**EGE UNIVERSITY GRADUATE SCHOOL OF NATURAL AND APPLIED SCIENCES**
**(M. Sc. THESIS)**

# ISOLATION PURIFICATION AND CHARACTERIZATION OF SECONDARY METABOLITES OF *Moenchia mantica* subsp. *caerulea*

**Yasin AKSOY**

**Supervisor: Assoc. Prof. Tamer KARAYILDIRIM**

**Department of Chemistry**
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| **Jüri Başkanı** | : Doç. Dr. Tamer KARAYILDIRIM | |
| **Raportör Üye** | : Prof. Dr. Özgen ALANKUŞ | |
| **Üye** | : Doç. Dr. Kadir AY | |

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E.Ü. Lisansüstü Eğitim ve Öğretim Yönetmeliğinin ilgili hükümleri uyarınca Yüksek Lisans Tezi olarak sunduğum "Isolation Purification and Characterization of Secondary Metabolites of *Moenchia mantica* subsp. *caerulea* (*Moenchia mantica* subsp. *caerulea*'nın Sekonder Metabolitlerinin İzolasyonu, Saflandırılması ve Karakterizasyonu)" başlıklı bu tezin kendi çalışmam olduğunu, sunduğum tüm sonuç, doküman, bilgi ve belgeleri bizzat ve bu tez çalışması kapsamında elde ettiğimi, bu tez çalışmasıyla elde edilmeyen bütün bilgi ve yorumlara atıf yaptığımı ve bunları kaynaklar listesinde usulüne uygun olarak verdiğimi, tez çalışması ve yazımı sırasında patent ve telif haklarını ihlal edici bir davranışımın olmadığını, bu tezin herhangi bir bölümünü bu üniversite veya diğer bir üniversitede başka bir tez çalışması içinde sunmadığımı, bu tezin planlanmasından yazımına kadar bütün safhalarda bilimsel etik kurallarına uygun olarak davrandığımı ve aksinin ortaya çıkması durumunda her türlü yasal sonucu kabul edeceğimi beyan ederim.

19 / 07 /2017

Yasin Aksoy

# ÖZET

## *Moenchia mantica* subsp. *caerulea*'NIN SEKONDER METABOLİTLERİNİN İZOLASYONU, SAFLANDIRILMASI VE KARAKTERİZASYONU

Yasin AKSOY

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Bu çalışmada ***Moenchia mantica* subsp. *caerulea*** türünden 15 bileşik izole edilmiş, izole edilen bileşiklerden 2 tanesinin yapısı spektral teknikler kullanılarak 5,7,4' -trihidroksiflavon 6-C-$\beta$-glukopiranozit ve izoskutellarein 6-C-$\beta$-glukopiranozit olarak belirlenmiştir. Kalan bileşiklerin yapı tayin çalışmaları devam etmektedir.

**Anahtar Kelimeler:** Caryophyllaceae, *Moenchia mantica* subsp. *caerulea*, flavanoid

## ABSTRACT

### ISOLATION PURIFICATION AND CHARACTERIZATION OF SECONDARY METABOLITES OF *Moenchia mantica* subsp. *caerulea*

AKSOY, Yasin

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In this thesis, 15 compounds were isolated from *Moenchia mantica* subsp. *caerulea* species and structures of the 2 of the isolated compounds determined as 5,7,4' -trihydroxyflavone 6-C-$\beta$-glucopyranoside and isoscutellarein 6-C-$\beta$-glucopyranoside by using spectroscopic techniques. Structure determination process of remaining compounds still continues.


**Keywords:** Caryophyllaceae, *Moenchia mantica* subsp. *caerulea*, flavanoids

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# 1. INTRODUCTION

## 1.1 Natural Products

Transforming and interconverting a large number of organic compounds is necessary for all organisms in order to make that compounds live, grow and multiply. They use energy in the form of ATP and construct their own tissues by the virtue of a supply of building blocks. To that end, a network of enzyme mediated and carefully regulated chemical reactions is combinated, as a whole termed as intermediary metabolism and the pathways applied are metabolic pathways.

Carbohydrates, proteins, fats, and nucleic acids are some of the significantly important molecules of life. These are polymeric materials with the exception of fats. Amino acids are the building block of proteins and nucleotides compose nucleic acids while sugar units are making up the carbohydrates. Organisms synthesize and transform chemicals and they vary considerably in this context. For the sake of example, plants synthesize compounds *via* photosynthesis from inorganic materials found in the environment in an effective manner. On the other hand, animals and microorganisms supply their raw materials in their diet, e.g. by consuming plants. From this point of view, majority of the metabolic pathways are responsible for degrading materials taken in as food while the others have need for synthesizing specialized molecules from the basic compounds so obtained. To the contrary the immensely varied characteristics of living organisms, it is observed that the pathways for generally modifying and synthesizing carbohydrates, proteins, fats, and nucleic acids are foundationally the same in all organisms, excepting minor revisions. All of these activities are indication of main unity of all living matter and described as primary metabolism, with the compounds involved in the pathways being termed primary metabolites.

Primary metabolic pathways synthesize, degrade, and generally interconvert compounds widely encountered in all organisms. In opposition to these primary metabolic pathways, there is another area of metabolism called secondary metabolites and these metabolites appertains with compounds which have a lot

fewer prevalance in nature. Secondary metabolites are an indication of individuality of species and are found in only specific organisms or groups of organisms.

Producing secondary metabolites in all circumstances is not required and the function of these compounds and their benefit to the organism is not yet known according to the great majority of the cases. Some are produced for comprehensible reasons for instance; as toxic materials providing defence against predators, as volatile attractants towards the same or other species, or as colouring agents to attract or warn other species, but it is necessary to take into consideration of the fact that all of them play a vital role in the welfare of the producer. Greater part of pharmacologically active natural products is mostly procured by this area of secondary metabolism.

For this reason it is apparent that the human diet could be unpalatable and significiently hazardous if all plants, animals, and fungi produced the same range of compounds.

The secondary metabolites of plants have no significant responsibility in the primary metabolism of the organism and they are compounds with disposition of restricted taxonomic distribution.

Alkaloids, anthraquinones, coumarins, essential oils (lower terpenoids and phenylpropanoids), flavonoids, steroids, and terpenoids (cardenolides, diterpenoids, iridoids, monoterpenoids, sesquiterpenoids [including sesquiterpene lactones], and triterpenoids are the most common plant secondary metabolites. (Cannell, 1998).

Plants, bacteria, fungi and some marine organisms such as sponges, tunicates, corals and snails are well recognized sources of secondary metabolites. Many of the secondary metabolites have considerably beneficial usage areas such as antibacterial or antifungal agents, anticancer drugs, cholesterol-lowering agents, immunosuppressants, antiparasitic agents, herbicides, diagnostics, and tools for research. It is seen that these functions of secondary metabolites make a

significant contribution in treatment or prevention of a multitude of biological disorders. Moreover, many of these disorders did not have any cure until these products were discovered.

Apart from their known activities and employment in combating disease, secondary metabolites also generate solutions to other diseases, some of which lack effective solutions. Additionally, most of the antibiotics, bacterial pigments and plant terpenoids are also widely used in medical applications for the reason that they have anti-HIV, antitumor, anti-ageing, immunosuppressant, antiprotozoal and anthelmintic properties. In an attempt to provide a lower fatality rate and to come up with new treatment modalities, emerging functions of known secondary metabolites are being improved and new secondary metabolite sources such as microbes, plants and higher animals are being investigated. By taking into consideration of effective functions of known secondary metabolites in the treatment of diseases, it is hoped that further importance will be attached to carry out these beneficial compounds in treatment of other forms of human disease (Vaishnav and Demain, 2010).

### 1.2. Phenolics

Secondary metabolites are aggregated in plants at different growing phases, dissociate in a widespread manner and vary chemically. These secondary metabolites are implicated in by phenolic compounds that also known as polyphenols (Macheix et al., 1990). Phenolics occurs in plantae extensively, have more than 8,000 structures presently recognized and they are seen both as simple molecules such as phenolic acids and as highly polymerized substances such as tannins. All of these functions make phenolics most abundant secondary metabolites of plants (Dai and Mumper, 2010).

Some of the compounds have an aromatic ring with one or more hydroxyl functional groups in their chemical structure. Phenolics comprise these compounds and they are separated into two groups as flavonoids and non-flavonoids. Flavonoids have the C6-C3-C6 skeleton, they are the most productive groups among the phenolic compounds and their central heterocyclic ring is

divided into different categories and these categories are dissociated from each other in terms of oxidation (Ribereau-Gayon, 1972). They are flavonols, flavones, isoflavones, flavonoids, anthocyanins and flavanols (catechins and tannins).

Tannins are examined in three categories: Hydrolysable tannins, non-hydrolysable tannins and condensed tannins (proanthocyanidins). Among the functions of the hydrolysable tannins, there is being the formation of esters of acids and sugars or their derivatives and so; they can give a sugar and a phenolic acid moiety on hydrolysis (Ribereau-Gayon, 1972). Sugar usually present as glucose or polysaccharide. At the same time, we can see the phenolic acids as gallic acid in the case of gallotannins or as ellagic acid in the case of ellagitannins (Santos-Buelga and Scalbert, 2000; Ribereau-Gayon, 1972).

Proanthocyanidins form coloured anthocyanidins and they are oligomers and polymers of flavan-3-ol monomeric units but this situation occurs only when they are exposed to acidic hydrolysis (Santos-Buelga and Scalbert, 2000; Ribereau-Gayon, 1972).

Non-flavonoids are: Simple phenols, phenolic acids, coumarins, xanthones, chalcones, stilbenes, lignins and lignans. Morover; phenolic acids are divided into two types of acid derivatives. They are benzoic acid derivatives and cinnamic acid derivatives. The difference between them is their skeletal structure. Benzoic acid derivatives are based on a C6-C1 skeleton, and cinnamic acid derivatives are based on a C6-C3 skeleton (Ribereau-Gayon, 1972).

The coumarins which are from non-flavonoids and they are phenolic acid derivatives. They have an oxygen heterocycle and benzene ring (Ribereau-Gayon, 1972). Xanthones have a C6-C1-C6 basic structure and stilbenes have a C6-C2-C6 skeleton. C6-C2-C6 skeleton of stilbenes contains various hydroxylation patterns (Andres-Lacueva et al., 2010). Chalcones have a C6-C3-C6 basic structure without a heterocyclic C-ring (Andres-Lacueva et al., 2010), lignins are polymers of C6-C3 units (Stafford, 1998) and lastly lignans are polymers of two phenylpropane units (Manach et al., 2004; Willför et al., 2006). Figure1.1 and 1. 2 show that how the general structures of each of these categories are. Each of the

phenolic groups in this category can be further subdivided into classes and the things that make this subdivision possible are hydroxylation patterns and stereochemistry.

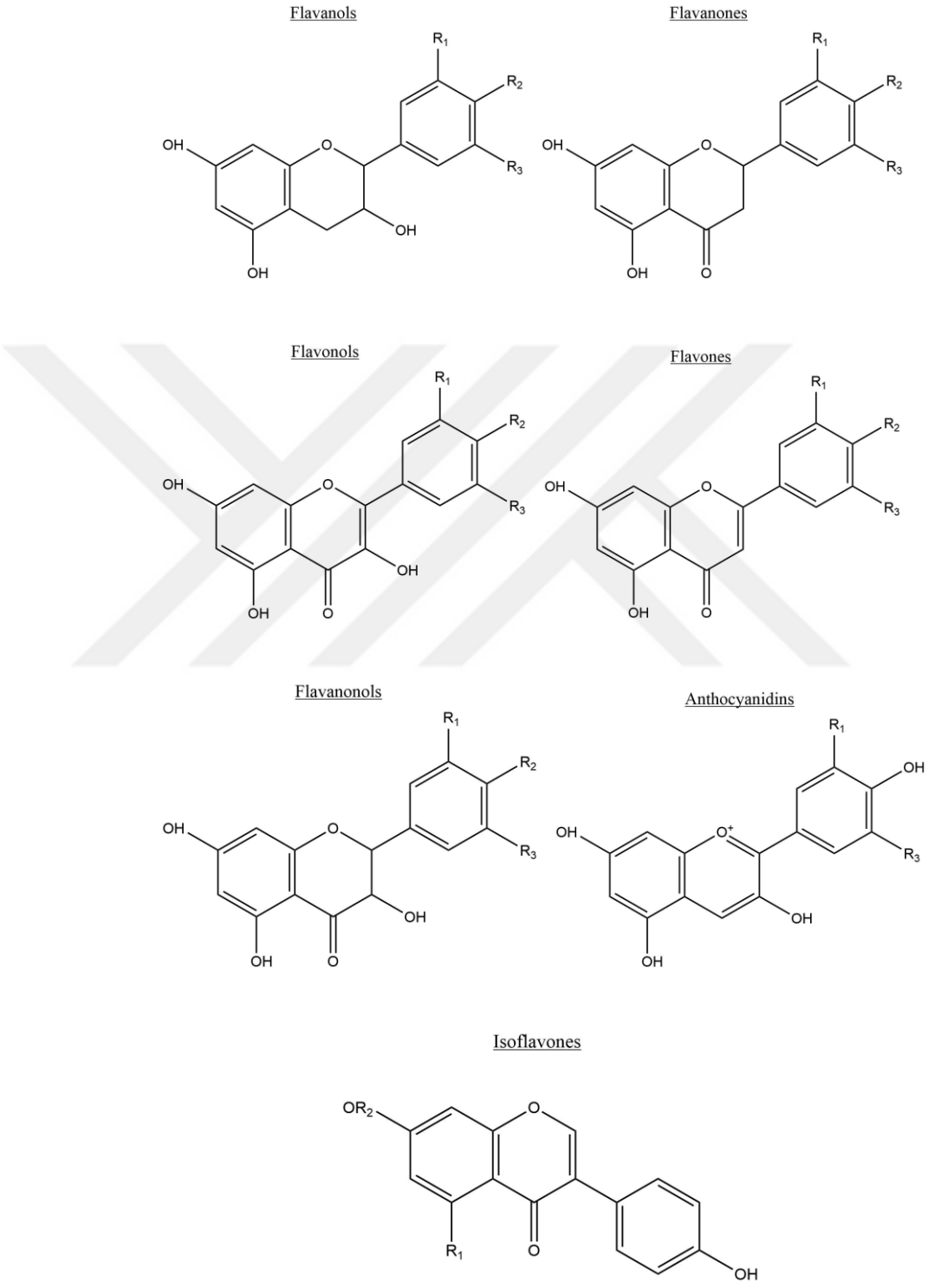



Figure 1. 1. Structure of flavonoid phenolic natural products

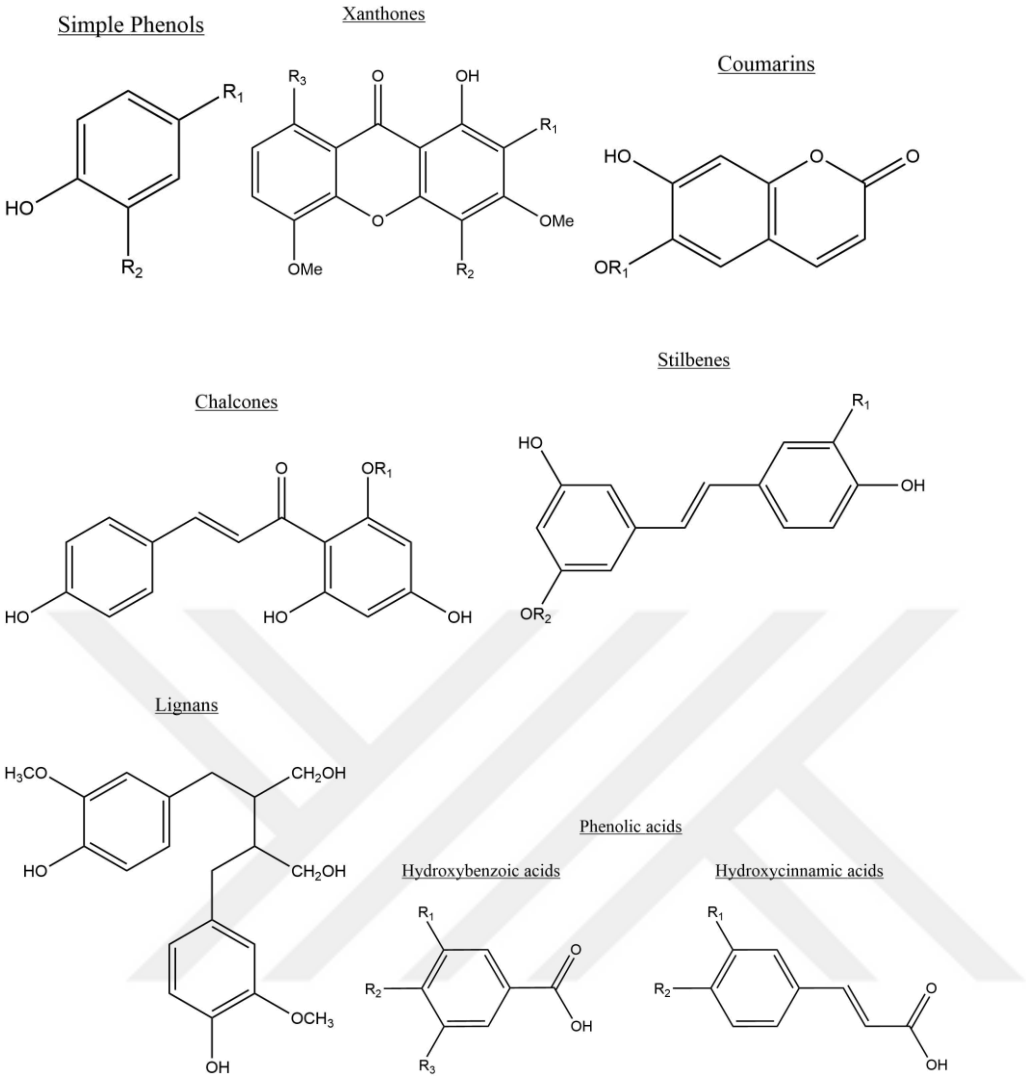



Figure 1. 2. Structure of non-flavonoid phenolic natural products

According to extensive researches, phenolic compounds are considerably active and fertile in terms of biodiversity. Inhibitation atherosclerosis through suppression of endothelin-1 (Corder et al., 2006) of red wine procyanidins and exhibition antioxidant activity in vivo (García-Marino et al., 2006) of grape seed procyanidins are good examples of this biological diversity. Another example is about green tea catechins. Their functions are antioxidant, anti-hypertensive, anti-depressant and anti-inflammatory (Huang et al., 1992; Jeong and Kong, 2004). When we examine A cacao pentameric procyanidin, we can see that it prevents the growth of human breast cancer cells (Ramljak et al., 2005). As for that proanthocyanidins, they tolerate the ultraviolet radiation or aggression by pathogens. (Manach et al., 2004; Santos-Buelga and Scalbert, 2000) Functions of

isoflavones are being anti-inflammatory (Kao et al., 2007), estrogenic and anti-proliferation effects (Zhang et al., 2007). And lastly, flavones, flavonols and anthocyanidins attract pollinating insects in flowers (Stalikas, 2007), and lignins and lignans are make plants grow and defense (Willför et al., 2006).

### 1.2.1. Flavonoids

Flavonoids belong to a group of polyphenolic compounds. They have an extensive coverage in plant kingdom. Known varieties of flavonoids are about 6000 but this data is valid for the end of the last century and new types of flenoids continue to be discovered (Harborne and Williams, 2000).

Many of flavonoids have a low toxic degree in mammals and play a big role in the preservation of capillary integrity. Thanks to their these functions, they contribute to the field of medicine (Cesarone et al., 1992). Among their features, there are being anti-hepatotoxic, anti-inflammatory and anti-ulcer activity (Bors et al., 1990; Colerige et al., 1980). Moreover; they have the ability of preventing enzymes such as aldose reductase, cycloxygenase, Ca+2-ATPase, xanthine oxidase, phosphodiesterase and lipoxygenase. They are quite effective in preventing oxidation, and they can remove from harmful and undesirable substances. Many of them are used as antiallergic, antiviral and protector against cardiovascular mortality (Clack et al., 1950; Hertog et al., 1993). It has been observed that they also have the ability of reducing tumor ceasing the growing of cancer cell lines in vitro (Mori et al., 1988).

#### 1.2.1.1. Pharmacological effects of flavonoids

**In vitro antioxidant activity of flavonoids**

Vitamins C and E are highly effective in preventing oxidation but many flavonoids outstrip even these vitamins in this regard (Prior and Cao, 2000). We can exemplify this as follows: Epigallocatechin gallate reduce one-electron at 550 mV under standard conditions. This value is lower than that of glutathione (920 mV) and it is possible to compare to that of α-tocopherol (480 mV) (Frei and Higdon, 2003; Maeta et al., 2007). Free radicals cause some injuries but

flavonoids can prevent this. While preventing these injuries, flavonoids use following mechanisms:

(1) direct scavenging of reactive oxygen species (ROS),

(2) activation of antioxidant enzymes (Nijveldt et al., 2001),

(3) metal chelating activity (Ferrali et al., 1997),

(4) reduction of α-tocopheryl radicals (Hirano et al., 2001; Heim et al., 2002),

(5) inhibition of oxidases (Heim et al., 2002; Cos et al., 1998),

(6) mitigation of oxidative stress caused by nitric oxide (Vanacker et al., 1995),

(7) increase in uric acid levels (Lotito and Frei, 2006),

(8) increase in antioxidant properties of low molecular antioxidants (Yeh et al., 2005).

**Antitumor effects of flavonoids**

Discussions about antitumor activity of flavonoids are still in progress and have not achieved a certainty. Performance of the antioxidant systems are under the required level most of the time. To make up this situation, it is suggested to involve the damage from reactive oxygen species in carcinogenesis (Loft and Poulsen, 1996; Pryor, 1997). In the event that reactive oxygen species damage DNA, mutations occur. Another reason of the mutations is division of cells with unrepaired or miss repaired damage. These mutations can occur in critical genes, such as oncogenes or tumor suppressor genes. Initiation or progression can be the result of this situation. An interference is necessary for signaling and growth of cells and reactive oxygen species can this interfere directly. Reactive oxygen species may demage cells from time to time. These damages can result in

inducing mitosis by invreasing the possibility of exposure of DNA to mutation and leading to mutations.

Flavonoids in the antioxidant category have the abilty of inhibiting carcinogenesis (De Stefani et al., 1999). Some of flavonoids prevent cell proliferation influentially. Fisetin, apigenin, and luteolin are the examples of this type of flavonoids (Fotsis et al., 1997). Flavonoid intake affects the incidence of lung cancer in an inverse proportion and an extensive madical exploration has been performed on this (Knekt et al., 1997). It has been determined that the causative agent of this situation is quercetin. Quercetin accounts for ninety-five percent of the flavonoids taken and this also has been proven by a number of studies. In the direction of experiment on mice, it has been established that the growth of tumor caused by deep melonacite cells are prevented by quercetin and apigenin and the invasive and metastatic efficiency is also depend on these (Caltagirone et al., 2000).

**Antiviral effects of flavonoids**

Another feature of flavonoids is preventing the formation of viruses. This feature has been proposed in a research by Wang et al (Wang et al., 1998). As examples of viruses of interest: Herpes simplex virus, respiratory syncytial virus, parainfluenza virus, and adenovirus. Quercetin is able to prevent the spread of diseases and has the non replicative ability. These have been proved by some researches. Virus replication cycle and how and at what stage flavonoids affect this cycle has been expressed in the previous sections (Kaul et al., 1985). As an example of this we can point that flavonoids have different effects on this feature. Some of them are related to intracellular replication of viruses on the other hand, some flavonoids cease the spreading of viruses. Until now, researches about this have been done in vitro and very little information is available on the antiviral effect of flavonoids in vivo. Flavonoids are present in the form of glycols and aglycons. Glycone form of flavonoids is more inhibitive on rotavirus infectivity (Eun-Ah et al., 2000).

**Anti-inflammatory effects of flavonoids**

Mechanisms of the anti-inflammatory activity of flavonoids: (a) antioxidative and radical scavenging activities, (b) regulation of cellular activities of inflammation-related cells, (c) modulation of the activities of arachidonic acid metabolism enzymes (phospholipase A2, cyclooxygenase lipoxygenase) and nitric oxide synthase, (d) modulation of the production of other proinflammatory molecules, (e) modulation of proinflammatory gene expression.

Many of the flavonoid molecules have anti-inflammatory functions and researchers have done researches and repeated many times. These functions have led to the research of anti-inflammatory activities of flavonols (quercetin, rutin and morin) and flavanones (hesperetin and hesperidin) on animal models which have acute and chronic inflammation (Rotelli et al., 2003). As a result of researches, only the chronic process and adjuvant arthritis were affected by Rutin. Another result shows that in case the xylene causes neurogenic inflammation, the only thing that could be effective is flavanones. Moreover, it has been seen that the subchronic inflammatory process is mostly affected by these flavonoids. Quercetin is the most important compound because of the feature of reducing paw edema induced by carrageenan (Rotelli et al., 2003). Injecting LPS to the mice, it has been proved that inflammatory reaction in the intestine and liver can be made lighter with a diet that includes an isoflavone, daidzin, glycitin, genistein and their glucosides (Paradkar et al., 2004). On the other hand, other flavonoids prevent the mice from adjuvant arthritis. This experimental model of inflammation shows that rutin, quercetin and hesperidin are the most effective ones in the chronic phase (Guardia et al., 2001).

**Anticancer effect of flavonoids**

Flavonoids have several mechanisms of action. Estrogenic/antiestrogenic activity, antiproliferation, induction of cellcycle arrest or apoptosis, prevention of oxidation, induction of detoxification enzymes, regulation of the host immune system, anti-inflammatory activity and changes in cellular signaling are some of these mechanisms (Birt et al., 2001). In cancer researches, the cellular signaling

pathways are used extensively. The reason of this is the ability of these pathways to regulate proliferation, survival and transformation of cells. Cell signaling pathways improve cell diversity and differentiation. Pathways are including the great part of molecular alterations associated with carcinogenesis. And these pathways are also including kinases such as MAPK, and protein kinases (PK). Both MAPK, and protein kinases (PK) have a significant role in inflammatory processes. These kinases and thkeir transcription factors act on cell growth. If this action is silenced or overactivated, cell growth can not be controlled and malignant transformation occurs (Fresco et al., 2006). Some of the flavonoids have the ability to regulate these pathways. In this way, gene expression is regulated and the inhibition of carcinogenesis is contributed by the pathways (Santangelo et al., 2007) . A summary of anti inflammatory mechanisms implicated in specific flavonoid chemoprevention is figured with table 1.

Table 1. 1. Summary of studies demonstrating some of the antiinflammatory mechanisms implicated in specific flavonoid chemoprevention

| Mechanism | Compound | Cancer Model |
|---|---|---|
| Antioxidant activity | Quercetin Genistein | Lung carsinogenesis Neutrophils |
| COX-2 inhibition | Naringin | Colon carsinogenesis |
| | Tricin | Adenoma in $APC^{min}$ mice |
| | Genistein | Human breast canser cells |
| | Apigenin | UVB induced mouse skin tumors |
| Inhibition of PKC | Apigenin | Mouse skin tumors |
| | Luteolin | Skin tumor cell line |
| | Quercetin | Skin tumor cell line |
| Modulation of MAPK | Genistein | Prostate cancer |
| | Apigenin | Prostate cancer cells |
| | Apigenin | Breast carcinoma cells |
| Modulation of NF-KB | Morin | Different tumor cell lines |
| | Genistein | Prostate, breast and pancreatic cancer cells |
| | Apigenin | Prostate cancer |

## 1.3 Natural Products Isolation Techniques

### 1.3.1 Extraction

If the plant is fresh or non dried, enzyme action can reduce the flavonoids. This situation is specially seen in glycosides. For this reason, plant material should be dry, iyophilized, or frozen. In order to make dry plant materials useable, they are pulverized. Solvents are used for extraction and this solvent is one of the functions of flavonoid types. At this point polarity has a great importance. Some of the flavonoids such as isoflavones, flavanones, methylated flavones, and flavonols have low polarity standard, do extraction with chloroform, dichloromethane, diethyl ether or ethyl acetate. On the other hand, extraction of flavonoid glycosides and more polar aglycones are carried out with alcohols or alcohol–water mixtures. Because of the high water solubility of glycosides, aqueous alcoholic solutions are quite efficient (Andersen and Markham, 2005).

### 1.3.2 Chromatography

Two types of phases appear here; a moving mobile phase and an immobile stationary phase. Both of them are involved by all of the chromatographic techniques. There is a difference between mobile phase and stationary phase in point of affinity toward the components and this effects the separation (Sarker et al., 2006).

Among the modern separation techniques, a variety of them can be observed: (Hostettmann et al., 1986; Marston and Hostettmann, 1991b) such as flash chromatography; DCCC, low-pressure liquid chromatography (LPLC), medium-pressure liquid chromatography (MPLC) and HPLC. Nevertheless conventional open column chromatography is still used in many of the separations. Especially the preliminary fractionation work is one of them.

### 1.3.2.1 Open column chromotography

Open-column chromatography is simple and valuable in point of being initial separation step. Consequently these make it still useful and efficient. Generally, İt is possible to only partially separate complex mixtures because of the low resolution. Irreversible adsorption that led the loss of material and the necessity of long period of time to separation are among the problems (Hostettmann and Marston, 1995)

Obtaining vast amount of flavonoids from crude plant extracts is an alternative way. Polyamide, cellulose, silica gel, Sephadex LH-20, and Sephadex G-10, G-25, and G-50 are support materials that can be used. To separate flavonoids, using sephadex LH-20 is most suitable one. It is known that except the size exclusion, organic solvents include adsorption and partition mechanisms for sephadex. Usage of methanol and ethanol as eluents is quite efficient way for proanthocyanidins. Despite this fact, acetone can be replaced them by virtue of its low molecular weight. Slow flow rates can also be anternative. Some of the open-column chromatographies have certain supports such as silica gel and polyamide. They are faced with the problem of irreversible adsorption of the solute on the column (Andersen and Markham, 2005).

### 1.3.2.2 Flash chromatography

Flash chromatography is a preparative pressure liquid chromatography method. This is a timesaver method by contrast with conventional open column chromatography. Driving eluent through a sorbent by compressed air or nitrogen by using glass column maximum pressure is provided at the top of the column. Resolution is quite high in this method. The reason for this is reducing the granulometry of the solvent due to the pressure on solvent. Flash chromatography is so fast method that a sample of 10 mg can be separated to 10 g only in 10 min. Consequently this method can be alternative of open-column chromatographic methods of preliminary fractionation.

### 1.3.2.3 Low pressure liquid chromatography (LPLC)

LPLC includes a mobile phase. This mobile phase is drained through a sorbent that is packed intensely. Choosing material for the stational phase is so important that it effects the absorption or size exclusion sufficiently that form the separation mechanism. Absorption contains silica gel, bonded-phase silica gel, alumina, polystyrene; size-exclusion: polyacrylamide and carbohydrates.

Stationary phase is choosen according to polarity of the sample. In order to separate the natural products, stationary phases are used in LPLC. Silica gel is the most commonly used one of them and it is possible to accept the silica as a typical polar sorbent. Range of 40–60 μm is enough to obtain high flow-rates without needing high pressures. Particle size of silis gel is at this value of range under normal conditions (Sticher, 2007).

Samples are fractionated and amount of them affects the amount of stationary phase. For 1 g of crude sample, 100–500 g of packing material is used. This is also used in the general guideline at these standards.

In order to manage the LPLC, 40–200-μm particles are used. This is out of question a considerable superiority of this flaw rate against the atmospheric pressure.

Formation of intermediate or final steps of purification can be generated by LPLC. Additively, other seperation methods are generally used with the LPLC (Sticher, 2007).

### 1.3.2.4 Medium-pressure liquid chromatography (MPLC)

“Medium-pressure liquid chromatography” (MPLC) is a closed column. The material of this column is generally glass and it runs with a connection to a compressed air source or a reciprocating pump. Wide range of column diameters, different materials of granulometry packing, different pressure levels and some commercially available systems are can be concluded to MPLC. There is a need for an alternative method in order to carry out the open-column chromatography

or flash chromatography. Owing to its higher resolution and shorter separation times, MPLC can answer this purpose. Loading capacity of MPLC is twenty five times higher than in terms of sample, packing and material ratio. This ratio is quite enough to saparate the flavonoids (Andersen and Markham, 2005).

In this method, operation of filling the columns is carried out by the users. It is suggested that to use between 25 and 200 μm size of particle in MPLC and the operation of packing can be achived in slurry or dry form. Ranges of 15 to 25, 25 to 40, or 43 to 60 μm are commonly used. In order to get a long column which has small internal diameter rather than shorter column which has larger internal diameter, process of increasing the resolution is applied. During this application, amount of stationary phase remains constant. Choosing suitable solvent system effects the efficiency of the running system and for this purpose, TLC or analytical HPLC can be used. Transposition to MPLC is straight forward and direct (Andersen and Markham, 2005).

#### 1.3.2.5 Vacuum liquid chromatography (VLC)

We can describe the vacuum liquid chromatography (VLC) as columnar preparative TLC. In an attempt to increase the eluent flow rate, vacuum is applied in VLC. Fractions are collected and the dry column is operated in that position. There is an opposite situation in flash chromatography. To apply VLC in constraction of natural products is easier now and this increases the usage of it. With this method up to 30 g of extract can be separated (Sticher, 2007).

This technique is used in order to increase the flaw rate of a mobile phase. During this procession, reduced pressure is used and the increase is sustained throughout a short bed of solvent. In the process of packing chromatography column, silica gel is used and this gel is generally at the TLC grade of 10-40 urn. After the packing the sample is eluted with solvent mixtures. Polarity of the solvent is low at the beginning of the sortation and then elution strenght is gradually increased. (Hostettmann and Marston, 1995).

Silica gel (both normal and reversed-phase), $Al_2O_3$, CN, diol and polyamide are the chromatographic supports of the VLC. RPC, MPLC, and HPLC are some of the other separation steps. It is necessary to fractionate the products before these steps and VLC is used for this purpose (Sticher, 2007).

### 1.3.2.6 High-performance liquid chromatography (HPLC)

HPLC can be used to separate the flovonoids with the preparative and analytical scales. This technique is becoming the most commonly used one in these days. In order to make this technique more popular and common, some technological methods are improved. These are; instrumentation, packing materials and column technology.

Analytical and preparative methods are different from each other. Analytical HPLC is not related to recovery of a sample. On the other hand the preparative HPLC is a purification technique and isolate the pure substance.

In general columns are used and these columns are packed with 10 μm (or smaller) particles in semiseparative HPLC. This value is for 1 to 100 mg sample sizes and 8 to 20 mm internal diameter of columns. It is possible to separate the large samples with preparative or even process-scale store equipments but this process increases the cost. For optimization, it is not necessary to wait for transposition to a semi preparative scale (Andersen and Markham, 2005).

Moderate polarity of free flavonoids and obvious polarity of their compounds provides the efficient separation with reversed-phase HPLC. During this course ODS C18 column by mobile phases of aqueous MeOH or ACN in various proportions with isocratic or gradient elution is used. Among the usage area of HPLC-PDA technique, there is detection and partial identification of flavonoids in plant extracts. The factor which provide this is the UV-active characteristic of compounds (Sticher, 2007)

## 1.4 Structural Elucidation

### 1.4.1 Nuclear Magnetic Resonance (NMR)

Nuclear Magnetic Resonance spectroscopy is enable to investigate the structure and some properties of molecules. NMR is one of the most useful method used for this purpose. NMR is used mainly in the researches of flavonoids, on the purpose of finding out uncharted compounds in structural manner. In spite of requirement for great amount of samples which are hard to find during the exploration, all proton and carbon signals using amounts of 1 mg could be assigned exactly in virtue of improved pulse programs and computing power (Fossen and Andersen, 2005).

#### 1.4.1.1 One dimensional NMR: $^{1}H$ and $^{13}C$

Among the NMR experiments, $^{1}H$ and $^{13}C$-NMR experiments are the most simple ones. Duty of each $^{1}H$ or $^{13}C$ nucleus in the molecule is ensuring the resonance frequency.

Inventory of chemical shifts (δ) and spin-spin couplings, is carried out by the 1H NMR experiments. Describing the latter one is made with the coupling constants (*J*). By the virtue of this, it is possible to know the relative number of hydrogens and their type. These informations are obtained with the comparison of recorded chemical shifts and compiled data. Determination of the aglycone type and the acyl groups attached to it, and identificition of the number and the anomeric configuration of the glycoside moieties attached to the aglycone are made possible by this. Consumnation of $^{1}H$-MNR data is practised bu the $^{13}C$-MNR data. $^{13}C$-MNR data compares the group types and compiled data and in consequence of this comparison types of groups are established. $^{13}C$-NMR (1.1 %) is less sensitive than $^{1}H$-NMR (99.9 %) because of the abundance (Claridge, 1999).

During the identification of aglycone types and substituent groups, $^{13}C$-NMR and $^{1}H$-NMR are can be used together. Accurate location of the various

groups should be explained in a structural manner and proved by various 2D experiments.

### 1.4.1.2 Homonuclear 2D NMR

The correlations between different nuclei in the molecules are shown in the contour maps. 2D NMR plays role in the constitution of this map. Homonuclearity and heteronuclearity are characteristics of 2D NMR and these features depend on the similarity or difference between nuclei in the molecules (Claridge, 1999). COSY(COrrelation SpectroscopY) is one of the precurses of multidimensional systems. Protons couple to each other two bonds apart ($^2J$HH), it can be three and four bonds apart ($^3J$HH and $^4J$HH) time to time. COSY crosspeaks are between these protons. The intensity of coupling is important here because of its effect on the intensity of the peak.

An experimentation is processed by creating correlations between all protons in a given spin system. This experimantation is known as TOCSY (TOtal Correlation Spectroscopy) and useful to identify protons on sugar rings on condition that there are couplings between every intervening protons. Each of the protons is in an interrelation with with all other protons from the same ring. After this phase, a transfer of magnezitation up to 5 or 6 bonds and interruption by small or null $^1$H-$^1$H couplings and hetero-atoms are carried out. It is possible to adjust the number of transfer steps with the changes made on the spin-lock time (Fossen and Andersen, 2005).

### 1.4.1.3 Heteronuclear 2D NMR

The nuclei of different elements are in a interrelation heteronuclear 2D NMR experimentation. 2D proton–carbon experiments HMQC/HSQC (Heteronuclear Multiple Quantum Coherence/Heteronuclear Single Quantum Coherence) and HMBC (Heteronuclear Multiple Bond Correlation) have the ability of dovetailing proton and carbon NMR data directly. This opportunity makes them one of the most powerful techniques.

Bond correlations between the protons of a molecule and the carbons are installed by HMQC and HSQ. They are attached to those carbons ($^{1}J_{CH}$). 1D $^{13}C$ experiments are not as sensitive as HMQC and HSQ. 1D experiment has low abundance of the isotope and thus signal-to-noise ratio is also low. Oppositely in the heteronuclear 2D experiment, transfer of initial magnetization to the $^{13}C$ atoms is carried out on the highly sensitive $^{1}H$ nuclei. In another NMR experiment it is seen that HMBC is more sensitive to 3-bond correlations than to 2-bond correlations. However there can be some changes according to overall signal-to-noise ratios and on the adjustable parameters of each experiment. $^{2}J_{CH}$,$^{3}J_{CH}$-HBMC is a new experiment carried out for the purpose of differentiate these two types of correlations (Claridge, 1999; Krishnamurthy, 2000; Fossen and Andersen, 2005).

In an attemp to assign the flavonoids to C atoms, HBMC is applied. These C atoms are nonprotonated from the aglycones and acyl groups. HMBC can also be used to determine the linkage points of heteroatomcontaining groups such as sugar residues. Reason of this is unstoppable characteristic of HMBC by heteroatoms. Another usage of HMBC is distinguishing the some classes of flavonoids. These flavonoids are distinguished as flavones from aurones, which have similar $^{1}H$- and $^{13}C$-NMR spectra. Cause of the usage HSQC and HMBC experiments, namely gradient enhanced (ge) is their higher sensitivity and capacity. They can establish strong intramolecular H bonding between the 4-oxo and 5-hydroxy groups in flavonoids (Exarchou et al., 2002; Kozerski et al., 2003).

In order to analize the flavonoids, a number of developments in NMR experiments are developed. While developing these new 2D and 3D techniques are used. 2D and 3D HSQC-TOCSY experiments have the ability to assign all $^{13}C$ signals of individual glycosides in polyglycosilated flavonoids (Fossen and Andersen, 2005).

# 2. MATERIALS and METHODS

## 2.1 Plant Material

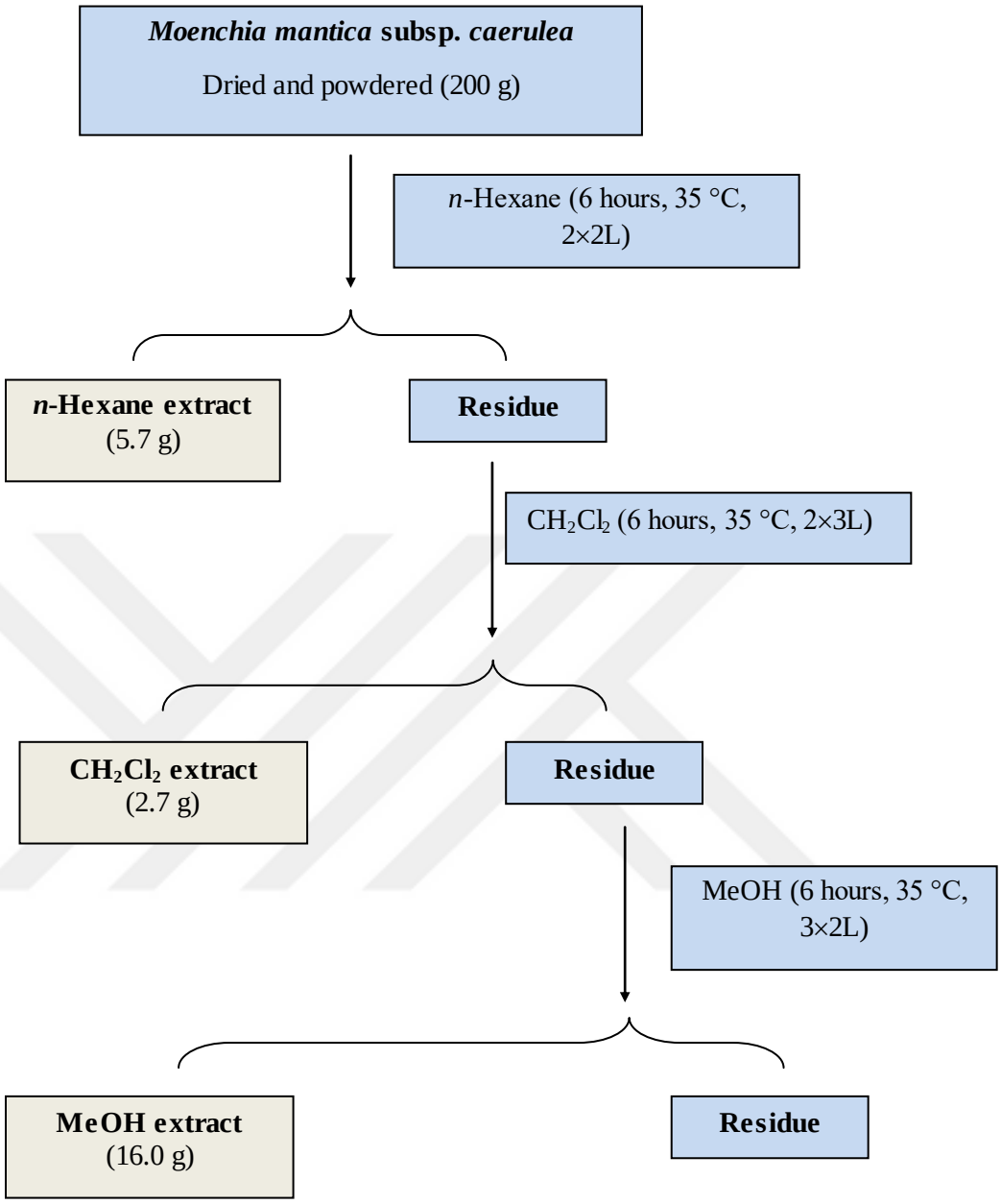
*Moenchia mantica* subsp. *caerulea* was collected from Mount Bozdağ, İzmir in May 2014 and plant material was identified by Assoc. Prof. Dr. Serdar G. Şenol (Department of Biology, Faculty of Science, Ege University). A voucher specimen was deposited in the Herbarium of Ege University.

## 2.2 Isolation and Purification

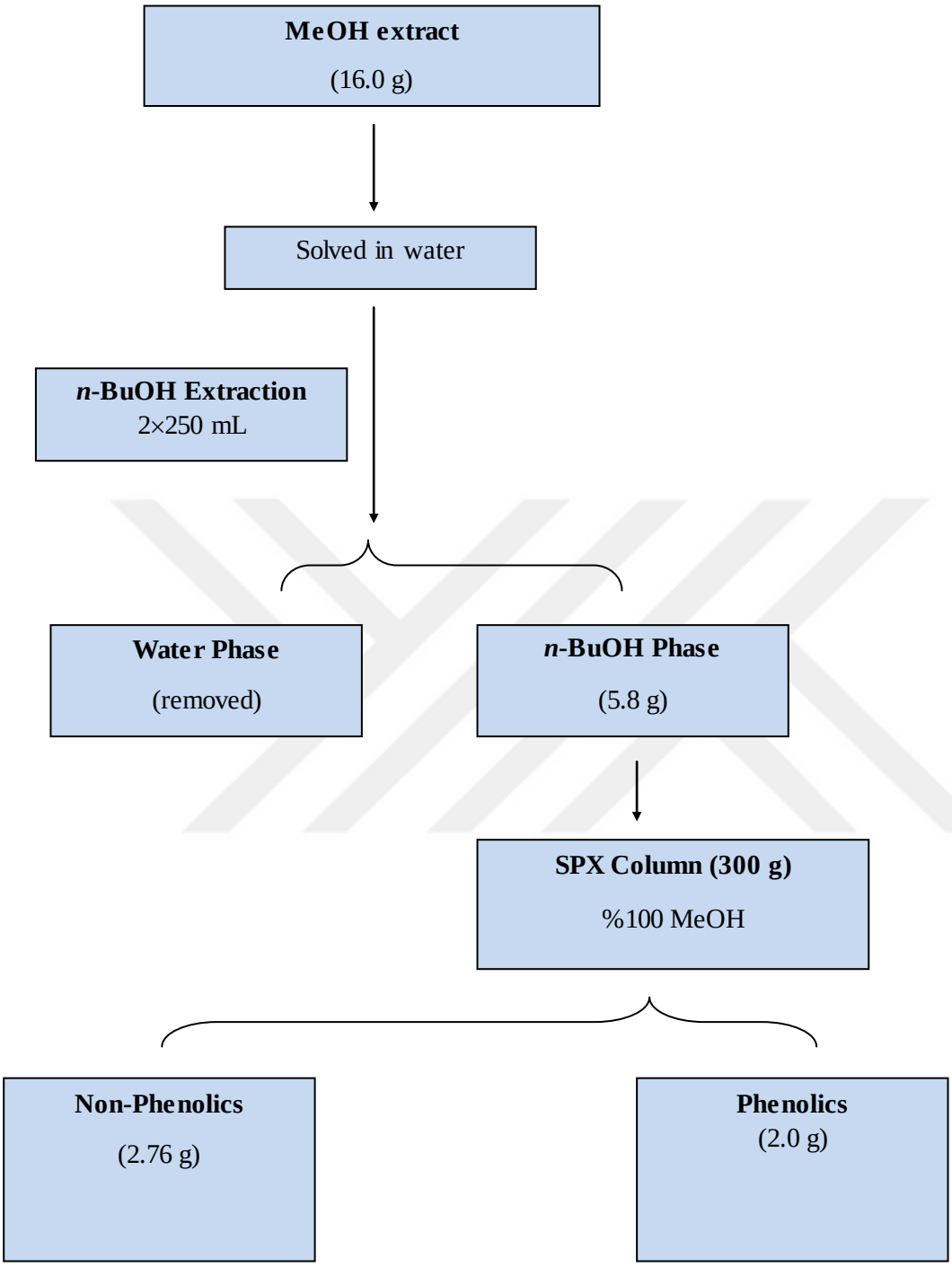
200 g of dried and grinded whole part of *Moenchia mantica* subsp. *caerulea* was extracted twice with 2 liter *n*-hexane, then residue extracted with 3 liter of dichloromethane twice, then residue extracted with 2 liter of methanol three times. After filtration, the solvent was removed by rotary evaporation yielding 16 gram of methanol extract. The MeOH extract was dissolved in 250 mL of saline water and successively partitioned with *n*-BuOH (2 × 250 mL). After the partition, 5.8 g of *n*-BuOH extract obtained (Scheme 2.1).0

5.8 gr of *n*-BuOH extract subjected to sephadex (LH-20 Amersham Biosciences, 300 g) column and eluted with MeOH. Fractions checked with TLC and it is observed that phenolics and non-phenolics are successfully seperated. (Scheme 2.2)

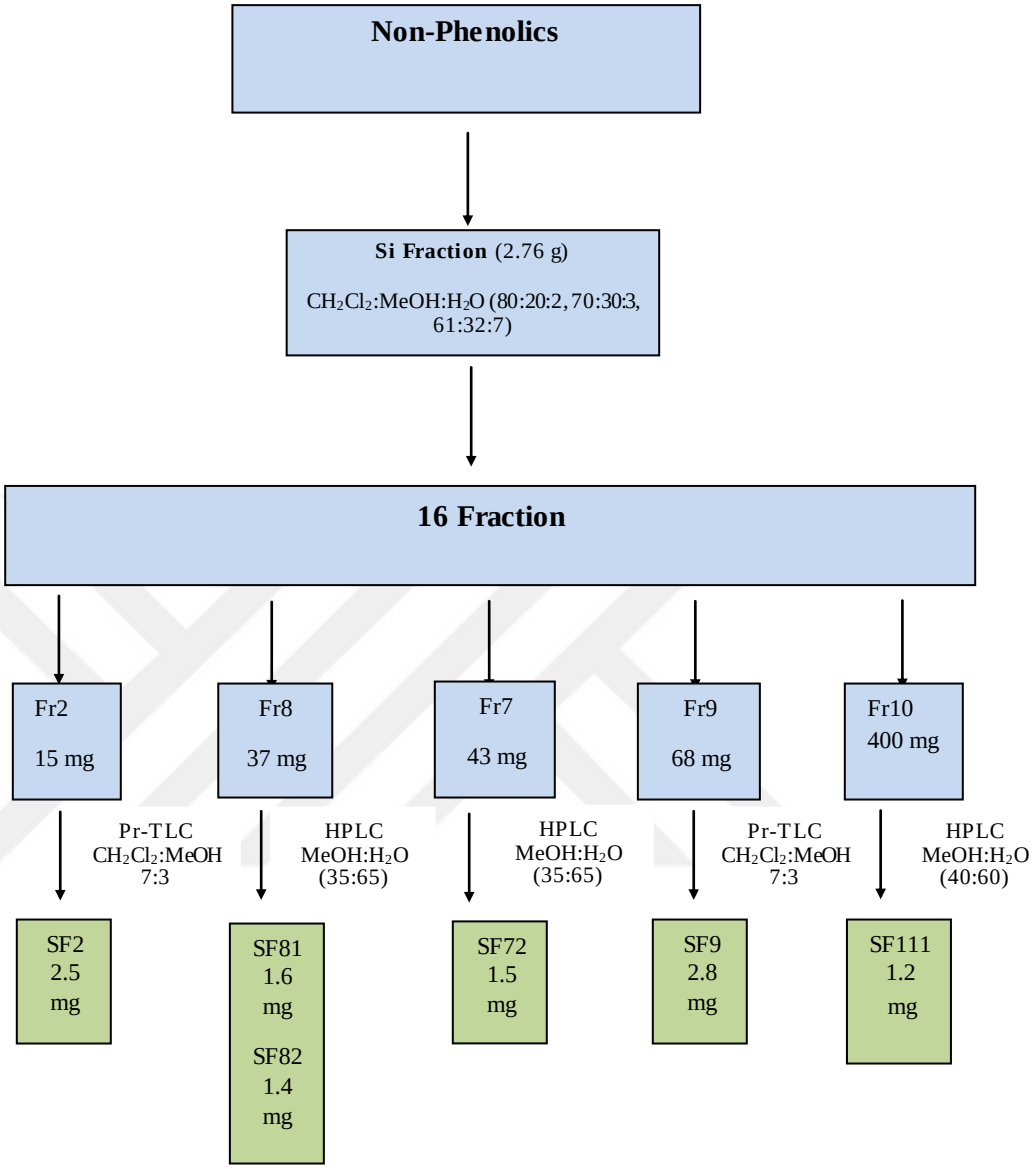
2 gr of phenolic rich fraction subjected to MPLC using reversed-phase material (Lichroprep RP-18, 25-40 μm) employing $H_2O$:MeOH (7:3; 2000 mL, 6:4 2000 mL) solvent system to obtain 4 main fractions. It's observed that the first fraction is püre and **RPF6** (280mg) was obtained as a pure compound. Fraction 2 applied to a preparative TLC, elution performed with $CH_2Cl_2$:MeOH:$H_2O$ (80:20:2) solvent system and as a result, **RPF8U** (2.4mg) was isolated. (Scheme 2.5)



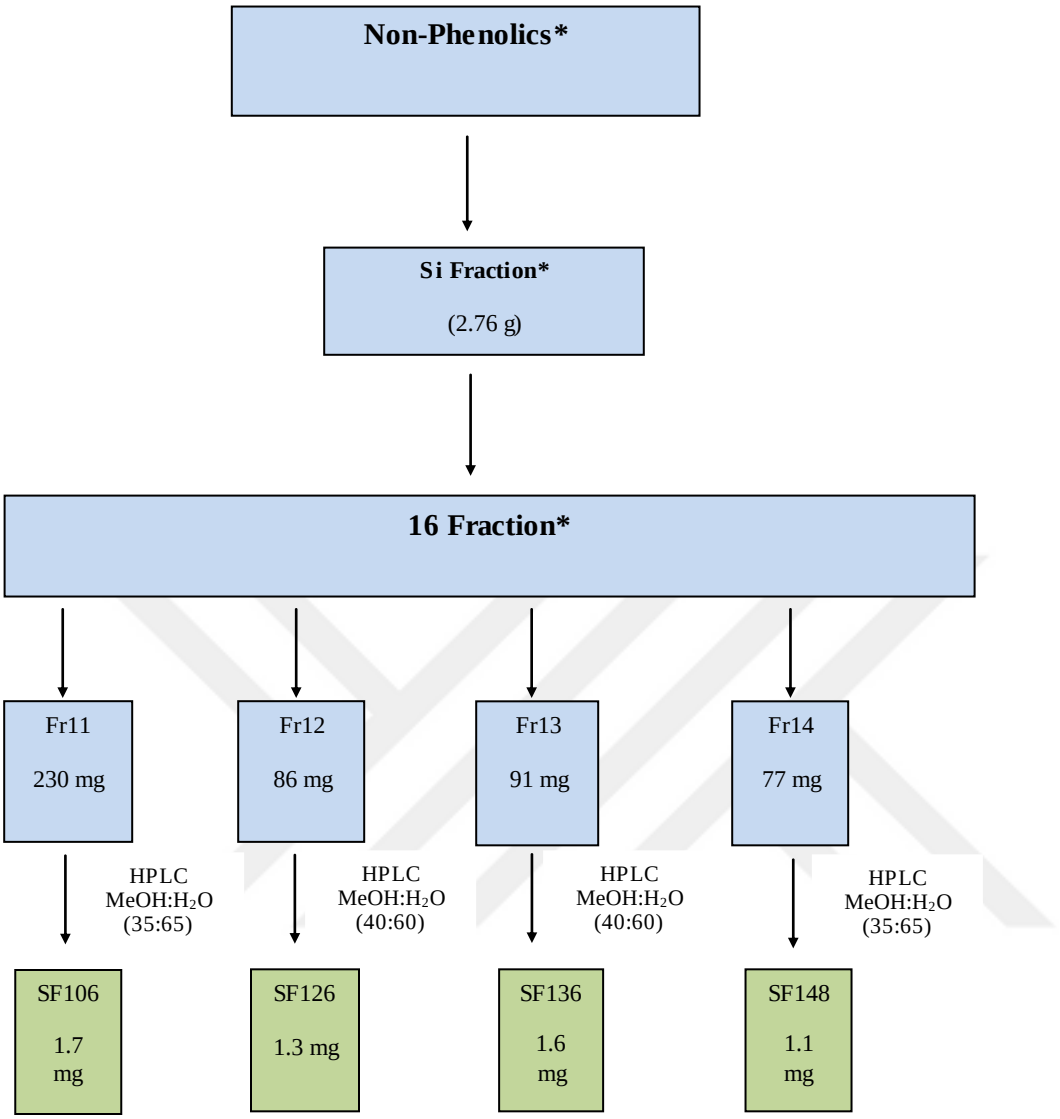

Scheme 2. 1. Extraction process *of Moenchia mantica* subsp. *caerulea*



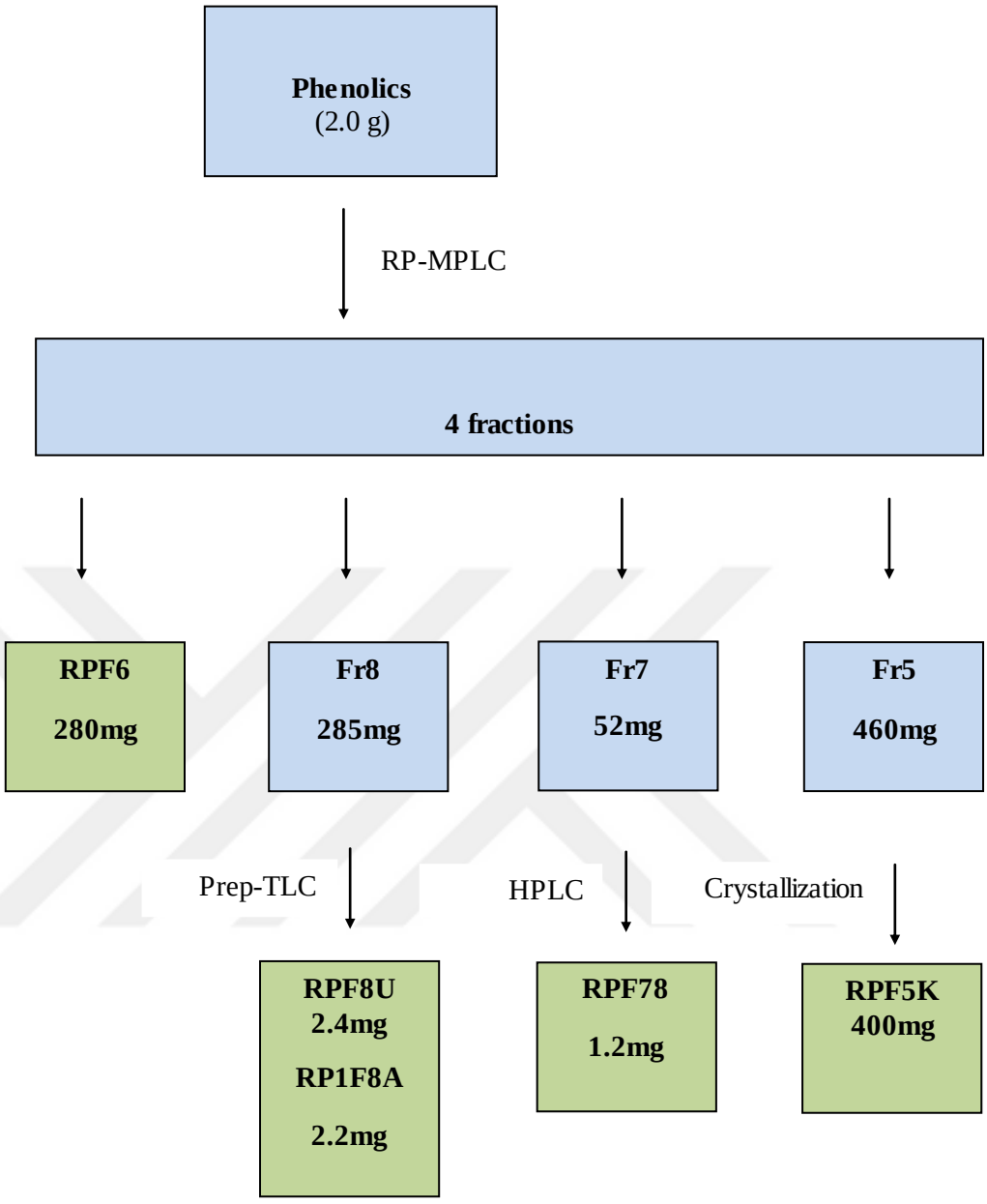

Scheme 2. 2. Extraction process of *Moenchia mantica* subsp. *caerulea*




Scheme 2. 3. Extraction process of *Moenchia mantica* subsp. *caerulea*




Scheme 2. 4. Extraction process of *Moenchia mantica* subsp. *caerulea*




Scheme 2. 5. Extraction process of *Moenchia mantica* subsp. *caerulea*

# 3. RESULTS and DISCUSSION

## 3.1 Structural Identification of Compound 1

The $^{1}H$ NMR spectrum of compound 1 was showed the characteristic resonances of an flavone proton at δ 6.60 (s, H-3) and an aromatic proton at δ 6.49 ppm (s, H-8) (Spectrum 3.1). In addition, the $^{1}H$ NMR resonances typical for AB system of two ortho-coupled aromatic protons of ring B were observed at δ 6.95 (2H, d, *J* = 8.4 Hz, H-3'/5') and δ 7.88 ppm (2H, d *J* = 8.4 Hz, H-2'/6') which were verified from the $^{13}C$ NMR resonance at δ 117.2 (C 3'/5') and δ 129.5 ppm (C 2'/6'). A further feature was the signal of an aromatic proton at δ 4.93 ppm (1H, d, *J* = 7.5 Hz) which correlated in the HSQC spectrum with a carbon signal at δ 75.2 ppm, is characteristic for a *C*-glycoside. Comparing with NMR data with literatüre, studies allowed us to identify the aglycone as 5,7,4' -trihydroxyflavone (Rigano et al., 2007)

The anomeric proton appearing at δ 4.93 ppm (1H, d, *J* = 7.5 Hz, H-1" , $\delta_C$ 75.2) together with the signals at δ 4.26 ppm (1H, dd, *J* = 7.5, 9.0, H-2", $\delta_C$ 72.3), δ 3.50 ppm (1H, dd, *J* = 9.0, 9.0 Hz, H-3" , $\delta_C$ 80.3), δ 3.53 ppm (1H, dd, *J* = 9.0, 9.0 Hz, H-4" , $\delta_C$ 71.4), δ 3.44 ppm (1H, m, H-5" , $\delta_C$ 82.4), δ 3.88 ppm (1H, dd, *J* = 2.0, 12.0 Hz, H-6a" , $\delta_C$ 63.0) and 3.77 ppm (1H, dd, *J* = 4.5, 12.0 Hz, H-6b" , $\delta_C$ 63.0) suggested the presence of a β-glucopyranosyl unit. The position of the glycosidation was deduced to be C-6 by an HMBC experiment which provided a key longrange correlation between the anomeric proton signal at δ 4.93 ppm and the carbon resonance at δ 109.6 ppm (C-6) (Spectrum 3.2).

The full assignment of the proton and carbon signals of the aglycone and sugar moieties of **1**, secured by COSY, HMQC, and HMBC spectra, indicated that **1** was 5,7,4' -trihydroxyflavone 6-C-*β*-glucopyranoside (Spectrum 3.3 and 3.4).

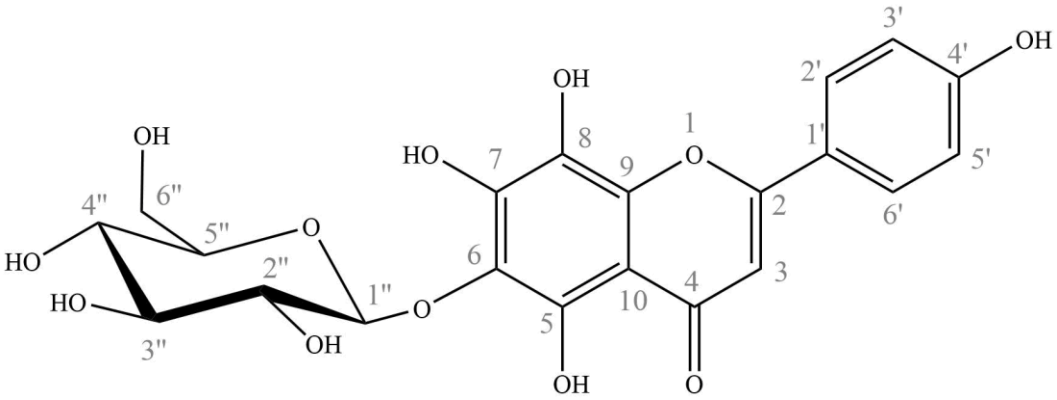
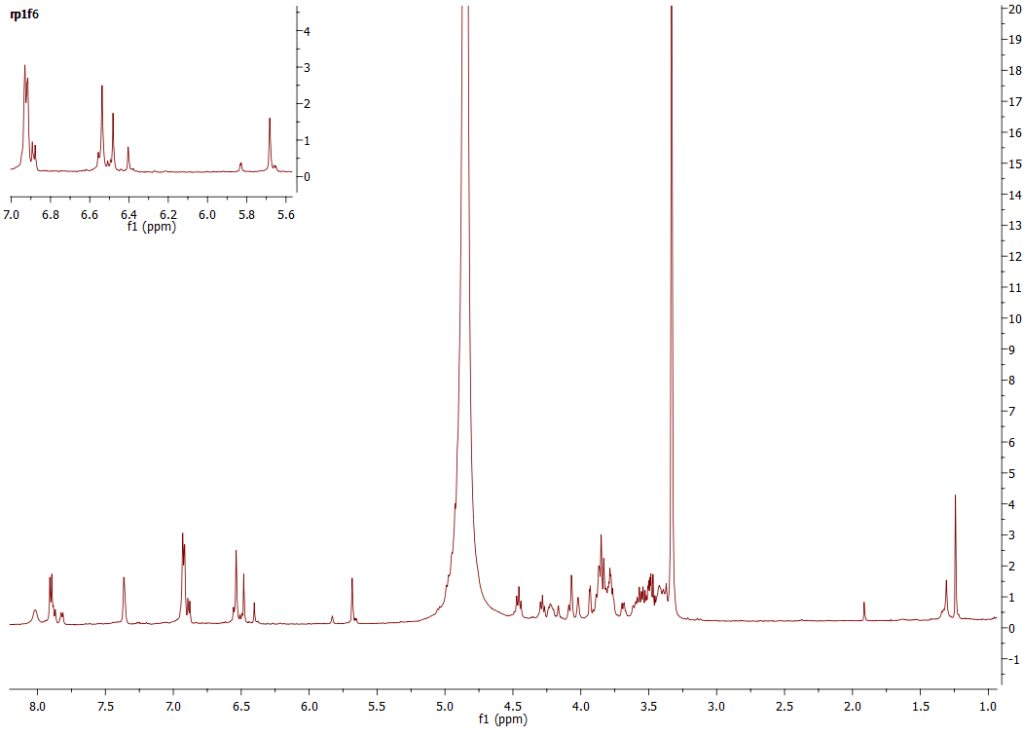
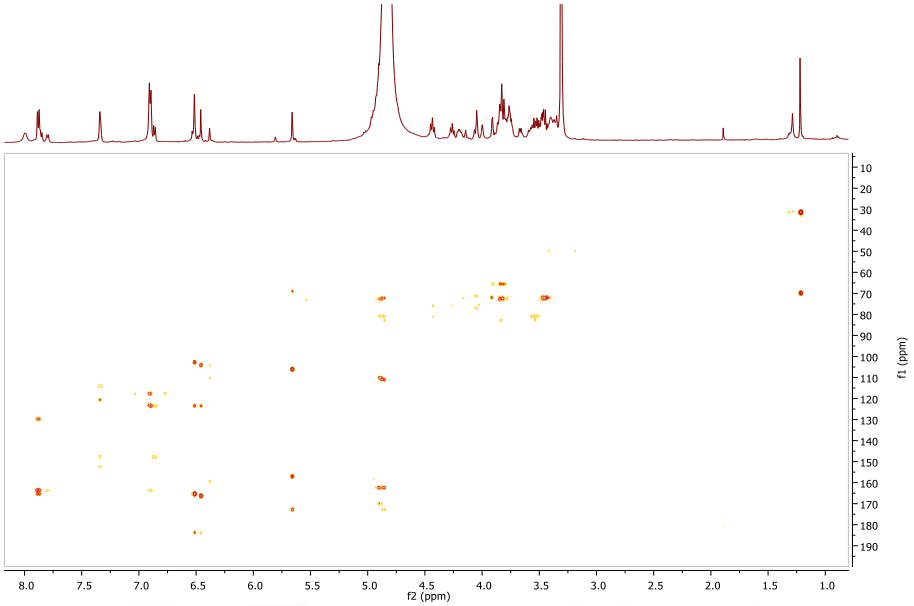


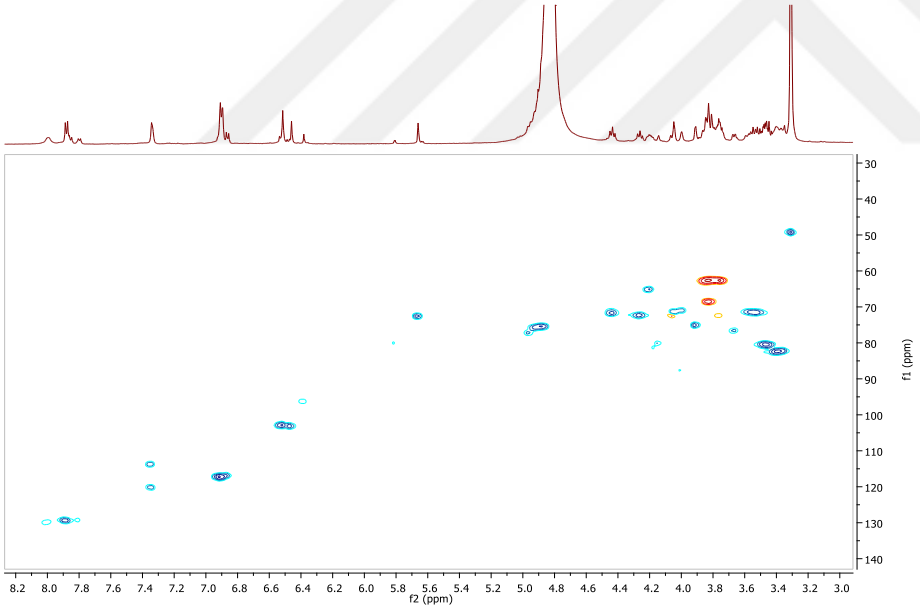

Figure 3. 1. Structure of Compound 1 (RPF6)



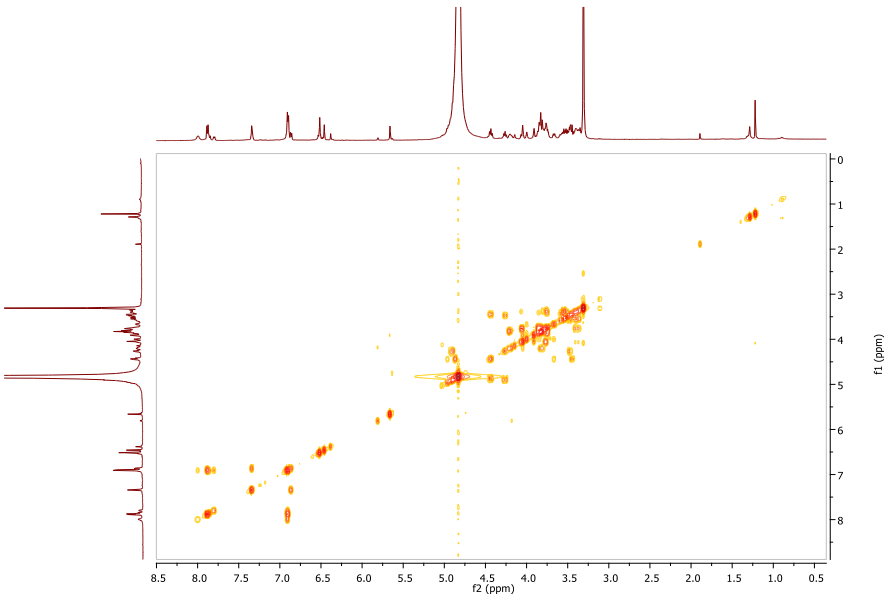

Spectrum 3. 1. $^{1}$H-NMR spectrum of Compound 1 (RPF6) in $CD_3OD$ (600 MHz)




Spectrum 3. 2. HMBC spectrum of Compund 1 in $CD_3OD$ (150 MHz)




Spectrum 3. 3. HSQC spectrum of Compund 1 in $CD_3OD$ (150 MHz)




Spectrum 3. 4. COSY spectrum of Compund 1 in $CD_3OD$ (150 MHz)

## 3.2 Structural Identification of Compound 2

A detailed analysis of the NMR data suggested that compound **2** differed from that of **1** only for the absence of a -OH group at C-8 position. Analysis of the 1D and 2D NMR data and comparing with literature are allowed the identification of the aglycone of 2 as the 5,7,8,4`-tetrahydroxyflavone, known as isoscutellarein, and the sugar as a $\beta$-glucopyranosyl unit (Rigano et al., 2007) Thus, compound **2** was identified as isoscutellarein 6-C-$\beta$-glucopyranoside (Spectrum 3.5- 3.8).

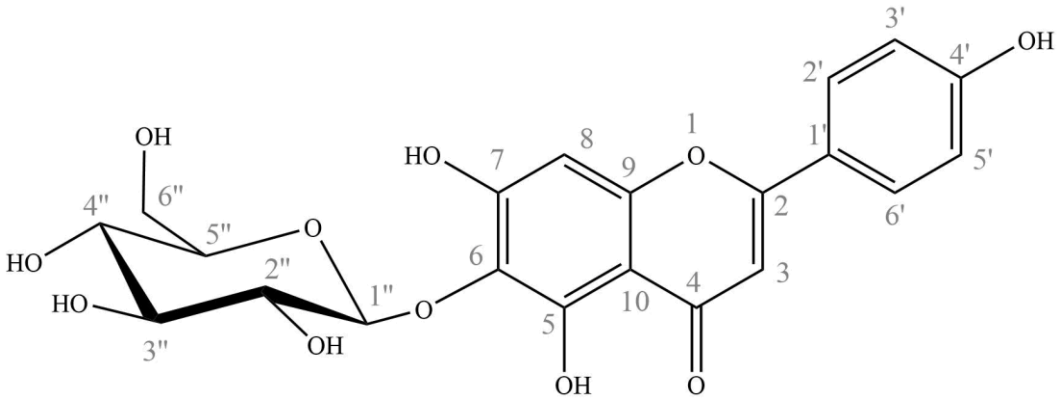
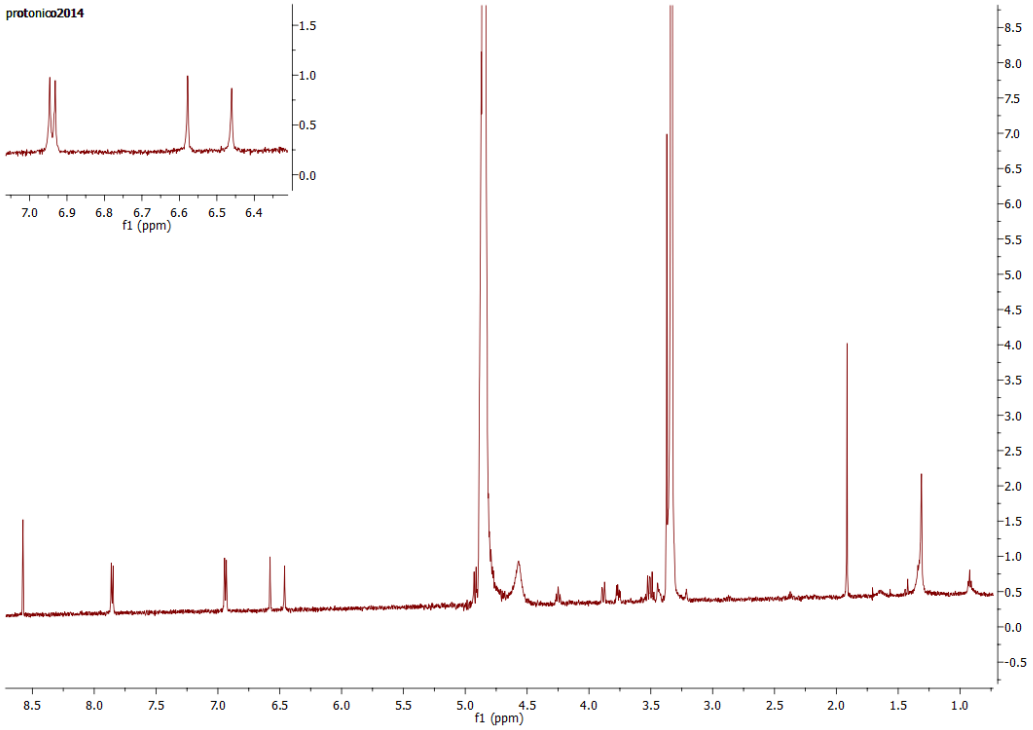
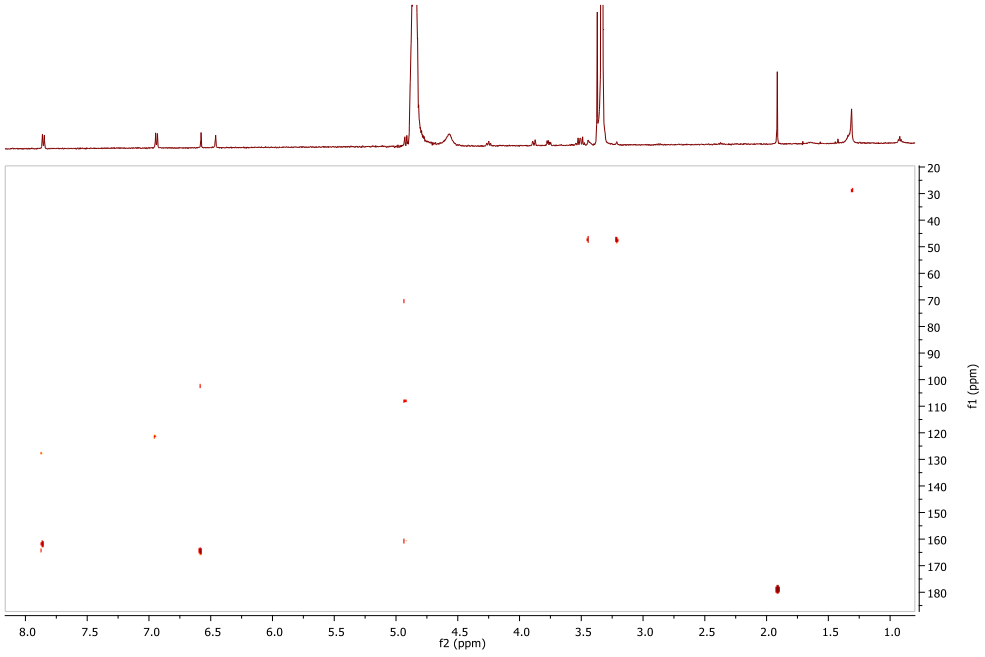


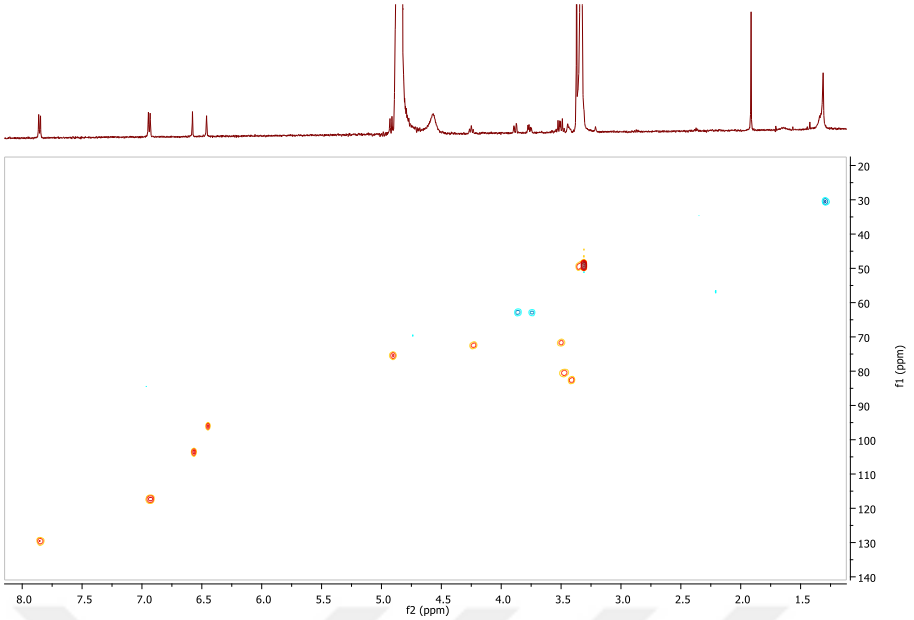

Figure 3. 2. Structure of Compound 2 (RP8U)



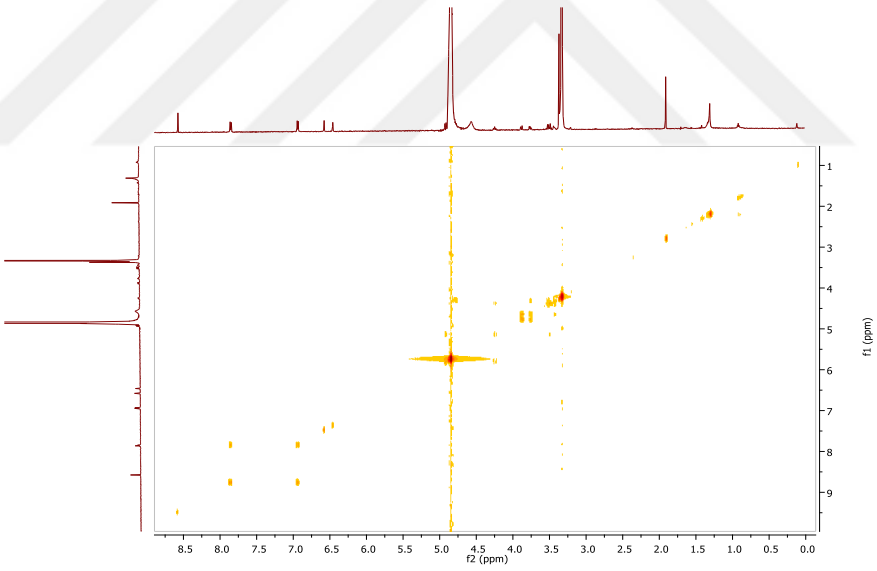

Spectrum 3. 5. $^{1}$H-NMR spectrum of Compound 2 (RP8U)




Spectrum 3. 6. HMBC spectrum of Compund 2 in $CD_3OD$ (150 MHz)




Spectrum 3. 7. HSQC spectrum of Compund 2 in $CD_3OD$ (150 MHz)




Spectrum 3. 8.COSY spectrum of Compund 2 in $CD_3OD$ (150 MHz)

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