Faculty of Graduate Studies

Chemical studies of flavonoids isolated from Palestinian plants

(Catmint, Crataegus and Fenugreek)

دراسة مركبات الفلافونويد المستخلصة من النباتات الفلسطينية

(القطرم، الزعرور والحلبة)

This Thesis was submitted in partial fulfillment of the requirements for the Master's Degree in Applied Chemistry from the Faculty of Graduate Studies at Birzeit University, Palestine

By

Maysaa Rabee

Under Supervision of

Dr. Saleh Rayyan and

Dr. Hijazi Abu Ali

June, 2018

Chemical studies of flavonoids isolated from Palestinian plants

(Catmint, Crataegus and Fenugreek)

By

Maysaa Rabee

This thesis was defended successfully on 20/06/2018 and approved by:

Committee Members	Signature
Dr. Saleh Rayyan **Supervisor** **Department of Chemistry, Birzeit University**	....
Dr. Hijazi Abu Ali **Supervisor** **Department of Chemistry, Birzeit University**	....
Dr. Ghassan Albarghouti **Member of thesis committee** **Department of chemistry, Birzeit University**	....
Dr. Imtiaz Khalid **Member of thesis committee** **Department of Chemistry, Birzeit University**	....

Acknowledgements

Thanks to Allah. Who granted me the power to finish this work. I would also like to express my deep appreciation to my two supervisors; Dr. Saleh Rayyan and Dr. Hijazi Abu Ali for excellent supervision, support, guidance and help during my thesis study.

Especially I want to thank my supervisor, Dr. Hijazi Abu Ali, who also give me this chance to reach master's degree. Thank you for your belief in me and thank you for always encouraging me to have belief in myself. I will always appreciate and be thankful for your enormous generosity and catching engagement. I would also like to thank Dr. Ghassan Albarghouti and Dr. Imtiaz Khalid for spending their precious time to read and discuss my work.

I am very pleased to gratitude every member of the department of chemistry who helped me throughout my study in particular, lab technicians; Mr. Azmi Dodeen, Mr. Ibrahim Shalash and Mr. Assem Mubarak. Also, all of thanks to lab technicians in biological department which allowed me to perform the antibacterial part of my thesis.

I wish to thank Professor Øyvind M. Andersen for allowing us to use his laboratory to do part of experimental work which was necessary to finish this work and Professor Torgils Fossen for his help in doing the NMR analysis.

My deepest gratitude goes to my family. My father and my mother, who have taught me the importance of having dreams and setting goals, my enjoyable twin brothers (Rami and Rama), who have taught me patience, happiness in dark day. Thank you all for constant support and interest. Finally, thanks to everyone who helped me & supported me during my study…

Maysaa, June, 2018

Contents

List of Figures…………………………………………………………………………VII

List of Tables……………………………………………………..……...................X

List of Schemes………………………………………………………………………. XI

Abbreviations…………………………………………………………….…...……… XII

Abstract……………………………………………………………….…………….. XIII

ملخص بالعربية ……………………………………………………………….………….....XIV

1.Introduction……………………………………………………………………….…....1

1.1 Flavonoids…………………………………………………………...……………….1

1.1.1 Flavonoid O- and C- glycosides……………………………………………........3

1.1.2 Occurrence of flavonoids……………………………………………………….….4

1.1.3 Functions of flavonoids in plants……………………………………………….…….4

1.1.4 Functions of flavonoids in humans…………………………………………………...5

1.1.5 Applications of flavonoids……………………………………………………………6

1.2 Plants treated in this research………………………………………………………...6

1.2.1 Isolation of flavonoids from Palestinian plants………………………………………7

1.2.1.1 Catmint………………………………………………………………………………...7

1.2.2 Modification of flavonoids…………………………………………………………...9

1.2.2.1 Fenugreek……………………………………………………………………………...9

1.2.2.2 Crataegus……………………………………………………………………….11

1.2.3 C- glycosylflavones in fenugreek and Crataegus………………………………………13

1.3 Anti-bacterial activity………………………………………………………………...….14

1.4 Aim of the research……………………………………………………………………14

2. Experimental……………………………………………………………………………16

2.1 Chemicals, plants and biological species……………………………………………...16

2.2 Instrumentation…………………………………………………………………….......16

2.3 Experimental methods used for isolation and structure elucidations……………………17

2.3.1 Extraction……………………………………………………………………………18

2.3.2 Sample fractionation and isolation of pure flavonoids………………………………...19

2.3.3 Characterization and structure elucidation……………………………………….......20

2.4 Isolation and identification of flavonoids in catmint……………………………….....24

2.5 Extraction of flavonoids content from fenugreek and Crataegus……………………..25

2.5.1 Fenugreek……………………………………………………………………………25

2.5.2 Crataegus…………………………………………………………………………….26

2.5.3 Modification of flavonoids to anthocyanidin (reductive reaction) ……………………26

2.5.4 Anti – bacterial activity……………………………………………………………...28

3. Results and discussion…………………………………………………………………29

3.1 Identification of flavonoids……………………………………………………………29

3.2 NMR spectroscopy……………………………………………………………….….33

3.2.1 Compound **1**…………………………………………………………….……33

3.2.2 Compound **2**…………………………………………………………….…40

3.3 Anti -bacterial activity……………………………………………………….....45

4. Conclusion……………………………………………………………………….....46

5. Recommendation………………………………………………………………...47

6. References………………………………………………………………………48

7. Appendices………………………………………………………………………52

Appendix A: 1D and 2D NMR spectra for compound **2**……………………………………….52

Appendix B: 1D and 2D NMR spectra for compound **3**……………………………………….56

Appendix C: 1D and 2D NMR spectra for compound **4**……………………………………….60

Appendix D: Mass spectra for compounds…………………………………………………….64

List of Figures

Figure 1.1: General flavones nucleus including the numbering system……………………......3

Figure 1.2: Parts of flavonoids structure…………………………………………………….…3

Figure 1.3: Structure of 8-methoxykaempferol 3-*O*-(6^{II}-malonyl-□-glucopyranoside) ……....4

Figure 1.4: Photograph of catmint (*Nepeta curviflora)* ………………………………………..7

Figure 1.5: Photograph of fenugreek *(Trigonella foenum-graecum L.)* ………………….......10

Figure 1.6: Photograph of Crataegus (hawthorn)……………………………………..…12

Figure 1.7: (A) Structure of apigenin 6-*C*-(2^{II}-*O*-β-xylopyranosyl-β-glucopyranoside) (R=H) and luteolin 6-*C*-(2^{II}-*O*-β-xylopyranosyl-β-glucopyranoside) (R=OH) (B) Structures of apigenin 6-*C*-(2^{II}-(6^{III}-O-*E*-caffeoyl-β-glucopyranosyl)-β-glucopyranoside (R=OH) and apigenin 6-*C*-(2^{II}-(6^{III}-*O*-*E*-*p*-coumaroyl-β-glucopyranoside)-β-glucopyranoside (R=H)…………………………...….. 14

Figure 2.1: Photograph of Agilent 8453 spectrophotometer …………………………….…......16

Figure 2.2: Photograph of the preparative HPLC…………….……………………………......16

Figure 2.3: Photograph of Bruker HDIII 850spectrophotometer…………………………......17

Figure 3.1: HPLC chromatogram of flavonoids from catmint (*Nepeta curviflora*) detected at 360 nm. See figure 3.2 for structures. The mobile phase consisted of formic acid: water (0.5:9.0, v/v; solvent A) and acetonitrile (solvent B) with the following elution profile: linear gradient was from 10% B in 0-2 min, 10-45% B from 5-17

min, 45-10% B from 17-18 min and 10% B from 18-20 min. The flow rate was 12.0 ml min^{-1}……………………………………………………….……..…….29

Figure 3.2: Derived structures of the novel flavonoids **1** and **2** and their known phenolic esters **3** and **4** as isolated from catmint (*Nepeta curviflora*)………………..…………...32

Figure 3.3: UV spectrum of compound **4** caffeic acid (ester form) recorded online during HPLC analysis……………..……………………………………..…………………..33

Figure 3.4: 1D ^{1}H NMR spectrum of apigenin 7-*O*-*β*-glucuropyranosyl-2''-*O*-(2'''-*O*-caffeoyl)-β-glucuropyranoside (**1**) isolated from catmint (*Nepeta curviflora*). The unsigned resonance belongs to methoxy group……………..……………………………..……...…34

Figure 3.5: Expanded parts of the 1D ^{13}C CAPT spectrum of apigenin 7-*O*-*β*-glucuropyranosyl-2''-*O*-(2'''-*O*-caffeoyl)-β-glucuropyranoside (**1**) isolated from catmint (*Nepeta curviflora*)…………………………………………….…………………….…....35

Figure 3.6: 2D DQF-COSY spectrum of apigenin 7-*O*-*β*-glucuropyranosyl-2''-*O*-(2'''-*O*-caffeoyl)-β-glucuropyranoside (**1**) isolated from catmint (*Nepeta curviflora*).. Arrows 1, 2, 3 and 4 points to the cross peaks between (2',6')/ (3',5'), β/α, 6''''/5'''' and 8/6, respectively. Arrows 5 and 8 points to the cross peaks between 1''/2'' and 4''/5'', respectively. Arrows 6, 7 and 9 points to the cross peaks between 1'''/2''', 2'''/3''' and 4'''/5''', respectively. ………………….36

Figure 3.7: 2D gs-HSQC spectrum of apigenin 7-*O*-*β*-glucuropyranosyl-2''-*O*-(2'''-*O*-caffeoyl)-β-glucuropyranoside (**1**) isolated from catmint (*Nepeta curviflora*)…………………………………………………..……...………37

Figure 3.8: 2D gs-HMBC spectrum of apigenin 7-*O*-*β*-glucuropyranosyl-2’’-*O*-(2’’’-*O*-caffeoyl)-β-glucuropyranoside (**1**) isolated from catmint *(Nepeta curviflora*)……………………………………………………..………….........38

Figure 3.9: 2D ROESY Spectrum of apigenin 7-*O*-*β*-glucuropyranosyl-2’’-*O*-(2’’’-*O*-caffeoyl)-β-glucuropyranoside (1) isolated from catmint (*Nepeta curviflora)*…………....39

Figure 3.10: Mass spectrum of of apigenin 7-*O*-*β*-glucuropyranosyl-2’’-*O*-(2’’’-*O*-caffeoyl)-β-glucuropyranoside (**1**) isolated from catmint (*Nepeta curviflora*)……………………………………………………………………….40

List of Tables

Table 1.1: Chemical structures and classification of flavonoids…………………………….…2

Table 1.2: Location of flavonoids in plants and their function……………………………....5

Table 1.3: Scientific classification of catmint…………………………………………………8

Table 1.4: Scientific classification of fenugreek……………………………………………...10

Table 1.5: Scientific classification of crataegus………………………………………………12

Table 1.6: The chemical constituents of hawthorn fruits, leaves, flowers……………………13

Table 2.1: Solvent consumption and elution volumes of fractions S1-S7 originating from partial purification on a 100×5 cm Sephadex column………….………………25

Table 3.1: Peak/compound correspondence as obtained LC-MS…………….……………30

Table 3.2: ^{1}H spectral data (δ in ppm and *J* in Hz) for compounds **1-4**dissolved in DMSO-D6 at 25°C……………………………………………..…………………...42

Table 3.3: ^{13}C spectral data (δ in ppm) for compounds **1-4** dissolved in DMSO-D_6 at 25°C……………………………………………………………..……..…43

Table 3.4: In-vitro anti-bacterial activity data for interest samples against gram-negative bacteria……………………………………………………………………..45

Table 3.5: In-vitro anti-bacterial activity data for interest samples against gram-positive bacteria……………………………………………………………………46

List of schemes

Scheme 2.1: Flow chart illustrating steps for isolation and characterization of flavonoids….18

Scheme 2.2: Preparation of zinc amalgam…………………………………………………27

Scheme 2.3: Synthesis of cyanidin 3-O-(6^{II}-O-α-rhamnopyranosyl)-β-glucopyranoside, (R_1=H, R_2=α-rhamnopyranosyl) and cyanidin 3,4^{I}-diglucoside (R_1=β-glucopyranoside, R_2=H)……………………………………………………………………….27

Abbreviations

1D-NMR	One- dimensional nuclear magnetic resonance spectroscopy
$^{1}J_{CH}$	One-bond correlation
2D-NMR	Two-dimensional nuclear magnetic resonance spectroscopy
ACN	Acetonitrile
br	Broad
CAPT	Compensated Attached Proton Test
CC	Column Chromatography
COSY	Correlation spectroscopy
d	Doublet
dd	Double of doublet
DMSO	Dimethyl sulfoxide
h	Hour(s)
HMBC	Gradient-selected heteronuclear multiple bond correlation spectroscopy
HPLC	High performance liquid chromatography
HSQC	Gradient-selected heteronuclear single quantum coherence
HZ	Hertz
IR	Infrared
J	Coupling constant in Hz
L	Liters
LC-MS	Liquid Chromatography- Mass Spectrometry
m/z	Mass to charge ratio
MeOH or CH_3OH	Methanol
mg	Milligram
MHz	Megahertz
min	Minute(s)
$^{m}J_{CH}$	Multiple bonds correlation
nm	Nanometer
NMR	Nuclear Magnetic Resonance
NOESY	Nuclear overhauser and exchange spectroscopy
ROESY	Rotating frame overhauser effect spectroscopy
s	Singlet
TFA	Trifluoroacetic acid
TOCSY	Total correlation spectroscopy
UV-Vis	Ultra violet -visible
v/v	Volume /volume ratio
w/w	Weight /weight ratio
δ	Chemical shift in ppm

Abstract

Catmint *(Nepeta curviflora)* is a commonly used medicinal plant native to the Middle East. In the first part of this research, *Nepeta curviflora* was extracted followed by chemical analysis and identification of its flavonoids. Currently, only limited information is available about the chemical constituents of this plant species. From aerial parts of catmint (*Nepeta curviflora*) the novel flavone *O*-glucuronides, ester forms of apigenin 7-*O*-*β*-glucuronopyranosyl-2''-*O*-(2'''-*O*-caffeoyl)-β-glucuronopyranoside (**1**) and luteolin 7-*O*-β-glucuronopyranosyl-2''-*O*-(2'''-*O*-(caffeoyl)-β-glucuronopyranoside (**2**) have been identified. In addition, ester derivatives of phenolic compounds of rosmarinic acid (**3**) and caffeic acid (**4**) have been identified. The isolated compounds were characterized using Ultra violet -visible spectroscopy (UV-Vis) and extensive use of one-dimensional nuclear magnetic resonance spectroscopy (1D-NMR) and two-dimensional nuclear magnetic resonance spectroscopy (2D-NMR).

In the second part of this research, activated zinc using ultrasound has been introduced as a new tool for reductive reaction to modify flavonoids of fenugreek and Crataegus to anthocyanins.

In-vitro, anti-bacterial activity of original fenugreek, modified fenugreek, original Crataegus and modified Crataegus were investigated against three gram-positive (Staphylococcus aureus, Bacillus subtilis and Staphylococcus epidermidis) and three gram-negative bacteria (Escherichia coli, Proteus mirabilis and Pseudomonas aeruginosa) using agar diffusion method. Unfortunately, all tested sample didn't show any anti-bacterial activity against all types of gram-negative and gram-positive bacteria.

ملخص بالعربية

نعنع القطط هو نبات طبي يستخدم عادة في الشرق الأوسط. في الجزء الأول من البحث، تم استخلاص وتحديد مركبات الفلافونويد في نبتة نعنع القطط. في الوقت الحاضر لا تتوفر سوى معلومات محدودة حول المكونات الكيميائية لهذه الأنواع النباتية. من الأجزاء الهوائية من هذه النبتة تم تحديد مركبين جديدين من عائلة الفلافونويد هما :

Apigenin 7-*O*-β-glucuronopyranosyl-2’’-*O*-(2’’’-*O*-caffeoyl)-β-glucuronopyranoside (**1**) and luteolin 7-*O*-β- ,glucuronopyranosyl-2’’-*O*-(2’’’-*O*-(caffeoyl)-β-glucuronopyranoside (**2**),

، بالإضافة إلى المشتقات الأسترية من هذه المركبات الفينولية المعروفة مثل حمض الروزمارينيك (3)، وحمض الكافيين (4). تم تحديد المركبات المعزولة بواسطة جهاز طيف الأشعة فوق البنفسجية والمرئية وجهاز مطياف الكتلة والاستخدام المكثف لجهاز الرنين المغناطيسي أحادي البعد وثنائي الأبعاد.

في الجزء الثاني من هذا البحث، تم إدخال الزنك المنشط باستخدام الموجات فوق الصوتية كوسيلة جديدة لتعديل التركيب الكيميائي للفلافونويدات من الحلبة والزعرورإلى الأنثوسيانينات المرادفة.

تم دراسة تأثير مستخلصات الحلبة الأصلي، الحلبة المعدلة، الزعرور الأصلي و الزعرور المعدّل على ثلاث أنواع من البكتيريا موجبة غرام (Staphylococcus aureus, Bacillus subtilis and Staphylococcus epidermidis) وعلى ثلاث أنواع من البكتيريا سالبة غرام (Pseudomonas aeruginosa, Proteus mirabilis and Escherichia coli) باستخدام طريقة الانتشار في الآجار.

لم تظهر جميع العينات التي خضعت للفحص أي نشاط مضاد للبكتيريا المستخدمة سالبة غرام وموجبة غرام .

1. Introduction

1.1 Flavonoids

Flavonoids (known also as bioflavonoids, from the Latin words flavus meaning yellow, their color in nature) are the largest group of secondary metabolites in plant kingdom.[1] Up- to- date there are more than 10,000 different flavonoids which have been isolated and identified all over the world.[2] Flavonoids family includes the following sub families; flavones (e.g., apigenin and luteolin), flavanones (e.g., hesperetin and naringenin), flavanols (e.g., catechin and epicatechin), flavonols (e.g., quercetin and kaempferol), flavanonols (e.g., taxifolin), isoflavones (e.g., genistein and daidzein) and anthocyanidins (e.g., cyanidin), which account for about 80 % of flavonoid as tabulated in (Table 1.1).[3,4,5]

The main part of the chemical structure of all flavonoids consists of a nucleus (aglycone) with the sequence C_6-C_3-C_6 (C_{15} skeleton) as shown in (Figure 1.1). The different flavonoids subfamilies mentioned earlier differ in the oxidation state of the C-ring. The aglycone nucleus of the different flavonoids can be glycosylated and hydroxylated in several positions. The hydroxyl groups can be methylated or glycosylated and the glycosyl moieties can be further glycosylated and/or acylated with aromatic and/or aliphatic acyl groups (Figure 1.2) shows parts of flavonoids structure.[6,7] Recent reports have shown complex flavonoids containing two aglycone and several glycosyl and acyl groups.[8,9]

Table 1.1: Chemical structures and classification of flavonoids.[5]

Sub-family of flavonoid	Structure backbone	Examples	
Flavones		Luteolin	Apigenin
Flavanones		Hesperetin	Naringenin
Flavanols		Catechin	Epicatechin
Flavonols		Quercetin	Kaempferol
Flavanonols		Taxifolin	
Isoflavones		Genistein	Daidzein
Anthocyanidins		Cyanidin	

Figure 1.1: General flavones nucleus including the numbering system.[10]

Sugar unit (s)

Acid unit(s)

Aglycone

Figure 1.2: Parts of flavonoids structure.

1.1.1 Flavonoid *O*- and *C*-glycosides

Flavonoids are present as a non-glycosylated form (aglycone) or attached to sugar unit(s) (glycoside). The glycoside residues can be attached through an O or C atoms of the aglycone, giving rise to *O*-glycosides, *C*-glycosides or *both at the same time*. Glycosylation or glycosidation makes the flavonoids less reactive and more water soluble. Most of the flavonoids occur as *O*-glycosides and less often as an acid resistant *C*-glycoside. Mono-, di-, tri- and higher level of glycosylations in which at least one of the hydroxyl groups are connected through hemiacetal bond(s) to sugar(s) have been isolated and identified.[8]

The most common glycosylation occurs at position 3, 5 and 7 in *O*-glycoside, while positions 6 and 8 of the aglycones are the most common for *C*-glycosylation.[11] The most widely identified sugar is glucose, however, rhamnose, galactose, xylose and arabinose are also common.

Sugars like allose, mannose, fructose, apiose and glucuronic acid are sometimes encountered. Sophorose, gentiobiose, rutinose, sambubiose and robinose are examples of the most common disaccharides in flavonoids.[12] (Figure 1.3) shows an example of a flavonol glycoside isolated from *Crataegi folium cum flore*.

Figure 1.3: Structure of 8-methoxykaempferol 3-*O*-(6^{II}-malonyl-β-glucopyranoside).[13]

1.1.2 Occurrence of flavonoids

Flavonoids are present in almost all types of plants. Within the plant they have been reported to exist in fruits, flowers, grains, barks, seeds, nuts, leaves, roots and stems.[14] Also they have been reported to exist in wings and bodies of butterflies. Our human body cannot synthesize these compounds and therefore consumption of vegetables and fruits are the main sources of flavonoids for our needs.[1]

1.1.3 Functions of flavonoids in plants

Flavonoids are synthesized in all parts of the plants within the cell or on the surface of its parts (e.g. leaves and stems) that have led to various functions. They play a significant role in providing the color, fragrance and taste to the fruits, flowers and seeds, making them attractive for insects, birds or mammals, which help in pollen or seed distribution.[15]

Flavonoids act as antioxidants, antimicrobials, photoreceptors, control of respiration and photosynthesis, visual attractors, growth regulates, sex determination, insect repellents and also protect plants from ultraviolet (UV) radiation damage.[16,17] (Table 1.2) Summarizes the location of flavonoids in plants and their function.

In general, flavonoids in food are responsible for color, taste, prevention of fat oxidation and protection of vitamins and enzymes.[18]

Table 1.2: Location of flavonoids in plants and their function.

Location of flavonoids	**Functions**
Flowers	Colors to attract insect to help in pollen or seed distribution.
Fruits and seeds	Taste, fragrance and protection from light and microbes.
Leaves	Physiological survival. Protect plants from fungal pathogens and UV-radiation.
Roots and stems	Antioxidants, antimicrobial, structure and growth regulators and sex determination.

1.1.4 Functions of flavonoids in humans

Besides their relevance in plants, flavonoids are important for human health because of their high biological activities including anti-microbial, anti-oxidant, anti-cancer, anti- human immunodeficiency virus (HIV), anti-platelet, anti-allergic, anti-hepatotoxic, anti-mutagenic, anti-ulcer actions and anti-inflammatory activity. Because of their pharmacological activities including

cytotoxic, antiviral, cardioprotective, atherosclerosis, neuroprotective, antimalarial, antileshmanial, antitrypanosomal, antiamebial, cardiovascular diseases, Alzheimer's disease (AD) and other age-related diseases.[6, 19-22]

These biological and pharmacological functions are attributed to their free radical scavenging efficacies, metal complexation capabilities and their ability to bind to proteins with high degree of specificity.[20]

1.1.5 Applications of flavonoids

In addition to their pharmaceutical, medicinal and cosmetic applications, flavonoids possess other applications in various fields of industry such as food industry as sweetening agents or colorant and taste enhances of common beverages, such as wine, beer and tea. In addition, flavonoids have been used in leather tanning industry and for other industrial applicatrion.[7]

1.2 Plants treated in this research

Medicinal plants are widely distributed in Palestine and are used in Traditional Palestinian Medicine. These plants include Arum palaestinum (Araceae), Urtica pilulifera (Urticaceae), Coridothymus capitatus (Labiatae), Majorana syriaca (Labiatae), Teucrium creticum (Lamiaceae) and Teucrium polium, (Lamiaceae).[23]

The abundance of more than 2900 species condensed on a very small geographical area is a major advantage of studying the Palestinian flora. This richness is due to the diversity of the soil and climatic conditions.[24] Medicines from natural resources including medicinal plants are quite popular nowadays, therefore, extracts of plants play a major role in developing many safe drugs for human avoiding side effects of manufactured drugs.[25] Amongst various plant families which have been known for their medicinal values are *Nepeta curviflora*, Crataegus and fenugreek.

1.2.1 Isolation of flavonoids from Palestinian plants

1.2.1.1 Catmint

Genus *Nepeta* (catnip or catmint), is a part of the mint family (Lamiaceae), which contains about 280 species common to Asia, North Africa and Europe, however, it is mostly abundant in the Mediterranean region, a photograph of *Nepeta curviflora* is shown in (Figure 1.4) and its scientific classification is presented in (Table 1.3).[26,27] It is commonly known as "Catnip or Catmint" due to its irresistible stimulated action on cats. The plant has a pleasant odor with beautiful flowers.

Nepeta curviflora, also known as Syrian Catnip, is native to Palestine, Lebanon and Syria.

Figure 1.4: Photograph of catmint (*Nepeta curviflora).*

Table1.3: Scientific classification of catmint.

Scientific classification	
Family	Labiatae (Lamiaceae)
Subfamily	Nepetoideae
Tribe	Mentheae
Genus	*Nepeta*
Species	*Nepeta curviflora*
common name	
Catmint	

Medicinally, the plant is famous for treating gastrointestinal and respiratory hyperactive disorders such as colic, diarrhea, cough, asthma and bronchitis and treating cardiovascular complaints, such as angina pectoris, cardiac thrombosis, tachycardia and heart weakness. It is also commonly used as a sedative, carminative, diuretic, antiseptic, antispasmodic agents and to treat colds, flu and fevers.[19,28]

Even though several reports discussed the composition of phenolics in *Nepeta* species,[29-35] few, if any, novel natural products have been extracted and identified from these species, which could have unique biological activities. According to previous reports, catmint is considered very rich in phenolic compounds including flavonoids and essential oil including monoterpenes, sesquiterpenes and sterols. One of the main active constituents which has been isolated is rosmarinic acid, an ester of caffeic acid. In addition, the plant also contains nepetalactones and alkaloids, such as actinidine and iridomyrmecine. Therefore, catmint is believed to have significant biological activities including antibacterial, antifungal, anti-nociceptive, analgesic, reduction of

serum lipids and anti-inflammatory effects.[28,36] Another study showed that extracted oil from catmint has antibacterial and antifungal properties, in addition to its powerful insect repellents.[37,38]

Mixtures of volatile constituents from *Nepata curviflora* have been reported to exhibit phytotoxic,[39] as well as nematicidal activity.[40] A methanolic extract of *Nepata curviflora* leaves showed efficacy against 88.8% of tested microorganisms,[41] however, only volatile chemical constituents have been reported from this species.[39,40,42,43]

The potential beneficial health effects associated with intake of *Nepeta species* have been suggested to be partly attributed to their content of essential oils and phenolics.[29,44-51] They have been reported as the major groups of secondary phenolic compounds which have been suggested to be responsible for a wide range of biological activities.[31,34,52-54] This group of secondary metabolites however, remains generally under-investigation in this genus.

1.2.2 Modification of flavonoids

The second part of this research focused on fenugreek and Crataegus because these plants are generally rich in bio-active proanthocyanidins, flavonoids and triterpenes.

1.2.2.1 Fenugreek

Fenugreek *(Trigonella foenum-graecum L.)* is one of the most important edible plants of the Fabaceae family and is also used in traditional medicine. It is a short-living annual plant that grows in Asia, Africa, Europe and Mediterranean countries. A photograph of fenugreek is shown in (Figure 1.5) and its scientific classification is presented in (Table 1.4).[55]

Figure 1.5: Photograph of fenugreek *(Trigonella foenum-graecum L.).*

Table1.4: Scientific classification of fenugreek.

Scientific classification	
Kingdom	Plantae
Family	Fabaceae
Genus Species	*Trigonella L.* *Trigonella foenum-graecum L.*
common name	
Fenugreek	

Seeds of fenugreek possess different constituents with variety of functions. They are rich in proteins such as globulin, histidine, albumin and lecithin, also contain essential oils and alkaloids which have been shown to be toxic to bacteria, parasites and fungi.[56,57] Leaves of fenugreek have been found to have ascorbic acid, β-carotene, fiber and high concentration of calcium, zinc and iron.[57]

Several studies showed that fenugreek contains several classes of secondary metabolites such as alkaloids, flavonoids, phenolic acids, polysaccharides, triterpenoids, steroidal sapogenins and nicotinic acid.[55,58] It is used in various fields including medicine, food, perfume, paints, beverages and cosmetics.[59]

Fenugreek has a long history as a traditional medicinal plant for treatment of diabetes, fever, anaemia, constipation and as a treatment of stomach disorders.[60] Fenugreek is known to have several pharmacological effects including hypocholesterolemia, chemopreventive, appetite stimulation, anti-obesity, antioxidant ,antiinflammatory, antimicrobial, ,antifungial, anti-allergic and anti-cancer effects.[55,61] Several studies have shown that fenugreek is a potent stimulator for breast milk production and it increases the blood circulation, making the body more active and energetic.[58]

Since 1971, there have been several reports dealing with the isolation of polyphenolic compounds from fenugreek.[60,62-68] During 2010, Rayyan et al, isolated 12 different C-glycosylflavones from seeds of *Trigonella foenum-graecum L.*.[69]

Sharma et al., studied the antimicrobial activity of fenugreek leaves, seeds and stem extracts against E. coli and Staphylococcus bacteria. The result showed that extract of fenugreek from different solvents have different antibacterial activities.[57]

1.2.2.2 Crataegus

Crataegus (hawthorn) is a member of the Rosaceae family, which is native to Europe, Africa and Asia. Commonly found as a shrub or small tree 5–10 m tall, a photograph of Crataegus is shown in (Figure 1.6) and its scientific classification is presented in (Table 1.5).[70]

Figure 1.6: Photograph of Crataegus (hawthorn).

Table 1.5: Scientific classification of Crataegus.

Scientific classification	
kingdom	Plantae
Family	Rosaceae
Genus	*Crataegus*
common name	
Crataegus	

Hawthorn contains different bioactive compounds, such as chlorogenic acid, flavonoids, triterpenes and phenolic acids, as presented in (Table 1.6).[70] These compounds are reported to have several pharmacological effects, including neuroprotective, hepatoprotective, cardioprotective and nephroprotective.[13,70] Furthermore, Crataegus (fruit, leaves and flowers) are being sold for the treatment of various cardiovascular diseases (hypertension, arrhythmias, congestive heart failure, coronary artery disease and myocardial weakness.[28]

Table 1.6: The chemical constituents of hawthorn fruits, leaves, flowers.[71]

Chemical constituents	Percentage	Location
Flavonoids	0.1% -1%	Fruits
Flavonoids	1% - 2%	Leaves and flowers
Oligomeric proanthocyanidins	1%-3%	Fruits or leaves with flowers
Triterpene acids	0.5%-1.4%	Fruits
Organic acids	2% - 6%	Fruits or leaves with flowers
Sterols, and trace amounts of cardio active amines	-	Fruits or leaves with flowers

1.2.3 *C*- glycosylflavones in fenugreek and Crataegus

Some medicinal plants contain important bioactive constituents are called *C*-glycosylflavones and show different biological activity such as antioxidant activity, antifungal and antimicrobial activity.[13,72] Fenugreek and Crataegus contain a significant amount of *C*-glycosylflavones and interest in investigating these compounds has increased recently for the following reasons:

(1) *C*-glycosylflavones, shown in (Figure 1.7), contain at least one non-hydrolysable glycosidic unit attached to the flavone aglycone with increased stability and potential impact on *in vivo* transportation properties.

(2) The recent transformation of these compounds to *C*-glycosylanthocyanins with the potential of overcoming problems related to hydrolytic instability of the naturally occurring anthocyanin-*O*-glycosides widely used as food colorants and nutraceuticals.[73]

Figure 1.7: (A) Structure of apigenin 6-*C*-(2^{II}-*O*-β-xylopyranosyl-β-glucopyranoside) (R= H) and luteolin 6-*C*-(2^{II}-*O*-β-xylopyranosyl-β-glucopyranoside) (R=OH). (B) Structures of apigenin 6-*C*-(2^{II}-(6^{III}-O-*E*-caffeoyl-β-glucopyranosyl)-β-glucopyranoside (R=OH) and apigenin 6-*C*-(2^{II}-(6^{III}-*O*-*E*-*p*-coumaroyl-β-glucopyranoside)-β-glucopyranoside (R=H).[8]

1.3 Anti-bacterial activity

Plants are frequently used as medicine in the treatment of various diseases. Several published reports showed that medicinal plants including fenugreek and Crataegus have antimicrobial activities.[74] Fenugreek and Crataegus appear to be a promising rich source of *C*-glycosylflavones, which can be modified and tested for their anti-bacterial activity and as a result, modification of similar compounds is a topic of interest and has been recently reported. The purified extracts obtained after Amberlite XAD-7 were modified according to the procedure reported by Bjorøy et al.[73,75] The semi-synthesized *C*-glycosylanthocyanins are tested for their biological activity as a mixture.

1.4 Aim of the research

The aim of the first part of this work is to isolate and identify natural products compounds (flavonoids) from medicinal plants (catmint). The main goal of the second part of this research is to semi-synthesize a new type of anthocyanins that are naturally unavailable by modification of the

XAD purified extract isolated from fenugreek and Crataegus. *O*-glycosylflavones and *C*-glycosylflavones are converted to *O*-glycosylanthocyanins and *C*-glycosylanthocyanins, respectively. The isolated compounds will be characterized using different analytical techniques including UV-Vis, 1D and 2D NMR. Moreover, the biological activity of the modified crude extract of fenugreek and Crataegus will be tested for antibacterial activity and the results will be compared to the original (non-modified) crude extract.

2. Experimental

2.1 Chemicals, plants and biological species

All chemicals and solvents were purchased from commercial sources. Plants were collected from different sources as described in the experimental section. Finally, all bacteria types were obtained from Biology and Biochemistry Department at Birzeit University.

2.2 Instrumentation

Monitoring of reductive reaction of Crataegus and fenugreek samples were recorded on Agilent 8453 photodiode array spectrophotometer in the 300-600 nanometer (nm) region using methanol (CH_3OH) as a solvent, a photograph of Agilent 8453 spectrophotometer is shown in (Figure 2.1).

Figure 2.1: Photograph of Agilent 8453 spectrophotometer.

Absorbance spectra were recorded on analytical and preparative High-Performance Liquid Chromatography (HPLC), a photograph of preparative HPLC is shown in (Figure 2.2).

Figure 2.2: Photograph of the preparative HPLC.

NMR spectra were recorded on a Bruker biospin 850 megahertz (MHz) and 600 MHz NMR for ^{1}H and ^{13}C nucleus. A photograph of Bruker HDIII 850 spectrophotometer is shown in (Figure 2.3).

Figure 2.3: Photograph of Bruker HDIII 850 MHz spectrophotometer.

2.3 Experimental methods used for isolation and structure elucidation

In general, separation, purification and structure identification of flavonoids are demanding and time-consuming processes. (Scheme 2.1) shows a typical procedure followed for obtaining pure flavonoids.

The main steps followed in separation and structure identification of pure flavonoids consist of:

1- Extraction of the plant material.
2- Separation of pure individual flavonoids.
3- Characterization and structure elucidation.

Flavonoids are known as relatively unstable; pH sensitive and must be treated carefully, stored in darkness and at low temperature to reduce hydration and degradation.

Scheme 2.1: Flow chart illustrating steps for isolation and characterization of flavonoids.

Extraction (0.5% TFA/MeOH: v/v) → HPLC analysis
Liquid-liquid partition → HPLC analysis
Amberlite-XAD → HPLC analysis
Sephadex LH-20
Preparative HPLC
HPLC analysis
MS & NMR analysis

2.3.1 Extraction

Extraction is the first technique for the recovery and isolation of flavonoids and other secondary metabolites components from plants, prior to analysis. Extraction technique depends on pH, temperature, analyte to-solvent volume ratio, and the number and time intervals of individual extraction steps. In almost all cases, extraction technique is repeated two or three times and individual extracts are combined before any further continuation.[76]

Extraction. Flavonoids were extracted with methanol containing 0.5% Trifluoroacetic acid (TFA). During the extraction process the extract was refrigerated at low temperature to avoid hydrolysis of the bond between the sugar and aliphatic acyl moieties and degradation. After the extraction process is completed, the individual extracts were combined and filtered and the organic solvent

was removed by evaporation under vacuum at relatively low temperatures (<35℃). Other types of flavonoids were extracted without adding acid as in fenugreek.[77,78]

Liquid – liquid partition or solvent extraction. After evaporation of the organic solvent (methanol in this case), the plant extract often contains several types of compounds such as chlorophylls, stilbenoids and other non-polar compounds must be removed by liquid – liquid partition with hexane or ethyl acetate.[77,78]

Amberlite XAD-7 (Adsorption Chromatography). The extracts obtained after the liquid -liquid partition step contain aromatic and non-aromatic water-soluble compounds. The non-aromatic water-soluble compounds (e.g. free sugars and low molecular aliphatic acids) were removed using Amberlite XAD-7 column chromatography (CC). Amberlite XAD-7 is an acrylic ester polymer that adsorbs the aromatic compounds such as flavonoids whereas free sugars and other polar non-aromatic compounds can be removed by washing with several liters (L) of distilled water. Then the adsorbed flavonoids were eluted using methanol containing 0.5% TFA (v/v) as mobile phase.[78]

2.3.2 Sample fractionation and separation of pure flavonoids

Column chromatography (CC) is very useful technique for separating mixture of compounds into its pure components. Sephadex LH-20 CC and preparative HPLC have been used in fractionation and separation of pure flavonoids.

Gel filtration column chromatography. Sephadex LH-20, which separates according to molecule size, was used as column material. Small particle size of Sephadex LH-20 which have a slow elution and high degree of gel filtration, provide excellent separation. This technique is generally characterized by simplicity, isocratic and fast elution for large molecules. First, the purified XAD-7 extracts were dissolved in a small amount of the initial mobile phase (ACN/H_2O/TFA; 10:90:0.5, v/v) followed by its application throughout the column. Separation was achieved by isocratic

elution or gradient elution using increasing amounts of methanol. Flavonoids are generally eluted in order of decreasing molecular mass.[79,80]

HPLC is considered as the most common technique for the separation of flavonoids, both on preparative and analytical scales. Constant improvements and modifications in instrumentation, packing materials and column technology which make the technique more attractive.[81]

Preparative HPLC. Preparative HPLC is a purification process which aims at the separation of a pure substance from a mixture in relatively short time.[81] The instruments used was a Gilson 321 pump equipped with UV detection (Dionex UltiMate 3000 Variable Wavelength Detector) (Dionex Corporation, Sunnyvale, CA, US). The system was equipped with an Econosil C18 column (250 mm x 22 mm; length x I.D., 10.0 µm). The elution protocol consists of solvents A, which is H_2O containing 0.5% TFA (v/v) and B, which is acetonitrile (ACN) containing 0.5% TFA (v/v). The following percent gradient was used: 100% A in 0-5 min, 10% B (linear gradient) from 5-52 min, 20% B from 53-65 min, 50% from 66-70 min then back to the starting conditions in 4 min. The flow rate was 12.0 ml min^{-1}. This technique has high resolving power in a relatively short time which is suitable for the separation of a relatively unstable flavonoids. Information about the flavonoids retention times, UV/Vis spectra and peak purities could be obtained.

2.3.3 Characterization and structure elucidation

Several chromatographic and spectroscopic techniques have been used to identify natural product compounds. In the past, due to the lack of different instrumentation, infrared (IR) spectroscopy which gives information related to the functional groups and the UV spectroscopy which reveals information related to the presence of sites of unsaturations in the structure were the main identification instruments. The IR technique became less important in structure elucidation of natural products due to the discovery of more powerful techniques like NMR and mass

spectrometry (MS), which are nowadays the major tools for the identification of natural products using only few milligrams (mg) of the isolated compounds.[81]

UV-Visible spectroscopy (UV-Vis). UV-Vis. spectra were obtained online for all the isolated compounds during the analytical HPLC analysis. Flavonoids usually absorb light in the region 300-550 nm which depends on the nature of the aglycone, the position of sugar substituents and the presence of aromatic acyl groups, however, aliphatic acyl groups are lacking significant UV-Vis absorption, and their presence cannot be directly detected by UV-Vis spectroscopy.

Mass spectrometry (MS). This technique was used basically to determine the molecular weight of the flavonoids and to obtain some information about the building blocks related to fragmented ions. However, the connection points between the different moieties cannot be determined using this technique.[81]

Nuclear Magnetic Resonance spectroscopy (NMR). NMR is a powerful method for the determination of the complete structure of flavonoids compounds. Both 1D ^{1}H-NMR and ^{13}C-NMR give useful informations about the chemical shift (δ) and coupling constants (J). 2D NMR spectroscopy including homo- and heteronuclear correlation cross peaks gives detailed information about the structure of isolated compounds. Other nuclei like ^{17}O- NMR has been used to study flavonoids only in a few cases.[81] Simple molecules can be identified using simple 1D-NMR, while more complicated molecules need 2D-NMR such as COSY, HMBC and HSQC.[82] The various NMR experiments used in this work are discussed below:

1D ^{1}H NMR. The proton spectra of flavonoids are used mainly to obtain qualitative information about proton chemical shifts, their coupling constants (J_{HH}) and provided quantitative information by integrating baseline-separated signals or selected spectral regions. Information about the nature

of the aglycone, type and number of sugar, acyl substituents and some limited connection points information can also be provided.[81]

1D ^{13}C NMR. Compensated Attached Proton Test (CAPT) has been used along with different 2D techniques to obtain the accurate carbon chemical shifts. The signals in ^{13}C-NMR experiments usually appear as singlets because of the decoupling of the attached protons. The signals of quaternary Carbon (C-C), tertiary carbon (C-H), secondary carbon ($C-H_2$) and primary carbon ($C-H_3$) are differentiated using different delays in the pulse-program. The C-C and $C-H_2$ signals can be distinguished from the C-H and $C-H_3$ signals by having opposite phases. However, due to the low abundance (1.1%) and the lesser favorable gyro magnetic ratio (γ) of ^{13}C compared to ^{1}H, this technique suffers from lack of sensitivity, in particular with respect to quaternary carbon. The ^{13}C-spectra have higher signal to noise levels than the corresponding ^{1}H spectra.[81]

2D ^{1}H- ^{1}H gradient selected, Double Quantum Filter, COrrelation SpectroscopY (gs-DQF-COSY). This technique is used to assign the different proton signals based on the couplings through bonds (*J*-coupling). COSY is a 2D homo-nuclear technique where the diagonal peaks represent the actual proton spectrum and the cross peaks show which protons are J-coupled to each other.[81]

2D ^{1}H-^{1}H TOtal Correlation SpectroscopY (2D TOCSY), also known as HOmo-nuclear HArtman-HAhn (HOHAHA). This is a two dimensional homonuclear NMR technique which has diagonal peaks, and identical proton chemical shift axes as the COSY technique. TOCSY is used to find the proton chemical shifts for all protons which belong to the same spin system (e.g. in a sugar unit, even if the protons are not directly *J*-coupled). This technique is very useful when the flavonoid structures are complex and contain more than one sugar unit. 1D TOCSY can be used as an alternative to this technique where coupling constant and accurate chemical shift values can be obtained for each spin system.[81]

2D ^{1}H-^{1}H Nuclear Overhauser and Exchange Effect SpectroscopY (2D NOESY). This is a 2D homo-nuclear technique based on coupling through space. The technique can provide information about the molecular geometry, conformation and connection points. Exchange cross peaks between analogous protons of different forms of the same compound that are in equilibrium with each other may be observed.[81]

2D ^{1}H-^{1}H Rotating frame Overhauser Effect SpectroscopY (2D ROESY). This is an alternative technique to the NOESY technique and is used to analyze compounds with molecular weight around 1000-3000 g/mole.[81]

2D ^{1}H- ^{13}C gradient- selected Heteronuclear Single Quantum Coherence (gs- HSQC). The inverse-detected 2D-heteronuclear experiment correlates ^{1}H and ^{13}C chemical shifts through single-bond heteronuclear couplings $^{1}J_{CH}$. The HSQC spectrum shows cross peaks between the chemical shifts of a proton and of a carbon that are directly bonded.[81]

*2D ^{1}H- ^{13}C gradient- selected Heteronuclear Single Quantum Coherence- TOtal Correlation SpectroscopY (*gs- HSQC-TOCSY). This technique is used mainly when the flavonoid structures are complex. It gives cross peaks for all the scalar-coupled proton and carbon nuclei belonging to the same spin system in addition to the one-bond correlated protons and carbons.[81]

2D ^{1}H- ^{13}C gradient- selected Heteronuclear Multiple Bond Correlation (gs- HMBC). The HMBC correlates ^{1}H and ^{13}C chemical shifts through multiple-bond heteronuclear couplings. The most important of which are $^{2}J_{CH}$ and $^{3}J_{CH}$, for which the strongest cross-peaks are observed. In addition, some $^{1}J_{CH}$ correlations and some long-distance correlations can be observed. In the heteronuclear multiple-bond correlation spectra, most quaternary carbon resonances may be assigned.[81]

2.4 Isolation and identification of flavonoids in catmint

Plant collection. The catmint was collected in May 2016 from Birzeit area, Ramallah-Palestine.

Extraction and purification of the flavonoids. Various parts of the plant including leaves, flowers and stems (755 g) were cut into pieces, dried and stored at room temperature for approximately one month. Next, the sample was extracted 3 times overnight at 4℃, first with 5 L 10 % Water/MeOH (v/v; containing 0.5 % TFA) then 2 times with 4L 0.5% TFA/MeOH (v/v). The filtered extract was concentrated under reduced pressure and re-dissolved in 200 ml water and then it was purified by partition against hexane (three times, 400 ml hexane each) and then subjected to Amberlite XAD-7 column chromatography (75.0*5.5 cm i.d.). The columns were washed with distilled water (10 L each) after which the flavonoids were eluted with methanol containing 0.5% TFA. The flavonoids were further purified and fractionated on a Sephadex LH-20 column (100*5 cm i.d.) using a step–wise gradient of ACN: TFA: H_2O (10:0.5:90 %) and MeOH: TFA: H_2O (70:0.5:30%) with flow-rate of 10 ml/min. 50 fractions were collected using different gradient as presented in (Table 2.1).

Fractions 25 and 26 contain the same compound and they were combined and further purified by preparative HPLC. Compounds **1**, **2** and **4** were further purified by preparative HPLC. Compound **3** was eluted after 927 mL of **S5**.

Table 2.1: Solvent consumption and elution volumes of fractions S1-S7 originating from partial purification on a 100×5 cm Sephadex column.

Solvents	Solvents ACN: H_2O: TFA	Vol. eluted ml
S1	10:90:0.5	280
S2	20:80:0.5	1015
S3	30:80:0.5	2047
S4	40:60:0.5	1043
Solvents	Solvents MeOH: H_2O: TFA	Vol. eluted ml
S5	70:30:0.5	1137
S6	80:20:0.5	904
S7	90:10:0.5	2304

2.5 Extraction of flavonoids content from fenugreek and Crataegus

2.5.1 Fenugreek

Plant material. Seeds of fenugreek was purchased from a local shop in Ramallah-Palestine.

Seeds of fenugreek (700 g) were soaked with distilled water, for 48 hour (h) in a refrigerator, followed by grinding the seeds to obtain the maximum amount of extract. The ground seeds were extracted 3 times with methanol at 4 ^{o}C. Then the extract was filtered. The filtrate extract was

concentrated under reduced pressure, and purified by partition against equal portions of hexane (4 times). The concentrated aqueous layer was applied on an Amberlite XAD-7 column. The flavonoids adsorbed on the column were washed with distilled water (9 L) before they were eluted with methanol.

2.5.2 Crataegus

Plant material. The Crataegus was collected in May 2017 from Birzeit area, Ramallah-Palestine. Leaves, stems and flowers (758 g) of Crataegus were cut into pieces and extracted 3 times with MeOH containing 0.5% TFA (v/v) for 48 h at 4 ^{o}C. The extract was filtered and concentrated under reduced pressure, purified by partition against hexane, and subjected to Amberlite XAD-7 column chromatography. The columns were washed with distilled water (14 L) before the flavonoids were eluted with methanol (2.5 L) containing 0.5% TFA.

2.5.3 Modification of flavonoids to anthocyanidin (reductive reaction)

General procedure. Zinc – amalgam was prepared as described by Elphimoff-Felkin and Sarda (1977).[83]

Amalgamated zinc. (10.3 g, 0.16 moles) of zinc powder was placed in a 1 L beaker, suspended with 12.5 ml of 10% hydrochloric acid and stirred for 2 min. The acid was then decanted and the solid was washed with distilled water. The washing process was continued until the water was neutral after testing with litmus paper. A warm solution of 2 g (0.0075 mole) of mercury chloride in 12.5 ml distilled water was then poured onto the zinc sample and the mixture was stirred gently for 10 min. After filtration, the powder was washed with 12.5 ml of distilled water followed by five portions of 95% methanol (12.5 ml each) and finally with five portions of anhydrous ether

(12.5 ml each). Drying under vacuum gave 9.8 g of zinc amalgam, (Scheme 2.2) shows the equation of preparation of zinc amalgam.

Scheme 2.2: Preparation of zinc amalgam

$$Zn + HgCl_2 \xrightarrow{HCl} ZnCl_2 + Hg$$

$$Zn + Hg \longrightarrow Zn(Hg)$$

Reductive reaction. Extract of flavonoids which was obtained after Amberlite XAD-7 column chromatography were dissolved in methanol containing 6% HCl (w/w) after through stirring in sonicator for 1- 4 h depending on the sample. Zinc – amalgam was added in portion (100-150 mg each). The reaction was monitored by measuring the absorbance each 30 min using UV-Vis spectroscopy at 520 nm. The reaction mixture was then filtered to remove unreacted Zn. Methanol was then evaporated under reduced pressure and the product was subjected to Amberlite XAD-7 column chromatography as described earlier. The product was then eluted using MeOH containing 0.5 % TFA by volume. The solvent was then evaporated and the product was further dried under reduced pressure in a vacuum oven at 30 ^{o}C. (Scheme 2.3) shows the equation of reduction of flavonols to the analogous anthocyanins.

Extract of flavonoids from Crataegus was treated following a similar procedure.

Scheme 2.3: Synthesis of cyanidin 3-O-(6^{II}-O-α-rhamnopyranosyl)-β-glucopyranoside, (R_1=H, R_2=α-rhamnopyranosyl) and cyanidin 3,4^{I}-diglucoside (R_1=β-glucopyranoside, R_2=H).

OH OR1 HO O HO OH O OH OH O O OR2

Zn

CH_3OH/HCl

OH OR1 HO O^+ HO OH O OH OH O OR2

2.5.4 Anti -bacterial activity

Original fenugreek, modified fenugreek, original Crataegus and modified Crataegus were screened against six kinds of bacteria, G+ bacteria (*S. aureus*, *S. epidermidis* and *B. Subtilis*) and G- bacteria (*P. mirabilis*, *P. aeruginosa* and *E. coli*) using agar diffusion method. In this method, single bacterial colonies were dissolved in sterile saline until the suspended cells reached the turbidity of McFarland 0.5 Standard. Then the bacterial inocula were spread on the surface of the Muller Hinton nutrient agar by means of a sterile cotton swab. Sterile glassy borer were used to make a 6 mm in diameter wells in the agar plate.[84] Samples were dissolved in dimethyl sulfoxide (DMSO) in concentrations equal to 6 mg/ml, then 25 µL of the test samples were introduced in the respective wells. DMSO solvent was used as negative control while gentamycin (G) and erythromycin (E) was used as positive control. The plate was incubated at 37 ^{o}C for 24 h. The anti-bacterial activity was determined by measuring the diameter inhibition zone of complete growth in millimeter (mm). The averages of three trials determined the results and are stated as average ± standard error.[85,86]

3. Results and discussion

3.1 Identification of flavonoids

The HPLC chromatogram of the methanolic crude extract of *Nepeta curviflora* showed four major and several minor flavonoids peaks, as well as other phenolic compounds as shown in (Figure 3.1). The methanolic extract of the aerial parts of *Nepeta curviflora* (stems, leaves and flowers) was purified by partition against hexane, followed by Amberlite XAD-7 absorption column chromatography. The flavonoids in the purified extract were further fractionated by Sephadex LH-20 column chromatography.

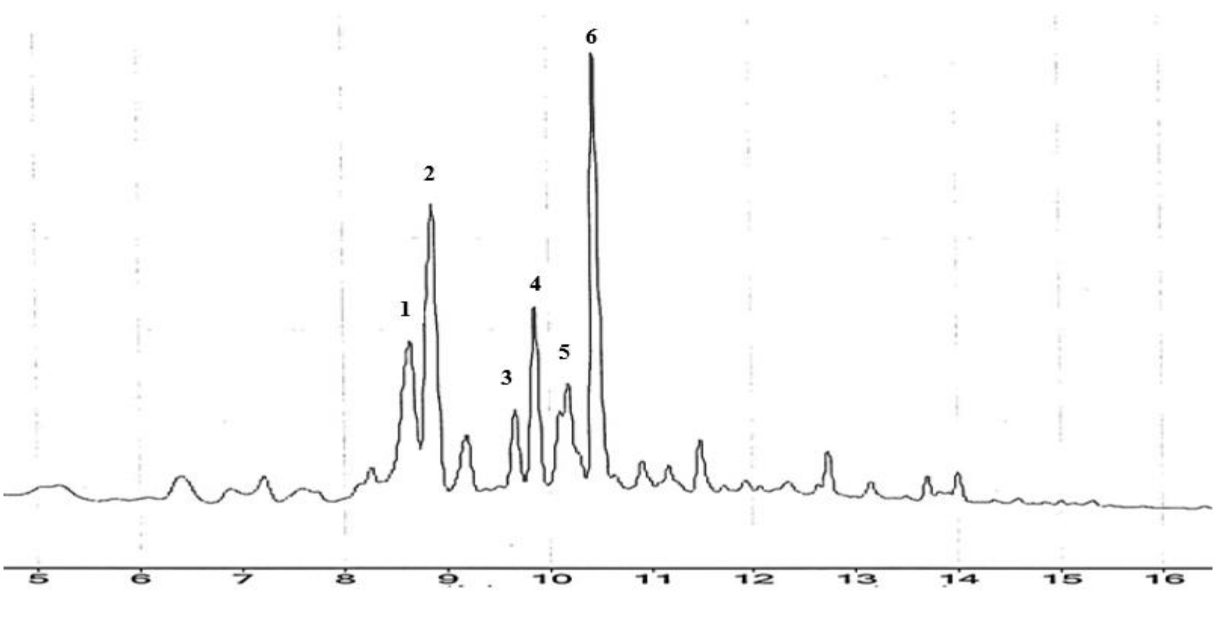


Figure 3.1: HPLC chromatogram of flavonoids from catmint (*Nepeta curviflora*) detected at 360 nm. See figure 3.2 for structures. The mobile phase consisted of formic acid: water (0.5:9.0, v/v; solvent A) and acetonitrile (solvent B) with the following elution profile: linear gradient was from 10% B in 0-2 min, 10-45% B from 5-17 min, 45-10% B from 17-18 min and 10% B from 18-20 min. The flow rate was 12.0 ml min^{-1}.

Each peak in HPLC chromatogram may indicates one compound or more. (Table 3.1) Summarizes the results as obtained from liquid chromatography- mass spectrometry (LC-MS).

Table 3.1: Peak/compound correspondence as obtained LC-MS.

Peak number	Retention time	Expected compound	$[M + H]^+$, g/mole
1	8.714	Unspecified	-
2	8.968	**10**	801.87
3	9.802	**1** or **8** or **9**	815.82
4	9.999	**7**	785.84
5	10.253	**8** or **9**	-
6	10.494	Unspecified	-

Compounds **1**,**5** and **6** are the ester forms of compound **7** which might have been synthesized during the work up procedure while compounds **2**,**8** and **9** are the ester forms of **10** which might have been synthesized during the work up procedures.

	R^1	R^2
1:	CH_3	CH_3
5:	CH_3	H
6:	H	CH_3
7:	H	H

	R^1	R^2
2:	CH_3	CH_3
8:	H	CH_3
9:	CH_3	H
10:	H	H

Figure 3.2: Derived structures of the novel flavonoids **1** and **2** and their known phenolic esters **3** and **4** as isolated from catmint (*Nepeta curviflora*).

compounds **3** and **4** were characterized (as the phenolic compounds of rosmarinic acid **3** and caffeic acid **4**, respectively), using NMR and UV-Vis spectroscopy as tabulated in (Tables 3.2 and 3.3). UV spectrum of compound **4** caffeic acid (ester form) is given in (Figure 3.3).

According to the obtained NMR spectra, compounds **3** and **4** were identified as the known compounds of rosmarinic acid and caffeic acid.

A

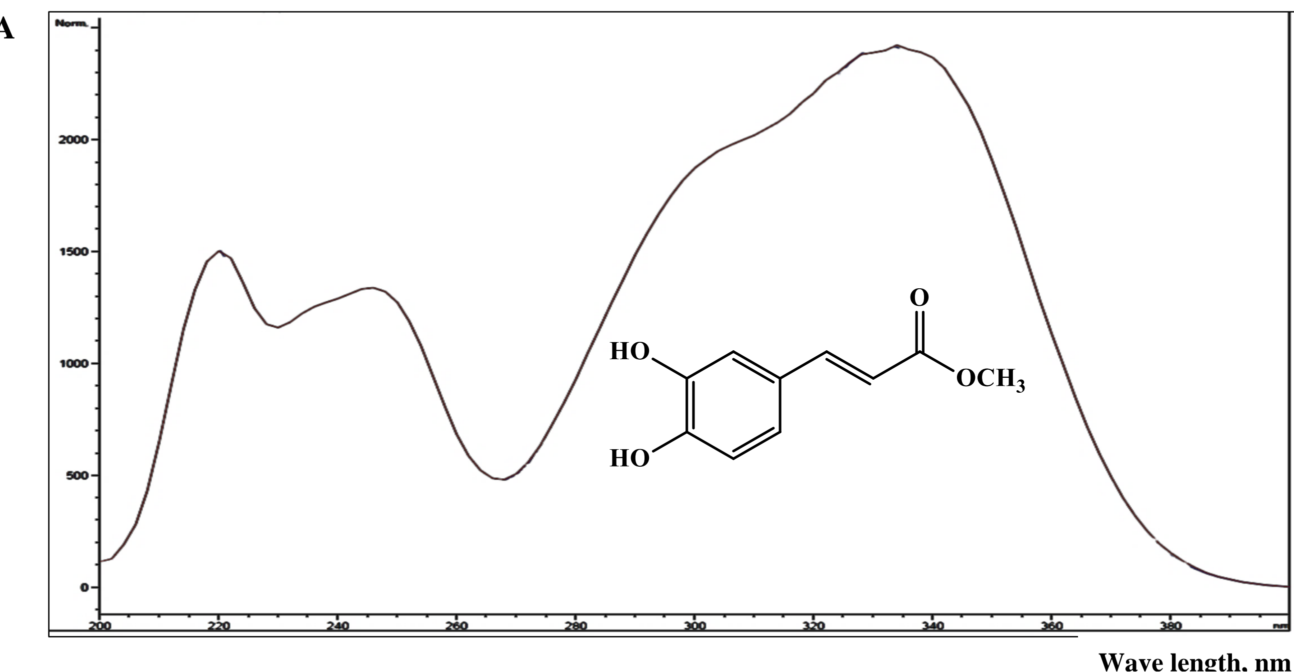


Figure 3.3: UV spectrum of compound **4** caffeic acid (ester form) recorded online during HPLC analysis.

3.2 NMR Spectroscopy

3.2.1 Compound 1

The downfield region of 1D ^{1}H NMR spectrum of **1** showed a 4H AA'XX' system at δ 7.94 (d, *J* = 8.8 Hz; H-2'/6') and δ 6.95 (d, *J* = 8.8 Hz; H-3'/5'), a 1H singlet at δ 6.84 (H-3) and a 2H AB system at δ 6.76 (d, *J* = 2.2 Hz; H8) and δ 6.39 (d, *J* = 2.2 Hz; H6) in accordance with a 7-*O*-substituted apigenin derivative.

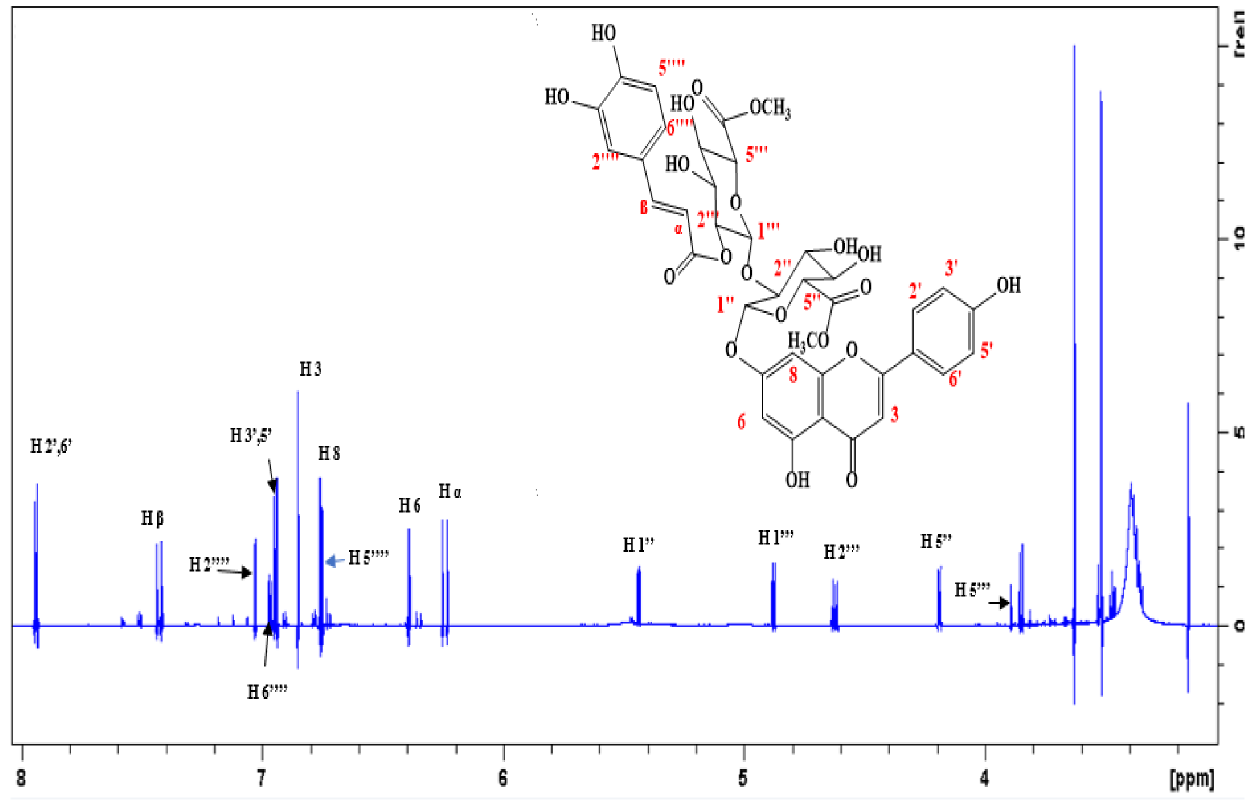


Figure 3.4: 1D ^{1}H NMR spectrum of apigenin 7-*O*-*β*-glucuropyranosyl-2’’-*O*-(2’’’-*O*-caffeoyl)-β-glucuropyranoside (**1**) isolated from catmint (*Nepeta curviflora*). The unsigned resonance belongs to methoxy group.

The 15 ^{13}C resonances belonging to the aglycone and the 9 ^{13}C resonances belong to the acyl moiety are indicated in the 1D ^{13}C CAPT spectrum of **1** as shown in the (Figure 3.5). The resonance was assigned by the observed cross peaks in the H2BC, HMBC and HSQC spectra.

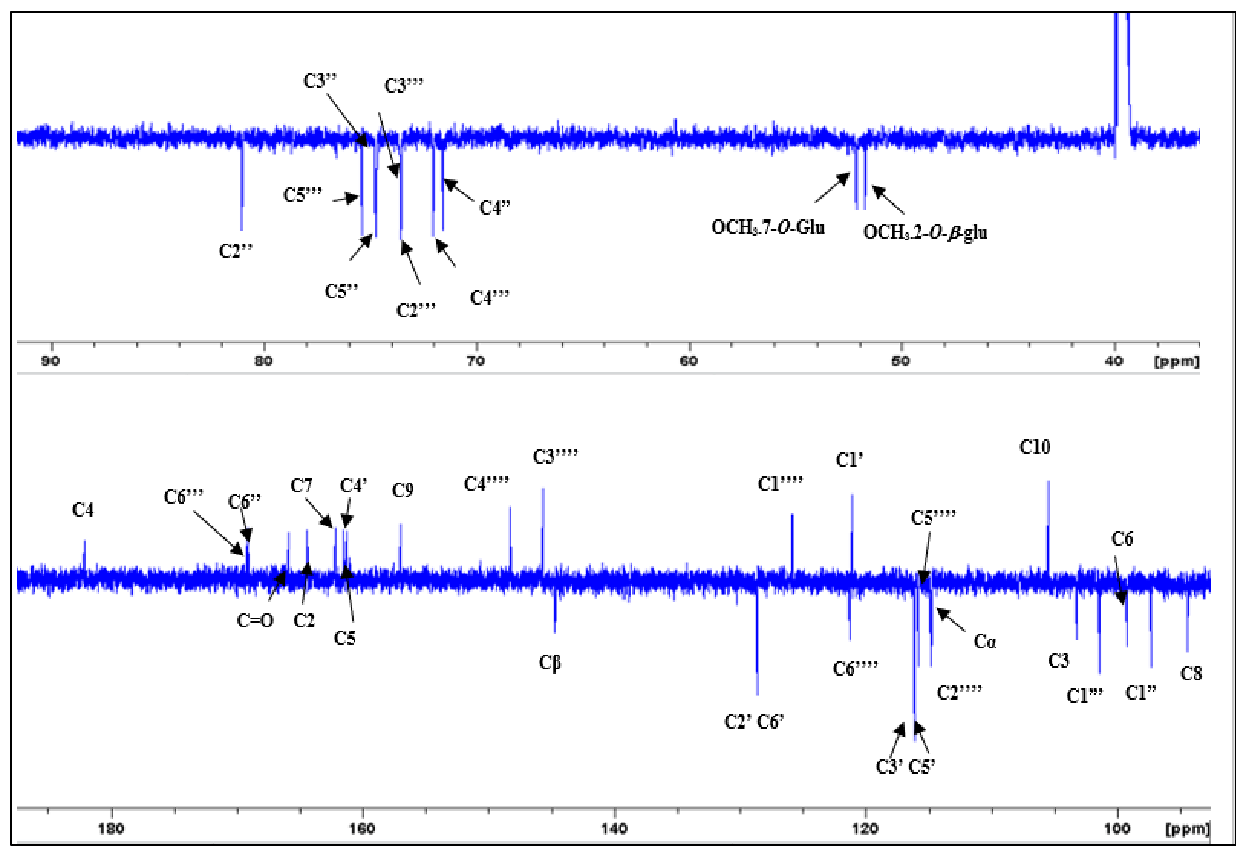


Figure 3.5: Expanded parts of the 1D ^{13}C CAPT spectrum of apigenin 7-*O*-*β*-glucuropyranosyl-2''-*O*-(2'''-*O*-caffeoyl)-β-glucuropyranoside (**1**) isolated from catmint (*Nepeta curviflora*)

The sugar regions of the 1D ^{1}H and 1D ^{13}C CAPT spectra of **1** indicated the presence of two glucuropyranosyl units as tabulated in (Tables 3.2 and 3.3). All ^{1}H sugar resonances were assigned by the 2D ^{1}H COSY experiment, and the corresponding ^{13}C resonances were then assigned by the ^{1}H -^{13}C HSQC experiment. The anomeric coupling constants 7.5 Hz and 8.2 Hz and the 12 ^{13}C resonances in the sugar region of the ^{13}C CAPT spectrum of **1** were in accordance with two *O*-*β*-glucuronopyranosyl units as shown in (Figure 3.6).[87]

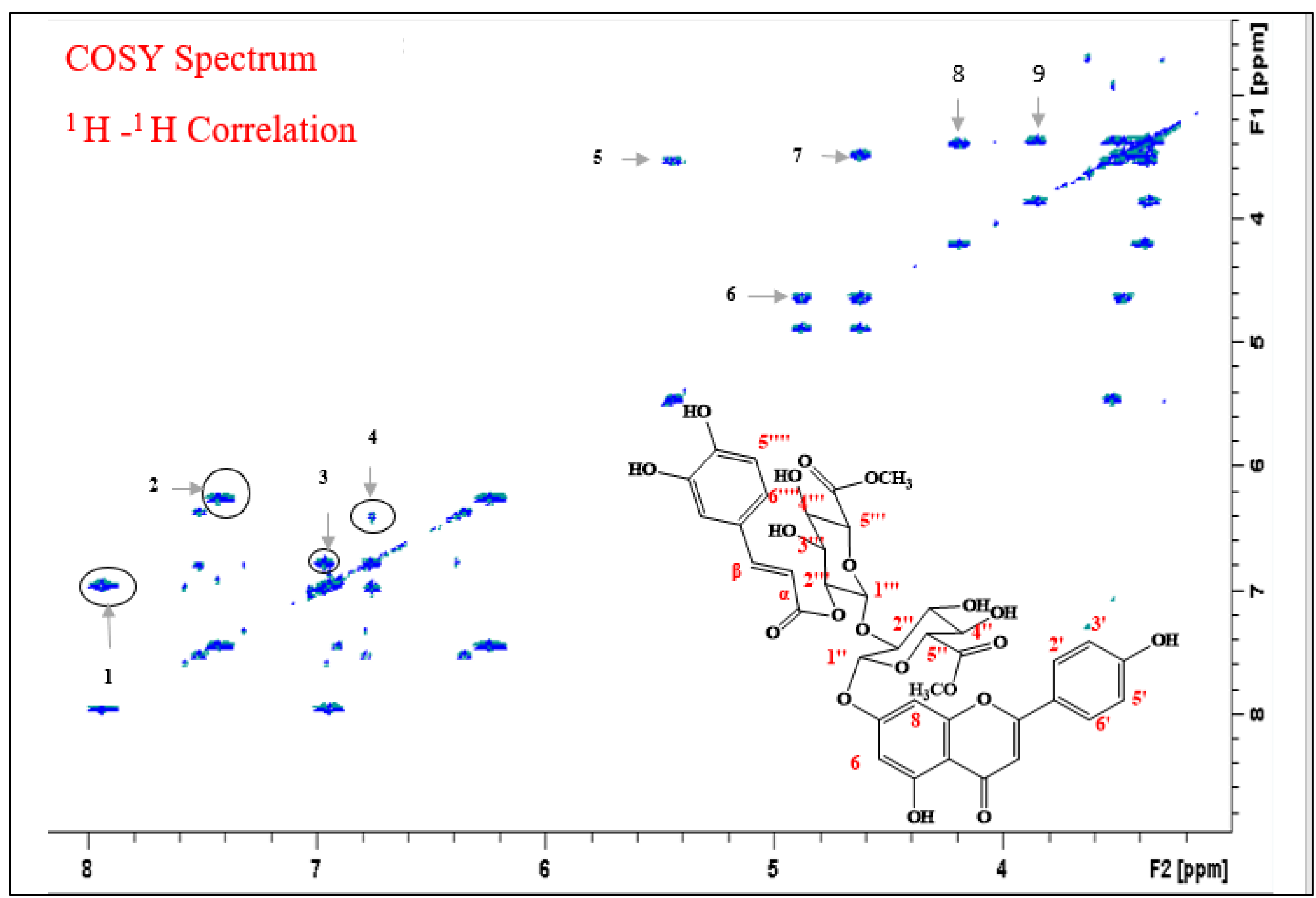


Figure 3.6: 2D DQF-COSY spectrum of apigenin 7-*O*-*β*-glucuropyranosyl-2''-*O*-(2'''-*O*-caffeoyl)-β-glucuropyranoside (**1**) isolated from catmint (*Nepeta curviflora*). Arrows 1, 2, 3 and 4 points to the cross peaks between (2',6')/ (3',5'), β/α, 6''''/5'''' and 8/6, respectively. Arrows 5 and 8 points to the cross peaks between 1''/2'' and 4''/5'', respectively. Arrows 6, 7 and 9 points to the cross peaks between 1'''/2''', 2'''/3''' and 4'''/5''', respectively.

The downfield shifts of H-2''' (δ 4.62) of the terminal glucuronosyl unit indicated the presence of acyl substitution. The presence of 3H AMX system at δ 7.03 (*J* = 2.1; H-2''''), δ 6.97 (*J* = 2.1, 8.5; H-6''''), and δ 6.76 (*J* = 8.5; H-5''''), and a 2H AX system at δ 7.43 (*J* =15.9 Hz, H-β) and δ 6.24 (H-α) confirmed the identity of the acyl-group to be (*E*)- caffeoyl acyl moiety.

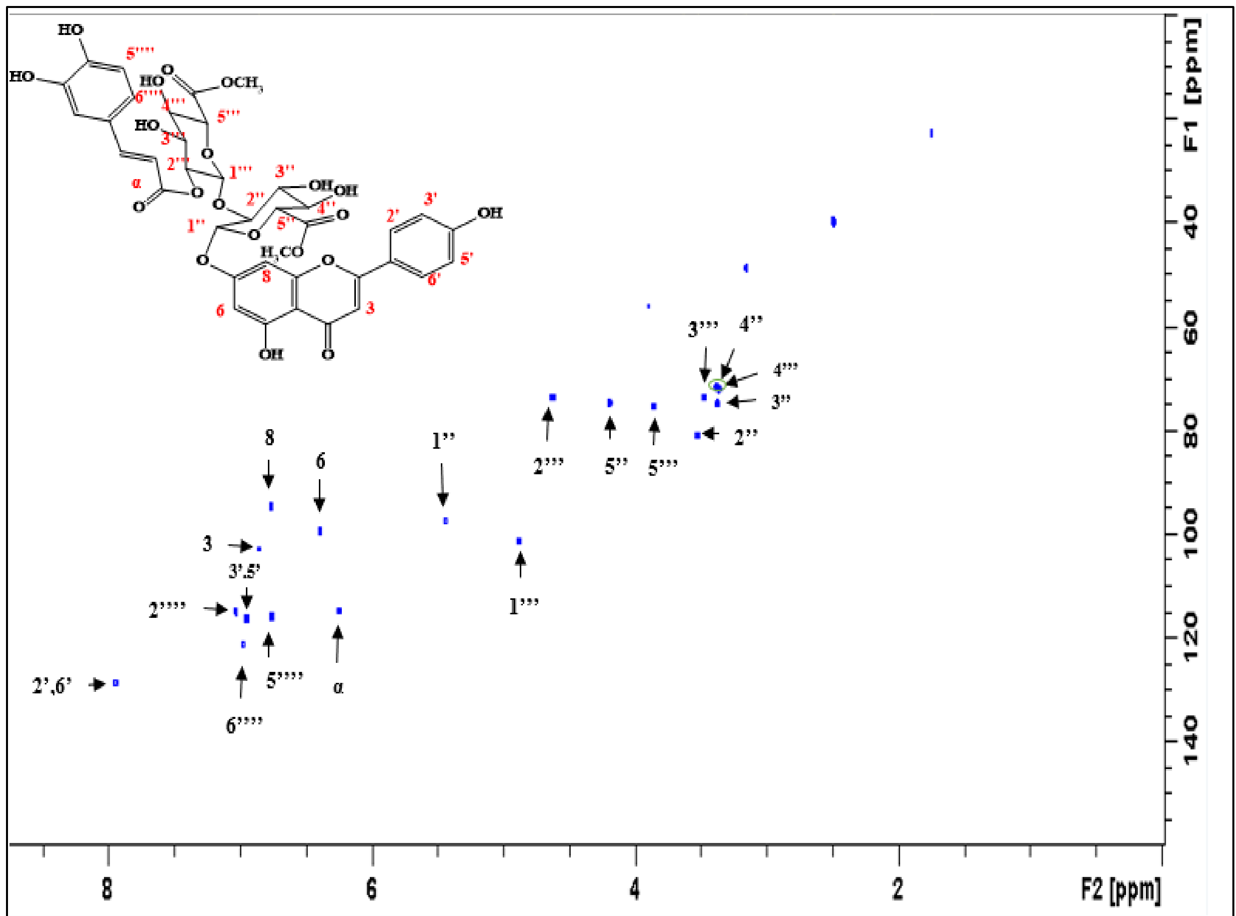


Figure 3.7: 2D gs-HSQC spectrum of apigenin 7-*O*-*β*-glucuropyranosyl-2''-*O*-(2'''-*O*-caffeoyl)-β-glucuropyranoside (**1**) isolated from catmint (*Nepeta curviflora*).

The linkages between the aglycone, sugar units and (*E*)- caffeoyl acyl moiety were determined by the long-range correlations in the 2D HMBC spectrum and ROESY spectrum as clearly indicated in (Figure 3.8) and (Figure 3.9).

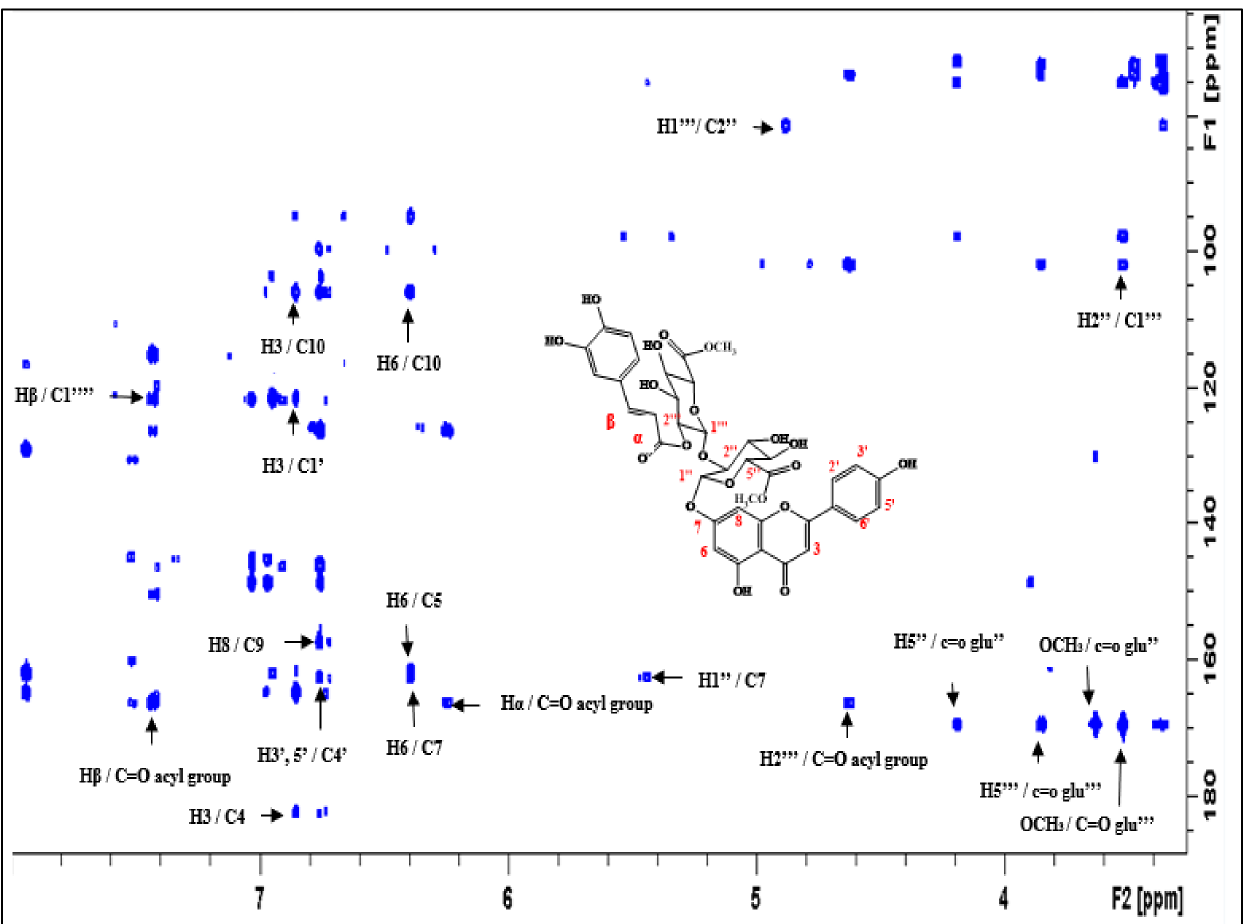


Figure 3.8: 2D gs-HMBC spectrum of apigenin 7-*O*-*β*-glucuropyranosyl-2''-*O*-(2'''-*O*-caffeoyl)-β-glucuropyranoside (**1**) isolated from catmint (*Nepeta curviflora*).

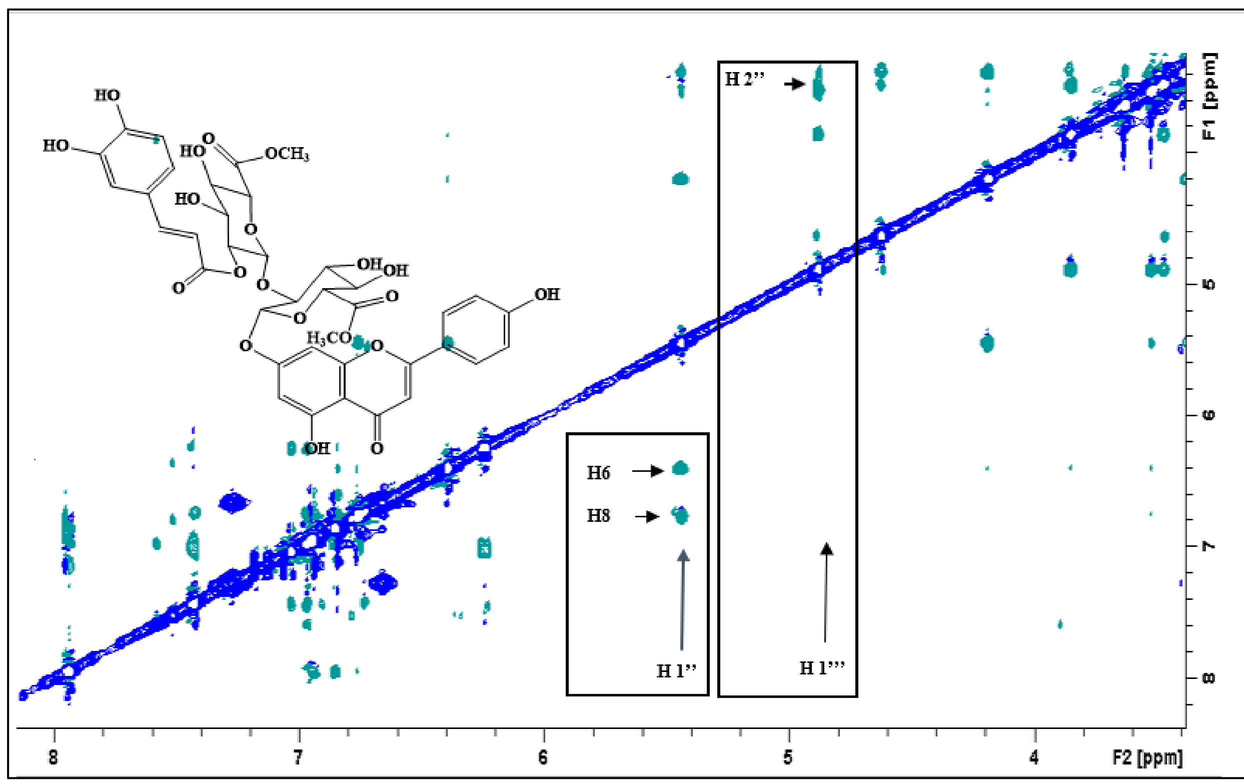


Figure 3.9: 2D ROESY Spectrum of apigenin 7-*O*-*β*-glucuropyranosyl-2''-*O*-(2'''-*O*-caffeoyl)-β-glucuropyranoside (1) isolated from catmint (*Nepeta curviflora*).

A molecular ion at *m/z* 815.82 g/mole was observed in the LC-MS spectrum (**1** + **H)**$^{+}$, confirmed the identity of **1** to be the novel compound apigenin 7-*O*-β-glucuronosyl-2''-*O*-(2'''-*O*-(caffeoyl)-β-glucuronide) present as an ester form of (**1**) as shown in (Figure 3.10).

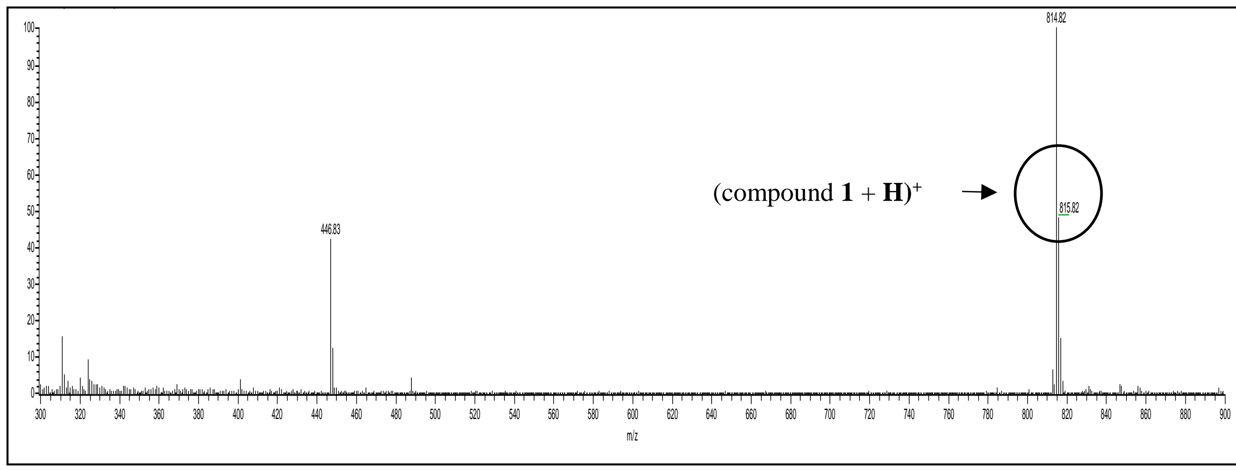


Figure 3.10: Mass spectrum of of apigenin 7-*O*-*β*-glucuropyranosyl-2''-*O*-(2'''-*O*-caffeoyl)-β-glucuropyranoside (**1**) isolated from catmint (*Nepeta curviflora*).

3.2.2 Compound 2

The downfield region of the 1D ^{1}H NMR spectrum of **2** showed a 3H AMX system at δ 7.43 (*J* = 2.2, 8.5 Hz; H-6'), and δ 6.92 (*J* = 8.5 Hz; H-5'), a 1H singlet at δ 6.73 (H-3) and a 2H AB system at δ 6.73 (d, *J* = 2.2 Hz; H6) and δ 6.39 (d, *J* = 2.2 Hz; H8) in accordance with a 7-*O*-substituted luteolin derivative. The 24 ^{13}C resonances belong to the aglycone and the acyl moiety in the 1D ^{13}C CAPT spectrum of **2** were assigned by the observed cross peaks in the H2BC, HMBC and HSQC spectra. The sugar regions of the 1D ^{1}H and 1D ^{13}C CAPT spectra of **2** showed the presence of two glucuronopyranosyl units as tabulated in (Tables 3.2 and 3.3). All ^{1}H sugar resonances were assigned by the COSY experiment, and the corresponding ^{13}C resonances were then assigned by the ^{1}H-^{13}C HSQC and the HSQC-TOCSY experiments. The anomeric coupling constants 7.5 Hz and 8.3 Hz and the 12 ^{13}C resonances in the sugar region of the ^{13}C CAPT spectrum of **2** were in accordance with two *O*-β-glucuropyranosyl units.[87,69] The downfield shifts of H-2''' (δ 4.63) belonging to the glucuronyl unit indicated the presence of acyl substitution. The

presence of a 3H AMX system at δ 7.04 (J = 2.2; H-2''''), δ 6.97 (J = 2.2, 8.1; H-6''''), and δ 6.77 (J = 8.1; H-5''''), and a 2H AX system at δ 7.44 (J =15.9 Hz, H-β) and δ 6.23 (H-α) confirmed the identity of the acyl-group to be (E)-caffeoyl. The linkages between the aglycone, sugar units and (E)-caffeoyl moiety were determined by the long-range correlations in the 2D HMBC spectrum. A molecular ion at *m/z* 830.87 g/mole in the LC-MS spectrum support the potential identity of **2** to be the novel compound luteolin 7-*O*-β-glucuronosyl-2''-*O*-(2'''-*O*-(caffeoyl)-β-glucuronoside) present as an ester form (**2**) as shown in (Figure 3.2).

Table 3.2: ^{1}H spectral data (δ in ppm and *J* in Hz) for compounds **1-4** dissolved in DMSO-D6 at 25 ºC.

	1	**2**		**3**
3	6.85 *s*	6.73 s	α	6.26 *d* 15.9
6	6.39 *d* 2.2	6.73 *d* 2.1	β	7.48 *d* 15.9
8	6.76 m	6.39 *d* 2.1	2	7.06 d 2.2
2’	7.94 *d* 8.8	7.43 br	5	6.77 *d* 8.2
3’	6.95 *d* 8.8		6	7.01 *dd* 2.2, 8.2
5’	6.95 *d* 8.8	6.92 *d* 8.5		
6’	7.94 *d* 8.8	7.43 *dd* 2.2, 8.5	α	5.12 dd 2.2, 7.7
	-*O*-Glu	7-*O*-Glu	$β_A$	2.95 *m*
1”	5.44 *d* 7.5	5.46 *d* 7.5	$β_B$	2.95 *m*
2”	3.52	3.53 *m*	2’	6.65 *d* 2.1
3”	3.37	3.37 *m*	5’	6.64 *d* 8.1
4”	3.37	3.37 *m*	6’	6.49 *dd* 2.1, 8.1
5”	4.19 *d* 7.5	4.19 *d* 9.4	OCH_3	-
	-*O*-*β*-glc	8-*C*-*β*-gal	**4**	
1”’	4.88 *d* 8.2	4.89 *d* 8.3	A	6.26 *d* 15.9
2”‘	4.62 d 8.2	4.63 dd 8.3, 9.6	B	7.48 *d* 15.9
3”‘	3.47	3.48 t 9.6	2	7.10 d 2.2
4”‘	3.36	3.36 t 9.6	5	6.77 *d* 8.2
5”‘	3.85 *d* 8.2	3.85 d 9.6	6	7.07 *dd* 2.2, 8.2
	2’’’-*O*-Caf	2’’’-O-Caf		
α	6.24 *d* 15.8	6.23 d 15.8		
β	7.43 *d* 15.8	7.44 d 15.8		
2’’’’	7.03 *d* 2.1	7.04 d 2.2		
5‘‘‘‘	6.76 m	6.77 d 8.1		
6‘‘‘‘	6.97 *dd* 2.1 , 8.5	6.97 dd 2.2 , 8.1		

Table 3.3: ^{13}C spectral data (δ in ppm) for compounds **1-4** dissolved in DMSO-D_6 at 25 °C.

	1	**2**		**3**
2	164.39	164.66	C=O	166.07
3	103.25	103.31	α	113.03
4	182.10	182.03	β	146.53
5	161.28	161.34	1	125.46
6	99.25	94.34	2	115.10
7	162.19	162.22	3	144.30
8	94.44	99.29	4	148.91
9	157.01	157.06	5	115.94
10	105.52	105.57	6	121.91
1′	121.12	121.50		
2′	128.66	119.25	C=O	170.11
3′	116.15	145.99	α	72.93
4′	161.50	150.14	βA	36.35
5′	116.15	116.18	βB	
6′	128.66	121.36	1′	126.82
	7-*O*-Glu	7-*O*-Glu	2′	116.82
1″	97.35	97.37	3′	145.78
2″	81.01	81.07	4′	144.16
3″	74.73	74.77	5′	115.61
4″	71.54	71.58	6′	120.25
5″	74.70	74.75	OCH_3	52.15
6″	169.10	169.15	**4**	

OCH₃	52.09	52.14	C=O	166.07
	2-*O*-*β*-glu	2-*O*-*β*-glu	α	113.03
1”’	101.42	101.46	β	146.53
2”‘	73.52	73.58	1	125.46
3”‘	73.56	73.61	2	115.10
4”‘	72.02	72.08	3	144.30
5”‘	75.37	75.42	4	148.91
6”’	169.17	169.22	5	115.94
OCH_3	51.71	51.75	6	121.91
	2”-O-caffeoyl	2”-O-caffeoyl		
C=O	165.87	165.94		
α	114.73	114.75		
β	144.72	144.79		
1““	125.86	125.89		
2““	114.83	114.86		
3““	145.65	145.72		
4““	148.29	148.36		
5““	115.82	115.87		
6““	121.30	121.36		

3.3 Anti-bacterial activity

The in-vitro anti-bacterial activity for original fenugreek, modified fenugreek, original Crataegus and modified Crataegus were tested against gram-positive and gram-negative bacteria using agar diffusion method. The results are summarized in (Table 3.4 and 3.5) gram-negative and gram-positive bacteria, respectively.

Table 3.4: In-vitro anti-bacterial activity data for interest samples against gram-negative bacteria.

Samples	*E. coli* (mm)	*P. aeruginosa* (mm)	*P. mirabilis* (mm)
Gentamycin	13.7 ± 1.2	11.7 ± 0.6	14.0 ± 1.0
Erythromycin	11.3 ± 0.6	10.7 ± 1.2	9.3 ± 1.2
DMSO	-	-	-
Fenugreek	-	-	-
Modified fenugreek	-	-	-
Crataegus	-	-	-
Modified Crataegus	-	-	-

Table 3.5: In-vitro anti-bacterial activity data for interest samples against gram-positive bacteria.

Samples	*S. aureus*(mm)	*S. epidermidis* (mm)	*B. Subtilis* (mm)
Gentamycin	13.3 ± 0.6	19 ± 3.0	13.7 ± 1.5
Erythromycin	19.3 ± 0.6	20.7 ± 1.2	18.3 ± 1.2
DMSO	-	-	-
Fenugreek	-	-	-
Modified fenugreek	-	-	-
Crataegus	-	-	-
Modified Crataegus	-	-	-

Unfortunately, all the tested plant extracts didn't show anti-bacterial activity against all gram-negative bacteria and gram-positive bacteria compared with standard of gentamycin and erythromycin samples Which encourages testing other bacteria because some compounds showed positive activity against some kinds of bacteria and negative against other.

4. Conclusion

The two novel flavone *O*-glucuronides, apigenin 7-*O*-*β*-glucuronopyranosyl-2''-*O*-(2'''-*O*-caffeoyl)-β-glucuronopyranoside (**1**) and luteolin 7-*O*-β-glucuronopyranosyl-2''-*O*-(2'''-*O*-(caffeoyl)-β-glucuronopyranoside (**2**), in addition to the known phenolic compounds rosmarinic acid (**3**) and caffeic acid (**4**) were isolated from aerial parts of catmint (*Nepeta curviflora*). The isolated compounds were fully characterized using UV-visible spectroscopy, LC-MS and extensive use of 1D and 2D NMR spectroscopy.

Modification of flavonoids in fenugreek seeds and Crataegus were modified to anthocyanidins using activated Zinc by ultrasound.

All tested samples of original fenugreek, modified fenugreek, original Crataegus and modified Crataegus didn't show any anti-bacterial activity against all types of gram-negative bacteria and gram-positive bacteria.

5. Recommendation

For the first part, two new compounds which were isolated from catmint should be investigated against antibacterial and antioxidant activities in further study.

For the second part, an antibacterial activity might be observed when considering the following:

1- Separate individual compounds by different technique, then investigate each compound for antibacterial activity.
2- Investigate a wide range of positive and negative bacteria other than those which have been used in this research.

6. References

1. Watson, R. R.; Preedy, V. R. *Nutrition and heart disease: Causation and prevention*; CRC Press, 2003.
2. Paulson, D. S. *Frontiers in Clinical Drug Research: Anti-Infectives* **2016**, *2*, 137.
3. Hamamatsu, S.; Yabe, K.; Nawa, Y. *Food Sci Technol Res* **2007**, *10*, 239.
4. Chua, L. S.; Latiff, N. A.; Lee, S. Y.; Lee, C. T.; Sarmidi, M. R.; Aziz, R. A. *Food Chem* **2011**, *127*, 1186.
5. Kumar, S.; Pandey, A. K. *Sci World J.* **2013**, *2013*.
6. Narayana, K. R.; Reddy, M. S.; Chaluvadi, M.; Krishna, D. *Indian J Pharmacol* **2001**, *33*, 2.
7. Brodowska, K. M. *Eur J Biol Res* **2017**, *7*, 108.
8. Rayyan, S.; Fossen, T.; Andersen, Ø. M. *J. Agric. Food. Chem.* **2005**, *53*, 10057.
9. Fossen, T.; Rayyan, S.; Holmberg, M. H.; Nimtz, M.; Andersen, Ø. M. *Phytochemistry* **2007**, *68*, 652.
10. Wang, T.-y.; Li, Q.; Bi, K.-s. Asian *j. res. pharm. sci.* **2017**.
11. Meskin, M. S.; Randolph, R. K.; Bidlack, W. R. *Phytochemicals: aging and health*; CRC press, 2008.
12. Kim, B. G.; Yang, S. M.; Kim, S. Y.; Cha, M. N.; Ahn, J.-H. *Appl. Microbiol. Biotechnol.* **2015**, *99*, 2979.
13. Rayyan, S.; Fossen, T.; Nateland, H. S.; Andersen, Ø. *Phytochem. Anal* **2005**, *16*, 334.
14. Laghari, A. Q.; Memon, S.; Nelofar, A.; Laghari, A. H. *Am J Analyt Chem* **2011**, *2*, 871.
15. Koes, R. E.; Quattrocchio, F.; Mol, J. N. *BioEssays* **1994**, *16*, 123.
16. Pietta, P.-G. *J. Nat. Prod.* **2000**, *63*, 1035.
17. Shohaib, T.; Shafique, M.; Dhanya, N.; Divakar, M. *Hygeia JD Med* **2011**, *3*, 1.
18. Yao, L. H.; Jiang, Y.; Shi, J.; Tomas-Barberan, F.; Datta, N.; Singanusong, R.; Chen, S. *Plant Foods Hum Nutr* **2004**, *59*, 113.
19. Panche, A.; Diwan, A.; Chandra, S. *J Nutr* Food *Sci.* **2016**, *5*.
20. Alzand, K. I.; Mohamed, M. *J. Pharm. Res* **2012**, *5*, 4013.
21. Tapas, A. R.; Sakarkar, D.; Kakde, R. *Trop J Pharm Res* **2008**, *7*, 1089.
22. Es-Safi, N.-E.; Ghidouche, S.; Ducrot, P. H. *Molecules* **2007**, *12*, 2228.

23. Ali-Shtayeh, M.; Jamous, R. M. *Til, Nablus, Palestine, Biodiversity and Environmental Research Center, BERC* **2008**.

24. Ali-Shtayeh, M.; Yaghmour, R. M.-R.; Faidi, Y.; Salem, K.; Al-Nuri, M. *J. Ethnopharmacol.* **1998**, *60*, 265.

25. Omar, G.; Abdallah, L. A.; Ismail, S.; Almasri, M. Y. *Inter J Indig Med Plants* **2013**, *46*, 1299.

26. Mutlu, S.; Atici, Ö.; Esim, N.; Mete, E. *Acta Physiologiae Plantarum* **2011**, *33*, 943.

27. Kraujalis, P.; Venskutonis, P. R.; Ragazinskiene, O. In *Proceedings of the 6th Baltic Conference on Food Science and Technology* 2011.

28. Hemaia, M.; Motawe, L.; Ibrahim, F.; Ibrahim, M.; Mahmoud, E.; Aly, H. *Int J Pharmtech Res* **2015**, *8*, 10.

29. Jamzad, Z.; Grayer, R. J.; Kite, G. C.; Simmonds, M. S.; Ingrouille, M.; Jalili, A. *Biochem. Syst. Ecol.***2003**, *31*, 587.

30. Lee, S. Y.; Lee, C. Y.; Eom, S. H.; Kim, Y. K.; Park, N. I.; Park, S. U. *Sci. Res. Essays* **2010**, *5*, 1122.

31. Mihaylova, D.; Georgieva, L.; Pavlov, A. *Int. J. Agr. Sci. Tech.* **2013**, *1*, 74.

32. Mišić, D.; Šiler, B.; Gašić, U.; Avramov, S.; Živković, S.; Nestorović Živković, J.; Milutinović, M.; Tešić, Ž. *Phytochem. Anal* **2015**, *26*, 72.

33. Modnicki, D.; Tokar, M.; Klimek, B. *Acta pol pharm* **2007**, *64*, 247.

34. Proestos, C.; Boziaris, I.; Nychas, G.-J.; Komaitis, M. *Food Chem.* **2006**, *95*, 664.

35. Trivellini, A.; Lucchesini, M.; Maggini, R.; Mosadegh, H.; Villamarin, T. S. S.; Vernieri, P.; Mensuali-Sodi, A.; Pardossi, A. *Ind Crops Prod* **2016**, *83*, 241.

36. Gilani, A. H.; Shah, A. J.; Zubair, A.; Khalid, S.; Kiani, J.; Ahmed, A.; Rasheed, M.; Ahmad, V. U. *J Ethnopharmacol* **2009**, *121*, 405.

37. Bourrel, C.; Perineau, F.; Michel, G.; Bessiere, J. *J. Essent. Oil Res.* **1993**, *5*, 159.

38. Tzakou, O.; Harvala, C.; Galati, E.; Sanogo, R. *Flavour Fragr J* **2000**, *15*, 115.

39. Mancini, E.; Apostolides Arnold, N.; De Feo, V.; Formisano, C.; Rigano, D.; Piozzi, F.; Senatore, F. *J. Plant Interact* **2009**, *4*, 253.

40. Musso, L.; Scaglia, B.; Haj, G. A.; Arnold, N. A.; Adani, F.; Scarì, G.; Dallavalle, S.; Iriti, M. *J Essent Oil Bear Pl* **2017**, *20*, 1424.

41. Barbour, E. K.; Al Sharif, M.; Sagherian, V. K.; Habre, A. N.; Talhouk, R. S.; Talhouk, S. N. *J Ethnopharmacol* **2004**, *93*, 1.

42. Senatore, F.; Arnold, N. A.; Piozzi, F. *J. Essent. Oil Res.***2005**, *17*, 268.

43. Barhoumi, L. M.; Al-Jaber, H. I.; Abu Zarga, M. H. *Jordan J* Chem. **2017**, *12*, 101.
44. Bernardi, M. M.; Kirsten, T. B.; Salzgeber, S. A.; Ricci, E. L.; Romoff, P.; Guilardi Lago, J. H.; Lourenço, L. M. *Psychology & Neuroscience* **2010**, *3*, 251.
45. Mahboubi, M.; Kazempour, N.; Ghazian, F.; Taghizadeh, M. *Herba polonica* **2011**, *57*, 62.
46. Nestorović, J.; Mišić, D.; Šiler, B.; Soković, M.; Glamočlija, J.; Ćirić, A.; Maksimović, V.; Grubišić, D. *Fitoterapia* **2010**, *81*, 621.
47. Rabbani, M.; Sajjadi, S.; Mohammadi, A. *Evidence-Based Complementary and Alternative Medicine* **2008**, *5*, 181.
48. Saeidnia, S.; Gohari, A.; Hadjiakhoondi, A. *J Med Plant Res.* **2008**, *1*, 54.
49. Shafaghat, A.; Oji, K. *Natural product communications* **2010**, *5*, 625.
50. Sonboli, A.; Salehi, P.; Yousefzadi, M. *Zeitschrift für Naturforschung C* **2004**, *59*, 653.
51. Zomorodian, K.; Saharkhiz, M. J.; Rahimi, M. J.; Shariatifard, S.; Pakshir, K.; Khashei, R. *J Dent (Tehran, Iran)* **2013**, *10*, 329.
52. Sood, A.; Boutard, B.; Chadenson, M.; Chopin, J.; Lebreton, P. *Phytochemistry* **1976**, *15*, 351.
53. Cigremis, Y.; Ulukanli, Z.; Ilcim, A.; Akgoz, M. *Eur Rev Med Pharmacol Sci.* **2010**, *14*, 661.
54. Seladji, M.; Bekhechi, C.; Beddou, F.; Dib, H.; Bendimerad, N. *J Appl Pharm Sci.* **2014**, *4*, 012.
55. Venkata, K. C. N.; Bagchi, D.; Bishayee, A. *Mol Nutr Food Res* **2017**.
56. Nandagopal, S.; Dhanalakshmi, D.; Kumar, A. G.; Sujitha, D. *J Pharm Res* **2012**, *5*, 413.
57. Sharma, V.; Singh, P.; Rani, A. *Eur J Exp Biol* **2017**, *7*.
58. Laila, O.; Murtaza, I. *Int J Food Nutr Sci* **2015**, *4*, 149.
59. Azab, A. E.; Albasha, M. O.; Elsayed, A. S. I. *Yangtze Medicine* **2017**, *1*, 235.
60. Benayad, Z.; Gómez-Cordovés, C.; Es-Safi, N. E. *Int J Mol Sci* **2014**, *15*, 20668.
61. Ahmad, A.; Alghamdi, S. S.; Mahmood, K.; Afzal, M. *Saudi J Biol Sci* **2016**, *23*, 300.
62. Adamska, M.; Lutomski, J. *Planta medica* **1971**, *20*, 224.
63. Seshadri, T.; Sood, A.; Varshney, I. *Indian J* Chem. **1972**.
64. Seshadri, T.; Varshney, I.; Sood, A. *Cur Sci* **1973**.
65. Wagner, H.; Iyengar, M. A.; Hörhammer, L. *Phytochemistry* **1973**, *12*, 2548.
66. Kawashty, S.; Abdalla, M.; El Din, E. G.; Saleh, N. *Biochem. Syst. Ecol.* **1998**, *26*, 851.
67. Han, Y.; Nishibe, S.; Noguchi, Y.; Jin, Z. *Phytochemistry* **2001**, *58*, 577.
68. Yuldashev, M. *Chem. Nat. Compd.* **2002**, *38*, 291.

69. Rayyan, S.; Fossen, T.; Andersen, Ø. M. *J. Agric. Food. Chem.* **2010**, *58*, 7211.
70. Nabavi, S. F.; Habtemariam, S.; Ahmed, T.; Sureda, A.; Daglia, M.; Sobarzo-Sánchez, E.; Nabavi, S. M. *Nutrients* **2015**, *7*, 7708.
71. Chang, Q.; Zuo, Z.; Harrison, F.; Chow, M. S. S. *J Clin Pharmacol* **2002**, *42*, 605.
72. Andersen, Ø.; Markham, K. *Flavonoids: chemistry, biochemistry and applications.* **2006**.
73. Bjorøy, Ø.; Rayyan, S.; Fossen, T.; Andersen, Ø. M. *J. Agric. Food. Chem.* **2009**, *57*, 6668.
74. El Nour, M. E.; Ali, A. M.; Saeed, B. *Int J Curr Microbiol Appl Sci* **2015**, *4*, 147.
75. Bjorøy, Ø.; Rayyan, S.; Fossen, T.; Kalberg, K.; Andersen, Ø. M. *Phytochemistry* **2009**, *70*, 278.
76. Stalikas, C. D. *J. Sep. Sci.* **2007**, *30*, 3268.
77. Davies, K. *Plant Pigments and their Manipulation*, 2004; Vol. 14.
78. Hurst, W. J. *Methods of analysis for functional foods and nutraceuticals*; CRC Press, 2008.
79. Tesso, H., 2005.
80. Jordheim, M., 2007.
81. Andersen, O. M.; Markham, K. R. *Flavonoids: chemistry, biochemistry and applications*; CRC press, 2005.
82. Lenny, S.; Barus, T.; Marpaung, L.; Nasution, M. P. *Malaysian J. Anal. Sci.* **2013**, *17*, 255.
83. Elphimoff-Felkin, I.; Sarda, P. *Organic Syntheses* **1977**, 101.
84. Parada, J.; ATRIA, A.; Wiese, G.; Rivas, E.; Corsini, G. *J Chil Chem Soc* **2014**, *59*, 2636.
85. Ali, H. A.; Darawsheh, M. D.; Rappocciolo, E. *Polyhedron* **2013**, *61*, 235.
86. Ali, H. A. *Polyhedron* **2016**, *107*, 97.
87. Fossen, T.; Andersen, Ø. M. *Spectroscopic techniques applied to flavonoids*, 2006.

7. Appendices

Appendix A: 1D and 2D NMR spectra for compound 2.

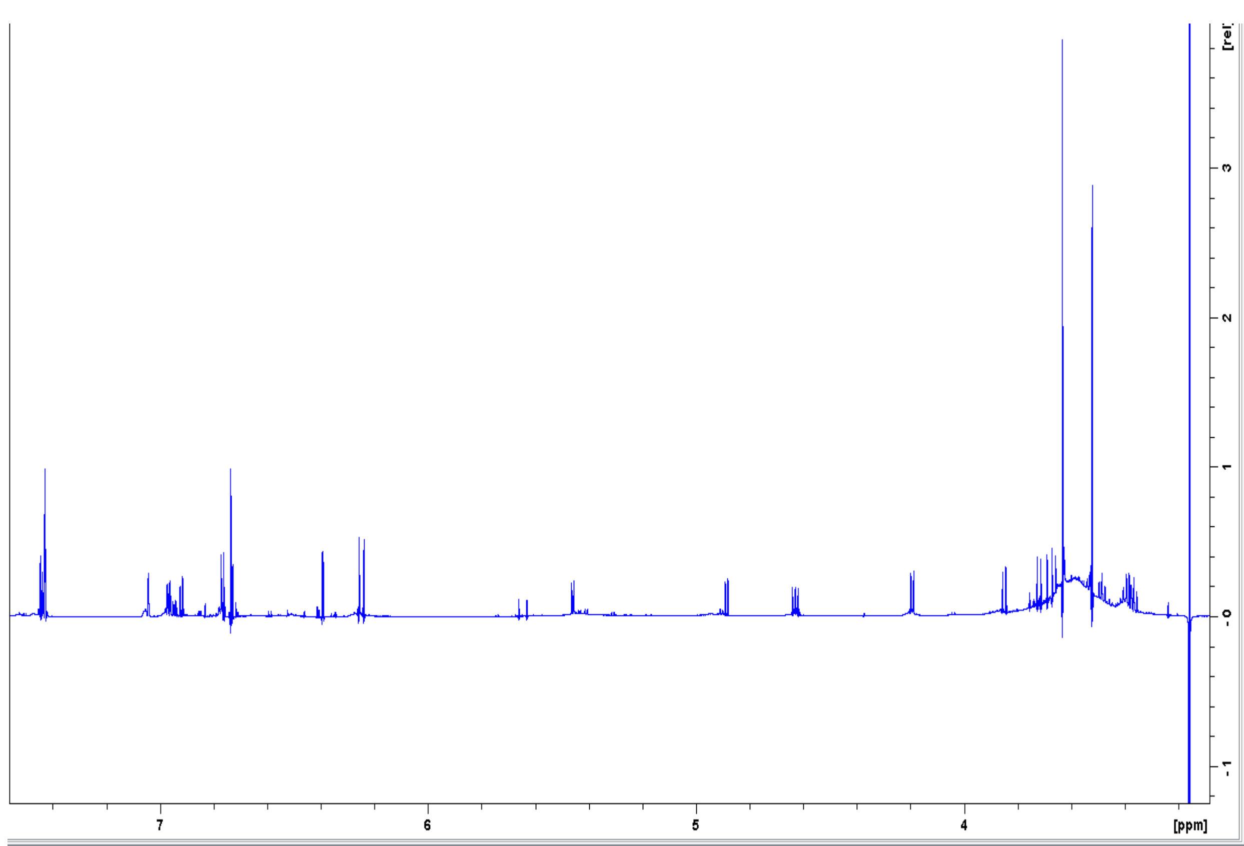
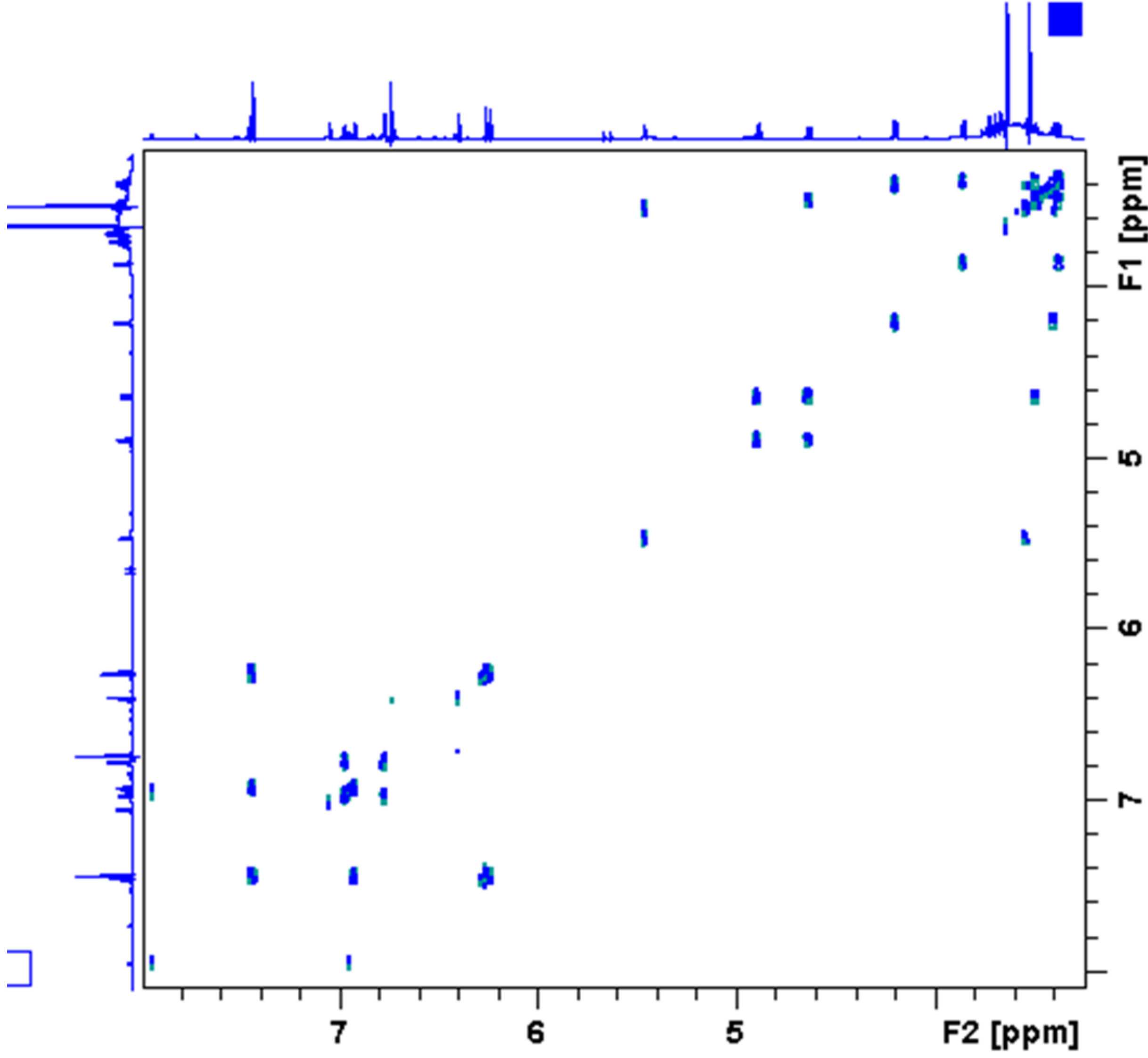


Figure (1): 1D ^{1}H NMR spectrum of luteolin 7-*O*-β-glucuronopyranosyl-2''-*O*-(2'''-*O*-(caffeoyl)-β-glucuronopyranoside (**2**) isolated from catmint (*Nepeta curviflora*).



Figure(2): 2D DQF-COSY spectrum of luteolin 7-*O*-β-glucuronopyranosyl-2''-*O*-(2'''-*O*-(caffeoyl)-β-glucuronopyranoside (**2**) isolated from catmint (*Nepeta curviflora*).

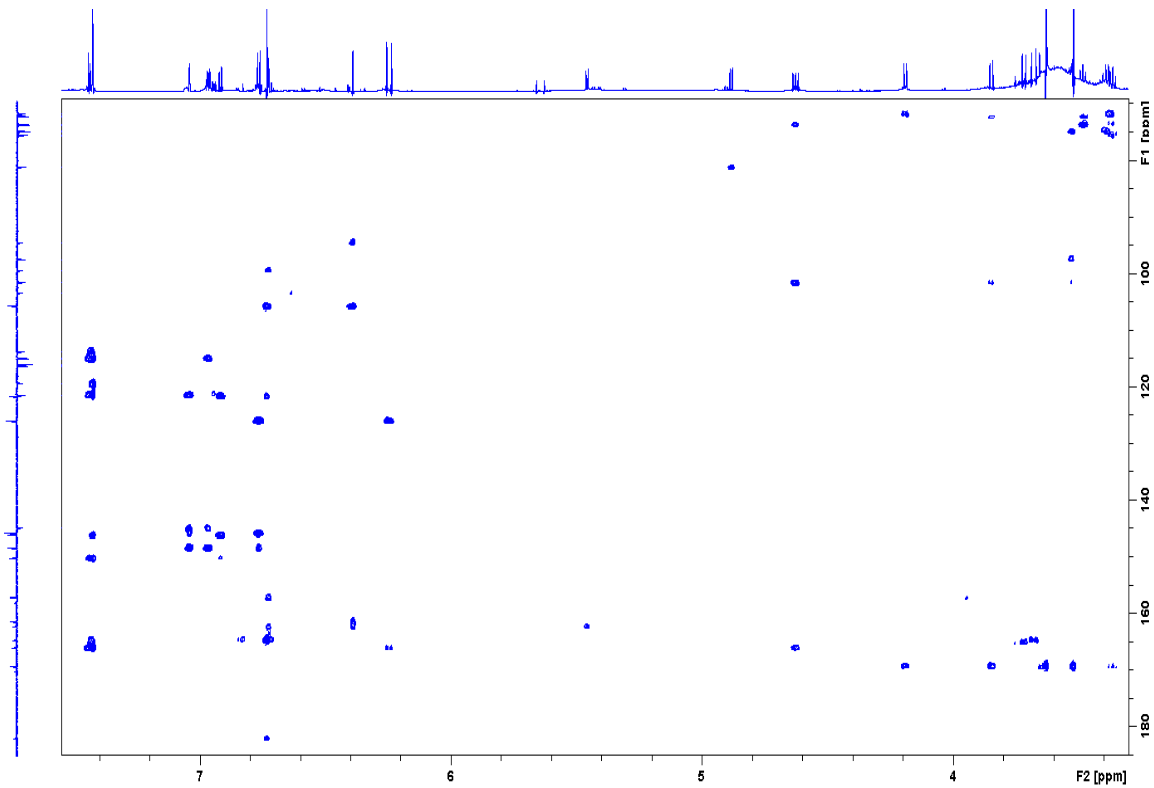


Figure (3): 2D gs-HMBC spectrum of luteolin 7-*O*-β-glucuronopyranosyl-2''-*O*-(2'''-*O*-(caffeoyl)-β-glucuronopyranoside (**2**) isolated from catmint (*Nepeta curviflora*).

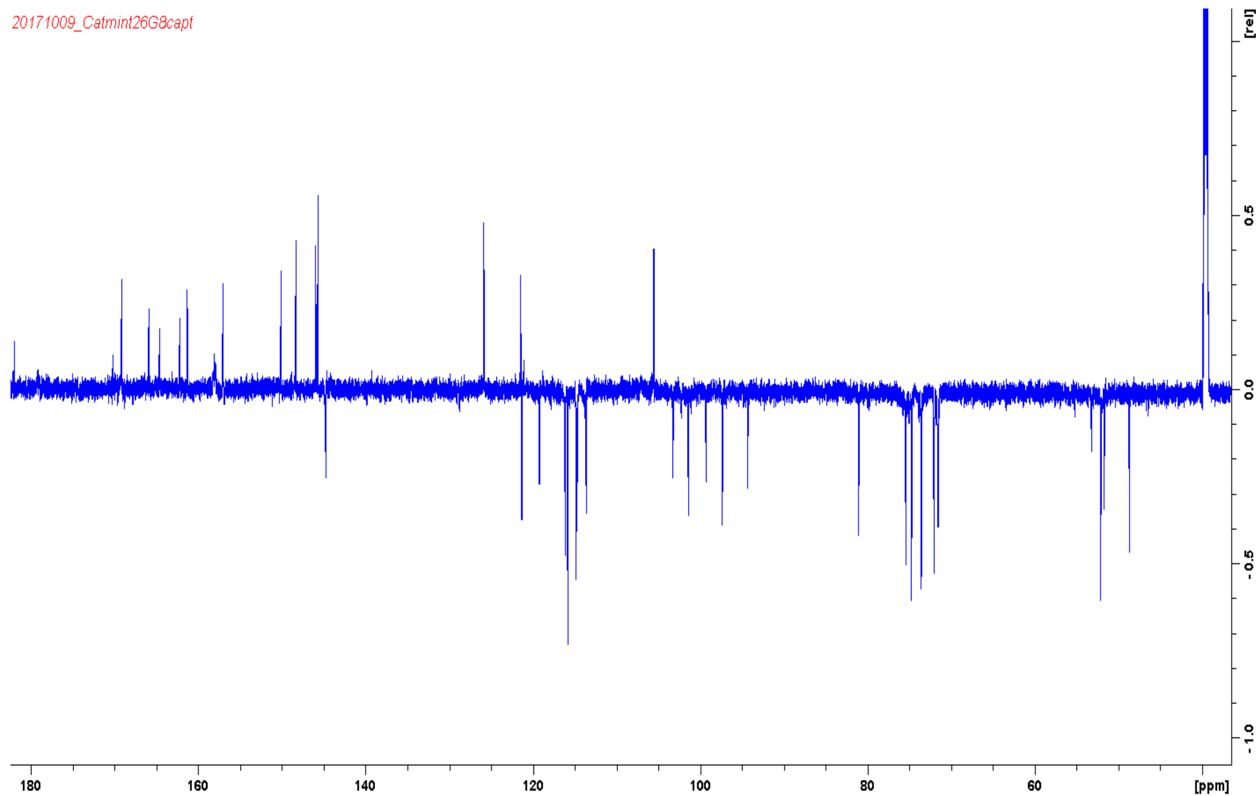


Figure (4): 1D ^{13}C CAPT spectrum of luteolin 7-*O*-β-glucuronopyranosyl-2’’-*O*-(2’’’-*O*-(caffeoyl)-β-glucuronopyranoside (**2**) isolated from catmint (*Nepeta curviflora*).

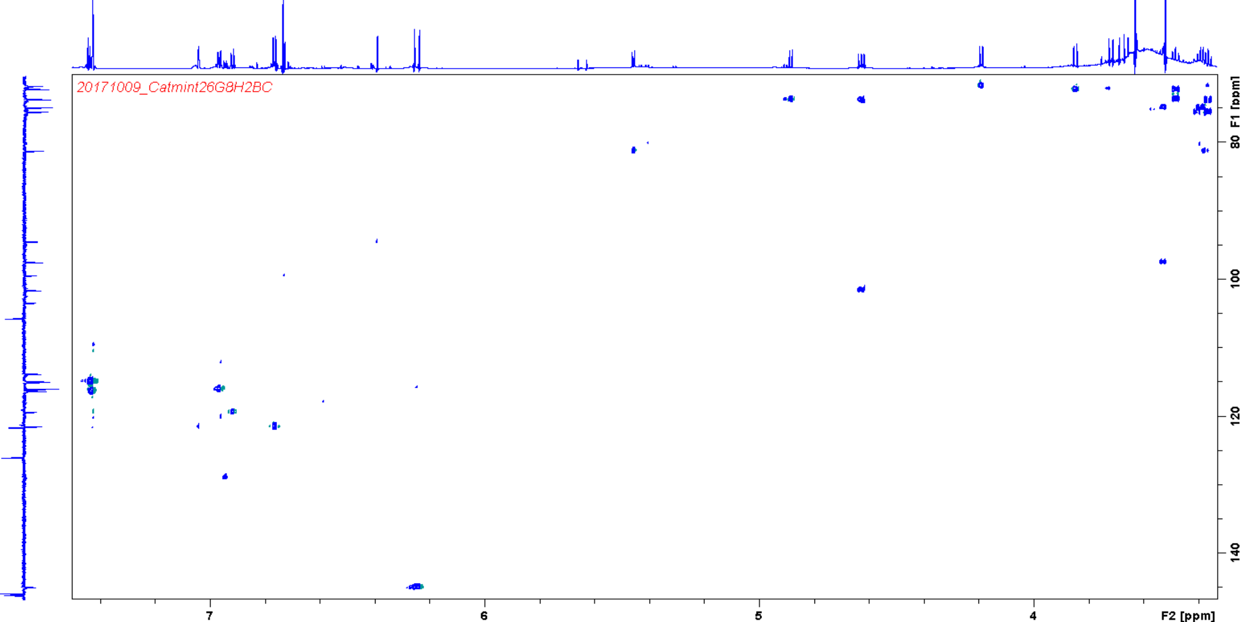


Figure (3): 2D gs-H2BC spectrum of luteolin 7-*O*-β-glucuronopyranosyl-2’’-*O*-(2’’’-*O*-(caffeoyl)-β-glucuronopyranoside (**2**) isolated from catmint (*Nepeta curviflora*).

Appendix B: 1D and 2D NMR spectra for compound 3.

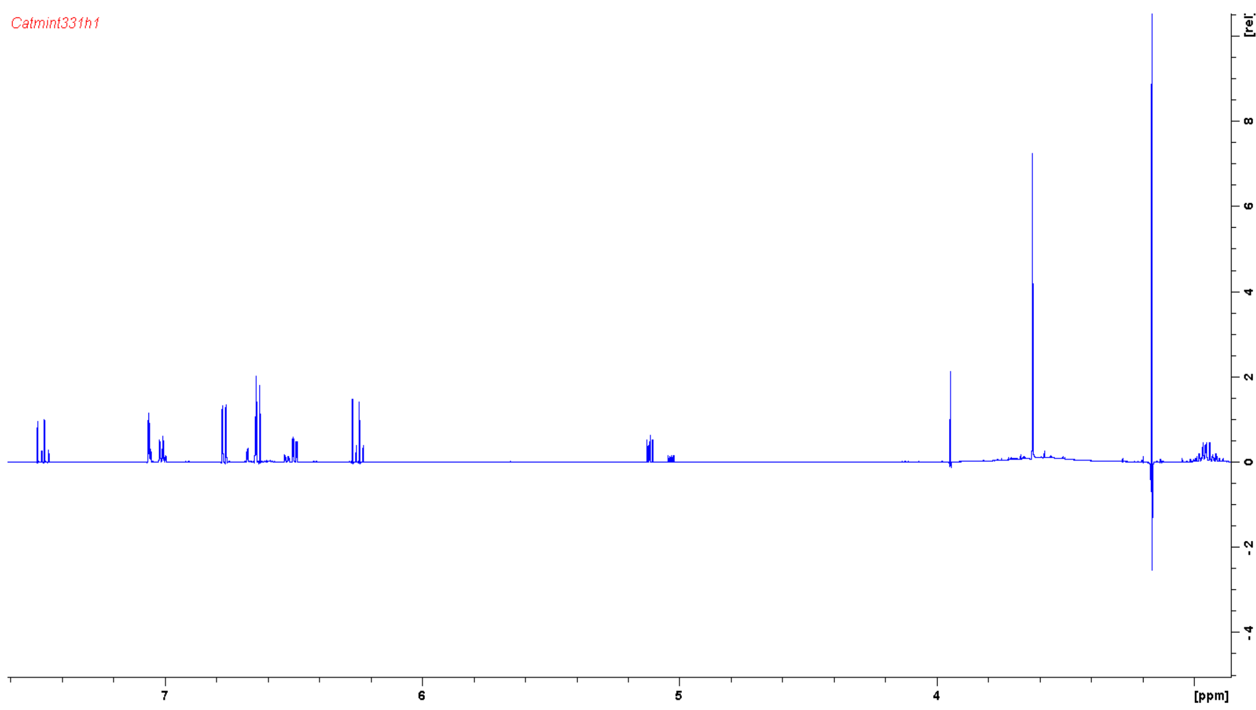


Figure (1): 1D ^{1}H NMR spectrum of ester derivatives of phenolic compounds of rosmarinic acid (**3**) isolated from catmint (*Nepeta curviflora*).

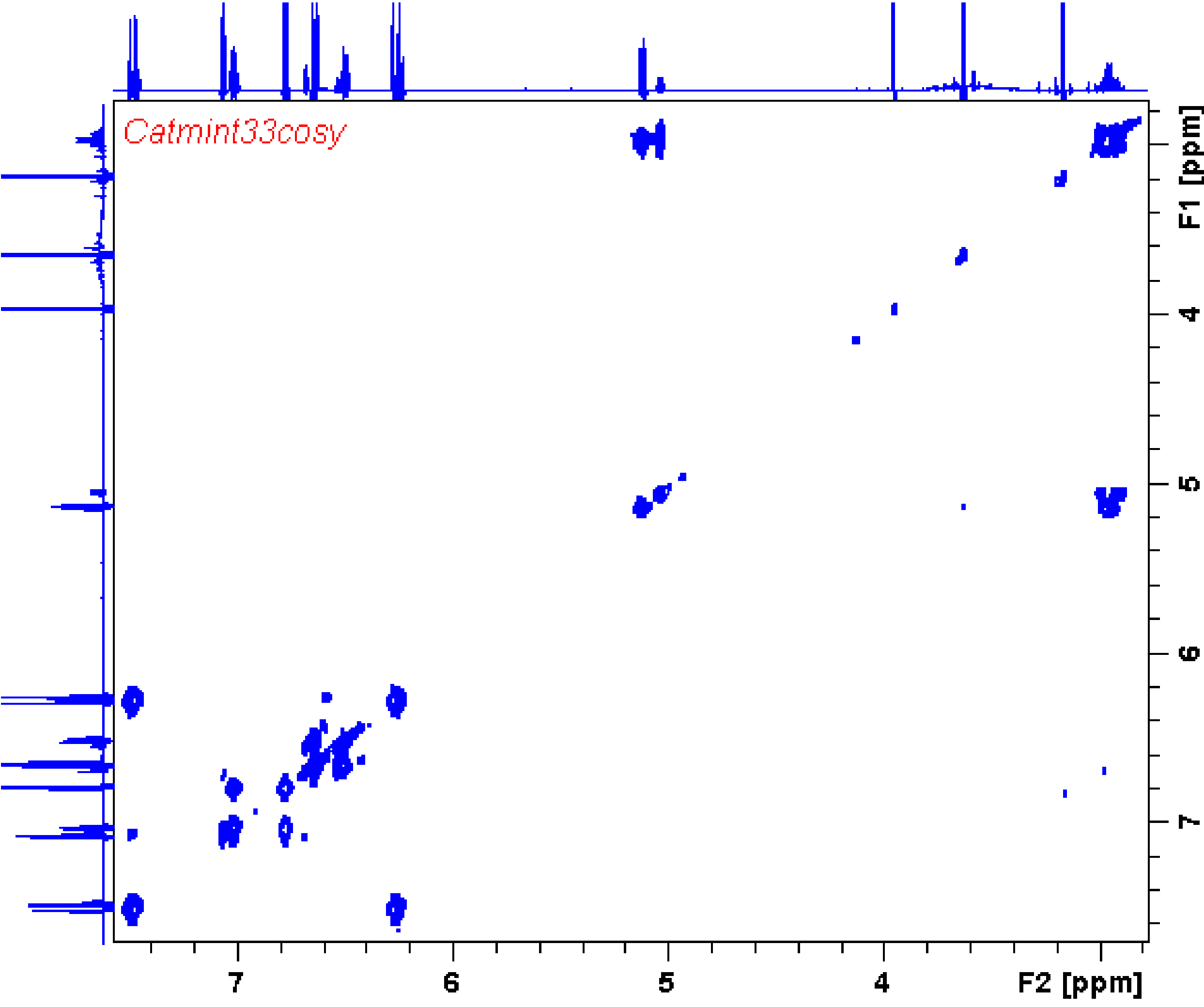


Figure (2): 2D DQF-COSY spectrum of ester derivatives of phenolic compounds of rosmarinic acid (**3**) isolated from catmint (*Nepeta curviflora*).

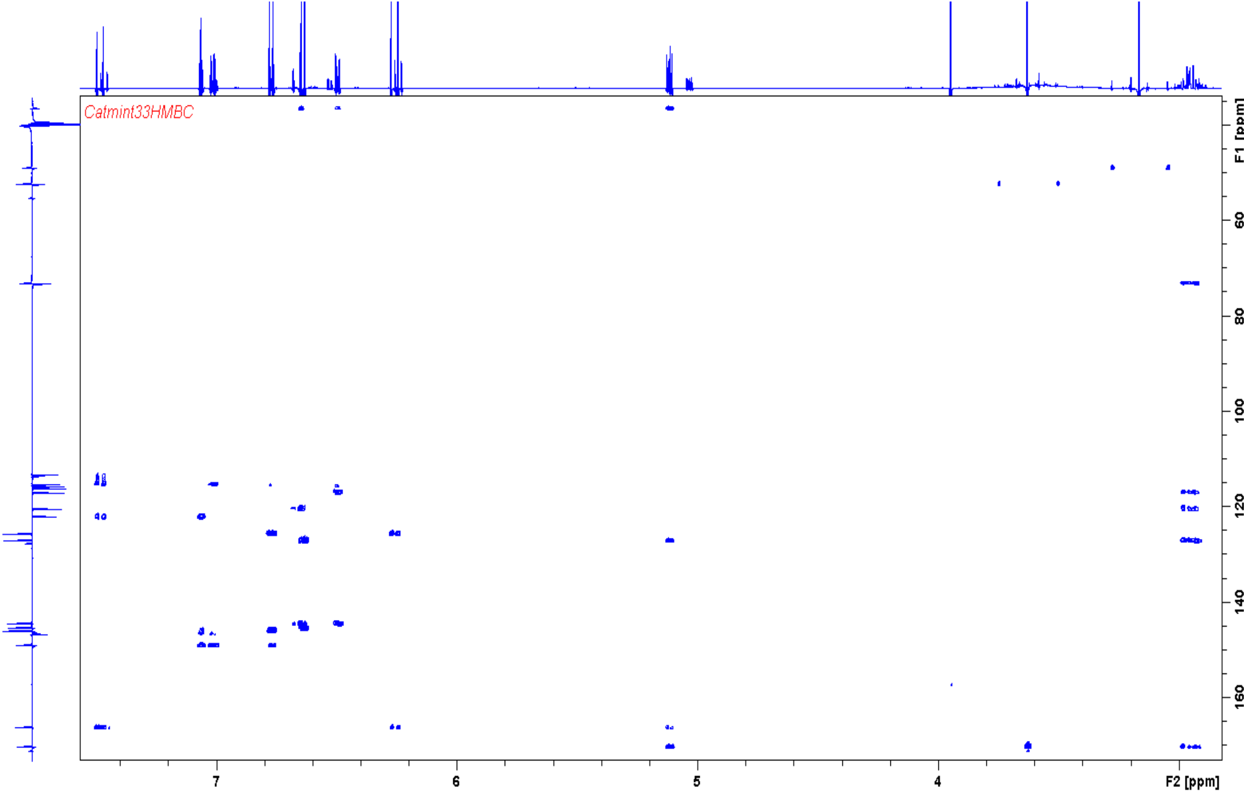


Figure (3): 2D gs-HMBC spectrum of ester derivatives of phenolic compounds of rosmarinic acid (**3**) isolated from catmint (*Nepeta curviflora*).

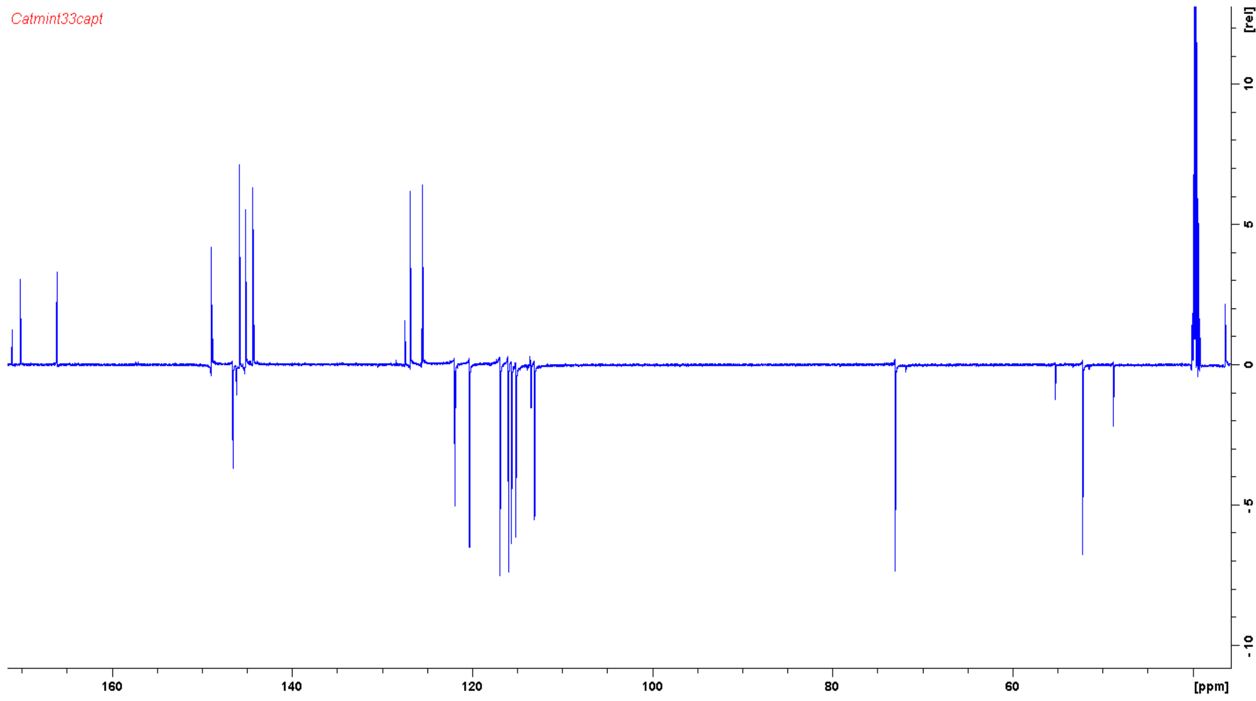


Figure (4): 1D ^{13}C CAPT spectrum of ester derivatives of phenolic compounds of rosmarinic acid (**3**) isolated from catmint (*Nepeta curviflora*).

Appendix C: 1D and 2D NMR spectra for compound 4.

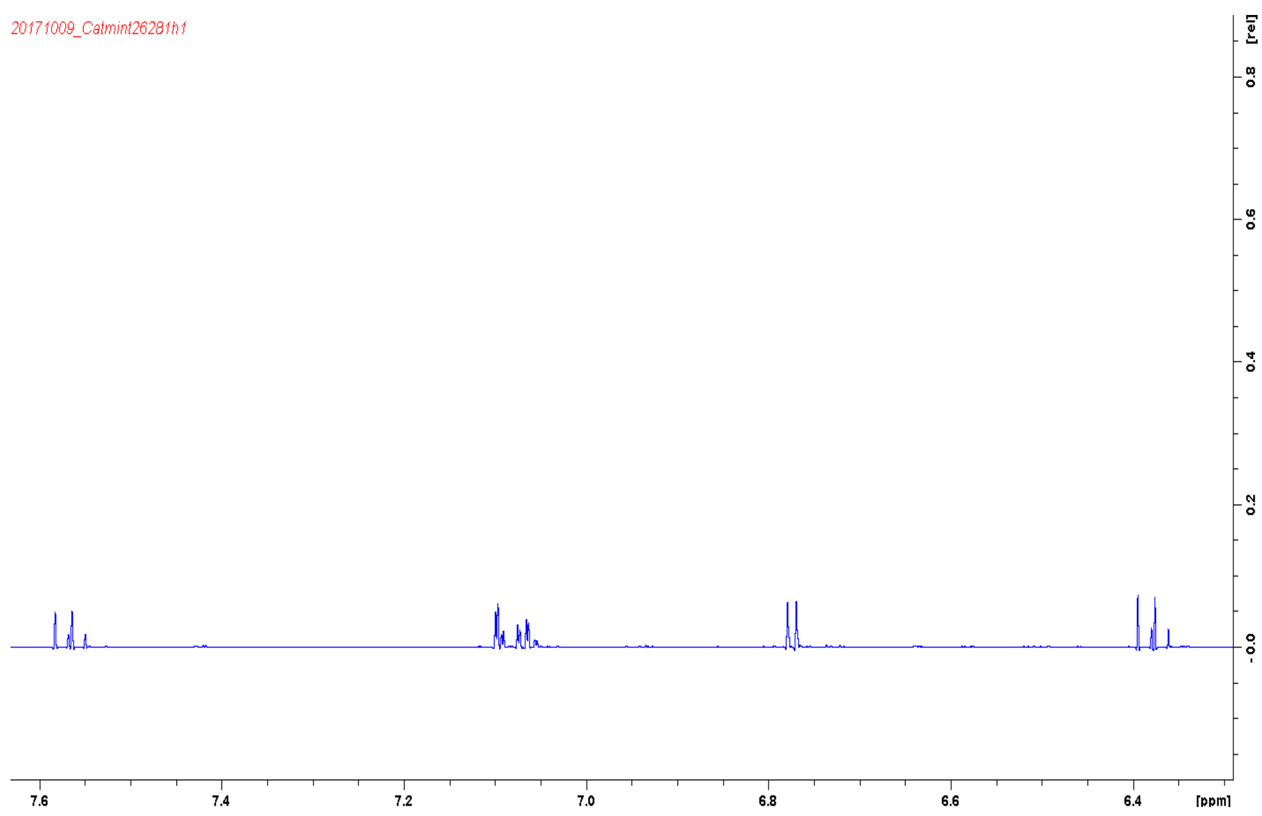


Figure (1): 1D ^{1}H NMR spectrum of ester derivatives of phenolic compounds of caffeic acid (**4**) isolated from catmint (*Nepeta curviflora*).

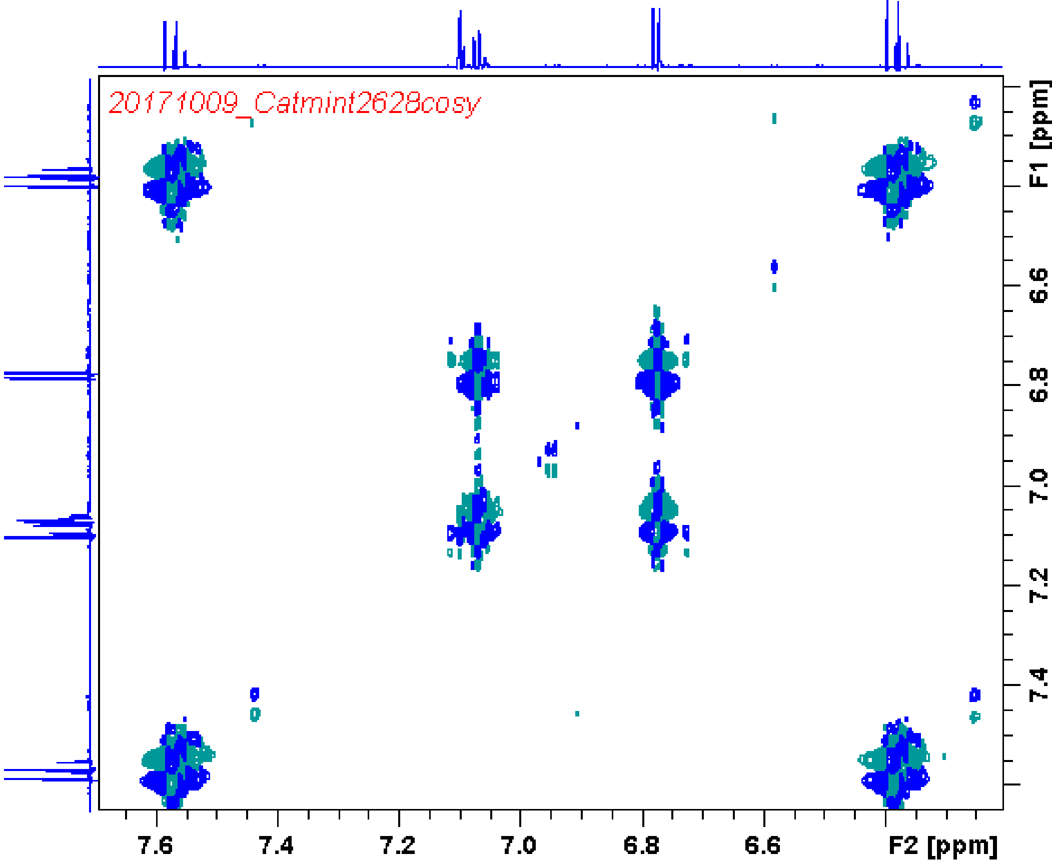


Figure (2): 2D DQF-COSY spectrum of ester derivatives of phenolic compounds of caffeic acid (**4**) isolated from catmint (*Nepeta curviflora*).

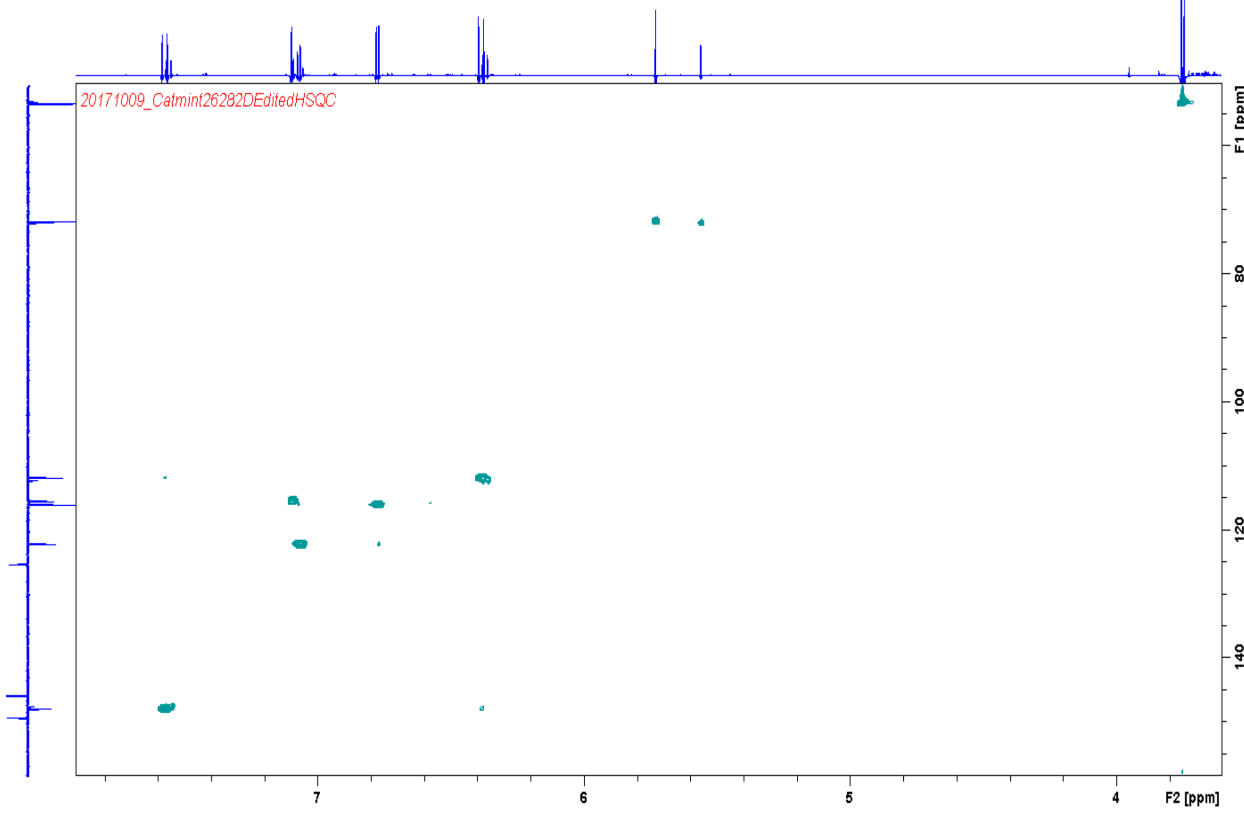


Figure (3): 2D gs-HSQC spectrum of ester derivatives of phenolic compounds of caffeic acid (**4**) isolated from catmint (*Nepeta curviflora*).

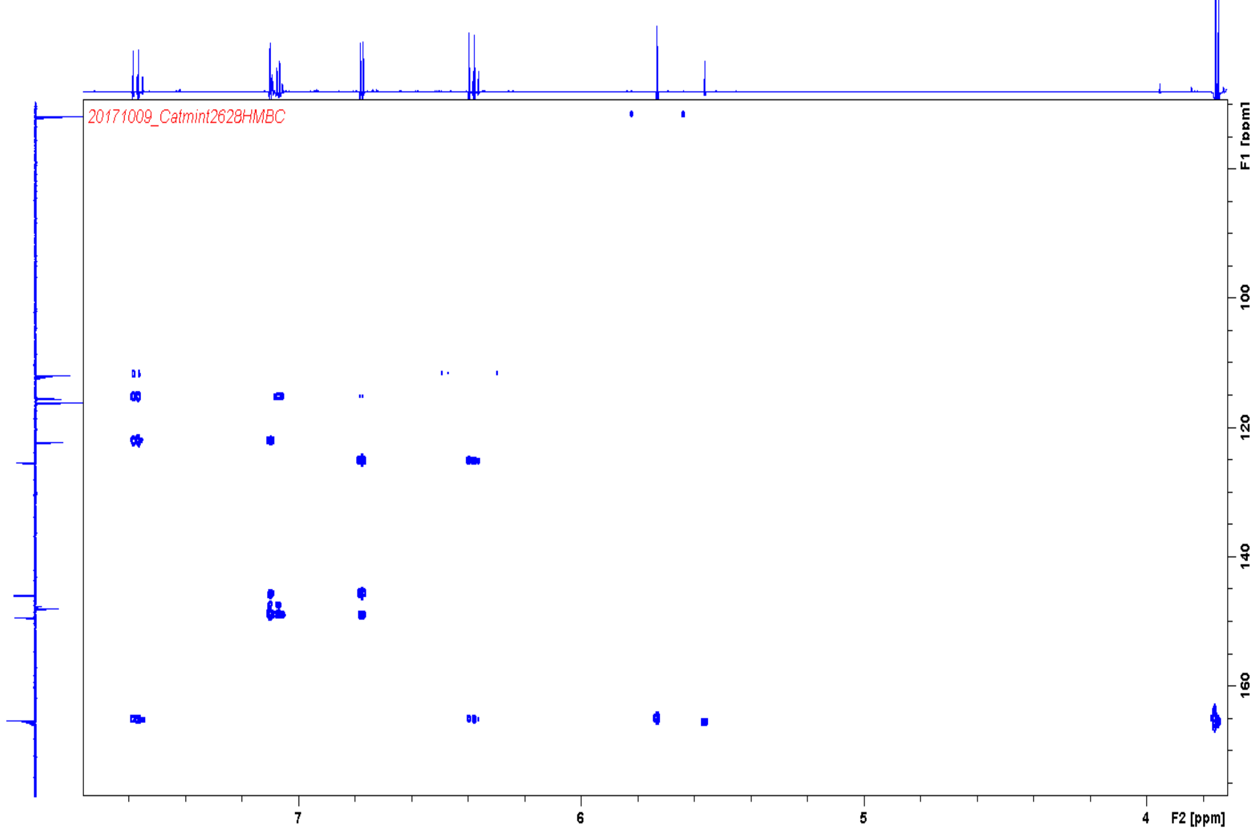


Figure (4): 2D gs-HMBC spectrum of ester derivatives of phenolic compounds of caffeic acid (**4**) isolated from catmint (*Nepeta curviflora*).

Appendix D: Mass spectra for compounds.

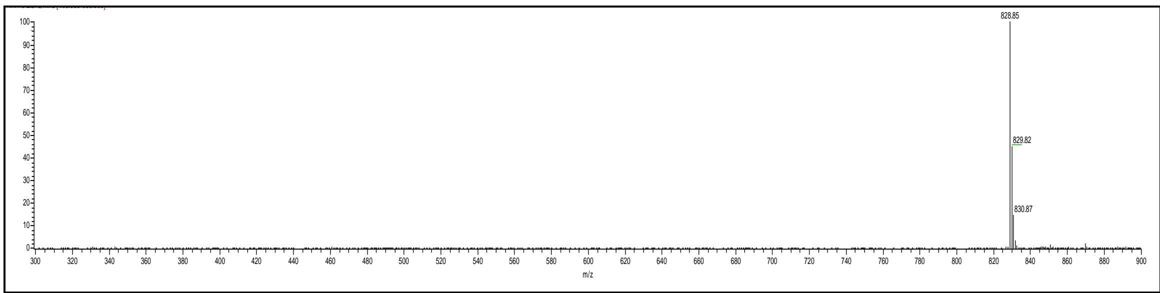


Figure (1): Mass spectrum for compound **2**.

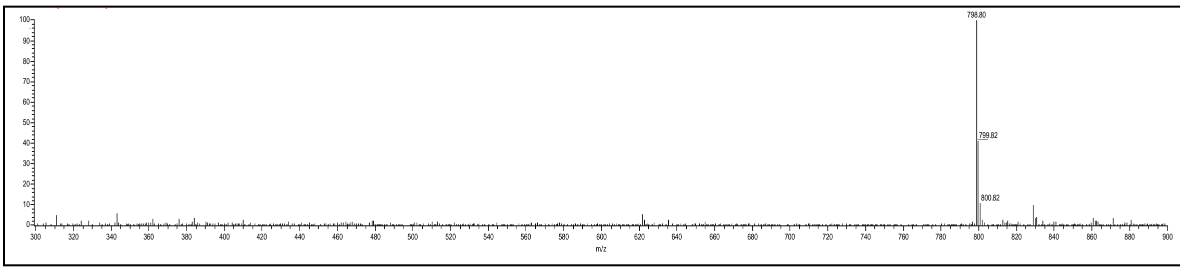


Figure (2): Mass spectrum for compounds **5** or **6**.

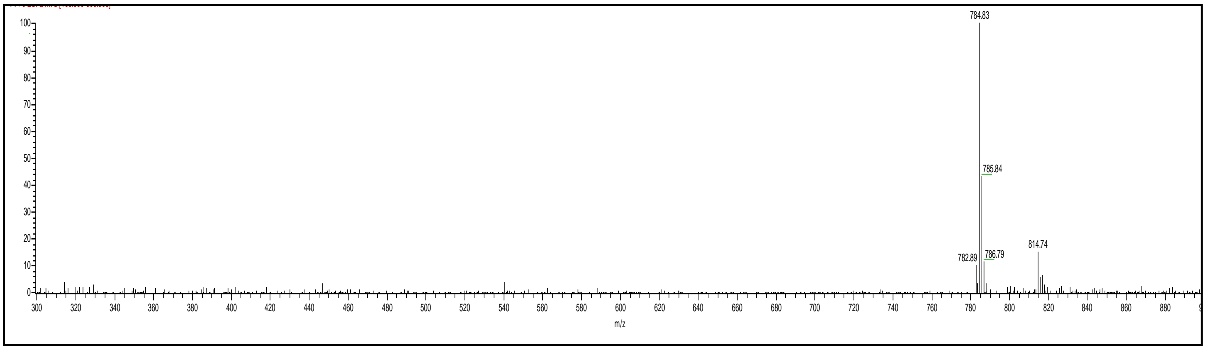


Figure (3): Mass spectrum for compound **7**.

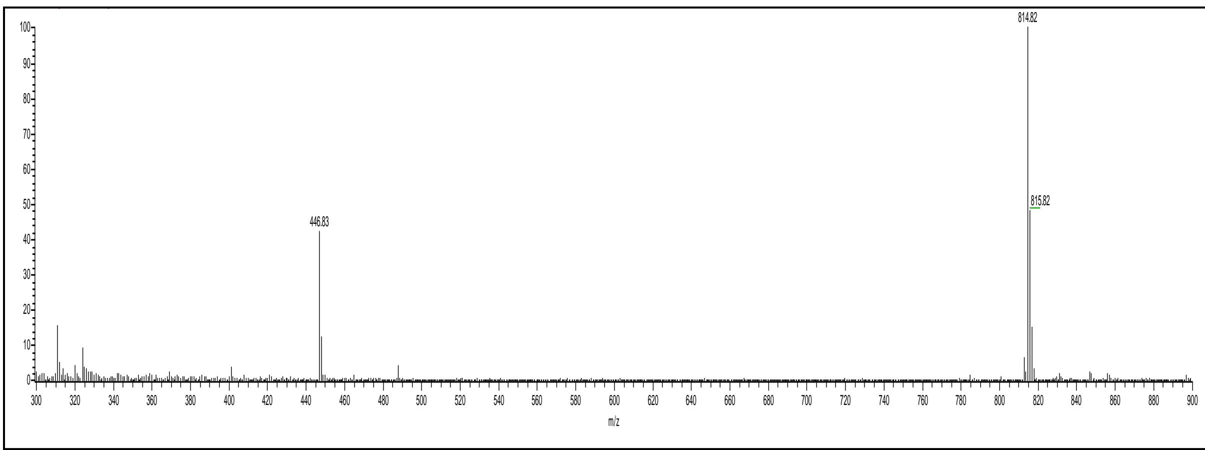


Figure (4): Mass spectrum for compounds **8** or **9.**

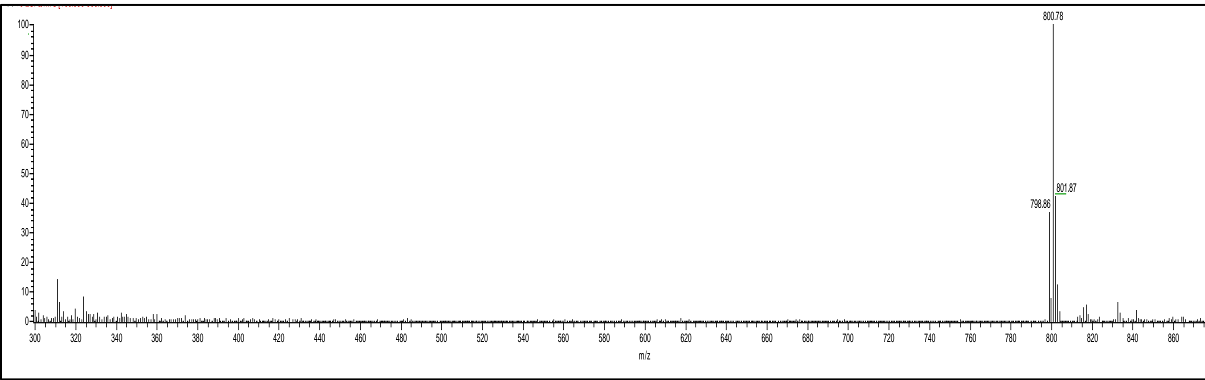


Figure (5): Mass spectrum for compound **10.**