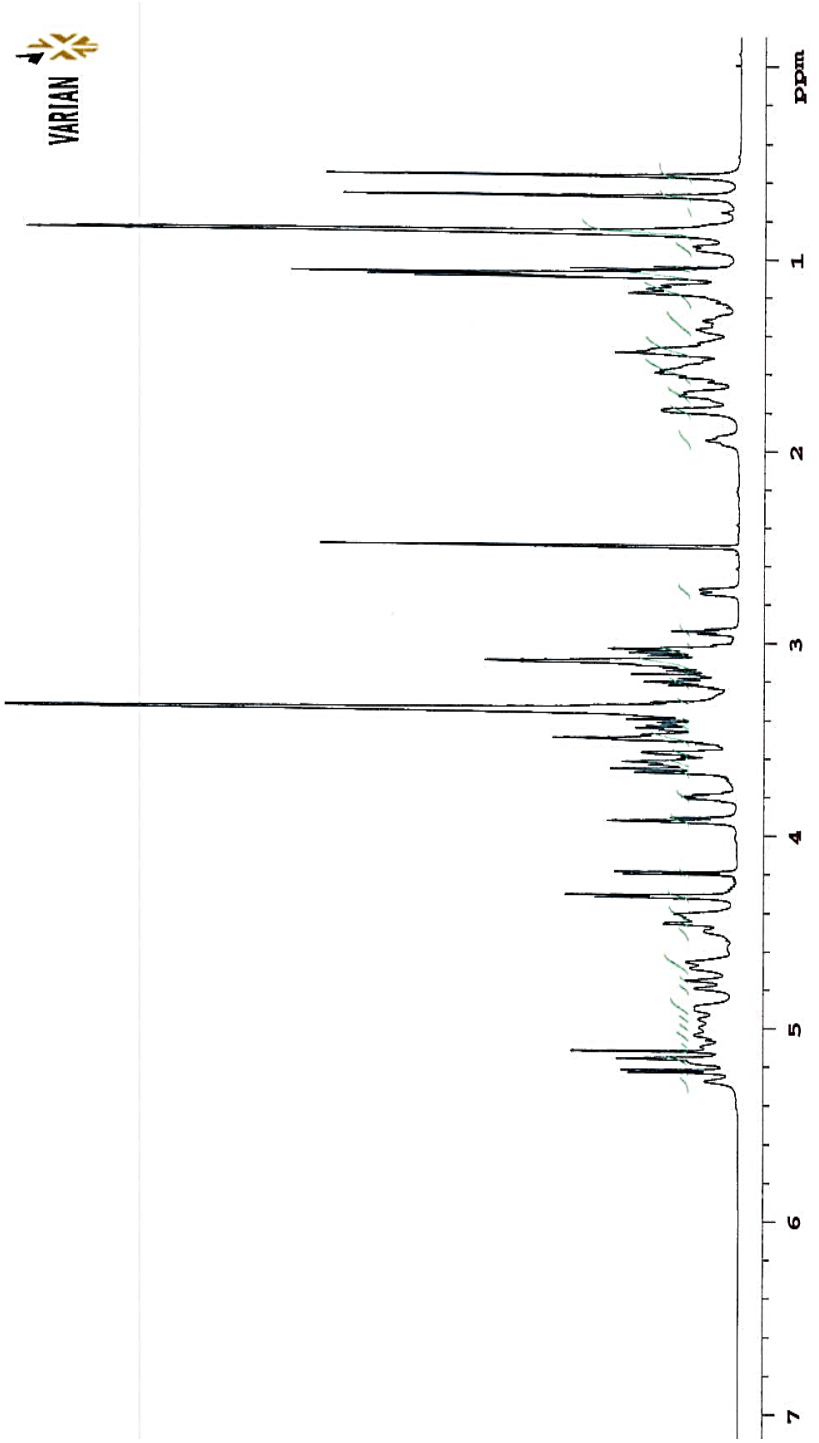


Figure 3.49 ^{1}H-NMR (600 *MHz*, DMSO-d_6) spectrum of compound **12**

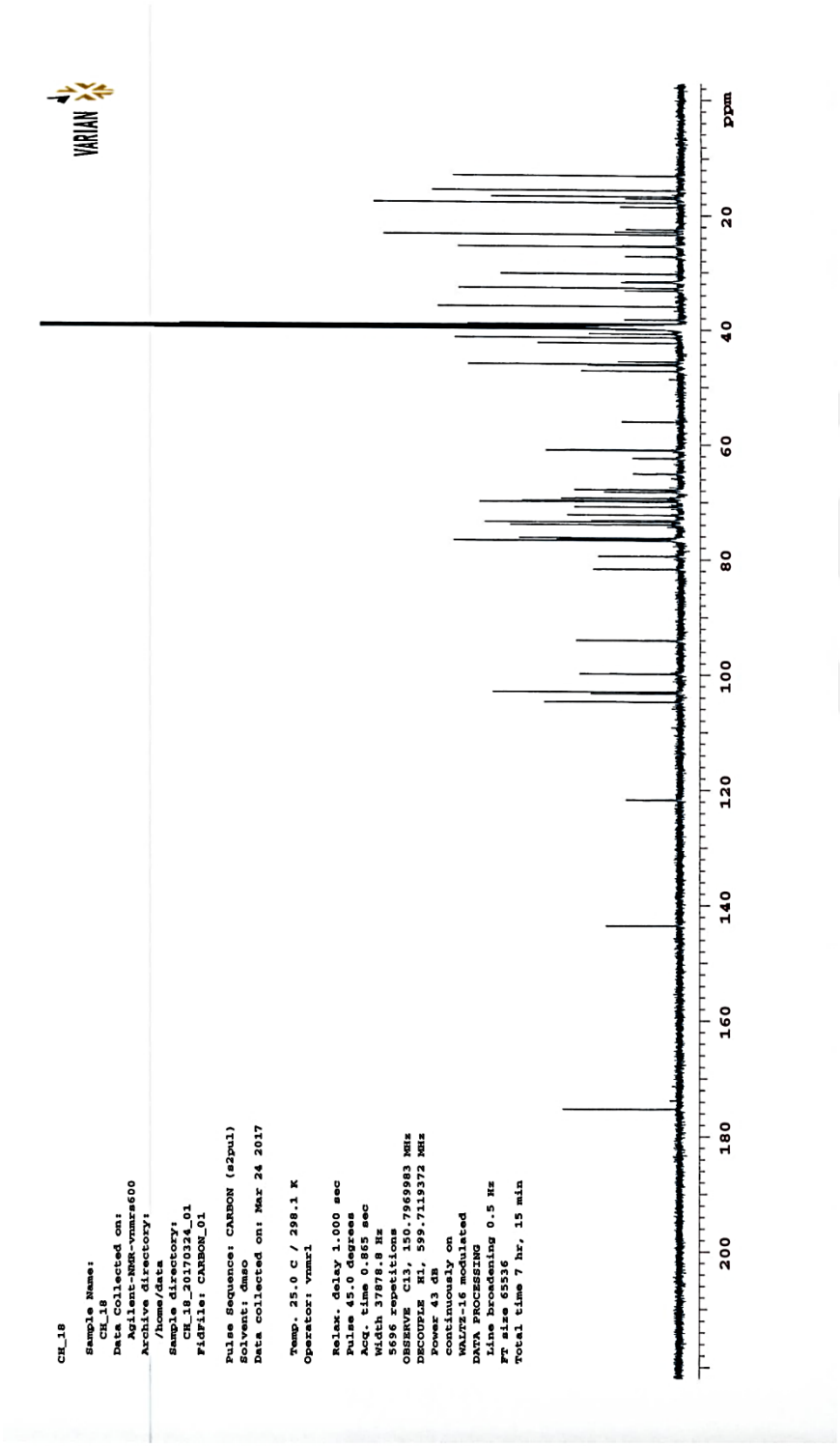


Figure 3.50 ^{13}C-NMR (150 *MHz*, DMSO-d_6) spectrum of compound **12**

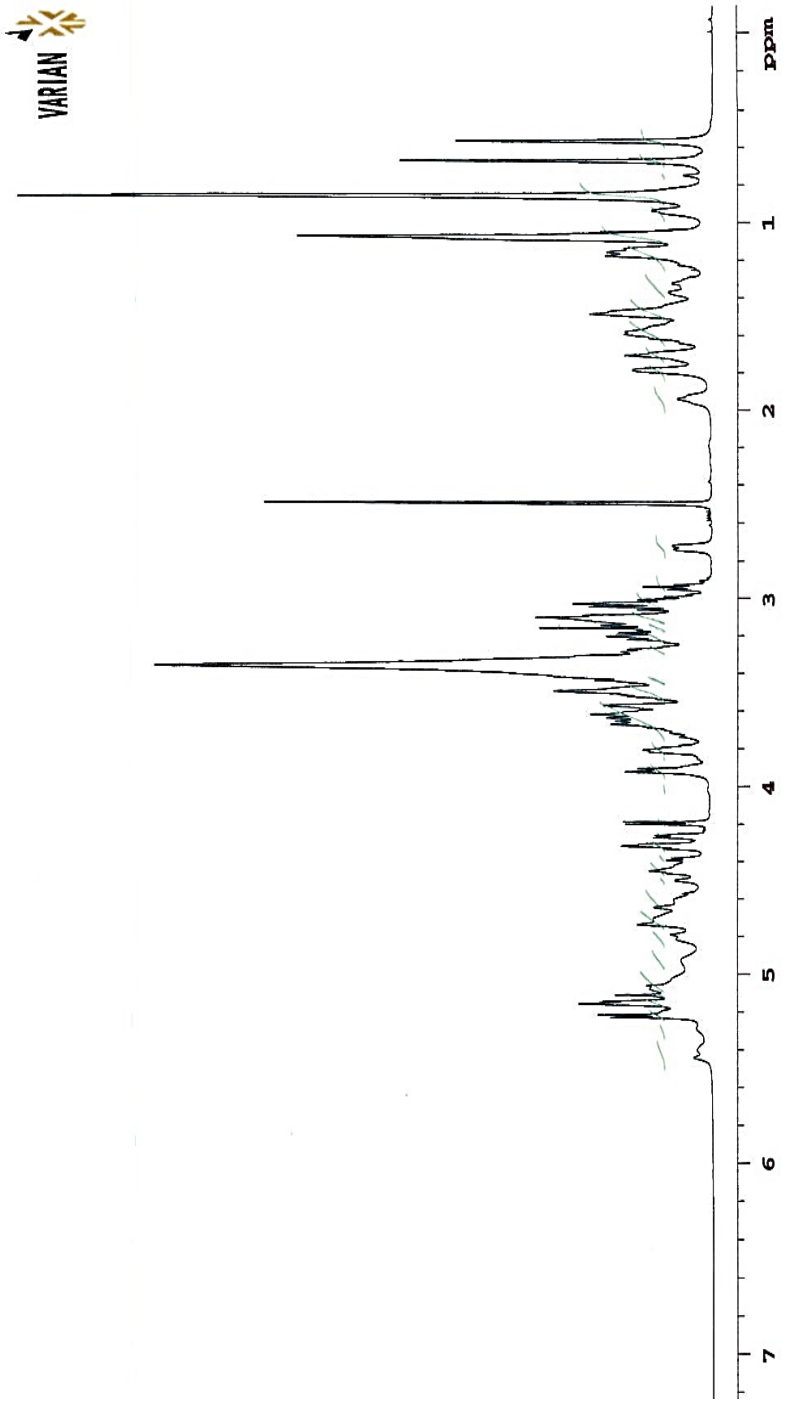


Figure 3.51 ^{1}H-NMR (600 *MHz*, DMSO-d_6) spectrum of compound **13**

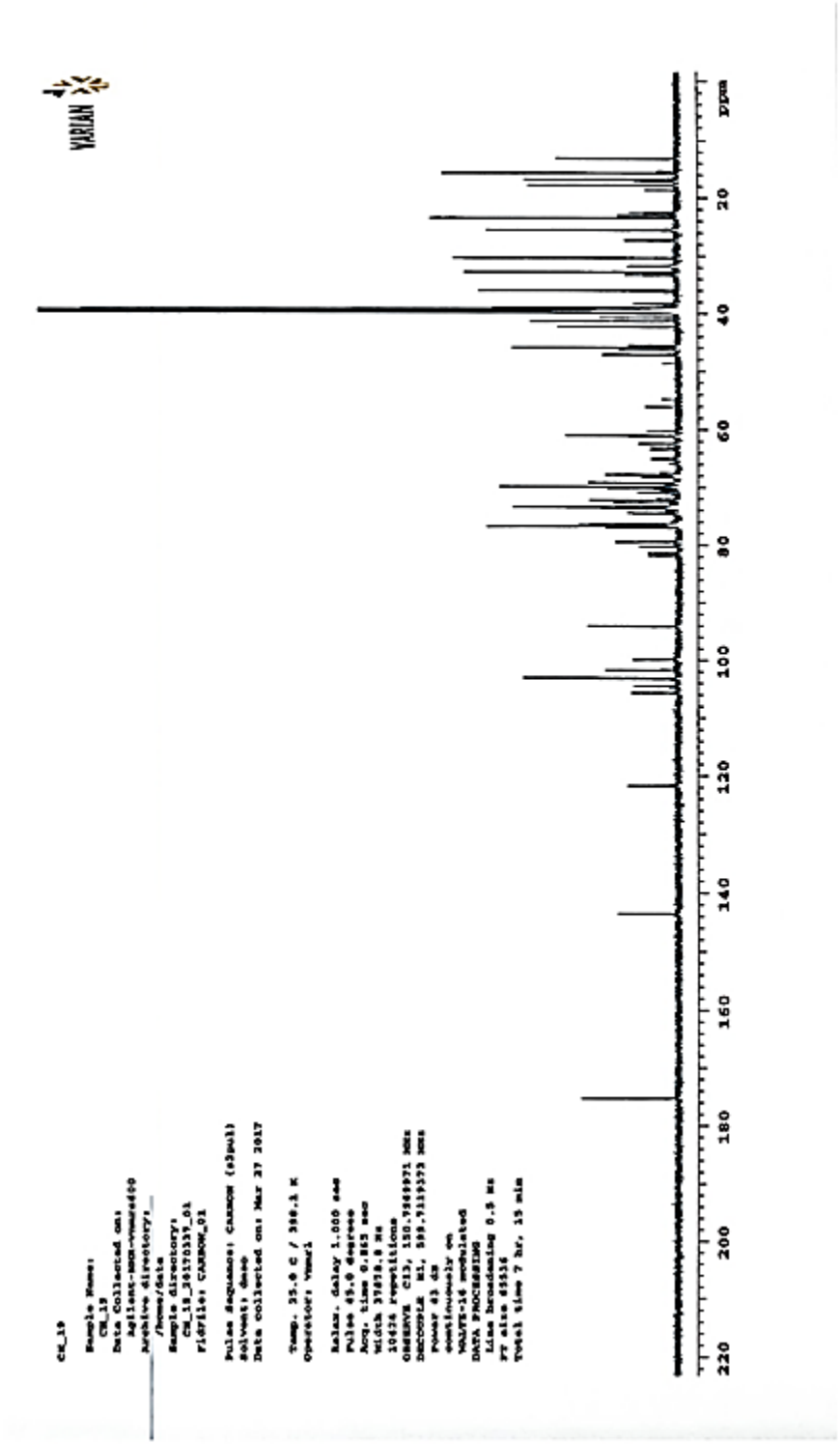


Figure 3.52 ^{13}C-NMR (150 *MHz*, DMSO-d_6) spectrum of compound **13**

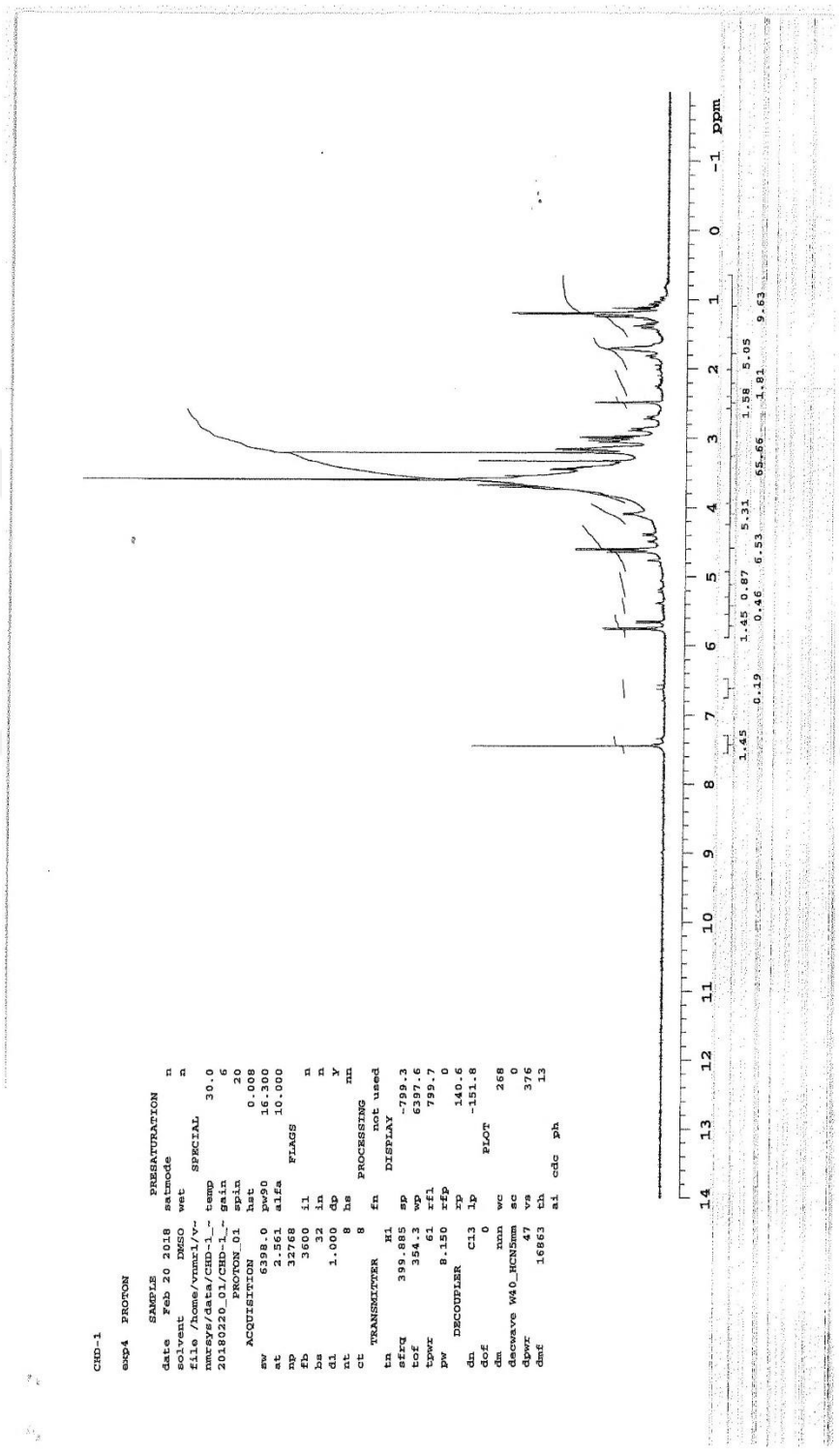


Figure 3.53 ^{1}H-NMR (400 *MHz*, DMSO-d_6) spectrum of compound **14**

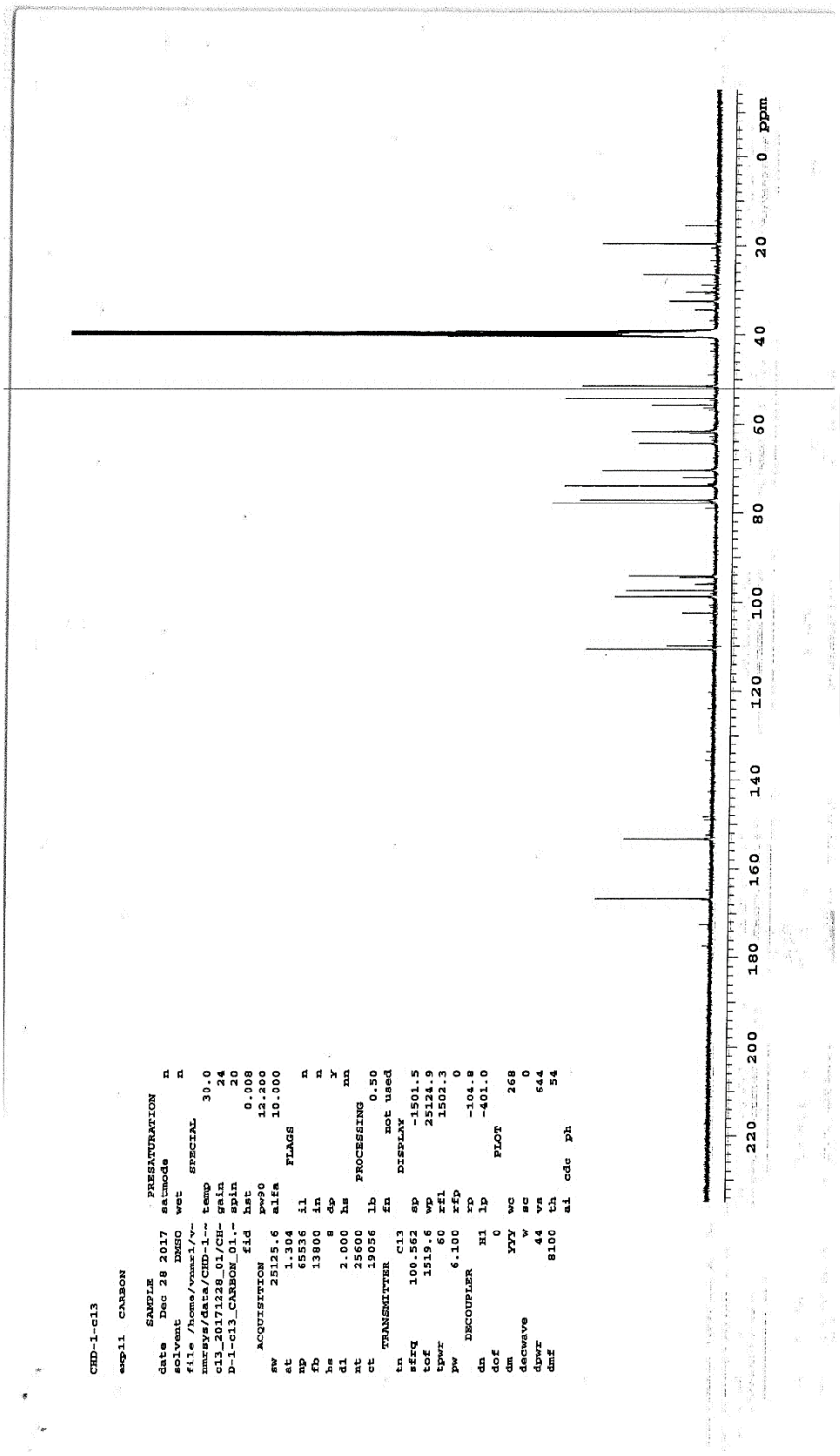


Figure 3.54 ^{13}C-NMR (100 *MHz*, DMSO-d_6) spectrum of compound **14**

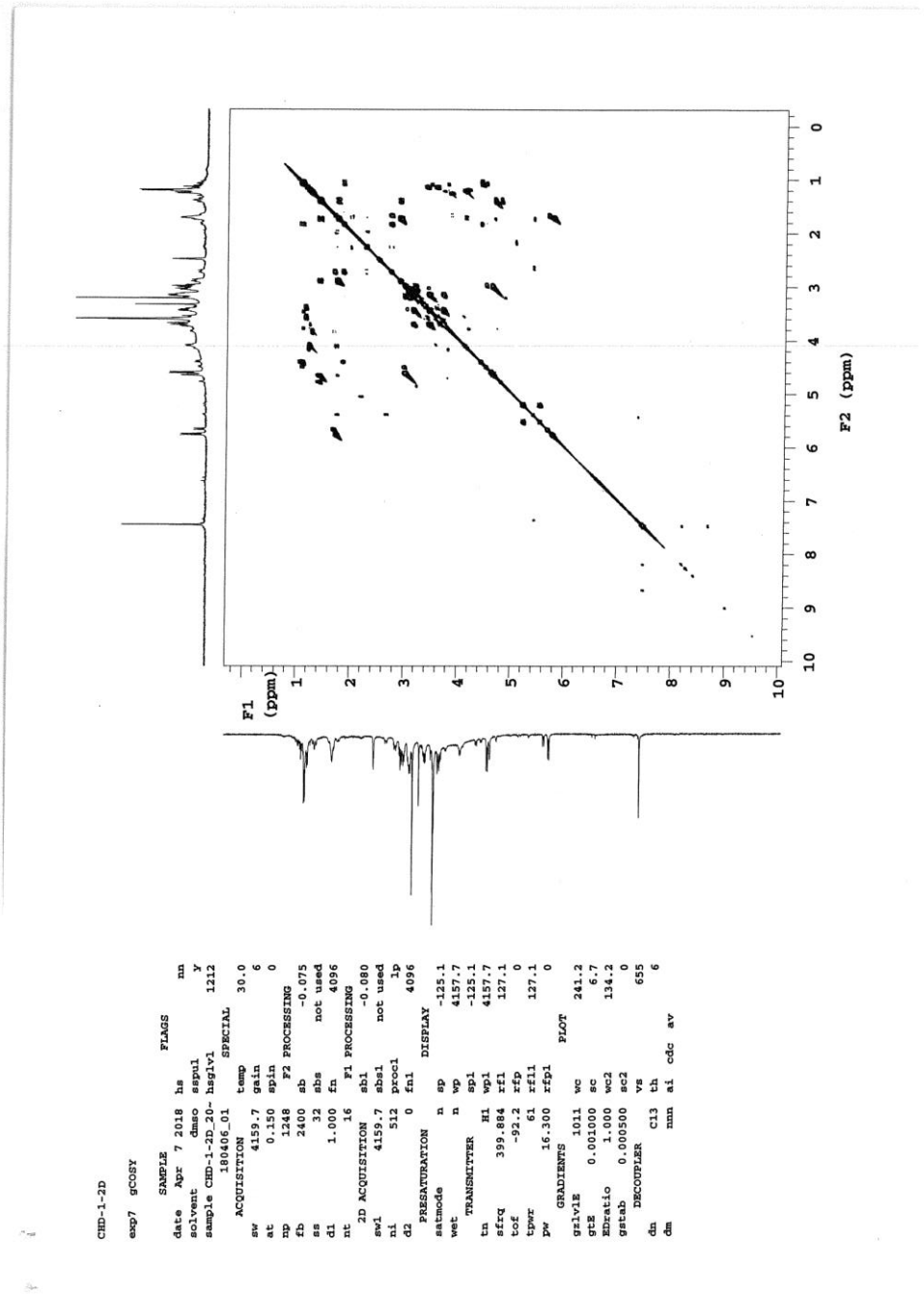


Figure 3.55 COSY (400 *MHz*) spectrum of compound **14**

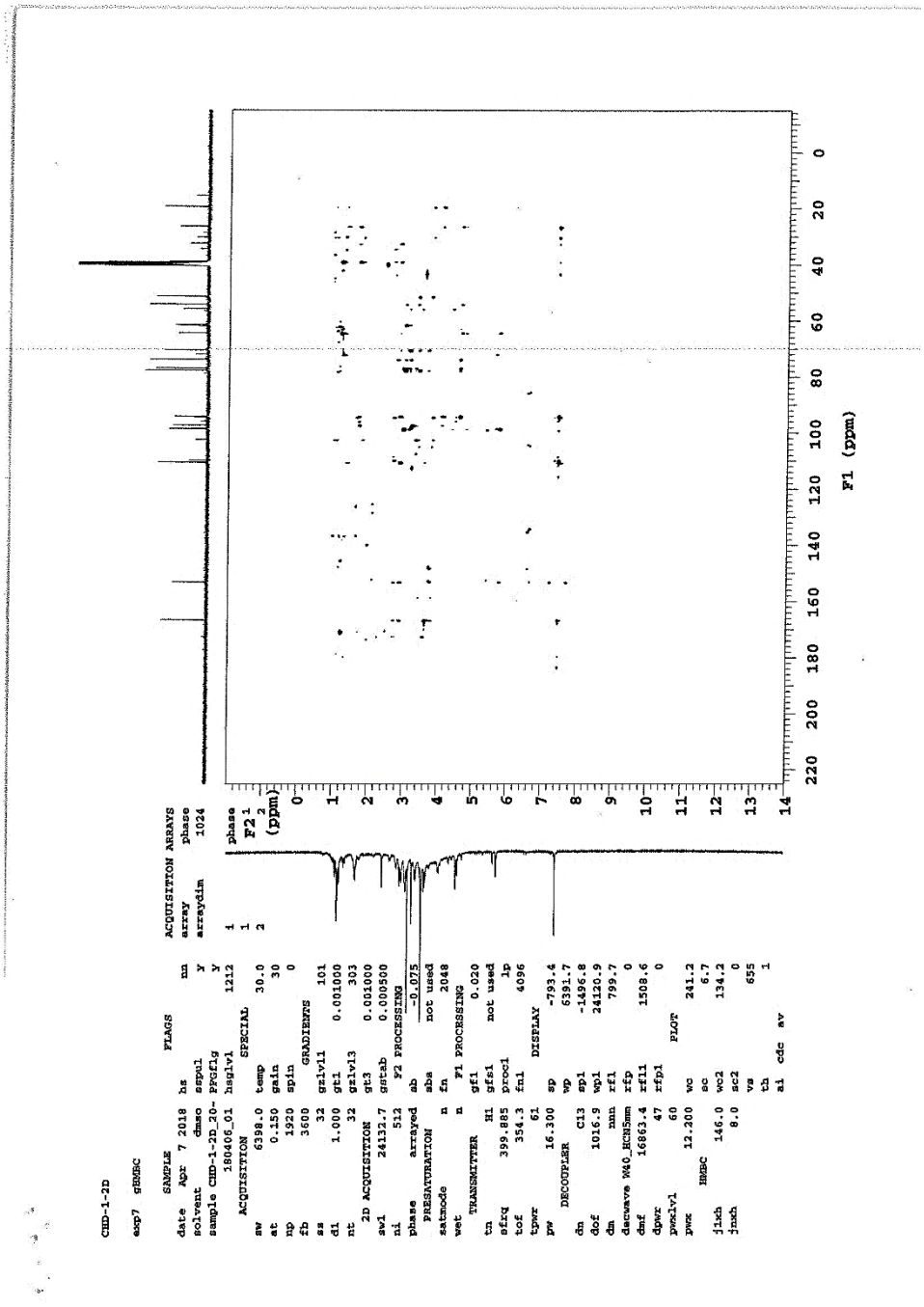


Figure 3.56 HMBC (400 *MHz*) spectrum of compound **14**

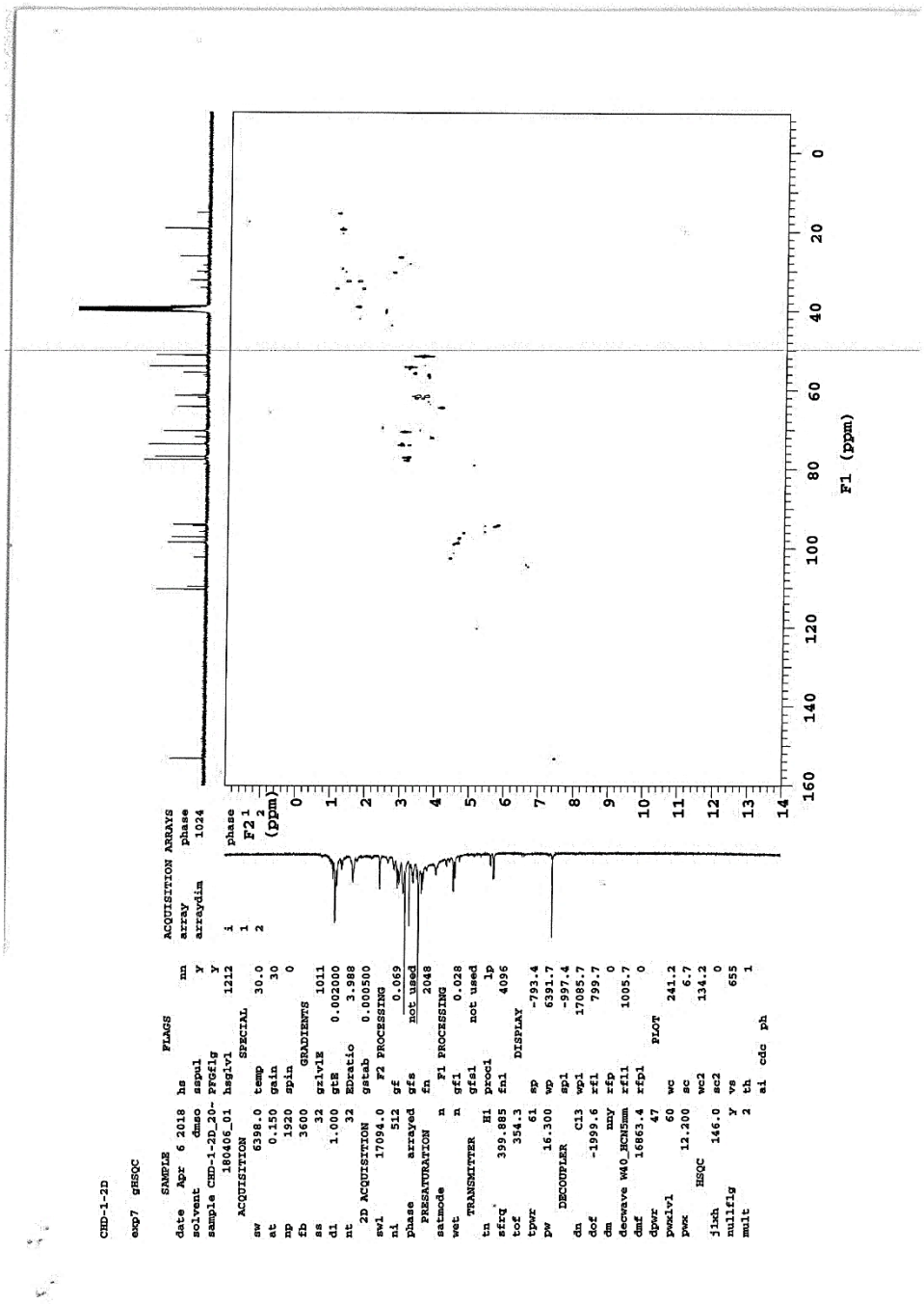


Figure 3.57 HSQC (400 *MHz*) spectrum of compound **14**

3.1.2 *Cephalaria procera*

Isolation and purification process on *Cephalaria procera* Fisch. & Avé-Lall (Caprifoliacea) was concluded that 8 pure triterpene glycosides were obtained. These compounds are identified as Akebia saponin D (**15**), *α*-Hederin (**16=2**), Collinsonidin (**17**), 3-*O*-*α*-L-rhamnopyranosyl-(1→2)-*α*-L-arabinopyranosyl hederagenin 28-*O*-*β*-D-glucopyranosyl ester (**18=3**), 3-*O*-*β*-D-galactopyranosyl-(1→3)-*α*-L-arabinopyranosyl hederagenin 28-*O*-*β*-D-glucopyranosyl-(1→6)-*β*-D-glucopyranosyl ester (**19**), Macranthoside A (**20=5**), Akebia saponin F (**21**) and Macranthoidin A (**22=12**).

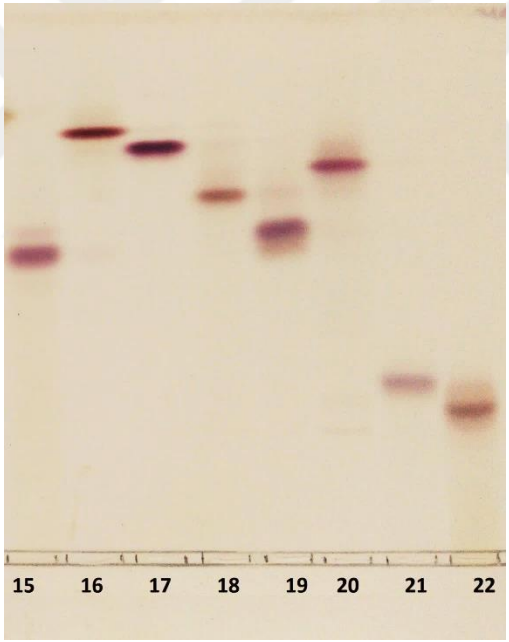


Figure 3.58 TLC spots of isolated pure compounds from *C. procera*

Compound 15 (**Akebia Saponin D**): A white, amorphous powder (120.0 mg), bisdesmosidic triterpenoid saponin, ^{1}H-NMR (DMSO-d_6, 400 *MHz*) and ^{13}C-NMR (DMSO-d_6, 150 *MHz*), see Figure 3.63 and 3.64 (Mukhamedziev et al., 1971).

According to the report, compound **15** (**Akebia Saponin D**) had the neuroprotective effect to object A$\beta_{25\text{-}35}$-induced cytotoxicity in PC 12 cells (Zhou et al., 2009). Hepatoprotection effect of Akebia Saponin D was tested by MTT and LDH assay in BRL cells (Gong et al., 2014). Besides, antiosteoporosis and cardioprotection were reported in various reports (Shen et al., 2016). The molecular structure of this compound was presented as in Figure 3.59.

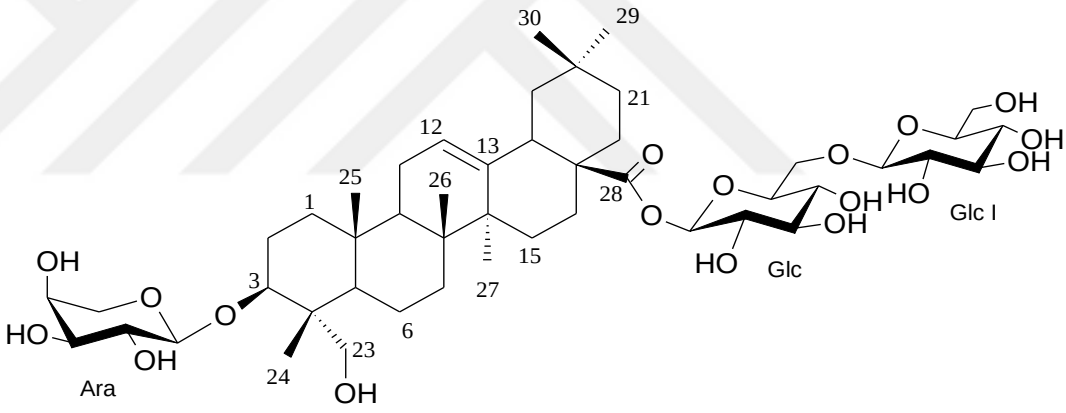


Figure 3.59 Structure of compound **15**

Compound 16 (***α*-Hederin = Comp. 2**): A white, amorphous powder (100.0 mg); ^{1}H-NMR (DMSO-d_6, 400 *MHz*) and ^{13}C-NMR (DMSO-d_6, 100 *MHz*), see Figure 3.65 and 3.66 (Aliev and Movsumov, 1976). The molecular structure of this compound was presented as in Figure 3.3.

Compound 17 (**Collinsonidin**): A white, amorphous powder (60.0 mg) monodesmosidic triterpenoid saponin; ^{1}H-NMR (DMSO-d_6, 400 *MHz*) and ^{13}C-NMR (DMSO-d_6, 100 *MHz*), see Table 3.2 and Figure 3.67 and 3.68 (Joshi et al., 1992). This compound was identified in Caprifoliceae family for the first time.

According to the report, compound **17** (**Collinsonidin**) exhibited potent cytotoxicities against all of the tested cell lines: A549, SK-OV-3, SK-MEL-2, XF498 and HCT15 (IC_{50} 5.1—7.4 μg/mL). Cytotoxicity of compound **17** was tested by SRB assay and inhibitory effect on the formation NO in LPS-activated macrophage 264.7 cells performed by using a nitrite assay (Jung et al., 2004). Another study showed that Compound **17** displayed significant inhibitory effects on Aβ42 induced fibrillogenesis (Chowdhury et al., 2017). Cytotoxic activity of Compound **17** against KB cells was reported in another study (IC_{50}(μM): 12.8) and quantitative structure-activity relationship (QSAR) study on the cytotoxic activity was performed (Luo et al., 2011). By the caspase 3/7 assay apoptotic activity of Compound **17** was reported that this compound has cytotoxic and apoptosis-inducing properties (Pawar et al., 2006). The molecular structure of this compound was presented as in Figure 3.60.

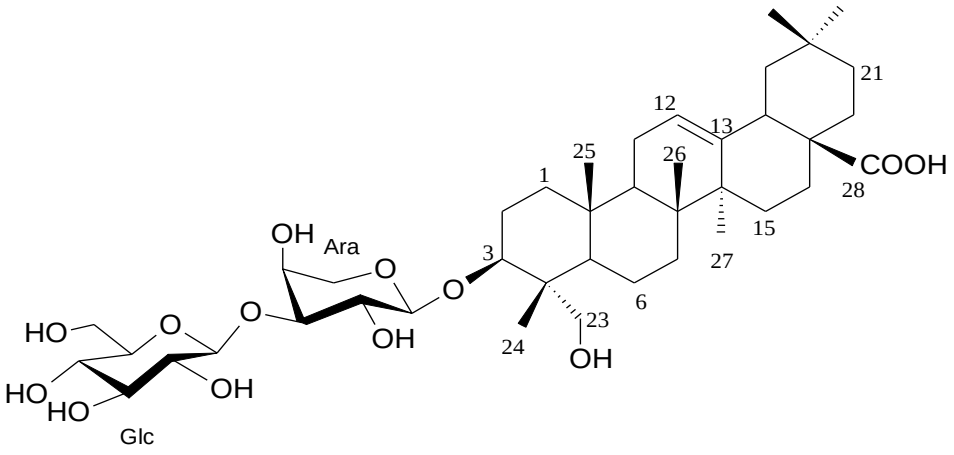


Figure 3.60 Structure of compound **17**

Table 3.2 ^{1}H and ^{13}C-NMR data for compound **17** [a-d]

Position	^{13}C-NMR	^{1}H-NMR	Position	^{13}C-NMR	^{1}H-NMR
				Ara	
1	38.4	0.82, 1.46, m	1	102.9	4.43, d (4.8)
2	25.5	1.58, 1.69, m	2	71.7	3.60, m
3	80.4	3.45, m	3	79.2	3.58, m
4	42.8	-	4	66.8	3.65, s
5	46.6	1.14, d (12.0)	5	63.6	3.29, m, 3.61, d (8.0)
6	17.7	1.18, 1.40, m			
7	32.6	1.41, 1.55, m		**Glc**	
8	38.9	-	1	104.2	4.32, d (7.6)
9	47.5	1.45, m	2	74.9	2.95, m
10	36.4	-	3	76.8	3.11, m
11	23.4	*nd*, 1.78, m	4	70.3	3.07, m
12	122.0	5.14, br s	5	77.2	3.07, m
13	144.3	-	6	61.4	3.45, 3.61, m
14	41.8	-			
15	27.7	0.97, 1.67, m			
16	23.1	1.44, 1.89, m			
17	45.9	-			
18	41.3	2.72, m			
19	46.2	1.02, 1.58, m			
20	30.8	-			
21	33.8	1.11, 1.26, m			
22	32.4	1.14, 1.42, m			
23	63.3	3.07, 3.37, d (7.2)			
24	13.1	0.56, s			
25	16.0	0.85, s			
26	17.3	0.69, s			
27	26.1	1.08, s			
28	179.0	-			
29	33.3	0.85, s			
30	23.8	0.85, s			

[a] ^{1}H and ^{13}C-NMR data (δ) were measured in DMSO-d_6 at 400 and 100 *MHz*, respectively.
[b] The assignments are based on COSY, HMQC and HMBC experiments.
[c] Coupling constants (*J*) in *Hz* are given in parentheses.
[d] nd: not detected

Compound 18 (=Comp. 3): (3-*O*-*α*-L-rhamnopyranosyl-(1→2)-*α*-L-arabinopyranosyl hederagenin 28-*O*-*β*-D-glucopyranosyl ester), A white, amorphous powder (90.0 mg); ^{1}H-NMR (DMSO-d_6, 400 *MHz*) and ^{13}C-NMR (DMSO-d_6, 100 *MHz*), see Figure 3.72 and 3.73 (Kawai et al., 1988). The molecular structure of this compound was presented as in Figure 3.4.

Compound 19 (3-*O*-*β*-D-galactopyranosyl(1→3)-*α*-L-arabinopyranosyl hederagenin 28-*O*-*β*-D-glucopyranosyl-(1→6)-*β*-D-glucopyranosyl ester): A white, amorphous powder (110.0 mg) bisdesmosidic triterpenoid saponin, ^{1}H-NMR (DMSO-d_6, 400 *MHz*) and ^{13}C-NMR (DMSO-d_6, 100 *MHz*), see Table 3.3 and Figure 3.74 and 3.75 (Quang et al., 2011). This compound was identified in Caprifoliceae family for the first time.

According to reports, compound **19** showed transactivational activity in HepG2 cells. The effects of compound **19** on the activation of PPARa were evaluated using a GAL-4-PPARa chimera assay (Quang et al., 2011). The molecular structure of this compound was presented as in Figure 3.61.

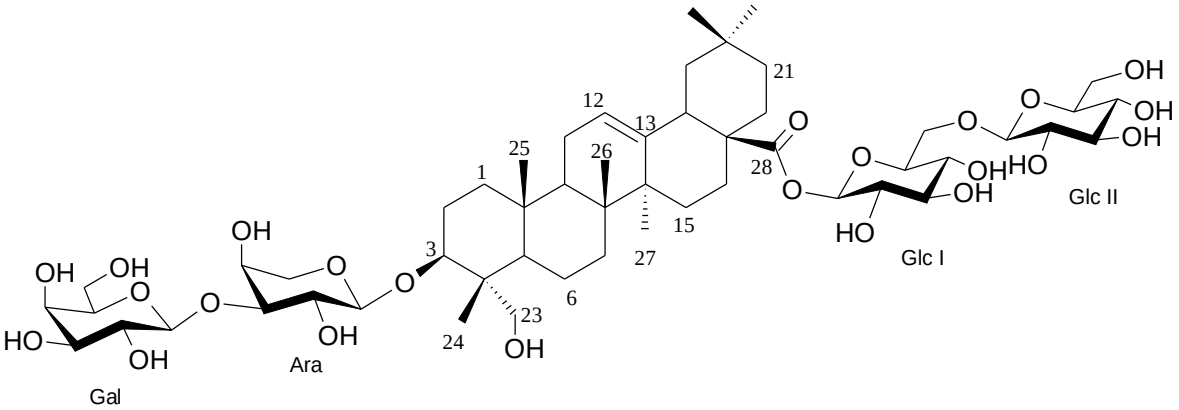


Figure 3.61 Structure of compound **19**

Table 3.3 ^{1}H and ^{13}C-NMR data for compound **19** [a-d]

Position	^{13}C-NMR	^{1}H-NMR	Position	^{13}C-NMR	^{1}H-NMR
				Ara at C-3	
1	38.4	0.83, 1.46, m	1	102.9	4.42
2	27.6	0.90, 1.71, m	2	74.8	3.46, m
3	80.5	3.46, m	3	79.3	3.61, m
4	42.8	-	4	66.8	3.65, m
5	46.6	1.09,m	5	63.7	3.31;3.69
6	18.2	*nd*, 1.06, m			
7	32.1	1.49, 1.55, m		**Gal**	
8	41.8	-	1	101.6	4.48, m
9	47.6	1.46, m	2	71.7	3.8, s
10	36.3	-	3	72.8	3.08, m
11	23.4	*nd*, 1.79, m	4	67.8	3.26, m
12	122.1	5.14, br s	5	76.6	3.32, m
13	143.9	-	6	61.1	3.43, 3.62, m
14	42.8	-			
15	29.2	*nd*, 1.20, m		**Glc I at C-28**	
16	22.9	1.57, 1.90, m	1	94.5	5.20, d (8.0)
17	46.4	-	2	74.9	2.98, m
18	41.1	2.72	3	76.7	3.12, m
19	45.9	1.06, 1.59, m	4	69.9	3.12
20	30.7	-	5	77.1	3.19, m
21	33.7	1.14, 1.32, m	6	65.3	3.32, 3.69, m
22	32.3	*nd*, 1.14, m			
23	63.2	3.42, m; 3.08, d (8.0)		**Glc II**	
24	13.4	0.58, s	1	104.1	4.33, d (7.2)
25	16.0	0.85, s	2	73.2	3.30, m
26	17.2	0.64, s	3	77.2	3.06, m
27	26.0	1.06	4	70.3	3.12, s
28	175.7	-	5	78.0	3.13, m
29	33.2	0.84, s	6	61.4	3.43, 3.62, m
30	23.8	0.85, s			

[a] ^{1}H and ^{13}C-NMR data (δ) were measured in DMSO-d_6 at 400 and 100 *MHz*, respectively.
[b] The assignments are based on HMQC and HMBC experiments.
[c] Coupling constants (*J*) in *Hz* are given in parentheses.
[d] nd: not detected

Compound 20 (**Macranthoside A = Comp. 5**): A white, amorphous powder (40.0 mg), ^{1}H-NMR (DMSO-d_6, 600 *MHz*) and ^{13}C-NMR (DMSO-d_6, 150 *MHz*), see Figure 3.78 and 3.79 (Saito et al., 1990). The molecular structure of this compound was presented as in Figure 3.6.

Compound 21 (**Akebia Saponin F**): A white, amorphous powder (10.0 mg), bisdesmosidic triterpenoid saponin, ^{1}H-NMR (DMSO-d_6, 400 *MHz*) and ^{13}C-NMR (DMSO-d_6, 100 *MHz*), see Table 3.4 and Figure 3.80 and 3.81 (Higuchi et al., 1972). This compound was identified in *Cephalaria* genus for the first time.

According to study, compound **21** showed no cytotoxicity and differantion-inducing activity at $5x10^{-5}$ M concentration against mouse myeloid leukemia cell line (M1) (Umehara et al., 1992). Another report on saponins of *Lonicera japonica* showed that hemolytic activity of compound **21** was relatively low (hemolysis: 78%) according to monodesmosidic compounds (Kawai et al., 1988). Molecular structure was presented as in Figure 3.62.

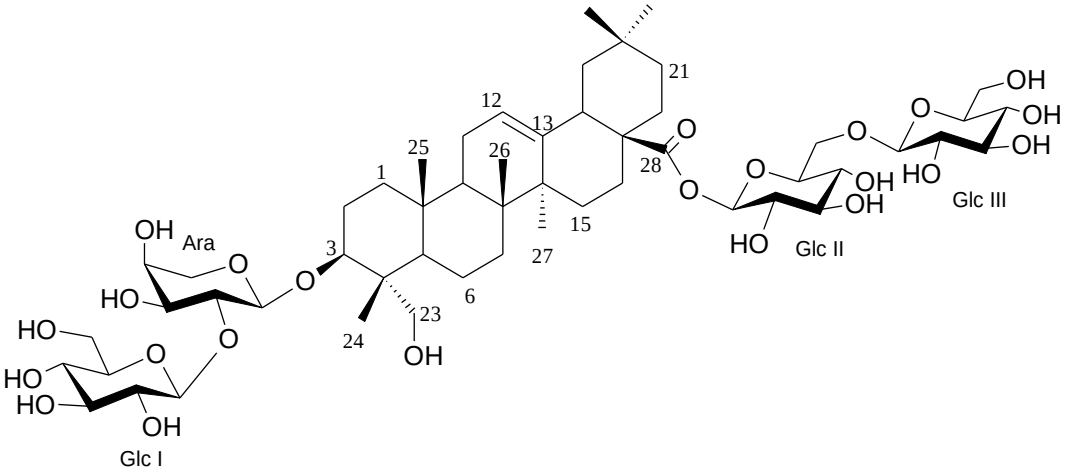


Figure 3.62 Structure of compound **21**

Table 3.4 ^{1}H and ^{13}C-NMR data for compound **21** [a-d]

Position	^{13}C-NMR	^{1}H-NMR	Position	^{13}C-NMR	^{1}H-NMR
				Ara at C-3	
1	38.5	*nd*, 0.82, m	1	102.9	4.43, d (3.2)
2	25.5	*nd*, 1.63, m	2	75.0	3.01, m
3	80.4	3.48, m	3	79.3	3.61, m
4	42.8	-	4	66.7	3.69, m
5	46.6	1.15, m	5	63.5	3.32, 3.65, m
6	17.6	*nd*, 1.06, m			
7	32.3	1.13, m		**Glc I**	
8	39.3	-	1	103.5	4.21, d (5.2)
9	47.6	1.48, m	2	71.1	3.05, m
10	36.4	-	3	76.8	3.19, m
11	23.0	1.57, 1.92, m	4	69.7	3.24, m
12	122.1	5.15, br s	5	76.9	3.16, m
13	143.7	-	6	61.4	3.45, 3.66, m
14	41.8	-			
15	32.1	*nd*, 1.44, m		**Glc II at C-28**	
16	23.4	*nd*, 1.79, m	1	94.5	5.20, d (8.0)
17	46.4	-	2	73.9	2.94, m
18	41.2	2.72, m	3	77.2	3.07, m
19	46.0	1.06, s	4	70.4	3.05, m
20	30.7	-	5	77.3	3.10, m
21	36.4	*nd*, 1.08, m	6	68.2	3.55, 3.92, d (8.0)
22	33.2	*nd*, 0.86, m			
23	66.7	*nd*, 3.69		**Glc III**	
24	13.2	0.53, s	1	104.2	4.31, d (8.0)
25	16.1	0.86, s	2	72.7	3.28, m
26	17.2	0.68, s	3	77.1	3.12, m
27	26.0	1.07, s	4	70.3	3.10, m
28	175.7	-	5	77.2	3.14, m
29	33.7	1.30, m	6	61.4	3.46, 3.66, m
30	23.9	0.86, s			

[a] ^{1}H and ^{13}C-NMR data (δ) were measured in DMSO-d_6 at 400 and 100 *MHz*, respectively.
[b] The assignments are based on HMQC and HMBC experiments.
[c] Coupling constants (*J*) in *Hz* are given in parentheses.
[d] nd: not detected

Compound 22 (**Macranthoidin A = Comp. 12**): A white, amorphous powder (25.0 mg), ^{1}H-NMR (DMSO-d_6, 600 *MHz*) and ^{13}C-NMR (DMSO-d_6, 150 *MHz*), see Figure 3.83 and 3.84 (Mao et al., 1993). The molecular structure of this compound was presented as in Figure 3.13.

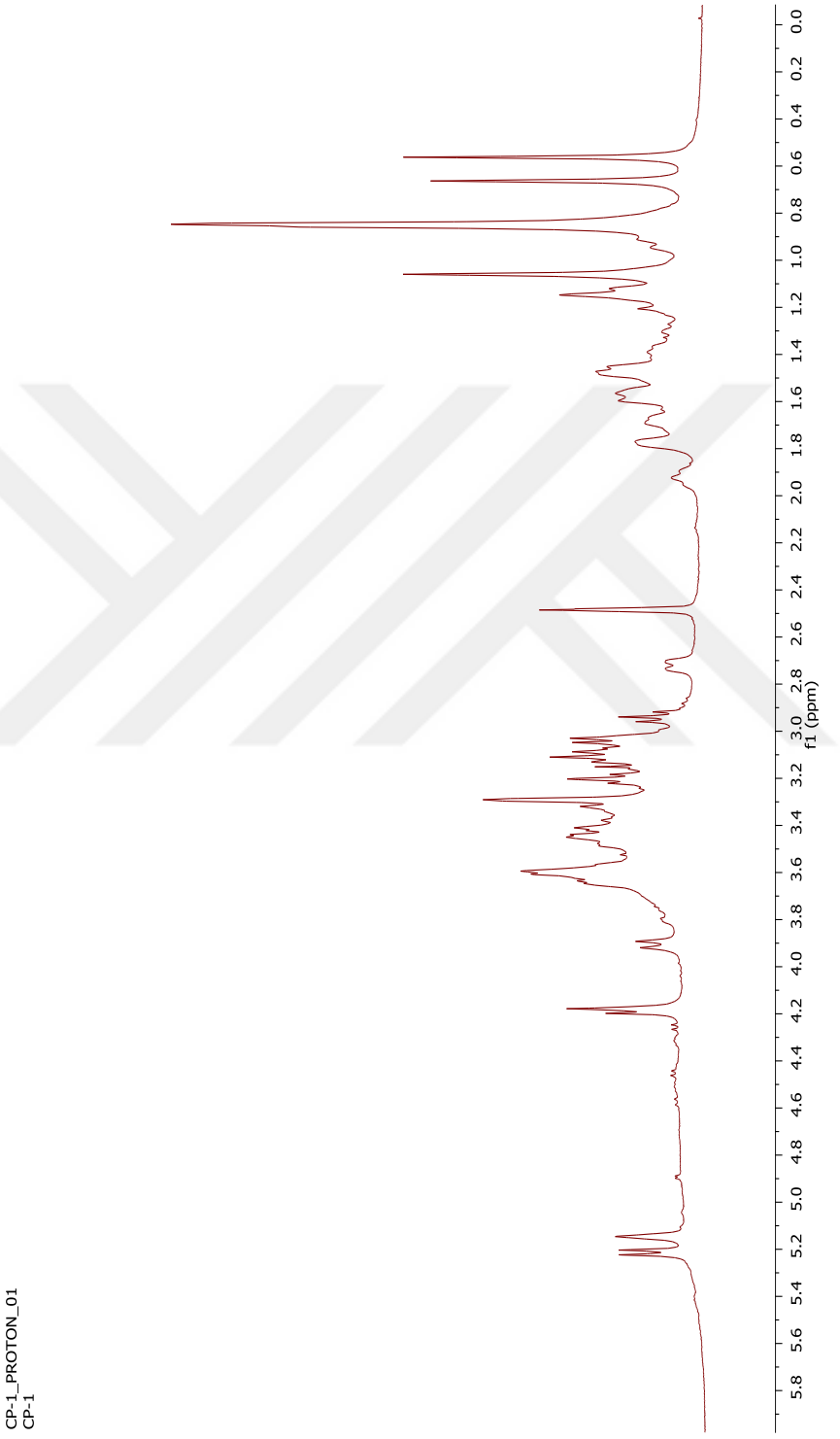


Figure 3.63 ^{1}H-NMR (400 *MHz*, DMSO-d_6) spectrum of compound **15**

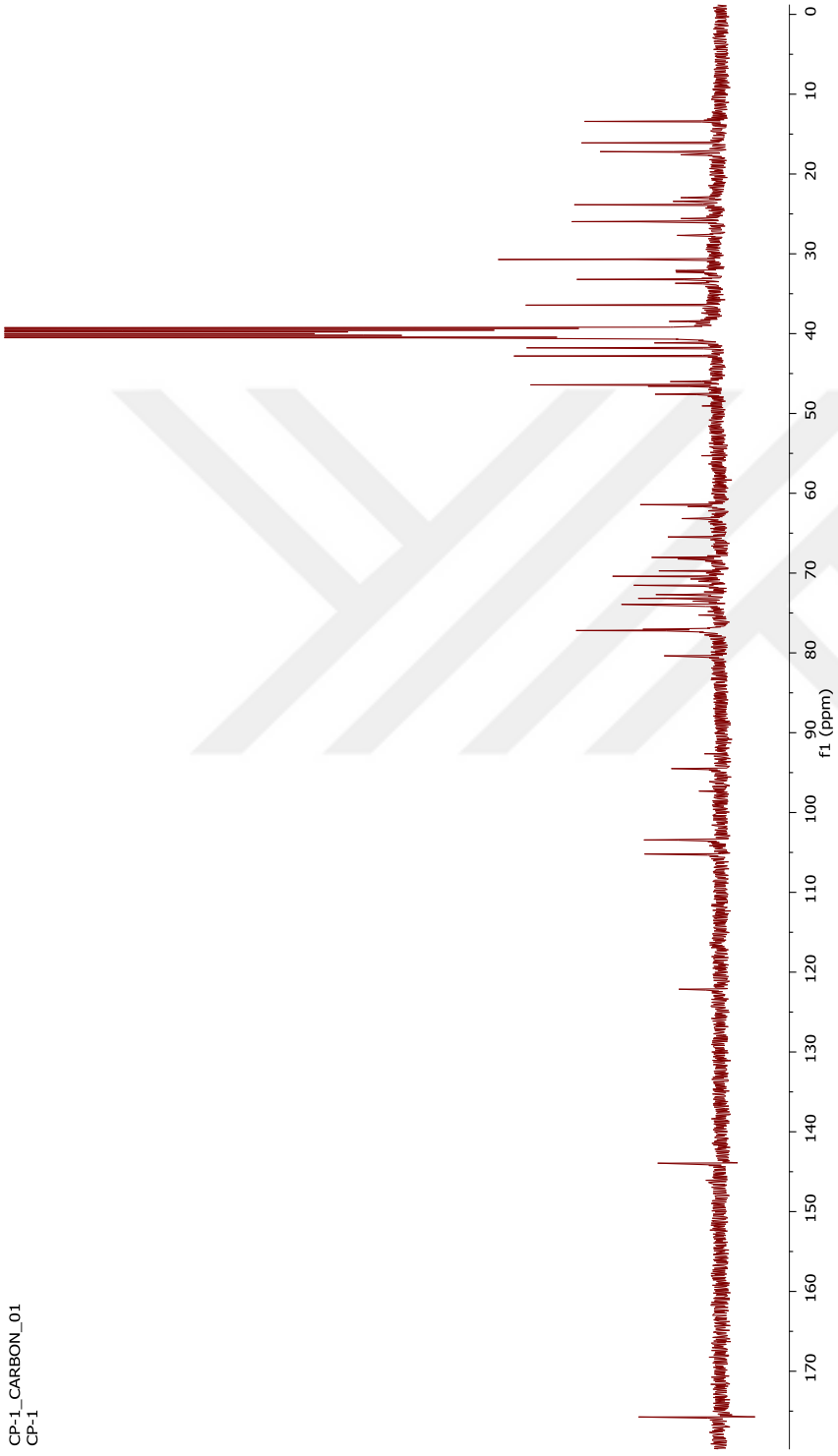


Figure 3.64 ^{13}C-NMR (150 *MHz*, DMSO-d_6) spectrum of compound **15**

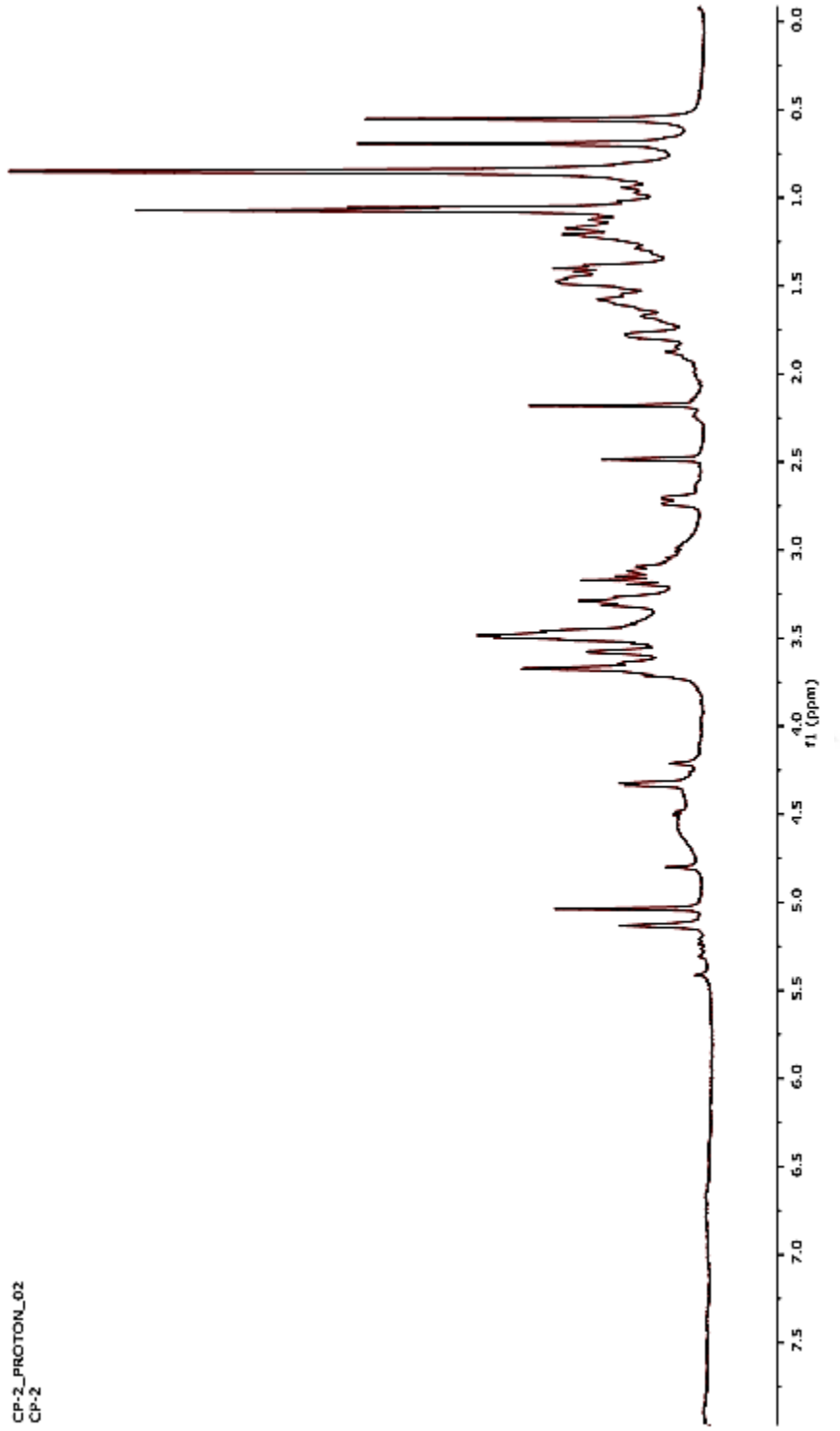


Figure 3.65 ^{1}H-NMR (400 *MHz*, DMSO-d_6) spectrum of compound **16**

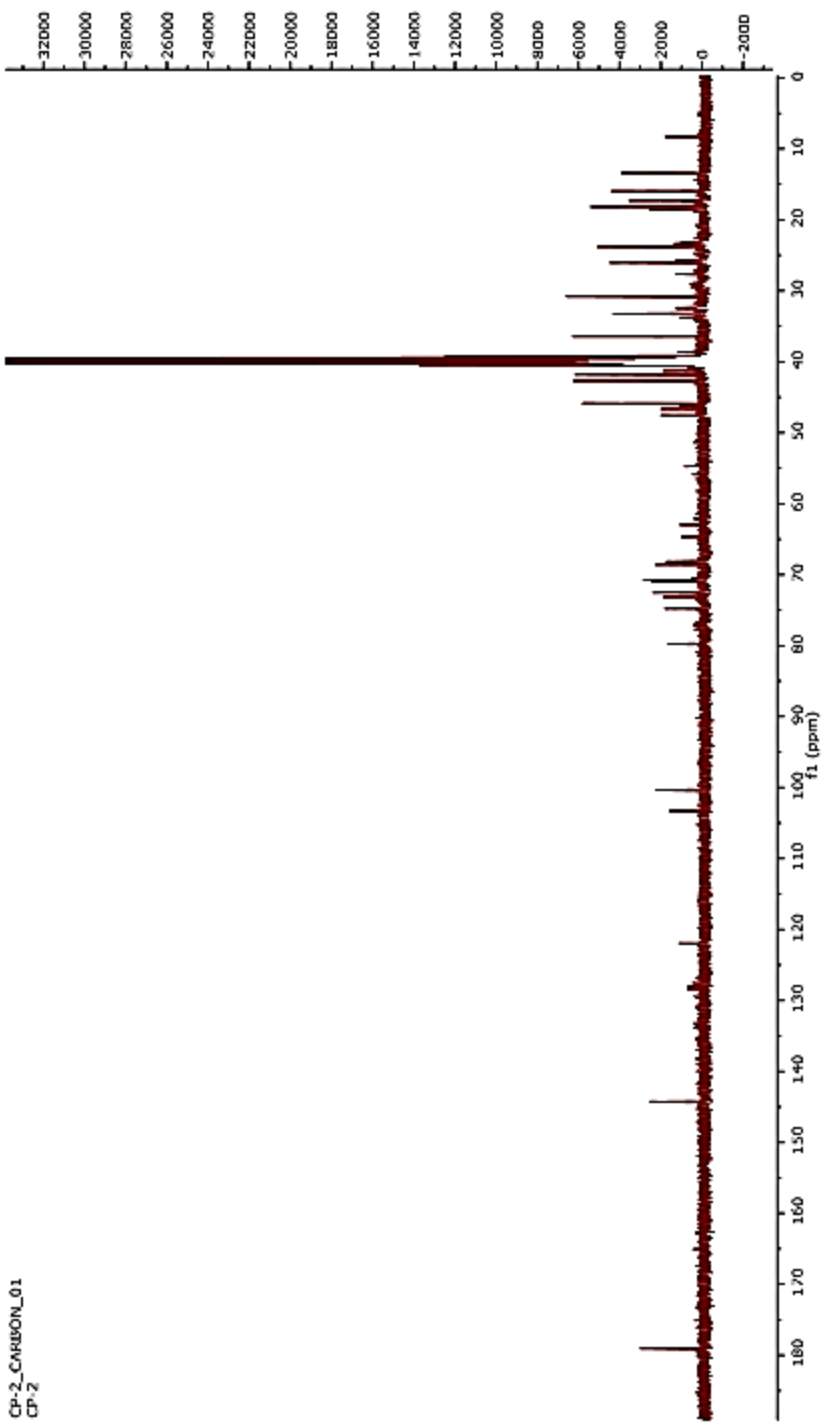


Figure 3.66 ^{13}C-NMR (100 *MHz*, DMSO-d_6) spectrum of compound **16**

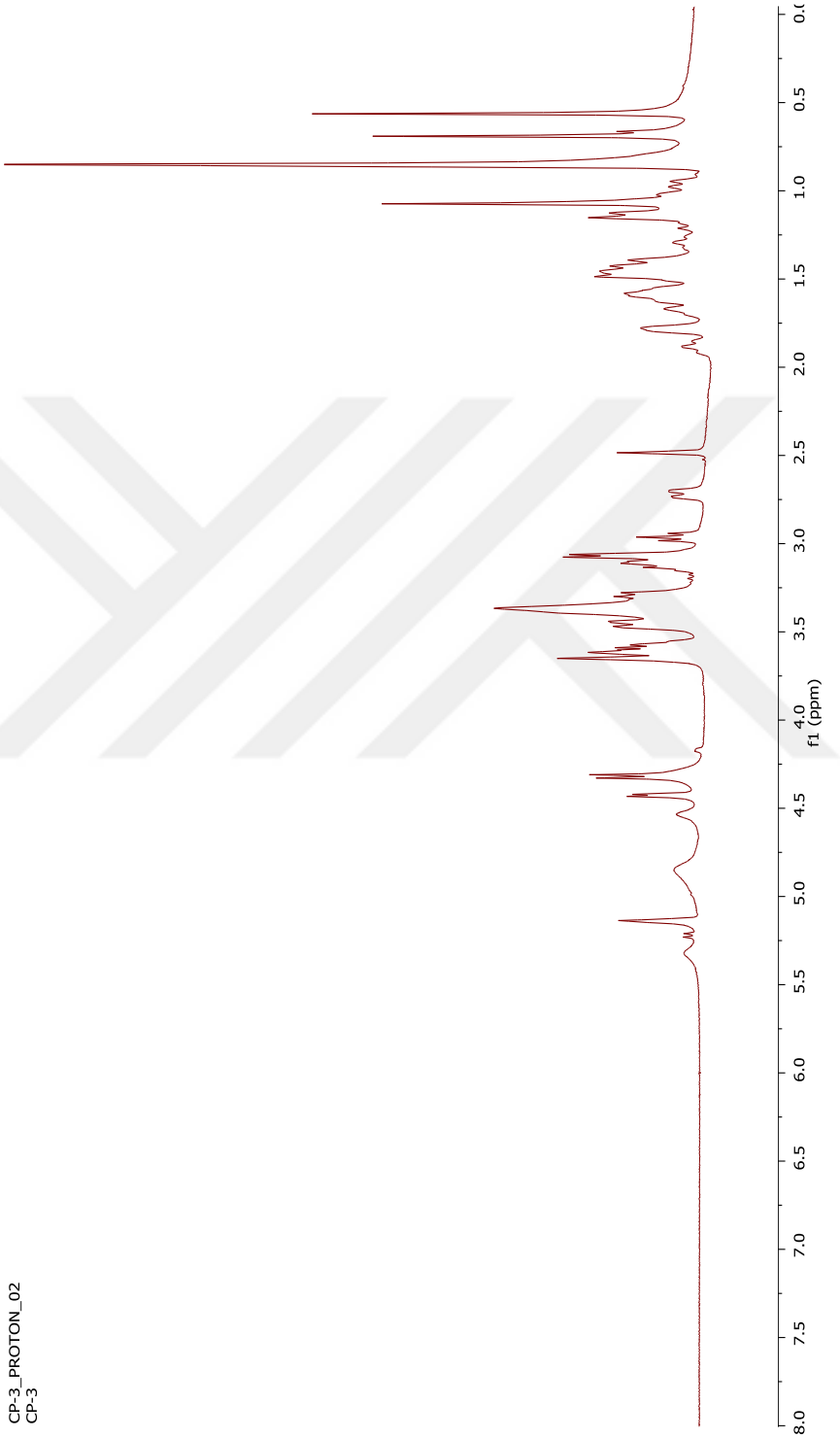


Figure 3.67 ^{1}H-NMR (400 *MHz*, DMSO-d_6) spectrum of compound **17**

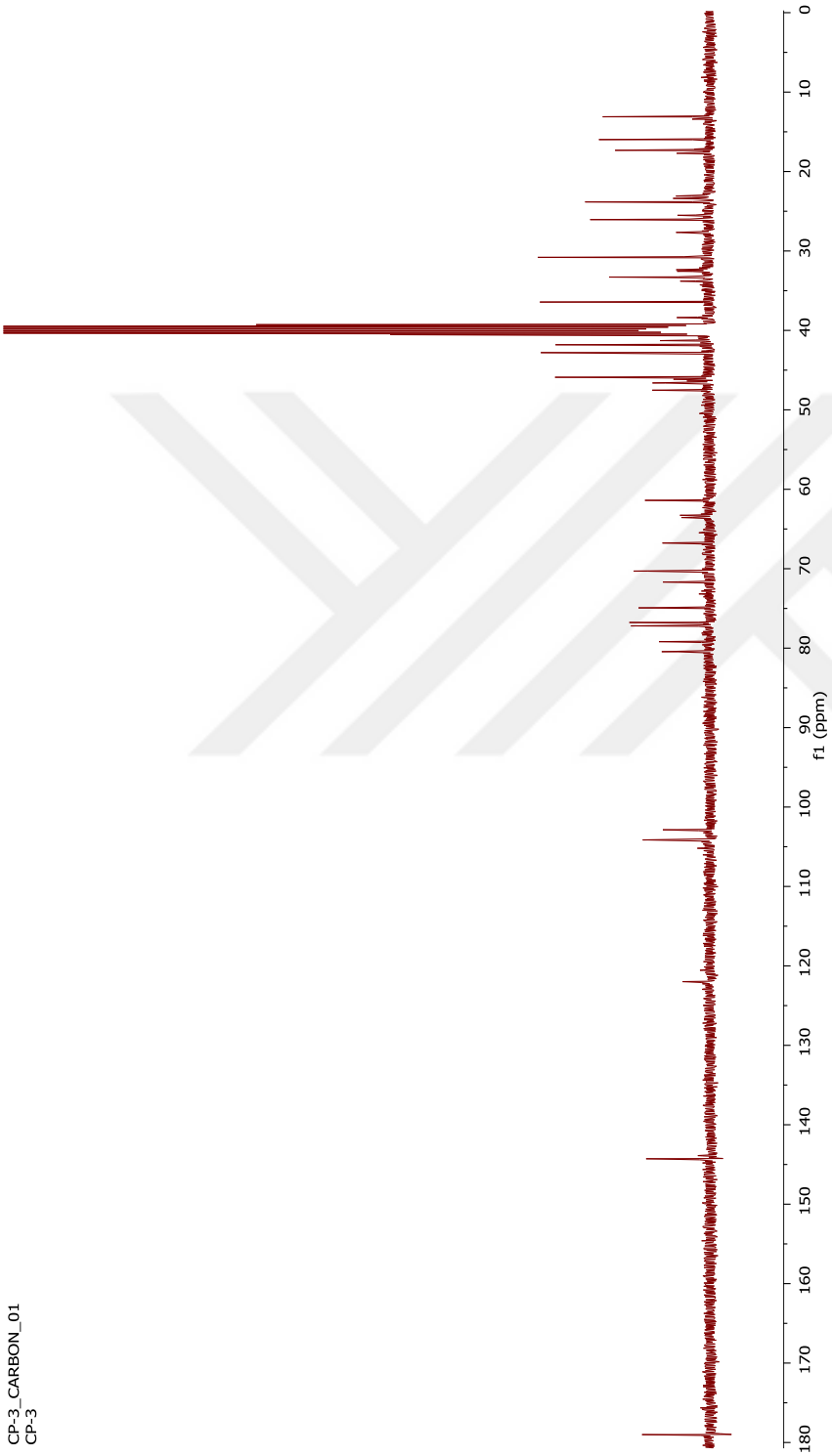


Figure 3.68 ^{13}C-NMR (100 *MHz*, DMSO-d_6) spectrum of compound **17**

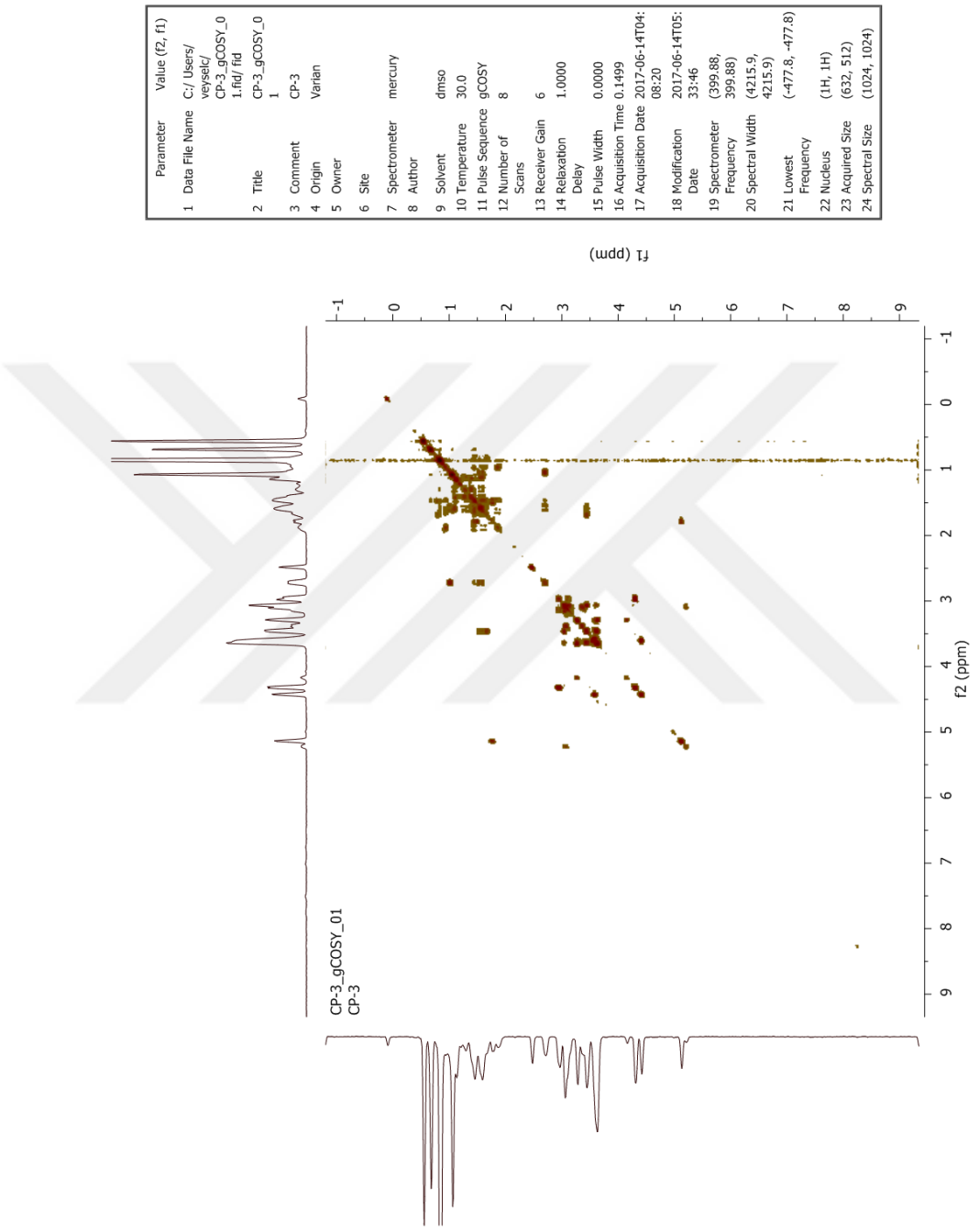


Figure 3.69 COSY (400 *MHz*) spectrum of compound **17**

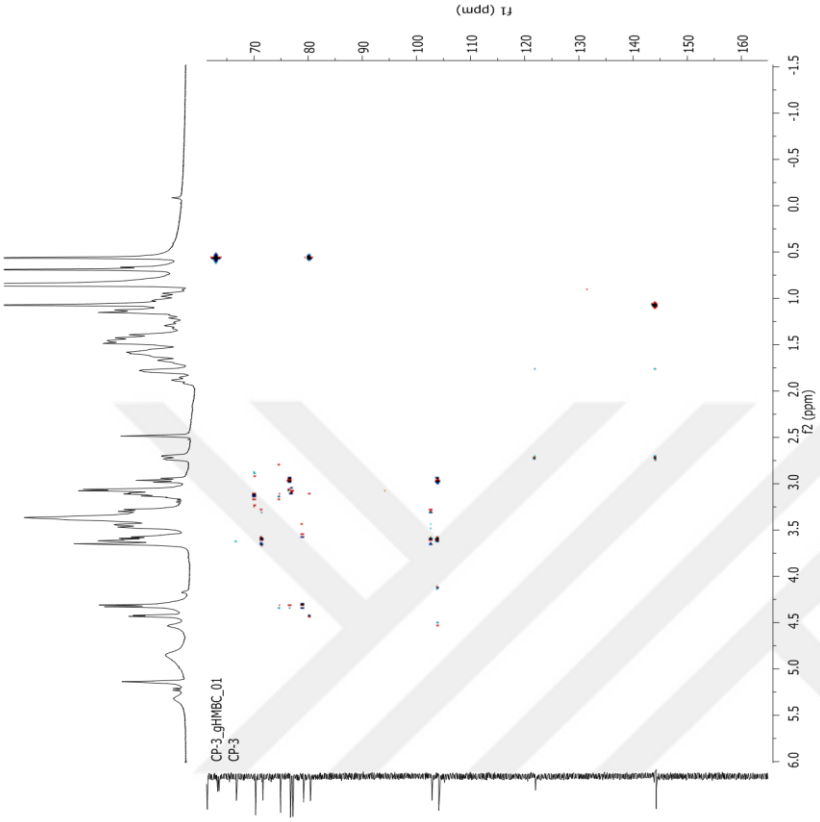


Figure 3.70 HMBC (400 *MHz*) spectrum of compound **17**

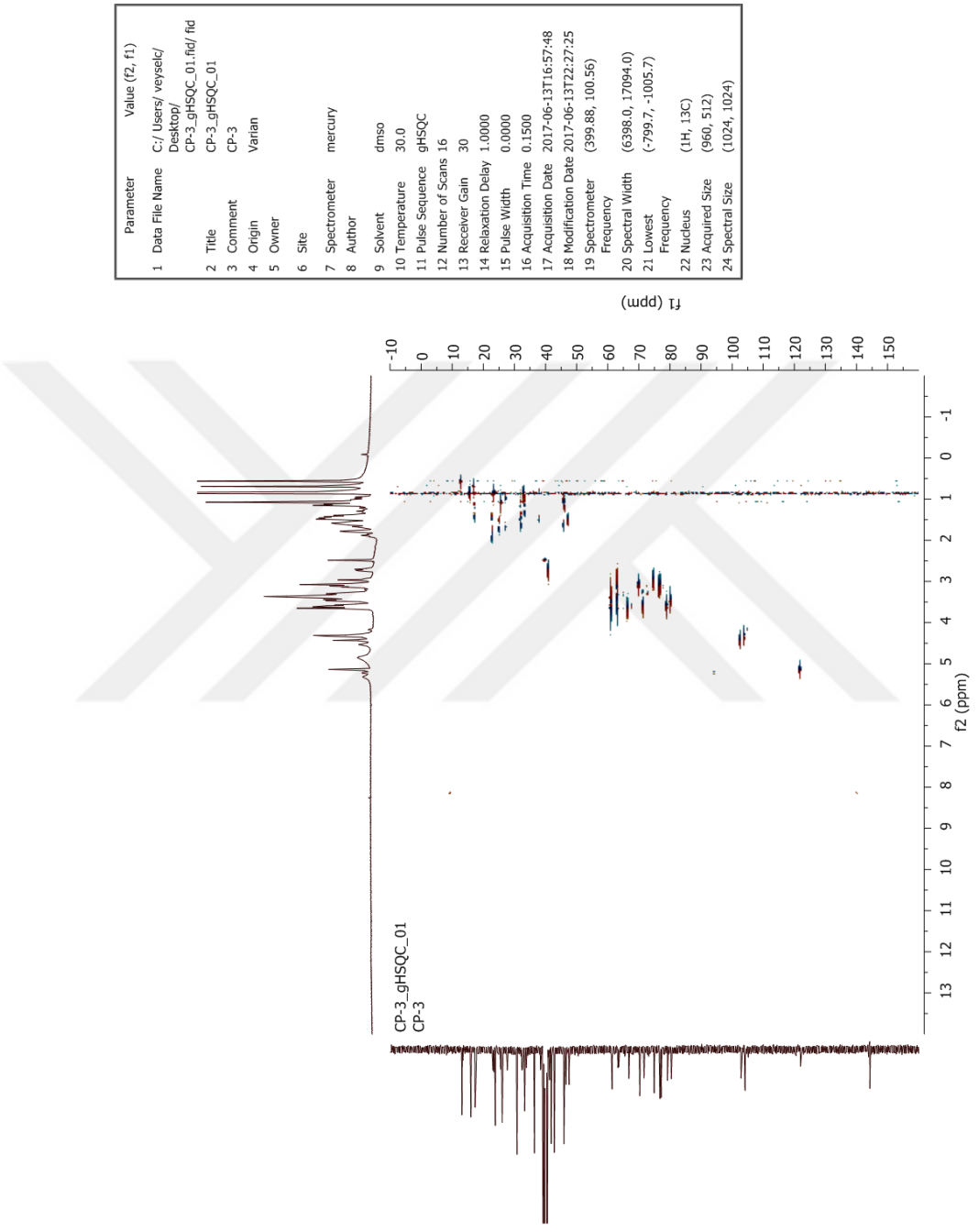


Figure 3.71 HSQC (400 *MHz*) spectrum of compound **17**

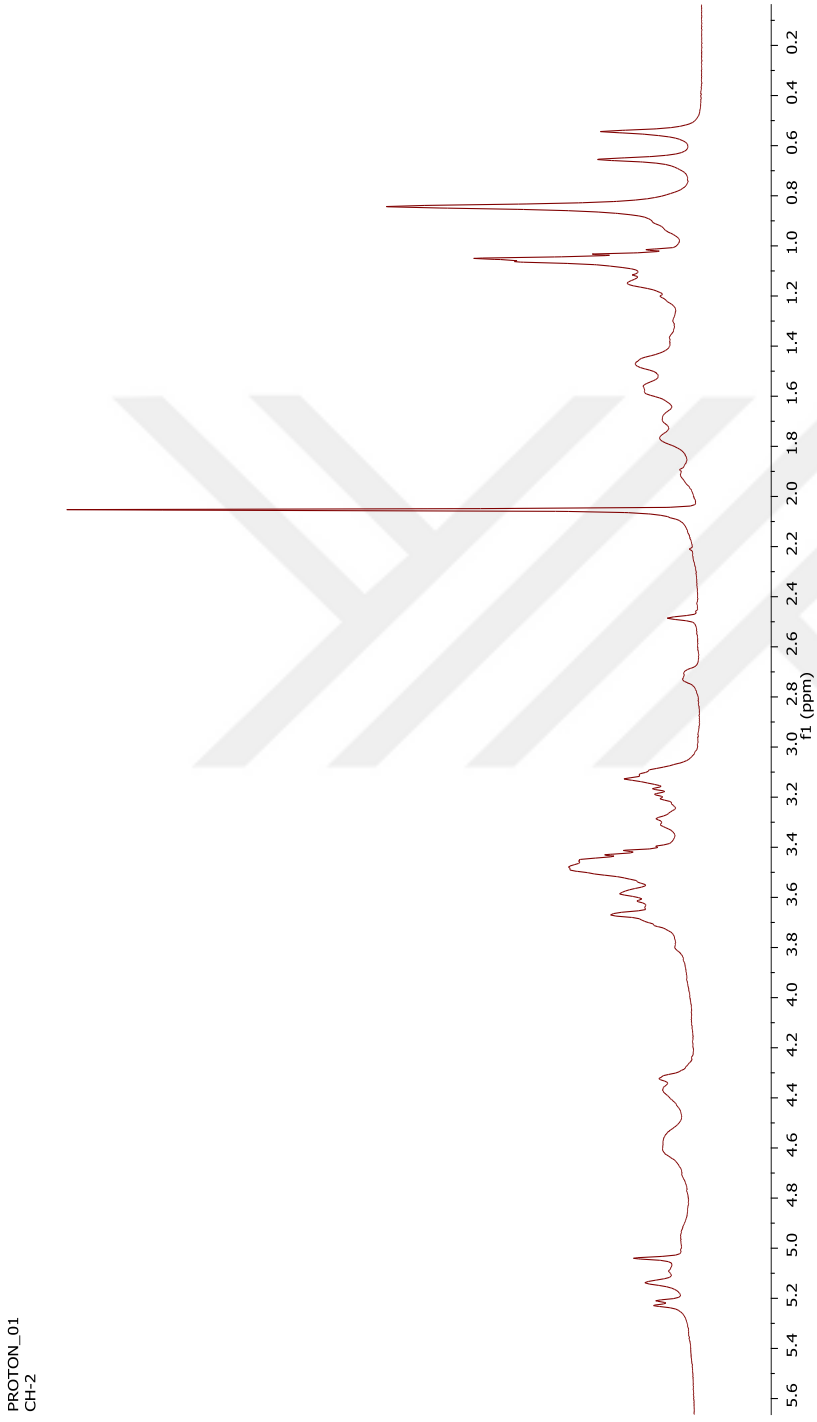


Figure 3.72 ^{1}H-NMR (400 *MHz*, DMSO-d_6) spectrum of compound **18**

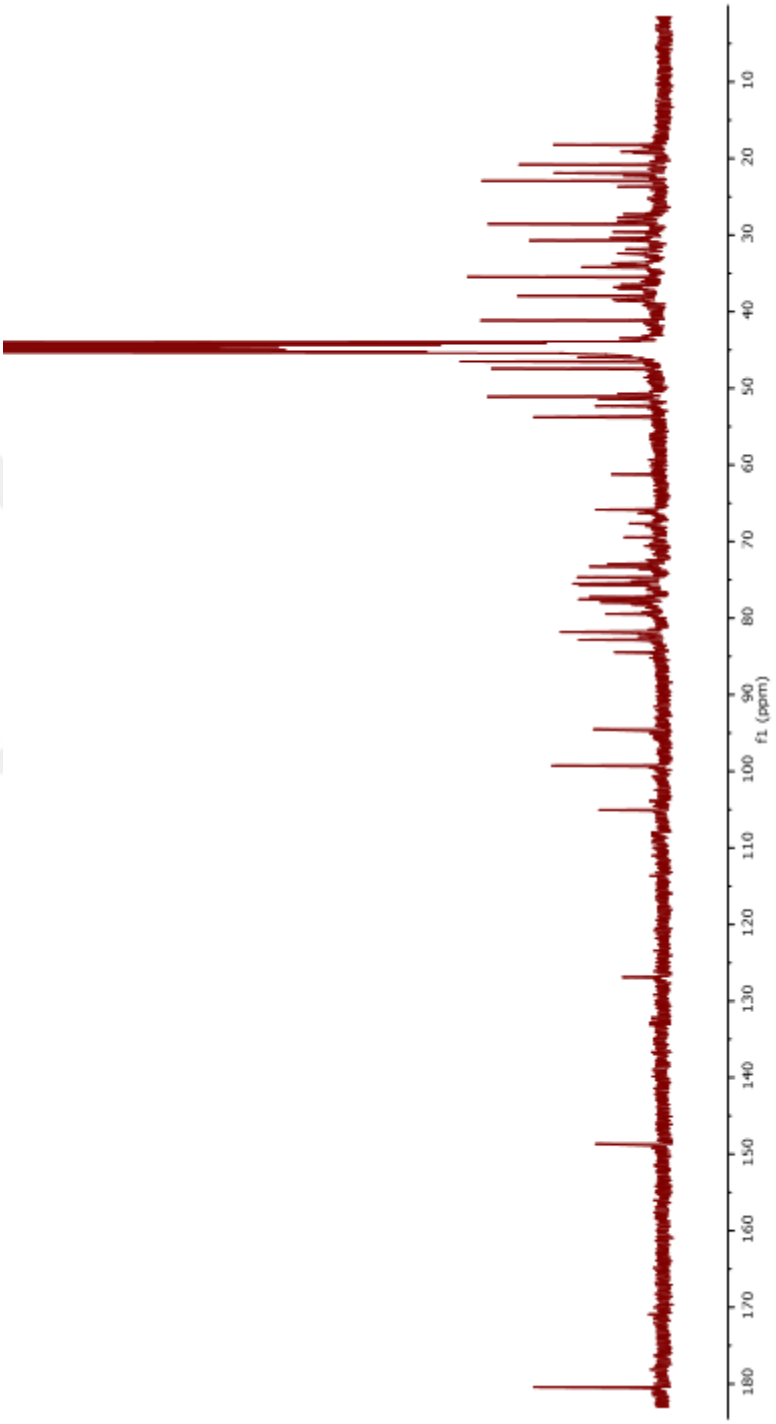


Figure 3.73 ^{13}C-NMR (100 *MHz*, DMSO-d_6) spectrum of compound **18**

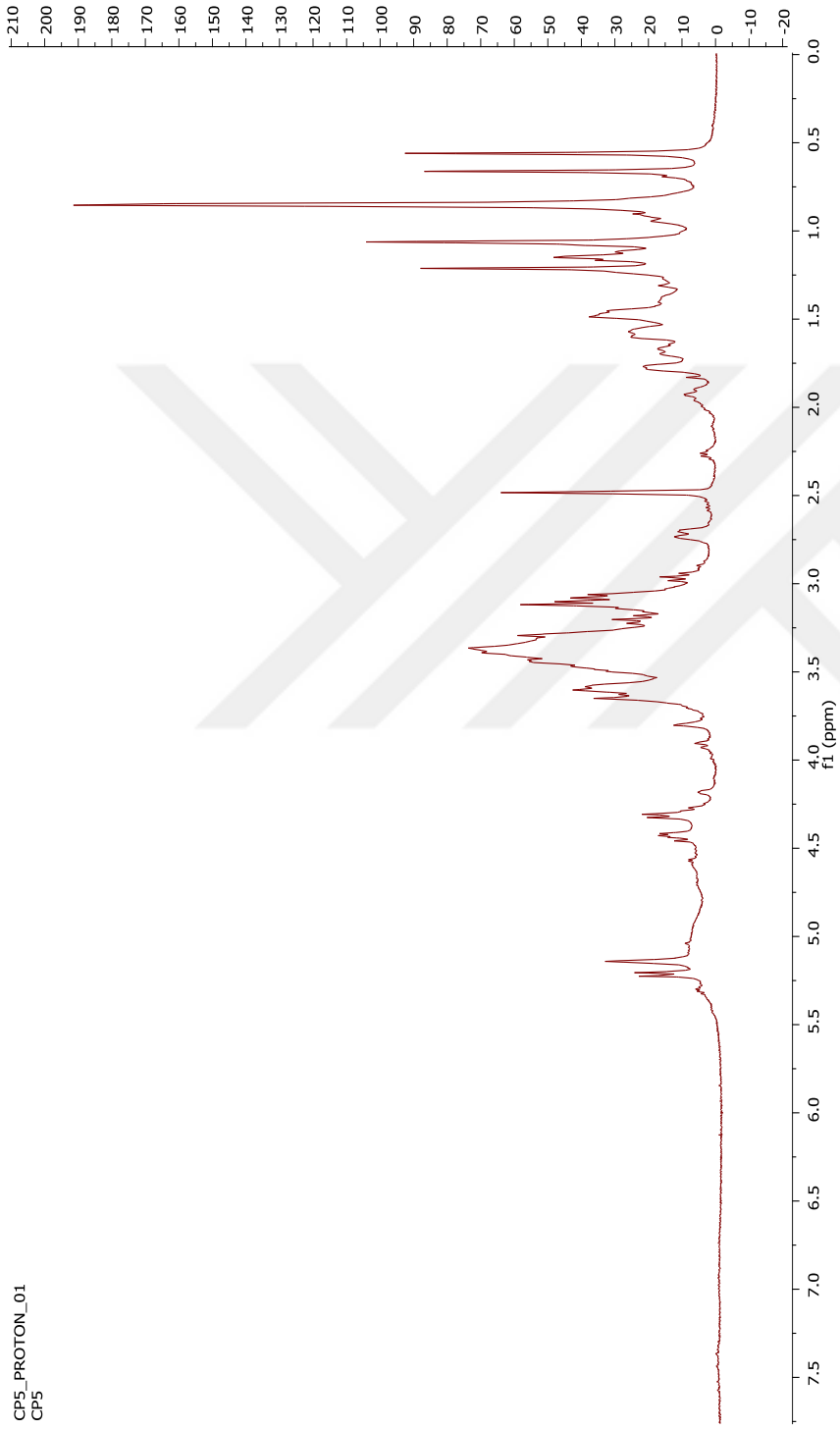


Figure 3.74 ^{1}H-NMR (400 *MHz*, DMSO-d_6) spectrum of compound **19**

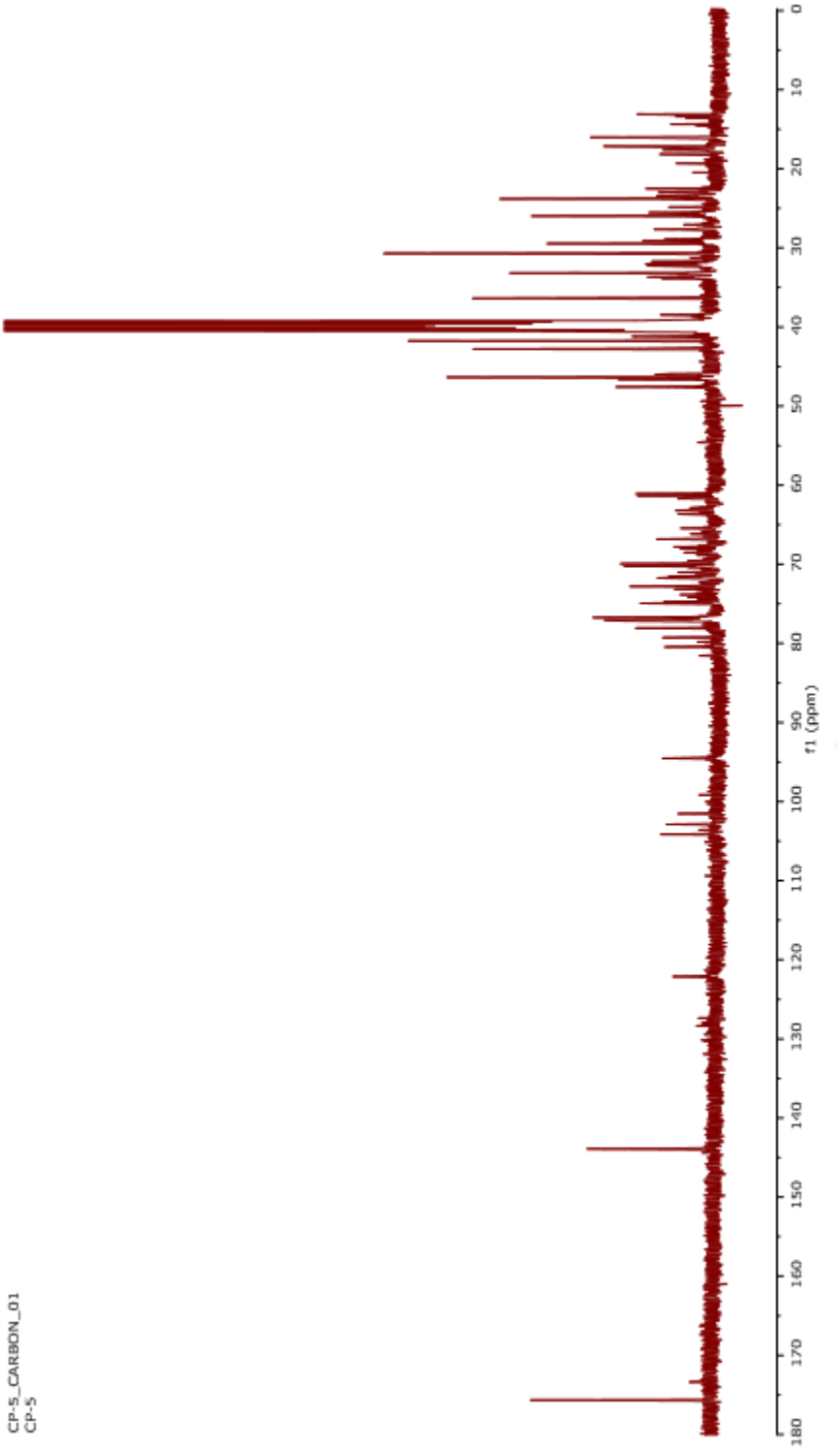


Figure 3.75 ^{13}C-NMR (100 *MHz*, DMSO-d_6) spectrum of compound **19**

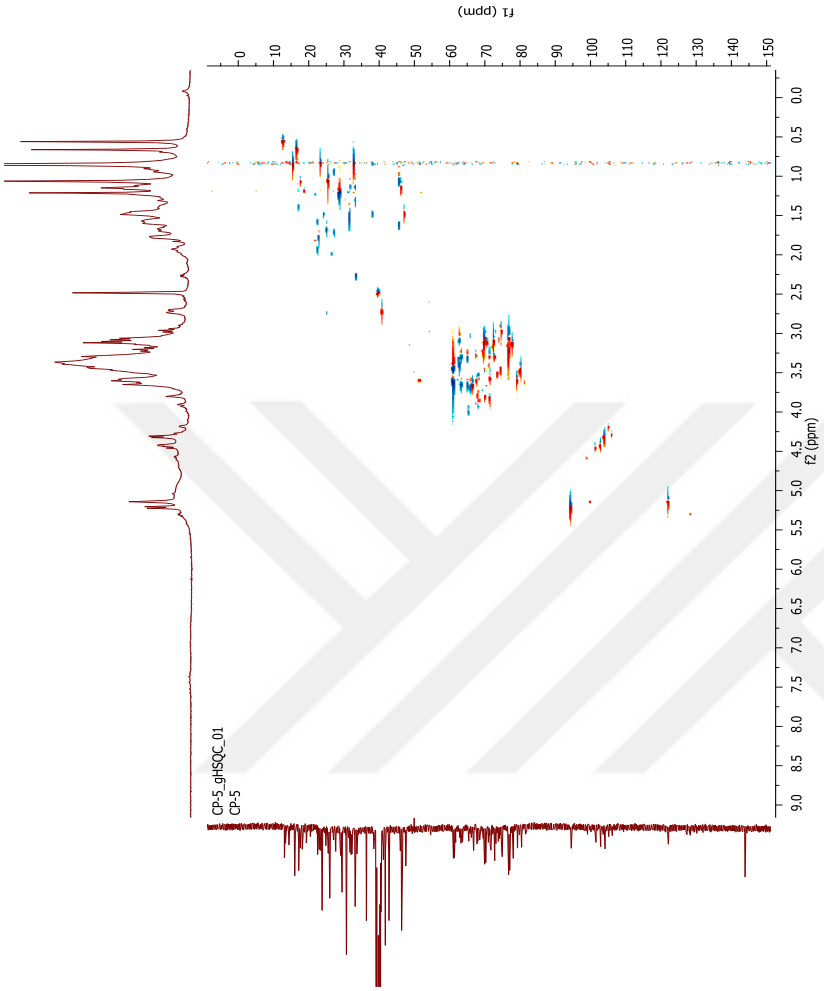


Figure 3.76 HSQC (400 *MHz*) spectrum of compound **19**

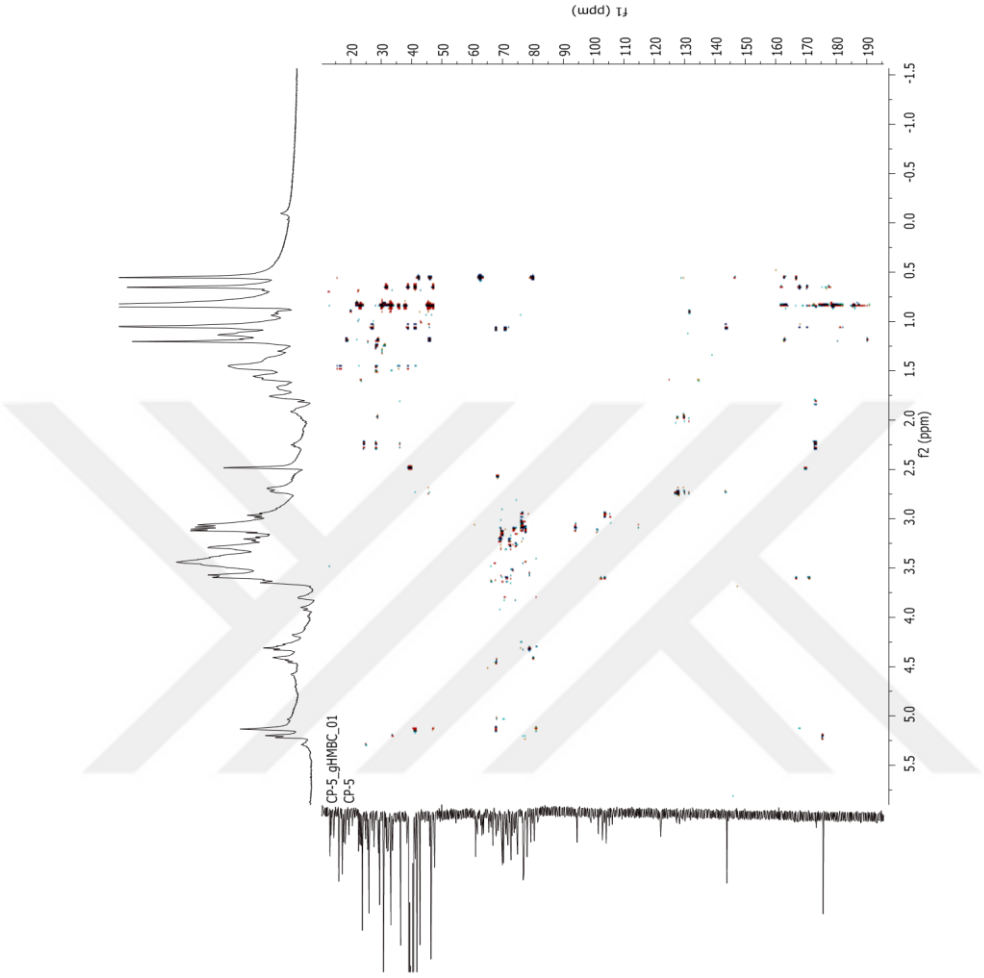


Figure 3.77 HMBC (400 *MHz*) spectrum of compound **19**

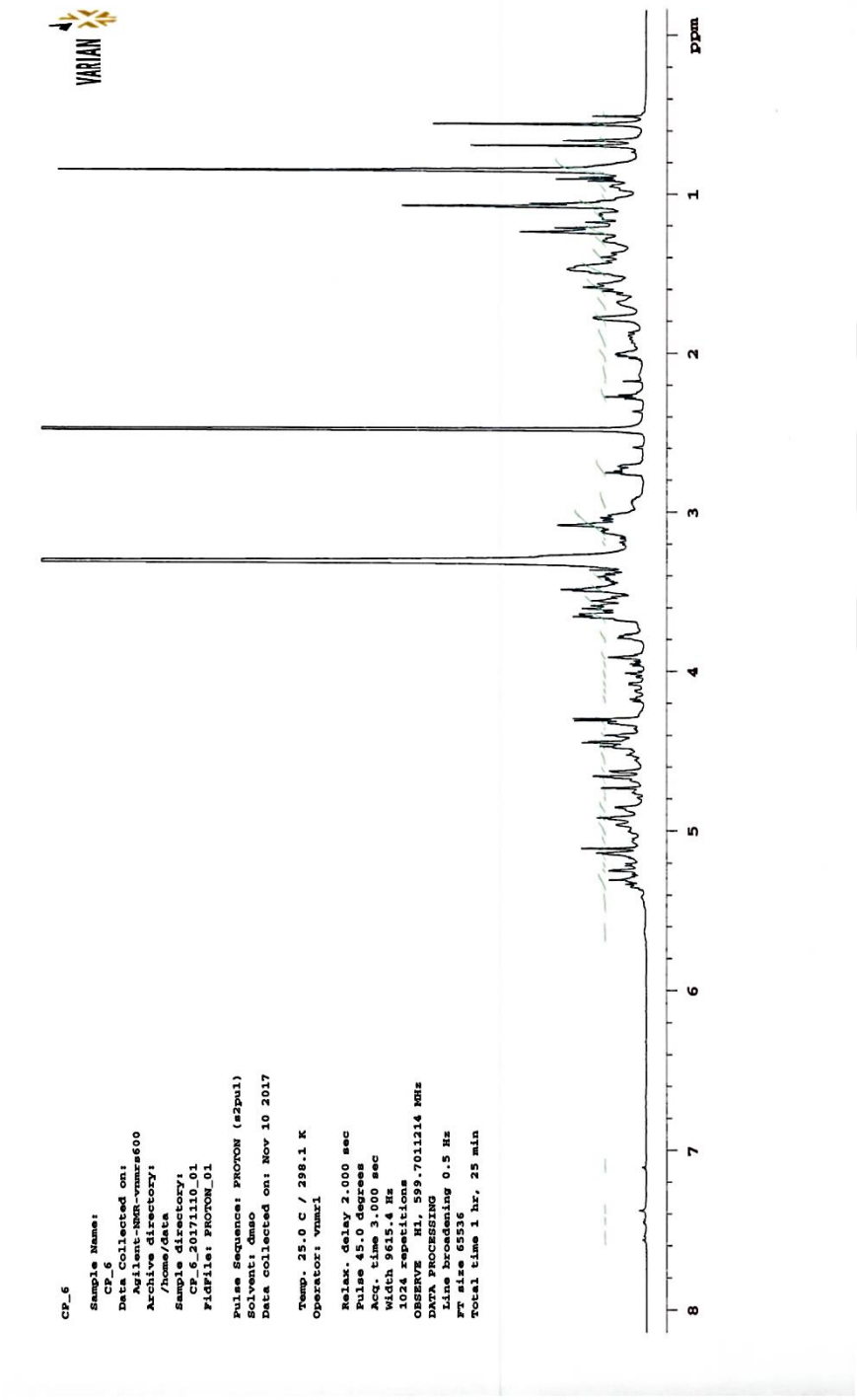


Figure 3.78 ^{1}H-NMR (600 *MHz*, DMSO-d_6) spectrum of compound **20**

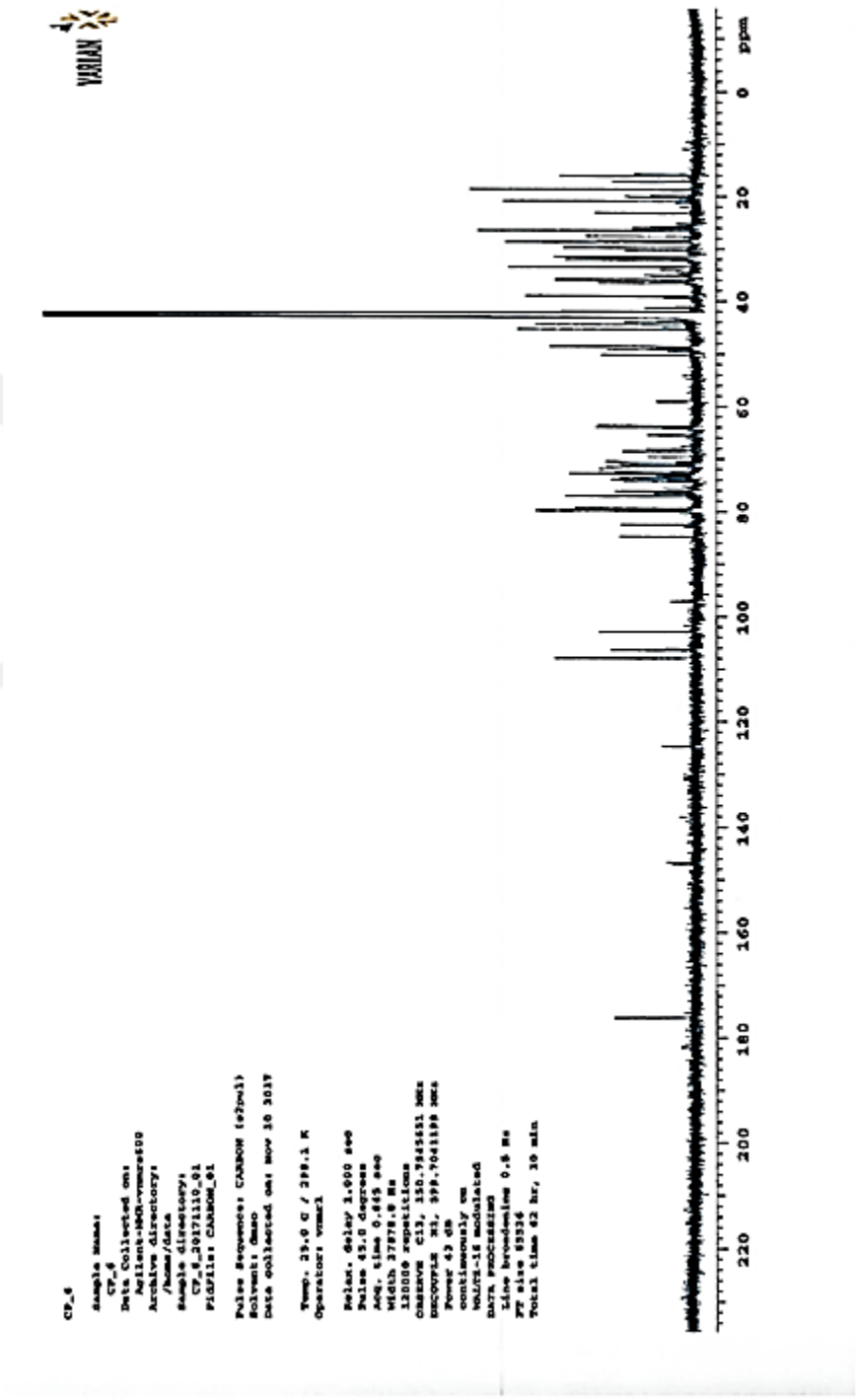


Figure 3.79 ^{3}C-NMR (150 *MHz*, DMSO-d_6) spectrum of compound **20**

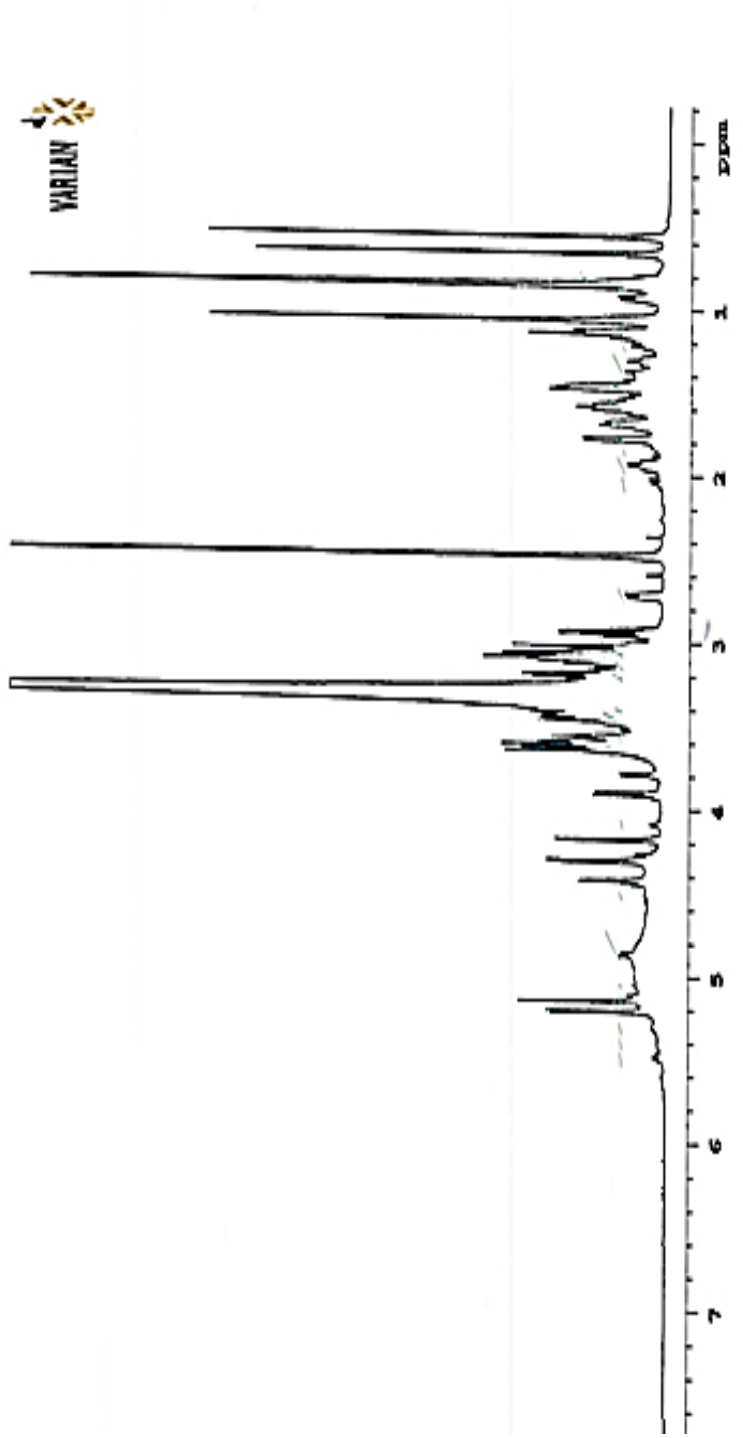


Figure 3.80 ^{1}H-NMR (400 *MHz*, DMSO-d_6) spectrum of compound **21**

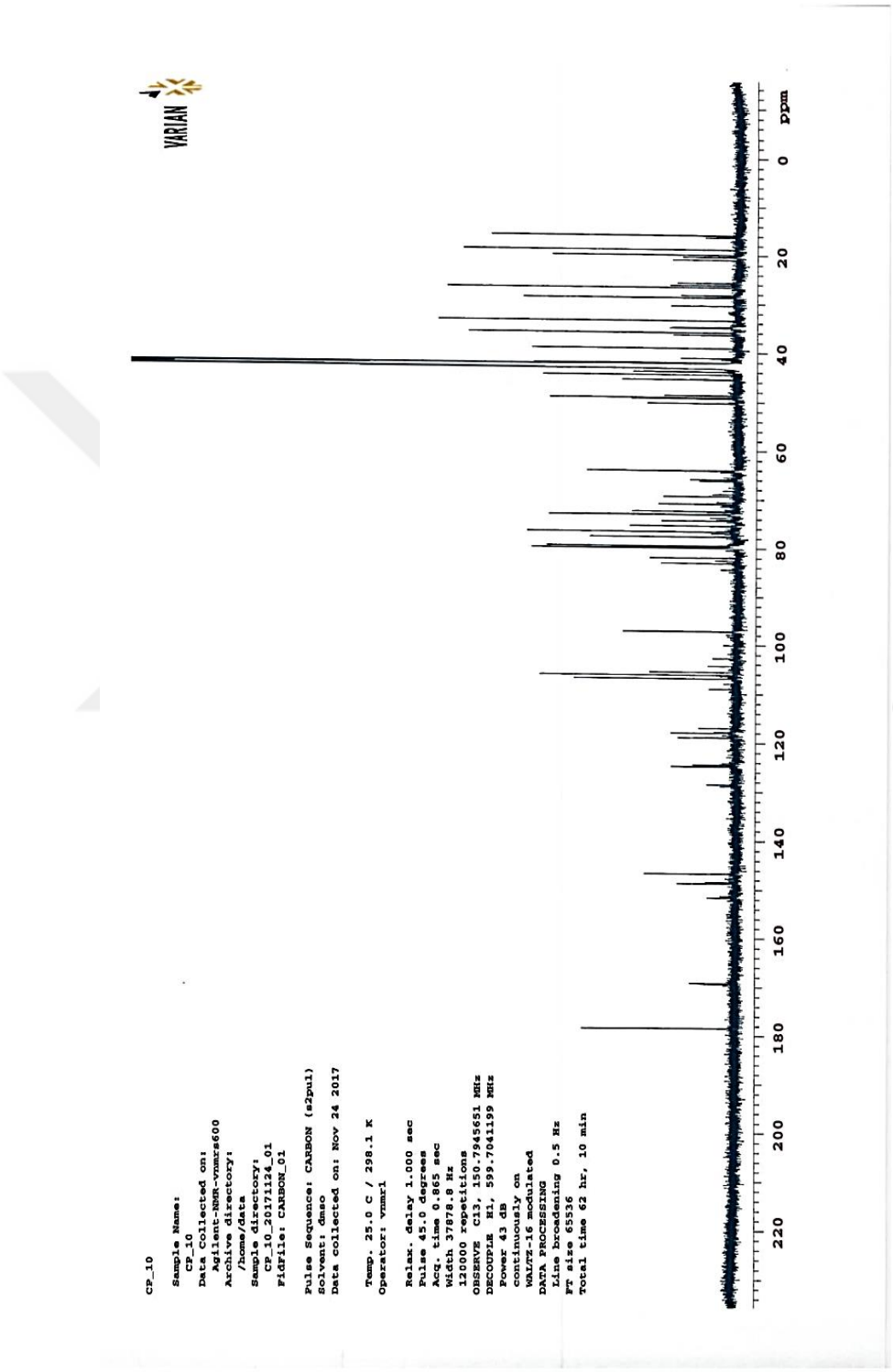


Figure 3.81 ^{13}C-NMR (100 *MHz*, DMSO-d_6) spectrum of compound **21**

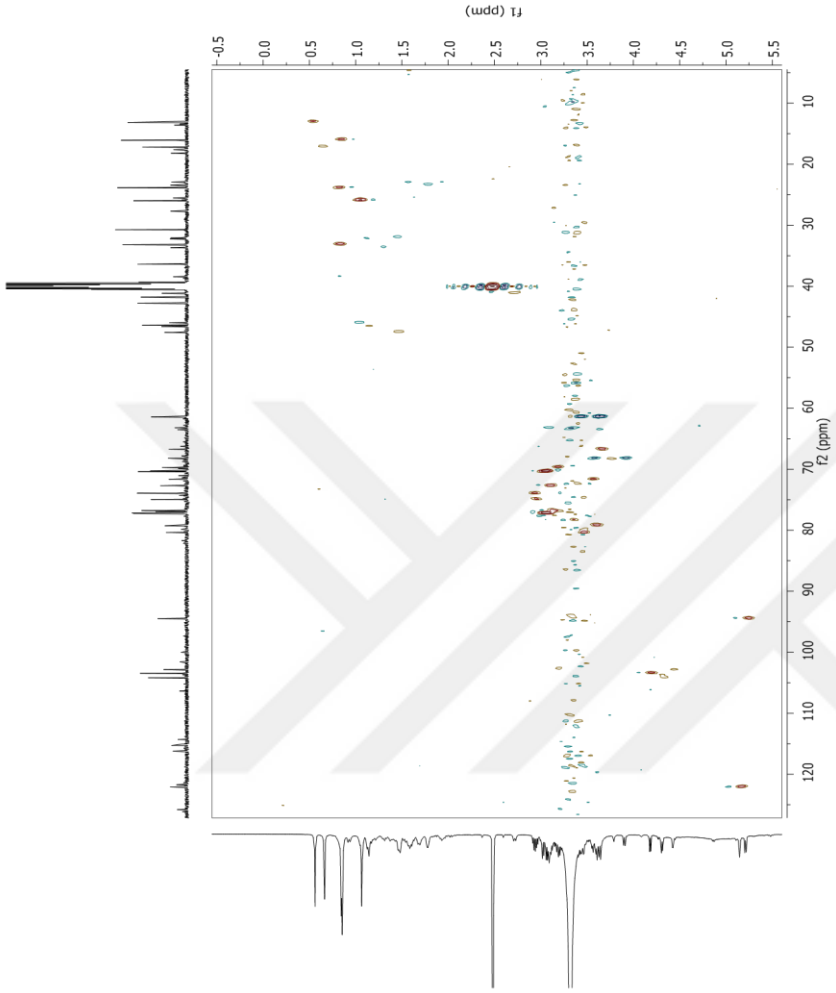


Figure 3.82 HSQC (400 *MHz*) spectrum of compound **21**

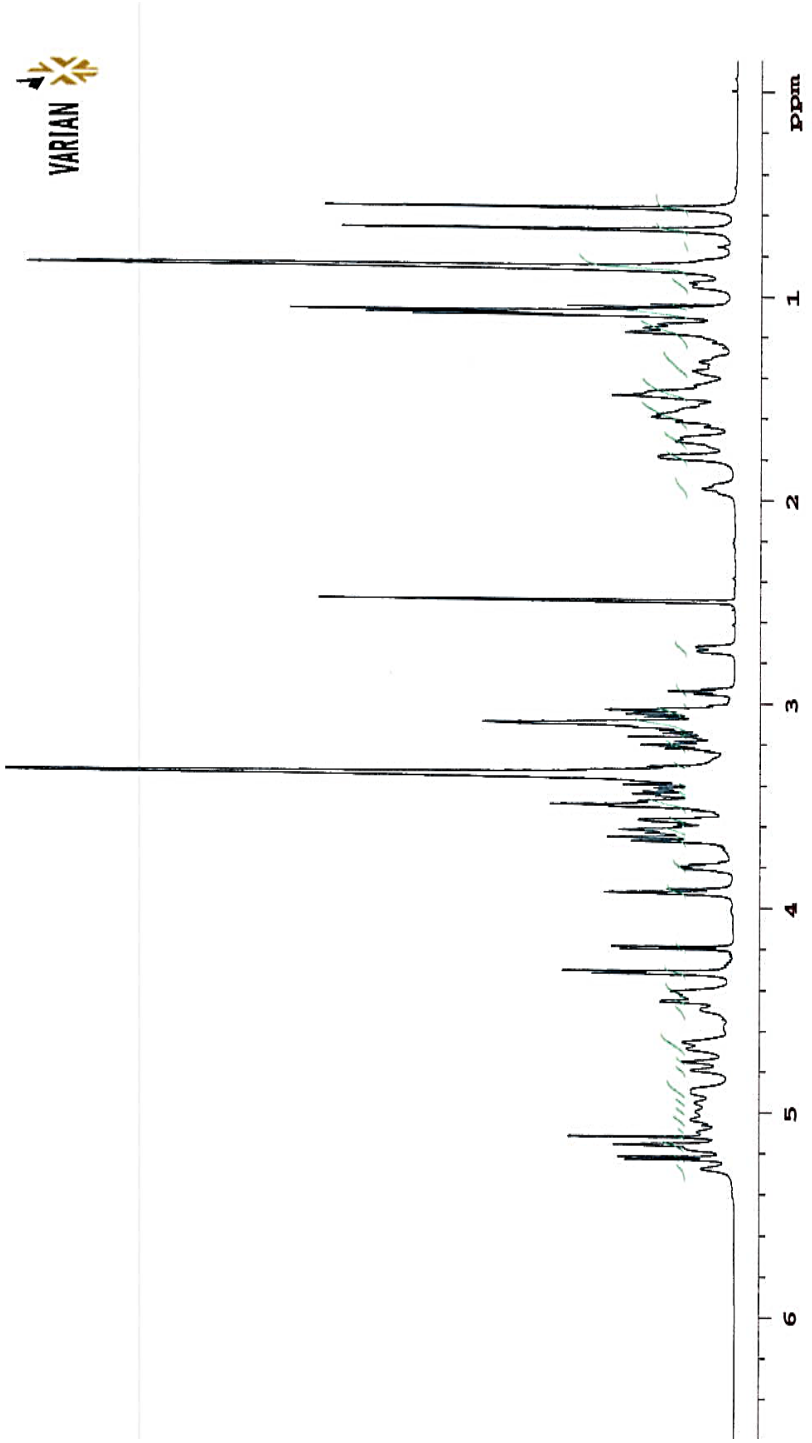


Figure 3.83 ^{1}H-NMR (600 *MHz*, DMSO-d_6) spectrum of compound **22**

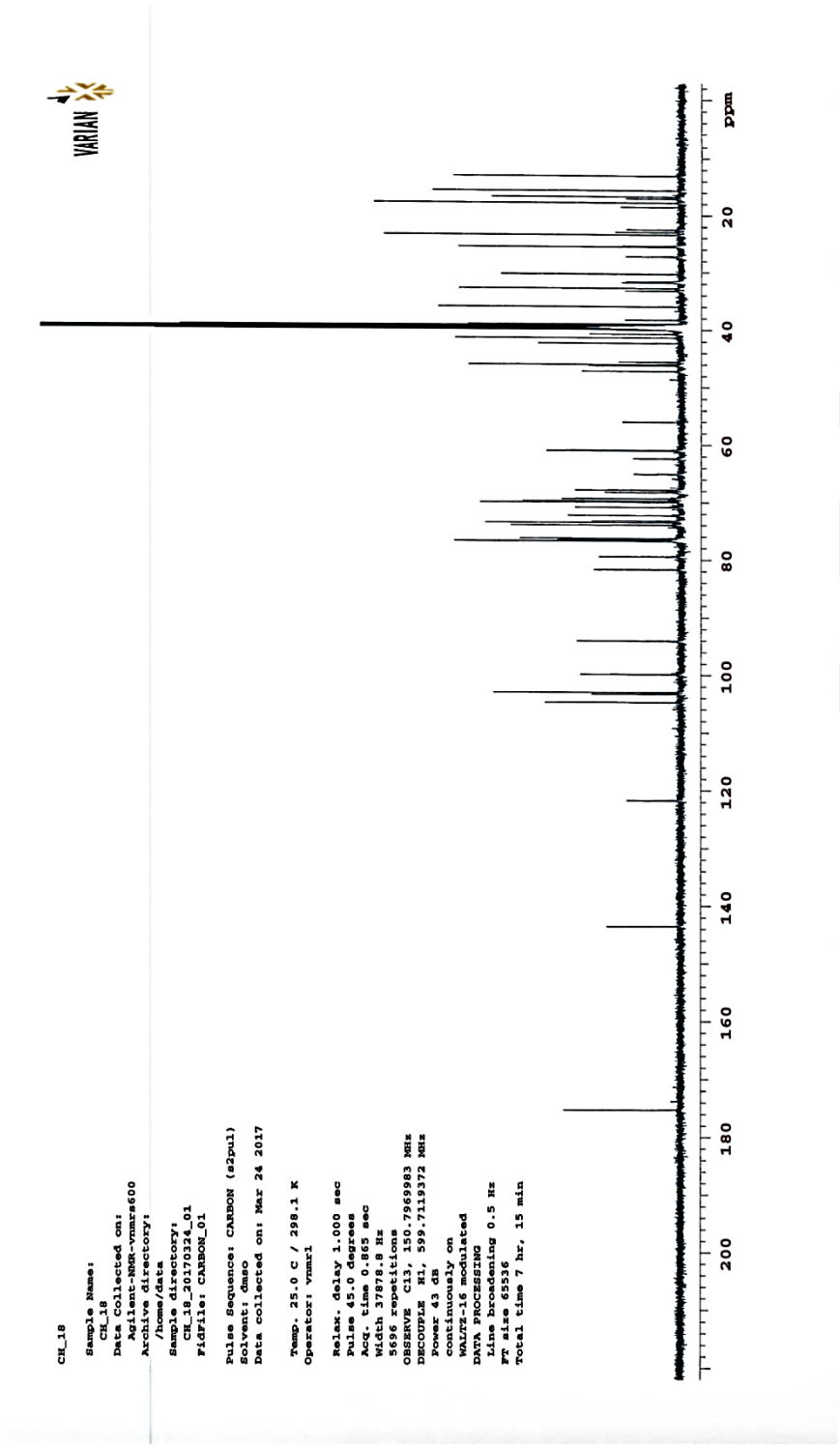


Figure 3.84 ^{13}C-NMR (150 *MHz*, DMSO-d_6) spectrum of compound **22**

3.1.3 *Cephalaria elazigensis* var. *elazigensis*

Isolation and purification process on *Cephalaria elazigensis* var. *elazigensis* Göktürk & Sümbül (Caprifoliacea) was concluded 1 pure triterpene aglycone namely 3-Epikatonic acid (**23**) which was identified in *Cephalaria* family for the first time.

Figure 3.85 TLC spot of isolated pure compound from *C. elazigensis* var. *elazigensis*

Compound 23 (3-Epikatonic acid): A white, amorphous powder (308.0 mg), triterpene aglycone, ^{1}H-NMR (DMSO-d_6, 400 *MHz*) and ^{13}C-NMR (DMSO-d_6, 100 *MHz*), see Table 3.5 and Figure 3.87 and 3.88 (Coxon and Wells, 1980). This compound was identified in Caprifoliceae family for the first time.

Cytotoxic effects of compound **23 (3-Epikatonic acid)** were evaluated in HepG-2 liver cancer cells using MTT assays. This oleanane type triterpene has no significant activity (IC_{50}: 95.5 μM) (Zhou et al., 2015). Another study showed that Compounds **23** compared to the concanavalin control group and showed an inhibitory effect on lymphocyte transformation by comparing with dexamethasone as reference compound. Inhibitiory effect of compound **23** (30 μg/mL) on lymphocyte was reported 70% inhibition (Shen et al., 2008). Another report explained that, compound **23** showed inhibitory activity against lymphocyte proliferation. Besides, cytotoxic activity of compound **23** was tested. This compound was inactive (IC_{50} > 25 μg/mL) against P388/S, P388/VCR(-), P388/VCR(+), P388/ADR(-) and P388/ADR(+) cell lines (Tanaka et al., 2001). The molecular structure of this compound was presented as (Figure 3.86).

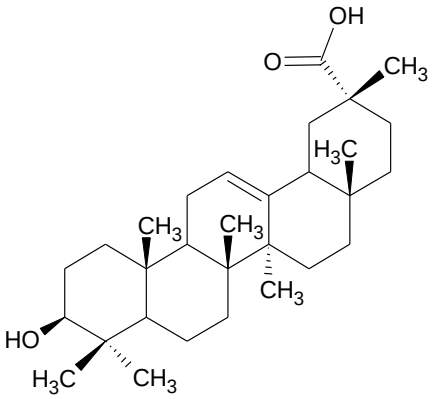


Figure 3.86 Structure of compound **23**

Table 3.5 ^{1}H and ^{13}C-NMR data for compound **23** [a-d]

Position	^{13}C-NMR	^{1}H-NMR
1	38.5	0.86, 1.48, m
2	27.4	1.44, m
3	77.3	2.98, m
4	37.1	-
5	55.3	0.66, m
6	18.5	1.30, 1.43, m
7	32.9	1.22, 1.37, m
8	40.0	-
9	47.6	1.45, m
10	38.8	-
11	23.4	1.45, 1,78, dd
12	122.0	5.14, br s
13	144.3	-
14	41.8	-
15	27.7	0.98, 1,64, m
16	46.2	1.02, 1.58, m
17	45.9	-
18	41.3	2.72, dd
19	32.6	1.40, 1.58, m
20	30.8	-
21	23.1	1.44, 1.90, m
22	33.8	1.10, 1.30, m
23	28.7	0.88, s
24	16.5	0.66, s
25	15.5	0.83, s
26	17.3	0.70, s
27	26.1	1.07, s
28	33.3	0.85, s
29	179.0	-
30	23.8	0.85, s

[a] ^{1}H and ^{13}C-NMR data (δ) were measured in DMSO-d_6 at 400 and 100 *MHz*, respectively.
[b] The assignments are based on COSY, HMQC and HMBC experiments.
[c] Coupling constants (J) in *Hz* are given in parentheses.
[d] nd: not detected

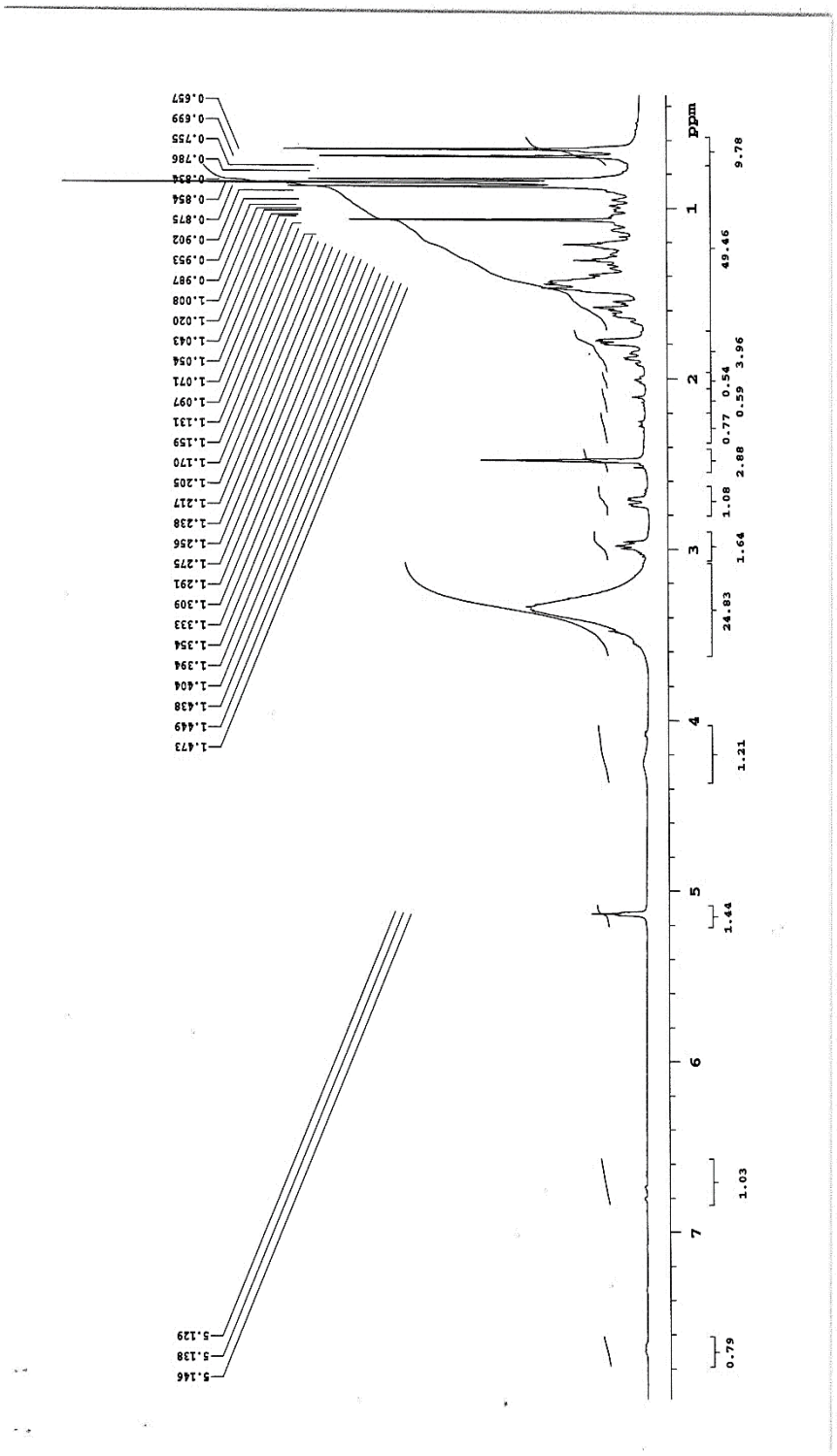


Figure 3.87 ^{1}H-NMR (400 *MHz*, DMSO-d_6) spectrum of compound **23**

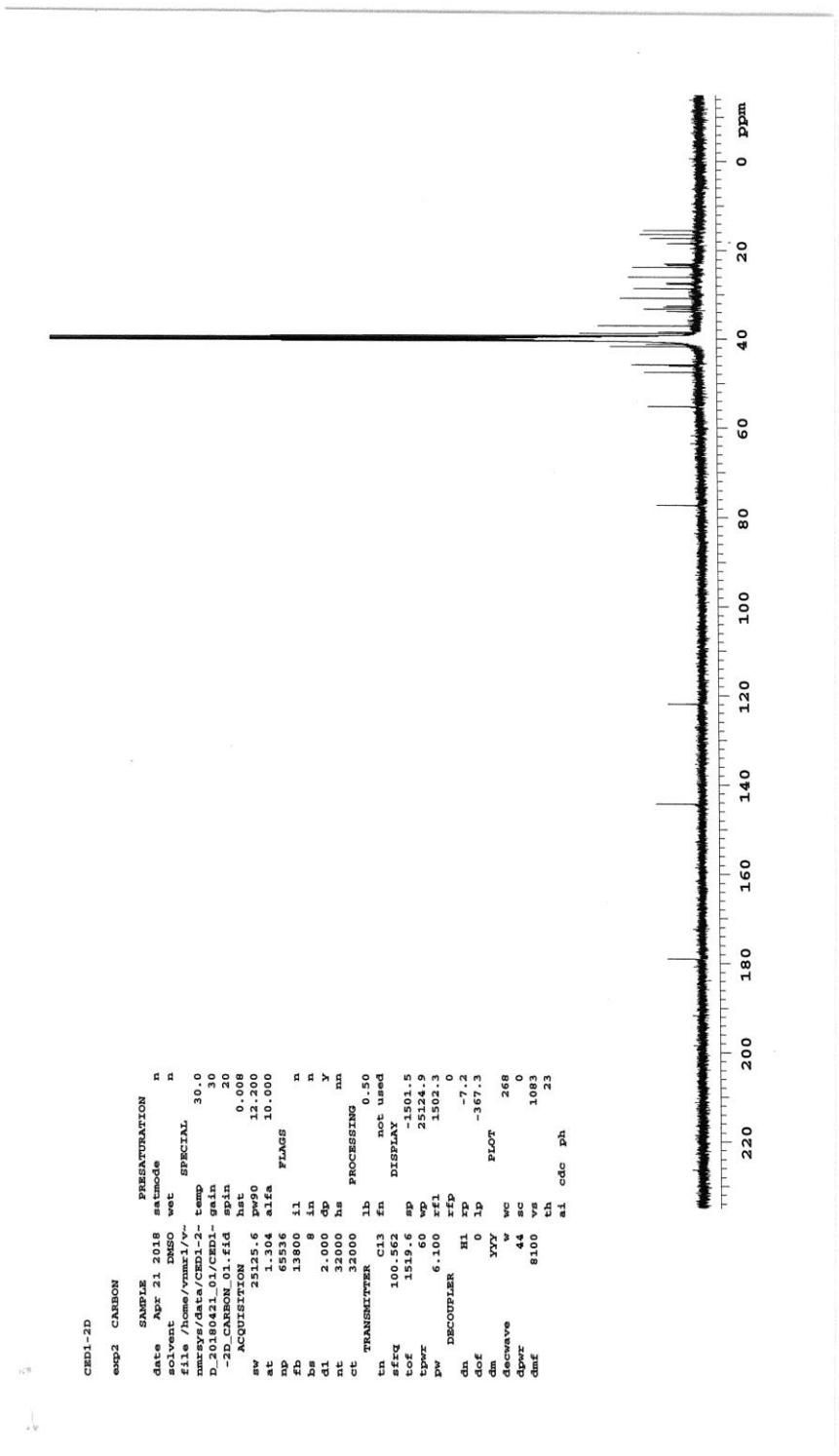


Figure 3.88 ^{13}C-NMR (100 *MHz*, DMSO-d_6) spectrum of compound **23**

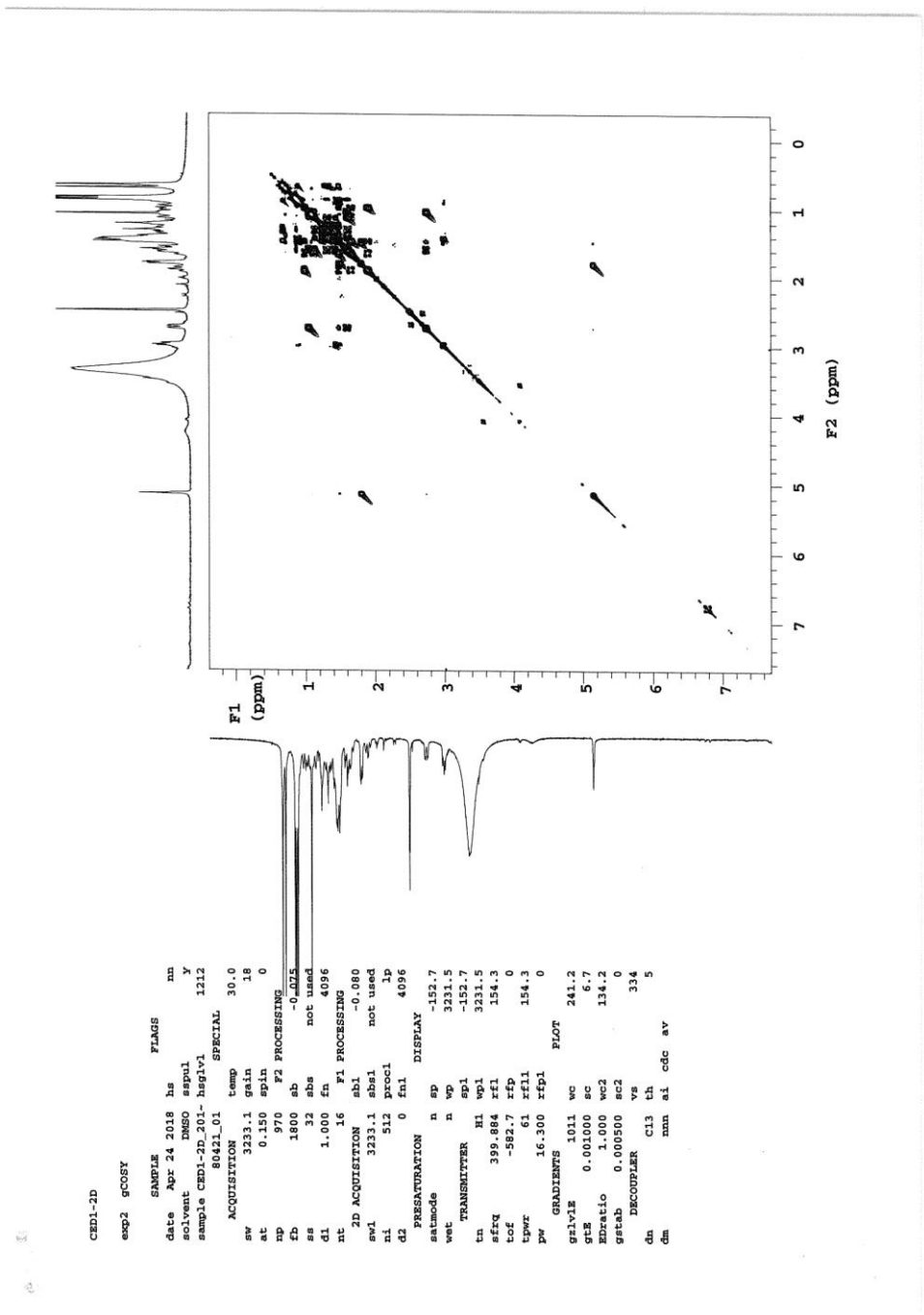


Figure 3.89 COSY (400 *MHz*) spectrum of compound **23**

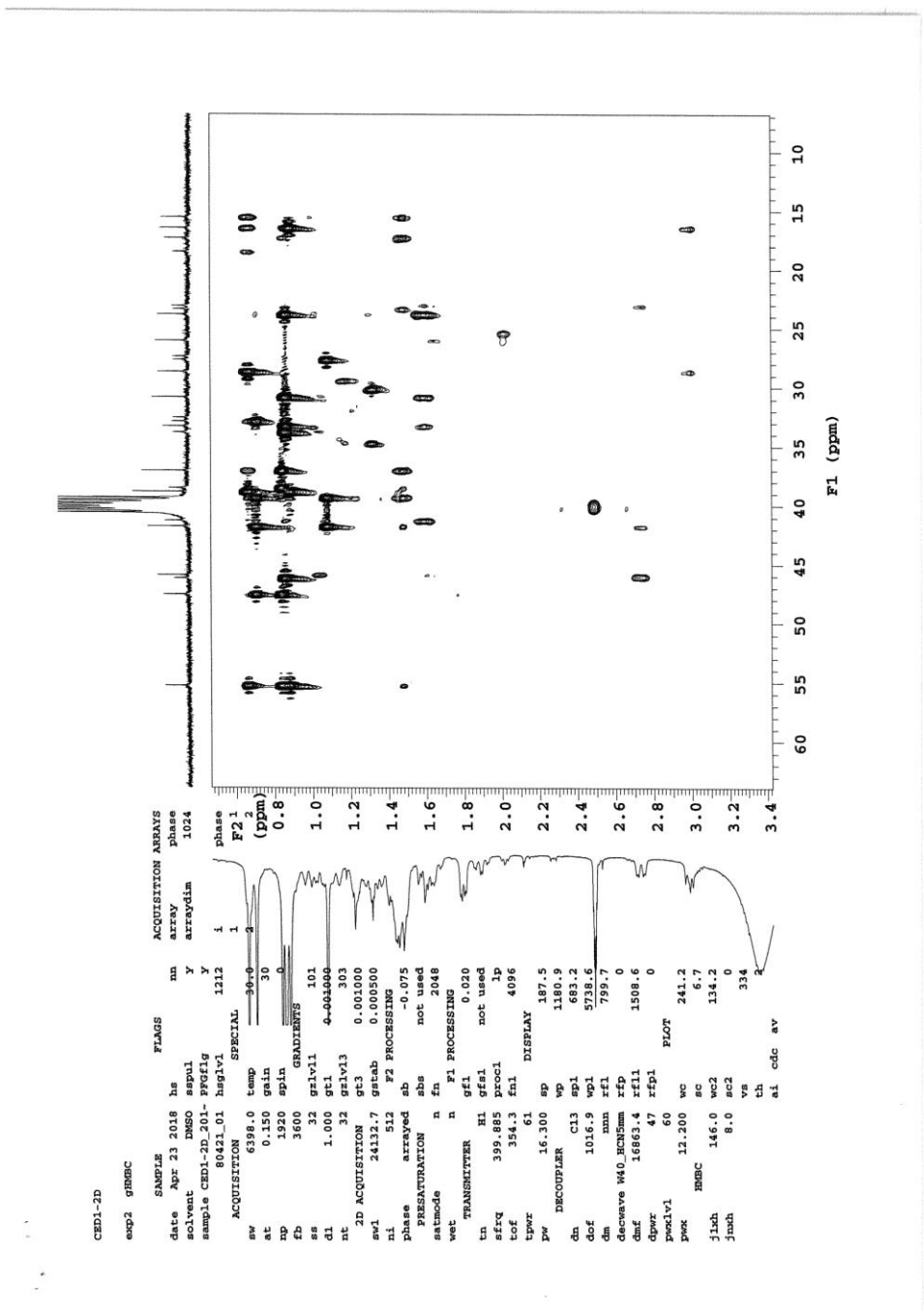


Figure 3.90 HMBC (400 *MHz*) spectrum of compound **23**

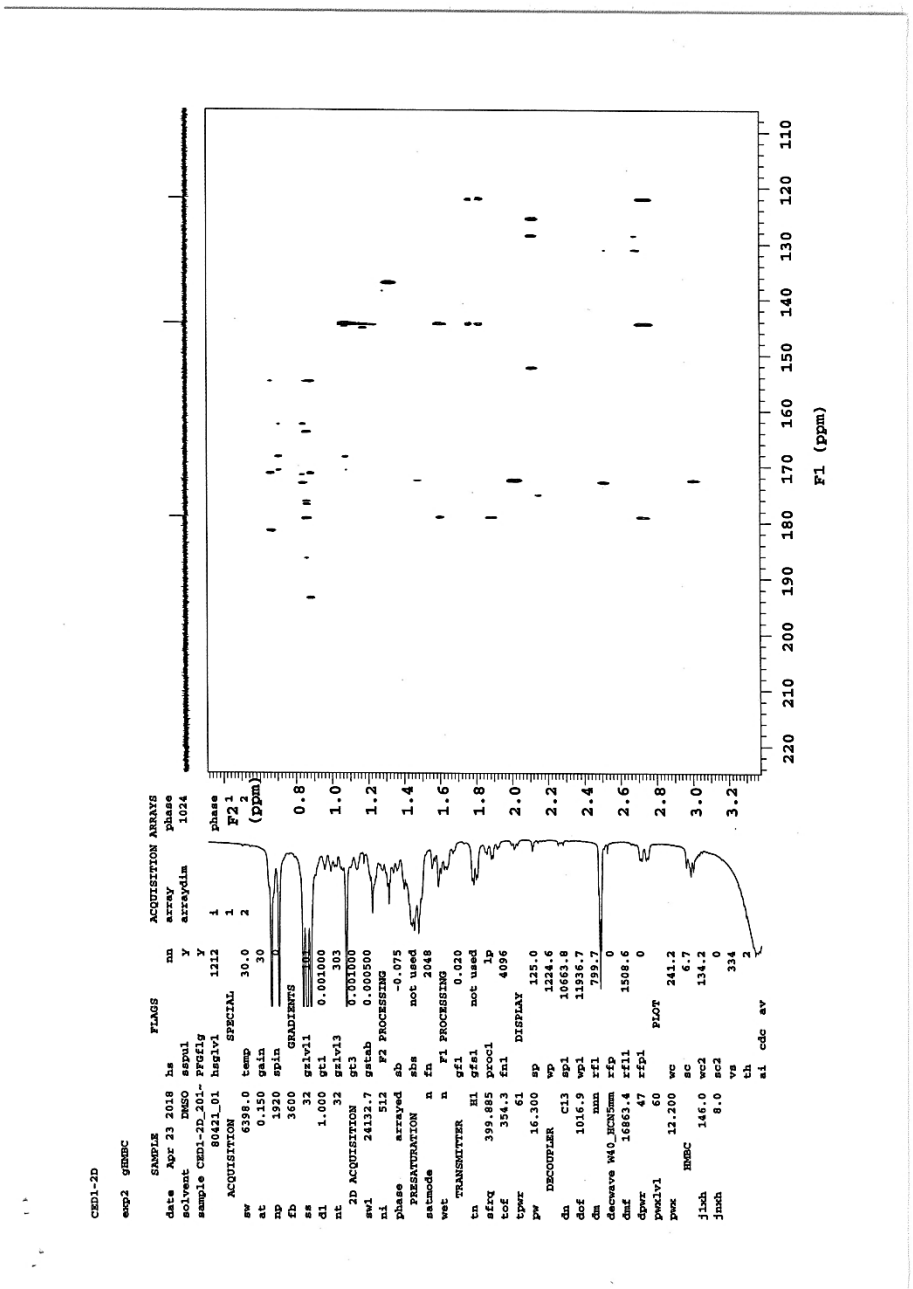


Figure 3.91 Expansion of HMBC (400 *MHz*) spectrum of compound **23**

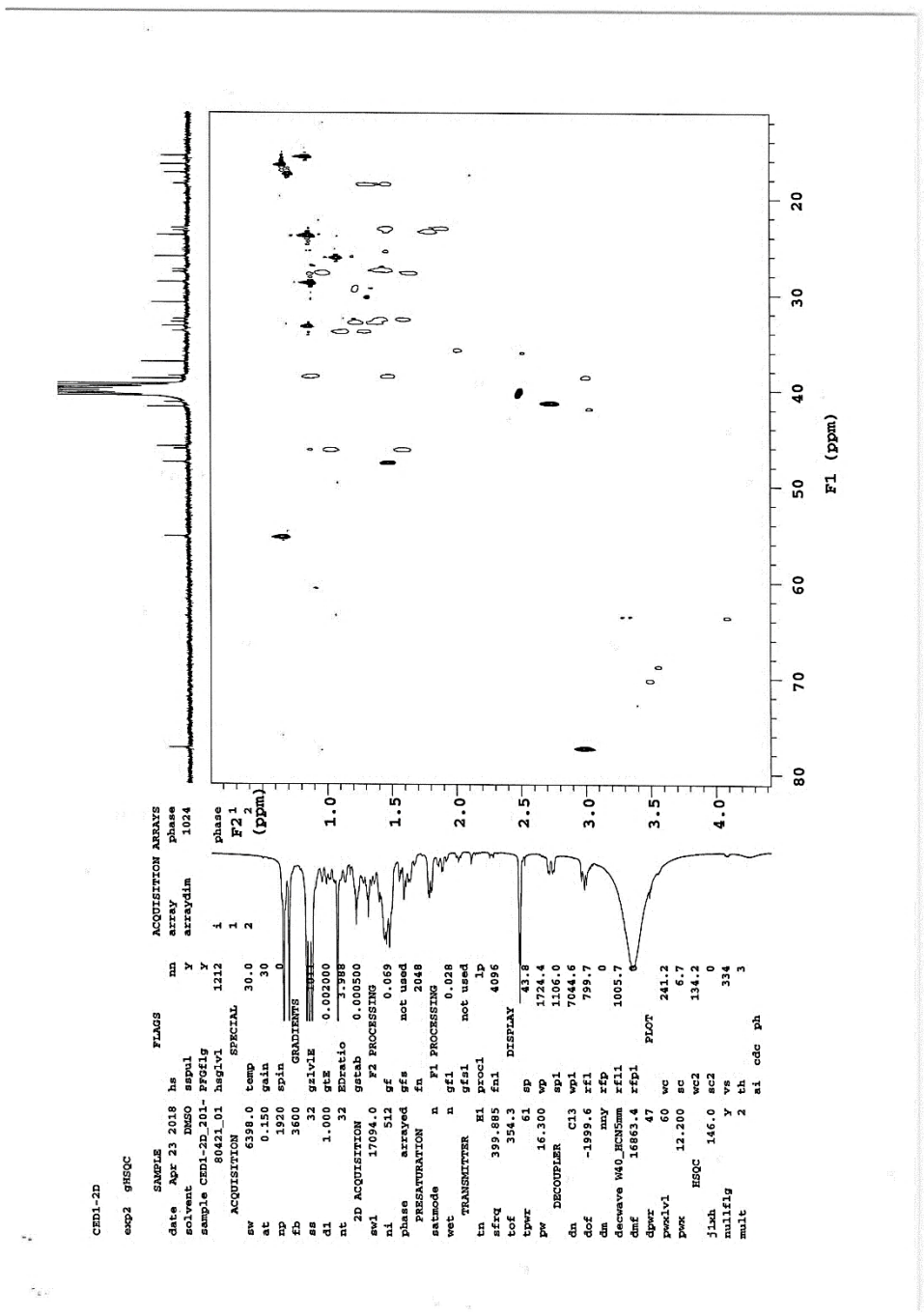


Figure 3.92 HSQC (400 *MHz*) spectrum of compound **23**

3.2 Determination of Triterpene Saponins by UPLC-ESI-MS/MS

Analyzing of triterpene saponins in crude *n*-BuOH extracts of *C. hirsuta, C. procera* and *C. elazigensis* var. *elazigensis* provides data before isolation and purification studies. This quick, easy, sensitive and time saving chromatographic method will ensure rapid identification of saponins in *n*-BuOH extracts of any other plants.

3.2.1 Optimization of UPLC conditions

UPLC conditions were optimized considering the peak shapes, resolutions and responses of reference substances. Varied UPLC parameters were examined and compared. Different brand chromatographic columns (same adsorbent, particle size, length and ID) didn't provide an advantage to the BEH C18 column. Methanol-water gradient was used as mobile phase which is more effective than acetonitrile-water gradient in resolving peaks. Under the optimized UPLC-MS/MS conditions, all compounds could be identified in the crude extract solutions by comparing their retention times and transition ions with reference solutions.

3.2.2 Application for UPLC-ESI-MS/MS

To identify *m/z* ratio of precursor and transition ions for quantitative analysis, forty individual reference substances were infused directly to the mass spectrometer in ESI positive mode. The cone energies were optimized individually according to peak heights and shapes for the precursor ions. The collision energies were adjusted to achieve adequate sensitivity for the transition ions and the list is given in Table 3.6. Mass values of the compounds found comparing with the standards which our group isolated previously. Cone energies were between 60V and 160V for all observed precursor ions. Collision energies were between 40V and 90V for all observed transition ions.

Table 3.6 Identification of saponins by UPLC-ESI-MS/MS

No	Compounds	References	RT (min)	Molecular Formula	Observed Precursor Ions (*m/z*)	Calculated Ions (*m/z*)	Observed Transition Ions (*m/z*)	Cone Energy (V)	Collision Energy (V)
1	Davisianoside A	Kayce et al., 2014	1.89	$C_{65}H_{106}O_{31}Na_2$	1406.0	1428.6508	347.12	100	80
							1081.5	100	70
2	Aytachoside A	Kayce et al., 2017b	2.05	$C_{59}H_{96}O_{26}Na$	1243.6	1243.6082	477.15	100	80
							1081.44	100	65
3	AnemoclemosidA	Li et al., 1995	2.70	$C_{35}H_{56}O_8$	627.2	604.3975	537.3	65	40
							583.43	65	40
4	Akebia saponin D	Higuchi et al., 1972	2.76	$C_{47}H_{76}O_{18}$	951.3	929.1000	347.16	100	60
							627.48	100	55
5	Gazipashoside B	Sarikahya and Kirmizigul, 2012b	2.82	$C_{64}H_{103}O_{31}$	1391.9	1367.6483	1067.81	120	75
							346.96	120	80
6	Aristatoside B	Sarikahya, 2014	2.82	$C_{70}H_{114}O_{35}Na$	1537.7	1537.7033	347.16	100	85
							1213.49	100	70
7	Cilicicoside I	Halay and Kirmizigul, 2010	2.83	$C_{71}H_{117}O_{36}Na$	1568.0	1568.2000	347.16	100	80
							1244.26	100	70
8	Macranthoidin B	Chen et al., 1995	2.84	$C_{65}H_{106}O_{32}Na$	1421.1	1399.5179	1097.55	120	70
							493.1	120	75
9	Aristatoside A	Sarikahya et al., 2014	2.84	$C_{71}H_{116}O_{36}Na$	1567.7	1567.7139	347.19	100	90
							639.13	100	90
10	Decaisoside E	Kong et al., 1993	2.87	$C_{58}H_{94}O_{26}Na$	1229.9	1229.5931	432.99	100	75
							346.92	100	75
11	Macranthoidin A	Mao et al., 1993	2.88	$C_{59}H_{96}O_{27}Na$	1259.6	1259.6031	935.4	120	70
							347.06	120	70
12	Elmalienoside C	Sarikahya and Kirmizigul, 2010	2.89	$C_{58}H_{94}O_{26}Na$	1230.5	1229.5926	905.87	130	70
							347.39	130	80
13	Elmalienoside A	Sarikahya and Kirmizigul, 2010	2.91	$C_{59}H_{96}O_{27}Na$	1259.6	1259.6031	935.39	160	70
							346.91	160	75
14	Balansoid D	Top et al., 2017	2.91	$C_{64}H_{104}O_{30}Na$	1375.6	1375.6505	347.21	130	75
							1051.48	130	70
15	Dipsacoside B	Mukhamedziev et al.,1971	2.92	$C_{53}H_{86}O_{22}Na$	1097.6	1097.5503	773.08	120	65

Table 3.6 Identification of saponins by UPLC-ESI-MS/MS (Continue)

No	Compounds	References	RT (min)	Molecular Formula	Observed Precursor Ions (*m/z*)	Calculated Ions (*m/z*)	Observed Transition Ions (*m/z*)	Cone Energy (V)	Collision Energy (V)
16	Elmalienoside B	Sarikahya and Kirmizigul, 2012a	2.92	$C_{53}H_{86}O_{22}Na$	1098.6	1097.5500	774.15	60	60
							347.84	60	72
17	Balansoid B	Top et al., 2017	2.93	$C_{64}H_{104}O_{30}Na$	1375.6	1375.6505	347.1	130	80
							1051.4	130	70
18	GazipashosideA	Sarikahya and Kirmizigul, 2012b	3.00	$C_{64}H_{103}O_{30}$	1375.5	1351.6540	347.19	130	75
							1051.55	130	60
19	Scoposide C	Sarikahya and Kirmizigul, 2010	3.01	$C_{69}H_{112}O_{34}Na$	1507.8	1507.6933	347.16	130	80
							1183.98	130	70
20	Balansoid C	Top et al., 2017	3.03	$C_{58}H_{94}O_{25}Na$	1213.6	1213.5976	447.19	130	73
							1051.19	130	70
21	Comp. 7	Braca et al., 2004	3.03	$C_{53}H_{86}O_{22}Na$	1097.6	1097.5503	935.39	100	60
							330.96	100	70
22	Scoposide B	Sarikahya and Kirmizigul, 2010	3.04	$C_{64}H_{104}O_{30}Na$	1375.7	1375.6505	1051.37	100	70
							347.03	100	80
23	Decaisoside D	Kong et al., 1993	3.05	$C_{52}H_{84}O_{21}$	1067.1	1045.2367	433.13	130	65
							905.37	130	55
24	Comp. 3	Kawai et al., 1988	3.07	$C_{47}H_{76}O_{17}Na$	935.5	935.4975	773.48	110	55
							184.97	110	60
25	Aristatoside C	Sarikahya, 2014	3.07	$C_{57}H_{92}O_{25}Na$	1200.1	1199.582	595.15	120	70
							1156.12	120	60
26	Scoposide F	Sarikahya et al., 2011	3.09	$C_{58}H_{93}O_{25}$	1213.5	1189.6011	889.3	100	60
							346.97	100	75
27	Sapindoside C	Chirva et al., 1970a	3.10	$C_{52}H_{84}O_{21}Na$	1068.4	1045.2367	1024.26	150	60
							595.66	150	60
28	Scoposide G	Sarikahya et al., 2011	3.15	$C_{58}H_{94}O_{25}Na$	1213.6	1213.5976	1051.19	100	60
							346.88	100	75
29	Scoposide A	Sarikahya and Kirmizigul, 2010	3.17	$C_{53}H_{101}O_{20}Na$	1082.5	1057.6892	757.59	120	60
							347.51	120	65

Table 3.6 Identification of saponins by UPLC-ESI-MS/MS (Continue)

No	Compounds	References	RT (min)	Molecular Formula	Observed Precursor Ions (*m/z*)	Calculated Ions (*m/z*)	Observed Transition Ions (*m/z*)	Cone Energy (V)	Collision Energy (V)
30	*α*-Hederin	Aliev and Movsumov, 1976	3.20	$C_{41}H_{66}O_{12}Na$	773.4	773.4440	729.04	100	45
							583.46	100	50
31	Scoposide D	Sarikahya and Kirmizigul, 2010	3.22	$C_{52}H_{84}O_{20}Na$	1051.5	1051.5448	889.4	120	55
							845.47	120	55
32	Isacoside	Kayce and Kirmizigul, 2010	3.22	$C_{64}H_{104}O_{30}Na$	1375.6	1375.6505	625.14	100	90
							771.4	100	85
33	Sapindoside B	Chirva et al., 1969	3.24	$C_{46}H_{74}O_{16}Na$	905.5	905.4869	861.25	110	55
							583.34	110	55
34	Lycicoside II	Halay and Kirmizigul, 2010	3.24	$C_{52}H_{83}O_{20}Na$	1051.7	1027.5483	889.29	120	55
							301.07	120	65
35	MacranthosideA	Saito et al., 1990	3.26	$C_{47}H_{76}O_{17}Na$	935.5	935.4975	462.93	150	60
							330.95	150	60
36	DavisianosideB	Kayce et al., 2014	3.26	$C_{53}H_{86}O_{21}Na$	1082.6	1081.5554	609.34	100	65
							477.35	100	65
37	Balansoid A	Top et al., 2017	3.27	$C_{52}H_{84}O_{20}Na$	1051.8	1051.5448	447.12	120	60
							579.12	120	60
38	Hederagenin	Braca et al., 2004	3.36	$C_{30}H_{48}O_4$	495.3	472.6997	451.4	70	24
							-	-	-
39	Scoposide E	Sarikahya and Kirmizigul, 2010	3.43	$C_{46}H_{73}O_{15}$	890.5	865.4949	845.87	100	50
							-	-	-
40	*β*-Sitosterol glucoside	Chirva et al., 1970b	3.96	$C_{35}H_{60}O_6$	1175.8	576.8500	599.03	100	50
							202.92	100	60

Comp. 7: 3-*O*-*β*-D-glucopyranosyl-(1→3)-*α*-L-rhamnopyranosyl-(1→2)-*α*-L-arabinopyranosyl hederagenin 28-*O*-*β*-D- glucopyranosyl ester

Comp. 3: 3-*O*-*α*-L-rhamnopyranosyl-(1→2)-*α*-L-arabinopyranosyl hederagenin 28-*O*-*β*-D-glucopyranosyl ester

3.2.1 Method validation

Method validation was applied with linearity, precision (repeatability), recovery, limit of detection (LOD) and limit of quantification (LOQ) parameters. Due to the saponin rich content of *n*-BuOH fraction, *Cephalaria hirsuta* plant was chosen to assess recovery, precision (repeatability), limit of detection (LOD) and limit of quantification (LOQ) parameters. These parameters are not needed for other fractions because of the same chemical properties.

3.2.1.1 Linearity

The linearity parameter for triterpene compounds were examined by analyzing standard solutions of six-point linear plot in the range of 0.025–1.0 mg/L. Correlation coefficients for all analytes in the working range were calculated as ≥ 0.9974. The linear regression equations and correlation coefficients are given in Table 3.7.

3.2.1.2 Recovery and precision (repeatability)

The recovery parameter was accomplished with *n*-BuOH fraction of *C. hirsuta* at 500.0 mg/kg and 1000.0 mg/kg concentration levels for each analytes. Due to content similarities between three species just *n*-BuOH fraction of *C. hirsuta* was performed as representative matrix. For the preparation of 500.0 mg/kg concentration level, 5 μL of the standard stock solution (100 mg/L) was added on 1.0 mg *n*-BuOH fraction. Then, 10 μL of the standard stock solution was added on 1.0 mg *n*-BuOH fraction to prepare 1000.0 mg/kg concentration level. Then, this spiked fractions was diluated to 10 mL with methanol in a volumetric flask (Dilution Factor: $1x10^4$). The unspiked *n*-BuOH fraction was also analyzed to determine the triterpene saponin concentrations in blank samples. The recoveries of each saponin at *n*-BuOH extract were evaluated.

Recoveries were calculated according to the following formula which changed in between 90.0% and 105.9% (Eq. 1).

$$\text{Recovery (\%)} = \frac{\text{Calculated Conc. - Blank Sample Conc.}}{\text{Spiked Conc.}} \times 100$$

Equation 1. Calculation of recovery parameter

For the precision (repeatability) parameter, *n*-BuOH extract spiked at two concentration levels (500.0 mg/kg and 1000.0 mg/kg) and analysed in six replicate during one day. The sample preparation was same as recovery procedure. Relative standard deviations (RSD) of precision were determined as between 1.2% and 8.9%. The representative data for recovery and RSD (%) are given in Table 3.7.

3.2.1.3 Limit of detection (LOD) and limit of quantification (LOQ)

The limit of detection (LOD) of triterpene saponins were obtained by multiplying the S/N ratio by 3. Then, LOQ values were calculated by multiplying the S/N ratio by 10. The calculated values for LOD and LOQ are given in Table 3.7.

3.2.1.4 Measurement of uncertainty

Linearity, repeatability (precision) and recovery parameters were evaluated to calculate uncertainty (EURACHEM/CITAC guide CG 4, 3rd edition, 2012). All results which have confidence level % 95 were given with measurement of uncertainty.

Table 3.7 Validation results of UPLC-ESI-MS/MS measurements

No	Compounds	Purity of Reference Compounds (%)	Linear regression equation	Correlation coefficients	LOD (*μ*g/kg)	LOQ (*μ*g/kg)	RSD (%)	Mean Reco-very (%)
1	Davisianoside A	97.5	y = 28284x - 124.09	0.9997	3.47	11.56	7.8	94.2
2	Aytachoside A	95.9	y = 51167x + 931.4	0.9995	0.04	0.14	4.5	95.8
3	Anemoclemoside A	99.2	y = 32238x - 239.51	0.9997	1.96	6.52	2.6	94.1
4	Akebia Saponin D	99.7	y = 353893x + 4581.7	0.9995	0.17	0.57	6.8	96.4
5	Gazipashoside B	100.0	y = 20045x + 82.039	0.9999	0.05	0.16	6.3	91.2
6	Aristatoside B	99.2	y = 25037x - 202.75	0.9989	0.16	0.54	6.2	94.6
7	Cilicicoside I	99.7	y = 18845x - 93.366	0.9995	0.32	1.07	1.5	100.2
8	Macranthoidin B	96.7	y = 8891.6x + 89	0.9998	0.05	0.16	3.8	94.6
9	Aristatoside A	98.8	y = 26522x - 71.643	0.9991	0.77	2.57	8.5	99.1
10	Decaisoside E	99.8	y = 182132x - 174	0.9999	0.02	0.06	2.9	92.1
11	Macranthoidin A	99.8	y = 151828x - 963.37	0.9994	0.51	1.71	7.1	95.7
12	Elmalienoside C	100.0	y = 47906x + 307.24	0.9998	0.41	1.35	2.4	94.8
13	Elmalienoside A	99.6	y = 179516x + 773.8	0.9999	0.06	0.21	3.9	96.8
14	Balansoid D	97.5	y = 144694x + 186.52	0.9997	0.08	0.28	4.4	96.1
15	Dipsacoside B	99.2	y = 105452x - 589.63	0.9996	0.31	1.02	7.7	101.9
16	Elmalienoside B	100.0	y = 21637x - 140.58	0.9998	1.94	6.47	8.7	105.9
17	Balansoid B	99.9	y = 120421x + 368.67	0.9999	0.23	0.75	8.9	92.6
18	Gazipashoside A	99.9	y = 142368x - 1421.6	0.9997	1.08	3.60	5.8	91.3
19	Scoposide C	99.6	y = 10400x + 43.638	0.9999	0.30	1.00	8.9	90.8
20	Balansoid C	98.5	y = 45076x - 0.6019	0.9999	0.04	0.15	8.2	91.1
21	Comp. 7	100.0	y = 125553x - 678.94	0.9995	1.90	6.33	4.0	97.8
22	Scoposide B	99.9	y = 69463x - 409.19	0.9987	0.61	2.04	7.2	95.2
23	Decaisoside D	99.0	y = 50598x - 259.08	0.9996	5.56	18.55	8.0	96.2
24	Comp. 3	99.7	y = 124869x - 130.93	0.9997	1.86	6.20	1.2	95.2
25	Aristatoside C	93.7	y = 7474.1x - 26.409	0.9996	6.16	20.53	6.0	92.1
26	Scoposide F	99.7	y = 70425x + 410.37	0.9998	0.87	2.91	5.1	93.0
27	Sapindoside C	99.8	y = 14230x + 19.968	0.9999	3.19	10.64	5.2	98.9
28	Scoposide G	99.6	y = 6524.1x - 8.9959	0.9999	0.37	1.22	4.5	97.1
29	Scoposide A	99.8	y = 4902.3x - 0.7102	0.9999	0.30	1.00	4.7	91.3
30	*α*-Hederin	100.0	y = 25324x - 85.064	0.9998	2.59	8.62	7.0	94.6
31	Scoposide D	92.1	y = 102197x + 624.96	0.9974	0.10	0.35	7.1	98.8
32	Isacoside	96.5	y = 17258x + 149.08	0.9993	4.53	15.10	2.4	91.3
33	Sapindoside B	100.0	y = 8193.6x - 16.119	0.9998	1.05	3.48	3.4	98.7
34	Lycicoside II	98.7	y = 100698x - 70.859	0.9998	0.52	1.75	4.0	100.5
35	Macranthoside A	98.6	y = 35428x - 4.7529	0.9999	0.72	2.39	4.5	100.9
36	Davisianoside B	97.6	y = 3496.6x + 7.6733	0.9998	2.88	9.59	6.2	92.5
37	Balansoid A	99.3	y = 18248x - 48.888	0.9999	1.82	6.08	8.4	90.0
38	Hederagenin	96.6	y = 4035.8x - 9.3593	0.9998	0.29	0.96	4.9	92.6
39	Scoposide E	99.4	y = 854.66x - 6.1706	0.9997	1.10	3.66	2.4	98.8
40	*β*-Sitosterol glucoside	99.0	y = 123352x - 186.79	0.9999	0.09	0.28	4.2	97.9

Comp. 7: 3-*O*-*β*-D-glucopyranosyl-(1→3)-*α*-L-rhamnopyranosyl-(1→2)-*α*-L-arabinopyranosyl hederagenin 28-*O*-*β*-D-glucopyranosyl ester

Comp. 3: 3-*O*-*α*-L-rhamnopyranosyl-(1→2)-*α*-L-arabinopyranosyl hederagenin 28-*O*-*β*-D-glucopyranosyl ester

3.2.1.5 Quantitative determination of saponins in *n*-BuOH fractions

Qualitative and quantitative determination of the compounds was applied by comparing references and *n*-BuOH extracts solutions based on retention times and transition ions. The appropriate two transition ions were selected for determination and the transition ions have more response were integrated to calculate concentrations of triterpene saponins in *n*-BuOH fractions. The concentrations of *n*-BuOH fractions were presented in Table 3.8.

According to the results, *C. hirsuta* was the richest species which has twenty-eight saponins. While scoposide G (10060.69 mg/kg) was the major compound for this species, aristatoside A (37.80 mg/kg) was the minor compound. Twenty-two compounds were detected in *n*-BuOH extract of *C. procera* which is resembled to *C. hirsuta* in terms of triterpene saponins. While dipsacoside B was the major compound (994.00 mg/kg), scoposide F (6.49 mg/kg), was the minor compounds in *n*-BuOH extract of *C. procera*. *C. elazigensis* var. *elazigensis* was the poorest species in terms of saponin content that we investigated. Only four saponins were detected in *n*-BuOH extract of this species. The major component was *β*-sitosterol glucoside (254.26 mg/kg) and the minor was macranthoside A (8.54 mg/kg). Figures (93-146) show the retention times and peaks of all triterpene saponins which were detected in *C. hirsuta*, *C. procera* and *C. elazigensis* var. *elazigensis*. In the present study, saponin contents of *C. hirsuta, C. procera and C. elazigensis var. elazigensis.* were determined by an UPLC-ESI-MS/MS method, for the first time. The method was applied in this study to perform a quantitative determination of 40 saponins in crude extracts. We identified and tentatively characterized 28 compounds in *C. hirsuta*, 22 compounds in *C. procera* and 4 compounds in *C. elazigensis* var. *elazigensis* based on their retention times and mass spectra in comparison with the data from reference standards which were isolated in our previous studies. This validated quantitative method presents content of triterpene saponins as mg/kg in *n*-BuOH fractions. These results prompted us to consider on the saponin contents of these

Cephalaria species which were not studied yet. As a result of this study, we found that among three species *C. elazigensis* var. *elazigensis* has only 4 saponins in its *n*-butanol fraction. Because it is known that isolation procedures are a difficult, long and costly way, the studies on this plant are not preferable. On the contrary, a more detailed investigation on the *C. hirsuta* and *C. procera* species including 28 and 22 saponins, respectively is needed. So, analysis of these compounds in crude extracts provides an idea before isolation and purification studies. This easy, sensitive and time saving chromatographic method provides rapid identification of saponins in *n*-BuOH extracts of other plants.

Table 3.8 Contents of saponins as mg/kg in *n*-BuOH fractions of *C. hirsuta*, *C. procera* and *C.elazigensis* var. *elazigensis*

Peak No	Compounds	*C. hirsuta*	*C. procera*	*C. elazigensis var. elazigensis*
1	Davisianoside A	<LOQ	<LOQ	<LOQ
2	Aytachoside A	<LOQ	<LOQ	<LOQ
3	Anemoclemoside A	<LOQ	<LOQ	<LOQ
4	Akebia Saponin D	<LOQ	<LOQ	<LOQ
5	Gazipashoside B	545.35±49.6	36.90±3.4	<LOQ
6	Aristatoside B	88.05±8.5	<LOQ	<LOQ
7	Cilicicoside I	<LOQ	<LOQ	<LOQ
8	Macranthoidin B	<LOQ	46.89±3.0	<LOQ
9	Aristatoside A	37.80±1.8	<LOQ	<LOQ
10	Decaisoside E	336.33±18.8	146.23±8.2	<LOQ
11	Macranthoidin A	229.83±15.6	151.91±10.3	<LOQ
12	Elmalienoside C	439.69±42.2	131.33±12.6	<LOQ
13	Elmalienoside A	108.54±4.9	22.33±1.0	<LOQ
14	Balansoid D	<LOQ	<LOQ	<LOQ
15	Dipsacoside B	1063.22±62.7	994.00±58.6	<LOQ
16	Elmalienoside B	308.35±12.0	290.93±11.3	<LOQ
17	Balansoid B	<LOQ	<LOQ	<LOQ
18	Gazipashoside A	111.68±6.6	<LOQ	<LOQ
19	Scoposide C	43.43±3.7	<LOQ	<LOQ
20	Balansoid C	<LOQ	<LOQ	<LOQ
21	3-*O*-*β*-D-glucopyranosyl-(1→3)-*α*-L-rhamnopyranosyl-(1→2)-*α*-L-arabinopyranosyl hederagenin 28-*O*-*β*-D- glucopyranosyl ester	148.40±13.9	92.71±8.7	<LOQ
22	Scoposide B	81.25±7.2	62.20±5.5	<LOQ
23	Decaisoside D	733.27±64.5	242.28±21.3	<LOQ
24	3-*O*-*α*-L-rhamnopyranosyl-(1→2)-*α*-L-arabinopyranosyl hederagenin 28-*O*-*β*-D-glucopyranosyl ester	227.33±19.8	120.57±10.5	<LOQ
25	Aristatoside C	200.30±16.2	<LOQ	<LOQ
26	Scoposide F	972.94±47.7	6.49±0.3	8.84±0.4
27	Sapindoside C	95.95±5.5	13.02±0.7	<LOQ
28	Scoposide G	10060.69±814.9	651.58±52.8	<LOQ
29	Scoposide A	3062.46±303.2	214.00±21.2	41.23±4.1
30	*α*-Hederin	243.35±21.7	152.69±13.6	<LOQ
31	Scoposide D	152.96±119	8.57±0.7	<LOQ
32	Isacoside	<LOQ	<LOQ	<LOQ
33	Sapindoside B	386.30±22.8	170.89±10.1	<LOQ
34	Lycicoside II	208.42±11.0	15.73±0.8	<LOQ
35	Macranthoside A	629.21±42.2	214.70±14.4	8.54±0.6
36	Davisianoside B	66.71±3.3	<LOQ	<LOQ
37	Balansoid A	<LOQ	<LOQ	<LOQ
38	Hederagenin	<LOQ	<LOQ	<LOQ
39	Scoposide E	156.44±9.9	<LOQ	<LOQ
40	*β*-Sitosterol glucoside	1024.20±88.1	166.97±14.4	254.26±21.9

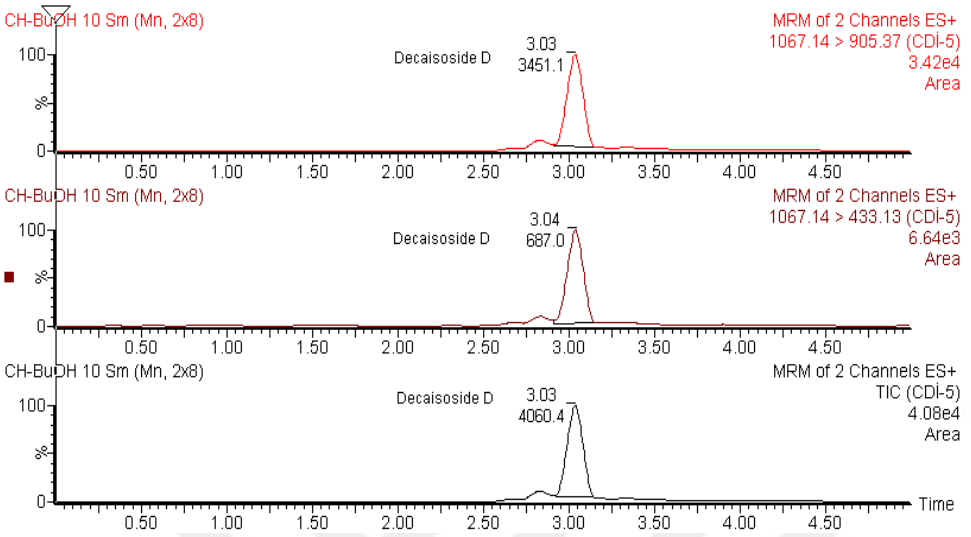


Figure 3.93 Chromatograms of Decaisoside D (*m/z*: 1067.14 → 905.37, 1067.14 → 433.13 and TIC), obtained by UPLC-ESI-MS/MS in the positive ion mode for crude extract solution in *C. hirsuta*

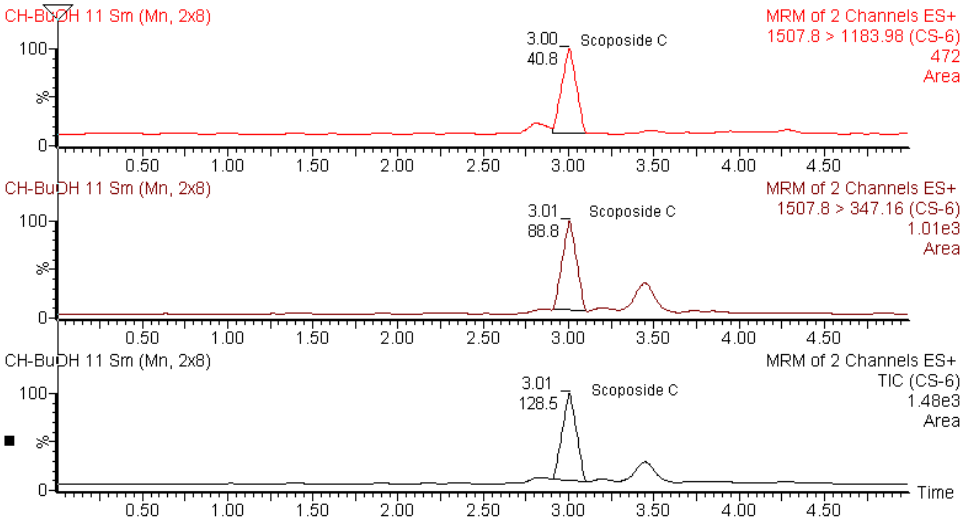


Figure 3.94 Chromatograms of Scoposide C (*m/z*: 1507.80 → 1183.98, 1507.80 → 347.16 and TIC), obtained by UPLC-ESI-MS/MS in the positive ion mode for crude extract solution in *C. hirsuta*

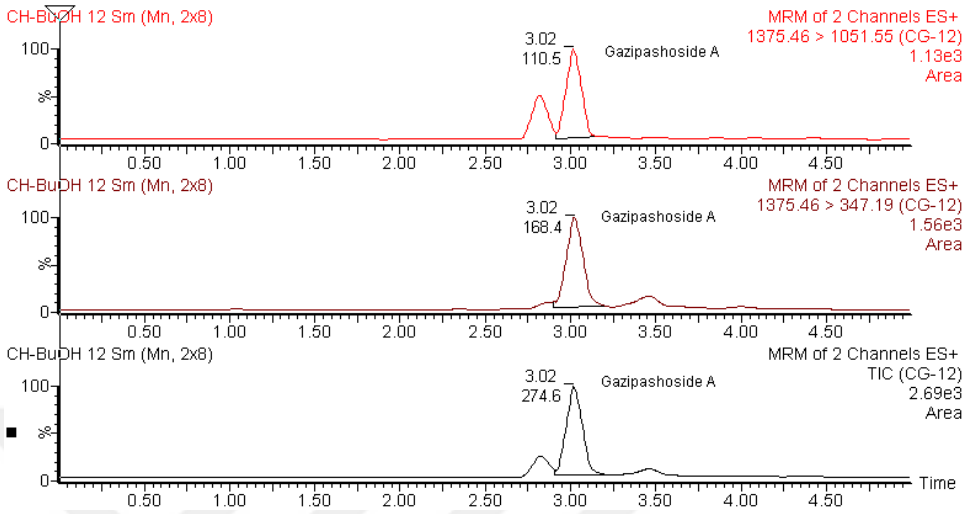


Figure 3.95 Chromatograms of Gazipashoside A (*m/z*: 1375.46 → 1051.55, 1375.46 → 347.19 and TIC), obtained by UPLC-ESI-MS/MS in the positive ion mode for crude extract solution in *C. hirsuta*

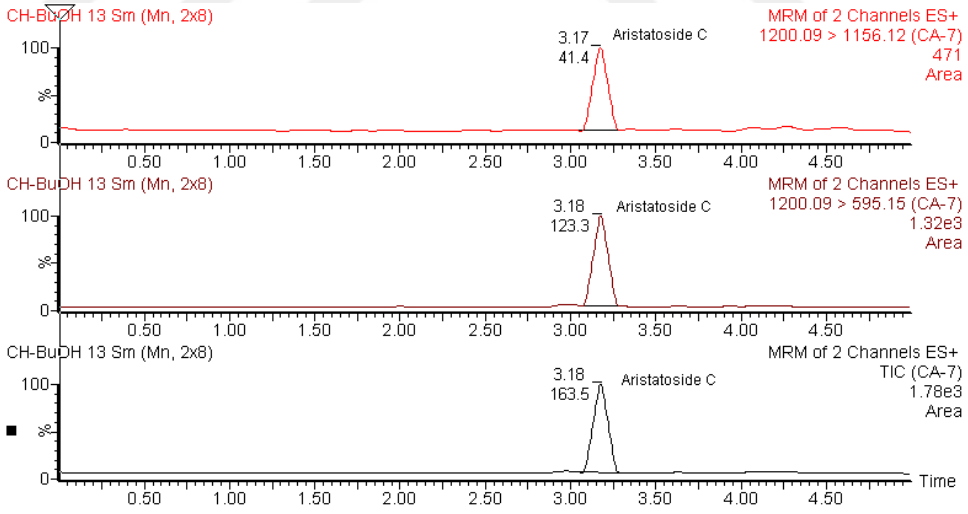


Figure 3.96 Chromatograms of Aristatoside C (*m/z*: 1200.09 → 1156.12, 1200.09 → 595.15 and TIC), obtained by UPLC-ESI-MS/MS in the positive ion mode for crude extract solution in *C. hirsuta*

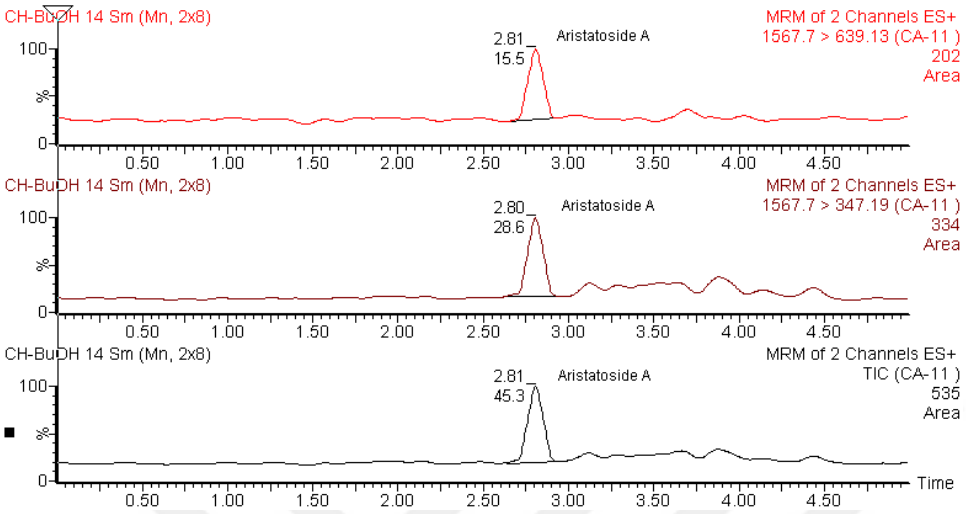


Figure 3.97 Chromatograms of Aristatoside A (*m/z*: 1567.70 → 639.13, 1567.7→ 347.19 and TIC), obtained by UPLC-ESI-MS/MS in the positive ion mode for crude extract solution in *C. hirsuta*

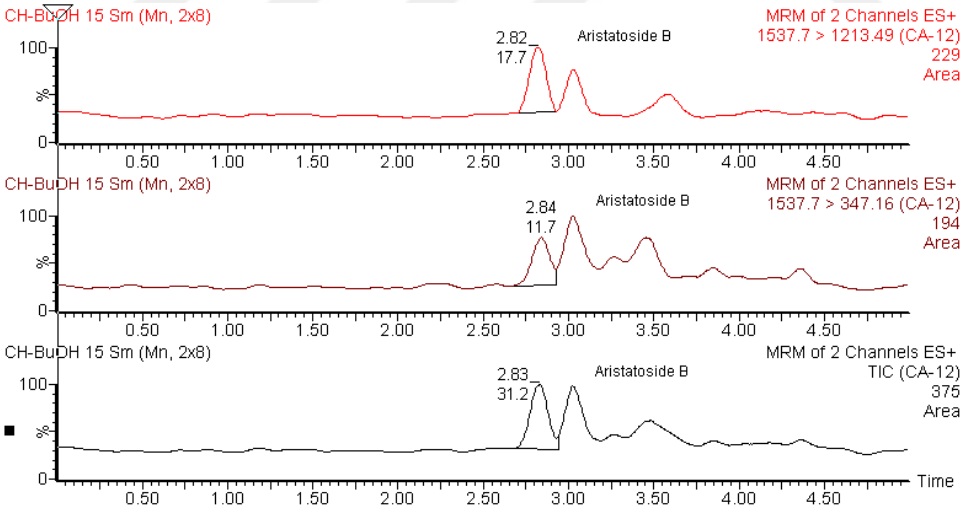


Figure 3.98 Chromatograms of Aristatoside B (*m/z*: 1537.70 → 1213.49, 1537.70 → 347.16 and TIC), obtained by UPLC-ESI-MS/MS in the positive ion mode for crude extract solution in *C. hirsuta*

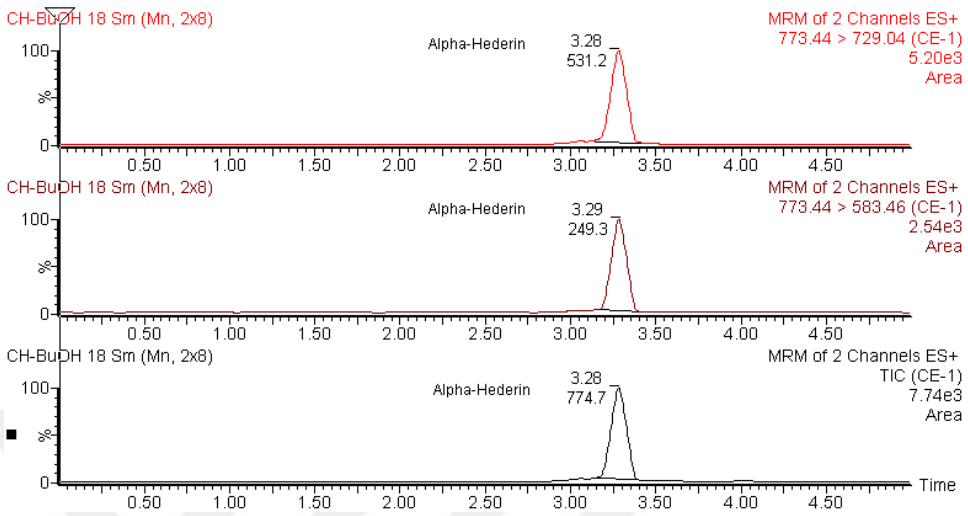


Figure 3.99 Chromatograms of *α*-Hederin (*m/z*: 773.44 → 729.04, 773.44 → 583.46 and TIC), obtained by UPLC-ESI-MS/MS in the positive ion mode for crude extract solution in *C. hirsuta*

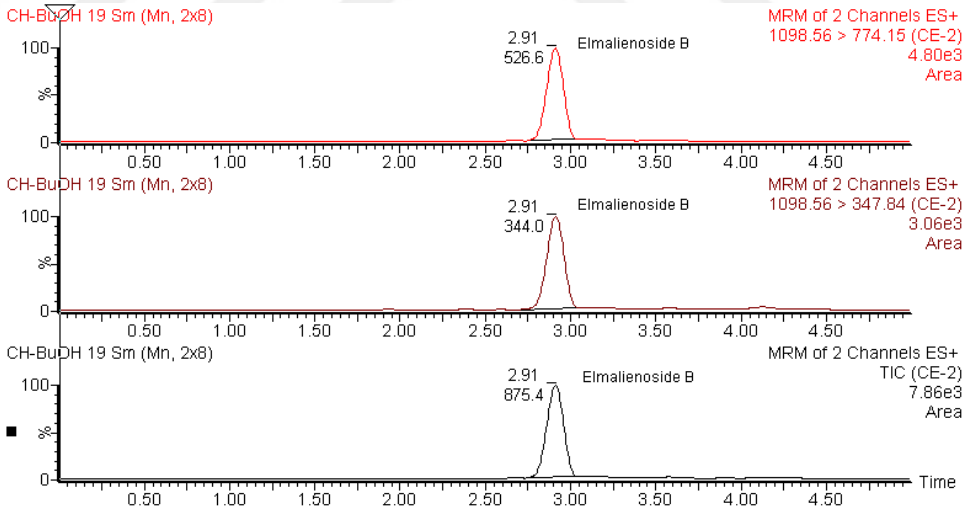


Figure 3.100 Chromatograms of Elmalienoside B (*m/z*: 1098.56 → 774.15, 1098.56 → 347.84 and TIC), obtained by UPLC-ESI-MS/MS in the positive ion mode for crude extract solution in *C. hirsuta*

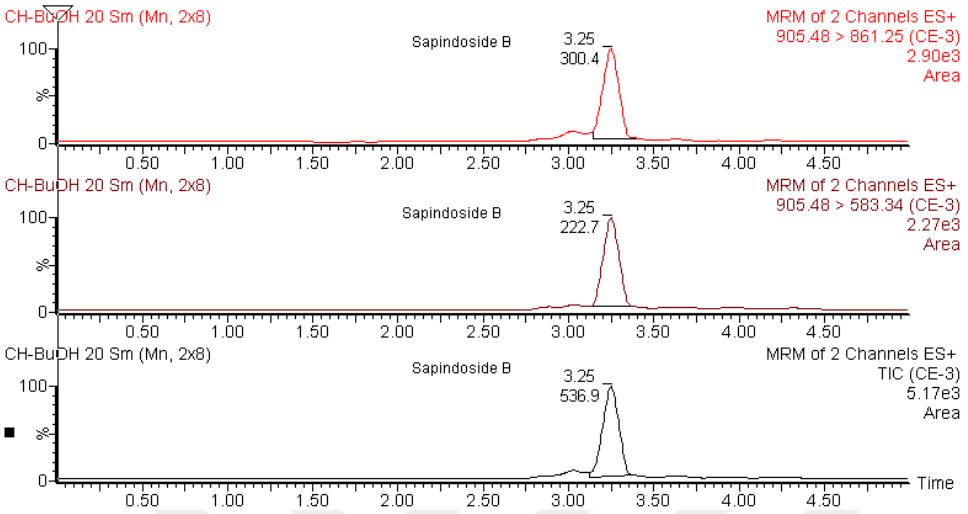


Figure 3.101 Chromatograms of Sapindoside B (*m/z*: 905.48 → 861.25, 905.48 → 583.34 and TIC), obtained by UPLC-ESI-MS/MS in the positive ion mode for crude extract solution in *C. hirsuta*

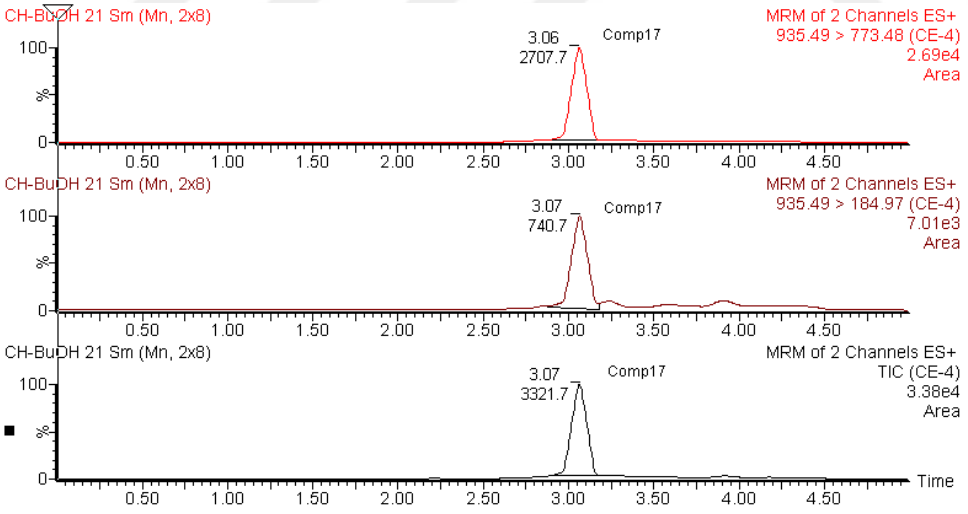


Figure 3.102 Chromatograms of 3-*O*-*α*-L-rhamnopyranosyl-(1→2)-*α*-L-arabinopyranosyl hederagenin 28-*O*-β-D-glucopyranosyl ester (*m/z*: 935.49 → 773.48, 935.49 → 184.97 and TIC), obtained UPLC-ESI-MS/MS in the positive ion mode for crude extract solution in *C. hirsuta*

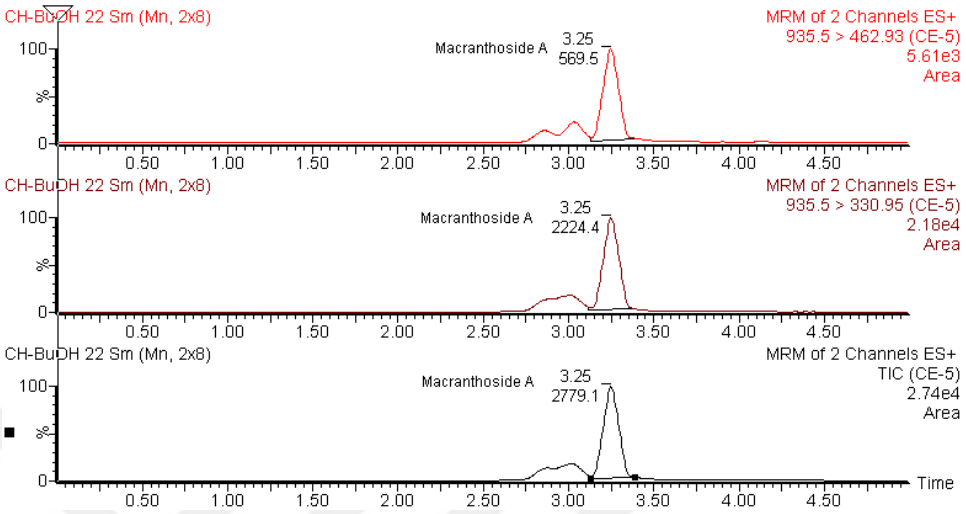


Figure 3.103 Chromatograms of Macranthoside A (*m/z*: 935.50 → 462.93, 935.50 → 330.95 and TIC), obtained by UPLC-ESI-MS/MS in the positive ion mode for crude extract solution in *C. hirsuta*

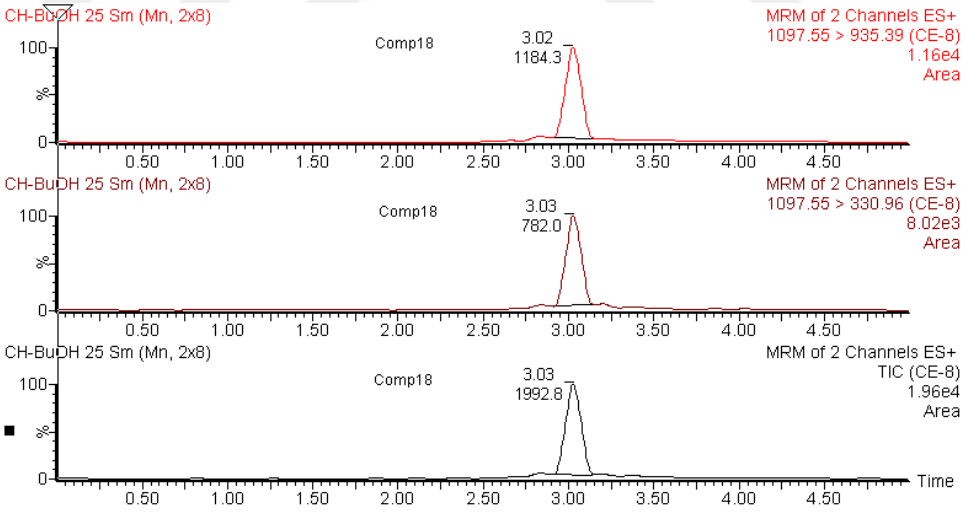


Figure 3.104 Chromatograms of 3-*O*-β-D-glucopyranosyl-(1→3)-*α*-L-rhamnopyranosyl-(1→2)-*α*-L arabinopyranosyl hederagenin 28-*O*-β-D- glucopyranosyl ester (*m/z*: 1097.55 → 935.39, 1097.55 → 330.96 and TIC), obtained by UPLC-ESI-MS/MS in the positive ion mode for crude extract solution in *C. hirsuta*

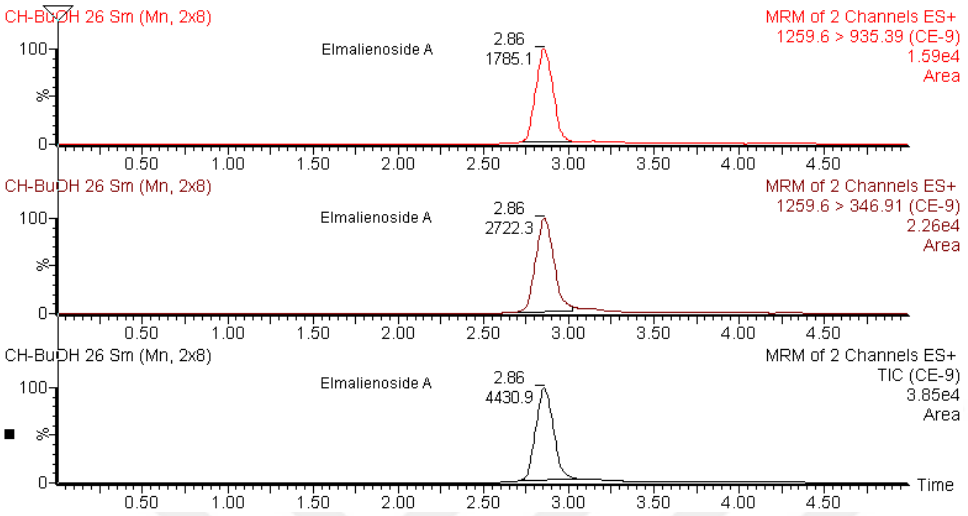


Figure 3.105 Chromatograms of Elmalienoside A (*m/z*: 1259.6 → 935.39, 1259.60 → 346.91 and TIC), obtained by UPLC-ESI-MS/MS in the positive ion mode for crude extract solution in *C. hirsuta*

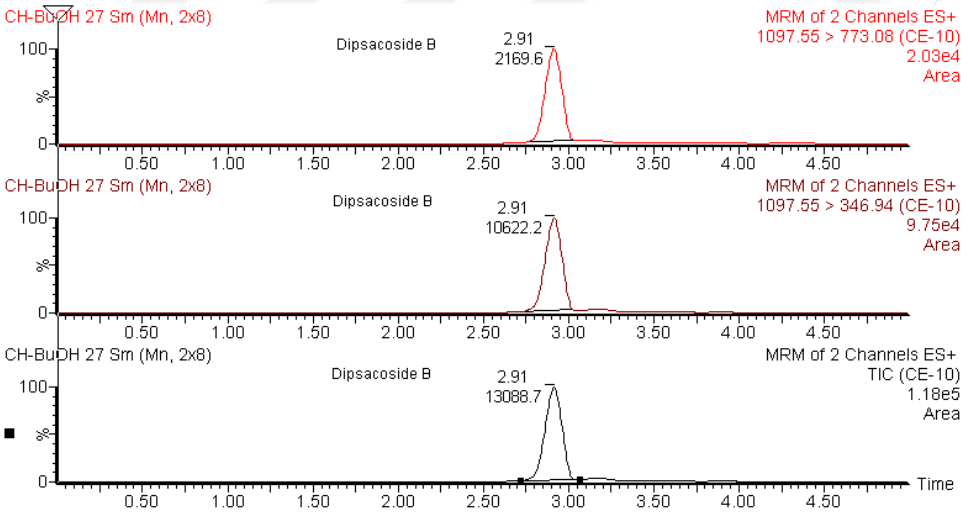


Figure 3.106 Chromatograms of Dipsacoside B (*m/z*: 1097.55 → 773.08, 1097.55 → 346.94 and TIC), obtained by UPLC-ESI-MS/MS in the positive ion mode for crude extract solution in *C. hirsuta*

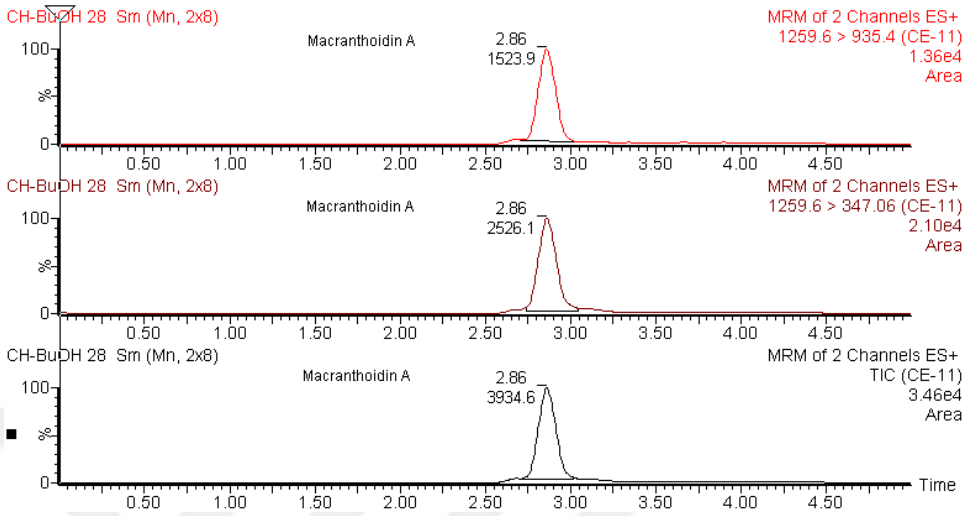


Figure 3.107 Chromatograms of Macranthoidin A (*m/z*: 1259.60 → 935.40, 1259.60 → 347.06 and TIC), obtained by UPLC-ESI-MS/MS in the positive ion mode for crude extract solution in *C. hirsuta*

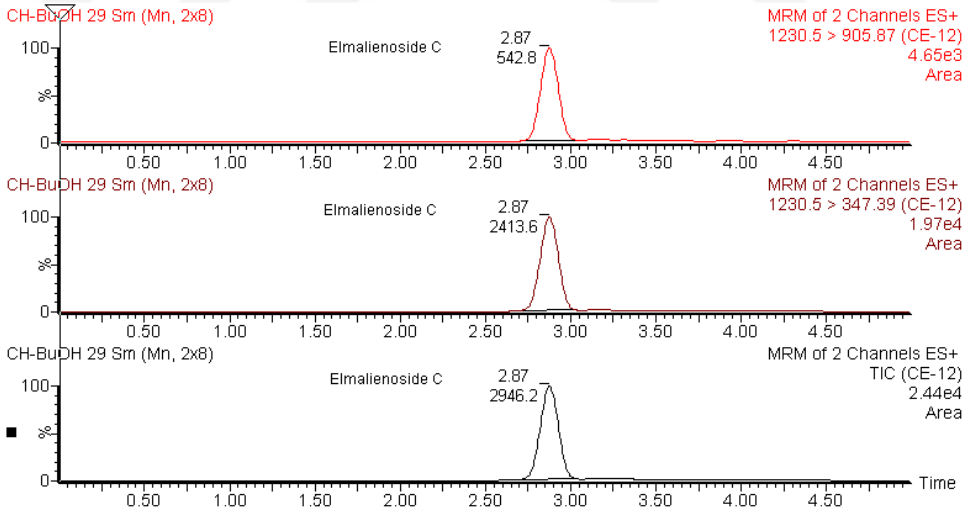


Figure 3.108 Chromatograms of Elmalienoside C (*m/z*: 1230.50 → 905.87, 1230.50 → 347.39 and TIC), obtained by UPLC-ESI-MS/MS in the positive ion mode for crude extract solution in *C. hirsuta*

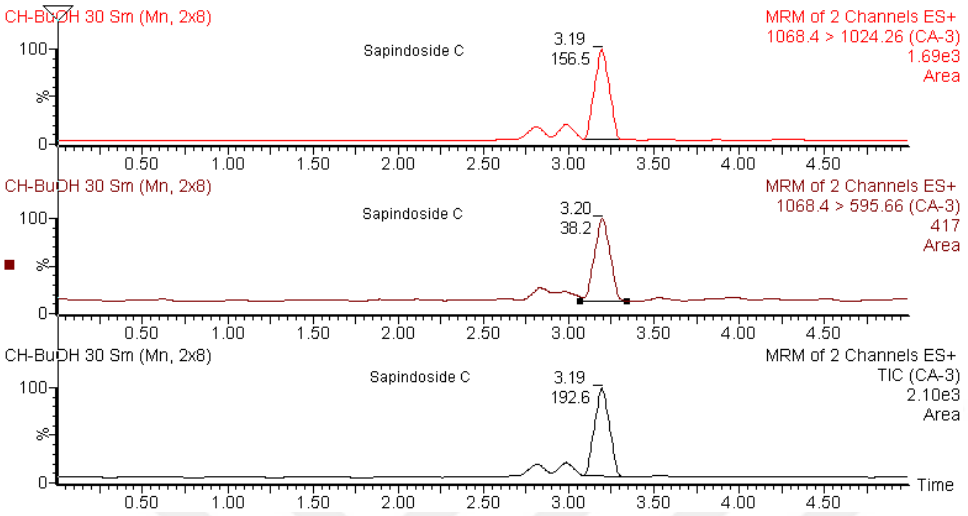


Figure 3.109 Chromatograms of Sapindoside C (*m/z*: 1068.40 → 1024.26, 1068.40 → 595.66 and TIC), obtained by UPLC-ESI-MS/MS in the positive ion mode for crude extract solution in *C. hirsuta*

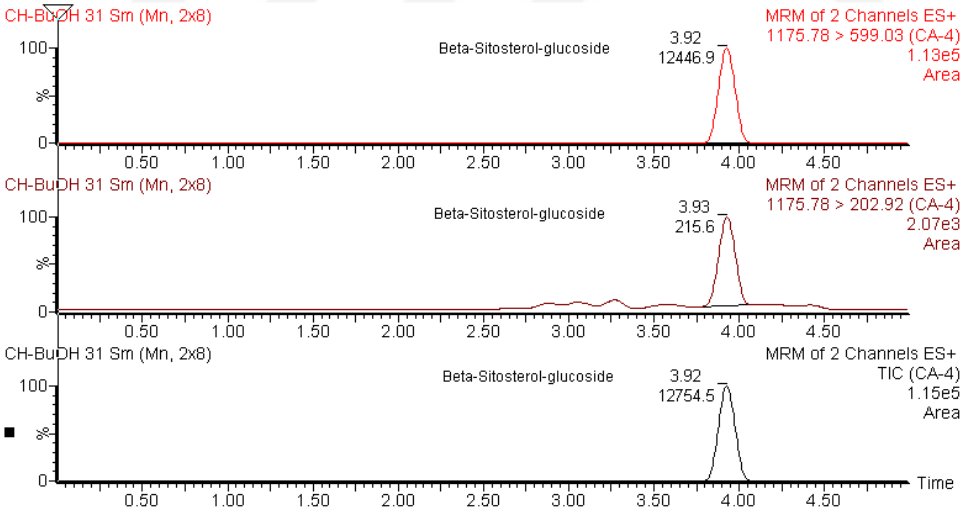


Figure 3.110 Chromatograms of *β*-Sitosterol-Glucoside (*m/z*: 1175.78 → 599.03, 1175.78 → 202.92 and TIC), obtained by UPLC-ESI-MS/MS in the positive ion mode for crude extract solution in *C. hirsuta*

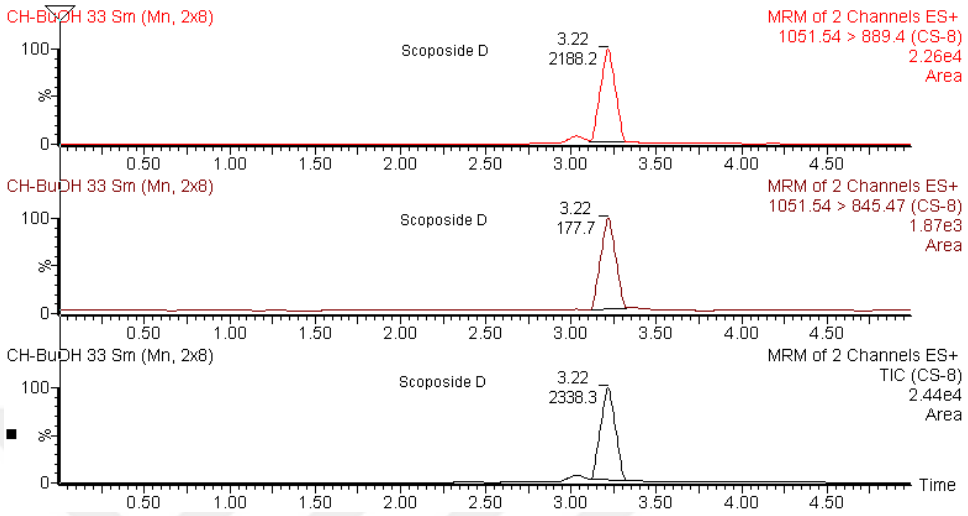


Figure 3.111 Chromatograms of Scoposide D (*m/z*: 1051.54 → 889.40, 1051.54 → 845.47 and TIC), obtained by UPLC-ESI-MS/MS in the positive ion mode for crude extract solution in *C. hirsuta*

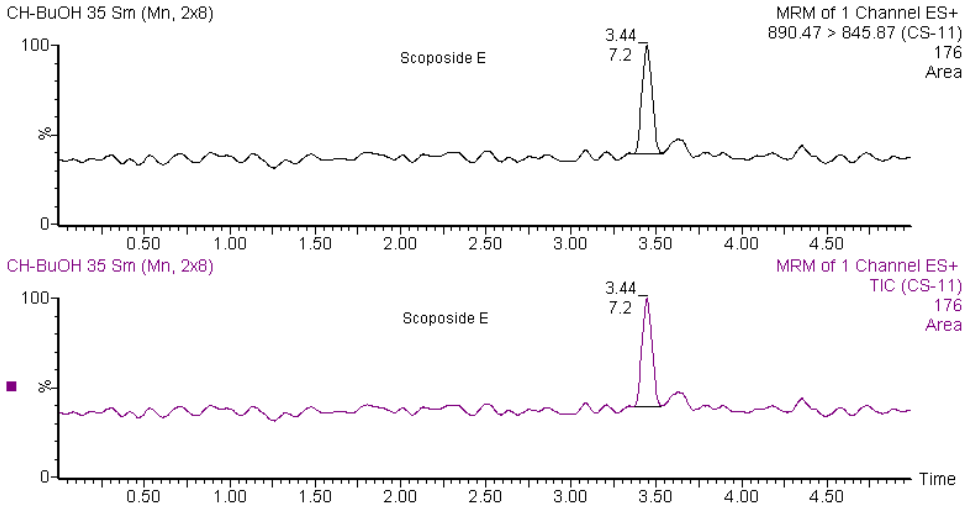


Figure 3.112 Chromatograms of Scoposide E (*m/z*: 890.47 → 845.87 and TIC), obtained by UPLC-ESI-MS/MS in the positive ion mode for crude extract solution in *C. hirsuta*

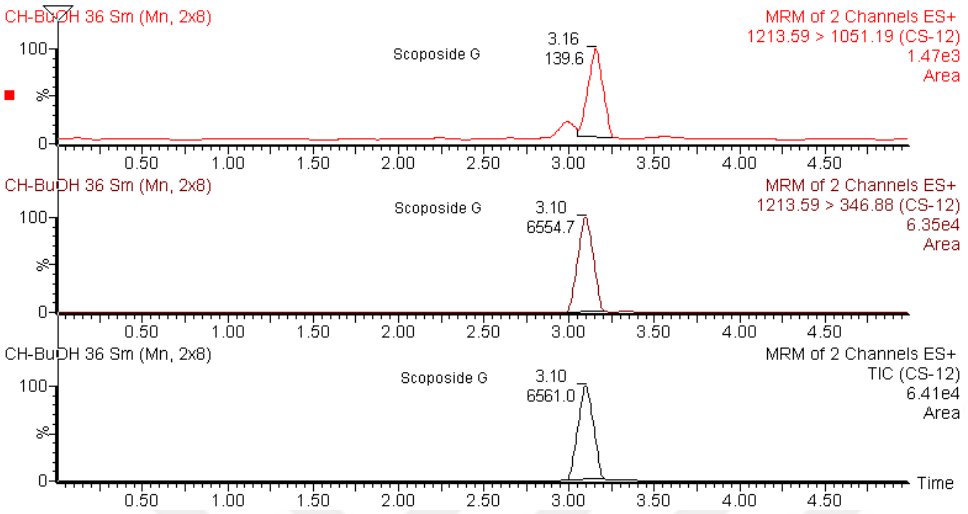


Figure 3.113 Chromatograms of Scoposide G (*m/z*: 1213.59 → 1051.19, 1213.59 → 346.88 and TIC), obtained by UPLC-ESI-MS/MS in the positive ion mode for crude extract solution in *C. hirsuta*

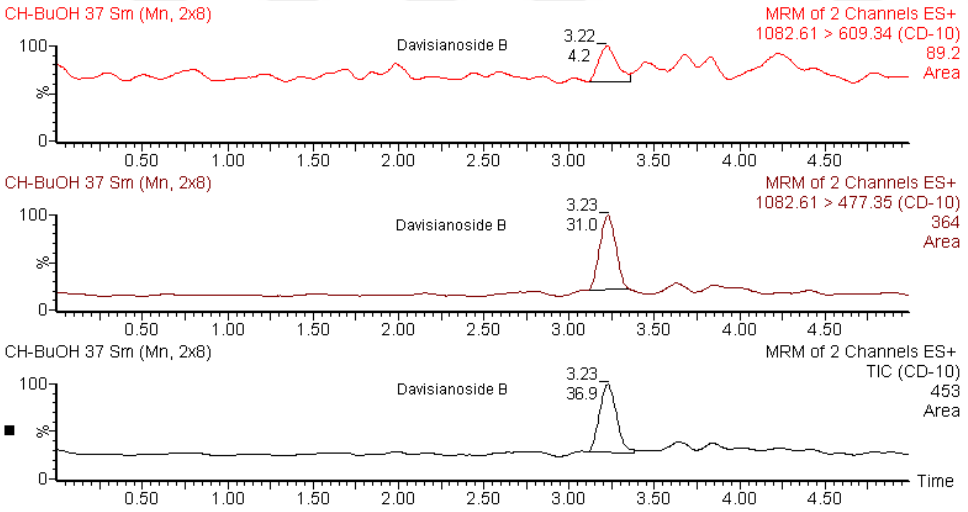


Figure 3.114 Chromatograms of Davisianoside B (*m/z*: 1082.61 → 609.34, 1082.61 → 477.35 and TIC), obtained by UPLC-ESI-MS/MS in the positive ion mode for crude extract solution in *C. hirsuta*

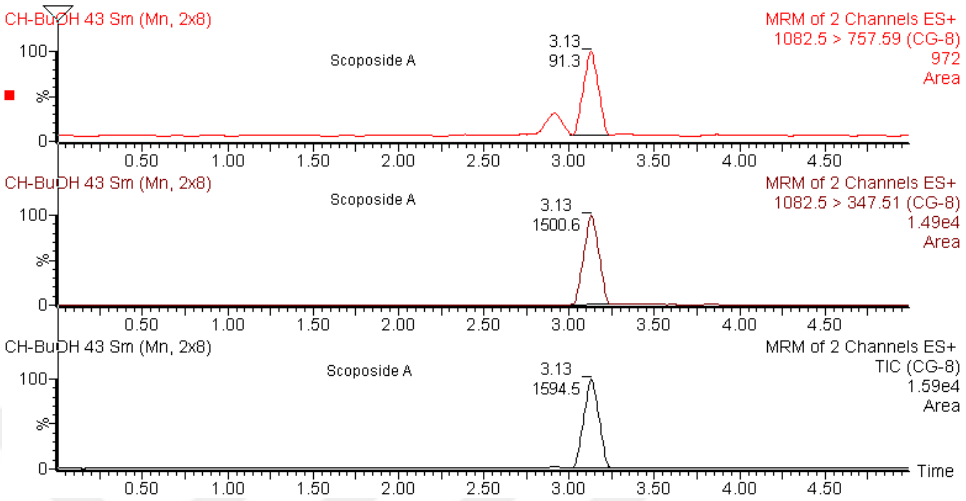


Figure 3.115 Chromatograms of Scoposide A (*m/z*: 1082.50 → 757.59, 1082.50 → 347.51 and TIC), obtained by UPLC-ESI-MS/MS in the positive ion mode for crude extract solution in *C. hirsuta*

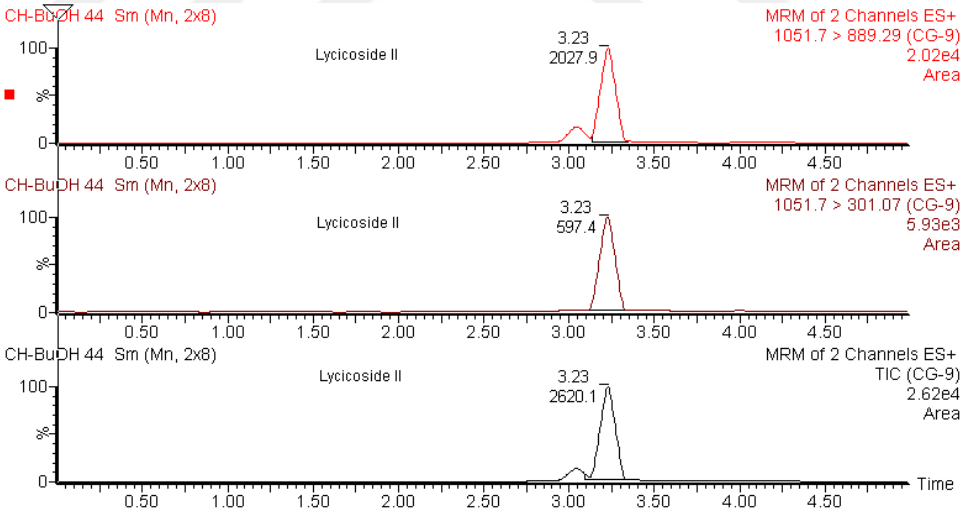


Figure 3.116 Chromatograms of Lycicoside II (*m/z*: 1051.70 → 889.29, 1051.70 → 301.07 and TIC), obtained by UPLC-ESI-MS/MS in the positive ion mode for crude extract solution in *C. hirsuta*

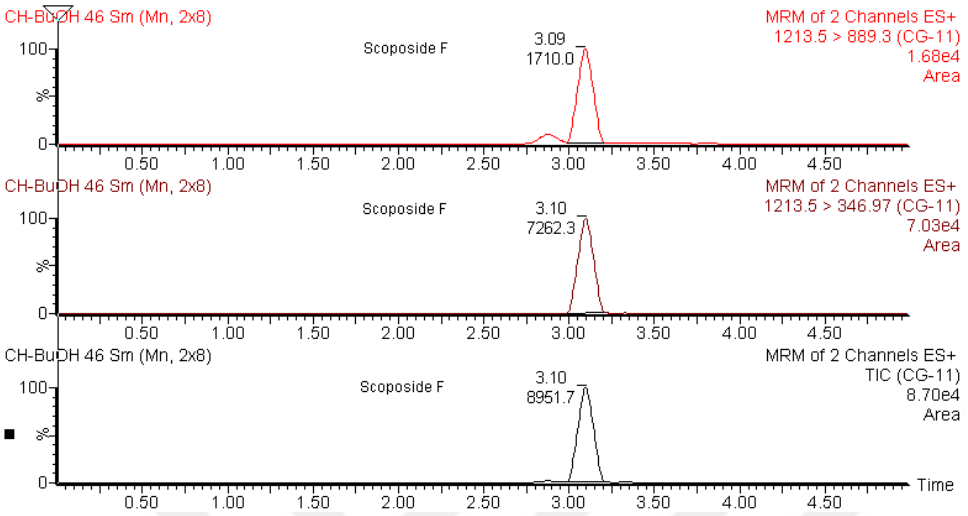


Figure 3.117 Chromatograms of Scoposide F (*m/z*: 1213.50 → 889.30, 1213.50 → 346.97 and TIC), obtained by UPLC-ESI-MS/MS in the positive ion mode for crude extract solution in *C. hirsuta*

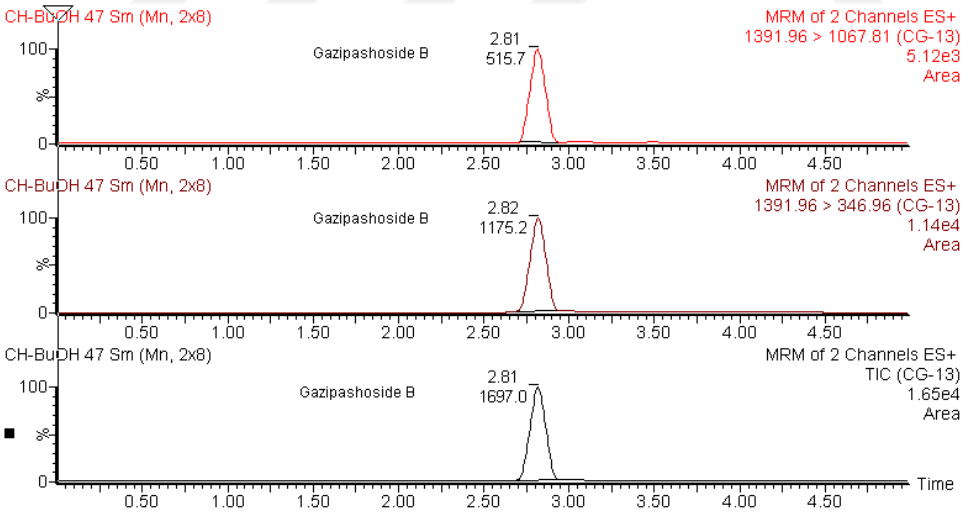


Figure 3.118 Chromatograms of Gazipashoside B (*m/z*: 1391.96 → 1067.81, 1391.96 → 346.96 and TIC), obtained by UPLC-ESI-MS/MS in the positive ion mode for crude extract solution in *C. hirsuta*

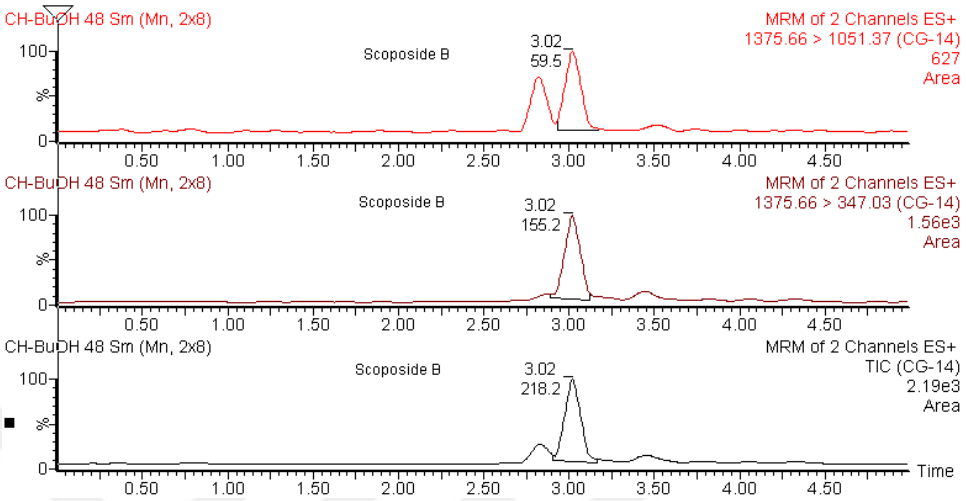


Figure 3.119 Chromatograms of Scoposide B (*m/z*: 1375.66 → 1051.37, 1375.66 → 347.03 and TIC), obtained by UPLC-ESI-MS/MS in the positive ion mode for crude extract solution in *C. hirsuta*

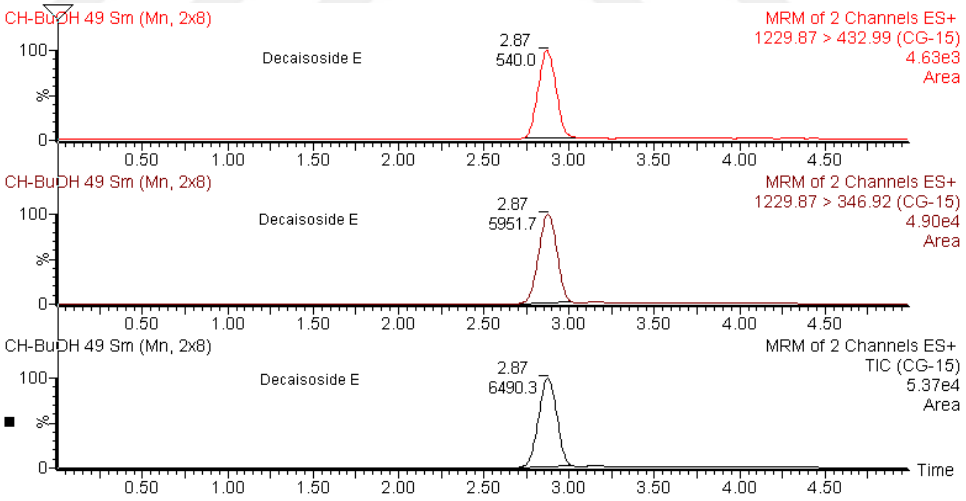


Figure 3.120 Chromatograms of Decaisoside E (*m/z*: 1229.87 → 432.99, 1229.87 → 346.92 and TIC), obtained by UPLC-ESI-MS/MS in the positive ion mode for crude extract solution in *C. hirsuta*

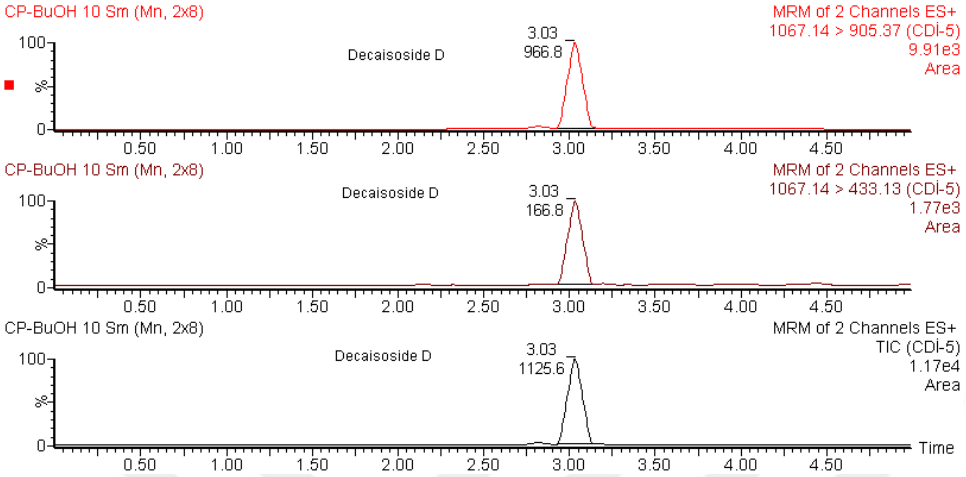


Figure 3.121 Chromatograms of Decaisoside D (*m/z*: 1067.14 → 905.37, 1067.14 → 433.13 and TIC), obtained by UPLC-ESI-MS/MS in the positive ion mode for crude extract solution in *C. procera*

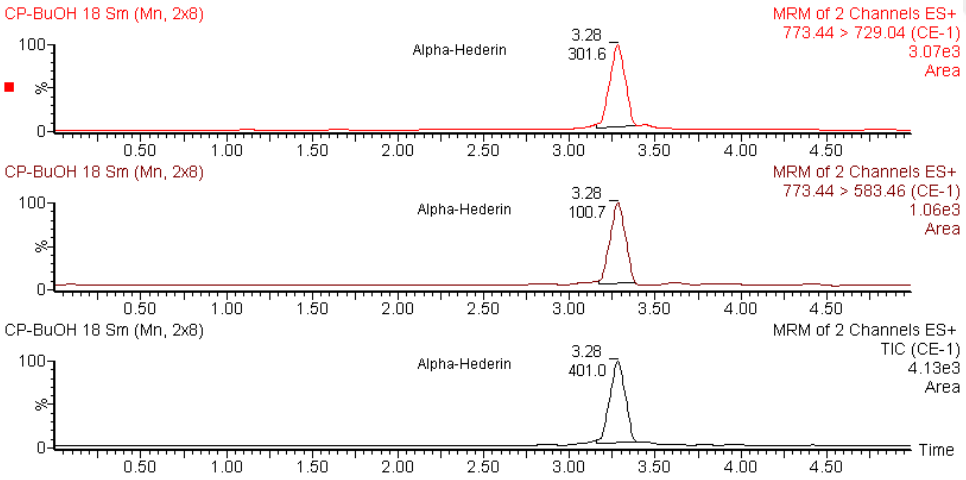


Figure 3.122 Chromatograms of *α*-Hederin (*m/z*: 773.44 → 729.04, 773.44 → 583.46 and TIC), obtained by UPLC-ESI-MS/MS in the positive ion mode for crude extract solution in *C. procera*

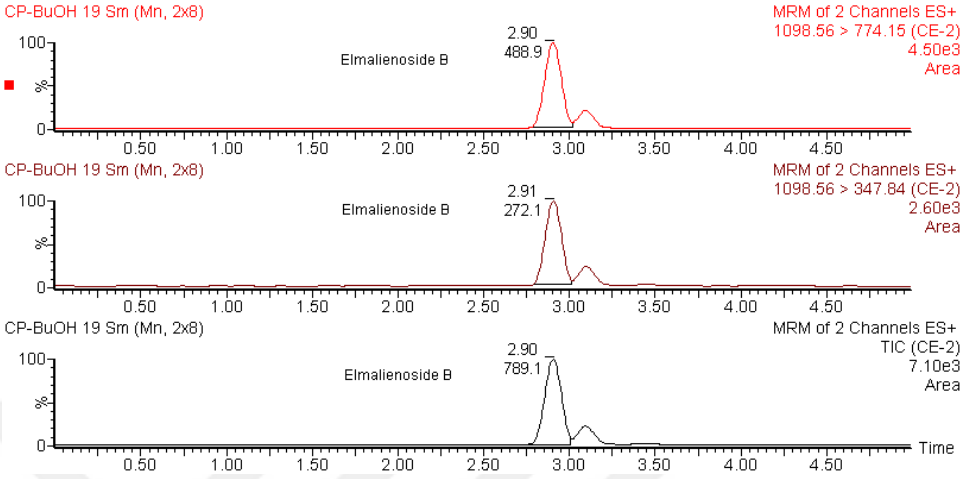


Figure 3.123 Chromatograms of Elmalienoside B (*m/z*: 1098.56 → 774.15, 1098.56 → 347.84 and TIC), obtained by UPLC-ESI-MS/MS in the positive ion mode for crude extract solution in *C. procera*

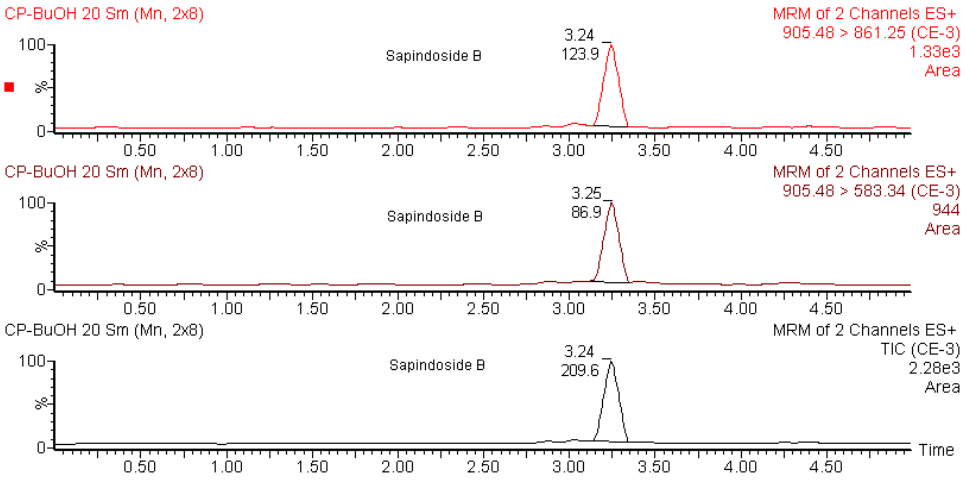


Figure 3.124 Chromatograms of Sapindoside B (*m/z*: 905.48 → 861.25, 905.48 → 583.34 and TIC), obtained by UPLC-ESI-MS/MS in the positive ion mode for crude extract solution in *C. procera*

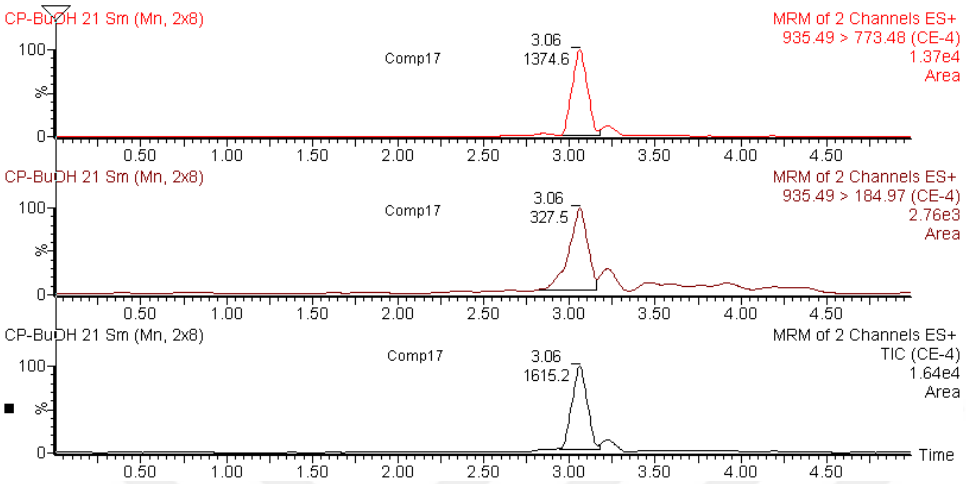


Figure 3.125 Chromatograms of 3-*O*-*α*-L-rhamnopyranosyl-(1→2)-*α*-L-arabinopyranosyl hederagenin 28-*O*-β-D-glucopyranosyl ester (*m/z*: 935.49 → 773.48, 935.49 → 184.97 and TIC), obtained by UPLC-ESI-MS/MS in the positive ion mode for crude extract solution in *C. procera*

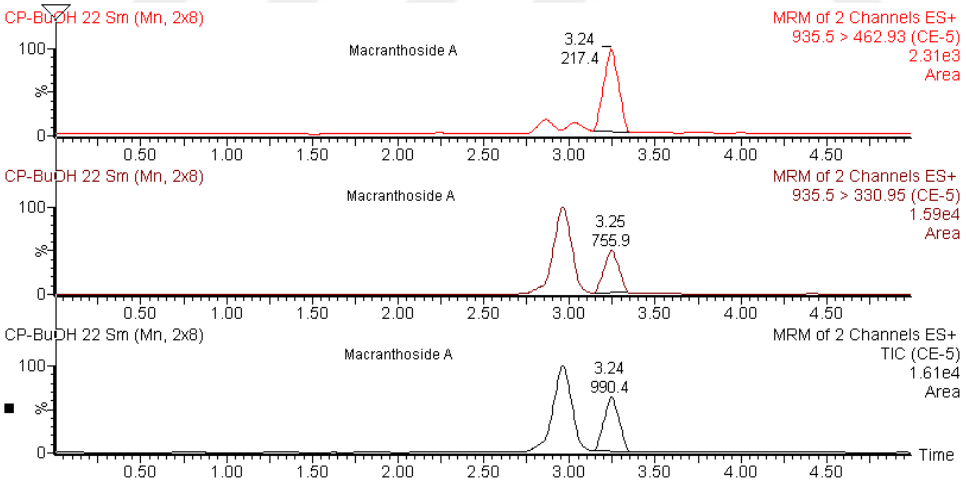


Figure 3.126 Chromatograms of Macranthoside A (*m/z*: 935.50 → 462.93, 935.50 → 330.95 and TIC), obtained by UPLC-ESI-MS/MS in the positive ion mode for crude extract solution in *C. procera*

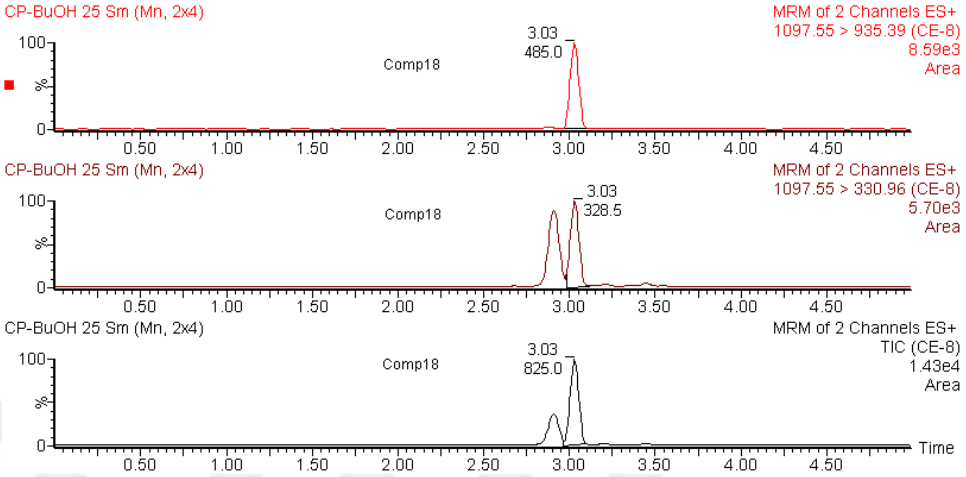


Figure 3.127 Chromatograms of 3-*O*-β-D-glucopyranosyl-(1→3)-*α*-L-rhamnopyranosyl-(1→2)-*α*-L-arabinopyranosyl hederagenin 28-*O*-β-D-glucopyranosyl ester (*m/z*: 1097.55 → 935.39, 1097.55 → 330.96 and TIC), obtained by UPLC-ESI-MS/MS in the positive ion mode for crude extract solution in *C. procera*

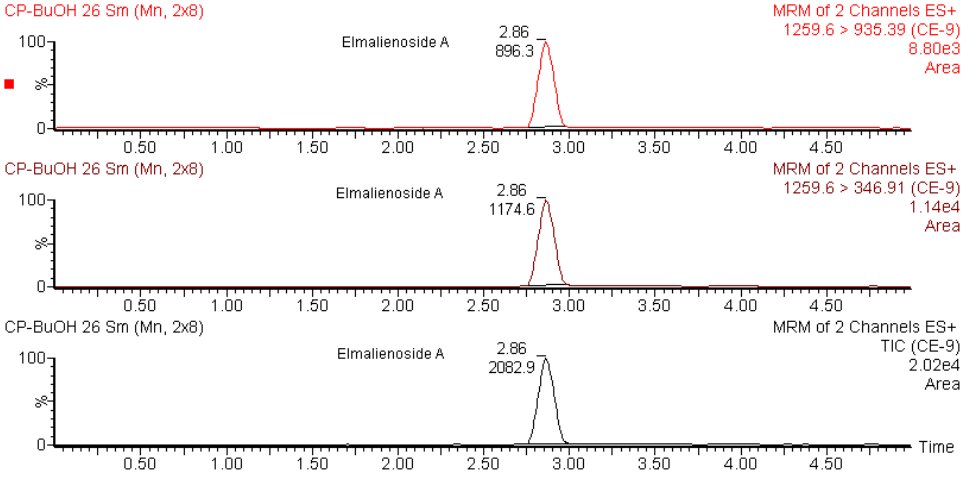


Figure 3.128 Chromatograms of Elmalienoside A (*m/z*: 1259.60 → 935.39, 1259.60 → 346.91 and TIC), obtained by UPLC-ESI-MS/MS in the positive ion mode for crude extract solution in *C. procera*

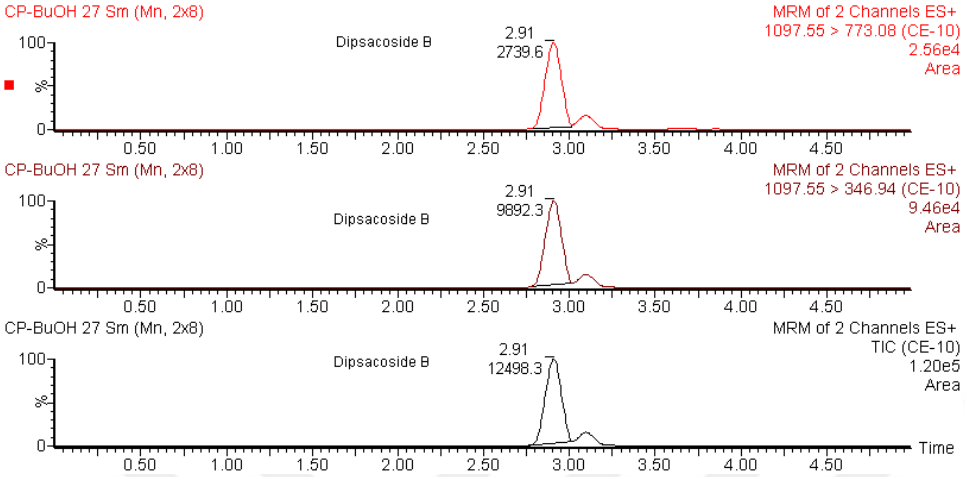


Figure 3.129 Chromatograms of Dipsacoside B (*m/z*: 1097.55 → 773.08, 1097.55 → 346.94 and TIC), obtained by UPLC-ESI-MS/MS in the positive ion mode for crude extract solution in *C. procera*

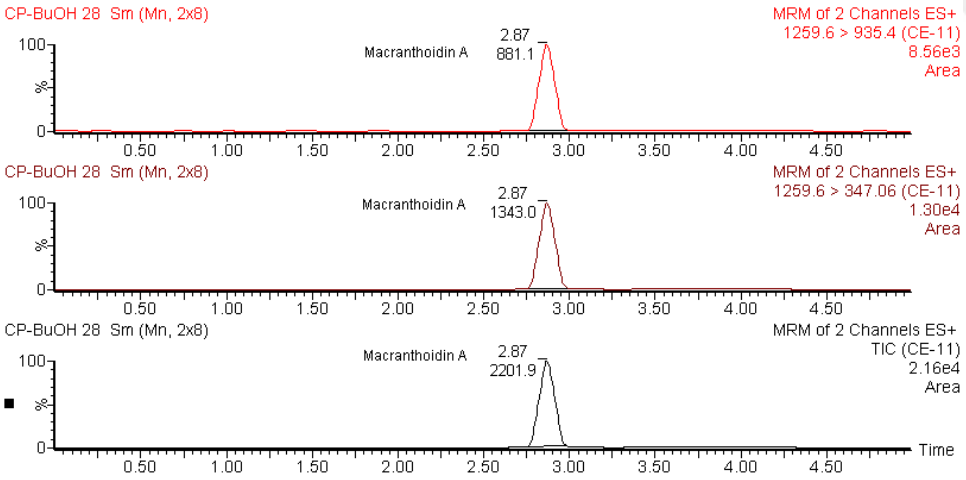


Figure 3.130 Chromatograms of Macranthoidin A (*m/z*: 1259.60 → 935.40, 1259.60 → 347.06 and TIC), compound obtained by UPLC-ESI-MS/MS in the positive ion mode for crude extract solution in *C. procera*

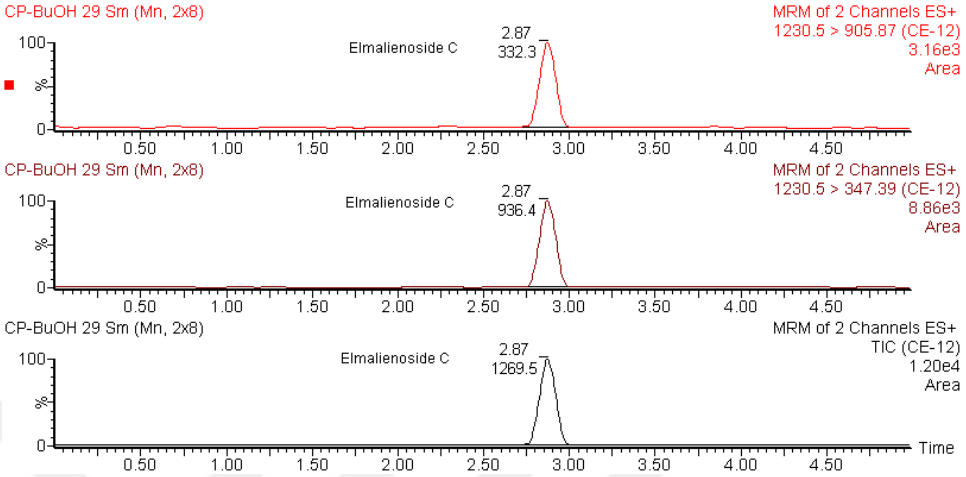


Figure 3.131 Chromatograms of Elmalienoside C (*m/z*: 1230.50 → 905.87, 1230.50 → 347.39 and TIC), obtained by UPLC-ESI-MS/MS in the positive ion mode for crude extract solution in *C. procera*

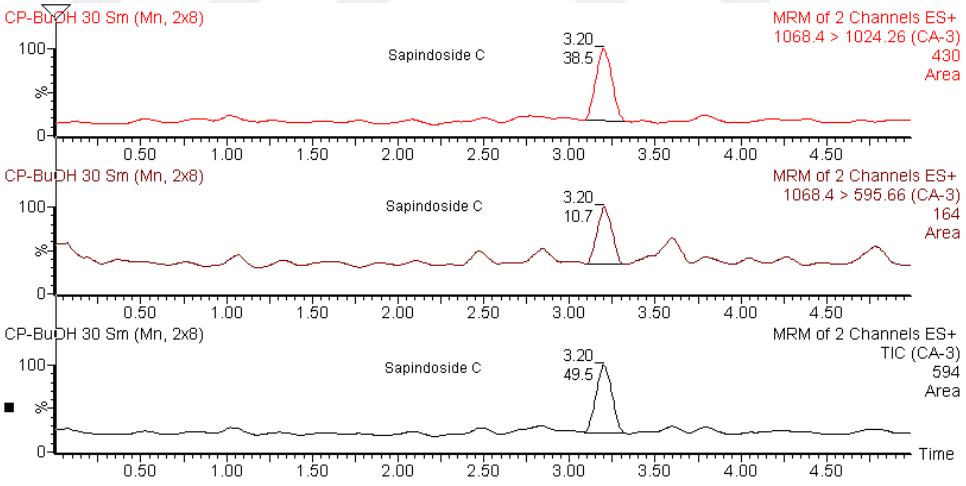


Figure 3.132 Chromatograms of Sapindoside C (*m/z*: 1068.40 → 1024.26, 1068.40 → 595.66 and TIC), obtained by UPLC-ESI-MS/MS in the positive ion mode for crude extract solution in *C. procera*

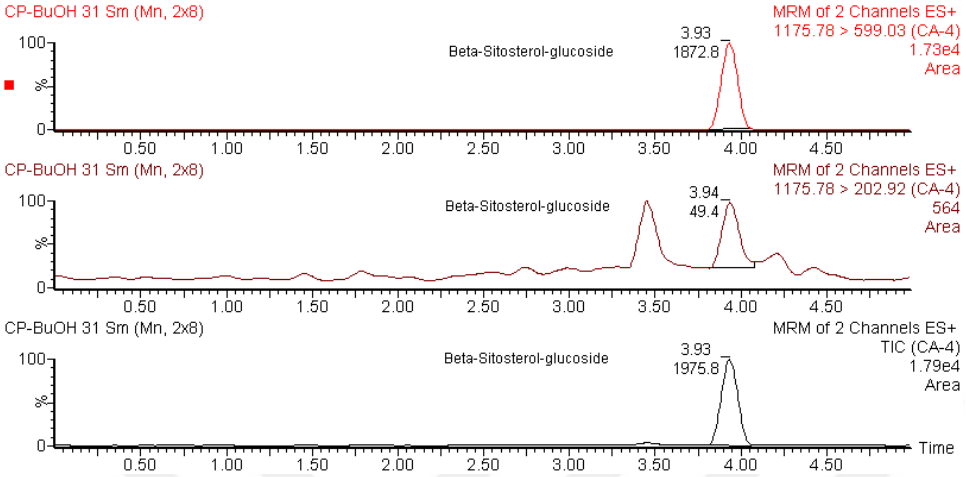


Figure 3.133 Chromatograms of *β*-Sitosterol-Glucoside (*m/z*: 1175.78 → 599.03, 1175.78 → 202.92 and TIC), obtained by UPLC-ESI-MS/MS in the positive ion mode for crude extract solution in *C. procera*

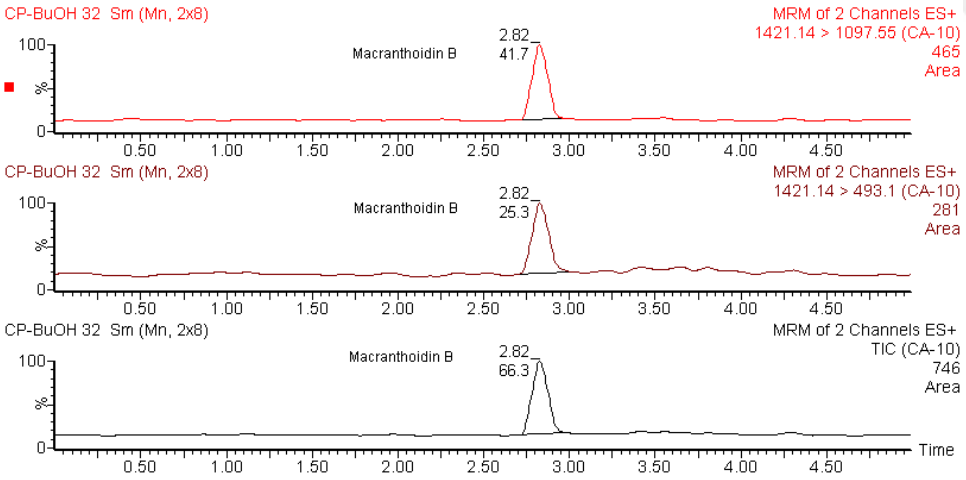


Figure 3.134 Chromatograms of Macranthoidin B (*m/z*: 1421.14 → 1097.55, 1421.14 → 493.1 and TIC), obtained by UPLC-ESI-MS/MS in the positive ion mode for crude extract solution in *C. procera*

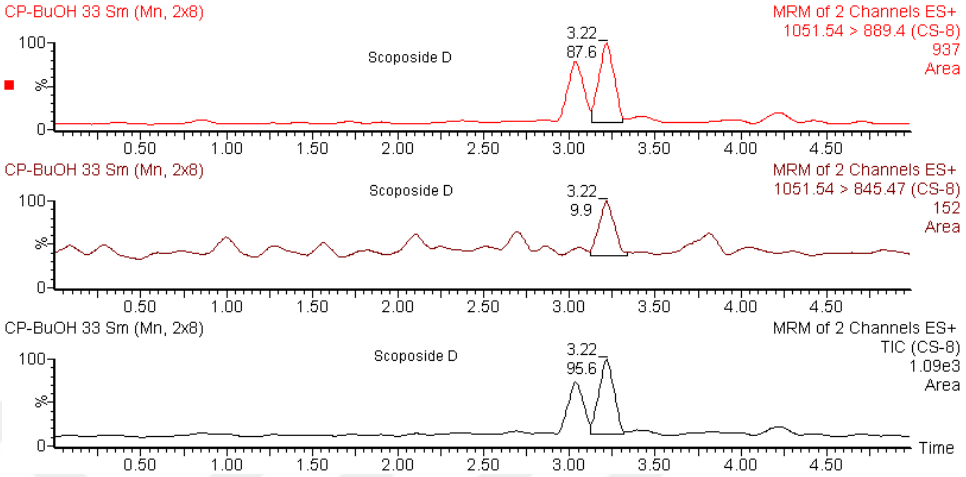


Figure 3.135 Chromatograms of Scoposide D (*m/z*: 1051.54 → 889.40, 1051.54 → 845.47 and TIC), obtained by UPLC-ESI-MS/MS in the positive ion mode for crude extract solution in *C. procera*

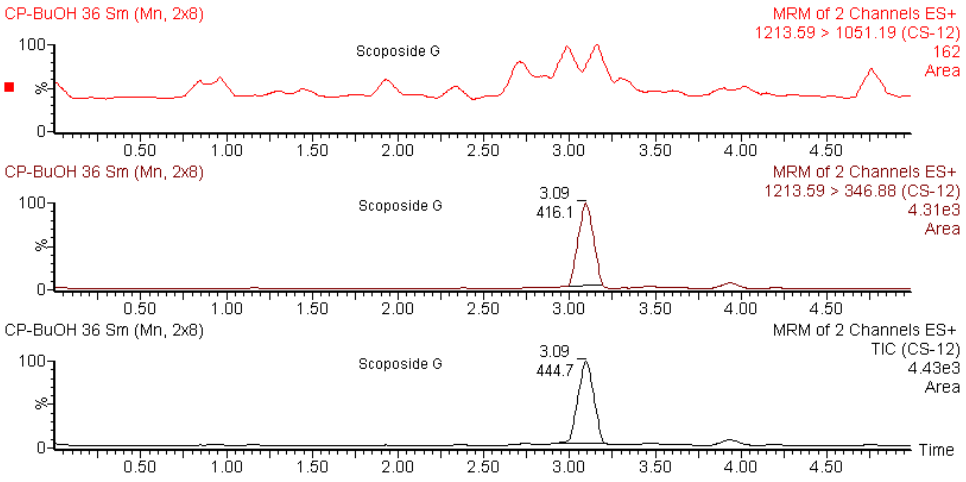


Figure 3.136 Chromatograms of Scoposide G (*m/z*: 1213.59 → 1051.19, 1213.59 → 346.88 and TIC), obtained by UPLC-ESI-MS/MS in the positive ion mode for crude extract solution in *C. procera*

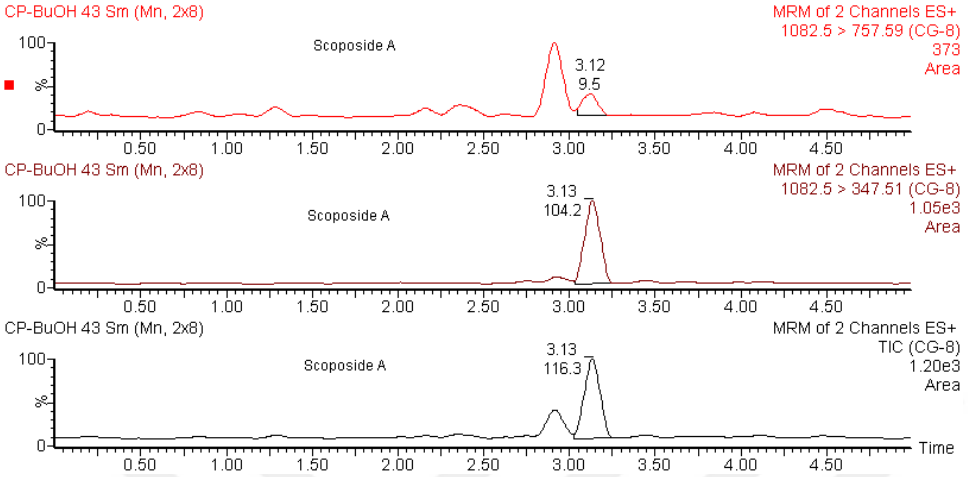


Figure 3.137 Chromatograms of Scoposide A (*m/z*: 1082.50 → 757.59, 1082.50 → 347.51 and TIC), obtained by UPLC-ESI-MS/MS in the positive ion mode for crude extract solution in *C. procera*

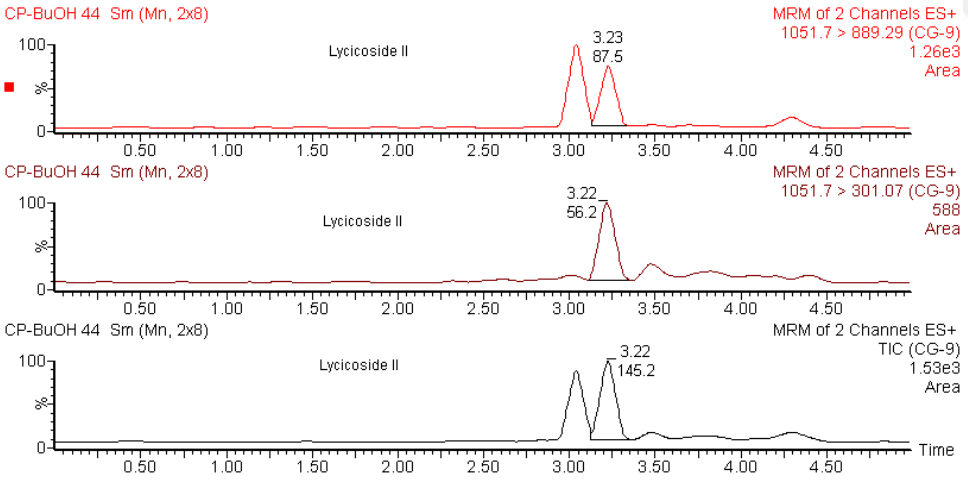


Figure 3.138 Chromatograms of Lycicoside II (*m/z*: 1051.70 → 889.29, 1051.70 → 301.07 and TIC), obtained by UPLC-ESI-MS/MS in the positive ion mode for crude extract solution in *C. procera*

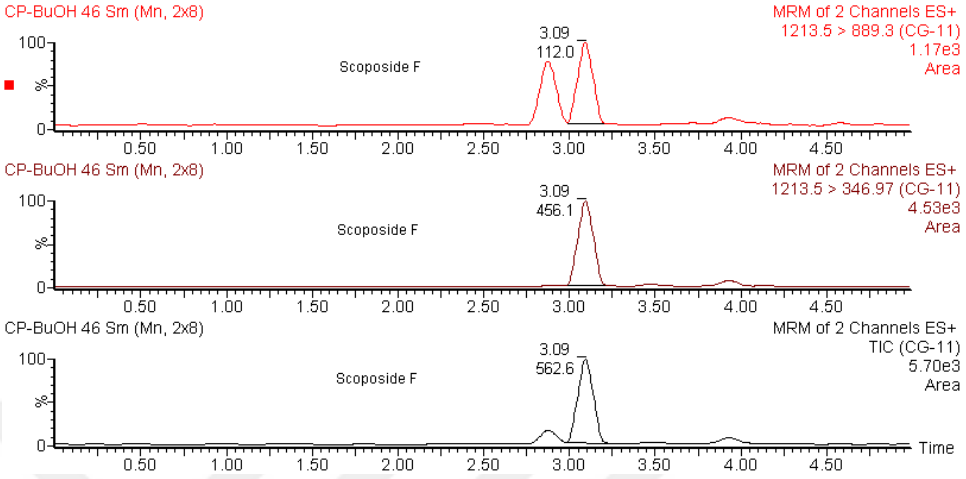


Figure 3.139 Chromatograms of Scoposide F (*m/z*: 1213.50 → 889.30, 1213.50 → 346.97 and TIC), obtained by UPLC-ESI-MS/MS in the positive ion mode for crude extract solution in *C. procera*

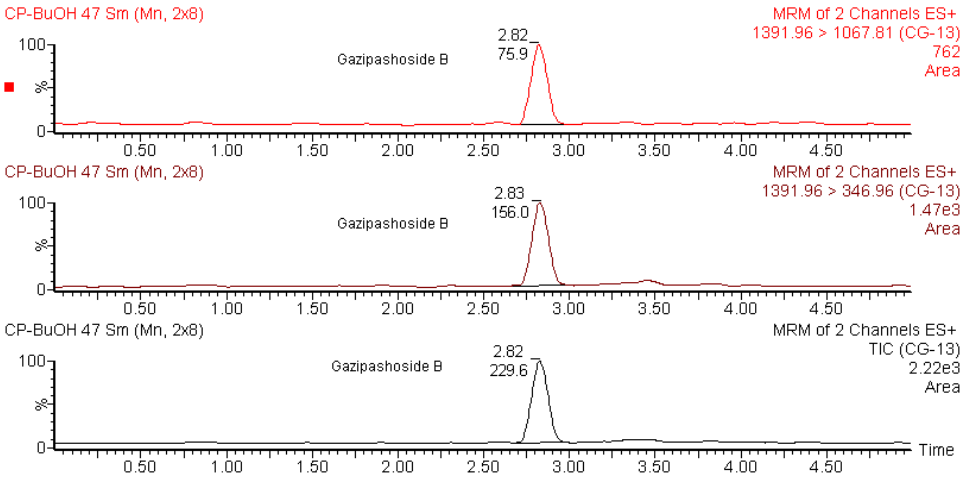


Figure 3.140 Chromatograms of Gazipashoside B (*m/z*: 1391.96 → 1067.81, 1391.96 → 346.96 and TIC), obtained by UPLC-ESI-MS/MS in the positive ion mode for crude extract solution in *C. procera*

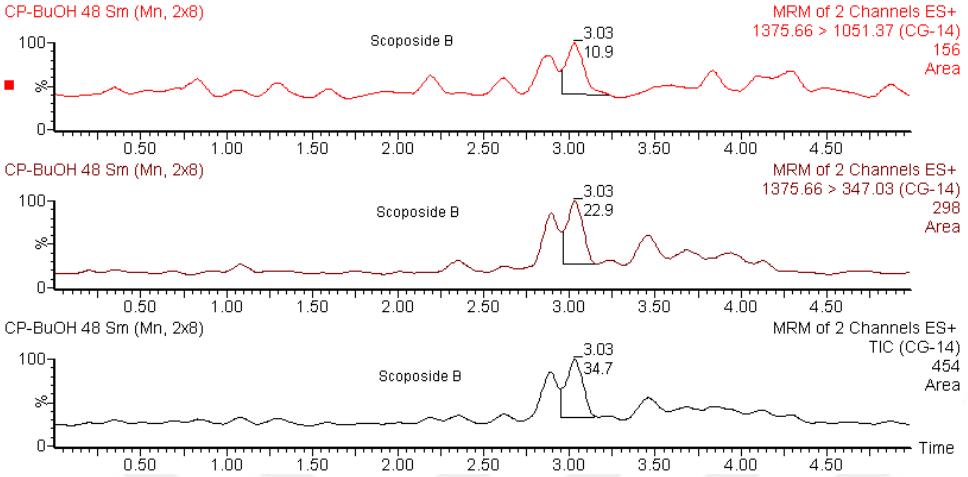


Figure 3.141 Chromatograms of Scoposide B (*m/z*: 1375.66 → 1051.37, 1375.66 → 347.03 and TIC), obtained by UPLC-ESI-MS/MS in the positive ion mode for crude extract solution in *C. procera*

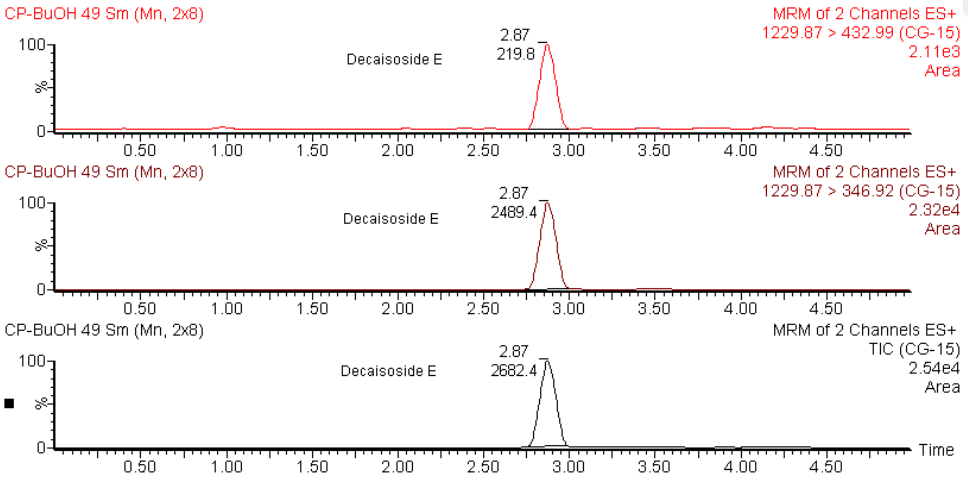


Figure 3.142 Chromatograms of Decaisoside E (*m/z*: 1229.87 → 432.99, 1229.87 → 346.92 and TIC), obtained by UPLC-ESI-MS/MS in the positive ion mode for crude extract solution in *C. procera*

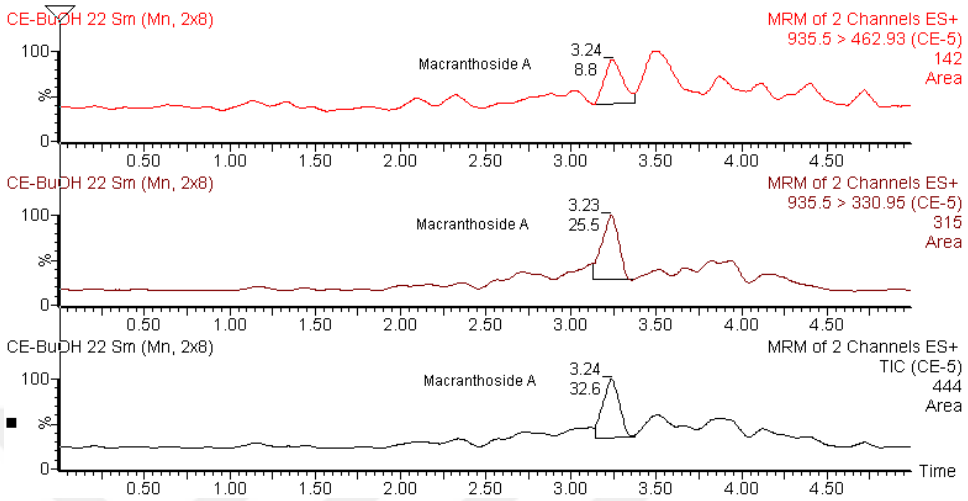


Figure 3.143 Chromatograms of Macranthoside A (*m/z*: 935.50 → 462.93, 935.50 → 330.95 and TIC), obtained by UPLC-ESI-MS/MS in the positive ion mode for crude extract solution in *C. elazigensis* var. *elazigensis*

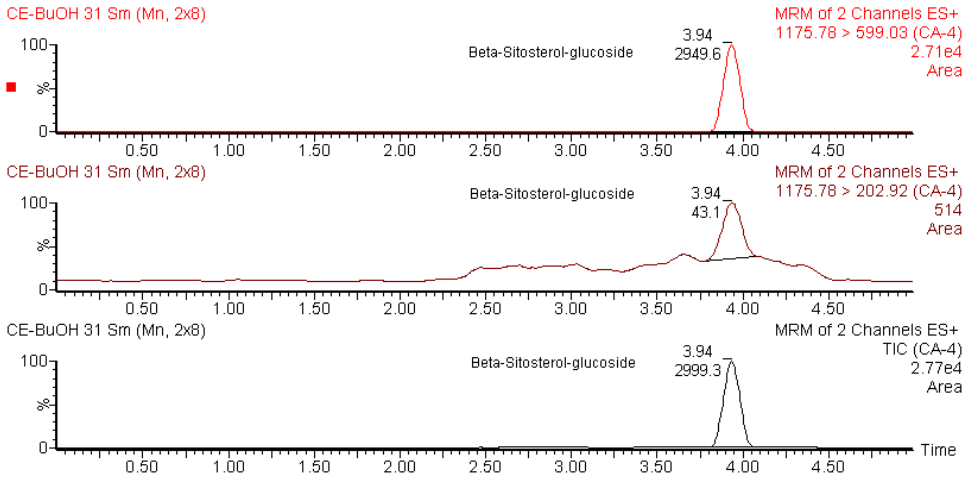


Figure 3.144 Chromatograms of *β*-Sitosterol-Glucoside (*m/z*: 1175.78 → 599.03, 1175.78 → 202.92 and TIC), obtained by UPLC-ESI-MS/MS in the positive ion mode for crude extract solution in *C. elazigensis* var. *elazigensis*

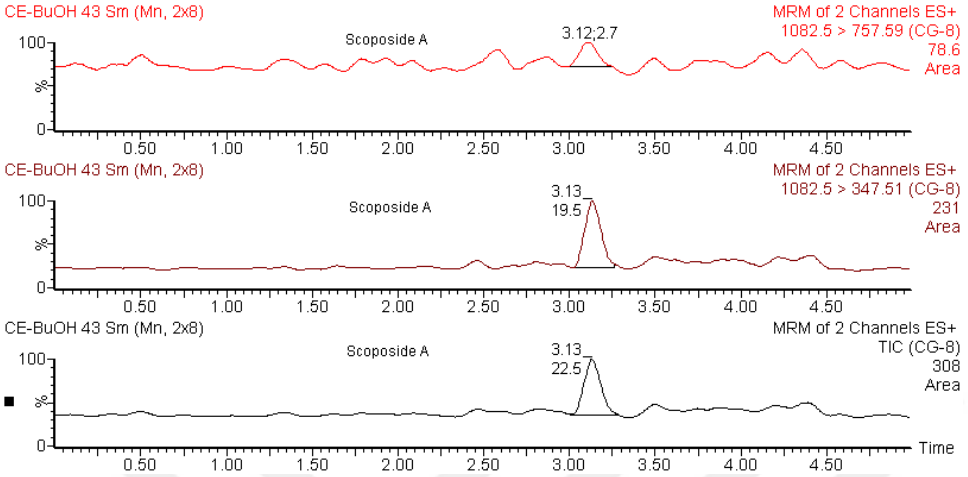


Figure 3.145 Chromatograms of Scoposide A (*m/z*: 1082.50 → 757.59, 1082.50 → 347.51 and TIC), obtained by UPLC-ESI-MS/MS in the positive ion mode for crude extract solution in *C. elazigensis* var. *elazigensis*

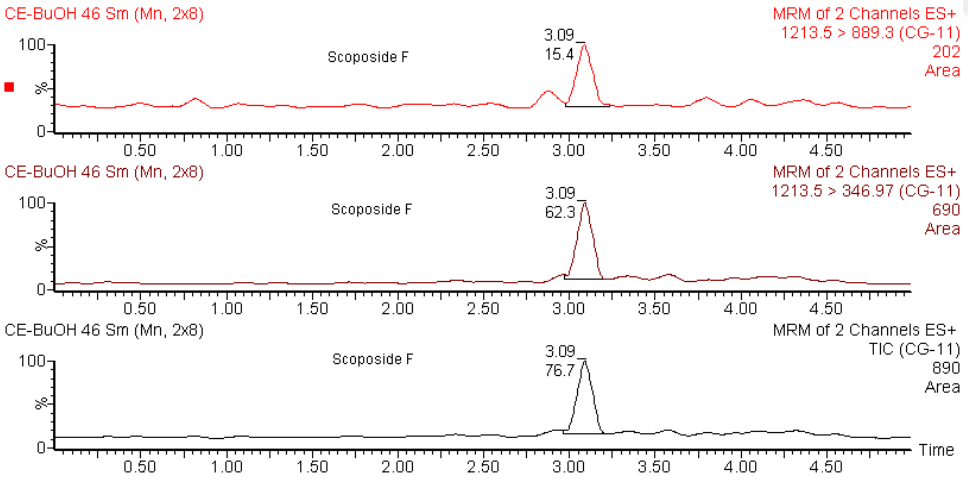


Figure 3.146 Chromatograms of Scoposide F (*m/z*: 1213.50 → 889.30, 1213.50 → 346.97 and TIC), obtained by UPLC-ESI-MS/MS in the positive ion mode for crude extract solution in *C. elazigensis* var. *elazigensis*

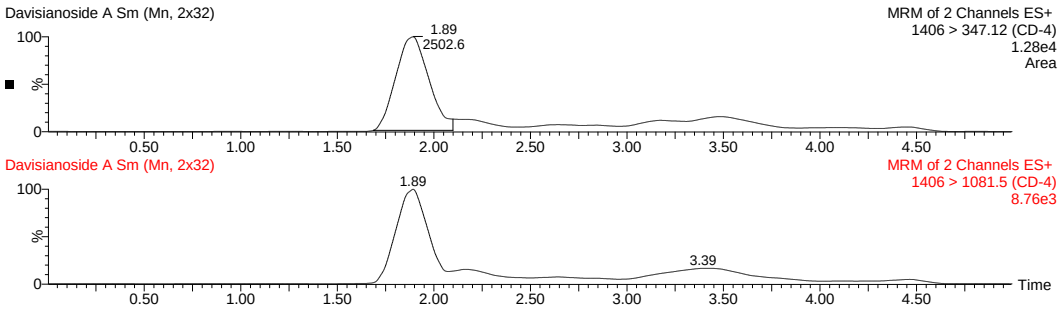


Figure 3.147 Chromatograms of Davisianoside A obtained by UPLC-ESI-MS/MS in the positive ion mode for standard solution (0.100 mg/L)

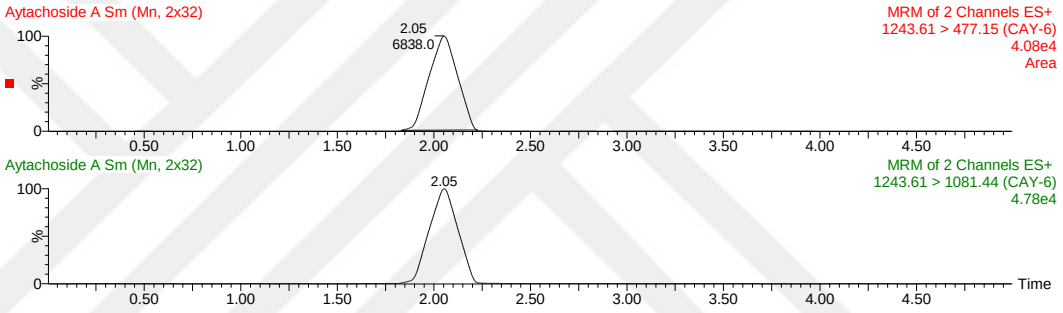


Figure 3.148 Chromatograms of Aytachoside A obtained by UPLC-ESI-MS/MS in the positive ion mode for standard solution (0.100 mg/L)

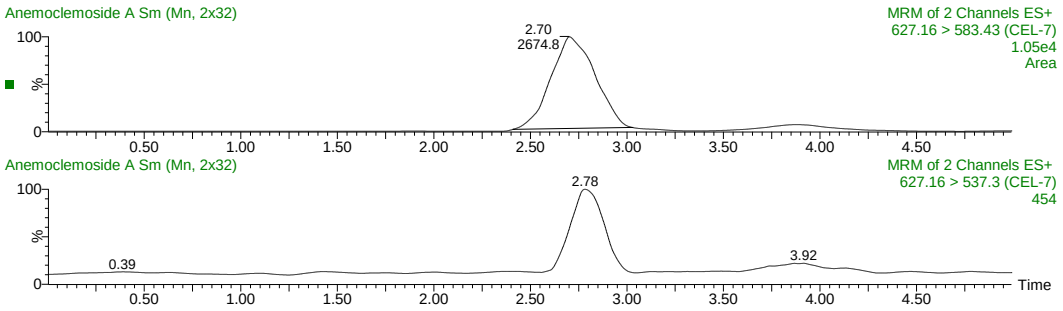


Figure 3.149 Chromatograms of Anemoclemoside A obtained by UPLC-ESI-MS/MS in the positive ion mode for standard solution (0.100 mg/L)

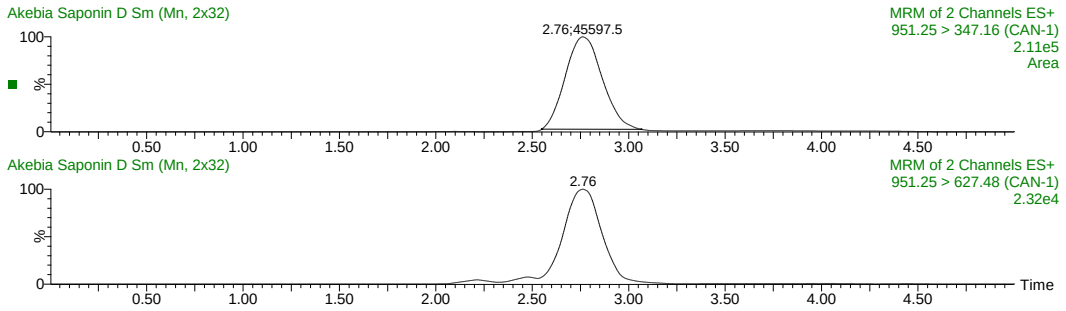


Figure 3.150 Chromatograms of Akebia Saponin D obtained by UPLC-ESI-MS/MS in the positive ion mode for standard solution (0.100 mg/L)

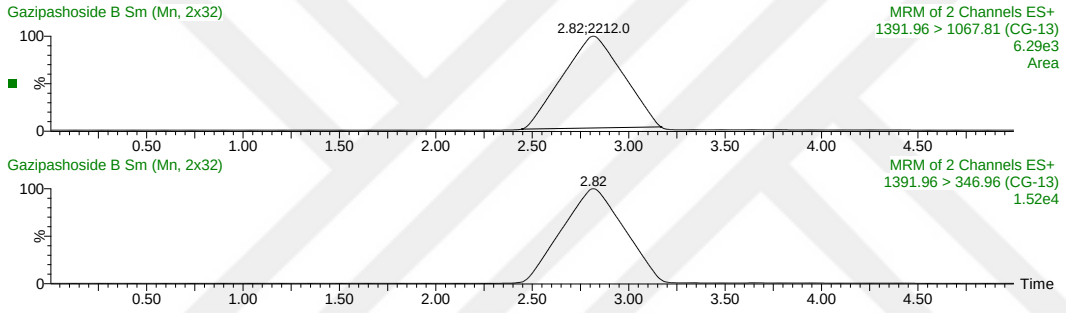


Figure 3.151 Chromatograms of Gazipashoside B obtained by UPLC-ESI-MS/MS in the positive ion mode for standard solution (0.100 mg/L)

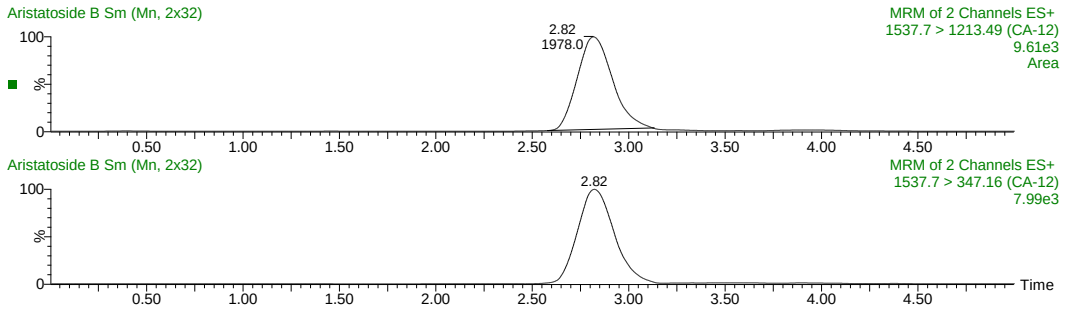


Figure 3.152 Chromatograms of Aristatoside B obtained by UPLC-ESI-MS/MS in the positive ion mode for standard solution (0.100 mg/L)

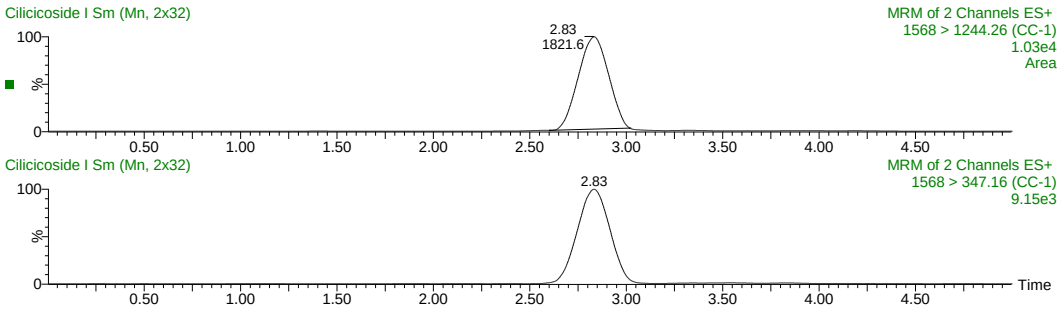


Figure 3.153 Chromatograms of Cilicicoside I obtained by UPLC-ESI-MS/MS in the positive ion mode for standard solution (0.100 mg/L)

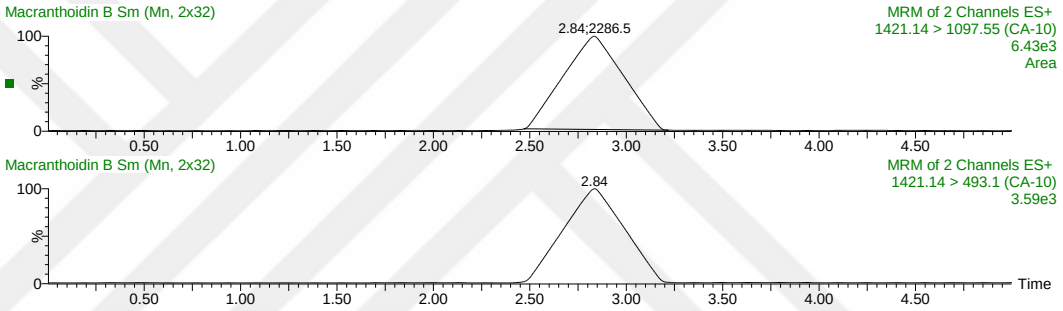


Figure 3.154 Chromatograms of Macranthoidin B obtained by UPLC-ESI-MS/MS in the positive ion mode for standard solution (0.100 mg/L)

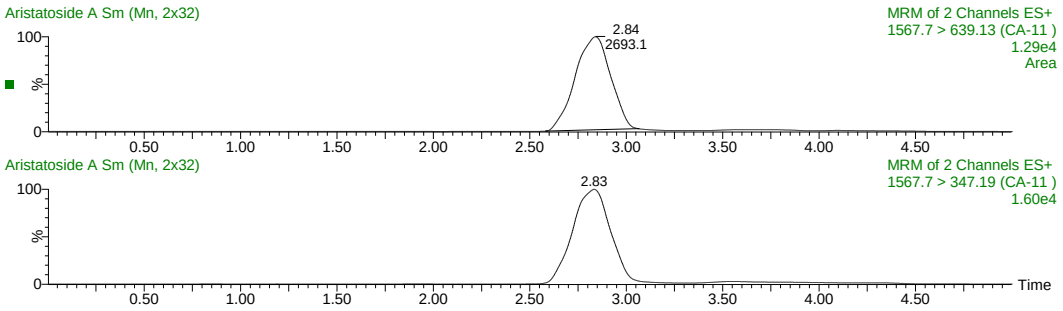


Figure 3.155 Chromatograms of Aristatoside A obtained by UPLC-ESI-MS/MS in the positive ion mode for standard solution (0.100 mg/L)

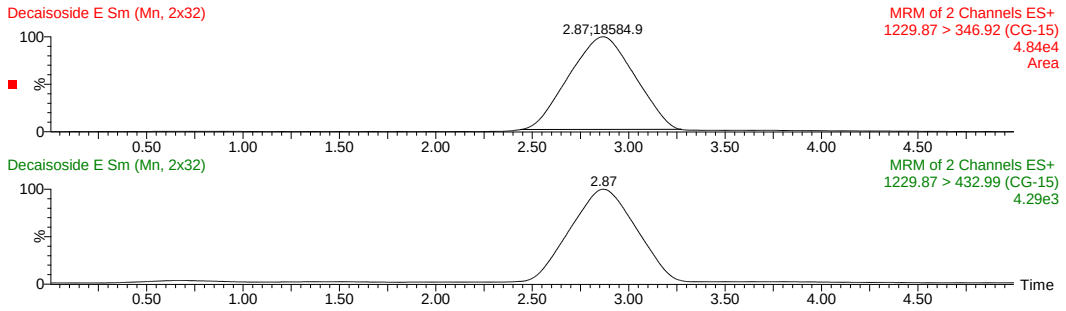


Figure 3.156 Chromatograms of Decaisoside E obtained by UPLC-ESI-MS/MS in the positive ion mode for standard solution (0.100 mg/L)

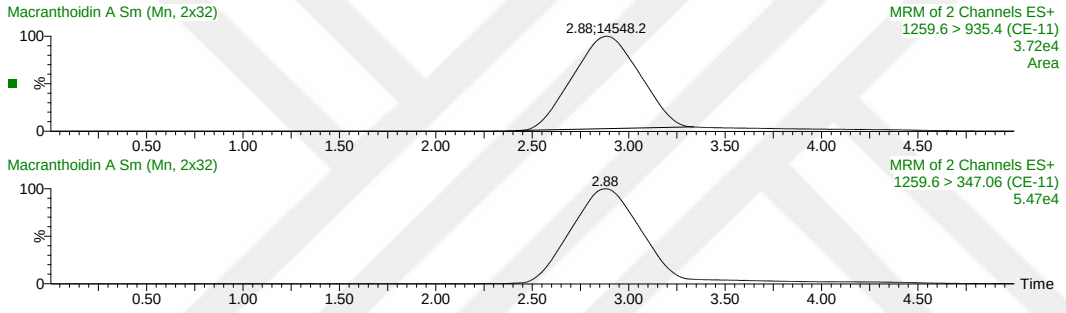


Figure 3.157 Chromatograms of Macranthoidin A obtained by UPLC-ESI-MS/MS in the positive ion mode for standard solution (0.100 mg/L)

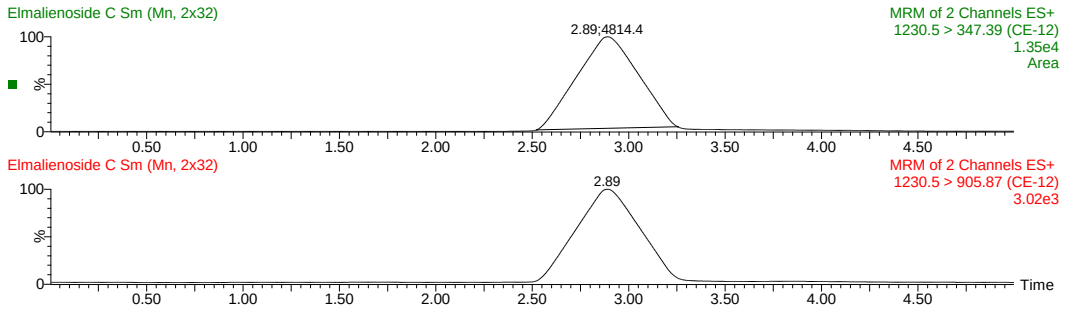


Figure 3.158 Chromatograms of Elmalienoside C obtained by UPLC-ESI-MS/MS in the positive ion mode for standard solution (0.100 mg/L)

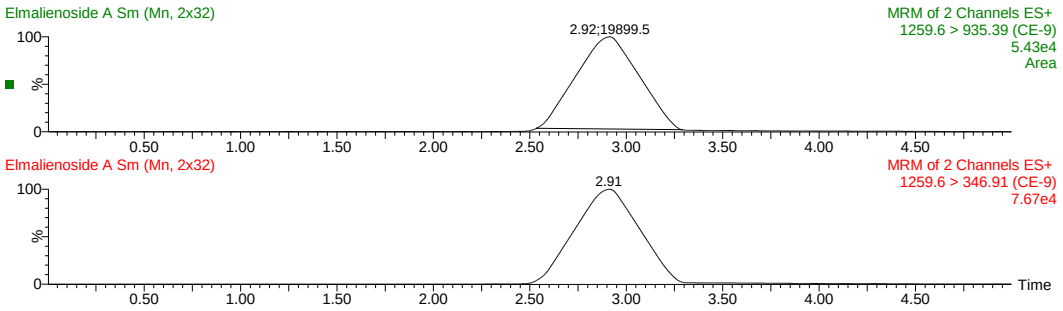


Figure 3.159 Chromatograms of Elmalienoside A obtained by UPLC-ESI-MS/MS in the positive ion mode for standard solution (0.100 mg/L)

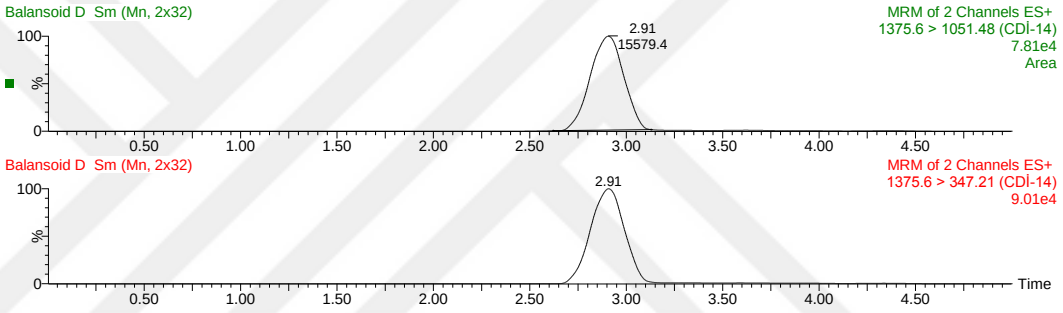


Figure 3.160 Chromatograms of Balansoid D obtained by UPLC-ESI-MS/MS in the positive ion mode for standard solution (0.100 mg/L)

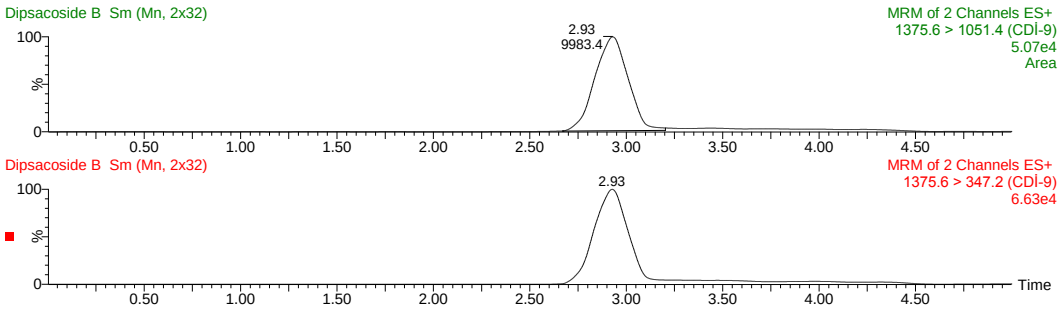


Figure 3.161 Chromatograms of Dipsacoside B obtained by UPLC-ESI-MS/MS in the positive ion mode for standard solution (0.100 mg/L)

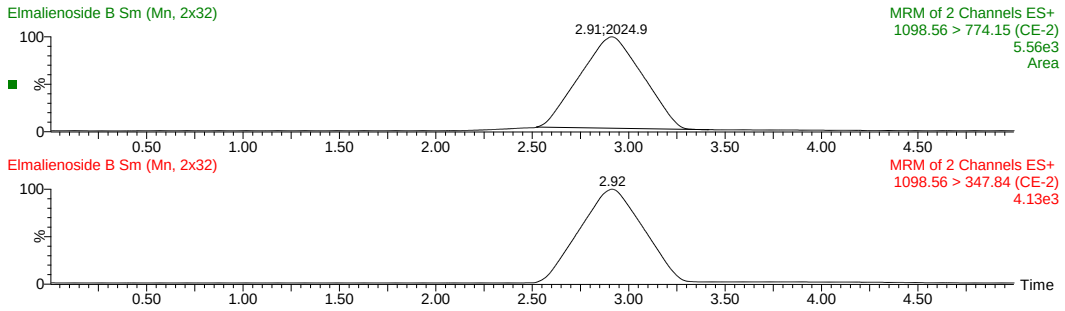


Figure 3.162 Chromatograms of Elmalienoside B obtained by UPLC-ESI-MS/MS in the positive ion mode for standard solution (0.100 mg/L)

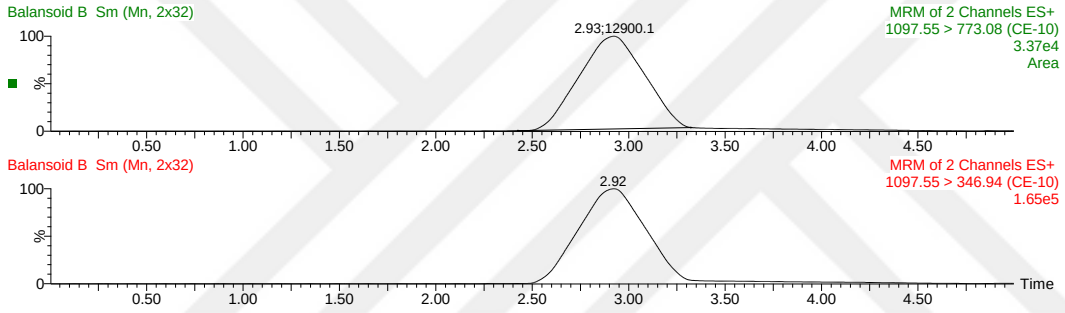


Figure 3.163 Chromatograms of Balansoid B obtained by UPLC-ESI-MS/MS in the positive ion mode for standard solution (0.100 mg/L)

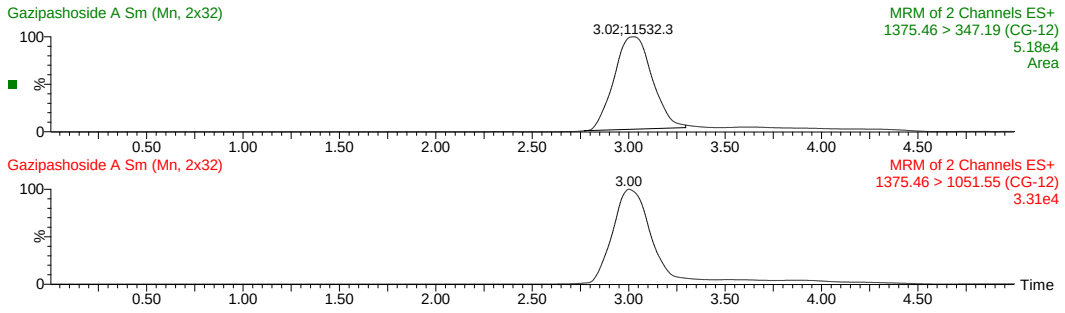


Figure 3.164 Chromatograms of Gazipashoside A obtained by UPLC-ESI-MS/MS in the positive ion mode for standard solution (0.100 mg/L)

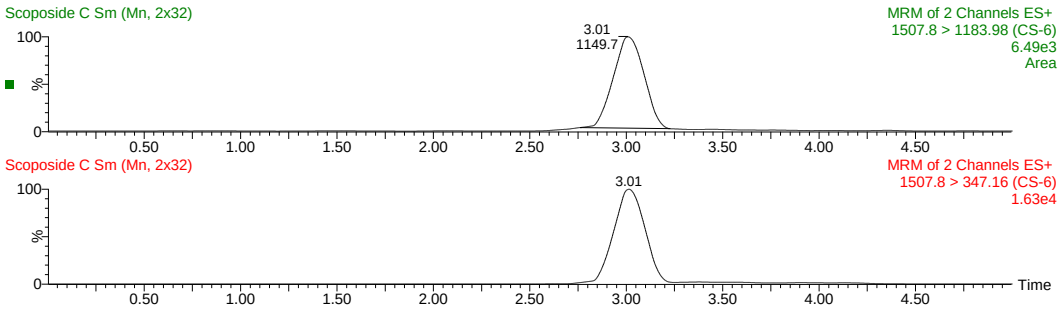


Figure 3.165 Chromatograms of Scoposide C obtained by UPLC-ESI-MS/MS in the positive ion mode for standard solution (0.100 mg/L)

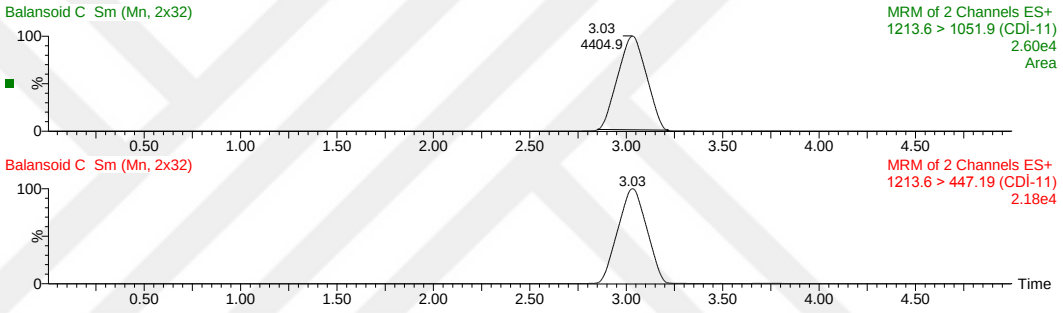


Figure 3.166 Chromatograms of Balansoid C obtained by UPLC-ESI-MS/MS in the positive ion mode for standard solution (0.100 mg/L)

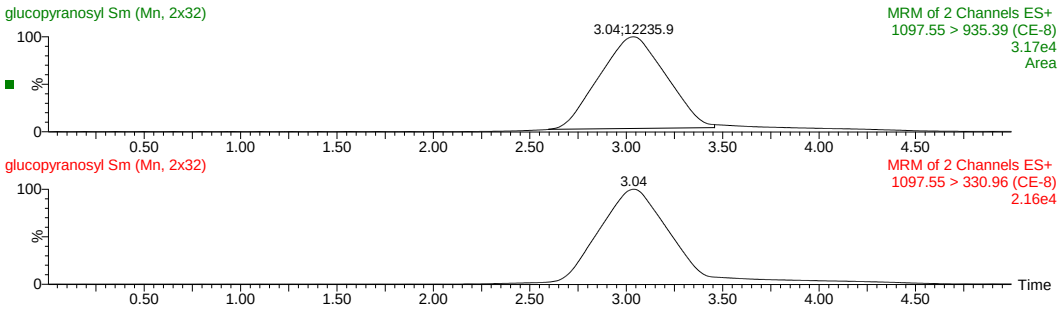


Figure 3.167 Chromatograms of 3-*O*-β-D-glucopyranosyl-(1→3)-*α*-L-rhamnopyranosyl-(1→2)-*α*-*L*-arabinopyranosyl hederagenin 28-*O*-β-D-glucopyranosyl ester obtained by UPLC-ESI-MS/MS in the positive ion mode for standard solution (0.100 mg/L)

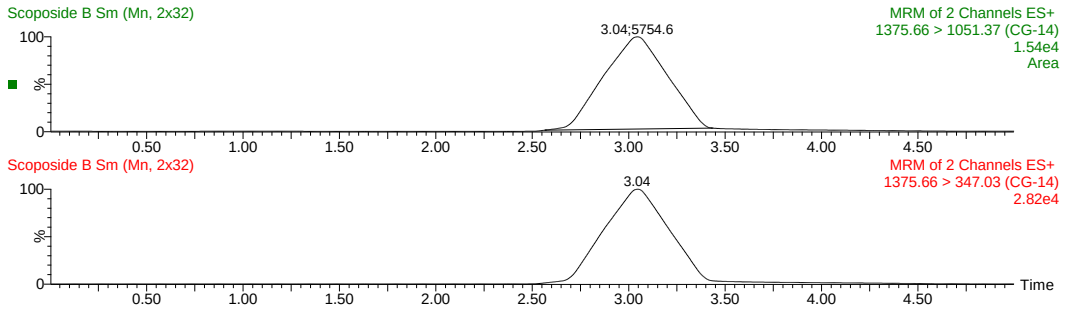


Figure 3.168 Chromatograms of Scoposide B obtained by UPLC-ESI-MS/MS in the positive ion mode for standard solution (0.100 mg/L)

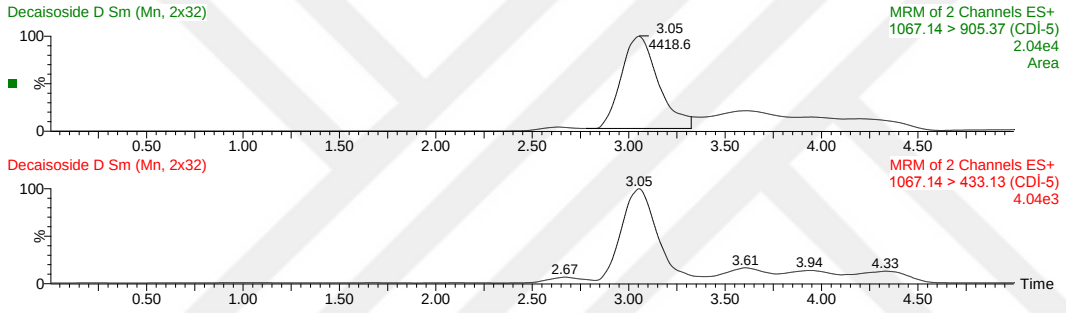


Figure 3.169 Chromatograms of Decaisoside D obtained by UPLC-ESI-MS/MS in the positive ion mode for standard solution (0.100 mg/L)

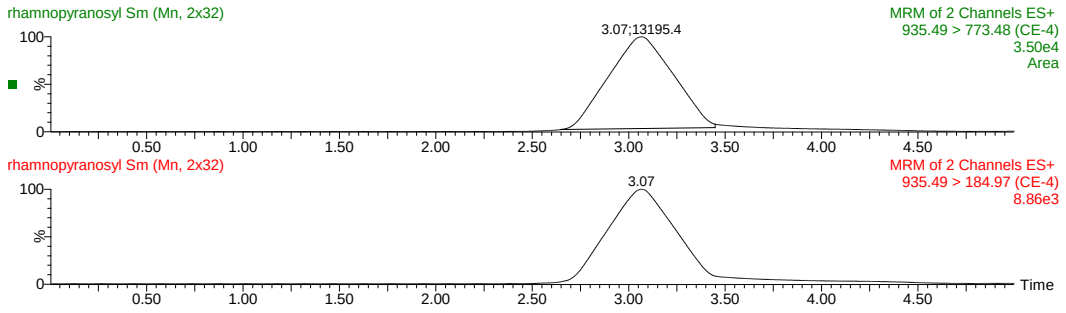


Figure 3.170 Chromatograms of 3-*O*-*α*-L-rhamnopyranosyl-(1→2)-*α*-L-arabinopyranosyl hederagenin 28-*O*-β-D-glucopyranosyl ester obtained by UPLC-ESI-MS/MS in the positive ion mode for standard solution (0.100 mg/L)

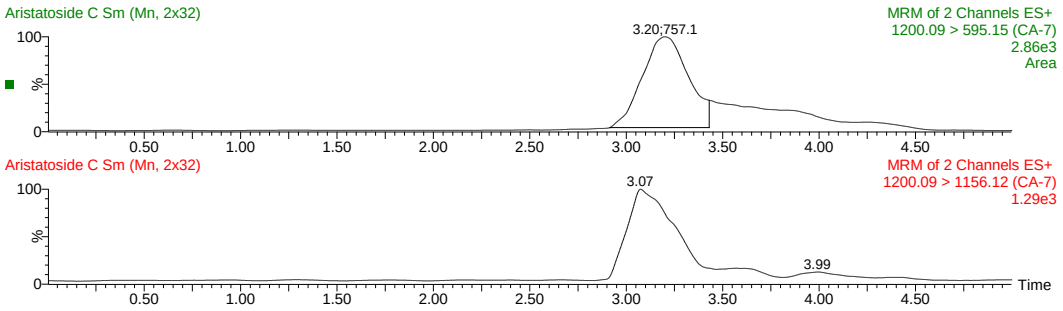


Figure 3.171 Chromatograms of Aristatoside C obtained by UPLC-ESI-MS/MS in the positive ion mode for standard solution (0.100 mg/L)

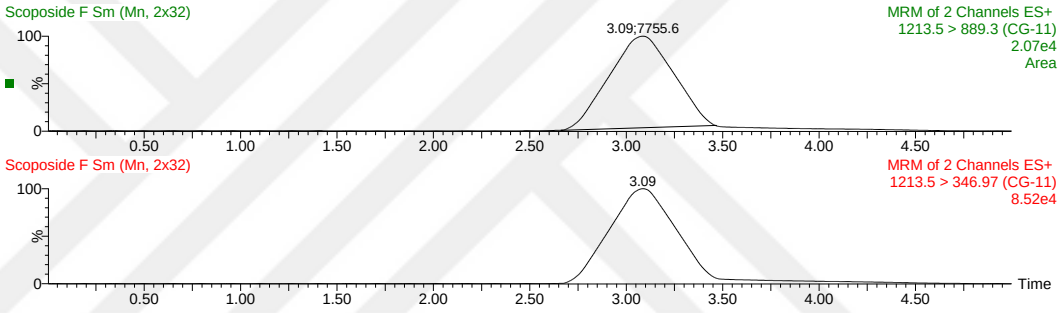


Figure 3.172 Chromatograms of Scoposide F obtained by UPLC-ESI-MS/MS in the positive ion mode for standard solution (0.100 mg/L)

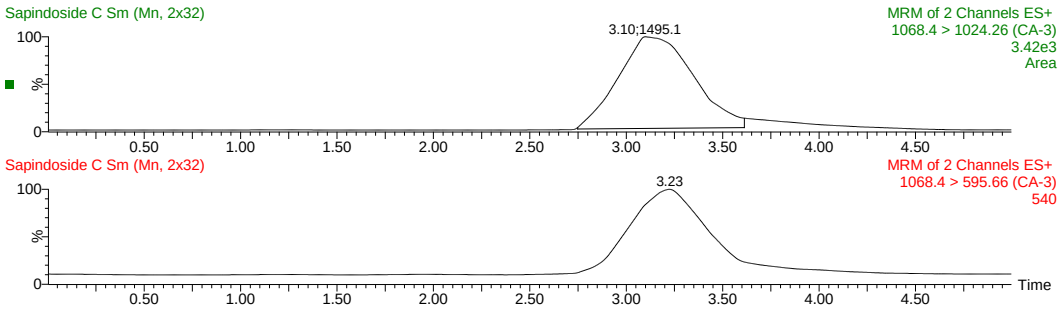


Figure 3.173 Chromatograms of Sapindoside C obtained by UPLC-ESI-MS/MS in the positive ion mode for standard solution (0.100 mg/L)

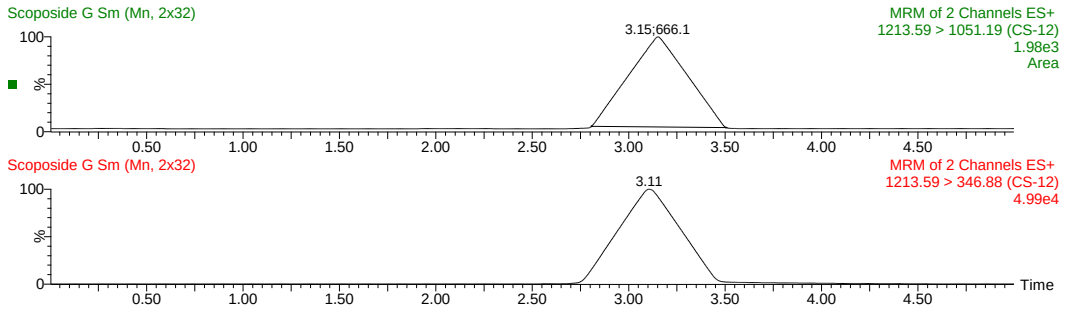


Figure 3.174 Chromatograms of Scoposide G obtained by UPLC-ESI-MS/MS in the positive ion mode for standard solution (0.100 mg/L)

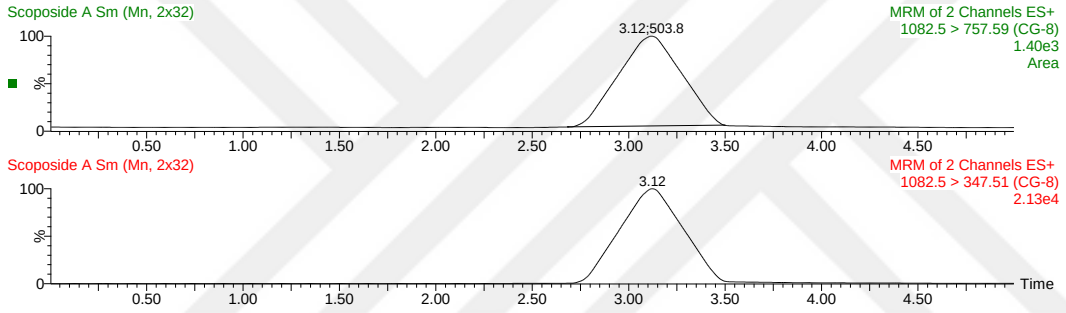


Figure 3.175 Chromatograms of Scoposide A obtained by UPLC-ESI-MS/MS in the positive ion mode for standard solution (0.100 mg/L)

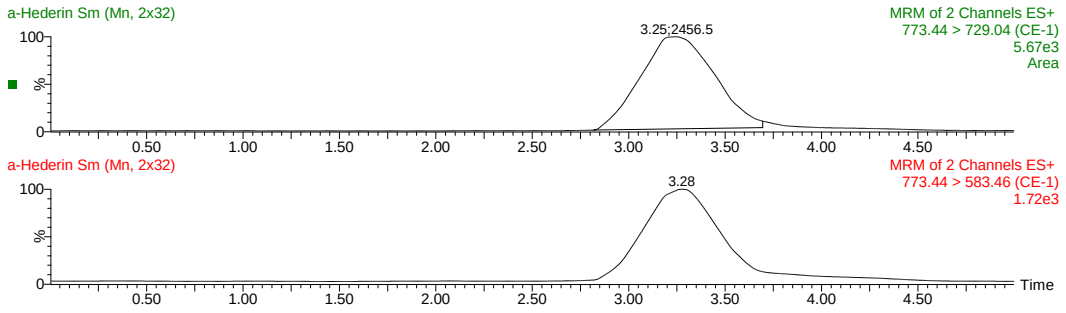


Figure 3.176 Chromatograms of α-Hederin obtained by UPLC-ESI-MS/MS in the positive ion mode for standard solution (0.100 mg/L)

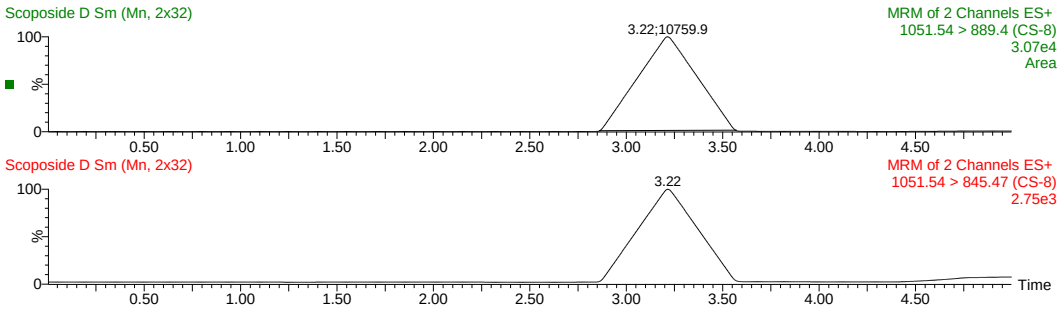


Figure 3.177 Chromatograms of Scoposide D obtained by UPLC-ESI-MS/MS in the positive ion mode for standard solution (0.100 mg/L)

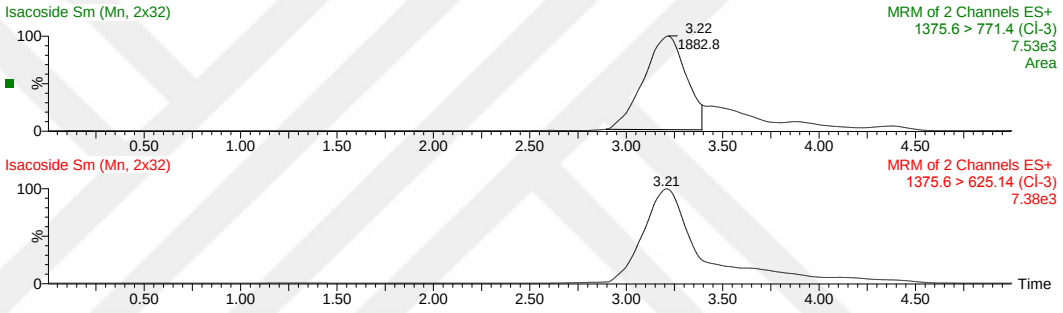


Figure 3.178 Chromatograms of Isacoside obtained by UPLC-ESI-MS/MS in the positive ion mode for standard solution (0.100 mg/L)

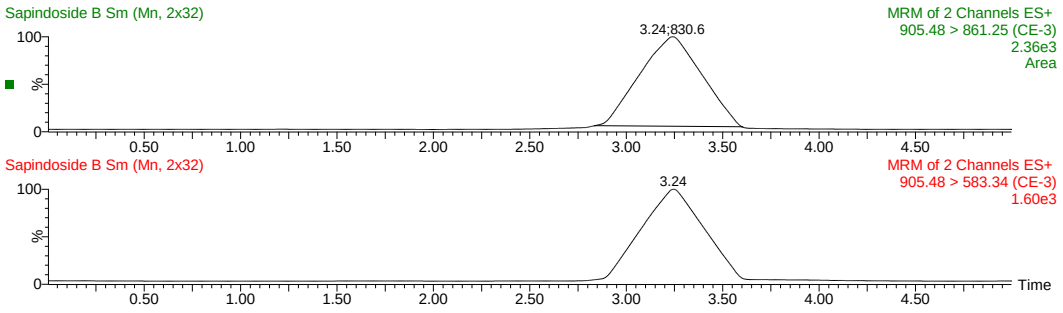


Figure 3.179 Chromatograms of Sapindoside B obtained by UPLC-ESI-MS/MS in the positive ion mode for standard solution (0.100 mg/L)

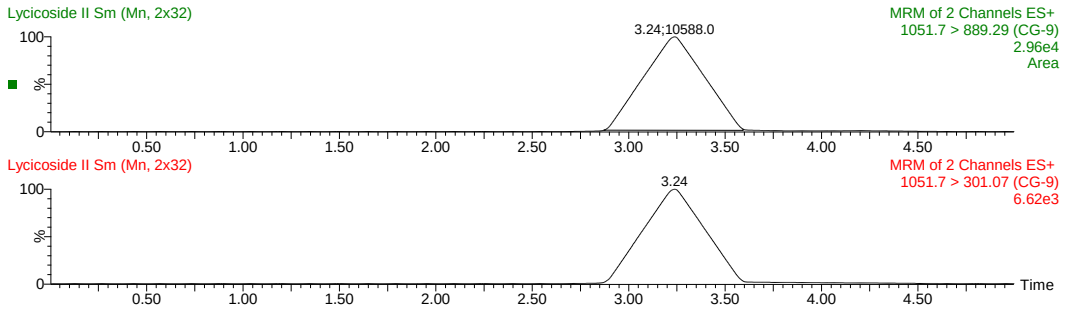


Figure 3.180 Chromatograms of Lycicoside II obtained by UPLC-ESI-MS/MS in the positive ion mode for standard solution (0.100 mg/L)

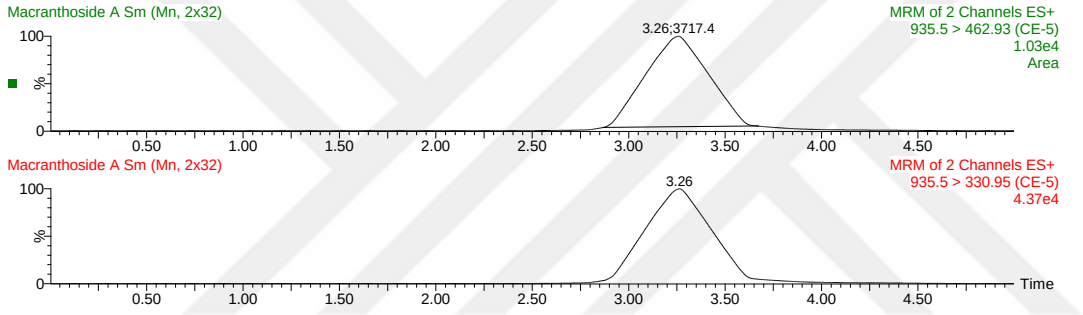
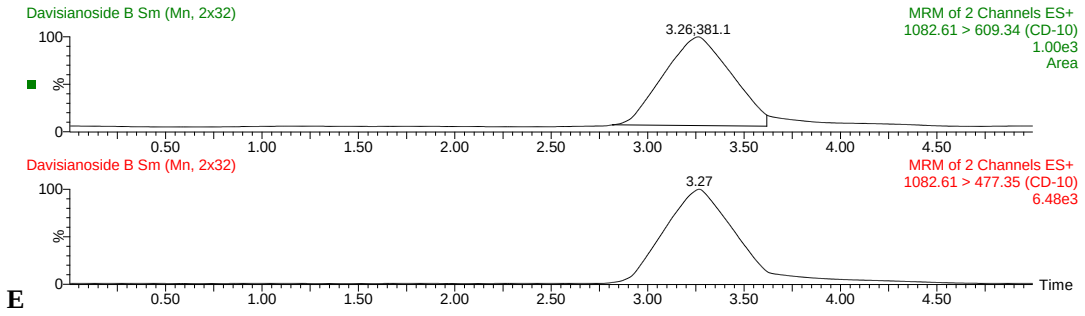


Figure 3.181 Chromatograms of Macranthoside A obtained by UPLC-ESI-MS/MS in the positive ion mode for standard solution (0.100 mg/L)



E

Figure 3.182 Chromatograms of Davisianoside B obtained by UPLC-ESI-MS/MS in the positive ion mode for standard solution (0.100 mg/L)

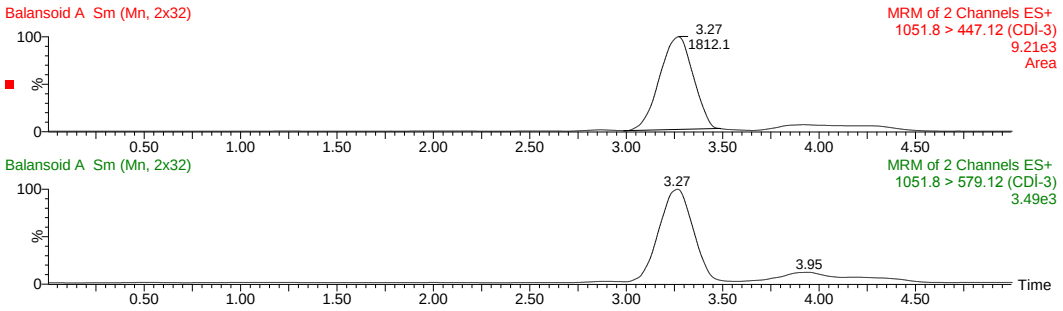


Figure 3.183 Chromatograms of Balansoid A obtained by UPLC-ESI-MS/MS in the positive ion mode for standard solution (0.100 mg/L)

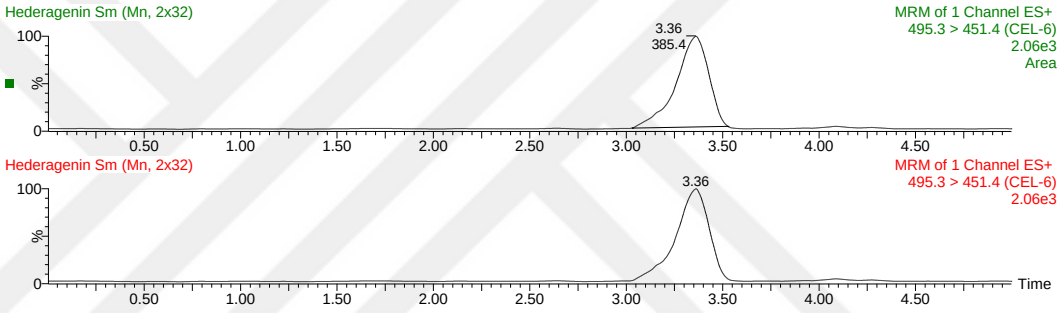


Figure 3.184 Chromatograms of Hederagenin obtained by UPLC-ESI-MS/MS in the positive ion mode for standard solution (0.100 mg/L)

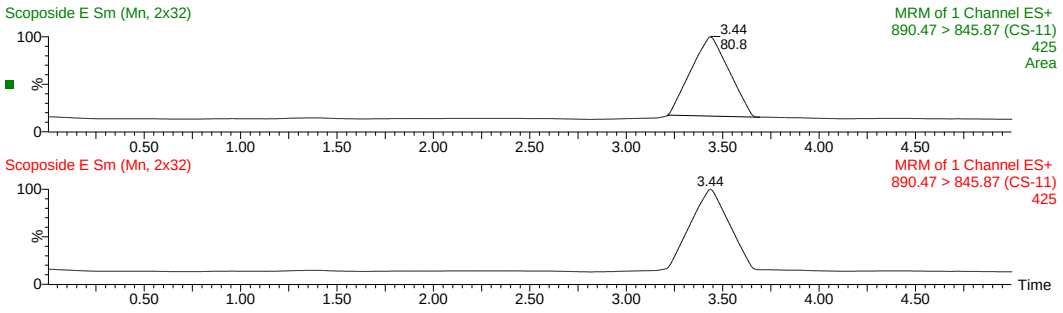


Figure 3.185 Chromatograms of Scoposide E obtained by UPLC-ESI-MS/MS in the positive ion mode for standard solution (0.100 mg/L)

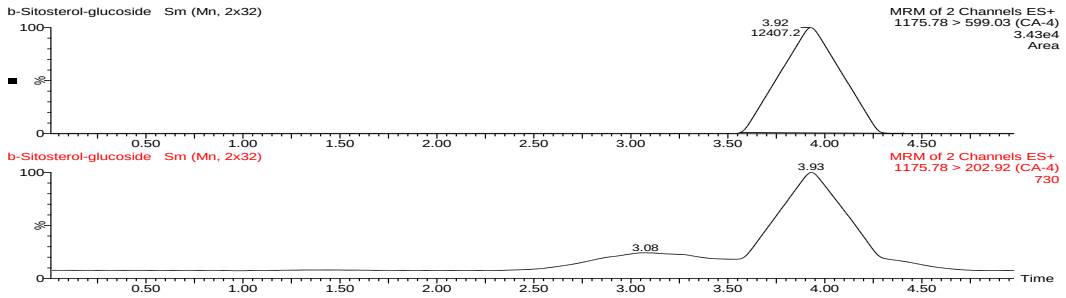


Figure 3.186 Chromatograms of *β*-Sitosterol glucoside obtained by UPLC-ESI-MS/MS in the positive ion mode for standard solution (0.100 mg/L)

3.3 Conclusion

As mentioned in introduction section, our aim was to investigate biologically active natural products, especially glycosidic ones, from three *Cephalaria* species using chromatographic, chemical and spectroscopic techniques from *n*-BuOH phases which are rich glycosidic compounds.

Isolation, purification and structural identification studies exhibited thirteen triterpene saponins and one iridoid glycoside from *C. hirsuta*, eight triterpene saponins from *C. procera* and one triterpenoid sapogenin from *C. elazigensis* var. *elazigensis*. Structures of twenty-three compounds were determined by 1D- and 2D- NMR techniques and literature data. According to the structural determination studies, Comp. **2** and Comp. **16**; Comp. **3** and Comp. **18**; Comp. **5** and Comp. **20**; Comp. **12** and Comp. **22** were found exactly same molecules.

Among the structurally designated ones, four compounds (Comp. **14**, **17**, **19**, **23**) in Caprifoliaceae family and one compound (Comp. **21**) in *Cephalaria* genus were identified, for the first time. When we interpret these five compounds, taking into consideration literature findings we saw that all identified compounds are possessed of different types of biological activities. Literature data exhibited that compounds **1**, **4**, **6**, **9**, **10** and **11** have antimicrobial activities in changing effects (Pasi et al., 2009; Sarikahya and Kirmizigul, 2010, 2012a; 2012b; Sarikahya, 2014). Compounds **2**, **7**, **8, 12**, **14**, **15**, **17** and **23** showed cytotoxcic and significant inhibitions on different cancer cell lines (Braca et al., 2004; Jung et al., 2004; Pawar et al., 2006; Godevac et al., 2006b; Shen et al., 2008; Cheng et al., 2014; Gong et al., 2014; Mohamed et al., 2015; Zhou et al., 2015; Chowdhury et al., 2017; Liu et al., 2018). While only compound **5** have antidiabetic activity (Kim et al., 1998), compounds **3**, **4**, **7**, **13**, **19** and **21** were evaluated for hemolytic activities with varied and important extent (Kawai et al., 1988; Umehara et al., 1992; Braca et al., 2004; Voutquenne et al., 2008; Quang et al., 2011 Sumer et al., 2017). This result was coincided with our previous reports in different

journals by means of structure-activity relationships (Kirmizigul et al., 2007, 2012; Sarikahya et al., 2010, 2011,2012a; 2012b, 2013, 2014, 2015a; 2015b, 2018; Kayce et al., 2010, 2014, 2017a; 2017b; Top et al., 2017; Ozer et al., 2018). From here, its clear to understand that this genus is rich by biologically active saponins.

In the light of all data, we can say that especially compounds **14**, **17**, **19**, **21**, and **23** admissible chemotaxonomic markers for Caprifoliaceae family and/or *Cephalaria* species. Additionally, these compounds may have an important source for the structural similarity to our previous results about their immunoactivity data for IL-1β (Sarıkahya et. al., 2018).

It is known that isolation and purification processes from plants need long and laborious ways to achieve the results. So, the qualitative and quantitative analyses of glycosidic compounds which are generally present in the *n*-BuOH phases of these plants were analyzed by UPLC-ESI-MS/MS instrument for the first time. This method has been proven by validation studies. This application resulted that among three species *C. hirsuta* and *C. procera* species have more saponins (28 and 22, respectively), than *C. elazigensis* var. *elazigensis* (4) in *n*-butanol fractions. With this application of the studied plants were performed with quick, easy, sensitive and timesaving method. This method can also be ensured rapid identification of the glycosidic compounds in *n*-BuOH fractions of any other plants.

These results prompted us to consider on the saponin contents of these *Cephalaria* species which were not studied yet. According to ongoing biological activity studies, saponins exhibit different activities such as immunomodulator, hemolytic and cytotoxic activities which will have important usage areas in the near future.

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