



EGE UNIVERSITY



MASTER'S THESIS

**ELUCIDATION AND CHARACTERIZATION
OF GLYCOSIDIC COMPOUNDS FROM
CEPHALARIA TUTELIANA (DIPSACACEAE)**

Merve DAĞLI

Supervisor : Assoc. Prof. Nazlı SARIKAHYA

Chemistry Department

Presentation Date: 16.01.2018

Bornova-İZMİR

2018

EGE UNIVERSITY GRADUATE SCHOOL of
NATURAL and APPLIED SCIENCES

(MASTER OF SCIENCE THESIS)

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Sayın Merve Dağlı tarafından Yüksek Lisans tezi olarak sunulan “Elucidation and characterization of glycosidic compounds from *Cephalaria tuteliana* (Dipsacaceae)” başlıklı bu çalışma EÜ Lisansüstü Eğitim ve Öğretim Yönetmeliği ile EÜ Fen Bilimleri Enstitüsü Eğitim ve Öğretim Yönergesi'nin ilgili hükümleri uyarınca tarafımızdan değerlendirilerek savunmaya değer bulunmuş ve **16.01.2018** tarihinde yapılan tez savunma sınavında aday oybirliği/oyçokluğu ile başarılı bulunmuştur.

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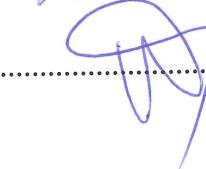
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EGE ÜNİVERSİTESİ FEN BİLİMLERİ ENSTİTÜSÜ
ETİK KURALLARA UYGUNLUK BEYANI

EÜ Lisansüstü Eğitim ve Öğretim Yönetmeliğinin ilgili hükümleri uyarınca Yüksek Lisans Tezi olarak sunduğum “Elucidation and characterization of glycosidic compounds from *Cephalaria tuteliana* (Dipsacaceae)” 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 verdiğim, 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 planlanmasıdan yazımına kadar bütün safhalarda bilimsel etik kurallarına uygun olarak davranışığımı ve aksinin ortaya çıkması durumunda her türlü yasal sonucu kabul edeceğini beyan ederim.

16 / 01 / 2018

Merve DAGLI





ÖZET

CEPHALARIA TUTELIANA (DIPSACACEAE) TÜRÜ ÜZERİNDEKİ GLIKOZİDİK BİLEŞİKLERİN KARAKTERİZASYONU VE YAPILARININ AYDINLATILMASI

DAĞLI, Merve

Yüksek Lisans Tezi, Kimya Anabilim Dalı

Tez Danışmanı: **Doç. Dr. Nazlı SARIKAHYA**

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Bu çalışmada endemik *Cephalaria tuteliana* (Dipsaceae) bitkisinden biyolojik aktif bileşenlerin izolasyonu, saflandırılması, yapı tayinleri ve biyolojik aktivitelerinin araştırılması amaçlanmıştır.

Araştırmaya konu olan bitki materyali çiçeklenme döneminde toplanıp uygun şartlar altında kurutularak, çalışmaya hazır hale getirilmiştir. Kuru bitki materyali metanol ile ekstrakte edildikten sonra *n*-butanol:su ekstraksiyonu yapılmıştır. Saponin içeriğinin zengin olduğu bilinen *n*-butanol fazı yağısı ve apolar kısımları uzaklaştırmak için *n*-hegzan ile ekstraksiyon yapılmıştır. *n*-butanol fazına kromatografi yöntemlerinden vakum likit kromatografisi, orta basıncılı sıvı kromatografisi, açık kolon kromatografisi ve ince tabaka kromatografisi uygulanarak 2 sapogenin, 1 iridoit glikozit ve 10 triterpen saponin olmak üzere toplam 13 adet saf bileşik elde edilmiştir. İzole edilen bileşiklerin yapıları NMR (1 ve 2 boyutlu) kullanılarak belirlenmiştir.

Bileşiklerden iki sapogenin (**1-2**) ve bir saponin (**12**), *Cephalaria* cinsinin dahil olduğu Caprifoliaceae familyasında ilk kez bulunmuştur. Elde edilen iki aglikonun sitotoksik aktiviteleri yapı aktivite ilişkisini belirlemek için farklı hücre panellerinde test edilmiştir.

Anahtar Kelimeler: Caprifoliaceae, *C. tuteliana*, fitokimya, izolasyon, kromatografi, saponin, sitotoksik aktivite



ABSTRACT**ELUCIDATION AND CHARACTERIZATION OF
GLYCOSIDIC COMPOUNDS FROM
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Supervisor: **Assoc. Prof. Dr. Nazli SARIKAHYA**

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In this study, it is aimed isolation, purification, structural determination studies and to investigate biological activities of endemic *Cephalaria tuteliana* (Dipsacaceae).

The plant material was collected during flowering period and dried under suitable conditions to be ready for research. The dried plant material was extracted with methanol and then separated into fractions by *n*-butanol:water extraction. The *n*-butanol phase, known to be rich in saponins, was extracted with *n*-hexane to remove apolar and oily parts. The chromatographic techniques such as vacuum liquid chromatography, medium pressure liquid chromatography, open column chromatography and thin layer chromatography were applied to butanol phase and 2 triterpenoid saponins, 1 iridoid glycoside and 10 saponin glycosides, totally 13 compounds were obtained. The exact structures of the isolated compounds were determined using (1D- and 2D-) NMR.

Among isolated compounds, two sapogenins, (**1-2**) and a saponin glycoside (**12**) were obtained from Caprifoliaceae family which include *Cephalaria* species, for the first time. The cytotoxic activities of two obtained saponins were examined against different cell lines for discussing structure activity relationship.

Key words: Caprifoliaceae, *C. tuteliana*, phytochemistry, isolation, chromatography, saponin, cytotoxic activity

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ABBREVIATIONS

UV	Ultraviolet
NMR	Nuclear Magnetic Resonance
COSY	Correlation (^1H - ^1H) Spectroscopy
HMQC	Heteronuclear Multiple Quantum Coherence
HMBC	Heteronuclear Multiple Bond Correlation
DMSO	Dimethylsulfoxide
TLC	Thin Layer Chromatography
CC	Column Chromatography
VLC	Vacuum Liquid Chromatography
MPLC	Medium Pressure Liquid Chromatography
TMS	Tetramethylsilan
HMDS-TMCS	Hexamethyldisilazane–Trimethylchlorosilane
HEK-293	Human Embryonic Kidney Cell 293
A-549	Adenocarcinomic Human Alveolar Basal Epithelial Cells
HeLa	Human Uterine Cervical Carcinoma
PANC1	Pancreas Ductal Adeno Carcinoma
SHSY5Y	Human Neuroblastoma Cells

1. INTRODUCTION

1.1. Importance of Natural Products

Natural compounds are important compounds that form themselves in living metabolism. In ancient times, people benefited from natural sources to make their lives easier. Old people have endorsed from them by applying them on their skin, adding them to their foods, giving them to their harvest and their animals. People used plants, animals, microorganisms and marine animals for cosmetics, agriculture, foods, textile, dyes and especially folk medicine since ancient times to todays. The usage areas and sources of natural products were given in detail under sub-headings.

1.1.1. Natural Products as Cosmetics

Since ancient times, people have been searching for ways to stay young. They have tried beauty rituels by using natural resources to prevent aging (see Figure1.1). For example, Old Greek goddess Aphrodite, has been greased with olive oil to protect her skin. Honey was also mixed with olive oil to help lighten the appearance of skin. Almond oils, apple circles, sea salt honey and milk were indispensable for the beauty rituals of Cleopatra, the last active pharaoh of Egypt (Stoecker, 2017).



Figure 1.1. Cosmetic applicatons of natural products

Nowadays, many cosmetic products have kinetin component due to aging retarding effect. Kinetin, (Figure 1.2) a plant growth hormone of the cytokinin type, is a molecule that supports cell division. (Levy, 2017) It is a compound first isolated from herring sperm DNA in 1955 (Miller et.al., 1955).

There are still many patented literatures about the cosmetic activity of kinetin and its derivatives (Kumari et. al., 2015; Xie et.al., 2008).

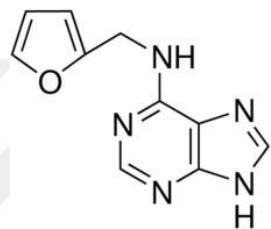


Figure 1. 2. Structure of Kinetin

1.1.2. Natural Products as Textiles and Dyes

Plants and animals have been used as dyes since centuries. Some examples for these, indigo is a fabric that has been used since ancient times.

The medicinal *Roccella tinctoria* in the Mediterranean sea seaweed also contains stain (Robiquet, 1829). *Dactylopius coccus* is an insect species that survives as parasitic in different cacti from the Dactylopiidae family. It is a bug that is known and used in ancient times, giving a red color (Greenfield, 2005).

In ancient Egyptian mummies, indigo blue stained wraps were found. The source of this dye is the tropical *Indigofera tinctoria* (see Figure 1.3.) plant found in Eastern India (Kamal and Mangla, 1993).

Today, Indigo (see Figure 1.4.) blue is the color of jeans that find a great place among our clothes and in recent years, the annual production of synthetic indigo has reached thousands of tons in the world.



Figure 1.3. *Indigofera tinctoria*

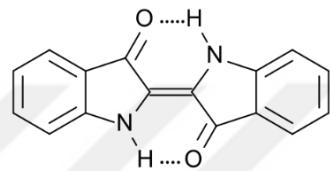


Figure 1.4. Indigo chemical structure

1.1.3. Natural Products in Agriculture

Since ancient times, people have benefited from natural sources to preserve and protect their crops and fields. It is known that plant powders and extracts were used as insecticides during the Ancient Roman period. In the epoch of the Persian king Xerxes, the powder of the *Pyrethrum* plant was used to control the head lice in children (Addor, 1995).

If we look again today, plant-derived pyrethrin is still used as an insecticide in agriculture (Aydin and Mammadov, 2017) (Figure 1.5).

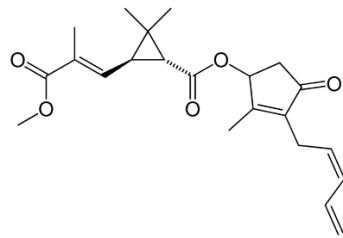


Figure 1.5. Pyrethrin

Nowadays, one of these protection methods is to use against diseases and harmful plants by using plants and plant extracts (Erler, 2000). For example, every part of the *Nimba* plant is active on humans and microorganisms which is used as a household pesticide (Prajapati, 2016).

1.1.4. Natural Products as Folk Medicine;

One of the earliest examples of the use of natural compounds as a medicine is the willow tree (see Figure 1.6.). In ancient Egypt, Assyrian and Greek manuscripts, former doctors Galen, Hippocrat and Dioscorides have described pain reliever and fever reducing properties of this tree. Native Americans used it for headache, fever, rheumatism, tremor and muscle pain, as well. In 1700s, it was also used in the treatment of malaria disease. Another example is aspirin which is known that the source of it is still the willow tree today (Stone, 1763).



Figure 1.6. Willow tree

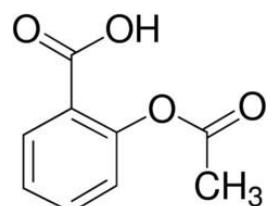


Figure 1.7. Acetylsalicylic acid

Many other studies on the use of natural compounds as medicine have been reported (Cragg and Newman, 2014). Humans have benefited not only plants but also many different natural sources, to get rid of their diseases. Some examples of different sources, isolated active compounds and their using areas are listed in Table 1.1.

Table 1.1. Some biological active compounds and their source

Source	Source's Name	Compound	Using area
Plant	Pacific yew tree	Paclitaxel	Breast and ovarian cancer
	<i>Camptotheca acuminata</i>	Camptothecin	Cancer treatment
	<i>Papaver somniferum</i>	Morphine	Pain Reliever
	<i>Parthenium integrifolium</i>	Quinnine	Malaria treatment
Microorganisms	<i>Penicillium notatum</i>	Penicillin	Infactions
	<i>Cephalosporium acremonium</i>	Cephalosporins	Infactions
Animals	<i>Epipedobates anthony</i>	Epibatidine	Pain Reliever
	<i>Bothrops jararaca</i>	Captopril	Hypertension, congestive heart failure
Marines	<i>Tethya crypta</i>	Spongouridine	Cytostatic activity on cancer treatment
	<i>Discodermia species</i>	Discodermolide	Cancer treatment

1.2. Primary and Secondary Metabolites

Metabolites are molecules that are spontaneously formed in living metabolism, usually in a small structure. The compounds required for the formation and maintenance of viability are called **primary metabolites**. These compounds are responsible for the basic needs of the organism such as growth, development and reproduction. Unlike primary metabolites, **secondary metabolites** do not directly affect life but in long-term decline or absence, affect adapt to life, fertility and aesthetics (Cooper, 2015).

The seconder metabolites play an important role in the vital defense and they have a limited distribution in the plant kingdom. This property separates these compounds from the primary metabolites (amino acids, sugars, nucleotides and acyl lipids) (Seigler, 1998). Because primary metabolites are found in all individuals of the plant kingdom, while secondary minerals are often found only in a certain species or close species.

1.2.1. Types of secondary metabolites

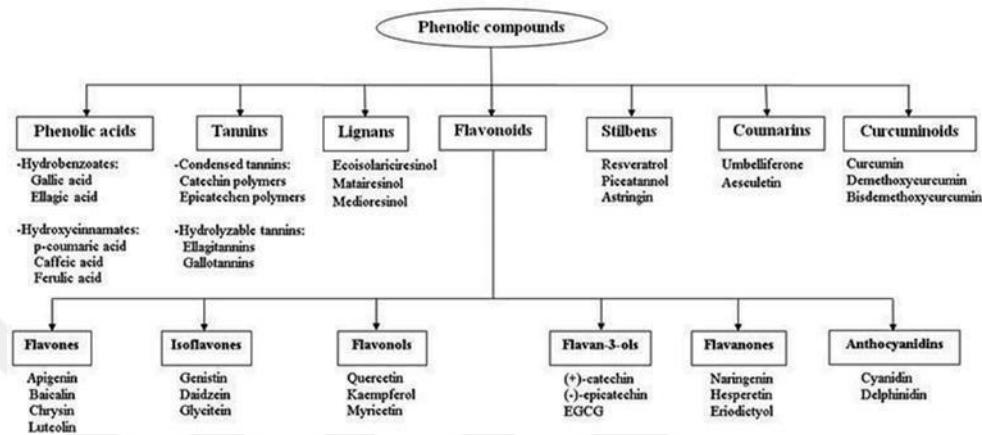
There are three main classes of secondary metabolites as given under sub-headings in detail.

1.2.1. Phenolics

Phenolics are compounds having one or more hydroxyl groups and aromatic rings. Generally, they can be classified (Table 1.2.) as phenolic acids and analogues, flavonoids, tannins, stilbenes, curcuminoids, coumarins, lignans, quinones (Fresco et.al, 2006).

Many vegetables, fruits and phenolic compounds found in medicinal plants have been the subject of many studies on the prevention of diseases due to their antioxidant properties (Cai et. al., 2004; Basli et. al., 2017).

Table 1.2. Classification of Phenolic Compounds



Phenolic compounds generally have antimicrobial and antibacterial activities due to the phenol in their structures. An example of this is the thymol molecule. Thymol has antimicrobial and antibacterial activities because of its phenolic structure. In the markets, this molecule which is a phenolic compound isolated from *Thymus vulgaris* plant species used in the treatment of diseases such as cough, asthma, bronchitis (Figure 1.8).

As well as the usage of it as a pesticide (European Commission Health & Consumers Directorate, 2013) and food additive (Commission Implementing Regulation, 2012).

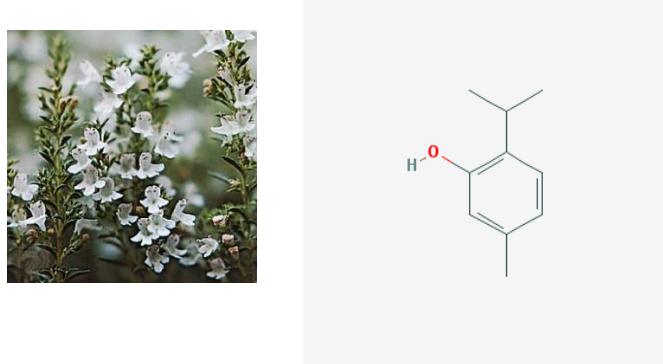


Figure 1.8.*Thymus vulgaris* and Thymol molecule

1.2.2. Nitrogen containing compounds 'Alkaloids'

In 1805 A German pharmacist Friederich VVilhelm named Serturner obtained a crystallized substance during the studies on *Papaver somniferum* (Figure 1.9.) (Busse et.al., 2006). This substance, called "morphinum" (Figure 1.10), showed a basic reaction, unlike what was known until then. The findings of Serturner have opened a new era in chemical research in plants. The Other, other basic substances were also obtained as well. All these substances are called alkaloids in a similar meaning alkaline. Subsequently, it was understood that this alkaline reaction was leading to molecular azotites and thus alkaloids were described as organic compounds containing nitrogen and alkaline reaction. A simple recipe was not enough to describe alkaloids. Because, this recognition, it would be necessary to count physiological amines (histamine, serotonin, etc.) from alkaloids obtained from plants or animals. The following studies showed that the alkaloids were nitrogen

containing heterocyclic those compounds. It was then necessary to make an addition to recognition (Tanker, 1990 ; Aniszewski, 2007).



Figure 1.9. *Papaver somniferum*

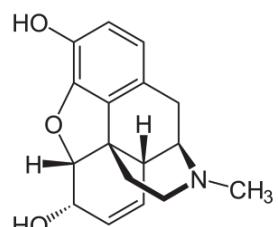


Figure 1.10. Structure of morphine

Alkaloids are substances from plants that are more or less basic reactive substances carrying strong physiological and pharmacodynamic activity, carrying one or more nitrogens in the ring (Mueller-Harvey, 1992). They are compounds that are commonly used as a drug starting ingredient, usually acting on the central nervous system. So that, some countries in which Turkey is also included have legalized alkaloid fields in particular as a starting material (Toprak Mahsulleri Ofisi Genel Müdürlüğü, 2001).

1.2.3. Terpenes

Turpentine is (Figure 1.12) a balsam obtained by cutting and carving the shells of various pine trees, is the basis of the word 'terpen' .Turpentine is a volatile liquid isolated from pine trees (Figure 1.11).

Simple mono and sesquiterpenes are essential oils obtained from various parts of plants. But di and triterpenes are not volatile, so they are derived from the resins of plants. Unlike these, tetraterpenes also form a separate group as carotenoids (Bano, 2007).



Figure 1.11. Pine tree



Figure 1.12. Turpentine

Terpenes are the largest class of secondary metabolites. They can be called natural compounds, which contain isoprene units on their surface. The number of isoprene units in the structure determines the type of terpenes can be seen in Table 1.3.

Terpenoids are used in many areas because of their biological activities. Many commercial uses are also the consequences of these activities such as terpenes have been found in many cosmetic products due to their pleasant smell, medicines which are important for human health and pest control agents (Zwenger, 2008).

Table 1.3. Classification of Terpenoids

<i>Isoprene units</i>	<i>Type of Terpenoids</i>	<i>Number of Carbons</i>
1	<i>Hemiterpene</i>	5
2	<i>Monoterpene</i>	10
3	<i>Sesquiterpene</i>	15
4	<i>Diterpene</i>	20
5	<i>Sesterterpene</i>	25
6	<i>Triterpene</i>	30
8	<i>Tetraterpene(Carotenoids)</i>	40
<i>N</i>	<i>Polyterpene</i>	5(<i>n</i>)

Paclitaxel (Figure 1.14) (trade name Taxol®) used in the treatment of breast, ovarian and lung cancer is a diterpenoid isolated from pacific yew tree (Figure 1.13). However, the level of paclitaxel in the bark of a tree will not be needed for treatment because the total amount of paclitaxel needed for treatment is 3 trees. This has also caused total or partial synthesis of the structure. So we can say that the isolation method provides a good reference for drug synthesis. (Sell, 2003)



Figure 1.13. Pacific Yew Tree

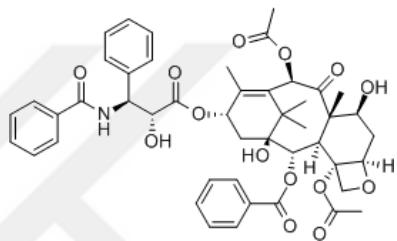


Figure 1.14. Structure of Paclitaxel

Besides all these, there are many benefits for vital functions within the plant. Studies have shown that pollinating beetles are attracted to plants by terpenoid release (Maimone et.al., 2007). There are many different modification products and derivatives generally characterized as terpenoids. These are mainly sterols, saponins and mero-terpenes (Kiyama, 2017).

In this thesis we mainly focused on the saponins which have become a source of hope for many issues, which are subject to much work.

1.3. Saponins

The word *saponin* is derived from *sapo*, Latin for “soap.” Because saponins form foams in aqueous solutions similar to soap. The cause of this foaming is the molecular structure of saponins. Because they contain both a hydrophobic (water-hating) sapogenin (non-sugar part) and a hydrophilic (water-loving) glycoside (sugar part) (see Figure 1.15) (Tamura et.al., 2012).

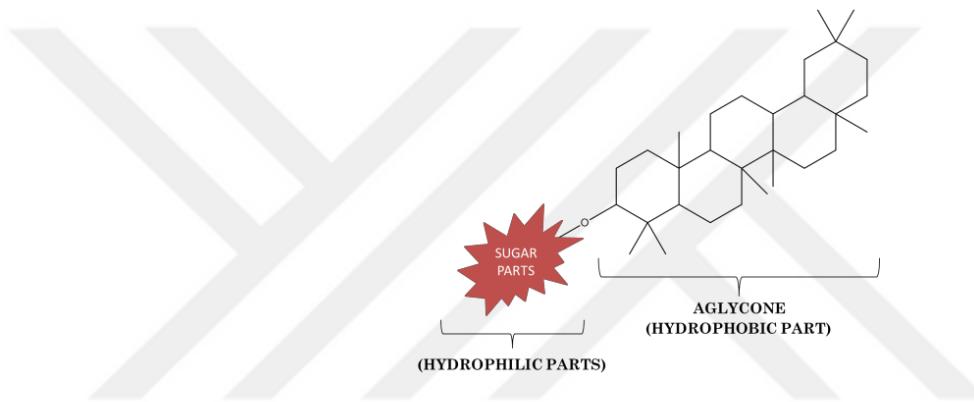


Figure 1.15. Parts of saponins

Saponins are phytochemicals found in many vegetables, beans and herbs. They play an important role in living metabolism. It can be said that the saponins in the 'triterpen' class of terpenes are triterpenoids which are generally have steroid or triterpenoid aglycone (see Figure 1.16) (Desai, 2009). In the past many plants have provided alternative methods due to the saponin content. Still, saponins continue to be used in different areas. Some of those are soaps, cosmetics, detergents, insecticides and pesticides. Even some plant extracts which are rich in saponins have been using in different industrial areas. Besides, saponins have become the targets of many researchers in medicine (Tamura, 2012; Moghimipour and Handali, 2015).

It was mentioned in the previous section that the saponins have a structure similar to soap. This also means that saponins can hold onto oils. This property allows saponins to emulsify oil soluble molecules in the digestive tract. It binds to bile acids and helps to throw them away. So it helps balance your cholesterol level in blood. A study found that giving a certain saponin extract to rats with high cholesterol reduced 'bad' (LDL) cholesterol without affecting 'good' (HDL) cholesterol (Malinow, 1977).

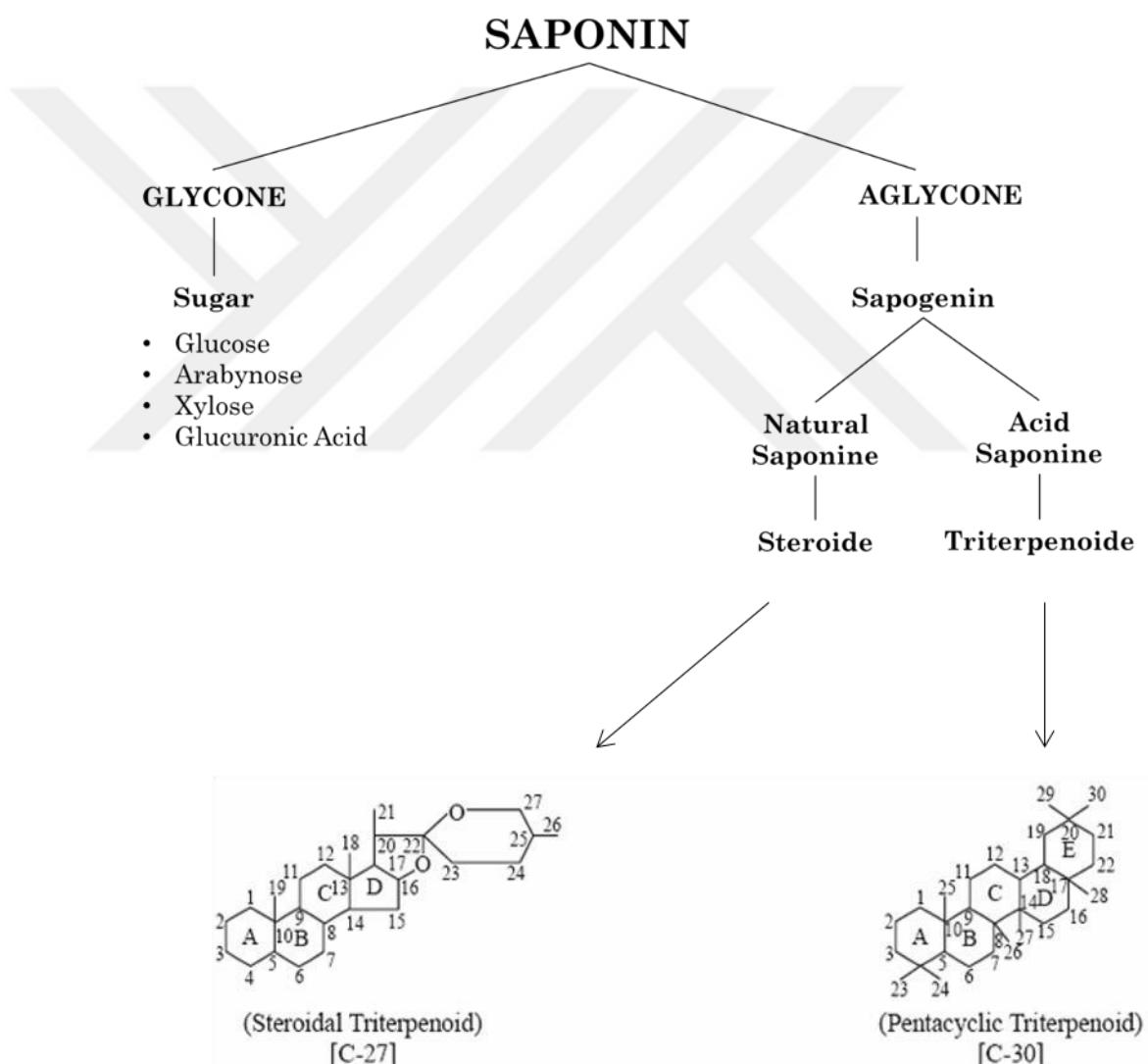


Figure 1.16. Glycone and Aglycone Parts of Saponins Structure of Steroidal (a) and Triterpenoidal (b) Aglycones

Cancer cell membranes are cholesterol type compounds. Saponins bind to cancer cell membranes as it binds cholesterol cells and prevent the reproduction of cancer cells (Rao, 1995; Yan et.al, 2009). In addition, many studies have supported the antimicrobial effect of saponins. Plants and other living organisms use saponins for defense against harm. Likewise, it is possible to play an important role for defense in human and animal metabolism, and a lot of research has been done for it.

As it interacts with cholesterol, it also causes hemolysis by forming complexes in the membranes of red blood cells. This property can also be used to enhance penetration of macromolecules (such as proteins) into the cell membrane. Saponins are also used as adjuvants in vaccines and serums (Simone, 2017; Sun, 2011).

1.4. The *Cephalaria* Species

Totally, there are 94 species of *Cephalaria* in the worldwide, located in Europe, the Eastern Mediterranean, East Asia, Central and North Africa. A total of 40 species found in Turkey and 24 of them are endemic (Davis, 1972).

As a result of many studies, some of these species has been reported to be used as dye (Szabó, 1940), food additive (Baytop, 1994), and folk medicine (Gunes and Ozhatay, 2011) in old times. Based on all these studies, it has been determined that this species are rich in secondary metabolites such as flavonoids (Godjevac et al., 2004; Movsumov et al., 2009), iridoids (Godjevac et al., 2000; Mustafaeva et al., 2008), alkaloids (Aliev et al., 1975) and especially saponins (Kayce et. al., 2014; Braca et.al., 2004).

Secondary metabolites abundance in *Cephalaria* species requires studies on the biological activities of this plant. As a result of these studies, it has been reported that essential oils of this plant species and compounds obtained as a result of isolation have cytotoxic, antimicrobial, antifungal, antioxidant, hemolytic and immunomodulatory properties (Sarıkahya et.al.,2018; Kayce and Kirmizigul, 2017; Sarıkahya et.al., 2015; Mutlu et.al., 2017).



2.MATERIAL & METHOD

2.1.General

Chromatographic methods have been used extensively for isolation and purification studies that make up a large part of the experimental part of our work. These methods are VLC (Vacuum Liquid Chromatography), MPLC (Medium Pressure Liquid Chromatography), TLC (Thin Layer Chromatography) and open column chromatography. In the first step, the VLC method was applied with a Lichroprep RP18 (Merck 9303-25- 40 μ m) silica filler to roughly fractionate. In the second step, Buchi brand pumps (C-605) and glass columns (15 / 460 and 49 / 230) were used for the MPLC method. Silica gel 60 (0.063-0.200, Merck 7734) was used as the fill material for MPLC and open column chromatography. Thin Layer Chromatography (TLC) was used to monitor the components, to determine if they were pure or not, and to identify them. TLC plates (F₂₅₄ Merck 5554 silica gel) were run with different polarities (CHCl₃ / MeOH / H₂O). In the dried layers, the UV active components were observed under the UV lamp at wavelengths of 254 nm and 366 nm. Components were visualized with 20% H₂SO₄ solution and heated (120 °C).

The structures of the purified compounds were determined using spectroscopic methods. In the structure determination, values of *j* were recorded as Hz DMSO-*d*₆ as a solvent , and TMS as internal standard using Varian AS 400 MHz spectroscopy for 1D-(¹³C and ¹H) and 2D-NMR (HMBC, HMQC,COSY,TOCSY)analysis.

2.2. Plant Material

Cephalaria tuteliana (Caprifoliaceae) (Figure 2.1) (Kus and Gokturk, 2005) is an endemic species collected from Istanbul, Kirac, Bahcesehir in July 2012 at about 90 m by H. Sumbul and R.S. Gokturk (Akdeniz University Herbarium Research and Application Centre No:7526). Every part of the plant was studied above ground.



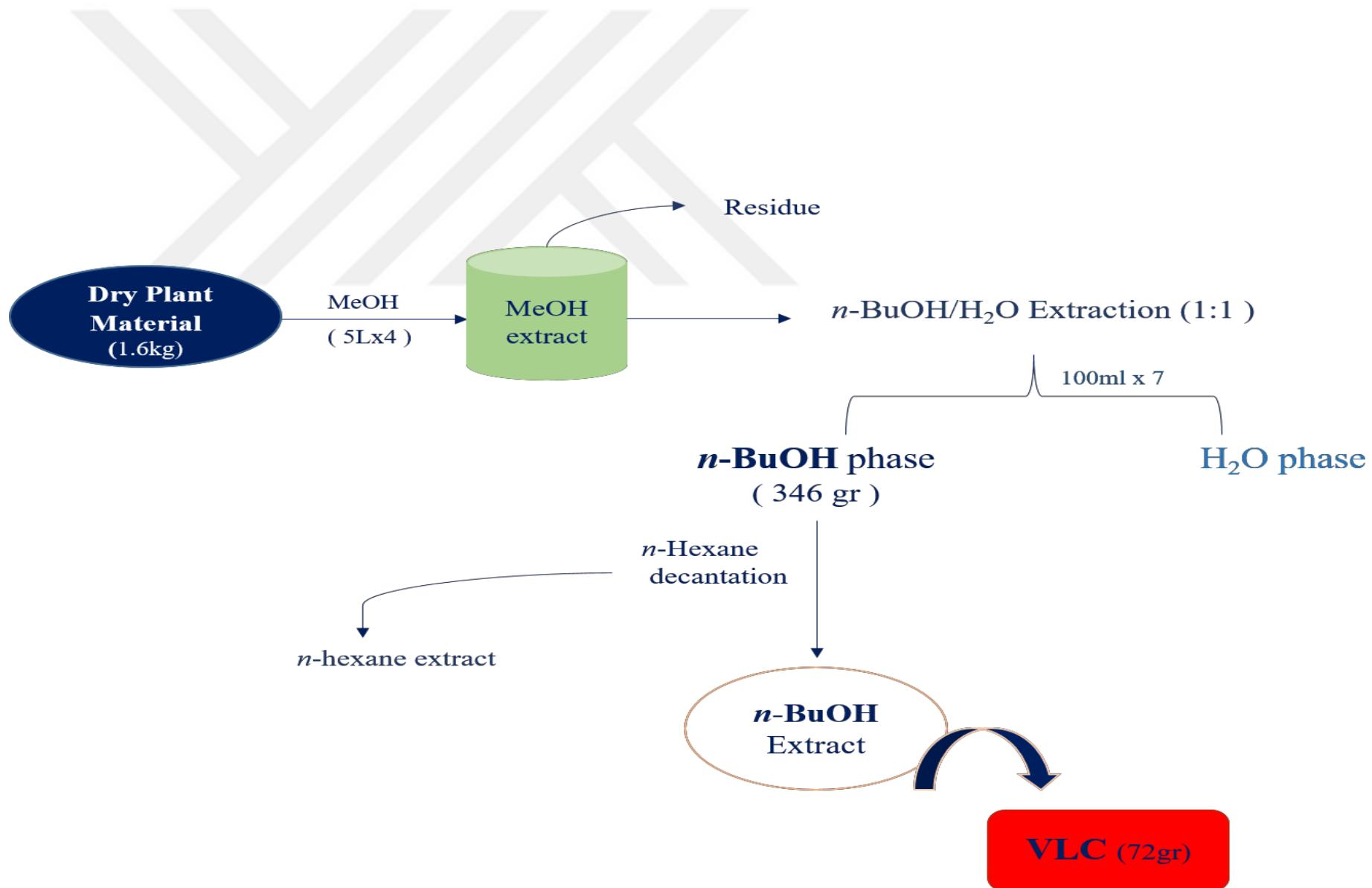
Figure 2.1. *Cephalaria tuteliana*

2.3. Extraction, Isolation and Purification

The *C. tuteliana* plant material was dried in dark and dry conditions and grinded. The plant material (1.6 kg) was mixed with MeOH at room temperature to prepare MeOH extract (5L x 4). MeOH was evaporated and then the concentrated material (388 g) was kept for the next step. In the second step, *n*-BuOH / H₂O(1:1) extraction was performed to separate the biologically active components. With a few repetitions, the two phases were completely separated from each others (100 mL x 7). The BuOH phase riched in secondary materials, was evaporated and an intense consistency was achieved (346 g). The *n*-BuOH fraction was decanted with *n*-hexane (100mL x 20) to separate oily substances and then

the dried BuOH phase (72 g) was applied to VLC (Scheme 2.1). RP silicagel and MeOH / H₂O (gradient from 0% to 100% MeOH) solvent system were used for the VLC method (Scheme 2.2). After that, fractions 20:80 and 10:90 (H₂O:MeOH) were combined (25 g) and applied to open column chromatography with suitable solvent system with CHCl₃:MeOH:H₂O (from 90:10:0.5 to 61:32:7). During this column 30 subfractions were obtained. Compound **1** (150 mg), compound **2** (58 mg), compound **5** (38 mg) and compound **6** (175 mg) were obtained purely directly from this column (Scheme 2.3). The fractions 22nd (0.80 g), 23rd (0.41 g) and 25th (0.9 g) were separately subjected to another open column chromatography. Compounds **12** (40 mg), **7** (106 mg), **8** (63 mg) and **9** (108 mg) were obtained from these fractions by CC, respectively.

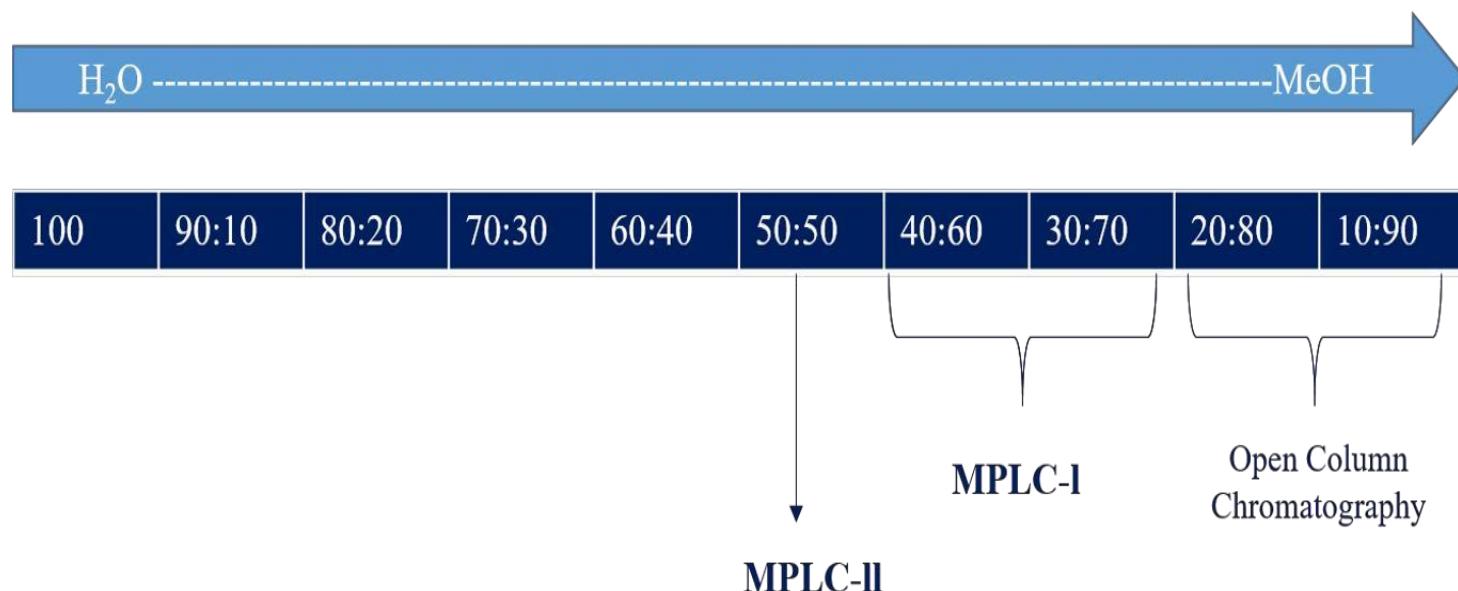
VLC fractions 40:60 and 30:70 were combined (9g) and MPLC was applied to this fraction. Fractionation was performed using the appropriate adsorbent (silicagel), buchi column (26 x 920 mm) and solvent system (90:10:0.5-61:32:7 / CHCl₃:MeOH:H₂O) flow rate 25 mL/min and max. pressure:40barr (Scheme 2.4). 13 subfractions were obtained and fractions 8-10 were combined (2.8 g) and subjected to open column chromatography. Compound **10** (110 mg) was obtained form directly from this column. After this column, the fractions 8-10 were subjected to a series of more open column chromatography. Compounds **3** (60 mg), **4** (47 mg) and **11** (26 mg) were obtained from these columns. Compound **13** (120 mg) was obtained by MPLC application of a 50:50 VLC fraction (12 g) (adsorbent:silicagel, column:26 x 920 mm, flow rate 25 ml/min and solvent system 90:10:1-61:32:7 / CHCl₃:MeOH:H₂O, max. pressure:40 bar).



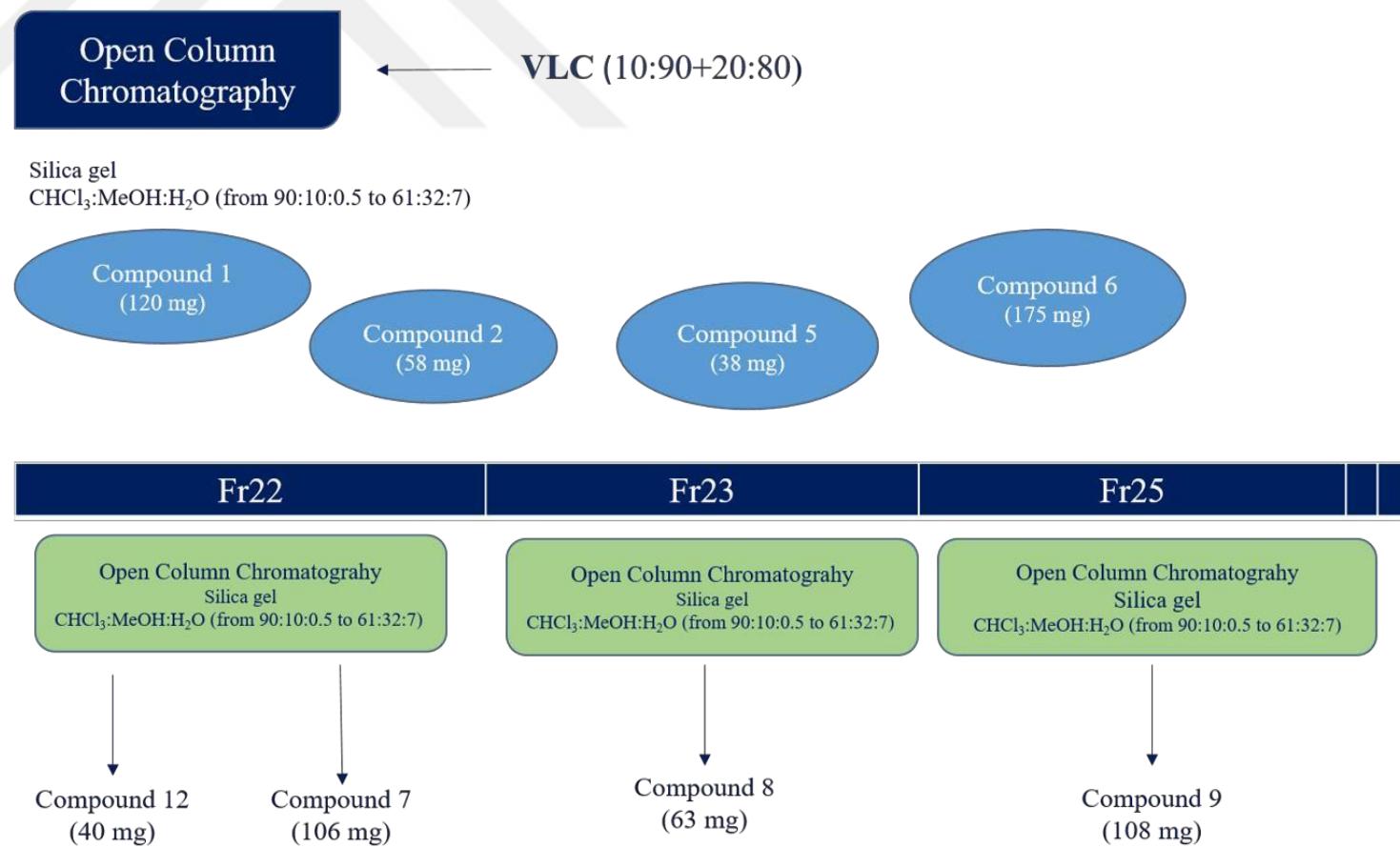
Scheme 2.1. Application steps for extraction

VLC (72gr)

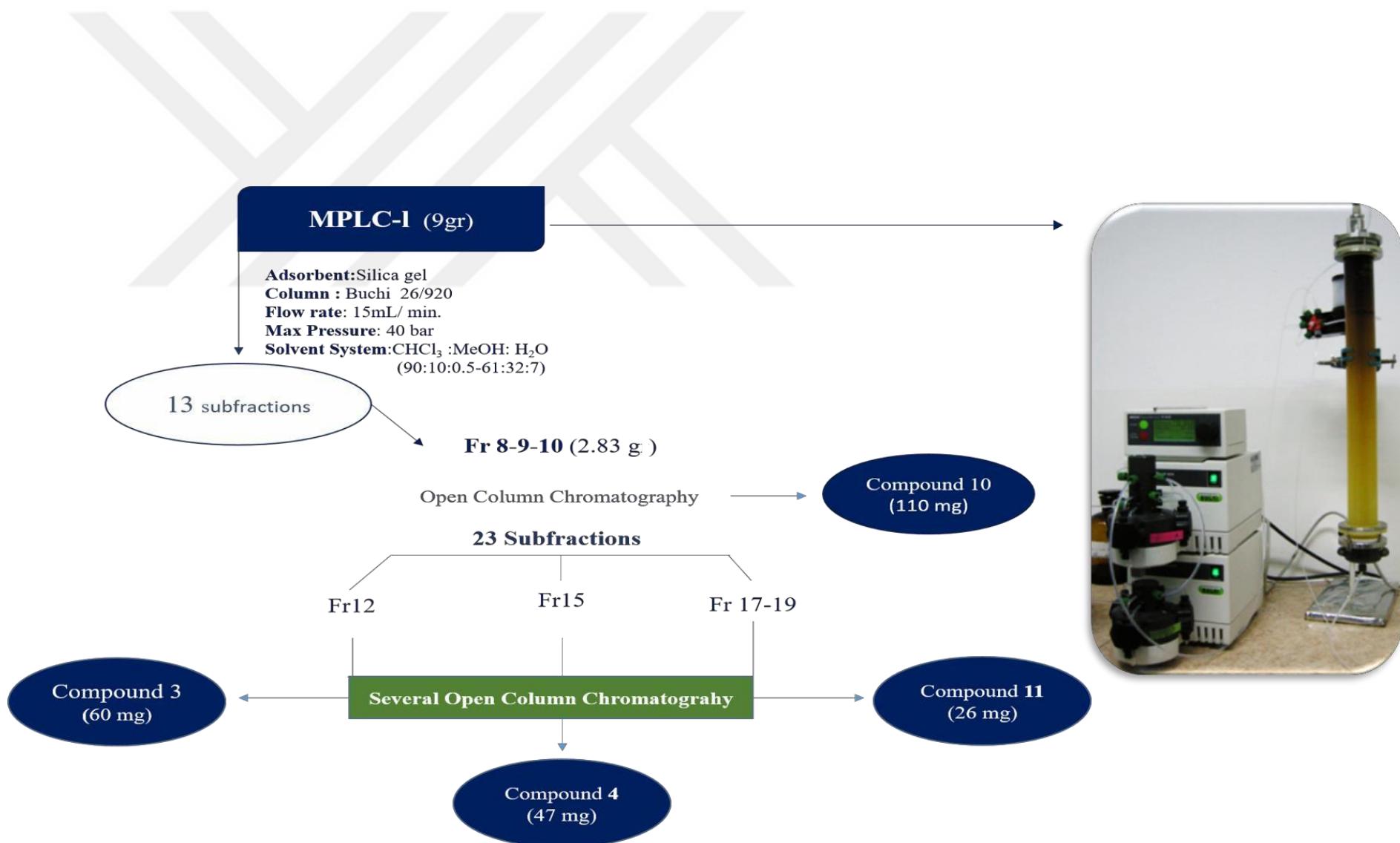
Adsorbent: RPSilica
Solvent system: H₂O:MeOH



Scheme 2.2. VLC application steps



Scheme 2.3. Application steps for Open CC



Scheme 2.4. Application steps for MPLC

2.4. Cytotoxic activity assays

A549, Hela, PANC1, SHSY5Y and a normal cell line HEK293 were used for testing cytotoxicity. All cell lines were purchased from American Type Culture Collection (ATCC, Manassas, VA, U.S.A.). The cell lines were maintained in Dulbecco's modified Eagle's medium F12 (DMEM/F12), supplemented with 10 % fetal bovine serum (FBS), 2 mM L-glutamine, 100 U/mL of penicillin, and 100 µg/mL of streptomycin (Gibco, NY, U.S.A.). The cells were incubated at 37 °C in a humidified atmosphere of 5 % CO₂. The cells were subcultured twice a week, and cells in the exponential growth phase were used in the experiments.

Cytotoxicity of *n*-butanol extract, compounds **1-2**, hederagenin and oleanoic acid was determined using a modified MTT assay, which detects the activity of mitochondrial reductase of viable cells. The assay principle is based on the cleavage of MTT that forms formazan crystals by cellular succinate-dehydrogenases in viable cells. DMSO is added to the wells to dissolve the formazan crystals. Briefly, all cell lines were cultivated for 24 h in 96-well microplates with an initial cell numbers of 1×10^5 cells/mL in a humidified atmosphere with 5 % CO₂, at 37 °C. Then, the cultured cells were treated with different concentration of compounds (0.5, 5, 50 µg/mL) followed by incubation for 48 h at 37 °C. Doxorubicin (Sigma, St. Lois, MO, U.S.A.) was used as a positive control. The optical density of the dissolved material was measured at 570 nm with UV-vis spectrophotometer (Thermo Multiskan Spectrum). The viability (%) was determined by the following formula:

$$\% \text{ viable cells} = [(\text{absorbance of treated cells with compound}) - (\text{absorbance of blank})] / [(\text{absorbance of control}) - (\text{absorbance of blank})] \times 100.$$

The mean IC₅₀ is the concentration of agent that reduces cell growth by 50 % under the experimental conditions and it is the average from at least three independent measurements that will be reproducible and statistically significant. The IC₅₀ values were reported at ± 95 % confidence intervals (± 95 % CI). This analysis was performed with Graph Pad Prism 5 (San Diego, CA, U.S.A.).



3. RESULTS and DISCUSSION

The secondary metabolite content of *Cephalaria tuteliana* was examined, for the first time. 2 sapogenins, 1 iridoid glycoside and 10 saponins, totally 13 known compounds were isolated, purified and structurally determined. Two sapogenins named pomolic acid (**1**) (Numata et.al., 1989), tormentic acid (**2**) (Villar et.al., 1986), ten known triterpene saponins which was named elmalienoside A (**3**) (Sarikahya and Kirmizigul, 2012), davisianoside A (**4**) (Kayce et.al., 2014), α -hederin (**5**) (Aliev and Movsumov, 1976), elmalienoside B (**6**) (Sarikahya and Kirmizigul, 2012), 3- O - α -L-rhamnopyranosyl-(1 \rightarrow 2)- α -L-arabinopyranosyl hederagenin 28- O - β -D-glucopyranosyl ester (**7**) (Kawai et. al., 1988), davisianoside B (**8**) (Kayce et.al., 2014), 3- O - β -D-glucopyranosyl- (1 \rightarrow 3)- α -L-rhamnopyranosyl-(1 \rightarrow 2)- α -L-arabinopyranosyl hederagenin 28- O - β - D-glucopyranosyl ester (**9**) (Braca et.al., 2004), dipsacoside B (**10**) (Mukhamedziev et.al., 1971), macranthoidin A (**11**) (Mao et.al., 1993), 3- O - β -[α - L-rhamnopyranosyl-(1 \rightarrow 3)- β -D-glucopyranosyl] hederagenin (**12**) (Lemos et.al., 1992) and one iridoid glycoside namely laciniatoside I (**13**) (Kocsis et.al. 1993) were obtained. The structures of all compounds were elucidated by spectral methods including IR, 1D and 2D NMR methods Among these compounds pomolic acid (**1**), tormentic acid (**2**) and 3- O - β -D-glucopyranosyl-(1 \rightarrow 4)-[α -L-rhamnopyranosyl-(1 \rightarrow 2)]- α -L-arabinopyranosyl hederagenin (**12**) were detected in *Cephalaria* species and Caprifoliceaea family as well, for the first time. The cytotoxic activities of *n*-butanol extract, compounds **1-2** were examined against cancerous cells A549, Hela, PANC1, SHSY5Ycells and noncancerous cell HEK293 by MTT method. We compared the cytotoxicity results with common aglycones hederagenin and oleanoic acid to discuss structure activity relationship

Table 3.1 .

Table 3.1. The cytotoxic activities of *n*-butanol extract, compounds **1-2** and aglycones hederagenin, oleanoic acid

Sample	Cell Lines (μM)				
	A549	HeLa	PANC1	SHSY5Y	HEK293
pomolic acid (1)	-	-	>50	79.35±4.037	-
tormentic acid (2)	-	-	-	-	-
hederagenin	44.48±0.912	30.78±1.555	31.40±1.499	23.05±0.509	37.05±0.898
oleanoic acid	31.61±0.954	68.88±1.117	44.54±2.503	18.94±1.156	>50
<i>n</i> -butanol extract	-	-	-	-	-
Doxorubicin	13.97±0.639	10.45±0.308	15.89±1.988	4.69±0.036	25.85±3.973

- not active

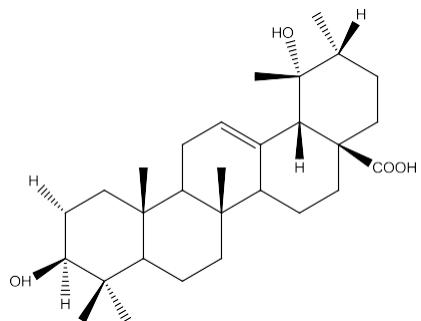
3.1. Compound 1 : Pomolic Acid (Numata et.al., 1989)

Figure 3.1. Pomolic acid

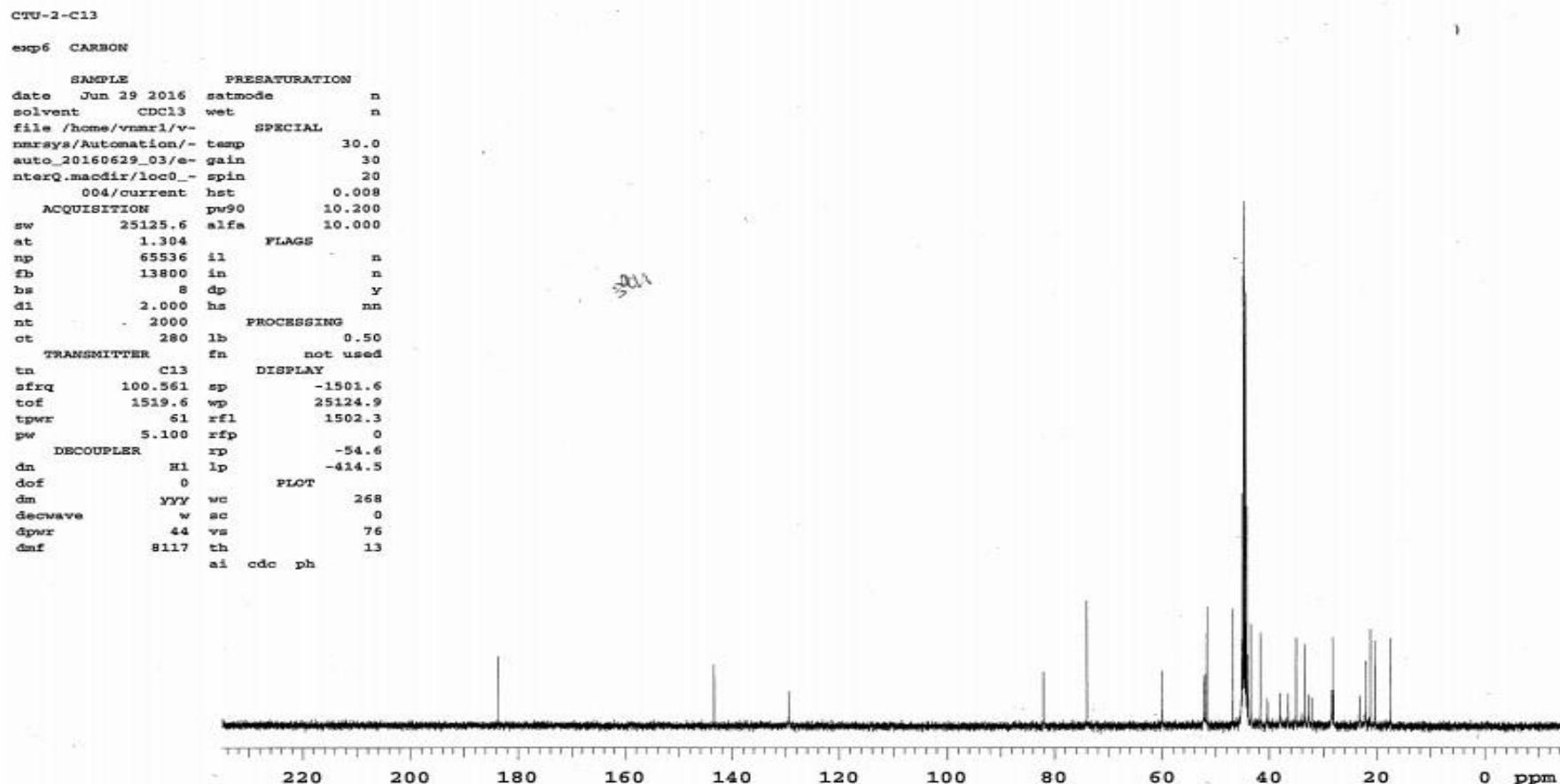
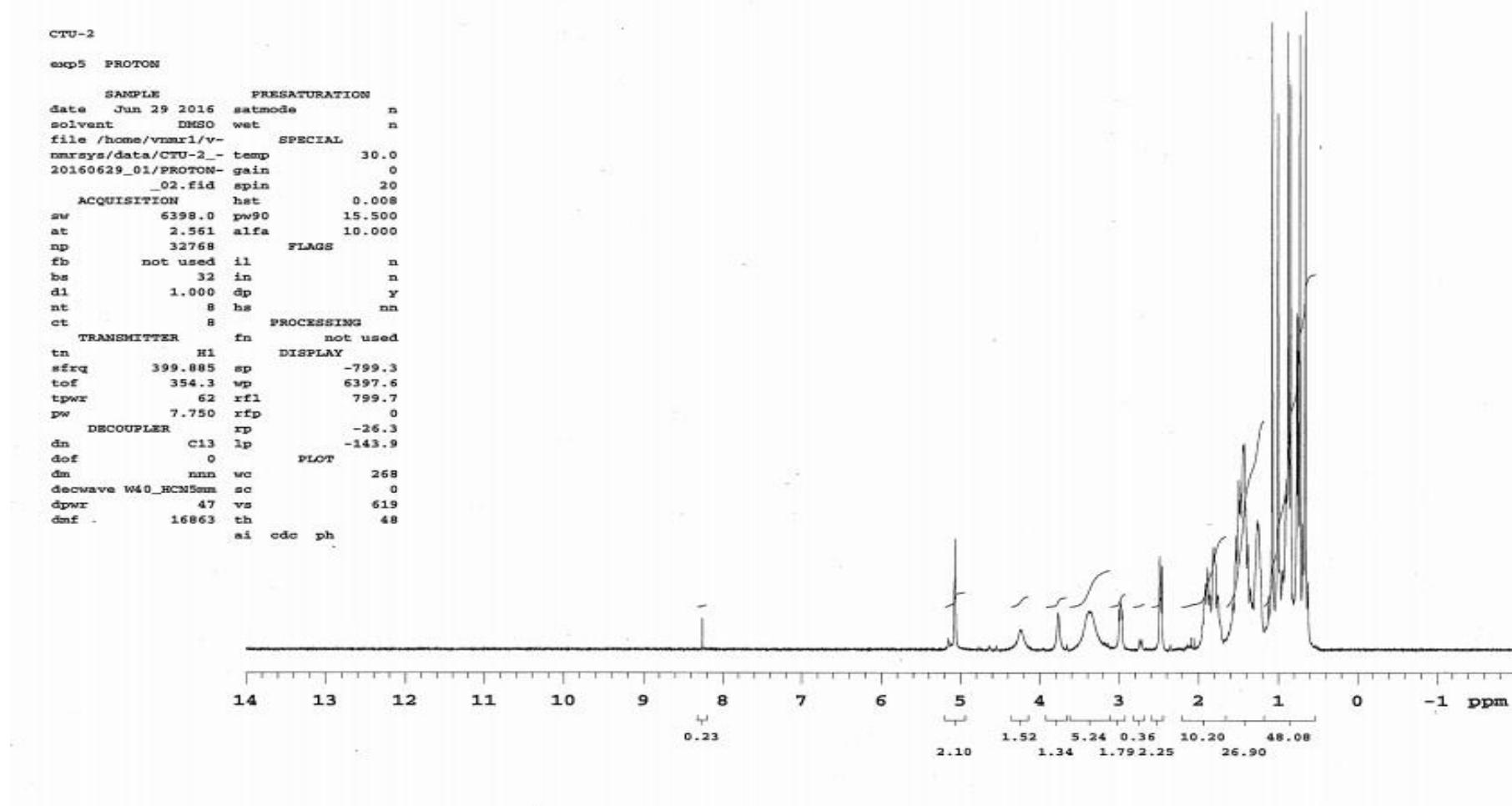


Figure 3.2. ^{13}C spectrum of Compound 1



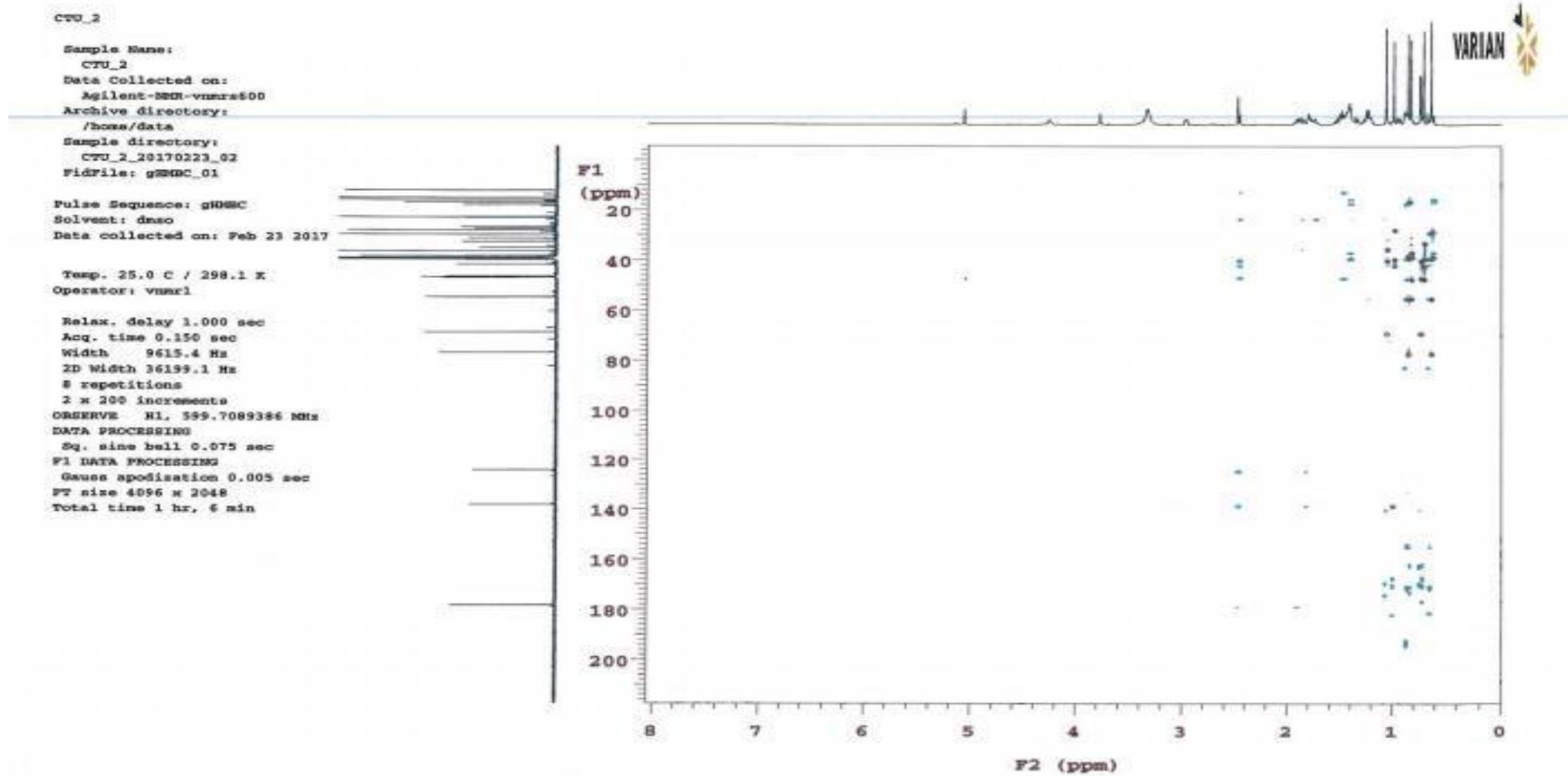


Figure3.4.HMBC spectrum of Compound 1

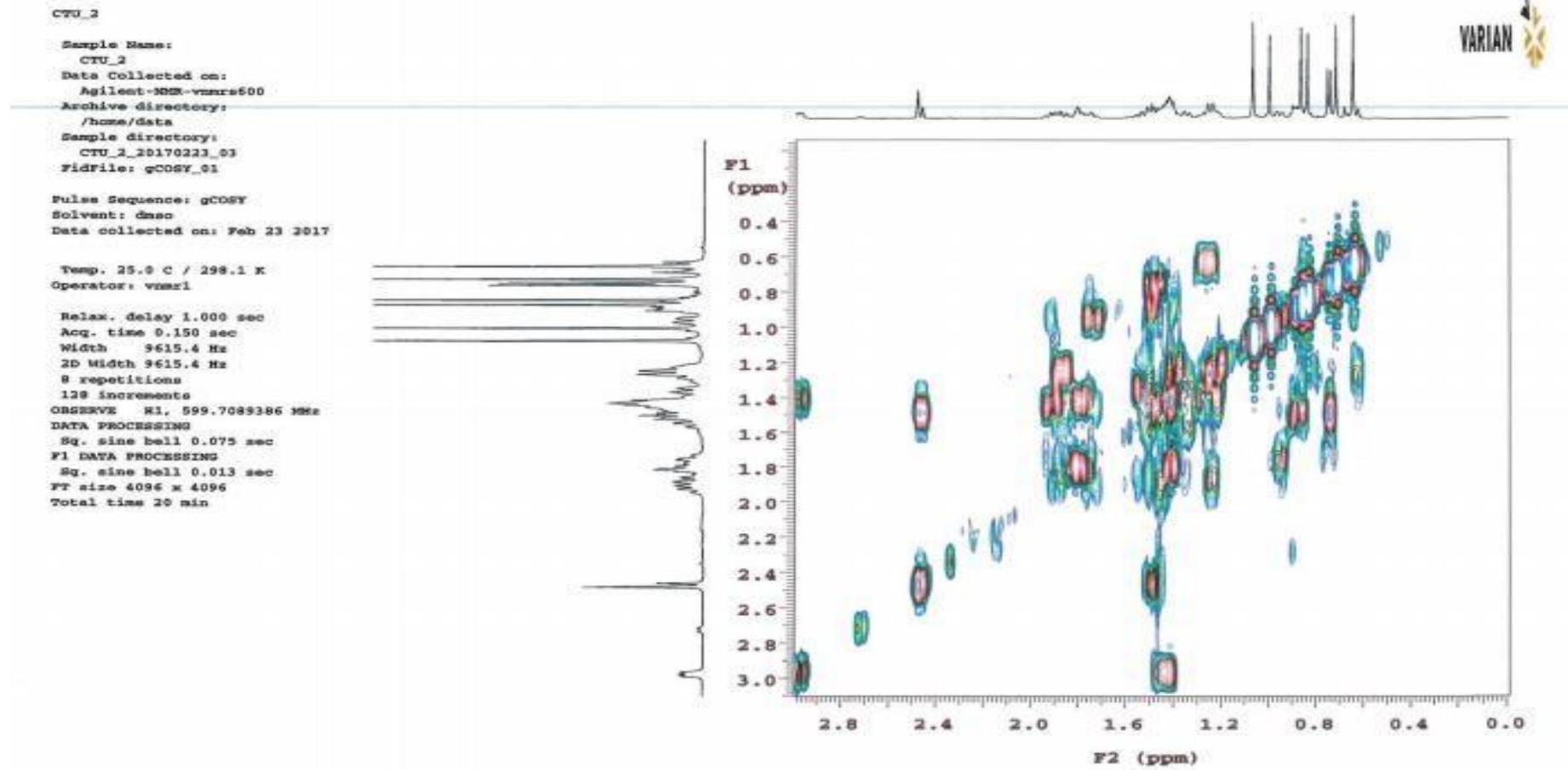


Figure 3.5. COSY spectrum of Compound 1

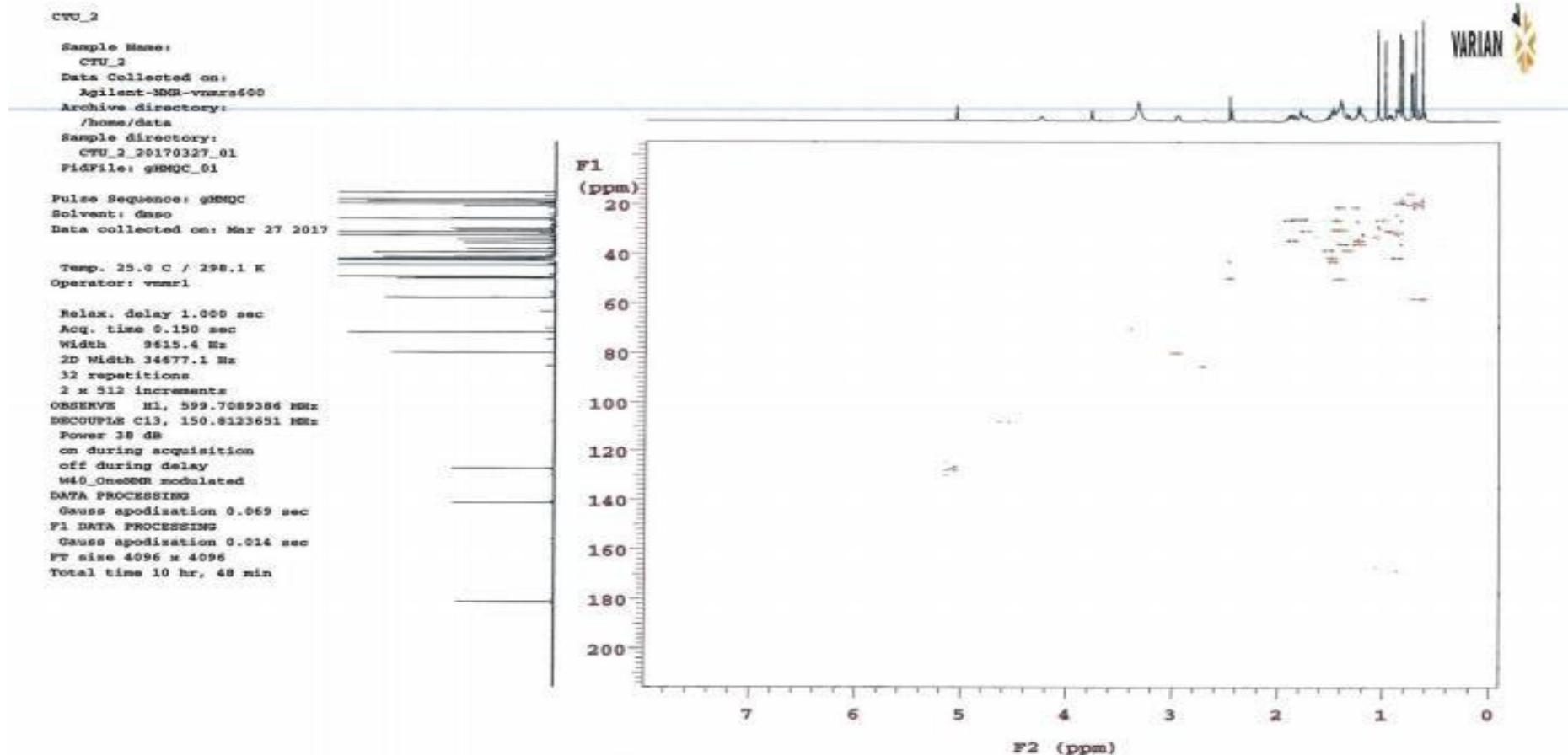


Figure 3.6. HMQC spectrum of Compound 1

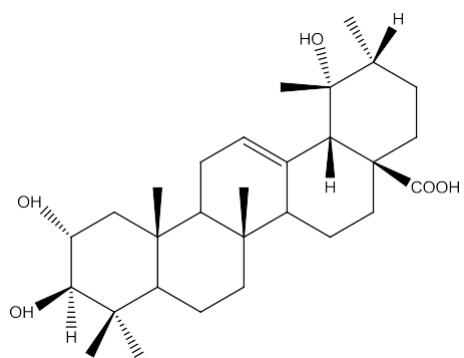
3.2. Compound 2 : Tormentic Acid (Villar et.al., 1986)

Figure 3.7. Tormentic Acid

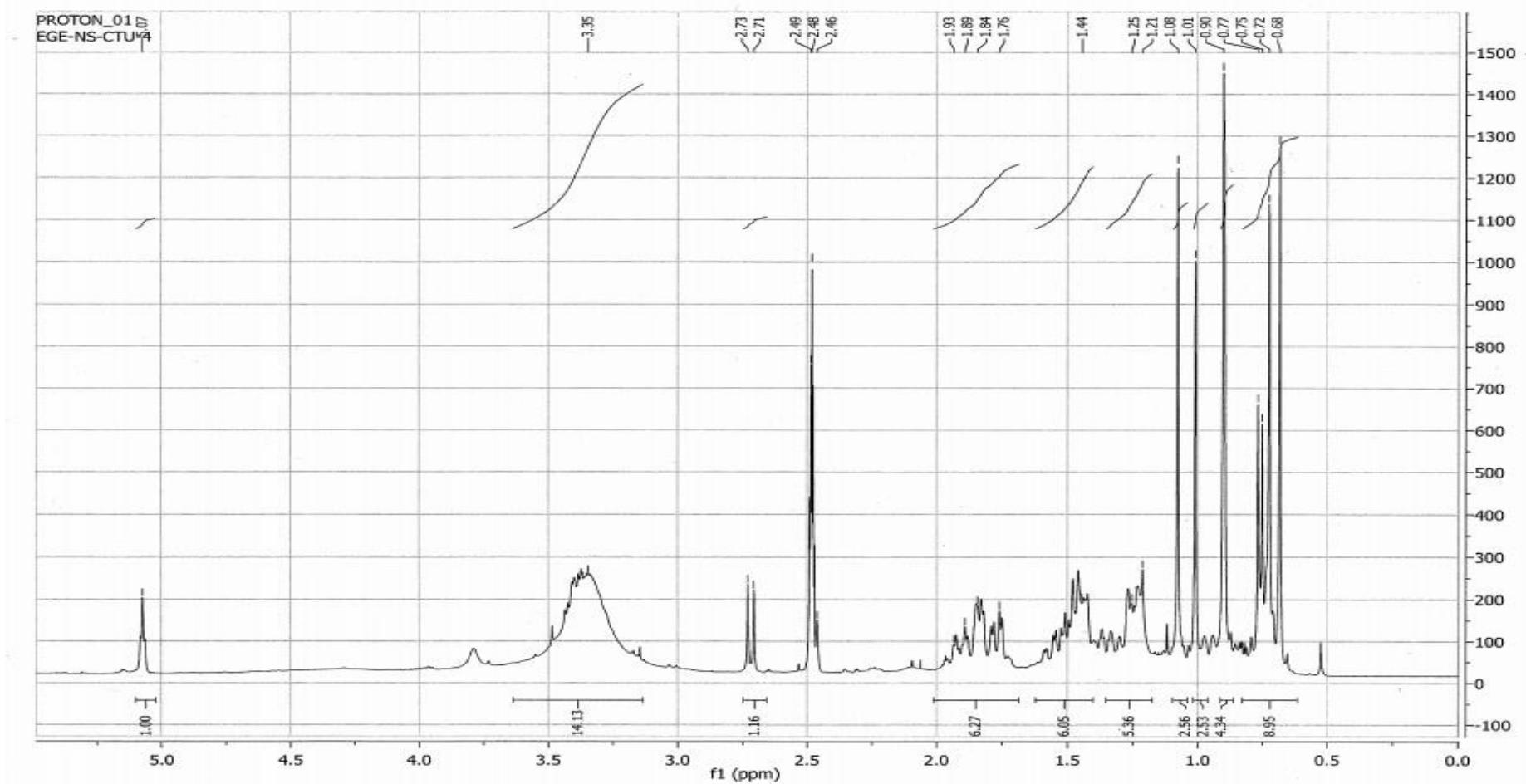


Figure 3.8. ^1H spectrum of Compound 2

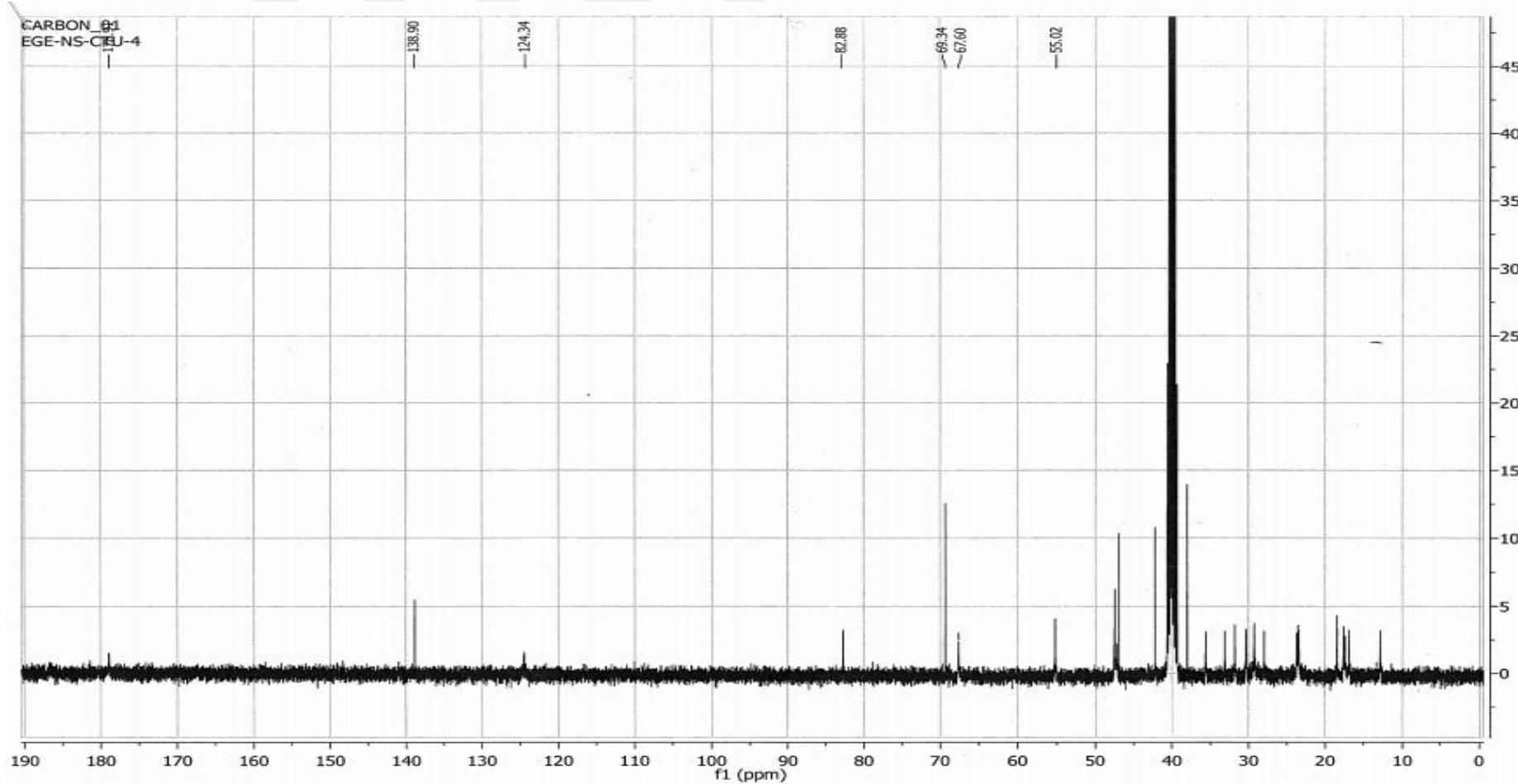


Figure 3. 9. ^{13}C spectrum of Compound 2

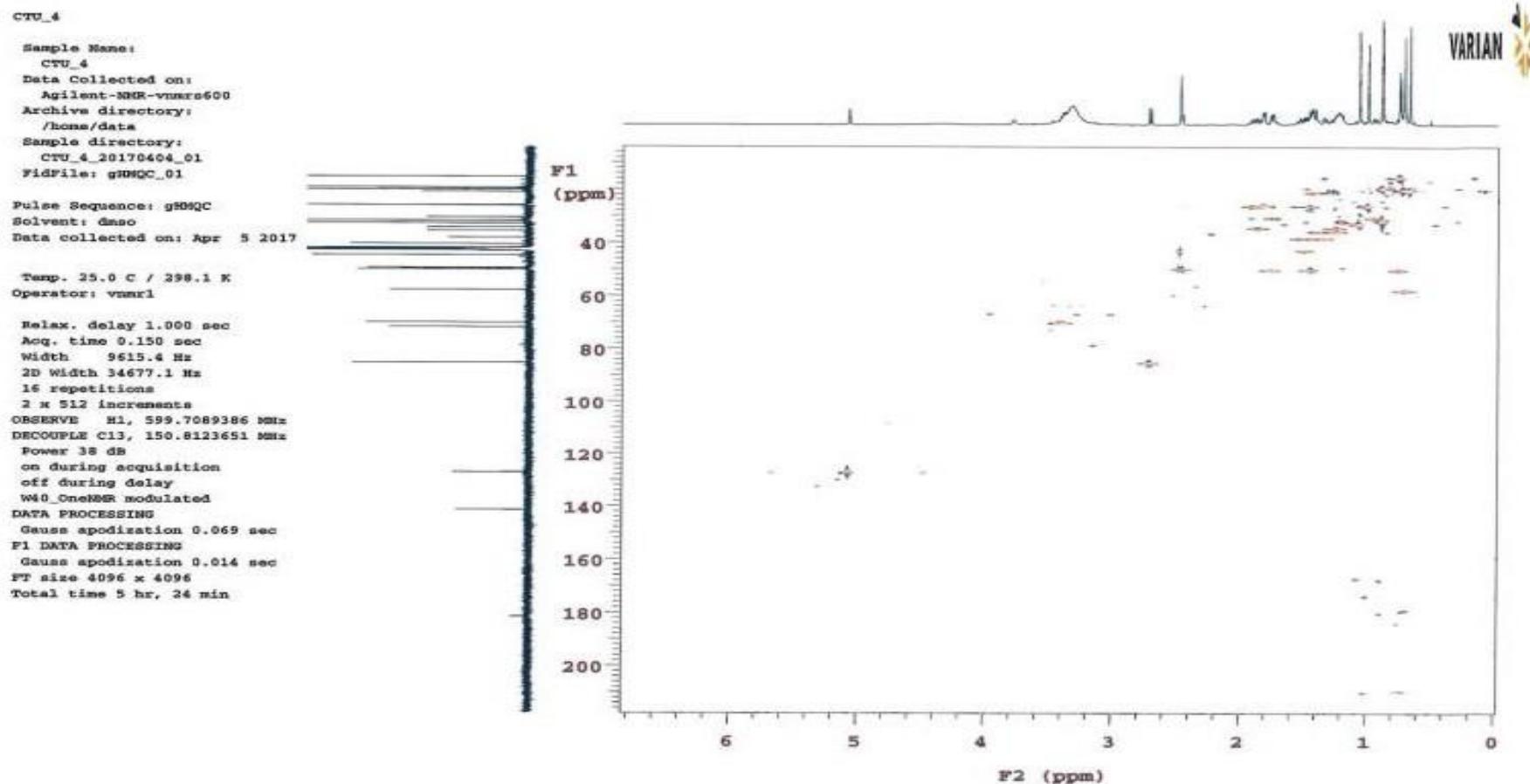


Figure 3.10.HMQC spectrum of Compound 2

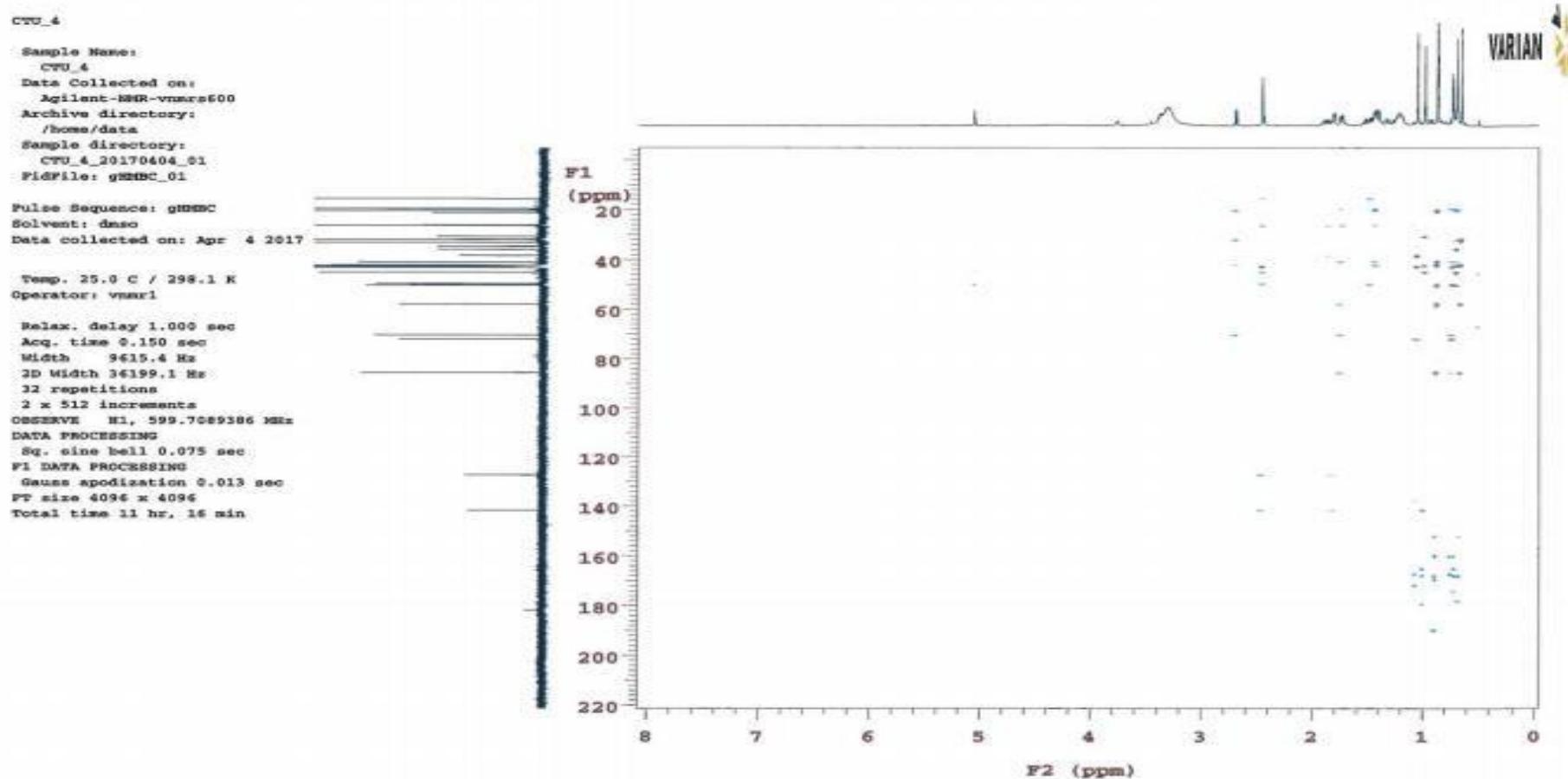


Figure 3.11. HMBC spectrum of Compound 2

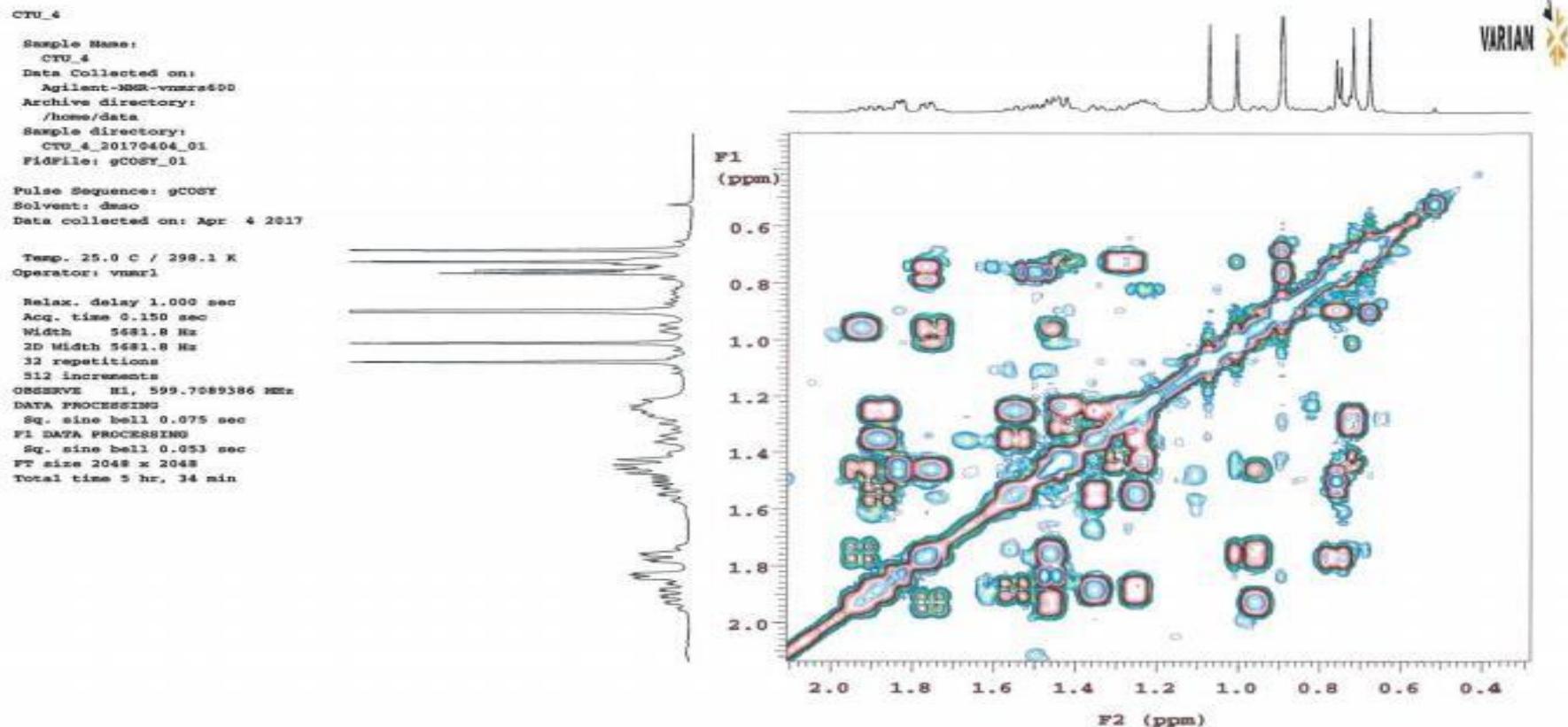


Figure 3.12. COSY spectrum of Compound 2

3.3. Compound 3: Elmalienoside A (Sarikahya and Kirmizigul, 2012)

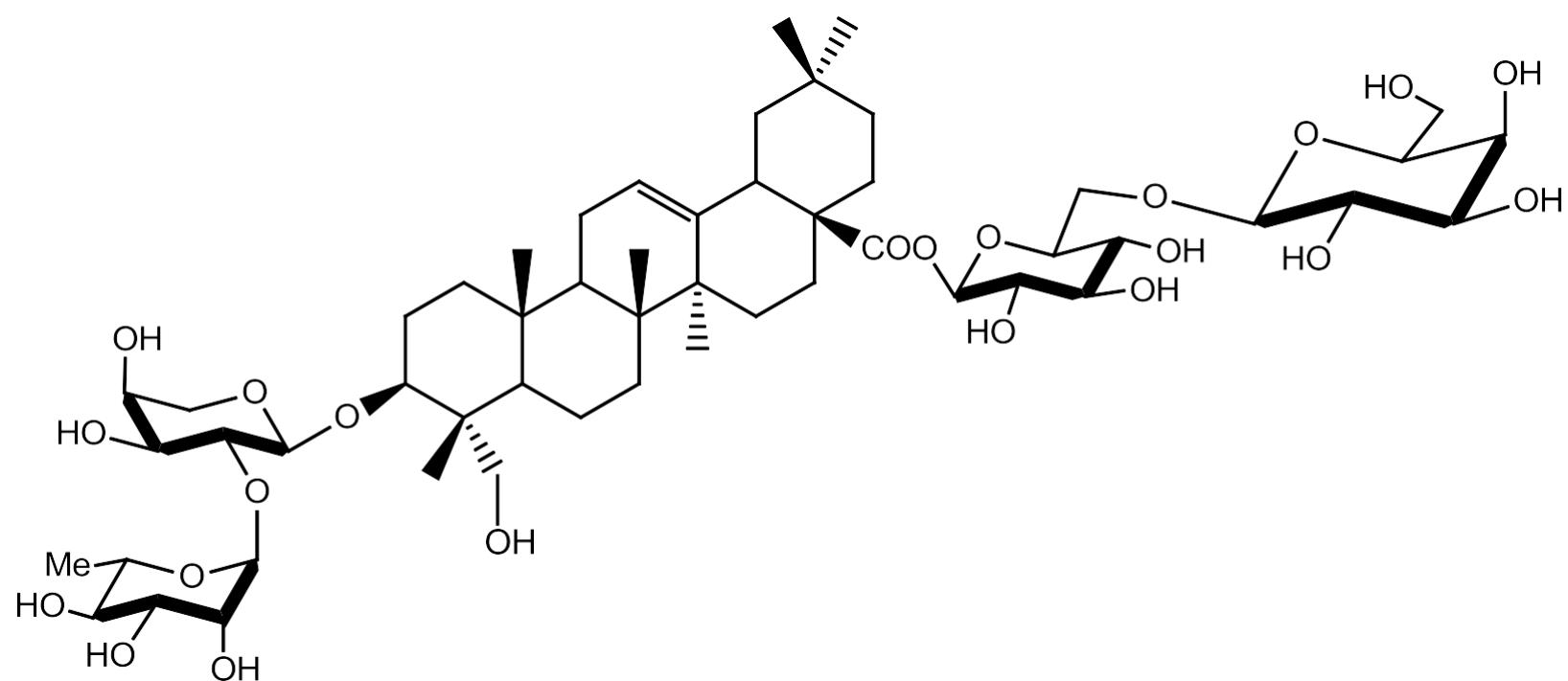


Figure 3.13. Elmalienoside A

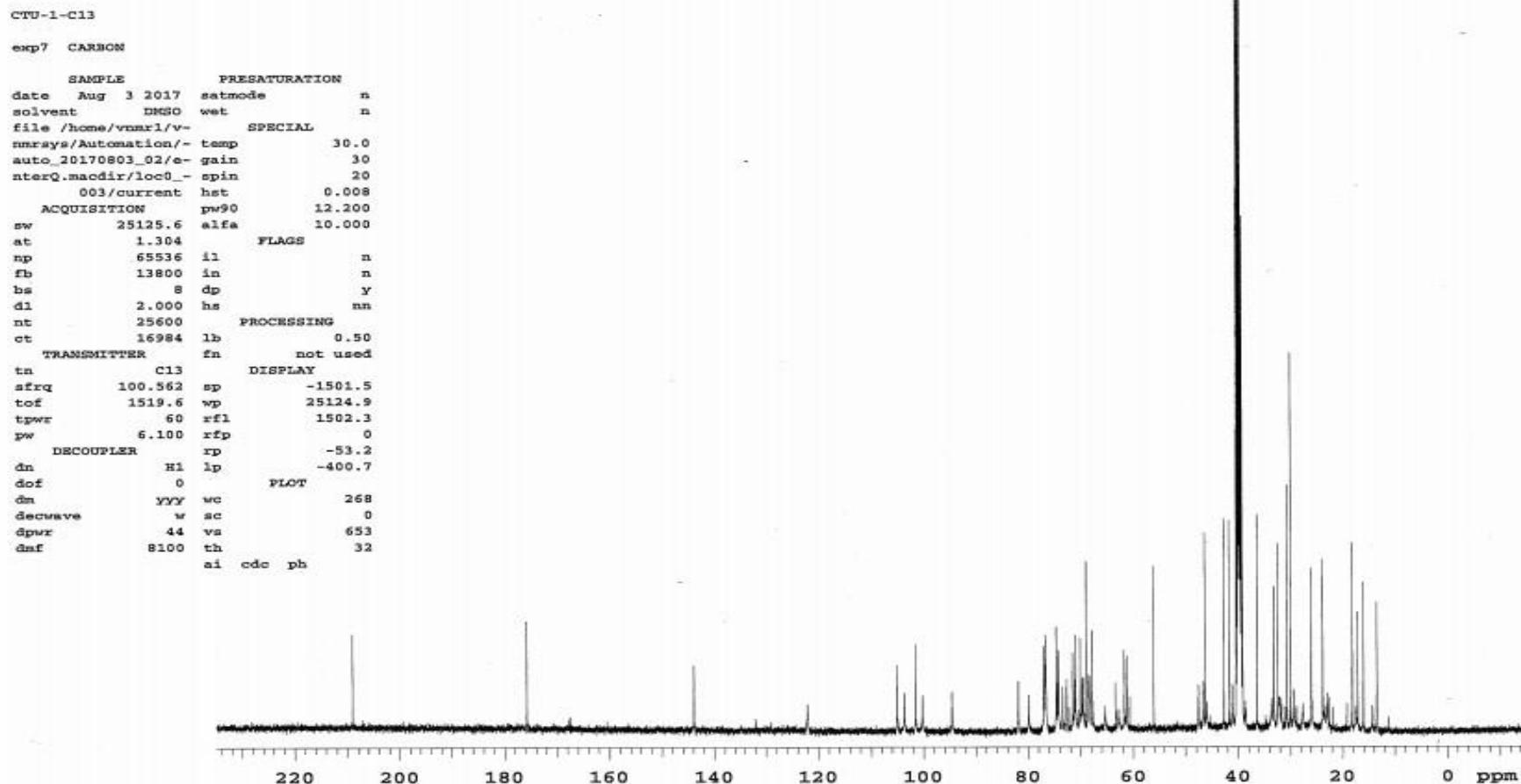


Figure 3.14. ^{13}C spectrum of Compound 3

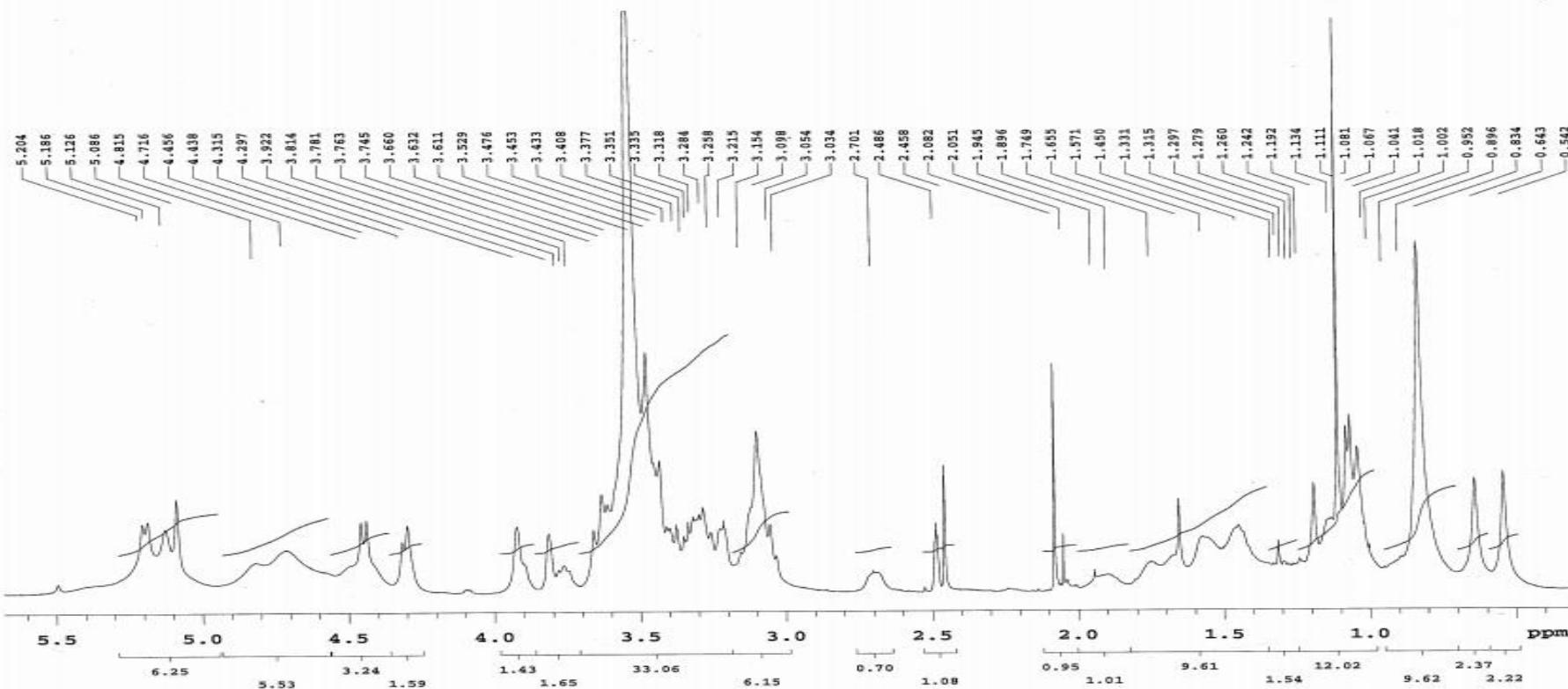


Figure 3.14. ^1H spectrum of Compound 3

3.4. Compound 4: Davisianoside A (Kayce et.al., 2014)

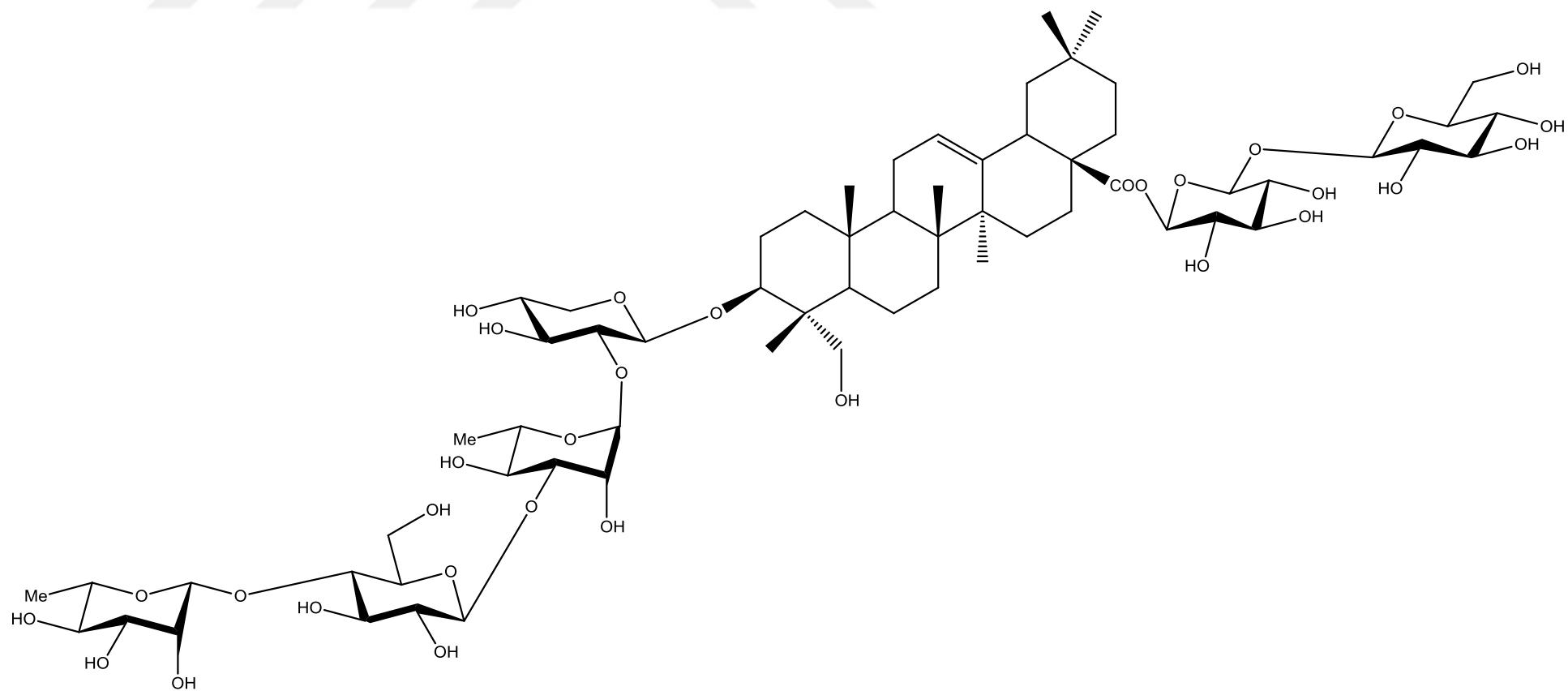
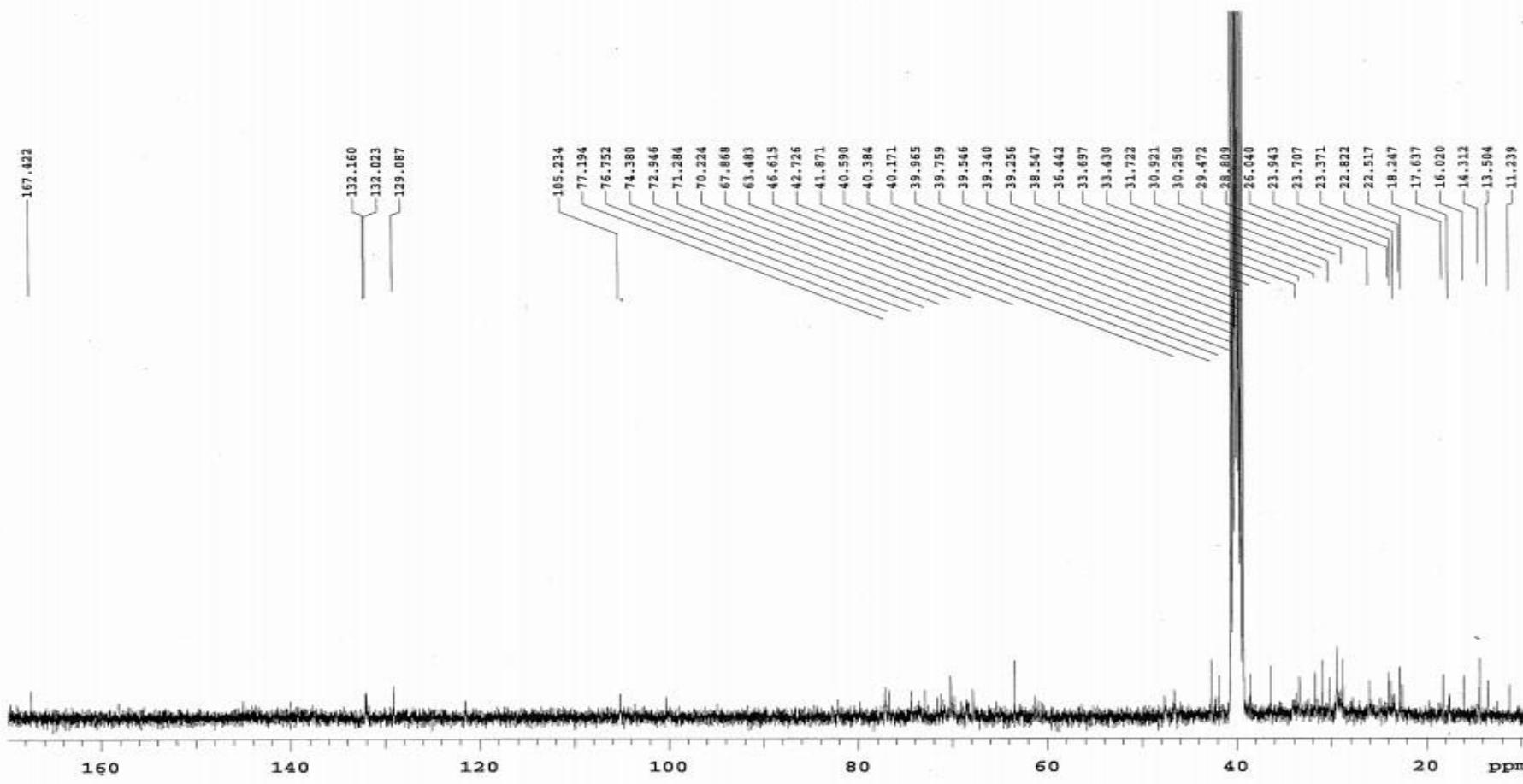


Figure 3.16.Davisianoside A



Şekil 3.16. ^{13}C spectrum of Compound 4

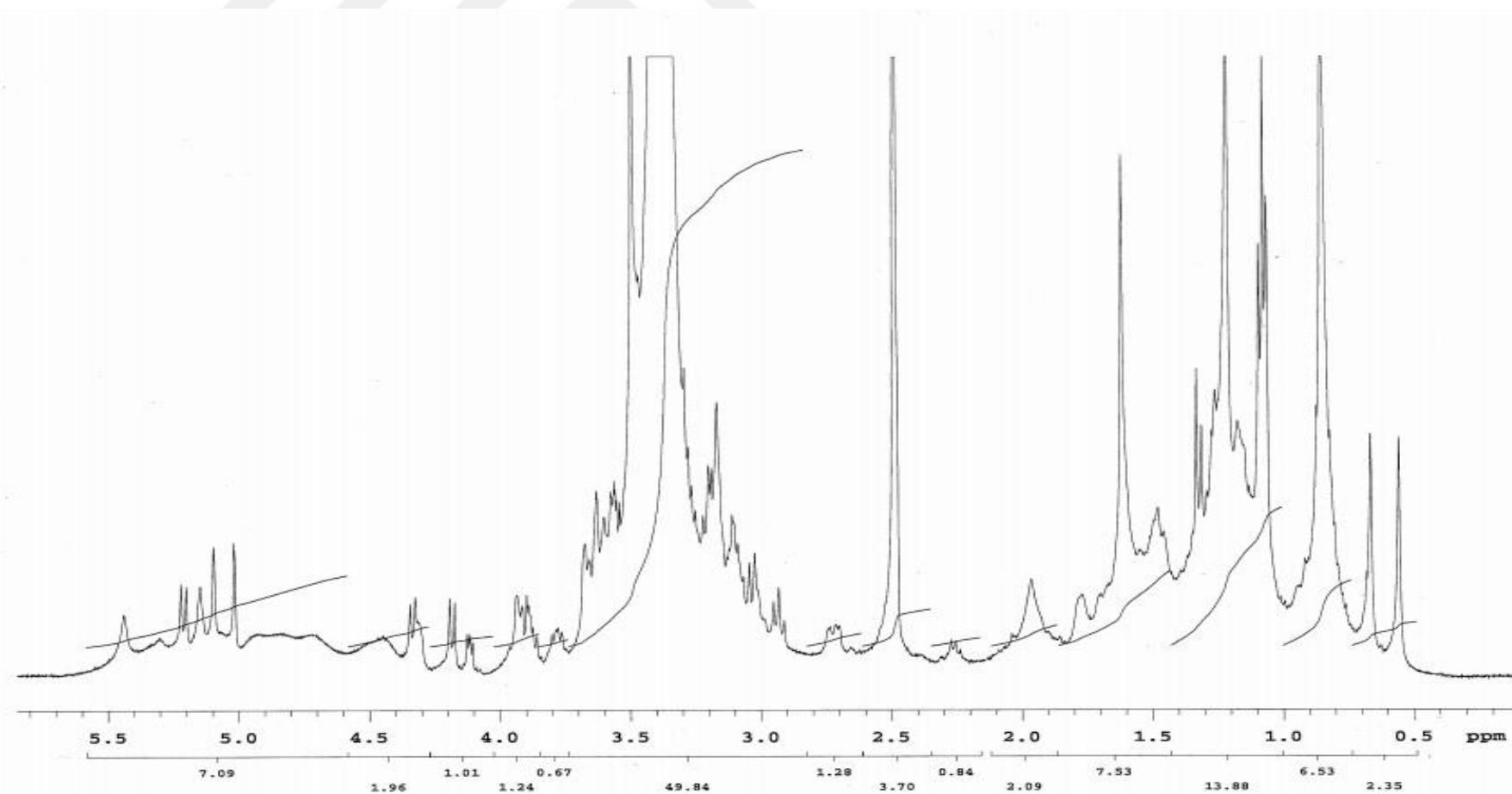
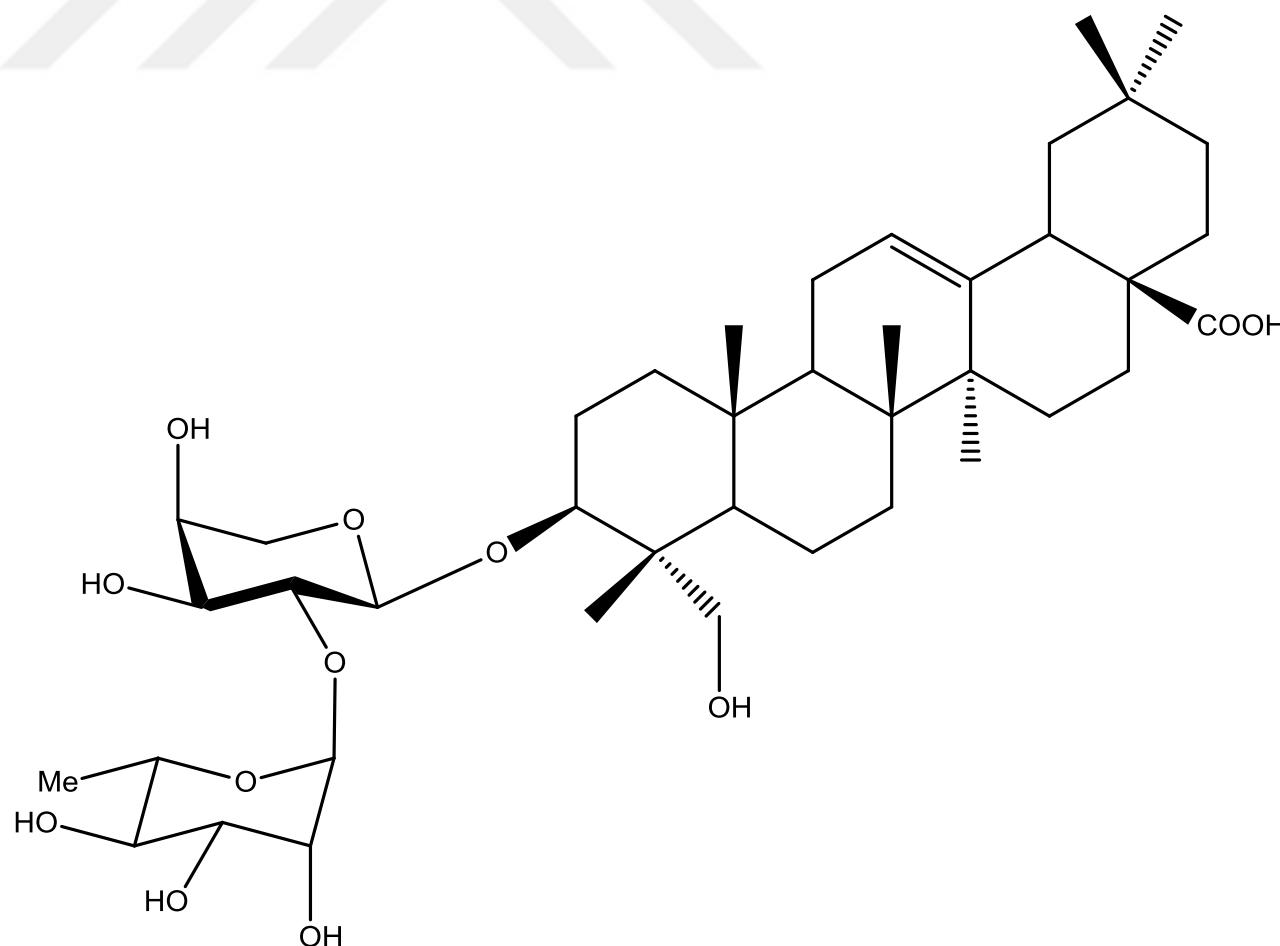


Figure 3.17. ^1H spectrum of Compound 4

3.5. Compound 5: Alpha Hederin (Aliev and Movsumov, 1976)**Figure 3.18.**Alpha Hederin

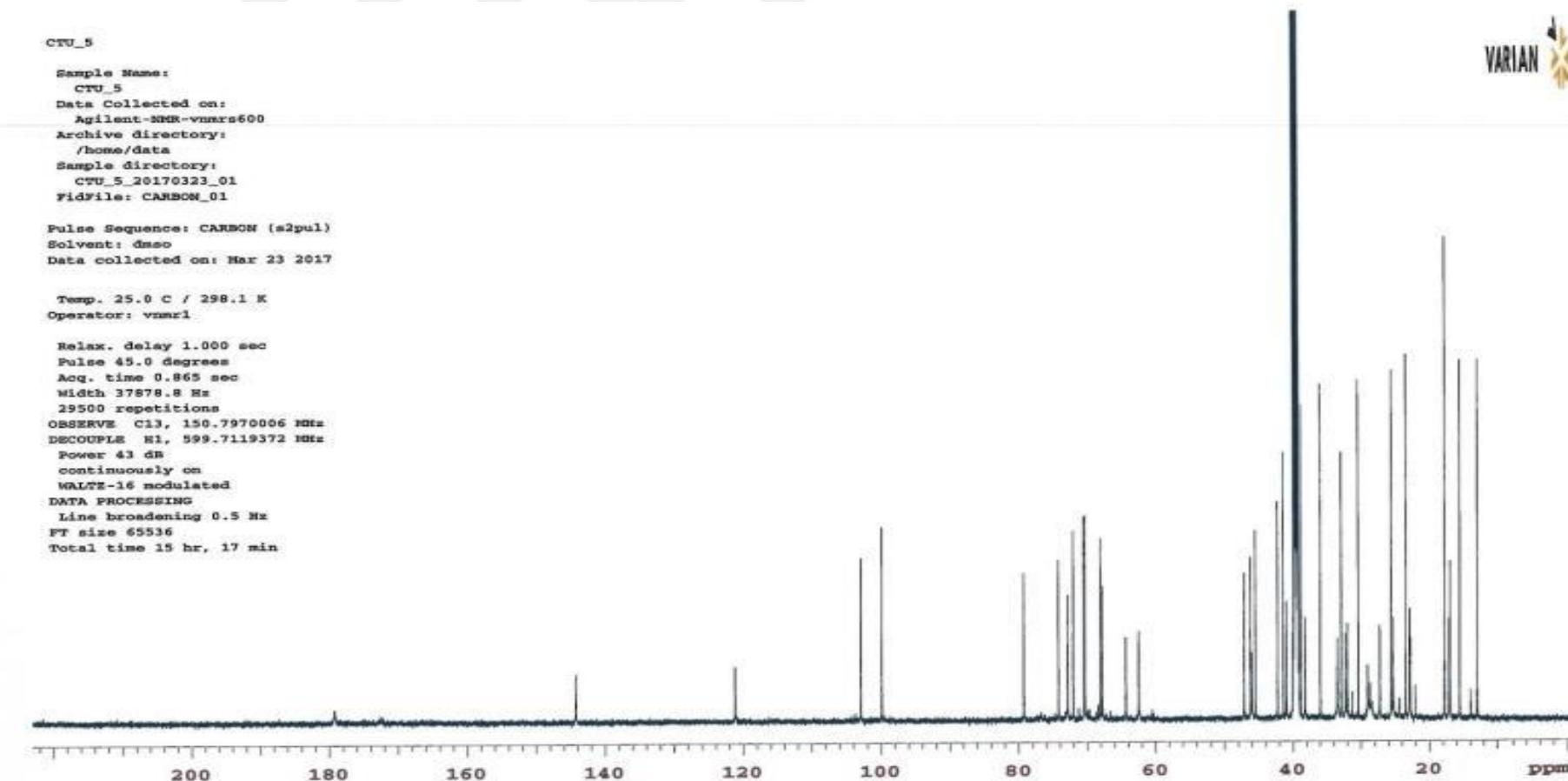


Figure 3.19. ^{13}C spectrum of Compound 5

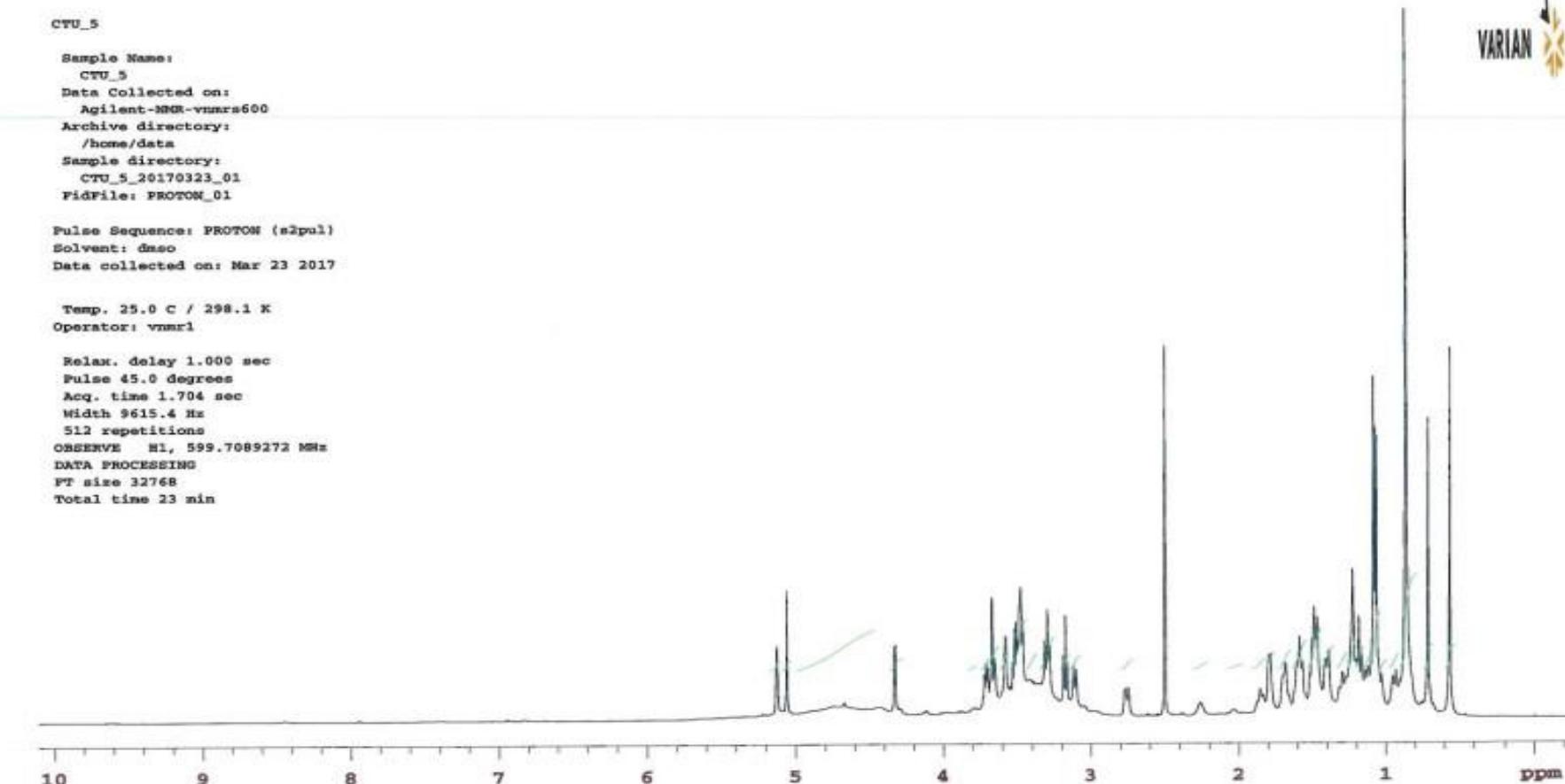
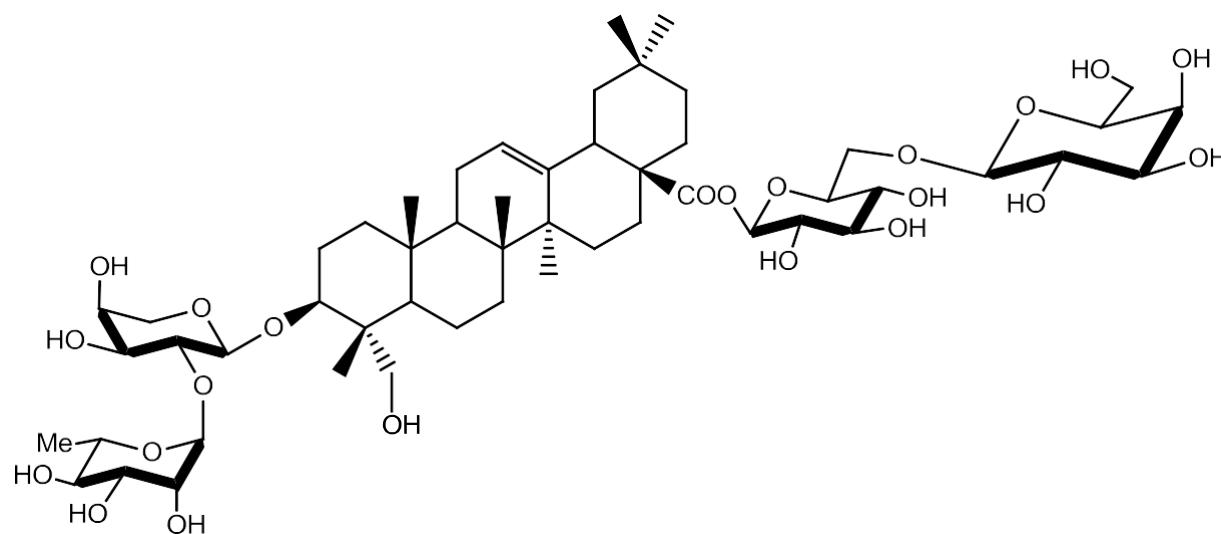


Figure 3.20. ^1H spectrum of Compound 5

3.6. Compound 6: Elmalienoside B (Sarikahya and Kirmizigul, 2012)**Figure 3.21. Elmalienoside B**

3.7. Compound 7: 3-*O*- α -L-rhamnopyranosyl-(1 \rightarrow 2)- α -L-arabinopyranosyl hederagenin 28-*O*- β -D-glucopyranosyl ester (Kawai et. al., 1988)

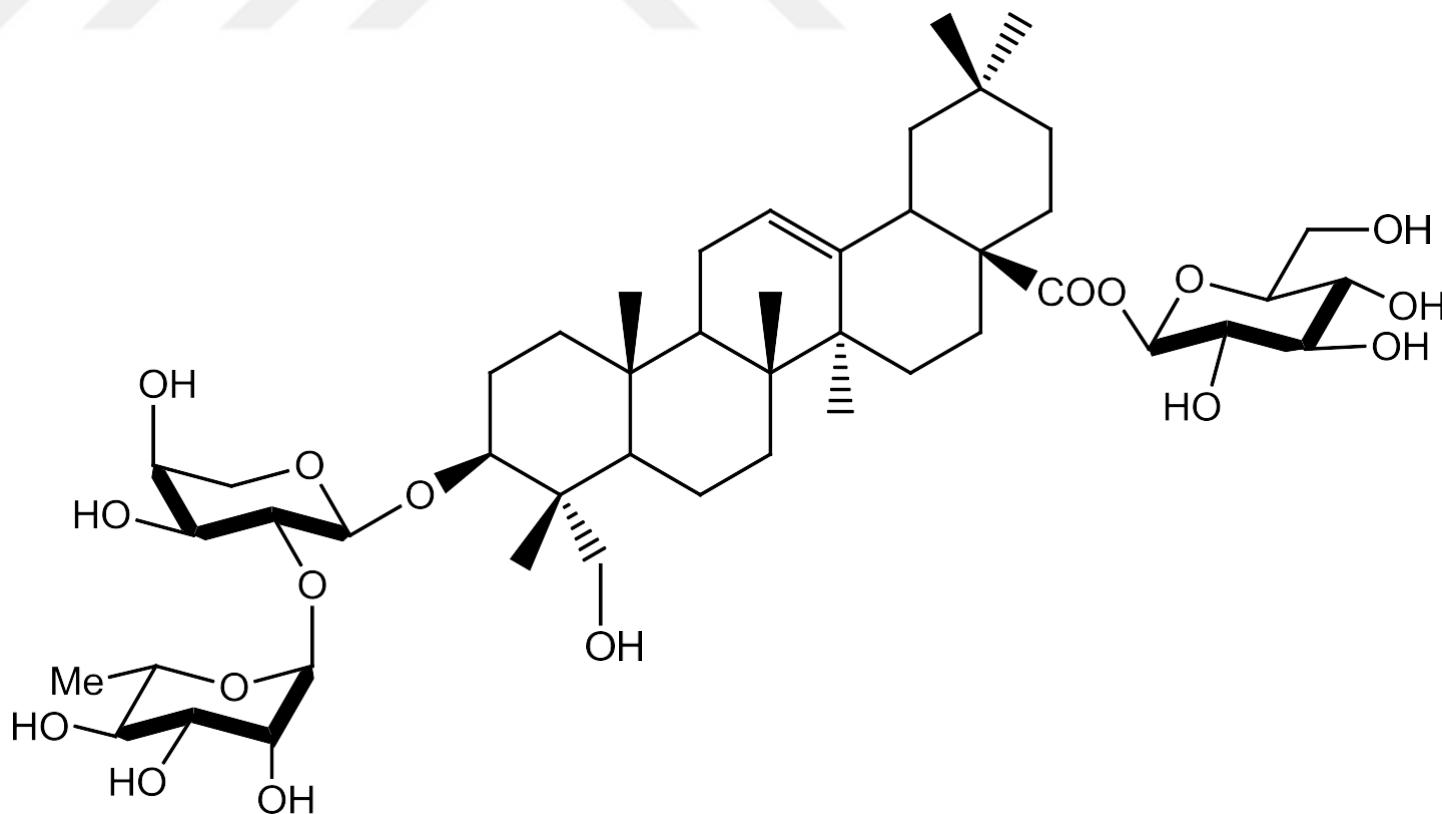


Figure 3.22. 3-*O*- α -L-rhamnopyranosyl-(1 \rightarrow 2)- α -L-arabinopyranosyl hederagenin 28-*O*- β -D-glucopyranosyl ester

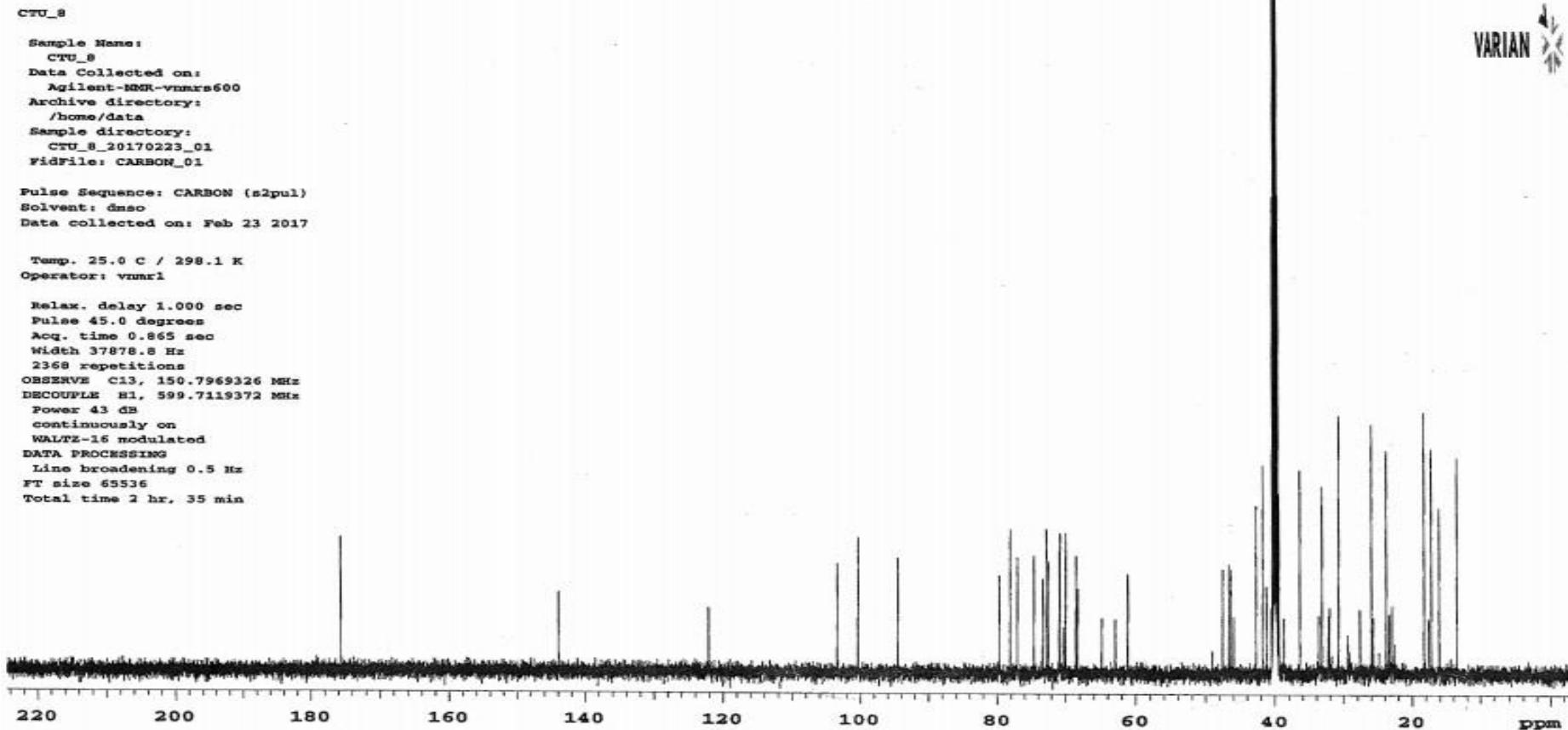


Figure 3.23. ^{13}C spectrum of Compound 7

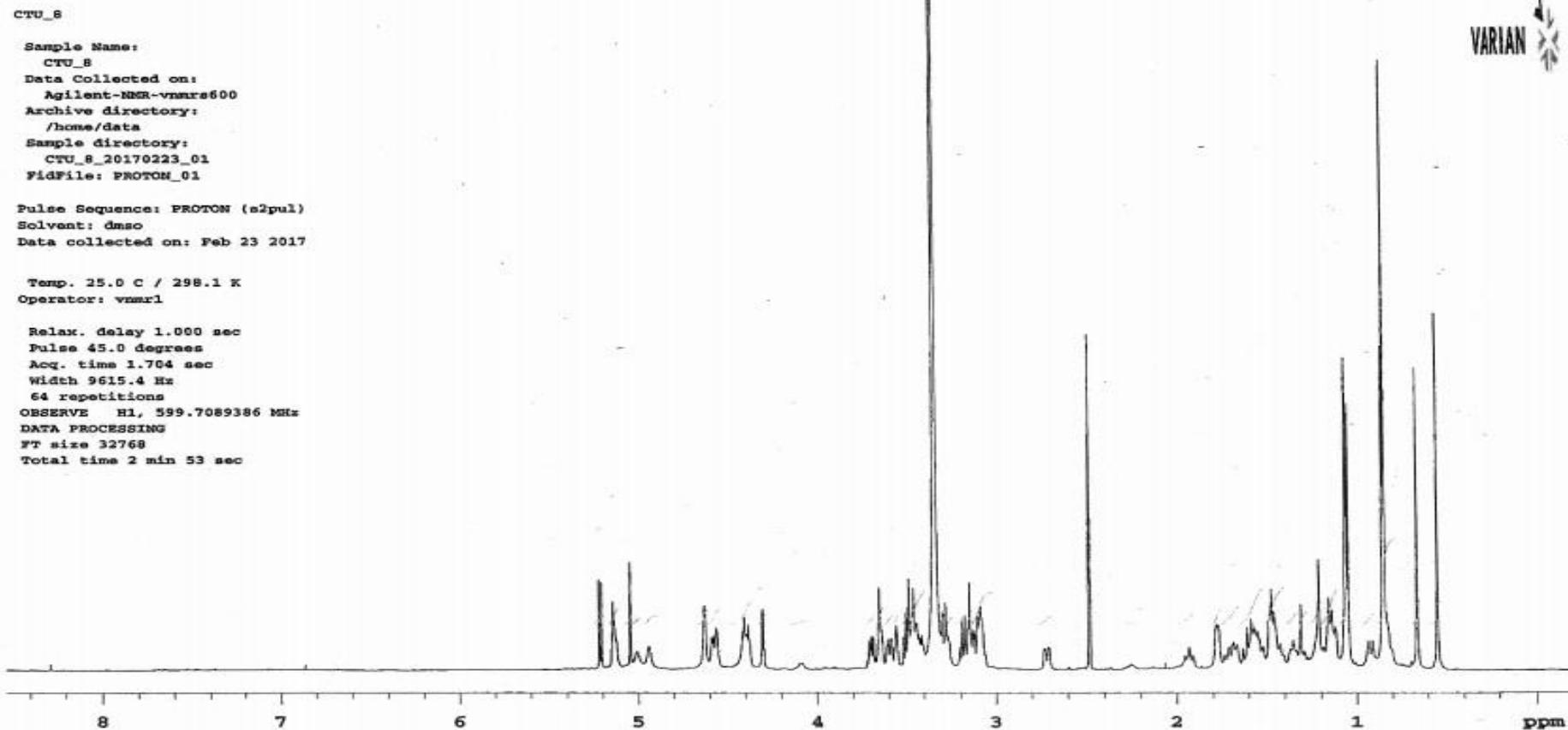
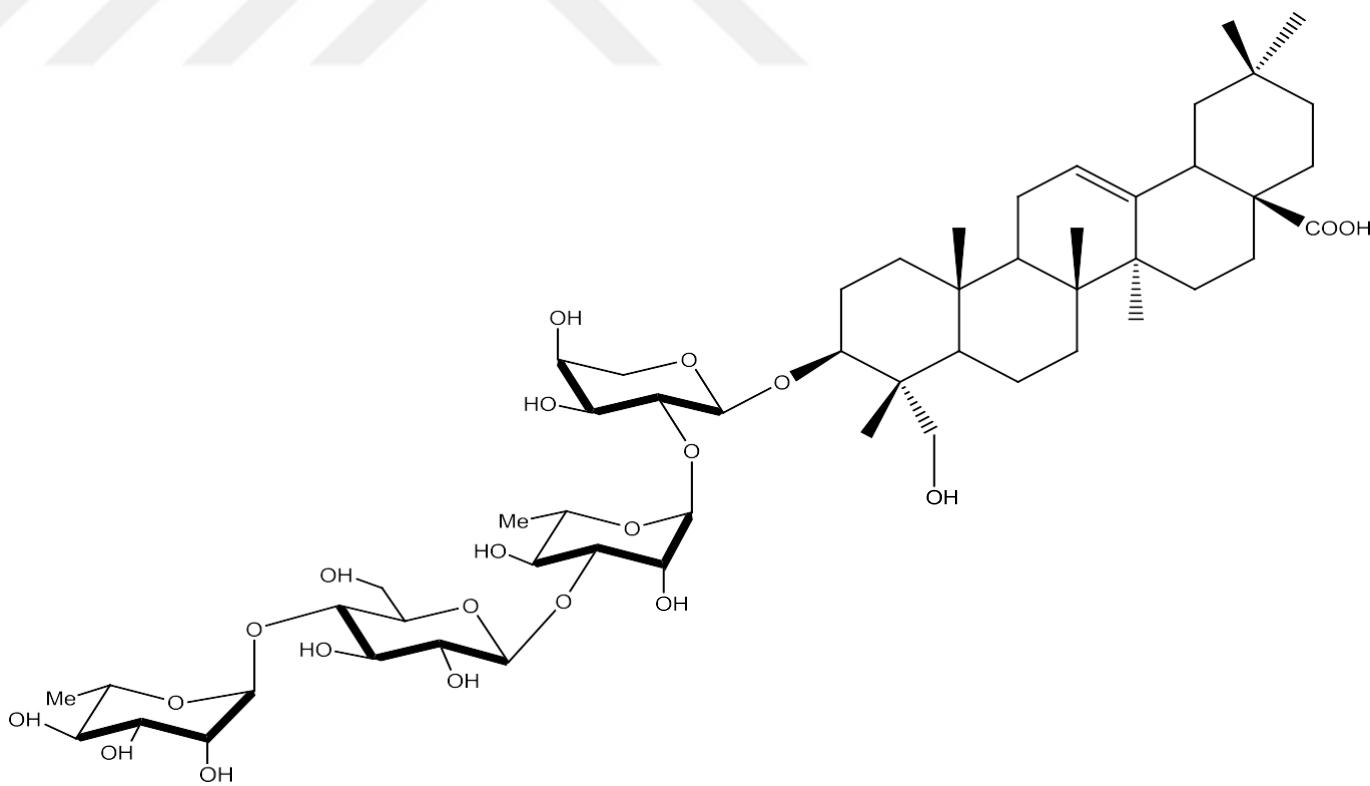


Figure 3.24. ^1H spectrum of Compound 7

3.8. Compound 8: Davisianoside B (Kayce et.al., 2014)**Figure 3.25.**Davisianoside B

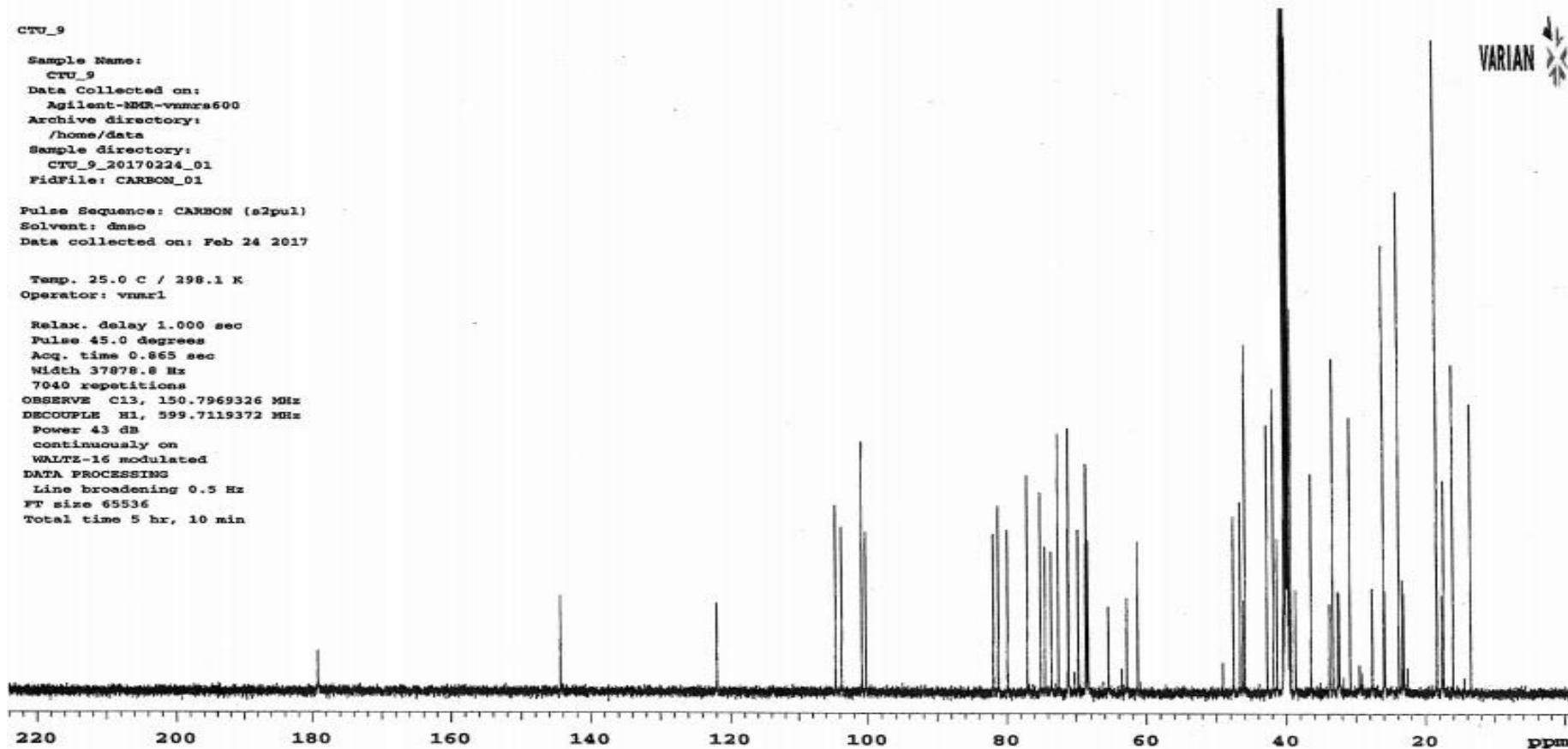


Figure 3.26. ^{13}C spectrum of Compound 8

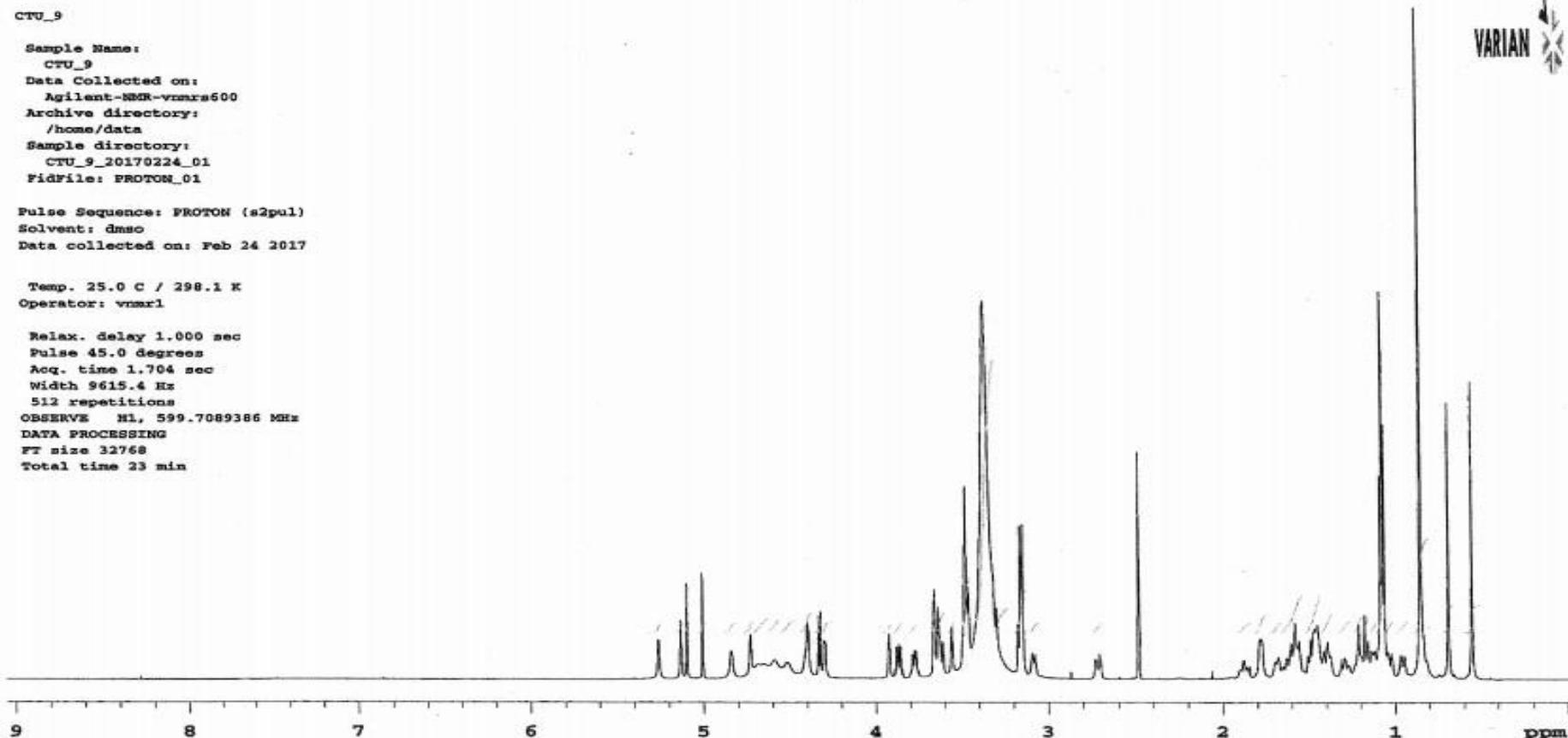


Figure 3.27. ^1H spectrum of Compound 8

3. 9. Compound 9: 3-*O*- β -D-glucopyranosyl-(1 \rightarrow 3)- α -L-rhamnopyranosyl-(1 \rightarrow 2)- α -L-arabinopyranosyl hederagenin 28-*O*- β -D-glucopyranosyl ester. (Braca et.al., 2004)

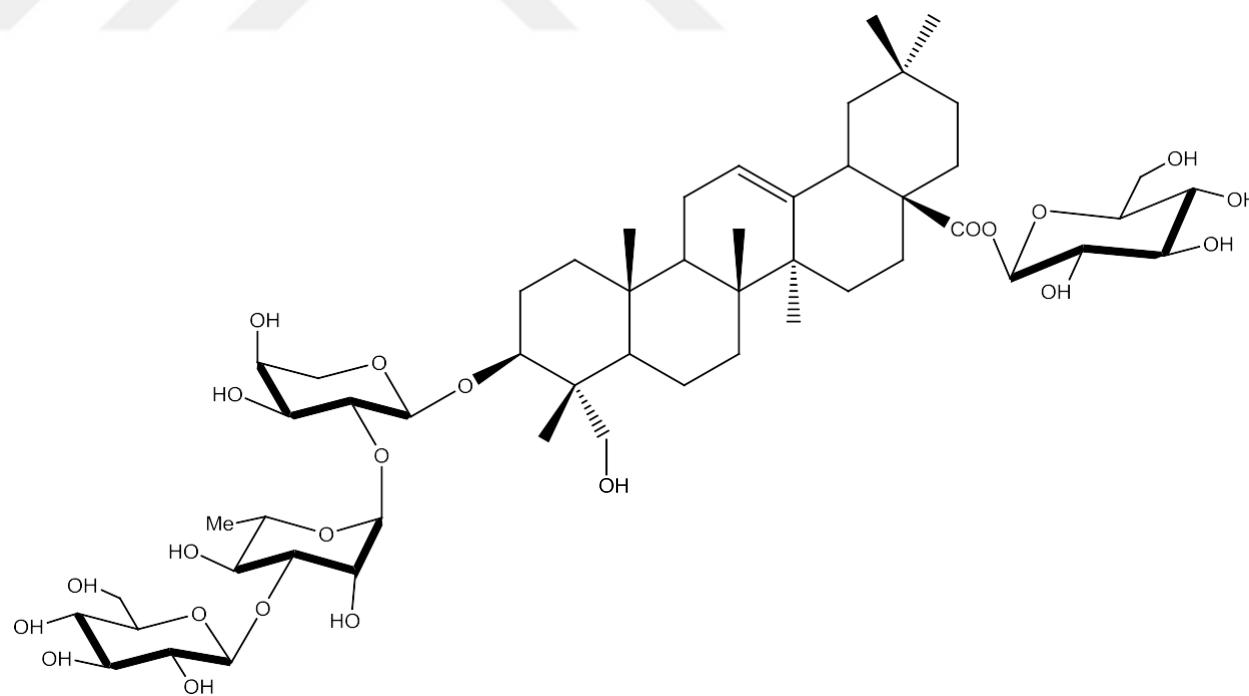


Figure 3.28. 3-*O*- β -D-glucopyranosyl-(1 \rightarrow 3)- α -L-rhamnopyranosyl-(1 \rightarrow 2)- α -L-arabinopyranosyl hederagenin 28-*O*- β -D-glucopyranosyl ester

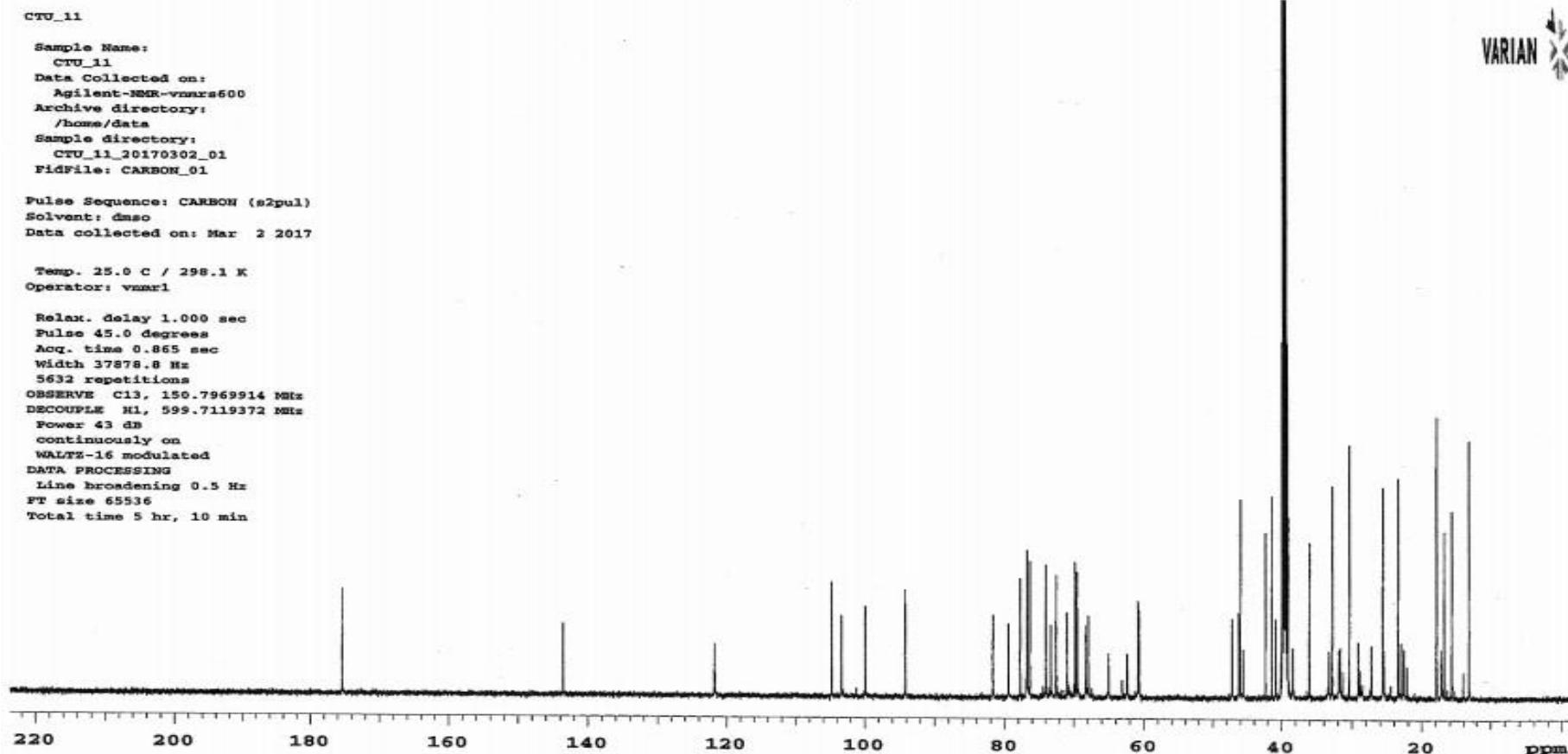


Figure 3.29. ^{13}C spectrum of Compound 9

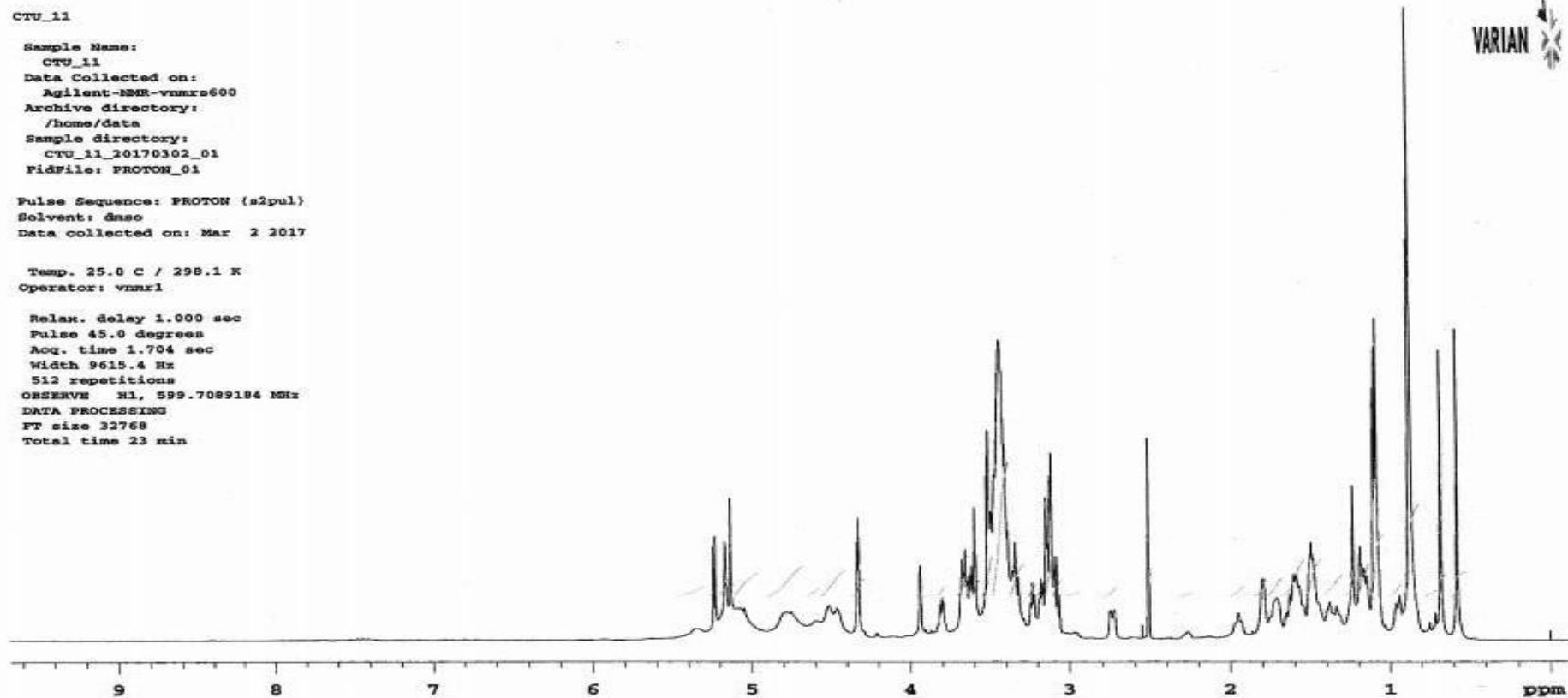
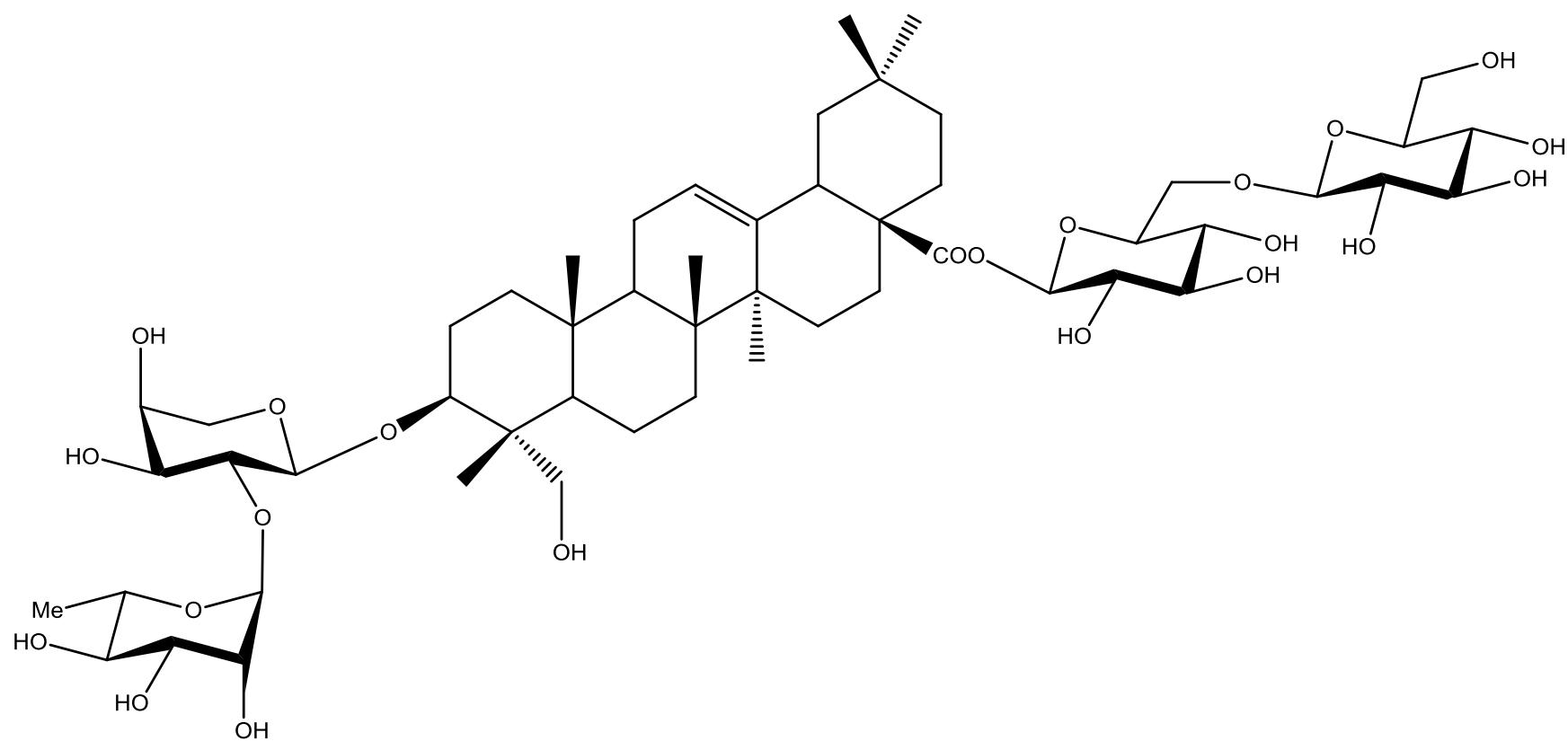


Figure 3.30. ^1H spectrum of Compound 9

3.10. Compound 10: Dipsacoside B (Mukhamedziev et.al., 1971)**Figure 3.31.**Dipsacoside B

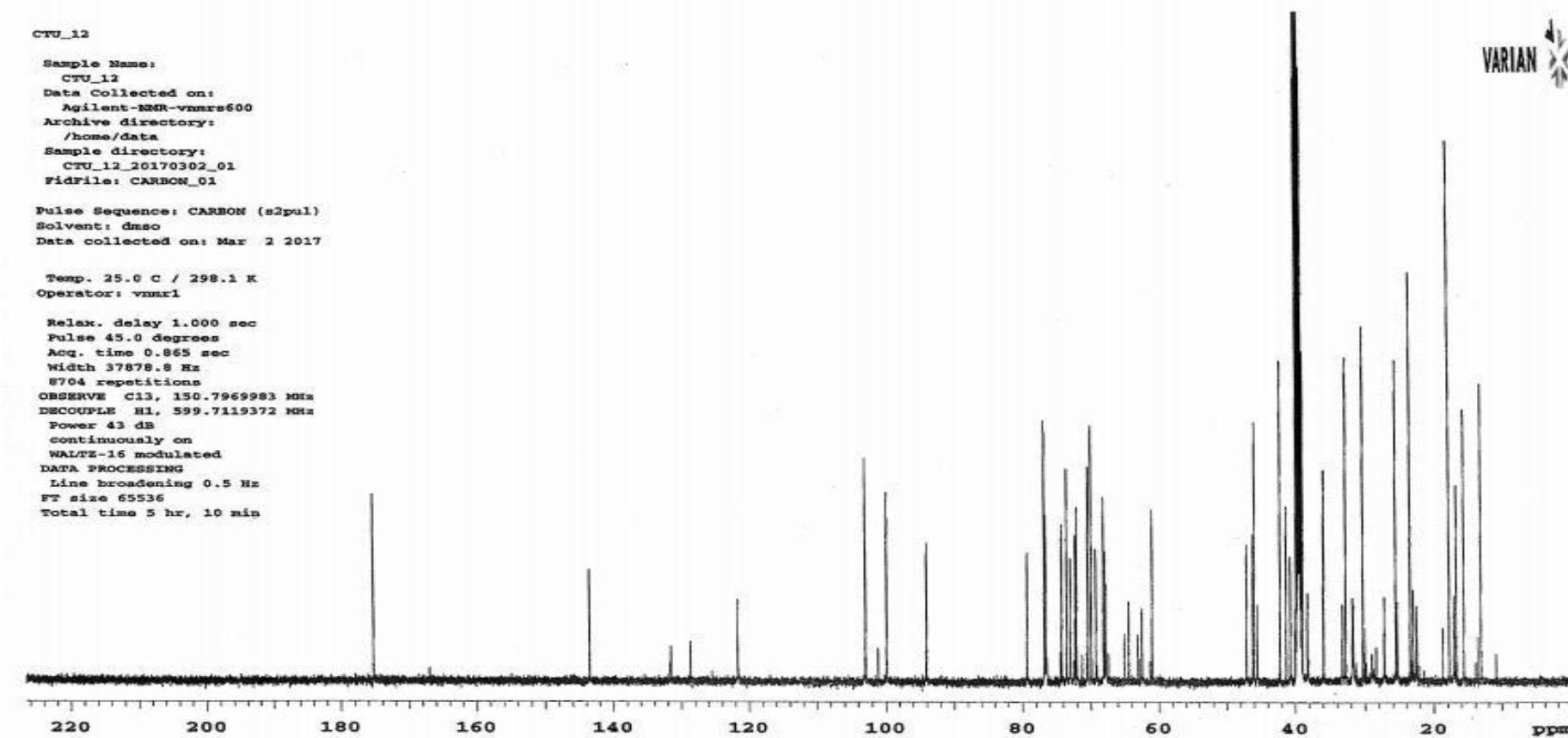


Figure 3.32. ^{13}C spectrum of Compound 10

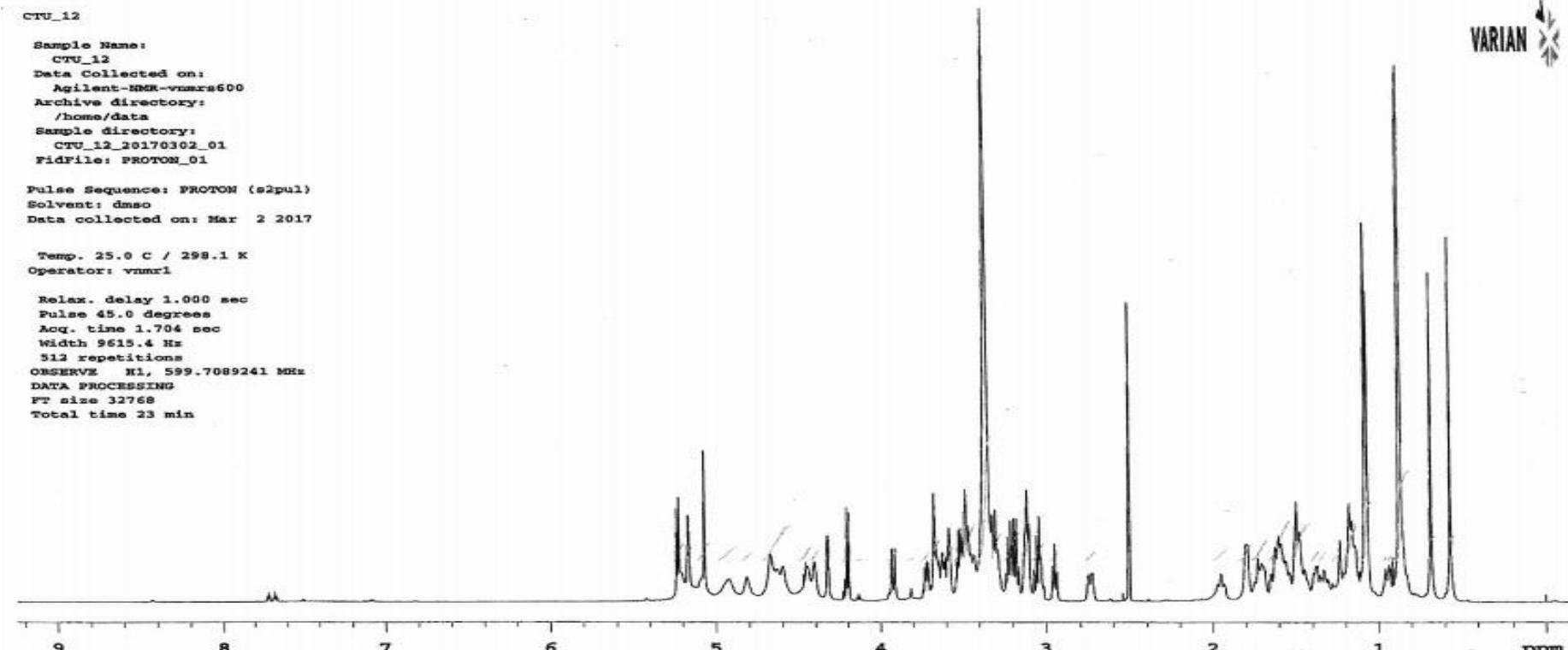
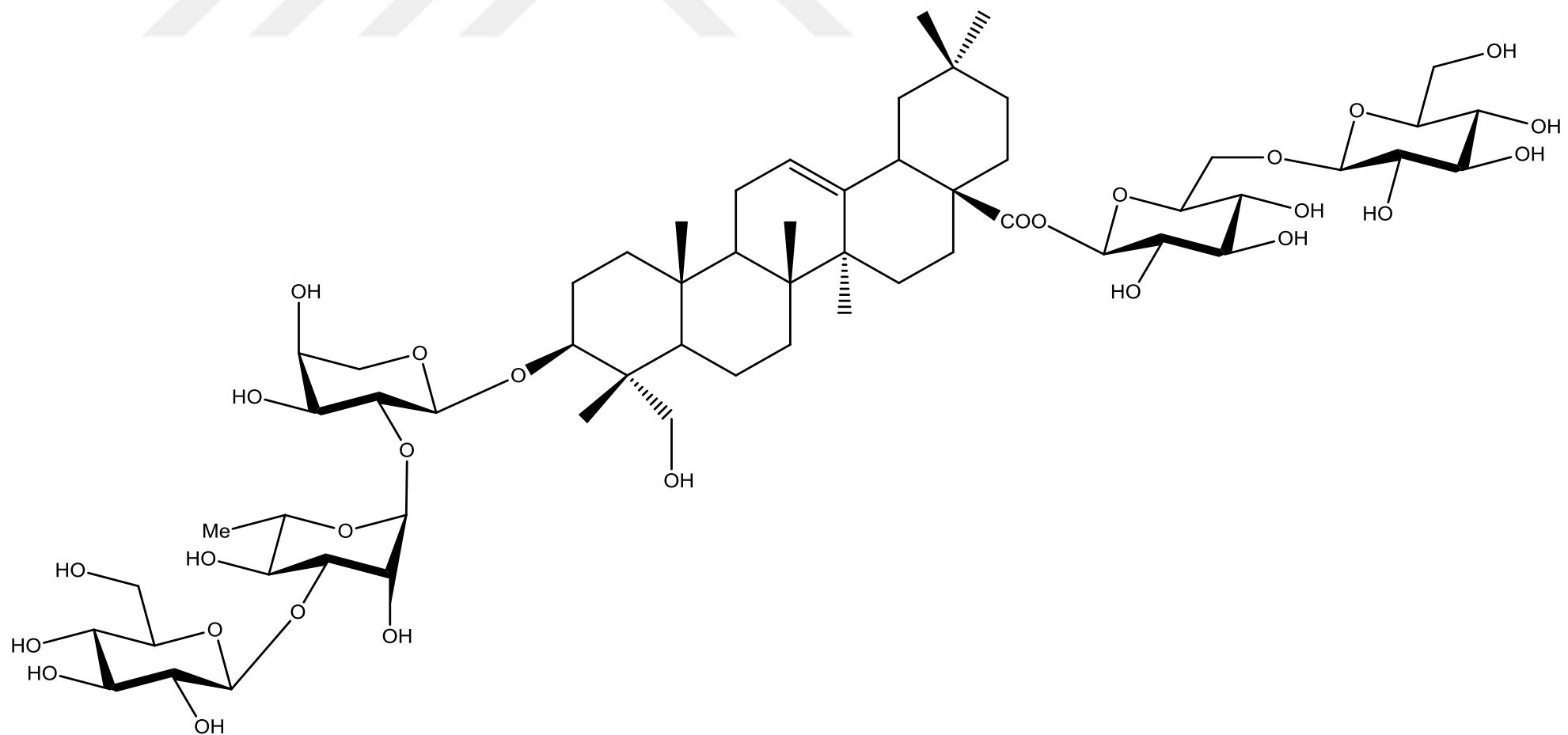


Figure 3.33. ^1H spectrum of Compound 10

3.11. Compound 11: Macranthoidin A (Mao et.al., 1993)**Figure3.34.** Macranthoidin A

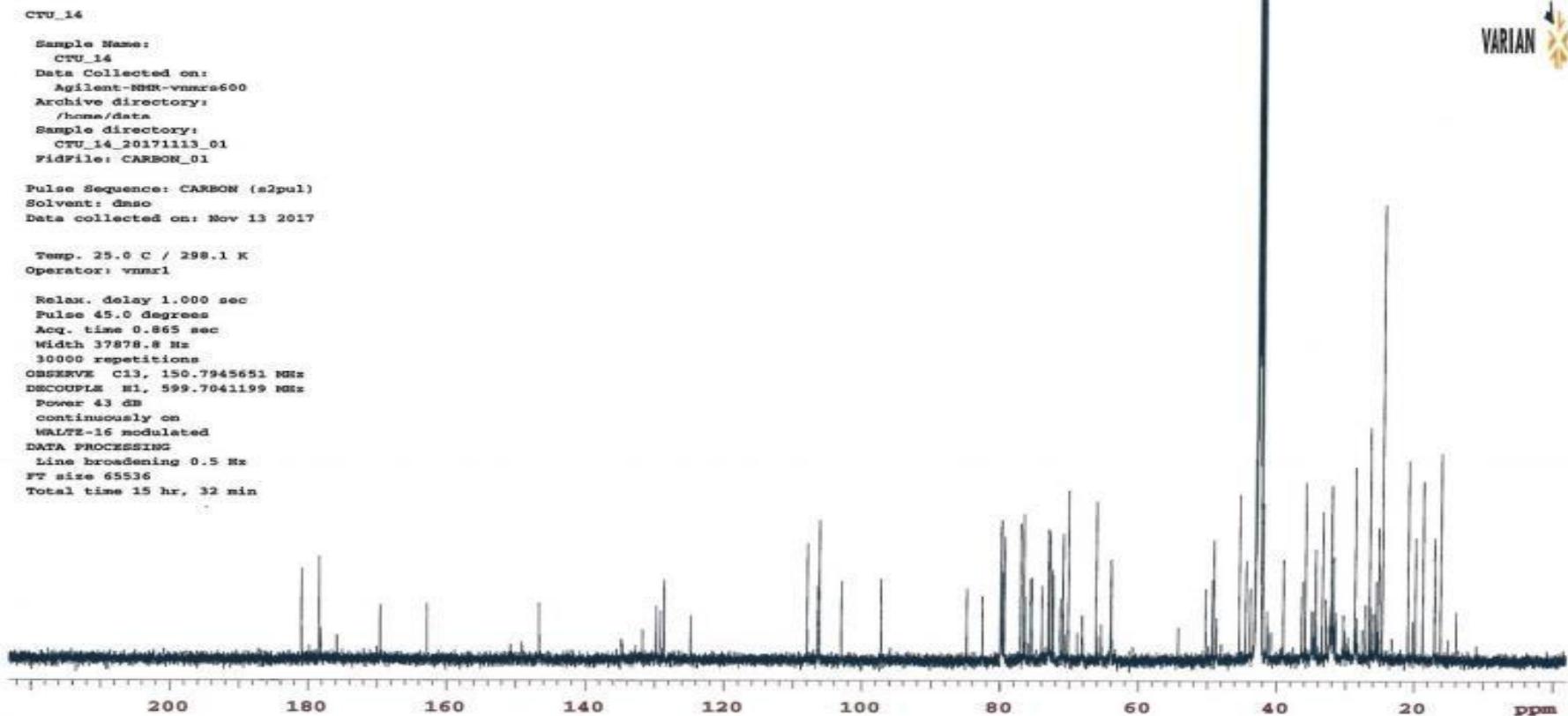


Figure 3.35. ^{13}C spectrum of Compound 11

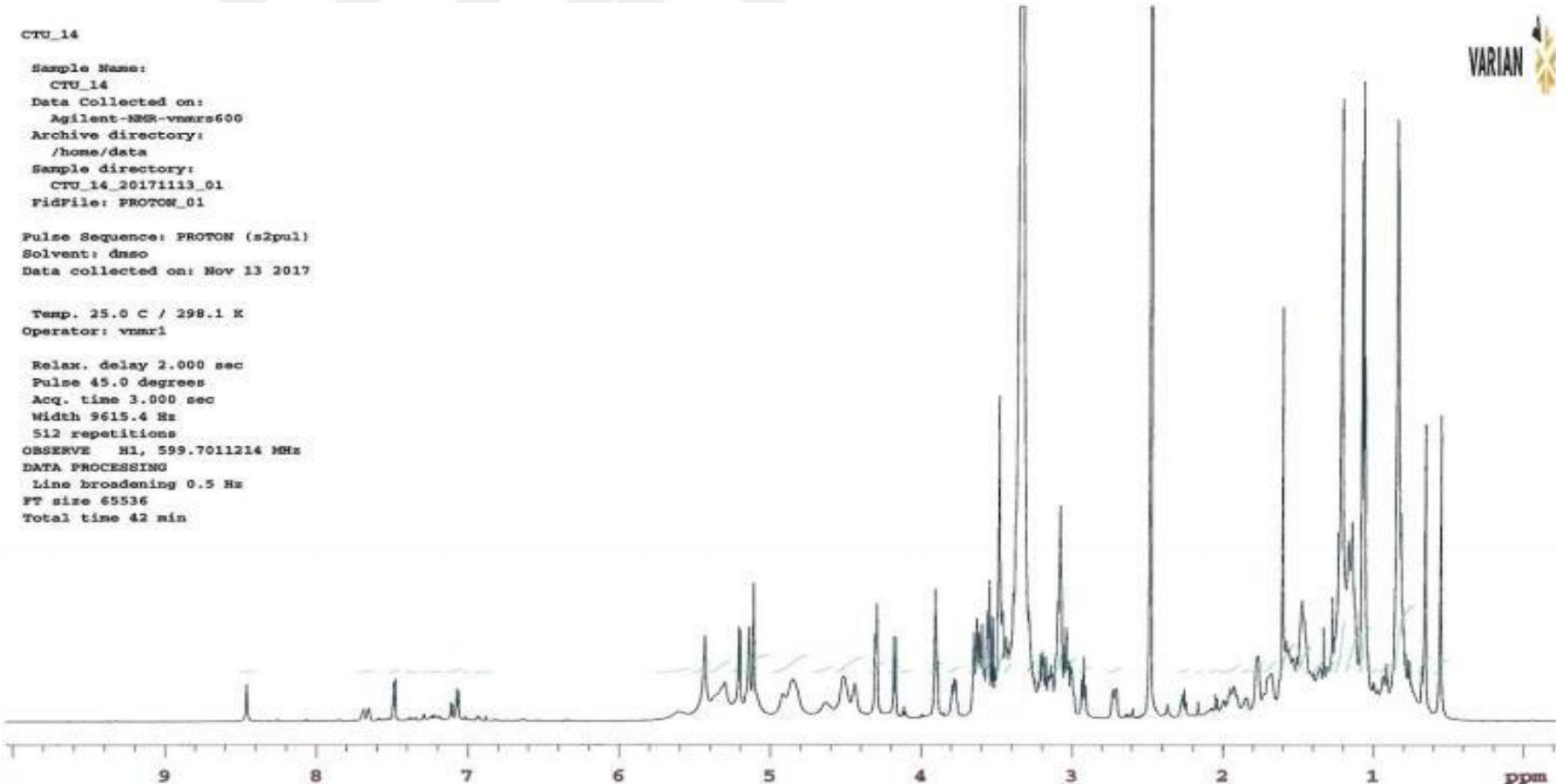
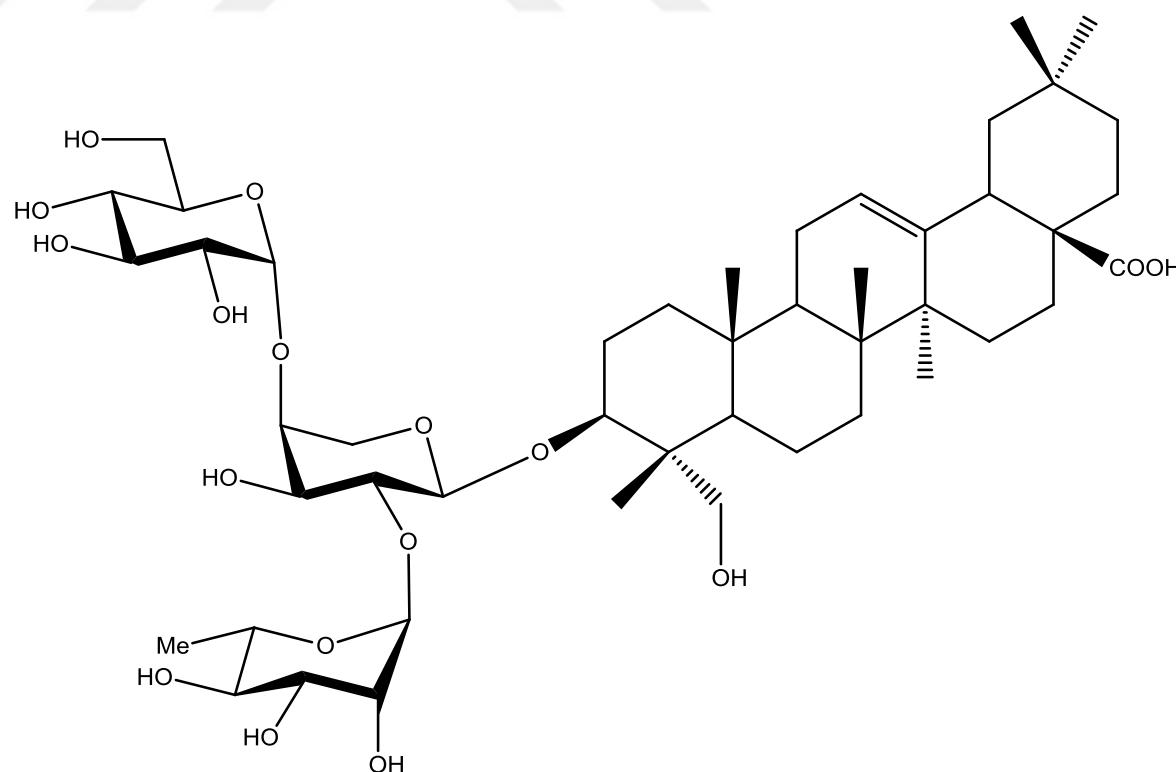


Figure 3.36. ^1H spectrum of Compound 11

3.12. Compound 12: 3-*O*- β -D-glucopyranosyl-(1 \rightarrow 4)-[α -L-rhamnopyranosyl-(1 \rightarrow 2)]- α -L-arabinopyranosyl hederagenin (Lemos et.al., 1992)**Figure 3.37.** 3-*O*- β -D-glucopyranosyl-(1 \rightarrow 4)-[α -L-rhamnopyranosyl-(1 \rightarrow 2)]- α -L-arabinopyranosyl hederagenin

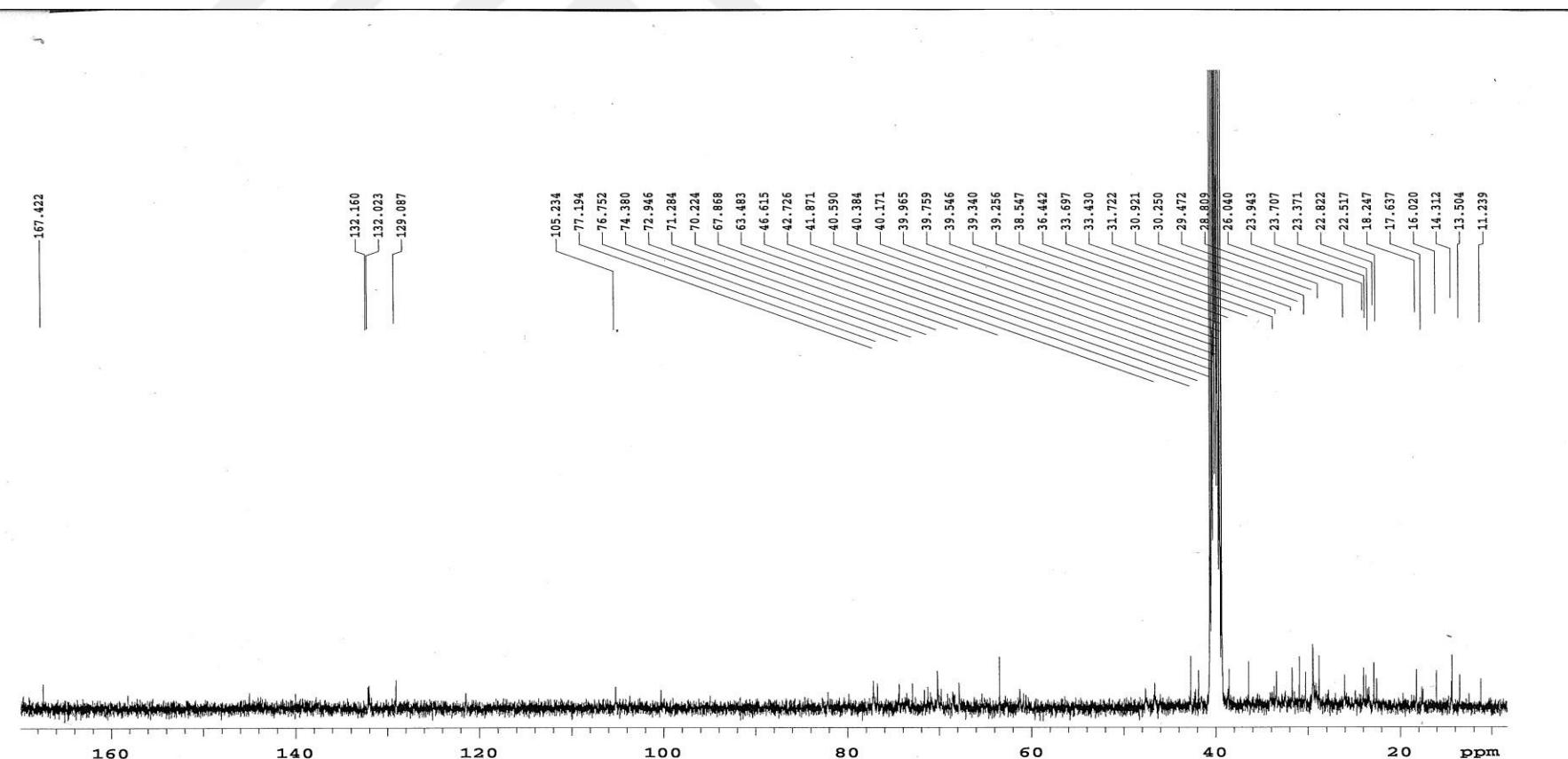


Figure 3.38. ^{13}C spectrum of Compound 12

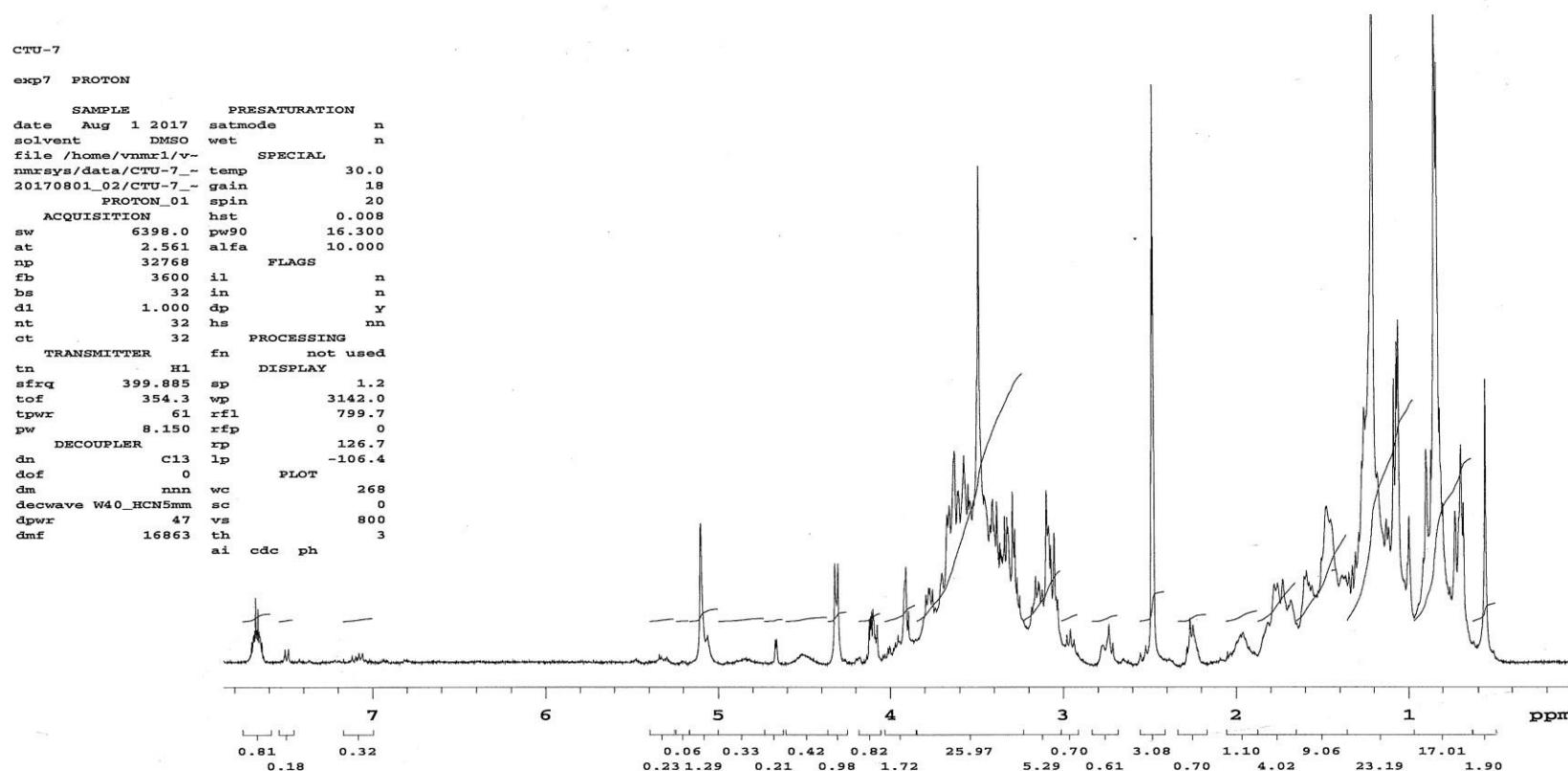


Figure 3.39. ^1H spectrum of **Compound 12**

3.13. Compound 13: Laciniatoside 1 (Kocsis et.al. 1993)

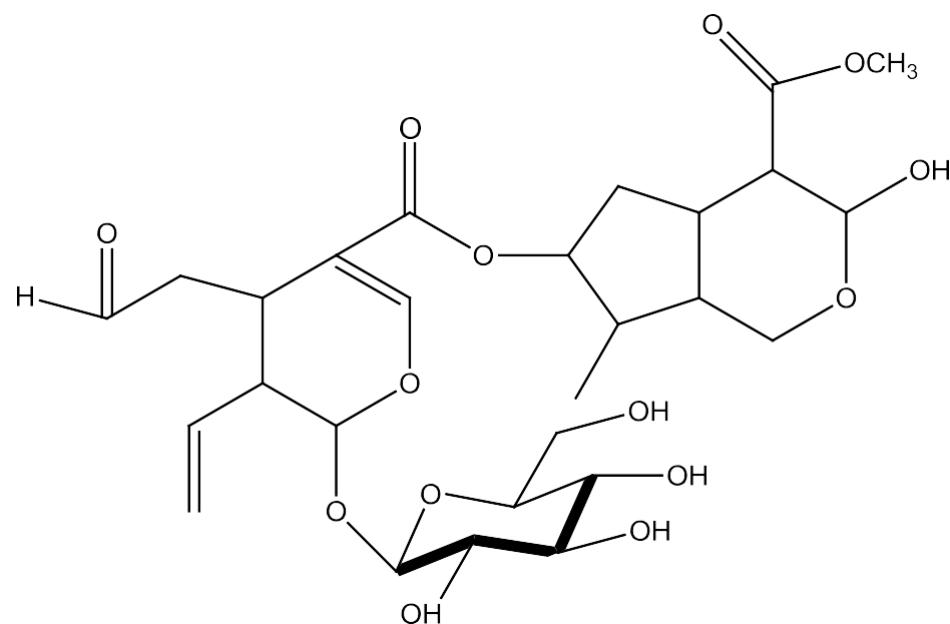


Figure 3.40. Laciniatoside 1

CTU_13
Sample Name:
CTU_13
Data Collected on:
Agilent-NMR-vnmrs600
Archive directory:
/home/data
Sample directory:
CTU_13_20170303_01
FidFile: CARBON_01

Pulse Sequence: CARBON (s2pul)
Solvent: dmso
Data collected on: Mar 3 2017

Temp. 25.0 C / 298.1 K
Operator: vmarl

Relax. delay 1.000 sec
Pulse 45.0 degrees
Acq. time 0.865 sec
Width 37878.8 Hz
8576 repetitions
OBSERVE C13, 150.7969867 MHz
DECOUPLE H1, 599.7119372 MHz
Power 43 dB
continuously on
WALTZ-16 modulated
DATA PROCESSING
Line broadening 0.5 Hz
FT size 65536
Total time 5 hr, 10 min

VARIAN

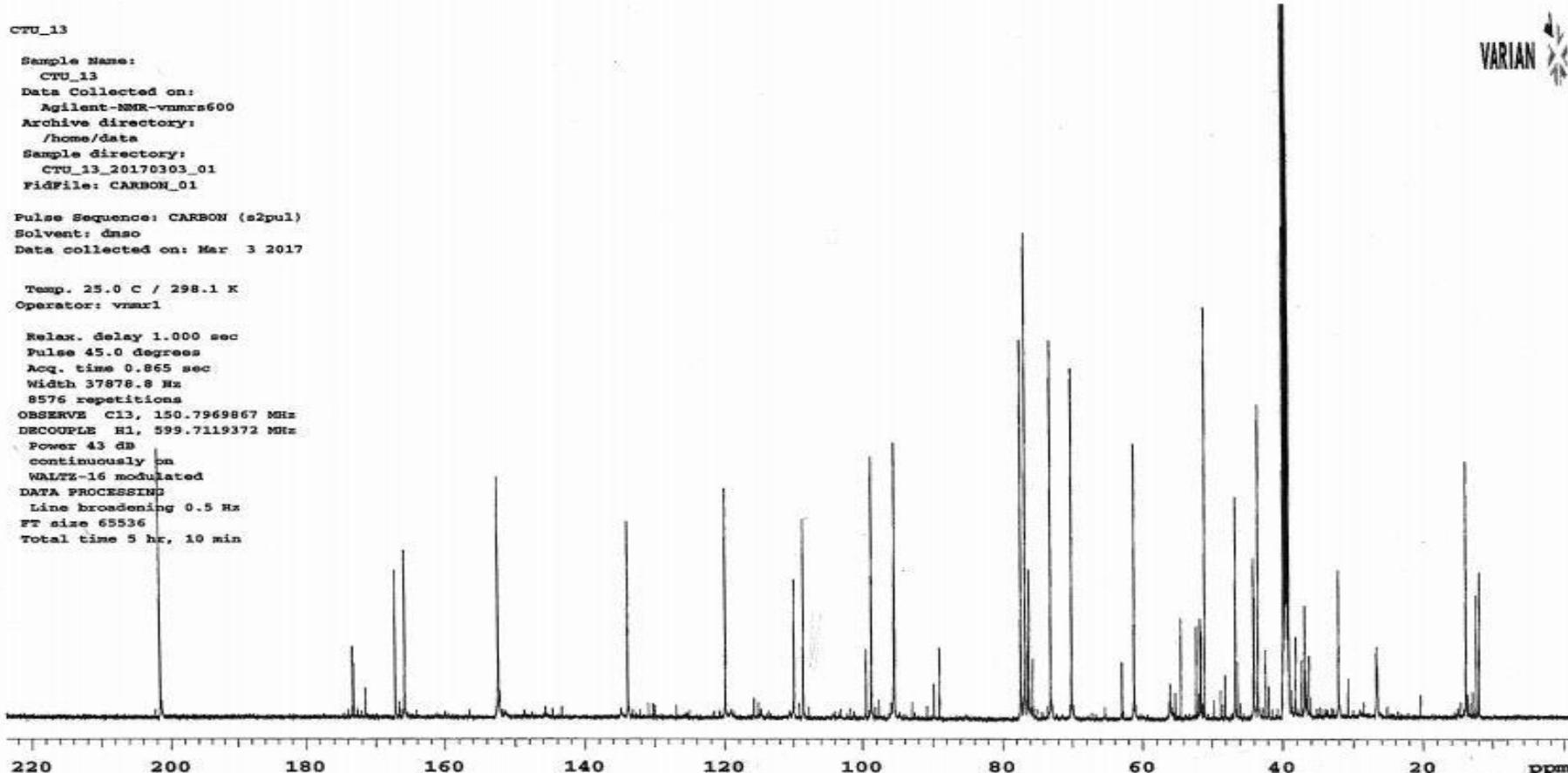


Figure 3.41. ^{13}C Spectrum of Compound 13

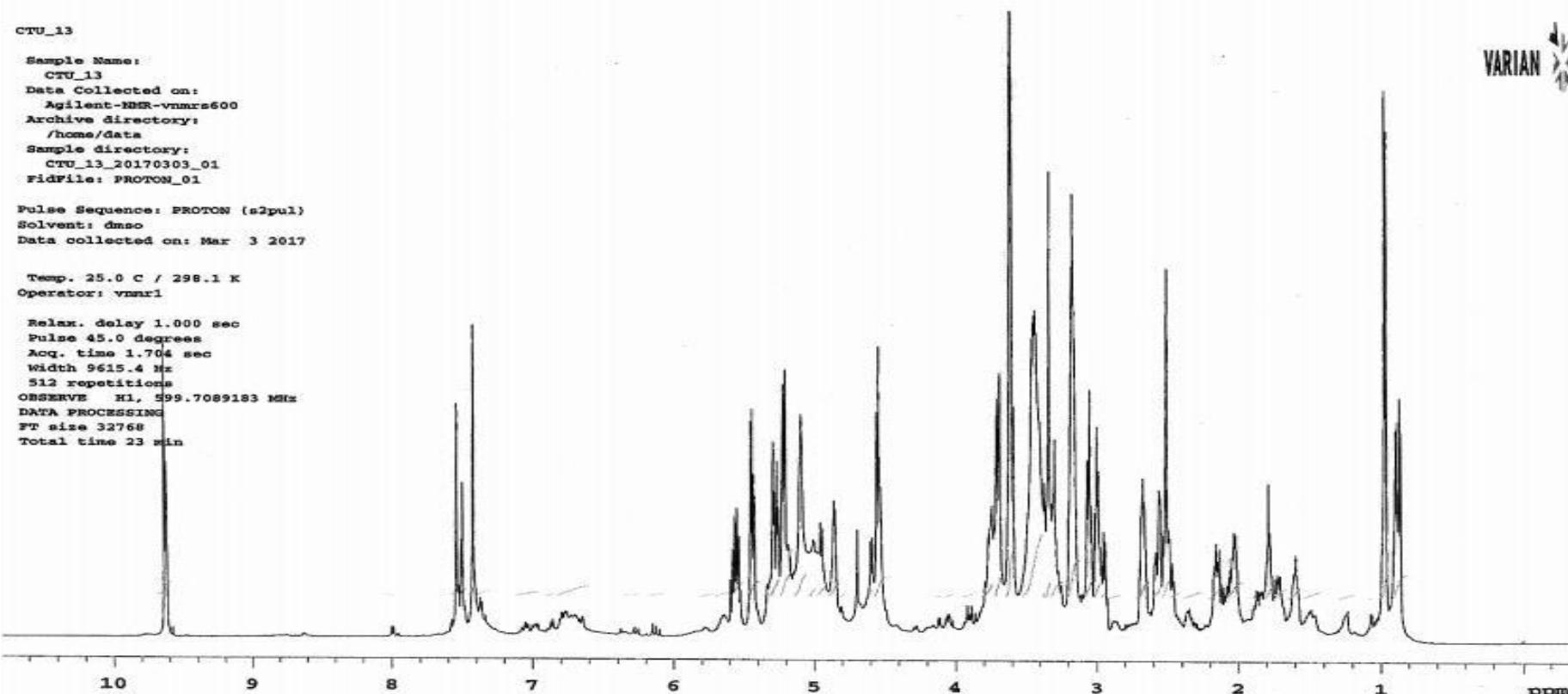


Figure 3.42. ^1H spectrum of Compound 13

4. CONCLUSION

The main purpose of our study is to examine the glycosidic compounds of *Cephalaria tuteliana* for the first time. According to bioassay-guided isolation and pre-chromatographic studies we focused on our phytochemical studies on *n*-butanol extract which were rich in triterpenoid glycosides.

Isolation and purification studies on this extract of *Cephalaria tuteliana* yielded 13 substances 3 of which were new in Caprifoliceae family. Two sapogenins named pomolic acid (**1**) (Figure 3.1), tormentic acid (**2**) (Figure 3.7), ten known triterpene saponins which was named elmalienoside A (**3**) (Figure 3.11), davisianoside A (**4**) (Figure 3.13), α -hederin (**5**) (Figure 3.18), elmalienoside B (**6**) (Figure 3.21), 3-*O*- α -L-rhamnopyranosyl-(1 \rightarrow 2)- α -L-arabinopyranosyl hederagenin 28-*O*- β -D-glucopyranosyl ester (**7**) (Figure 3.22), davisianoside B (**8**) (Figure 3.25), 3-*O*- β -D-glucopyranosyl-(1 \rightarrow 3)- α -L-rhamnopyranosyl-(1 \rightarrow 2)- α -L-arabinopyranosyl hederagenin 28-*O*- β -D-glucopyranosyl ester (**9**) (Figure 3.28), dipsacoside B (**10**) (Figure 3.31), macranthoidin A (**11**) (Figure 3.34), 3-*O*- β -[α -L-rhamnopyranosyl-(1 \rightarrow 3)- β -D-glucopyranosyl] hederagenin (**12**) (Figure 3.37) and one iridoid glycoside namely laciniatoside I (**13**) (Figure 3.40) were obtained. The structures of all compounds were elucidated by a combination of spectral methods including IR, 1D and 2D NMR methods. Among these compounds pomolic acid (**1**), tormentic acid (**2**) and 3-*O*- β -D-glucopyranosyl-(1 \rightarrow 4)-[α -L-rhamnopyranosyl-(1 \rightarrow 2)]- α -L-arabinopyranosyl hederagenin (**12**) were detected in *Cephalaria* species and Caprifoliceae family as well, for the first time.

Studies have demonstrated that saponins have many positive effects especially on cancer, stimulation of the immune system and blood cholesterol levels. According to these advantages, scientists have investigated many biological activities of saponins as seen in Table 4.1. (Hostettmann and Marston, 1995).

Table 4.1. Biological Activities of Saponins

Biological activities of saponins	
Antiulcer	Cytotoxic
Antioxidant	Diuretic
Adjuvant	Effects on ruminal fermentation
Analgesic activity	Effect on absorption of minerals and vitamins
Adaptogenic	Effect on animal growth (growth impairment), reproduction
Antiallergic	Effect on cognitive behavior
Antiedematous	Effect on ethanol induced amnesia
Antiexudative	Effect on morphine/nicotine induced hyperactivity
Antifeedant	Expectorant
Antifungal	Genotoxic
Anti-inflammatory	Haemolytic
Antimicrobial	Hepaprotective
Antigenotoxic	Hepatocytoprotective
Antihepatotoxic inhibitory effect on ethanol absorption	Hypocholesterolemic
Antiprotozoal	Hypoglycemic
Antimutagenic	Increase permeability of intestinal mucosa cells
Antiparasitic	Immunostimulatory effects
Antiobesity	Inhibit active nutrient transport
Antiviral	Molluscicidal
Antipsoriatic	Nootropic
Antiphlogistic	Neuroprotective
Antipyretic	Reductions in stillbirths in swine
Antispasmodic	Reduction in fat absorption
Antithrombotic (effect on blood coagulability)	Reduction in ruminal ammonia concentrations
Antitussive (relieving or preventing cough)	Ruminant bloat
Chemopreventive	Sedative
Clastogenic	Inhibit active nutrient transport

In this study, in the light of our ongoing studies about cytotoxic and immunomodulatory activities of triterpenic compounds (Sarikahya et al., 2018), we investigated cytotoxic properties of two different aglycones which were isolated from *Cephalaria*, for the first time. For this reason, the cytotoxicity of compounds **1** and **2** and hederagenin, oleanoic acid which are two common aglycones in *Cephalaria* were compared against cancerous cells A549, Hela, PANC1, SHSY5Ycells and noncancerous cell HEK293 by MTT method. As it is seen in Table 3.1 while pomolic acid and tormentic acid did not show any cytotoxicity, hederagenin and oleanoic acid found as slightly active. Hederagenin showed cytotoxic activity on cancerous A-549, Hela, PANC1, SHSY5Ycells and non-cancerous HEK-293 cell with IC₅₀ values of 44.48, 30.78, 31.40, 23.05 and 37.05 μ M, respectively. Oleanoic acid displayed cytotoxicity on cancerous A-549, Hela, PANC1, and SHSY5Ycells with IC₅₀ values of 31.61, 68.88, 44.54 and 18.94, respectively. Beside that it did not any cytotoxicity against non-cancerous HEK-293 cell. When it comes to structure activity relationship, it can be concluded that extra hydroxyl group (-OH) and different locations of methyl (-CH₃) groups on aglycone may not result more cytotoxicity. It can be seen clearly from the structures of four different aglycones in Figure 3.43.

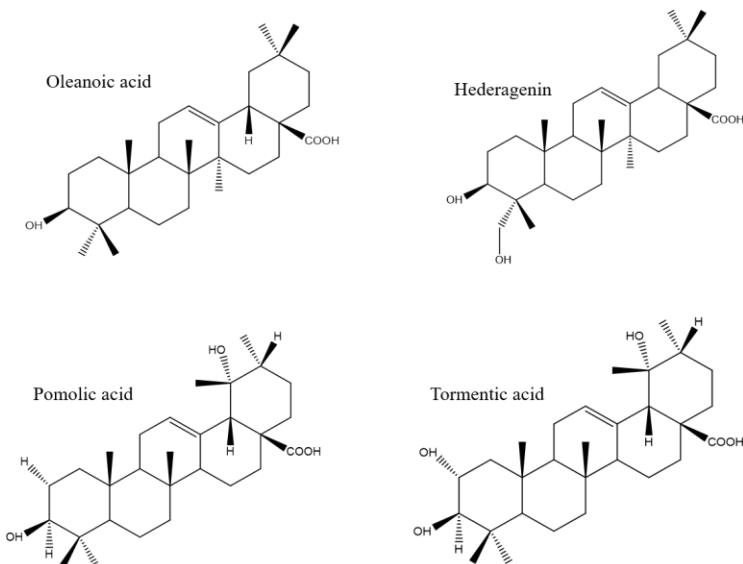


Figure 3.43. Oleanoic acid, Hederagenin, Pomolic acid and Tormentic acid

The use of plants and their extracts as a folk medicine has a long history. The plant-derived compounds have also a long history of clinical usage. To date, 35.000-70.000 plant species have been investigated for their possible medicinal use. Current drug innovation from plants still depends on on bioactivity-guided fractionation and isolation of many important biologically active pure compounds. As it was stated before in Introduction section, one of these the pure compounds triterpene saponins play an important role in modern drug development in terms of biological activities. As a conclusion, the results of this investigation clearly show that *Cephalaria tuteliana* contain a large number of saponins. Thus, the extract of *C. tuteliana* which contains these saponins can be a folk medicine and additive materials for different industrial areas.

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