DETERMINATION OF POLYPHENOLIC CONSTITUENTS OF SOME PLANTS GROWN IN BOLU

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DETERMINATION OF POLYPHENOLIC CONSTITUENTS OF SOME PLANTS GROWN IN BOLU

by

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IN

THE DEPARTMENT OF CHEMISTRY

AUGUST 2012

Approval of the Graduate School of Natural and Applied Science

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Prof. Dr. Yaşar Dürüst

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I certify that this thesis satisfies all the requirements as a thesis for the degree of Master Science.

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This is to certify that we have read this thesis and that in our opinion it is fully adequate in scope and quality as a thesis for the degree of Master of Sciences.

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ABSTRACT

DETERMINATION OF POLYPHENOLIC CONSTITUENTS OF SOME PLANTS GROWN IN BOLU

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AUGUST 2012, 107 Pages

Phytochemicals are the immune system of the plants. They protect plants from diseases and pests. Also they have protective effects on human body such as anti-carcinogenic, anti-bacterial and antioxidant. In this regard, phytochemical research have quite a lot of importance in all respects.

In this study, the polyphenolic constituents of flowers of some plants grown in Bolu were determined by qualitative and quantitative methods. Five plants were studied and analyzed. The source plants *Silene compacta FISCHE* (SC), *Digitalis lamarcki* (DL) , *Xeranthemum annuum L.* (XA), *Epilobium hirsutum L.* (EH), and *Epilobium angustifolium L.* (EA) used in this study are found widespread in Bolu province. Of these plants there is no report regarding the plant SC in the literature.

After collecting, drying and grinding process, the flowers of the plants were extracted by various methods and prepared for analysis.

In the plant SC; myricetin, gallic acid, kaempferol, *p*-hydroxybenzoic acid, and quercetin-3-O-rhamnoside; in DL*:* quercetin, caffeic acid, quercetin-3-O-glucoside, and myricetin-3-O-rhamnoside; in XA: ferulic acid, quercetin-3-O-glucoside, galangin, kaempferol, quercetin, protocatechuic acid, myricetin-3-O-rhamnoside, and luteolin; in EH: kaempferol, quercetin, caffeic acid, gallic acid, quercetin-3-O-glucoside, and quercetin-3-O-rhamnoside; in EA: kaempferol-3-O-rhamnoside, quercetin-3-O-glucoside, apigenin-7-0-glucoside, gallic acid, quercetin-3-O-rhamnoside, and vanillic acid were detected by LC-MS technique. TLC was also performed using different solvent systems for analysis of phenolic compounds.

The highest contents of benzoic acids (159.41 mg/100g, in EH*),* cinnamic acids (292 mg/100g, in SC), and flavanoids (3890.12 mg/100g, in EH*)* were detected by means of HPLC method.

Keywords: Phytochemical Analysis, Polyphenolic compounds, Quantitative Analysis, HPLC, LC-MS.

ÖZET

BOLU'DA YETİŞEN BAZI BİTKİLERİN POLİFENOLİK BİLEŞENLERİNİN TAYİNİ

Banko, Seray

Yüksek Lisans Tezi, Kimya Bölümü

Tez Danışmanı: Prof. Dr. Nedime DÜRÜST

AĞUSTOS, 107 Sayfa

Fitokimyasallar bitkilerin bağışıklık sistemidir. Bitkileri hastalıklardan ve zararlılardan korur. Ayrıca insan vücudunda antikarsinojen, antibakteriyel ve antioksidan gibi koruyucu etkileri de vardır. Bu bakımdan, fitokimyasal araştırmalar her yönden oldukça fazla öneme sahiptir.

Bu çalışmada, Bolu'da yetişen bazı bitkilerin polifenolik bileşenleri kalitatif ve kantitatif metodlar ile belirlendi. Beş adet bitki analiz edildi. Bu çalışmada kaynak bitki olarak Bolu ilinde yaygın olan *Silene compacta FISCHE* (SC), *Digitalis lamarcki* (DL), *Xeranthemum annuum L.* (XA), *Epilobium hirsutum L.* (EH), ve *Epilobium angustifolium L.* (EA) bitkileri kullanıldı. Literatürde, bu bitkilerden olan SC'ye ilişkin hiçbir bilgi bulunmamaktadır. Toplama, kurutma ve öğütme

işlemlerinden sonra çeşitli ekstraksiyon metodlarıyla ekstrakte edildi ve analiz için hazırlandı.

LC-MS tekniği ile SC bitkisinde: mirisetin, gallik asit, kemferol, *p*-hidroksibenzoik asit ve kuersetin-3-O-ramnosit; DL'de: kuersetin, kafeik asit, kuersetin-3-O-glikozit ve mirisetin-3-O-ramnosit; XA'da: ferulik asid, kuersetin-3-O-glikozit, galangin, kemferol, kuersetin, protokateşik acid, mirisetin-3-O-ramnosit, and luteolin; EH'de: kemferol, kuersetin, kafeik asit, gallik asit, kuersetin-3-O-glikozit ve kaempferol, quercetin, caffeic acid, gallic acid, quercetin-3-O-glucoside, and kuersetin-3-O-ramnosit; EA'da: kemferol-3-O-ramnosit, kuersetin-3-O-glikozit, apigenin-7-0-glikozit, gallik acid, kuersetin-3-O-ramnosit ve vanillik asid tespit edildi. Fenolik bileşiklerin analizi için farklı çözücü sistemleri kullanılarak TLC yapıldı.

HPLC methodu yardımıyla, en yüksek benzoik asit bileşenleri (159.41 mg/100g, EH'de), sinnamik asit bileşenleri (292 mg/100g, SC'de) ve flavonoid bileşenleri (3890.12 mg/100g, EH'de) tayin edildi.

Anahtar Kelimeler: Fitokimyasal Analiz, Polifenolik bileşikler, Kantitatif Analiz, HPLC, LC-MS.

To my father and family. . .

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1. INTRODUCTION

Plants, which contains phytochemical compounds play an important role in human's life. These compounds have some properties such as anti-aging, anti-carcinogenic, anti-imflammatory, anti-fungal, anti-bacterial and anti-oxidant. Because of these features, they have been utilized for 5,000 years to cure various diseases in different ways and also have been used as nutrient source. Lots of beneficial plants can be consumed in the form of herbal tea, and the active compounds in them can be used in pharmacological production, or these plants can also be used as an ointment or paste by mixing various proportion through primitive methods.

Thus, people recognized many new plants in their world, and so they seek answers to such questions as: Which plants are edible or not? Which plant is beneficial for what? How can one plant be distinguished from others? To answer such questions, people have done most of the research and at the end of this period, they thought that classification of the plants will make their research easier. In this respect, botanic science and phytochemistry which are the studies of plants and examining the plants in a certain way according to their types came into existence.

In ancient times people were interested in numerous plants that grew in their habitats. The plants have been categorized in accordance with their particular uses (i.e., for medicine, poisons, food, etc.). By the end of twentieth century, some ethnic people generated a classification scheme for the plants by making use of their day-to-day experiences.

Carl Linnaeus, the Swedish naturalist-physician, was known as the first person to apply two-word Latin names or binomial nomenclature to all plants. This Linnaeus's system has been recognized by scientific community and he is called the Father of Modern Taxonomy.

According to paleontologist George Gaylord Simpson, *" Systematics is the scientific study of the kinds and diversity of organisms and of any and all relationships among them."*

Taxonomy is generally considered as the study of classification, including its rules, theories, principles, and procedures (Woodland, 2000). Systematics involves the discipline of *taxonomy*, a term attached to the word *taxon.* Scientific names enables us to access to information about groups of organisms, and thus it is valuable to have one name by which all can refer to a group of plants (Judd et al., 2002).

Plants are called with two-word Latin names. The first word of Latin name is genus and the second one is species. Genus name is a noun which is always capitalized while species name is generally an adjective, sometimes a name and is not capitalized (Woodland, 2000). Name of a person who gives the Latin name to the plant firstly, is written at the end of these two-word Latin names which identify the plants as scientific. This person's name is written as both a full-name and an

abbreviated name. Generally more than three syllabic names are abbreviated (i.e., instead of BOISSIER to Boiss.) and more famous person's name is written with only one letter (i.e., instead of LINNAEUS or Linne to L.). This two-word Latin name of plant is written as italic version.(i.e. *Epilobium hirsutum L.).*

The source plants *Epilobium hirsutum L., Epilobium angustifolium L., Silene compacta FISCHE, Xeranthemum annuum L. and Digitalis Lamarcki* used in this study is widespread in Bolu province. The collecting process is very important. There are many points to consider when plants are to be collected. Appropriate conditions must be provided while collecting the plants.

Today, research is being done quite a lot about beneficial plants by scientists. Turkey is quite a rich country with regard to plant diversity. It is thought that there are more than 10.000 various type of plants in Turkey. Approximately, 30% of these species have aromatic properties, which means that they have their own smell and taste.

Phytochemical compounds are obtained from plants by several seperation methods. Many methods can be utilized to find phytochemicals in plants. These methods include qualitative and quantitative analysis. Seperation, purification, and identification of the many different components presented in plants require these methods in all operations (Harborne, 1998). However before all these methods, there are few preparation steps for the analysis processes. These preparation steps consist of three main processes such as collection, drying and grinding process. Almost all organs of the plant such as leaves, roots, fruits, flowers and also stems include important phytochemicals.

Phytochemicals are generally called as “secondary metabolites”. One group of the most important secondary metabolites are phenolic compounds which can be classified into three subgroups, namely, simple phenols and phenolic acids, hydroxybenzoic acids and hydroxycinnamic acid derivatives, and flavanoids.

1.1. PHYTOCHEMICALS

Phytochemicals (from the Greek word *phyto*, meaning plant) are biologically active and chemical compounds, which exist in the plants naturally. So far scientists have discovered more than 4000 species of phytochemicals and expected that they will discover many more.

Today, phytochemicals are used in many different areas. In many studies, it has been identified that they protect plants from hazardous impact of disease and also they contribute to the plant’s color aroma, and flavor. Effects of phytochemicals on human bodies are identified by scientists:

Impacts on cancer; phytochemicals can detoxicate substances that cause cancer. They neutralize free radicals, have a role in inhibiting enzymes that activate carcinogens, and have a role activating enzymes that detoxicate carcinogens (Miller, 2002).

Impacts on heart disease; phytochemicals can minimize the risk of coronary heart disease by preventing the oxidation of low-density lipoprotein (LDL) cholesterol, reducing the synthesis or absorption of cholesterol, normalizing blood pressure and clotting, and improving arterial elasticity (Miller, 2002).

The most important function of phytochemicals for human beings is to react with the free oxygen molecules or free radicals. Thus, phytochemicals make an antioxidant effect in human body. If free radicals are not removed from our bodies, they may damage the cells of us (Nyam News, 2005). Due to the health protection properties of these phytochemicals, they have quite a lot of importance in their application fields.

1.1.1. PHENOLIC COMPOUNDS

The term 'phenolic' or 'polyphenol' can be defined chemically as a substance which possesses an aromatic ring bearing one or more hydroxy substituents. These compounds usually occur in the form of glycosides or esters in plants. That is the reason for their tendency to be highly water-soluble (Proestos et al., 2004).

Phenolic compounds including simple phenols and phenolic acids, hydroxybenzoic acids and hydroxycinnamic acid derivatives, and flavonoids are bioactive substances occurring widely in plants. These phenolics are the most abundant secondary metabolites in plants and can be classified into non-soluble compounds such as condense tannins, lignins, cell-wall bound hydroxycinnamic acids, and soluble compounds such as phenolic acids, phenypropanoids, flavonoids and quinones (Rispail et.al., 2005).

In previous times, scientists dealt with plants due to their roles of polyphenols on plant physiology and their colour and flavor properties, but in recent

years they pointed out their effects on health, especially the functions of antioxidant and radical scavenging (Güven et al., 2010).

"Phenolic compounds possess redox properties, which allow them to act as reducing agents, hydrogen donators and singlet oxygen quenchers, finally leading them to be antioxidants." (Laghari et al., 2011). Phenolics represent the most abundant and widely spread class of plant natural products, playing important functions for the plant, including support of the plant body, protection against biotic and abiotic stresses.

For humans, plant phenolics are the basis of several plant-derived drugs and recently they have attracted much attention because of their protective features against cancer, cardiovascular and neurodegenerative diseases, associated with their antioxidant activity (Truong et. al., 2007).

Different parts of plants, especially their flower part, contains a great variety of natural antioxidants. These antioxidants are classified as phenolic acid, flavonoids, and many other phenolic compounds (Kaisoon et al., 2011).

1.1.1.1. Simple Phenols and Phenolic Acids

Phenolic acids are simple compounds of non-flavonoid family constituting a large group of phenolic compounds in plant kingdom. Recently, phenolic acids have gained attention due to their antioxidative, antiinflammatory, antimutagenic, and anticarcinogenic properties as well as their ability to modulate some key enzymatic functions in the cells (Liyana-Pathirana et. al., 2006).

Phenolic acids are found to be strong antioxidants capable of preventing, or delaying the rate of oxidation, a free radical chain reaction which takes place in autoxidisable materials (Tarnawski et al., 2006).

Phenolic acids may form both ester and ether linkages owing to their biofunctional nature through reactions involving their carboxylic and hydroxyl groups, respectively.

The antioxidant activities of phenolic acids and their ester forms depend on the amount of hydroxyl groups in molecules which being strong with steric effect (Yıldız, 2007). On the other hand, Hertog (1993) indicated that phenolic compounds show relieving effect on edema and antiallergic effect.

In case of taking overdose of phenolic compunds, they show toxic effect and cause to throat cancer but if they are taken steady, shows improving effects on protection mechanism of human body, decreasing risk of cancer and reducing toxicity (Yıldız&Baysal, 2003).

1.1.1.2. Hydroxybenzoic Acids and Hydroxycinnamic Acids Derivatives

Hydroxybenzoic and *hydroxycinnamic acids* are predominant phenolic acids found in plants. Phenolic acids, which are important derivatives of these compounds, form with connecting OH and OCH_3 groups to them. Differences between their derivatives consist in the different patterns of hydroxylations and methoxylations of their aromatic rings.

Hydroxybenzoic acids (BA) are commonly present in bound form. Hydroxybenzoic acids are also found in the form of sugar derivatives (Schuster and Hemann, 1985). The antioxidant effect of dihydroxybenzoic acid derivatives depend on position of hydroxyl groups. While *o*- and *m*- positions have higher activity (such as 2,3-dihydroxybenzoic acid), *m-p* positions (such as 3,4-dihydroxylbenzoic acid-protocathecuic acid) have lower activity. Gallic acid show the most active hdyroxybenzoic acid because of having three hydroxyl groups (Ho, 1992).

In general, *hydroxycinnamic acid* (CA) derivatives are the most common phenolic acids and serve as antioxidants. Hydroxycinnamic acids are found as free only in a very small amounts and mostly are found as acid derivatives.

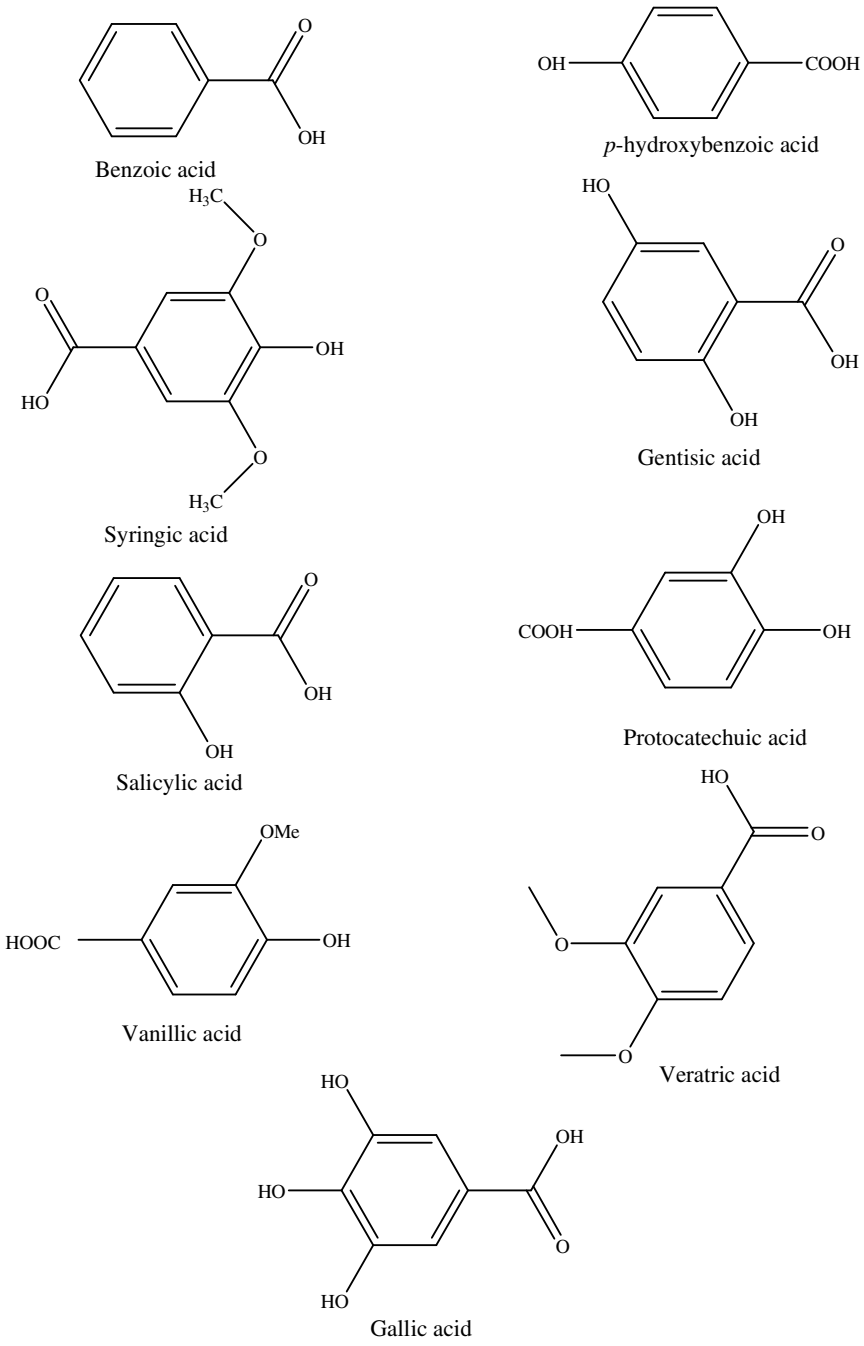


Figure 1.1. Structures of Hydroxybenzoic Acid Derivatives and their common names

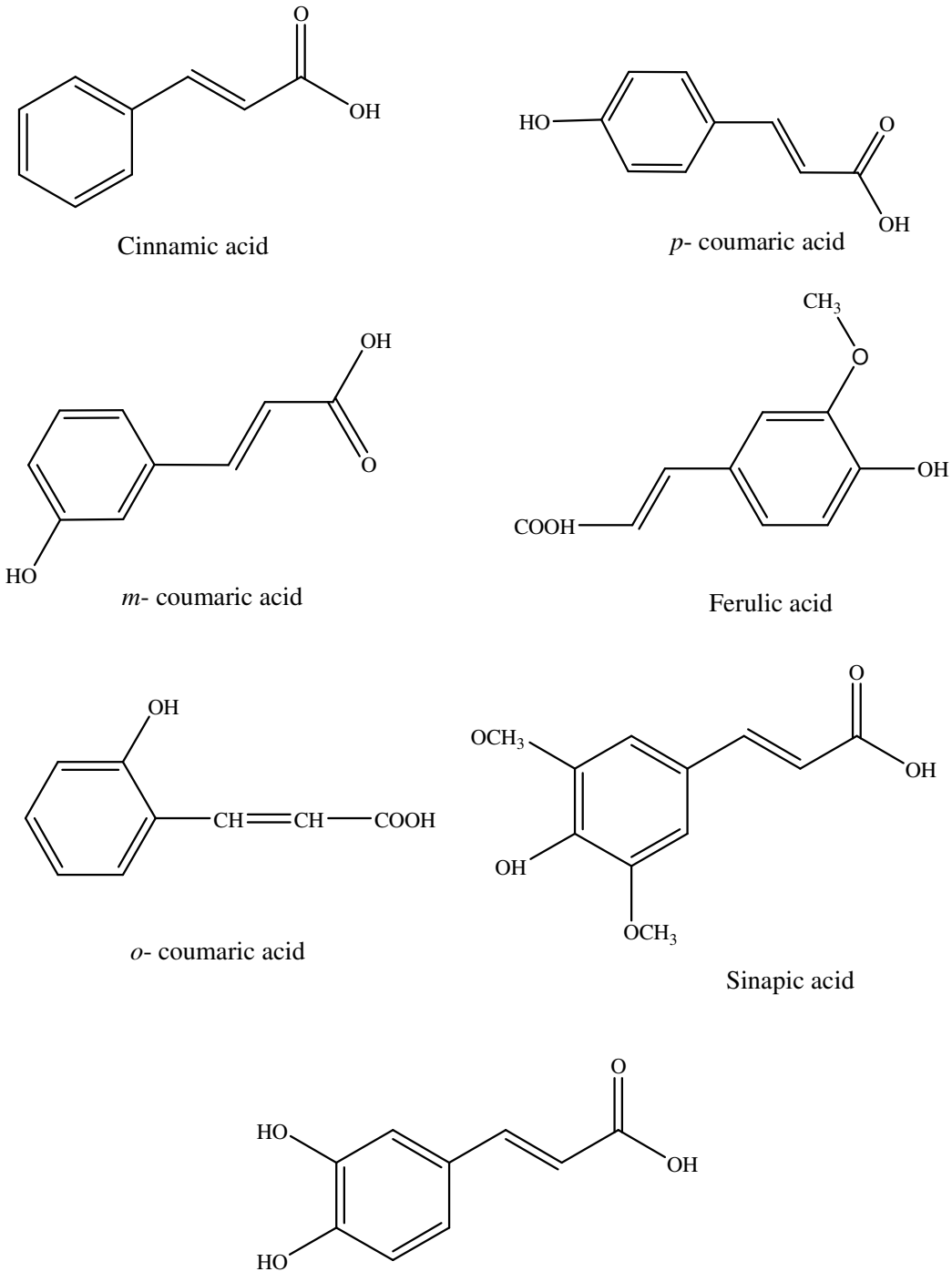


Figure 1.2. Structures of Hydroxycinnamic Acid Derivatives and their common names

1.1.1.3. Flavanoids

Since centuries, flavonoids are known as plants pigments. Flavonoids represent the most common and widely distributed group of plant phenolics. In all plants tissues, flavonoids are located in cells or surface of various plant organs. Flavonoids are one groups of secondary metabolites of plants and they occur from primary metabolites (such as carbohydrates and aminoacids) as a result of photosynthesis. The most common observed substituents in structure of flavonoids are hydroxyl groups. They can be alkylated or glycosylated easily because of their reactivity properties.

Therefore, the methoxy and glycosyl derivaties of flavonoids are often common in plants. The structure of methoxyflavonoids have methoxy groups from one to seven. In nature, the most common flavonoids are observed as a form of mono-, di-, trimethoxy.

Most of the flavonoid aglycones are found in a glycosylated form in a plant cell, this is assumed to protect them from degradation, to reduce their toxic effects and to aid their transport accross membranes by increasing their water solubility (Jones and Vogt, 2001).

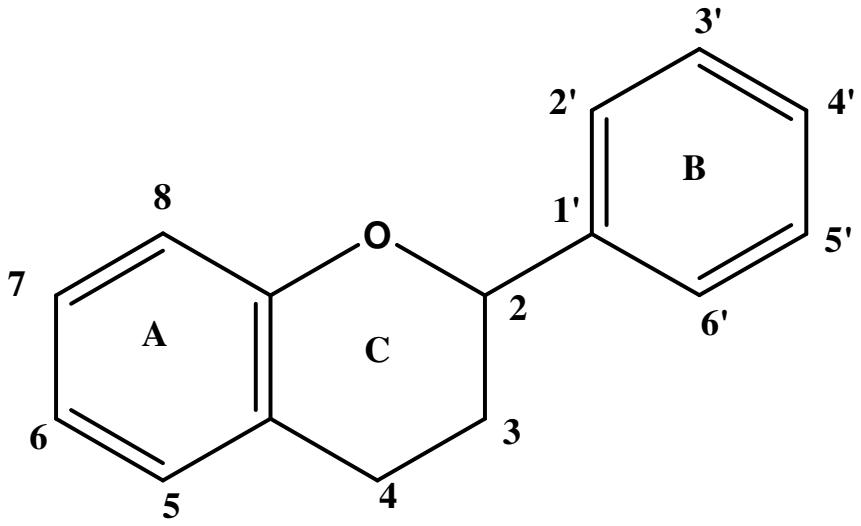


Figure 1.3. Basic flavonoid structure

The basic flavonoid structure is the flavan nucleus, which consists of 15 carbon atoms arranged in three rings (C6-C3-C6), which are labeled A, B, and C (Fig. 1.3). The major flavonoid classes include *anthocyanidins, flavan-3-ols (catechins), flavanones, flavones, flavonol, isoflavones, and flavanonols.* Differences between the different flavonoids; the number of connected hydroxyl groups, degree of unsaturation and due to oxidation level of triple carbon segment.

Anthocyanins, glycosides of anthocyanidins, are the most important water-soluble plant pigment classes which give the color (i.e. red, blue, and violet) to flowers and fruits (Yıldız, 2007). Anthocyanins are named and classified by the number of sugar molecules they contain. Compounds that bind to anthocyanidins are not limited just sugars. Sometimes hydroxycinnamic acids and aliphatic acids can be bind.

Flavanols are a colourless compounds and are derivatives of reducing *flavons*. Their dihydroxy derivatives are *flavanones.* The most common and biologically active dietary flavonol is *quercetin.* The *isoflavons* are isomers of

flavones. *Flavon* and *flavon glycosides* are a compounds has light yellow colour, found in almost every plants. *Flavanones* and *flavones* are generally found together and they are bonded with certain enzymes. It can be said that in flavon-rich plants hardly ever have anthocyanins (Yıldız, 2007).

Flavonoids may be monomeric, dimeric, or oligomeric. Polymeric flavonoids, known as tannins, are divided into two groups, condensed and hydrolysable. Condensed tannins are polymers of flavonoids while hydrolysable tannins contain gallic acid.

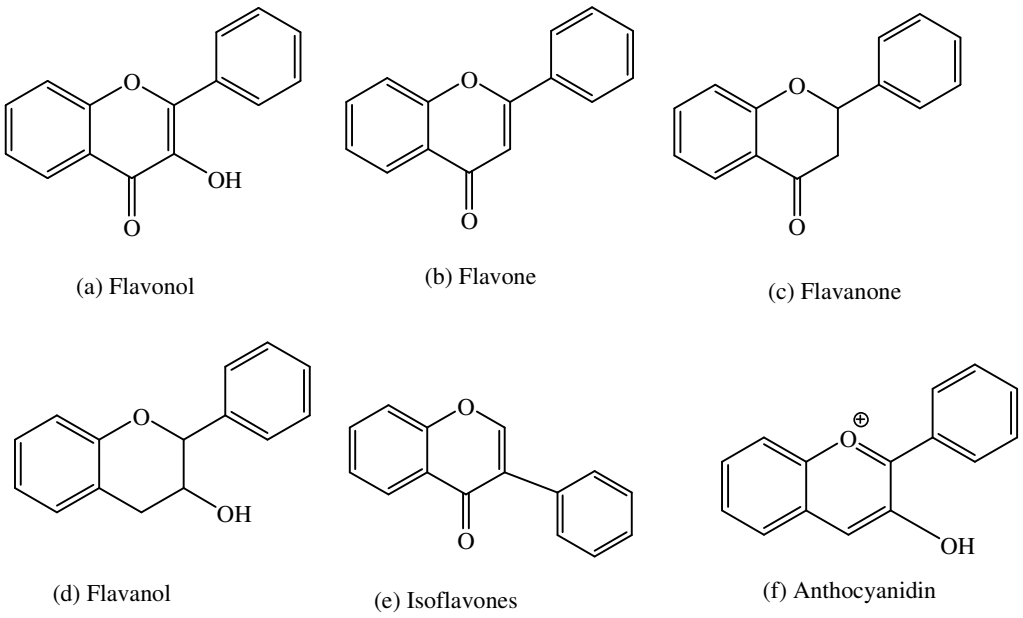


Figure 1.4. General formulas of major flavonoids classes

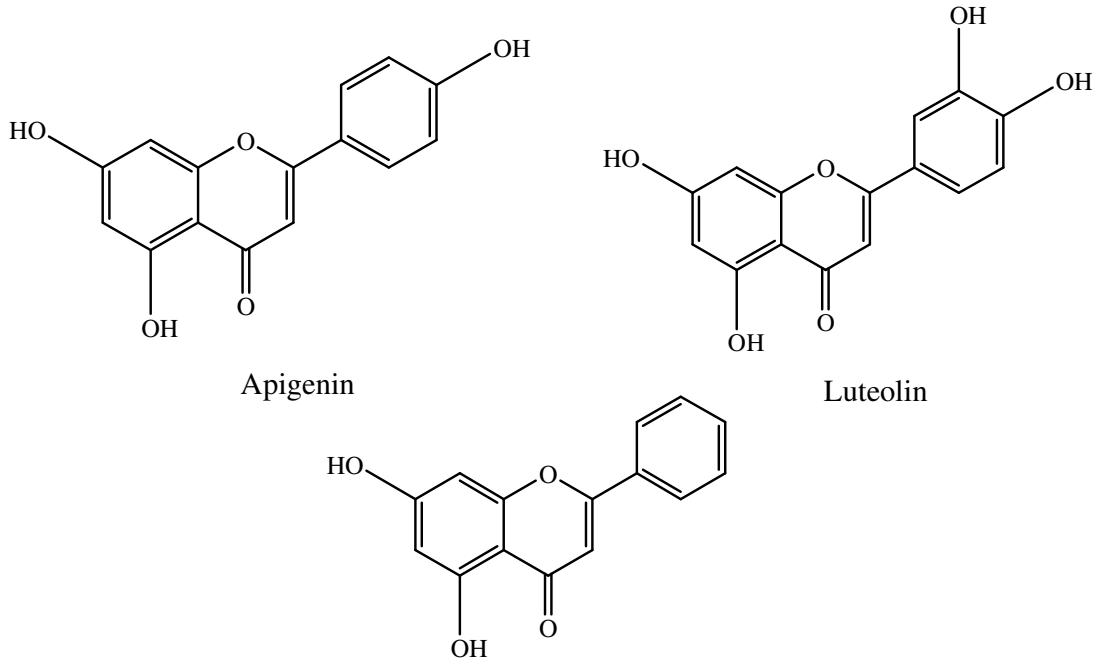
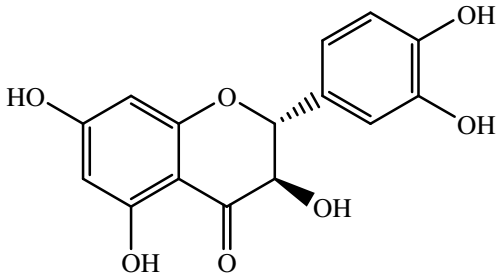
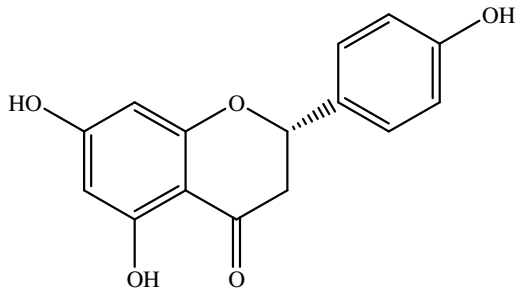


Figure 1.5. Structures of some known flavones and their common names

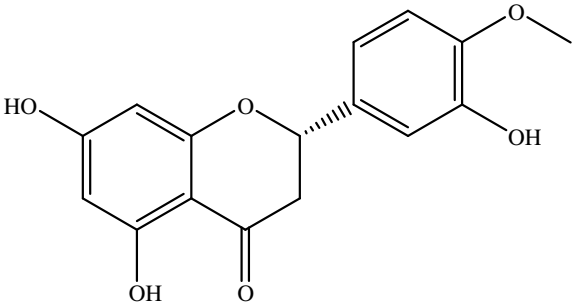


Taxifolin

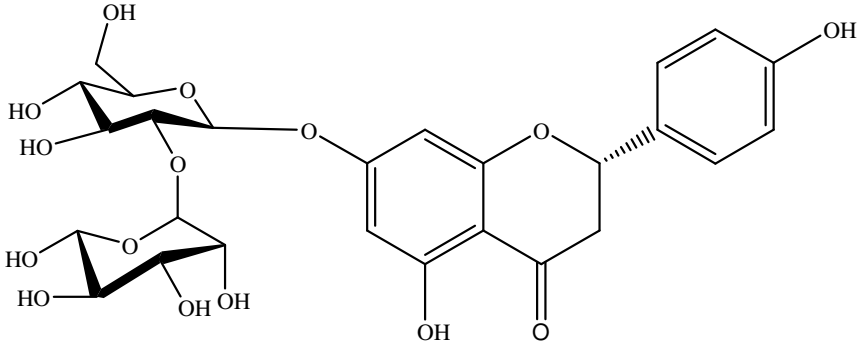
Figure1.6. Structure of a flavanonol and its common name



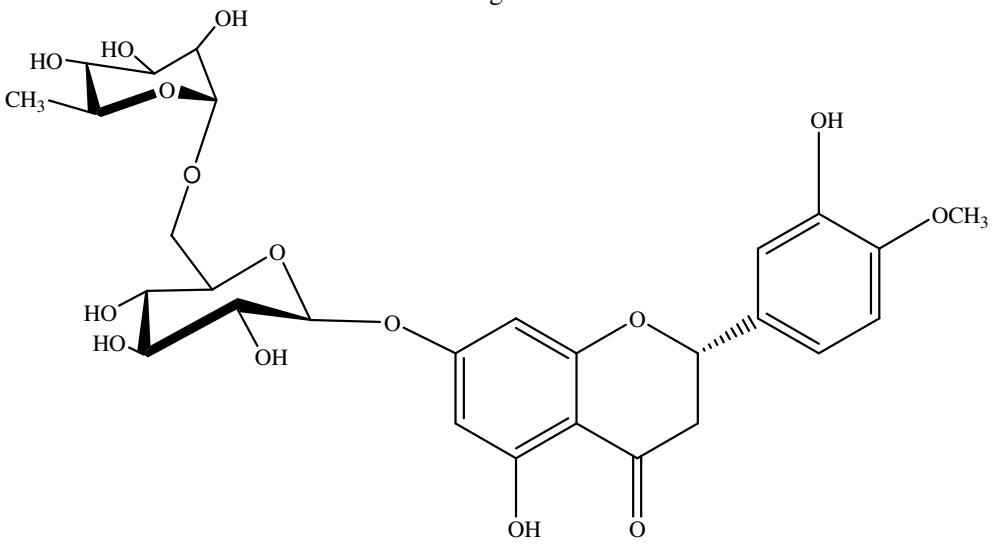
Naringenin



Hesperetin

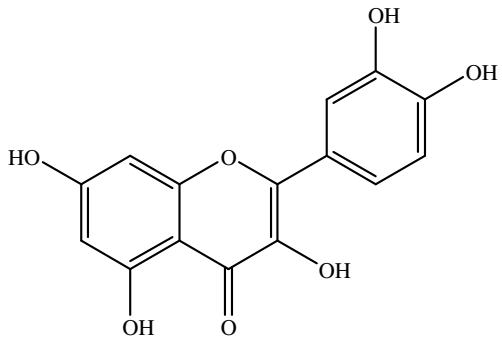


Naringin

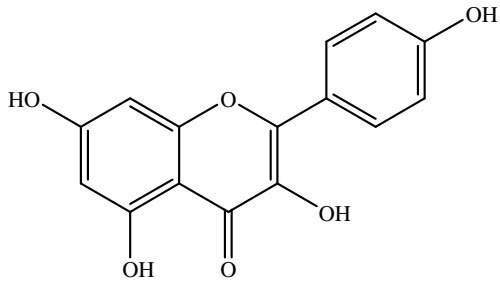


Hesperidin

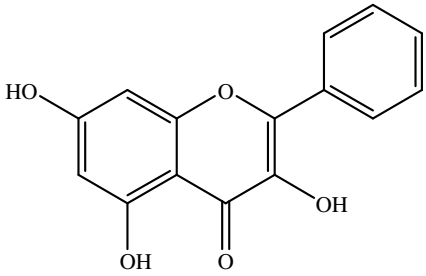
Figure 1.7. Structures of some known flavanones and their common names



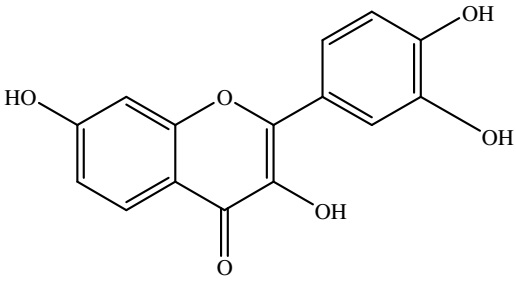
Quercetin



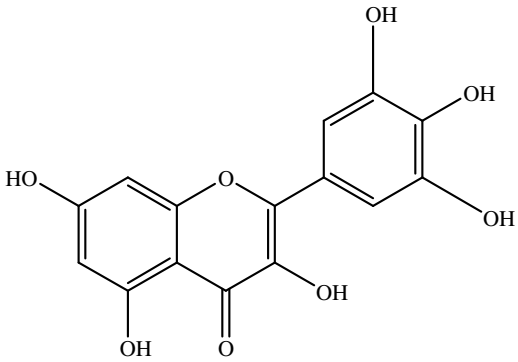
Kaempferol



Galangin



Fisetin



Myricetin

Figure 1.8. Structures of some known flavanols and their common names

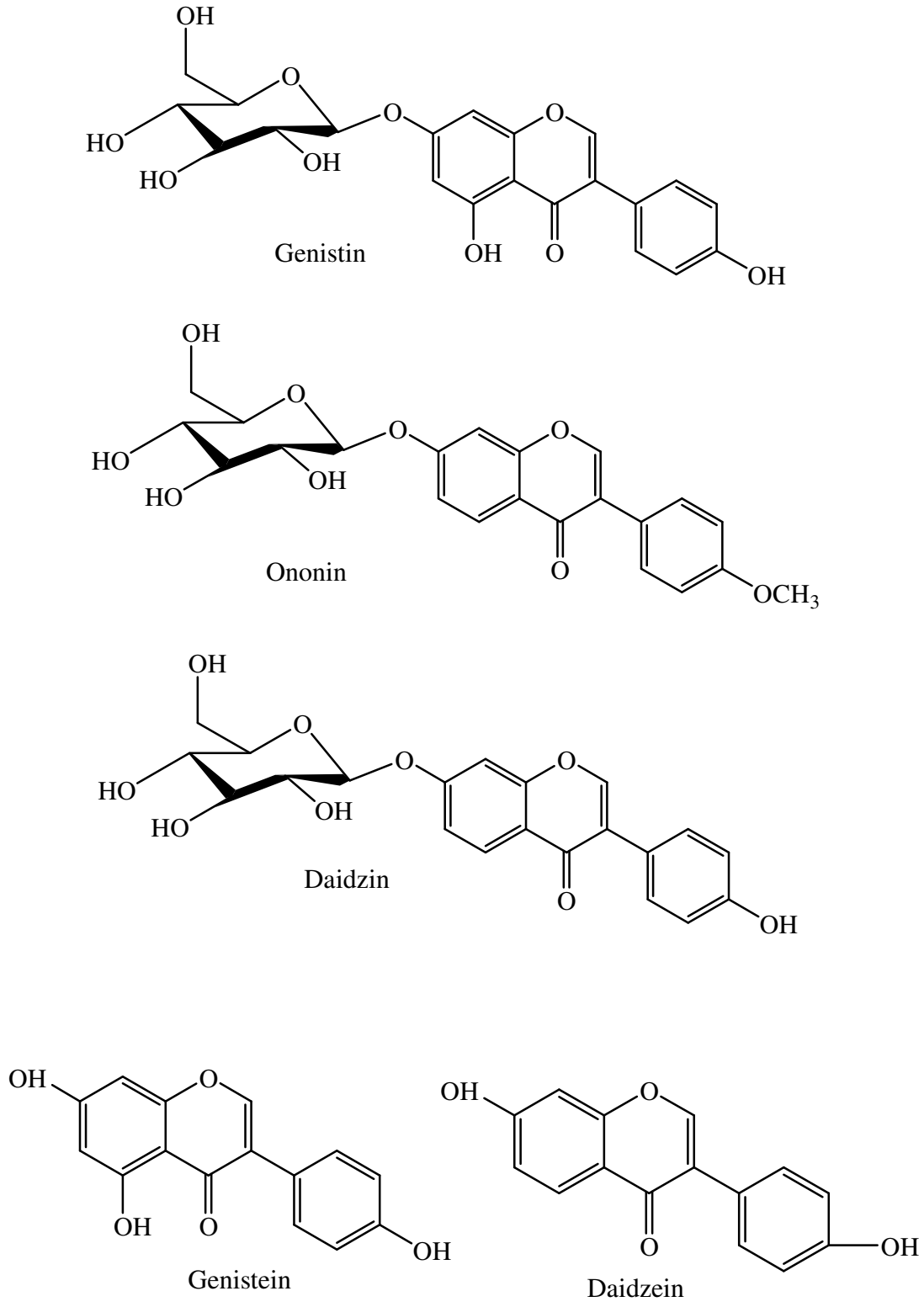


Figure1.9. Structures of some known Isoflavones and their common names

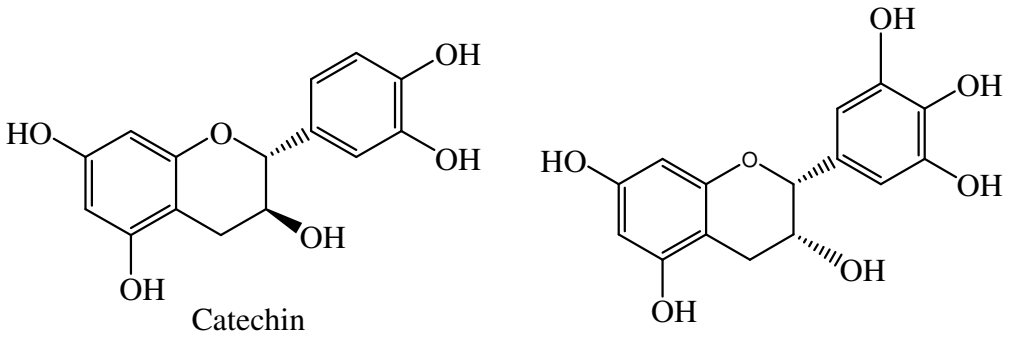


Figure 1.10. Structures of some known flavon-3-ols and their common names

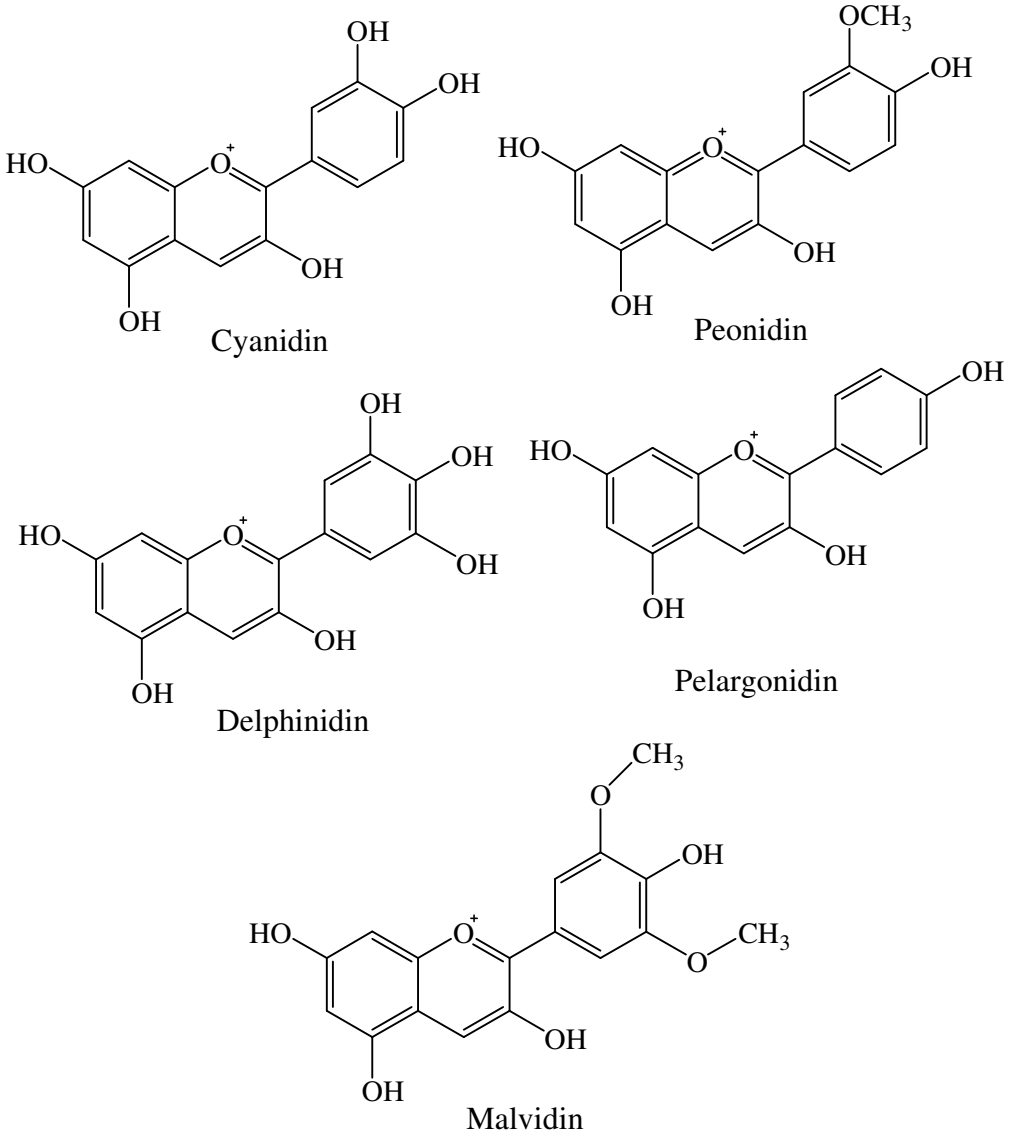


Figure 1.11. Structures of some known anthocyanidins and their common names

The first study of the biologically active flavonoids were published by Rusznyak and Szent-Gyorgyi in 1936 (Güven, 2010). Flavonoids are known as a chemical compounds in which slowing the oxidation effect of some substances. Flavanoids increase the effects of vitamin C and function as antioxidants. Biological active flavonoids (bioflavonoids) known as vitamin P compounds. At the same time these flavonoids have steric effect on fissure and bleeding in capillary vessel. (Nyam news, 2005). They protect our blood vessels particularly the tiny capillaries that carry oxygen and nutrients to our cells and are believed to slow down the development of cataracts in persons who have diabetes. They are also known to be biologically active against liver toxins, tumours, viruses and other microbes, allergies and inflammation. It was found that flavonoids functioned to reduce blood-lipid and glucose and to enhance human immunity (Atoui et al., 2005).

Table 1.1. Some functions of flavonoids

FLAVONOIDS	FUNCTIONS
Hesperidin	- prevents inflammation - is a pain reliever -rises blood levels of the "good" cholestreol and lowers the bad cholestreol.
Quercetin	- reduces inflammation associated with allergies - can inhibit the growth of head and neck cancers - protects the lungs from the harmful effects of pollutants and cigarette smoke.
Resveratol	- may reduce the risk of blood clots, cancer and heart disease
Anthocyanins	- potent antioxidants - improve balance coordination and short term memory in the elderly.
Anthocynanins	-helps to prevent urinary tract infections.

Due to their protection effect from UV rays properties, some flavonoids are utilized in cosmetic as an additive. Quantitative determination of individual flavonoid glycosides is difficult because most standards are not commercially available.

Flavonoids show flouresans in UV light and also they change colour with NH_3 due to their phenolic structures. Through these properties, they can be analyzed

with Thin Layer Chromatography and Paper Chromatography by UV light (254 and 366 nm) (IŞIK, 2005).

1.2. METHODS OF PLANT ANALYSIS

Because of the varied pharmacological activity of phenolic acids plants containing phytochemicals are widely used in phytotherapy. Therefore, their determination and identification in plant material is very important aspect of phytochemical analysis.

1.2.1 Collection, Drying and Grinding Process of Plants

Scientific field study, is the first work for a plant analysis. Informations obtained from this stage have a quality and prepare a gound for futher studies. Before going to collect plant, preliminary research must be done. Every prominent details mut be noted by plant collector during the plant collection process.

After collection process, collected plants are dried in the shade for a few days. Drying in the shade process is quite important point for prevention of phytochemicals in plants. If plants are not dry enough, they can be dried in an oven at about 40 ^{0}C for 72 hours.

The basic principle is to grind the plant material, which increases the surface area for extraction thereby increasing the rate of extraction. Before the

starting plant analysis, dried plant samples are ground in a mortar. So that the plant powders are obtained by grinding process. As long as plant samples are not dried enough, good grinding process can not be done.

1.2.2 Methods of Extraction and isolation

1.2.2.1.General Methods of Extraction

Extraction is a method to seperate any substance and remove dissolved and also unwanted impurity from solid or liquid mixtures. Fresh or dried plant materials can be used as a source for the extraction of secondary plant components.

Organic substances are usually more soluble in organic solvents than in water. Taking advantage of this, organic substances can be taken into any organic solvent. If electrolyte substances ($CaCl_2$, NaCl) are added into aqueous solution, the solubility of organic matter in aqueous solution decreases. Generally ether, chloroform, benzene, petroleum benzene are used as an organic solvent in extraction method. Selection of appropriate solvent depend on the solubility of the substance which are extracted. The basic purpose of extraction is that both seperates the organic subtance and expend less organic solvent. A large amount of substances can be taken into organic solvent phase by repeating extraction several times with the same amount of organic solvent each time.

If extraction process is done into seperatory funnel, its size must be equal to nearly two times volume of aqueous solution. During the extraction process, undesirable situation may occur such as emulsion. In this case,

- aqueous phase is saturated with sodium chloride
- add a few drops of organic solvent which is used.
- expect for a while
- air is blown into the aqueous phase by pipette.

Effect of extracted plant phytochemicals depends on;

1. The nature of plant material
2. Its origin
3. Degree of processing
4. Moisture content
5. Particle size

Results of some studies showed that the extraction yield of phenolic and flavonoid contents are greatly depending on the solvent polarity. Properties of a good solvent in plant extractions includes, low toxicity, ease of evaporation at low heat, promotion of rapid physiologic absorption of the extract, preservative action, inability to cause the extract to complex or dissociate. The choice of solvent is influenced by what is intended with the extract. The various solvents that are used in the extraction procedures are water, acetone, alcohol, chloroform, ether. Less polar flavonoids (e.g., isoflavones, flavanones, methylated flavones, and flavonols) are extracted with chloroform, dichloromethane, diethyl ether, or ethyl acetate, while

flavonoid glycosides and more polar aglycones are extracted with alcohols or alcohol–water mixtures. Glycosides have increased water solubility and aqueous alcoholic solutions are suitable. (Marston & Hostettmann, 2006).

Table 1.2. Solvents used for active component extraction (Tiwari et al., 2011).

WATER	ETHANOL	METHANOL	CHLOROFORM	ETHER	ACETONE
Anthocyanins	Tannins	Anthocyanins	Terpenoids	Alkaloids	Phenol
Starches	Polyphenols	Saponins	Flavonoids	Terpenoids	Flavonols
Tannins	Flavonol	Terpenoids		Coumarins	
Saponins	Terpenoids	Tannins		Fatty acids	
Terpenoids	Sterols	Flavones			
	Alkaloids	Polyphenols			

General characteristics of the most commonly used solvents which should be known in plant analysis are given in Table 1.3 :

Table 1.3. Properties of some solvent used in plant analysis

FLAMMABLE	VERY FLAMMABLE	TOXIC	EXPLOSIVE
Acetone	Petroleum benzene	Benzene	Ether
Benzene	Acetone	Carbontetrachloride	
Butanol	Ether	Chloroform	
Ethylacetate		Methanol	
Ethanol		Dichloromethane	
Methanol			
Petroleum benzene			
Toluene			

Different extraction techniques, such as infusion, maceration, Soxhlet extraction, digestion, *etc.*, are widely used for obtaining extractable substances from different parts of a number of plants .

1.2.2.1.1. Maceration

Samples are allowed to kept in a contact with organic solvent in a stoppered container for a defined period with frequent agitation until soluble matter dissolved. Generally this process is carried out at 15-20°C. Insoluble part removed by filtration.

1.2.2.1.2. Infusion

In general, infusion is a method for preparing herbal tea. Fresh infusions are prepared by macerating the solids for a short period of time with either cold or boiling water. In this process, boiling water is poured over the sample. Although infusion extraction is done by this way, actually it does not depend on the boiling. Very-fine partical does not preffered for infusion because it can be occur problem during the filtration step.

1.2.2.1.3. Digestion

This is a kind of maceration in which gentle heat is applied during the maceration extraction process. It is used when moderately elevated temperature is not objectionable and the solvent efficiency of the menstruum is increased thereby.

1.2.2.1.4. Decoction

In this process, the sample is boiled in a specified volume of water for a defined time; it is then cooled and strained or filtered. This procedure is suitable for extracting water-soluble, heat-stable constituents.

1.2.2.1.5. Percolation

This is the procedure used most frequently to extract active ingredients in the preparation of tinctures and fluid extracts. A percolator (a narrow, cone-shaped vessel open at both ends) is generally used. The solid ingredients are moistened with an appropriate amount of the specified menstrum and allowed to stand for approximately 4 h in a well closed container, after which the mass is packed and the top of the percolator is closed. Additional menstrum is added to form a shallow layer above the mass, and the mixture is allowed to macerate in the closed percolator for 24 h. The outlet of the percolator then is opened and the liquid contained therein is allowed to drip slowly. Additional menstrum is added as required, until the percolate measures about three-quarters of the required volume of the finished product. The marc is then pressed and the expressed liquid is added to the percolate. Sufficient menstrum is added to produce the required volume, and the mixed liquid is clarified by filtration or by standing followed by decanting.

1.2.2.1.6. Hot Continuous Extraction (Soxlet Extraction)

Soxhlet extractor is the most commonly used tool for extracting solid herbal samples. Soxhlet extraction is frequently used to isolate flavonoids from solid samples. (Stalikas, 2007). In most cases, aqueous methanol or acetonitrile is used as solvent. The materials required for soxhlet extraction; thimble, condenser, mantle, siphon tube and round bottom flask.

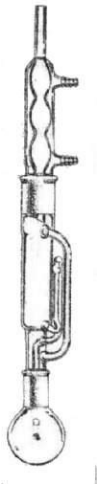


Figure 1.12. Soxhlet Extraction Apparatus

Powdered plant material can also be extracted in a Soxhlet apparatus, first with hexane, for example, to remove lipids and then with ethyl acetate or ethanol to obtain phenolics. This approach is not suitable for heat-sensitive compounds. (Marston & Hostettmann, 2006).

In this method, the finely ground plant sample is placed in a “thimble” made of strong filter paper. The extracting solvent in flask is heated, and its vapors condense in condenser. The condensed extractant drips into the thimble containing

the plant sample, and extracts it by contact. When the level of liquid rises to the top of siphon tube, the liquid siphons into flask. This process is continuous and is carried out until a drop of solvent from the siphon tube does not leave residue when evaporated.

The advantage of this method, compared to previously described methods, is that large amounts of plant can be extracted with a much smaller quantity of solvent. This effects tremendous economy in terms of time, energy and consequently financial inputs. Different solvents can be used in extraction process. Addition of co-solvents as a modifier to increase the polarity of liquid phase is possible. Soxhlet extraction mainly depends on characteristic of matrix and of size of particles as the internal diffusion may be a limiting step during extraction. The most important advantage of conventional Soxhlet extraction technique is its continuous character. The sample has a contact with the fresh portion of the solvent. After finishing the process of extraction no filtration is required. It is also very cheap and simple method where small experience is required.

The most significant disadvantages compared with other techniques are the long time of extraction, poor penetration of the matrix by the solvent, and the large amount of solvent required, which is very expensive and causes environmental problems.

1.2.2.2. Hydrolysis

Hydrolysis of the esters and glycosides to a free phenolic acid have been a strategy employed to simplify the analysis. There are two main procedures to cleave the ester and glycoside bond reported in the literature, acidic hydrolysis (for glycosides), and alkaline hydrolysis (for esters).

Although reaction time and temperature for the acidic hydrolysis condition vary a great deal, this general method involves treating the plant extract itself with inorganic acid (HCl) at reflux or above reflux temperatures in aqueous or alcoholic solvent (methanol being the most common, acid ranged from 1 to 2 M aqueous HCl, and the reaction times ranged from 30 min to 1 h) (Stalikas, 2007).

1.2.3. Methods of Seperations and Identification (Chromatographic Methods)

Chromatography is a method for separating different chemical compounds from a mixture. It is based on some principles such as adsorption, solubility, ion-exchange or molecular elimination. Substances which are separated by chromatography can be easily identified and isolated. Therefore this method also called as purification method. It can be said that common points are stationary phase (i.e, solid or liquid) and mobile phase (i.e, liquid or gas) in all chromatographic methods.

The nature of solvent plays an important role in the development of chromatography. The following criteria may be adopted for the choice of the solvent;

- The solvent should not react chemically with any of the components of the sample mixture
- The composition of solvent mixture should not change with time. It means that none of its components should be volatile.
- The solvent should not interfere with the detection of spots.
- The distribution ratio should be independent of solute concentration

There is a large variety of solvent systems, adsorbents, and chromatographic techniques employed in analysis of phenolic acids.

1.2.3.1. Surface (Planar) Chromatography

Chromatographic methods can be seen as rich analysis field with different application methods. Planar chromatography a separation technique in which the stationary phase is present as or on a plane. The plane can be a paper, serving as such or impregnated by a substrate as the stationary bed (paper chromatography, PC) or a layer of solid particles spread on a support e.g. a glass plate (thin layer chromatography, TLC).

1.2.3.1.1. Thin Layer Chromatography (TLC)

Thin layer chromatography is different from paper chromatography in that a flat surface of glass, metal or plastic coated with adsorbent such as silica gel, alumina

or any other material, is used.

Although a lot of methods have been reported for the investigation of phenolics, thin layer chromatography (TLC), owing to its numerous advantages, is still often employed. TLC is fast, inexpensive, and it can be used as a preliminary method in analysis of these compounds preceding the other methods, e.g., HPLC.

The mixtures of three, four, or even five solvents with various polarity were used as the mobile phases. Typically, nonpolar or weakly polar solvent (e.g., benzene, heptane, toluene), medium polar diluent such as chloroform or ethyl acetate, and strongly polar modifier (e.g., methanol, water) were mixed to obtain the best selectivity of separation. Selected mobile phase must not chemically affect or dissolve the stationary phase because this leads to modification of properties of the chromatographic system and it must not produce chemical transformations of the separated compounds.

The multicomponent mobile phase applied in TLC must be used only once, not repeatedly, because the volatility of solvents produces a continuous modification of quantitative composition of the mobile phase, which negatively affects the chromatographic repeatability. The mobile phase must be easily eliminated from the adsorbent layer and must be compatible with detection methods. The reproducibility can be greatly affected by the conditions and the time of preservation of the mobile phase solution.

Now-a-days, the thin layer plates and sheets incorporating fluorescent dyes in powdered adsorbent, are commercially available. When these are held under ultraviolet radiation, dark spots glow where sample spots occur due to fluorescent

substances. If it is not possible, a chemical reagent is sprayed onto TLC plate to make certain spots. In TLC, ultraviolet (UV) absorption of the mobile phase solvents does not play a significant negative role in detection and quantification of the analytes, because the mobile phase is evaporated from the plate prior to the detection.

Thin-layer chromatography (TLC) on silica gel is very favourable for the qualitative analysis of flavonoids and phenolic acids.

1.2.3.1.2. Paper Chromatography (PC)

It is one of the oldest and simplest techniques for qualitative analysis though it can also be used for quantitative determination. The technique is based on the movement of solvent phase in upward or downward direction by gravity and accordingly, it can be categorized as ascending or descending PC.

In paper chromatography, the sample mixture is applied to a piece of filter paper, the edge of the paper is immersed in a solvent, and the solvent moves up the paper by capillary action. Components of the mixture are carried along with the solvent up the paper to varying degrees, depending on the compound's preference to be adsorbed onto the paper versus being carried along with the solvent. The paper is composed of cellulose to which polar water molecules are adsorbed, while the solvent is less polar, usually consisting of a mixture of water and an organic liquid. The paper is called the stationary phase while the solvent is referred to as the mobile phase.

1.2.3.1.3. Preparative Layer Chromatography (PLC)

The procedure of PLC is similar to TLC, the major differences being the use of thicker layers. Layer thicknesses range from 500μm (0.5 mm) to 10 mm but layers measuring between 0.5 and 2 mm give the best results. PLC is faster and more convenient than classical column chromatography and it is much less expensive and less complicated High Performans Column Liquid Chromatography (HPLC).

1.2.3.2. Column Chromatography

1.2.3.2.1. Gas Chromatography

Gas chromatography (GC) is used mostly for analysis of volatile, unpolar (hydrophobe) compounds such as the components of essential oils and other unpolar constituents itself (different kind of terpenes).

Now it is usually coupled with mass spectrometry (GC–MS). This technique allows the measurement of the molecular weight of a compound and once a molecular ion has been identified, it is possible to measure this ion accurately to ascertain the exact number of hydrogens, carbons, oxygens, and the other atoms that may be present in the molecule. This will give a molecular formula. Several techniques are available in MS, of which electron impact is widely used. This techniques gives good fragmentation of the molecule and is useful for structure

elucidation as the fragments can be assigned to functional groups present in the molecule.

The disadvantage of this technique is that molecular ions are sometimes absent. Softer techniques such as chemical ionization (CI), electrospray ionization (ESI), and fast atom bombardment (FAB) mass spectrometry ionize the molecule with less energy; consequently, molecular ions are generally present, but with less fragmentation information for structure elucidation purposes.

1.2.3.2.2. High Performance Liquid Chromatography (HPLC)

HPLC is used routinely in phytochemistry to pilot the preparative-scale isolation of natural products and to control the final purity of the isolated compounds. The analytical sensitivity of the technique, particularly when is coupled with UV detection such as photodiode array (DAD), enables the acquisition of UV spectra of eluting peaks from 190 to 800 nm. The flow rates of this system are typically 0.5 –2.0 mL=min and sample loading in the analytical mode allows the detection and separation of plant extract components. With DAD UV detection, even compounds with poor UV characteristic can be detected.

HPLC is a highly sensitive technique when is coupled to electronic library searching of compounds with known UV spectra. Modern software enables the UV spectra of eluting peaks to be compared with spectra stored electronically, thereby enabling early identification of known compounds or, usefully, the comparison of novel compounds with a similar UV spectrum, which may indicate structural

similarity. It is also possible to increase the size of these electronic libraries and improve the searching power of the technique. HPLC is a powerful technique for fingerprinting of biologically active extracts and for comparisons that can be drawn with chromatograms and UV spectra stored in an electronic library. This is currently very important for the quality control of herbal medicines.

With HPLC, which can be run in fully automated mode, and carousel autosamplers, it is possible to analyze tens to hundreds of samples. HPLC apparatus are computer-driven and chromatographic run may be programmed. Computers are also used for the storage of the chromatographic data. Modern technology can be applied for column sorbents, including standard sorbents such as silica and alumina, reversed phase C18 C8, phenyl stationary phases, and modern sorbents such as polar bonded stationary phases (CN-, Diol-, aminopropyl-silica), chiral stationary phases, gel size–exclusion media, and ion exchangers. This versatility of stationary phase has made HPLC a highly popular method for bioassay-guided isolation.

Separation of chemical compounds is carried out by passing the mobile phase, containing the mixture of the components, through the stationary phase, which consists of a column packed with solid particles. The cause for retention is physical and chemical forces acting between the solute and the two phases, on the chromatographic column. The reason for retention is the difference in the magnitude of forces; this results in the resolution and hence separation of the individual solutes. The separation of compounds occurs by distribution of solutes between the two phases.

An HPLC system contains the following components:

Reservoir: This is meant for the mobile solvents. Acetonitrile, methanol, heptane, isopropanol and cyclohexane are the organic modifiers most commonly used. Trifluoroacetic acid, heptafl uorobutyric acid, phosphoric acid and triethylamine phosphate are ion-pairing reagents for beter chromatographic results. All tubing and fittings should be chemically inert. Solvent must be fi ltered through a 0.45-μm filter unit.

Degasser: In analytical operations, the mobile phase should be free of air bubbles. For this purpose, a degasser is used.

Pumps: These are devices that deliver the mobile solvent at a controlled flow rate to the separation system. HPLC uses reciprocating pumps: a pump with a single or multiple chambers, from which the mobile phase is displaced by reciprocating pistons or diaphragms. Binary gradients are created by the selected mixing of two solvents, on a single-headed two-pump system. Accurate gradient is maintained by microprocessor control.

Injector/autosampler: This device introduces a liquid sample into the mobile phase or onto the chromatographic bed. An autosampler can perform repeated functions without operator attendance, and thus is a labor-saving device.

Column: Silica and modified silica columns are available for various applications. Examples are octyl (C8), octadecyl (C18), phenyl (C6H5), and cyno (CN) columns.

Guard column: This is used to protect the main column.

Detectors: No universal detector is available for all molecules. However, according to the characteristic of the molecules investigated, various detectors are used.

Fraction collector: This device collects the fractions containing the molecules of interest during the chromatographic run.

Records: A computer is used for chromatographic data acquisition.

Numerous variables affect an HPLC analysis. *Mobile phase composition* is vital parameter that affects the resolution, retention time and peak area. Pumps contribute the most towards variations of results, as precise composition of mobile phase and flow rate can only be assured by accurate pumps. *Gases dissolved in the mobile phase* are a source of flow-rate inaccuracies and errors in detector response. *Retention time variations* are often discussed to know the tolerable limits. The retention time is affected by flow rate, column temperature, mobile phase composition and integration. An error in flow rate leads to changes in the retention time to the same extent. *Small variations in column temperature* have more significant effects on retention time. Several more factors, *like injection volume*, *connecting tubing, end fittings* and *detector volume*, also have bearings on the final results. Large injection volume and quantity of analyte result in broadening of the peak. Preparing the sample in the mobile phase produces the best result and should be taken as the first choice.

In the past 20 years the on-line combination of HPLC and MS has become a routinely applicable, extremely powerful and user-friendly analytical tool.

1.2.3.2.3. Capillary Elctrophoresis (CE)

Electrophoresis has been known for many years but gain widespread attention when Jorgenson and Lukacs demonstrated small quartz tubes. Since that time capillary electrophoresis (CE) has been very popular method for separation of active compounds. The separation is obtained by differential migration of solutes in an electric field, driven by two forces, the electrophoretic migration and the electro-osmotic flow. Migration into discrete zones is due to differences into electrophoretic mobilities which is related to the mass-to-charge ratio. In a typical CE instrument, analytes are introduced at the anode and are detected at the cathode.

The equipment for CE is very simple and cheap. A typical capillary tube is 25–75 cm long and made of silica. The most widely used detector for monitoring of plant metabolites is an ultra-violet (UV) spectrophotometer.

CE method permits the use of very small amounts of sample because of small dimensions of the capillary. The important advantage is large surface to volume ratio that allows for application of large electric potential across the capillary.

CE is a micro-analytical method which provides advantages in terms of speed, high efficiency, low cost, and simplicity. It can be applied for the separation of different classes of compounds using the same equipment, changing only the composition of the buffer. CE can be used on-line with other techniques such as HPLC, NMR, MS. It needs small amount of sample and buffer.

CE are used for seperation of proteins, peptides, amino acids, nucleic acids, inorganic ions, organic bases and organic acids.

1.2.4. Detection of Some Phytochemicals1

1.2.4.1. Detection of glycosides: Extracts were hydrolysed with dil. HCl, and then subjected to test for glycosides.

Modified Borntrager's Test: Extracts were treated with Ferric Chloride solution and immersed in boiling water for about 5 minutes. The mixture was cooled and extracted with equal volumes of benzene. The benzene layer was separated and treated with ammonia solution. Formation of rose-pink colour in the ammonical layer indicates the presence of anthranol glycosides.

Legal's Test: Extracts were treated with sodium nitropruside in pyridine and sodium hydroxide. Formation of pink to blood red colour indicates the presence of cardiac glycosides.

Liebermann's test: Crude extract was mixed with each of 2ml of chloroform and 2ml of acetic acid. The mixture was cooled in ice. Carefully concentrated H_2SO_4 was added. A colour change from violet to blue to green indicated the presence of steroidal nucleus, i.e., glycone portion of glycoside.

Salkowski's test: Crude extract was mixed with 2ml of chloroform. Then 2ml of concentrated H_2SO_4 was added carefully and shaken gently. A reddish brown colour indicated the presence of steroidal ring, i.e., glycone portion of the glycoside.

Keller-kilani test: Crude extract was mixed with 2ml of glacial acetic acid containing 1-2 drops of 2% solution of $FeCl_3$. The mixture was then poured into another test tube containing 2ml of concentrated H_2SO_4. A brown ring at the interphase indicated the presence of cardiac glycosides.

1.2.4.2. Detection of phenols

Ferric Chloride Test: Extracts were treated with 3-4 drops of ferric chloride solution. Formation of bluish black colour indicates the presence of phenols.

1.2.4.3. Detection of flavonoids

Alkaline Reagent Test: Extracts were treated with few drops of sodium hydroxide solution. Formation of intense yellow colour, which becomes colourless on addition of dilute acid, indicates the presence of flavonoids.

Lead acetate Test: Extracts were treated with few drops of lead acetate solution. Formation of yellow colour precipitate indicates the presence of flavonoids.

Shinoda test: Crude extract was mixed with few fragments of magnesium ribbon and concentrated HCl was added drop wise. Pink scarlet colour appeared after few minutes which indicated the presence of flavonoids.

CHAPTER II

2. MATERIAL AND METHODS

2.1. Plant Materials

The source plants *Silene compacta FISCHE, Digitalis lamarcki, Xeranthemum annuum L., Epilobium hirsutum L.* and *Epilobium angustifolium L.* used in this study is widespread in Bolu province.

Silene compacta FISCHE, Xeranthemum annuum L.and Epilobium angustifolium L. were collected from roadside of Bolu-Ankara province (Turkey), *Digitalis Lamarcki* was collected from nearby Kıbrıscık and *Epilobium hirsutum L.* was collected from roadside of Gölcük. (Kıbrıscık and Gölcük are districts of Bolu).

Plants were collected in July 2010 and 2011. The botanical identifications were made by Assist. Prof. Nursel İkinci (Department of Biology, Abant Izzet Baysal University, Bolu, Turkey).

Above-mentioned plant samples were seperated form their stalks and flower parts were dried in the shade. After drying process, shade-dried flower samples were grinded in the mortar and was stored properly for further analysis.

The genus *Epilobium* is widely distributed all over the world and consists of over 200 species, with the most common being *Epilobium angustifolium L.* (Onar et al., 2011). Many different members of the genus *Epilobium* have been utilize in folk medicine as curative for prostate disease (Hiermann et al., 1986). The herb is rich in polyphenolic compounds such as flavonol-3-*O*-glycosides, phenolic acids, and ellagitannins (Bazylko et al., 2007). Ellagitannins (plant polyphenols) are one of the classes of bioactive compounds present in *Epilobium* species (Schepetkin et al., 2009). The herb is rich in polyphenolic compounds such as flavonol-3-*O*-glycosides, phenolic acids and ellagitannins (Bazylko et al., 2007).

Figure 2.1.a. *Silene compacta FISCHE*

Note: Reference of fig. 4.1.a (http://yabanicicek.com/silene-compacta.php)

Figure 2.1.b. *Silene compacta FISCHE,* Bolu

Figure 2.2.a. *Digitalis lamarcki,* Bolu

Figure 2.2.b. *Digitalis lamarcki,* Bolu

Figure 2.3.a. *Xeranthemum annuum L.*

Note: Reference of fig. 4.3.a (http://bonnier.flora-electronica.com/menus/065-Composees/Xeranthemum%20annuum%205.html)

Figure 2.3.b. *Xeranthemum annuum L.,* Bolu

Figure 2.4.a. *Epilobium hirsutum L.*

Figure 2.4.b. *Epilobium hirsutum L.*

Note: Reference of fig. 4.4.a - 4.4.b (http://yabanicicek.com/epilobium-hirsutum.php)

Figure 2.5.a. *Epilobium angustifolium L.*

Note: Reference of fig. 4.5.a (http://yabanicicek.com/epilobium-angustifolium.php)

Figure 2.5.b. *Epilobium angustifolium L.,* Bolu

2.2. EXTRACTION PROCEDURES

2.2.1. Extraction for qualitative TLC tests and LC-MS studies

2.2.1.1. Soxhlet Extraction (MeOH)

Certain amount of dried and ground flowers were uniformly packed into a thimble and extracted with methanol for 48 h. The crude extract obtained in this way was taken in a beaker and dried till all the solvent got evaporated.

In the next step, certain amount of the crude extract was dissolved in 125 mL hot water and was taken into a seperatory funnel for doing extraction. The solution was extracted with petroleum benzine, chloroform ($CHCl_3$), followed by diethyl ether (Et_2O), ethyl acetate (EtOAc) and then butanol (BuOH). The obtained aqueous organic solutions were washed by a few drops of water and dried over Na_2SO_4 in a volumetric flask for a day. The obtained aqueous organic solutions (petroleum benzine, chloroform, ether, ethyl acetate and butanol) were evaporated to dryness in vacuum rotary evaporator at 40 ºC.

2.2.1.2. Acidic Hydrolysis with 8% HCl

To certain amount of dried and ground flowers, HCl (100 mL, 8%) was added and refluxed for 1 h. Obtained acidic mixture was filtered and cooled. Afterwards the acidic mixture was extracted with petroleum benzine, chloroform ($CHCl_3$), followed by diethyl ether (Et_2O), ethyl acetate (EtOAc) and then butanol (BuOH). The remaining aqueous organic solutions were washed by a few drops of water and dried over Na_2SO_4 in a volumetric flask for a day. The obtained organic solutions (petroleum benzene, chloroform, ether, ethyl acetate and butanol) were evaporated to dryness in vacuum rotary evaporator at 40 ºC.

In addition, before extraction process, driying process generally increases the efficiency of extract.

2.2.1.3. TLC

Preparation of Mobile Phase:

The mobile phase was prepared with different volatile organic solvents at different ratios. In this study; following solvent systems were used as mobile phase.

$CHCl_3$-MeOH-H_2O: 75: 25: 3

EtOAc-MeOH-H_2O: 100: 13.5: 10

$CHCl_3$-MeOH-H_2O: 65: 45: 12

Preparation of Developing Container:

Mobile phase was poured into TLC tank to a depth of just less than 0.5 cm. To aid in the saturation of the TLC tank with solvent vapors, tank was covered with a watch glass, was shaken gently and allowed it to stand about 30 minutes.

Preparation of TLC Plate:

Plant extracts were obtained as described in Section 2.2.1.1. and Section 2.2.1.2.

A pinch of plant extract dissolved in a sufficient volume of volatile organic solvent such as acetone, methanol, chloroform. Microcapillary tube was dipped into this obtained sample solution and then gently touched the end of it onto the proper location on the TLC plate.

After these preparation steps, TLC plate was placed into developing TLC tank carefully, watch glass was covered and left it undisturbed on our bench top. The solvent rose up the TLC plate by capillary action. Plate was allowed to develop until the solvent was about a centimeter below the top of the plate. Then plate was removed from the beaker and immediately marked the solvent front with a pencil and allowed to dry.

Visualization of the Spots:

For visualization of the spots UV Spectophotometer was used. Visible spots were circled by a pencil.

Notes:

- Due to the high diversity in chemical composition of secondary metabolites, different methods of extraction and assays were employed to determine the metabolites.

- As a rule of thumb, a concentration of 1% usually works well for TLC analysis. If the sample is too concentrated, it will run as a smear or streak. If it is not concentrated enough, you will see nothing on the plate. Sometimes you will need to use trial and error to get well-sized, easy to read spots.

- Don't allow the spot to become too large.

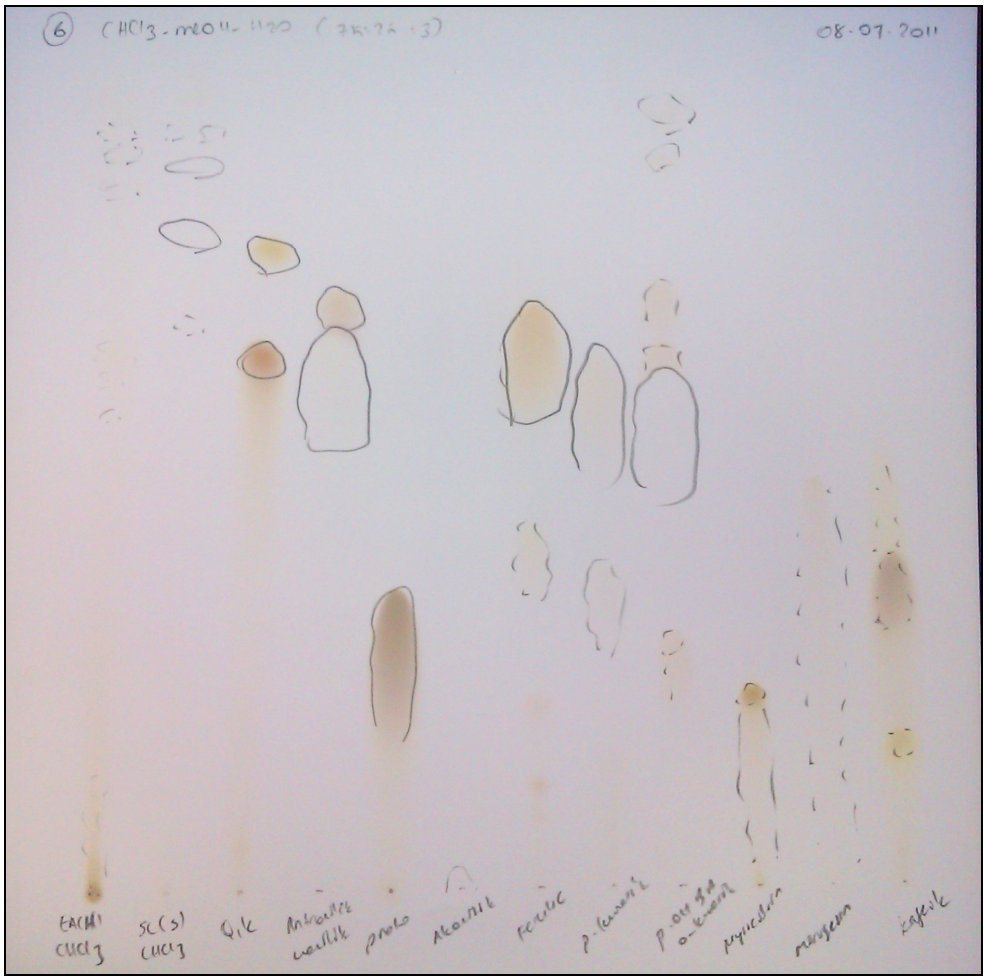


Figure 2. 6 TLC plate of *Silene compacta FISCHE* (SC) and *Epilobium angustifolium L.* (EA)

<u>*Solvent System:*</u> $CHCl_3$: MeOH: H_2O (75: 25: 3)

1. EA - $CHCl_3$ sample extract. (Acidic Hydrolysis)
2. SC - $CHCl_3$ sample extract .(Methanolic Extraction)
3. Quercetin and Kaempferol
4. Antranilic acid and Vanilic acid
5. Protocatechuic acid
6. Aconitic acid
7. Ferulic acid
8. *p*-coumaric acid
9. *p*-hydroxybenzoic acid and *o*-coumaric acid
10. Myricitrin
11. Myricetin
12. Caffeic acid

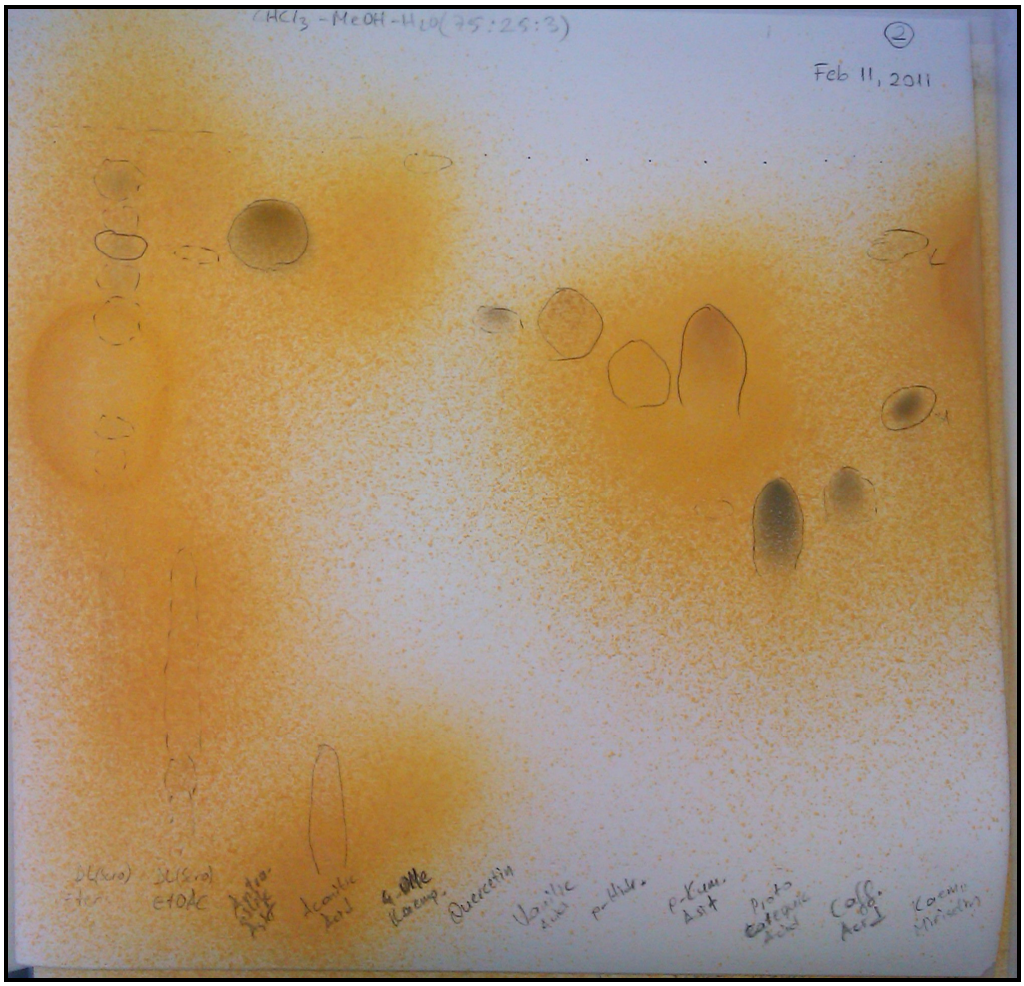


Figure 2.7. TLC plate of *Digitalis lamarcki* (DL)

<u>*Solvent System:*</u> $CHCl_3$: MeOH: H_2O (75: 25: 3)

1. DL - Et_2O sample extract. (Methanolic Extraction)
2. DL - EtOAc sample extract .(Methanolic Extraction)
3. Antranilic acid
4. *trans*- aconitic acid
5. 4'- methoxy kaempferol
6. Quercetin
7. Vanilic acid
8. *p*-hydroxybenzoic acid
9. *p*-coumaric acid
10. Protocatechuic acid
11. Caffeic acid
12. Kaempferol and myricetin

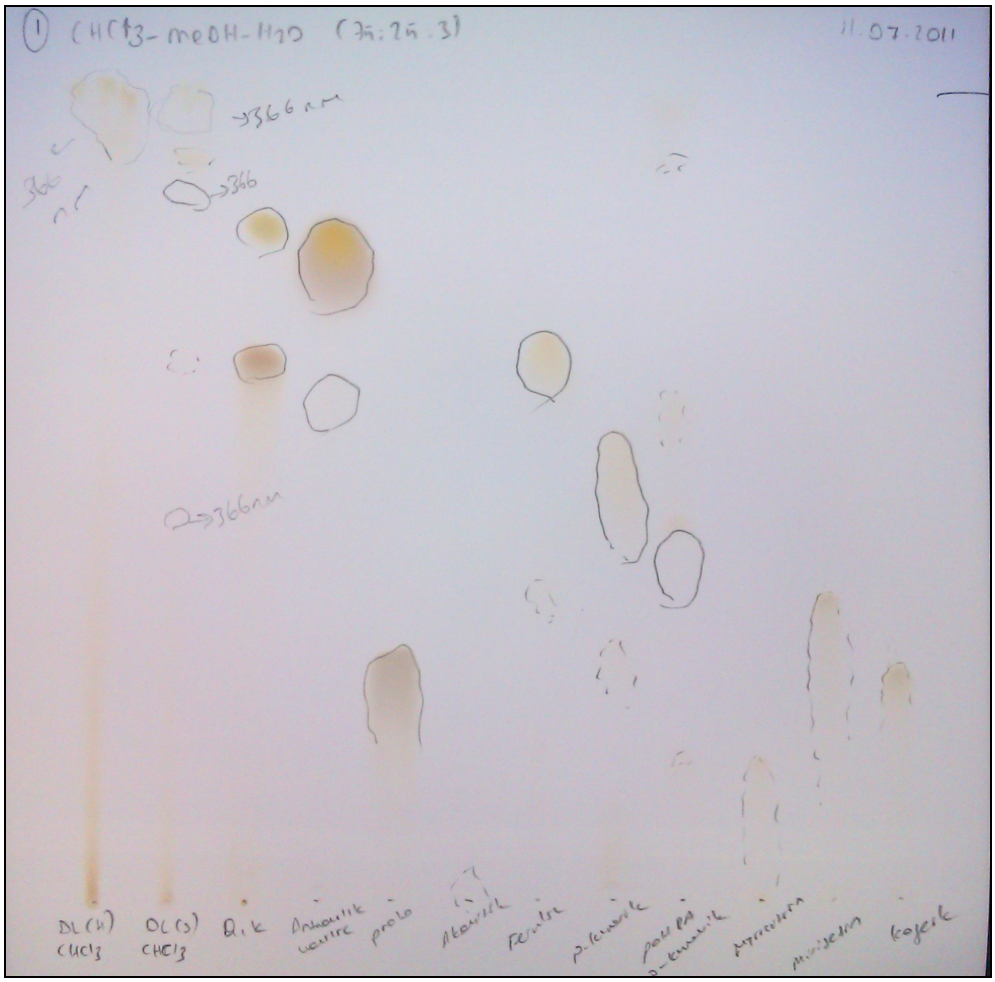


Figure 2.8. TLC plate of *Digitalis lamarcki* (DL)

Solvent System: $CHCl_3$: MeOH: H_2O (75: 25: 3)

1. DL - $CHCl_3$ sample extract. (Acidic Hydrolysis)
2. DL - $CHCl_3$ sample extract .(Methanolic Extraction)
3. Quercetin and Kaempferol
4. Antranilic acid and Vanilic acid
5. Protocatechuic acid
6. Aconitic acid
7. Ferulic acid
8. *p*-coumaric acid
9. *p*-hydroxybenzoic acid and *o*-coumaric acid
10. Myricitrin
11. Myricetin
12. Caffeic acid

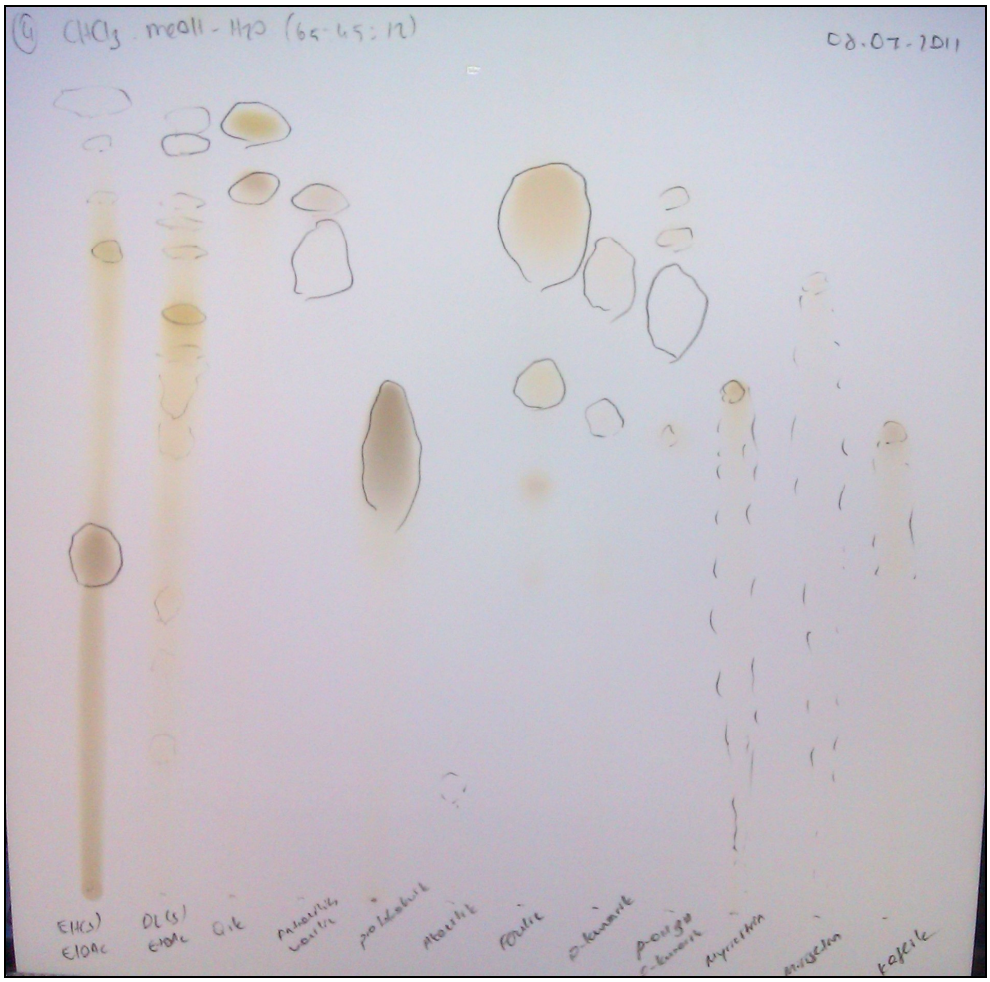


Figure 2.9. TLC plate of *Digitalis lamarcki* (DL) and *Epilobium hirsutum L.*(EH)

<u>*Solvent System:*</u> $CHCl_3$: MeOH: H_2O (65: 45: 12)

1. EH - EtOAc sample extract. (Methanolic Extraction)
2. DL - EtOAc sample extract .(Methanolic Extraction)
3. Quercetin and Kaempferol
4. Antranilic acid and Vanilic acid
5. Protocatechuic acid
6. Aconitic acid
7. Ferulic acid
8. *p*-coumaric acid
9. *p*-hydroxybenzoic acid and *o*-coumaric acid
10. Myricitrin
11. Myricetin
12. Caffeic acid

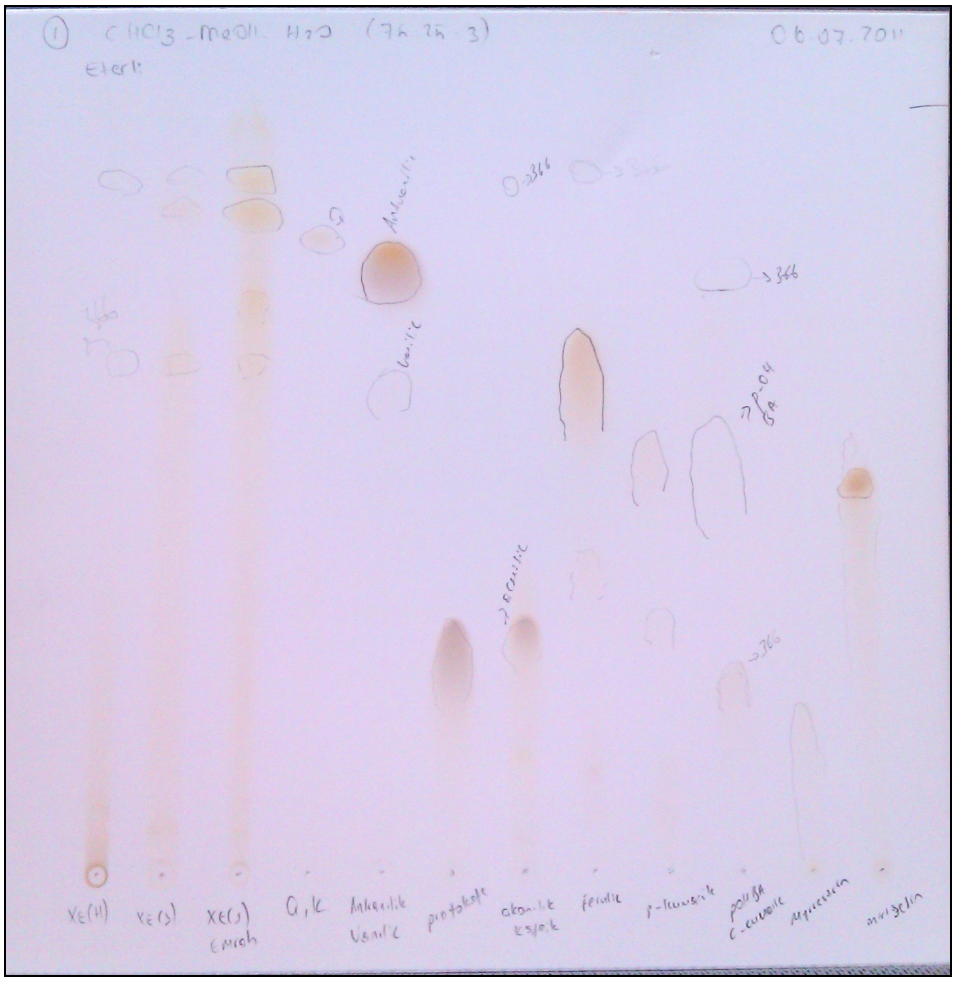


Figure 2.10. TLC plate of X*eranthemum annuum L.* (XA)

<u>*Solvent System:*</u> $CHCl_3$: MeOH: H_2O (75: 25: 3)

1. XA – Et_2O sample extract. (Acidic Hydrolysis)
2. XA - Et_2O sample extract .(Methanolic Extraction)
3. XA - Et_2O sample extract .(Methanolic Extraction)
4. Quercetin and Kaempferol
5. Antranilic acid and Vanilic acid
6. Protocatechuic acid
7. Aconitic acid and Caffeic acid
8. Ferulic acid
9. *p*-coumaric acid
10. *p*-hydroxybenzoic acid and *o*-coumaric acid
11. Myricitrin
12. Myricetin

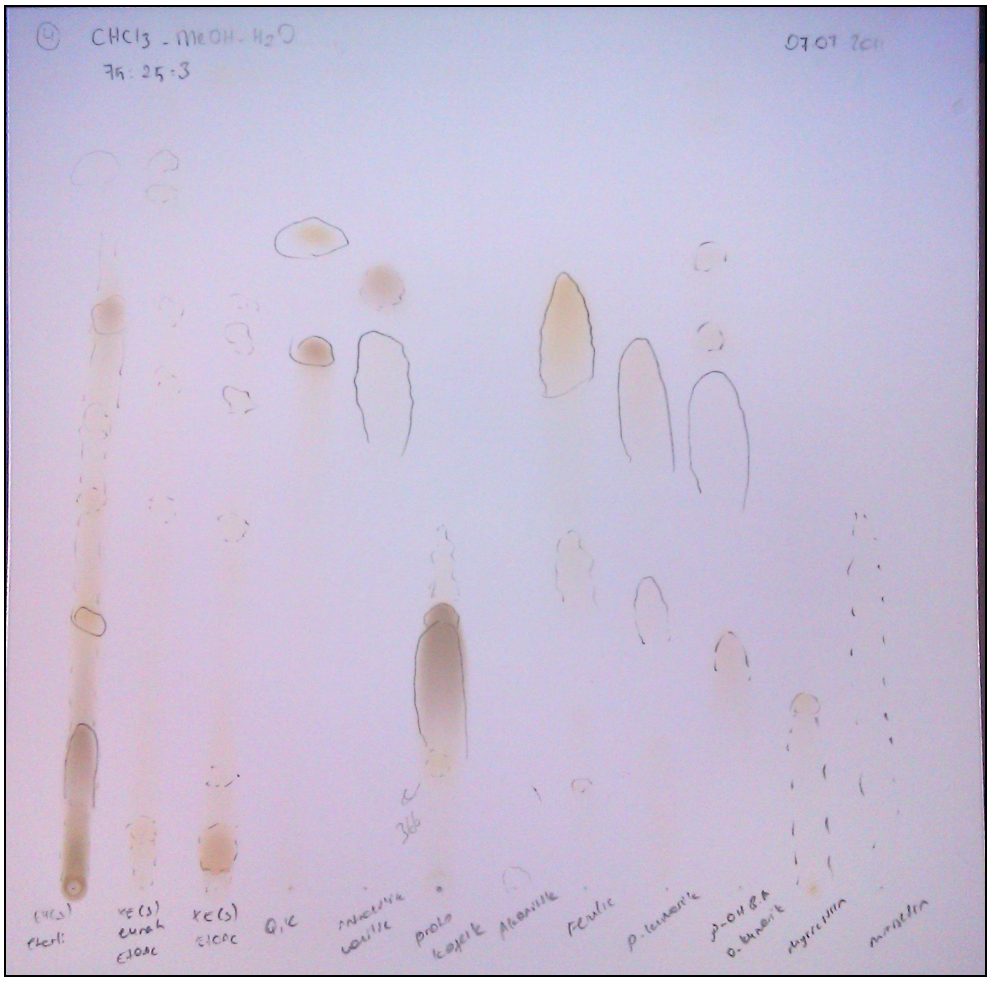


Figure 2.11. TLC plate of X*eranthemum annuum L.* (XA) and *Epilobium hirsutum L.* (EH)

<u>*Solvent System:*</u> $CHCl_3$: MeOH: H_2O (75: 25: 3)

1. EH - Et_2O sample extract. (Methanolic Extraction)
2. XA – EtOAc sample extract .(Methanolic Extraction)
3. XA - EtOAc sample extract .(Methanolic Extraction)
4. Quercetin and Kaempferol
5. Antranilic acid and Vanilic acid
6. Protocatechuic acid
7. Aconitic acid and Caffeic acid
8. Ferulic acid
9. *p*-coumaric acid
10. *p*-hydroxybenzoic acid and *o*-coumaric acid
11. Myricitrin
12. Myricetin

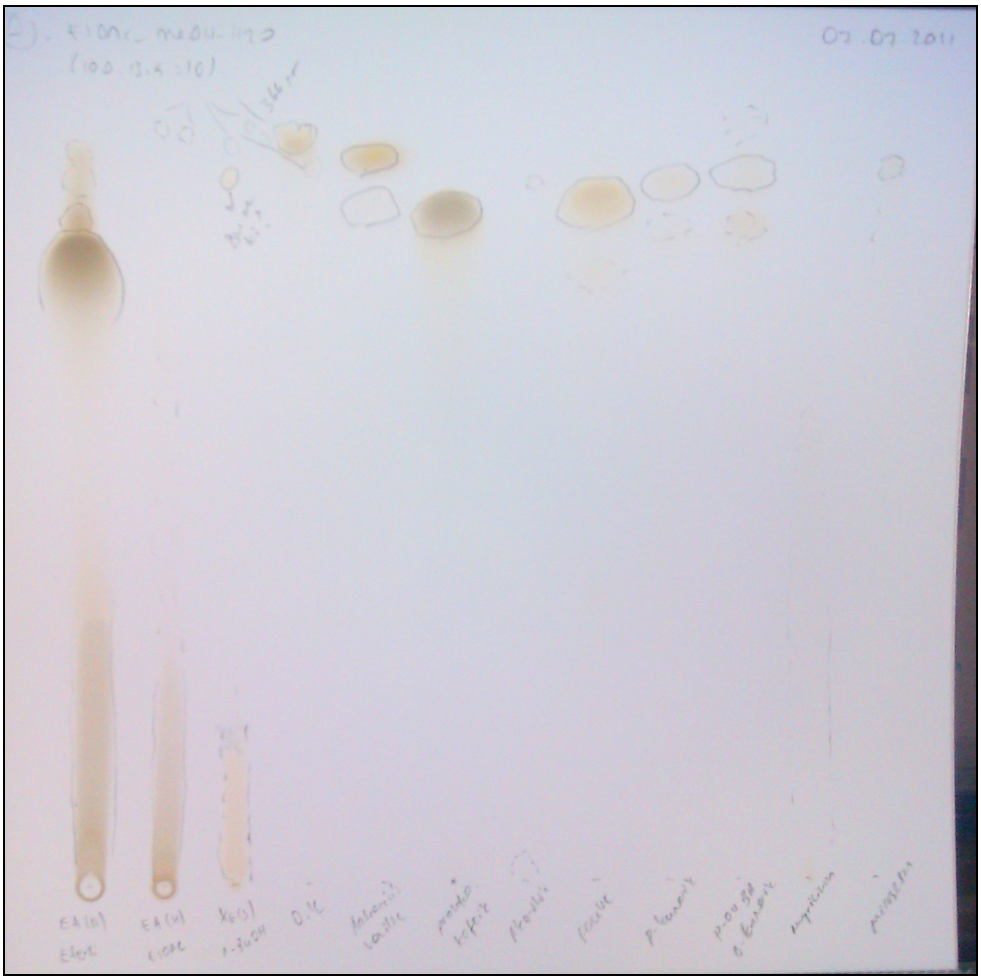


Figure 2.12. TLC plate of X*eranthemum annuum L.* (XA) and *Epilobium Angustifolium L.* (EA)

<u>*Solvent System:*</u> EtOAc: MeOH: H_2O (100: 13.5: 10)

1. EA – Et_2O sample extract. (Acidic Hydrolysis)
2. EA – EtOAc sample extract. (Acidic Hydrolysis)
3. XA – n-BuOH sample extract .(Methanolic Extraction)
4. Quercetin and Kaempferol
5. Antranilic acid and Vanilic acid
6. Protocatechuic acid and Caffeic acid
7. Aconitic acid
8. Ferulic acid
9. *p*-coumaric acid
10. *p*-hydroxybenzoic acid and *o*-coumaric acid
11. Myricitrin
12. Myricetin

-For Et_2O extracts of DL, SC and XA plants,

-For $CHCl_3$ extracts of DL, SC, XA, EH and EA plants,

-For EtOAc extracts of DL, SC, XA and EA plants,

$CHCl_3$: MeOH: H_2O (75: 25: 3) solvent system was found as suitable mobile phase.

-For Et_2O extracts of EH and EA plants, EtOAc: MeOH: H_2O (100: 13.5: 10) solvent system was found as suitable mobile phase.

-For EtOAc extracts of DL and EH plants, $CHCl_3$: MeOH: H_2O (65: 45: 12) solvent system was found as suitable mobile phase.

In SC $CHCl_3$ extract (methanolic extraction): kaempferol (Figure 2.6); in DL $CHCl_3$ extract (methanolic extraction): quercetin and myricetin (Figure 2.8); in DL Et_2O extract (methanolic extraction): quercetin (Figure 2.7); in XA Et_2O extract (methanolic extraction): quercetin, kaempferol and protocatechuic acid (Figure 2.10); in EH EtOAc extract (methanolic extraction): quercetin and kaempferol (Figure 2.9) were found by TLC tests.

2.2.2. Extraction For Quantitative Studies

2.2.2.1. Soxhlet Extraction (Petroleum Benzene)

Certain amount of dried and ground flower were uniformly packed into a thimble and extract with petroleum benzene for 8 h to remove oil and chlorophyll.

After that defatted powdered plant material was taken in a beaker and dried till all the solvent got evaporated. Dried defatted powdered plant material was kept for their future use in phytochemical analysis.

2.2.2.2. Extraction with 70% aqueous Methanol

Certain amount of dried defatted powdered plant material was heated with MeOH (250 mL, 70%) on a hot plate with continuous stirring at 40ºC for 30 minutes. After 30 minutes, the methanolic solution was decanted into a volumetric flask. This process was repeated three times and total methanolic solution was filtered through filter paper. The obtained methanolic solution was evaporated to dryness in the vacuum rotary evaporator at 40ºC. Then dried MeOH extract was dissolved in 75 mL hot water and this aqueous solution was extracted with 30 mL ethyl acetate for 10 times. The ethyl acetate organic solution was washed by a few drops of water and was suspended in seperatory funnel for a day. The day after, ethyl acetate organic solution was evaporated to dryness in vacuum rotary evaporator at 40ºC. Lastly, the ethyl acetate extract was weighed.

2.2.2.3. Acidic Hydrolysis with 1.2 M HCl

Certain amount of dried and ground flowers plant material were refluxed with 125 mL of 1.2 M HCl (diluted with 50% MeOH) at 80ºC for 1 h. Acidic mixture was filtered. After filtration, obtained acidic mixture was evaporated to

dryness in the vacuum rotary evaporator at 40 ºC. Dried HCl extract was dissolved in 75 mL hot water and this aqueous solution was extracted with 30 mL ethyl acetate for 10 times. The ethyl acetate organic solution was washed by a few drops of water and was suspended in seperatory funnel for a day. The day after, ethyl acetate organic solution was evaporated to dryness in vacuum rotary evaporator at 40ºC. Lastly, the ethyl acetate extract was weighed.

2.3. Chemical Instruments and Their Conditions

2.3.1. LC-MS

Instrument:	Waters Allians, Waters Micromass ZQ.
Used Method:	Negative Electrospray Ionization [ES(-)].
Used Column:	XTerra® MS C-18 (4.6 X250 mm,5 µm).
Used Mobile Phase:	Water: Acetonitrile: Methanol (5:85:10).
Flow Rate:	0,75 mL/min.
Dedector:	Photo-diode array (254 nm).

Analytical Conditions;

capillary voltage:	3.41 kV.
cone voltage:	26 V.

source temperature: 100^{o}C.

desolvation temperature: 350^{o}C.

CHAPTER III

3. RESULTS AND DISCUSSION

Phenolic compounds are a large group of phytochemicals found widespread in the plant kingdom. Depending on their structure they can be classified into phenolic acids, hydroxycinnamic acid derivatives and flavonoids. Phenolic compounds have received considerable attention for being potentially protective factors against cancer and heart and lung diseases, because of their potent antioxidative properties.

In this study, some phenolic acids, hydroxycinnamic acids and flavonoids from the flowers of five different plants grown in Bolu have been determined by chromatographic methods. These plants are *Silene compacta FISCHE* (SC), *Digitalis lamarcki* (DL), *Xeranthemum annuum L.* (XA), *Epilobium hirsutum L.* (EH), and *Epilobium angustifolium L.* (EA). Of these plants there is no report regarding the plant SC in the literature. As for the plant DL, there is too no study related to the quantitative analysis of polyphenolic compounds by means of HPLC and structure elucidation by mass spectrometric analysis. There is a number of study on the remaining three plants, XA, EH and EA, but however, more detailed quantitative analysis of the chemical components has been conducted in this study and in order to

get insight of the diversity and to perform a comparative study. The study consists of three parts: Qualitative TLC tests, LC-MS studies for structure identifications, and HPLC studies for quantitative determinations. For qualitative studies, after aplying Soxhlet extraction for 48h by MeOH to the dried and ground flowers, the crude extract obtained in this way has been fractionated by petroleum benzine, chloroform ($CHCl_3$), diethyl ether (Et_2O), and ethyl acetate (EtOAc), respectively. $CHCl_3$, Et_2O and EtOAc extracts have been utilized in the relevant analyses. By TLC inspection of these extracts in the various developing solvent systems repeatedly, we checked and had information about the phenolic acids and flavonoids which may be found in these plants. Then, LC-MS analysis was done and existing compounds were detected and structures of these compounds were idendified by comparing with the literature values and data. The compounds identified by LC-MS in the plants under investigation were shown in Table 3.1. LC-MS spectra of each extract related to the plants SC, DL, XA, EH and EA have been depicted respectively below, Figures 3.1-3.18.

Table 3.1. Phenolic compounds detected in the extracts of five different plants by using LC-MS.

Plant Name	CHCl3	Et_2O	EtOAc
SC[a]	Myricetin Gallic acid Kaempferol	*p*-hydroxybenzoic acid Gallic Acid	Quercetin-3-O-rhamnoside
DL[a]	Quercetin	Caffeic acid Quercetin-3-O-glucoside Myricetin-3-O-rhamnoside	Quercetin-3-O- glucoside Myricetin-3-O-rhamnoside
XA[a]	Ferulic acid Quercetin-3-O-glucoside Galangin	Kaempferol Quercetin Galangin Protocatechuic acid	Myricetin-3-O-rhamnoside Luteolin
EH[a]	Kaempferol Quercetin Caffeic acid	Quercetin Gallic acid	Quercetin-3-O-glucoside Gallic acid
EH[b]	-	-	Quercetin-3-O-rhamnoside Gallic acid Quercetin
EA[a]	Kaempferol-3-O-rhamnoside Quercetin-3-O-glucoside Apigenin-7-0-glucoside Gallic acid	-	Quercetin-3-O-rhamnoside
EA[b]	-	-	Vanillic acid

Notes: SC *(Silene compacta FISCHE*), DL(*Digitalis lamarcki*), XA *(Xeranthemum annuum L.*), EH (*Epilobium hirsutum* L.), EA(*Epilobium angustifolium* L.), [a] →Soxhlet Extraction (with MeOH), [b]→Acidic Hydrolysis (with 8% HCl.

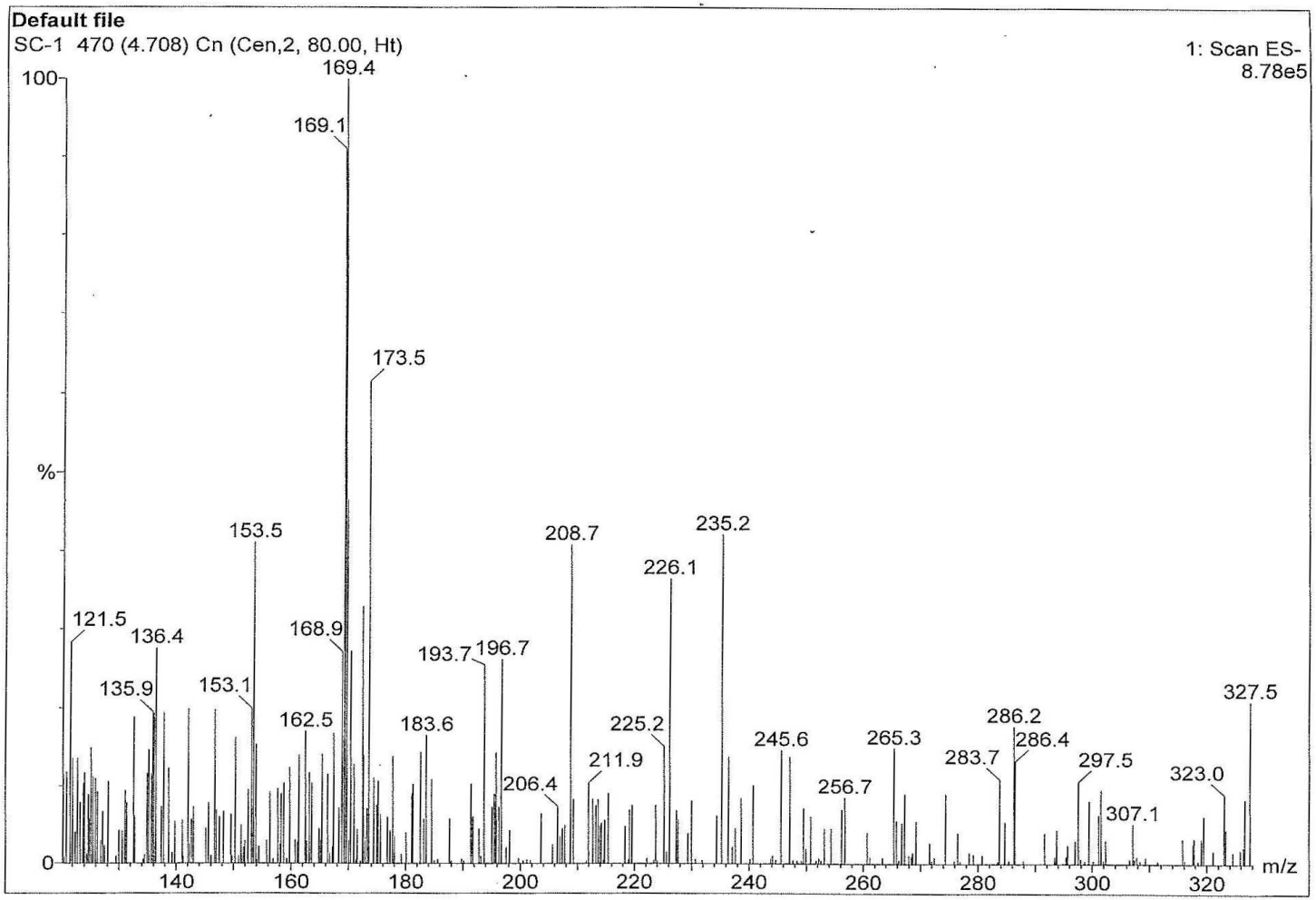


Figure 3.1. LC-MS spectrum of Gallic acid detected in the $CHCl_3$ extract of *Silene compacta FISCHE*

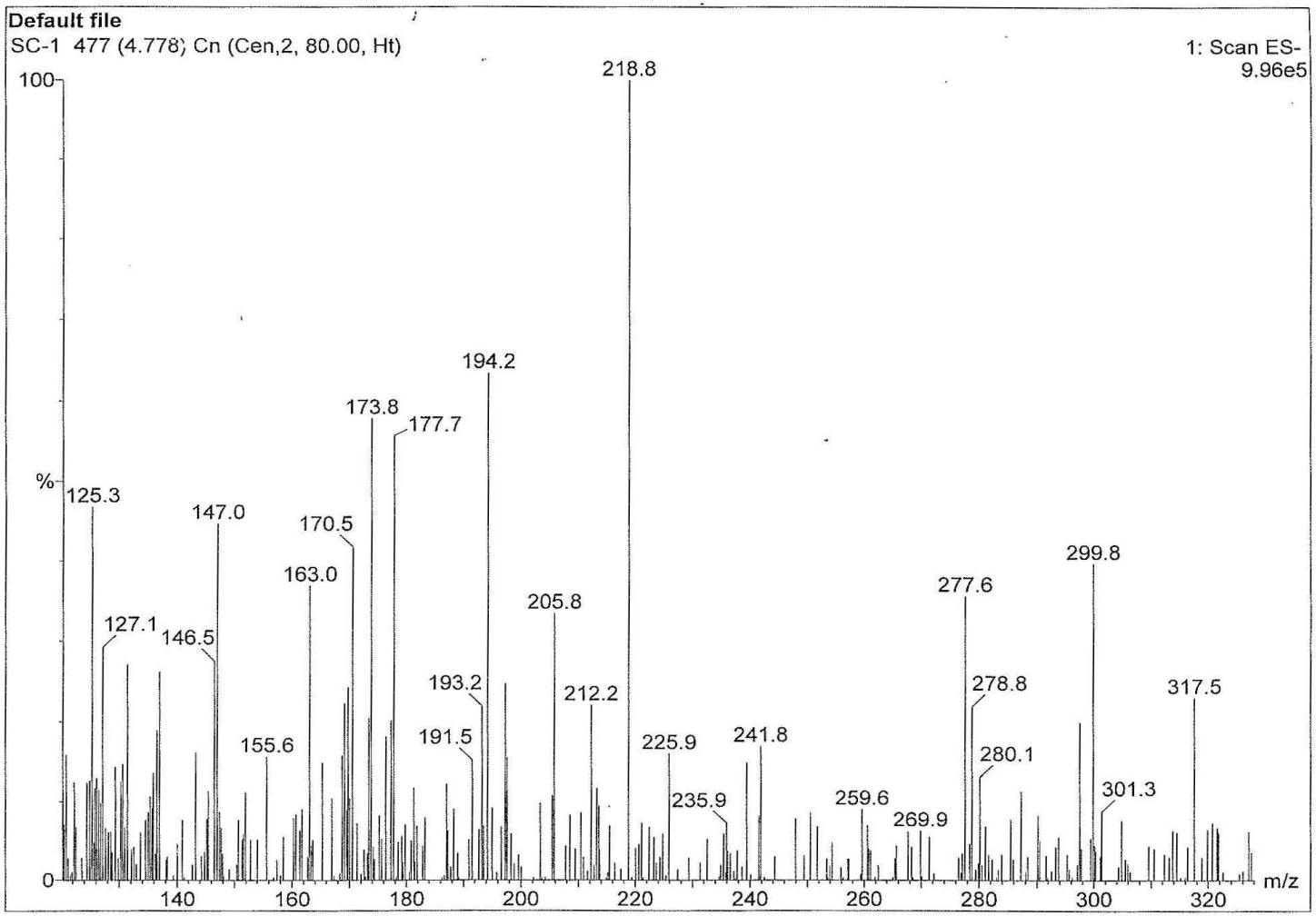


Figure 3.2. LC-MS spectrum of Myricetin detected in the $CHCl_3$ extract of *Silene compacta FISCHE*

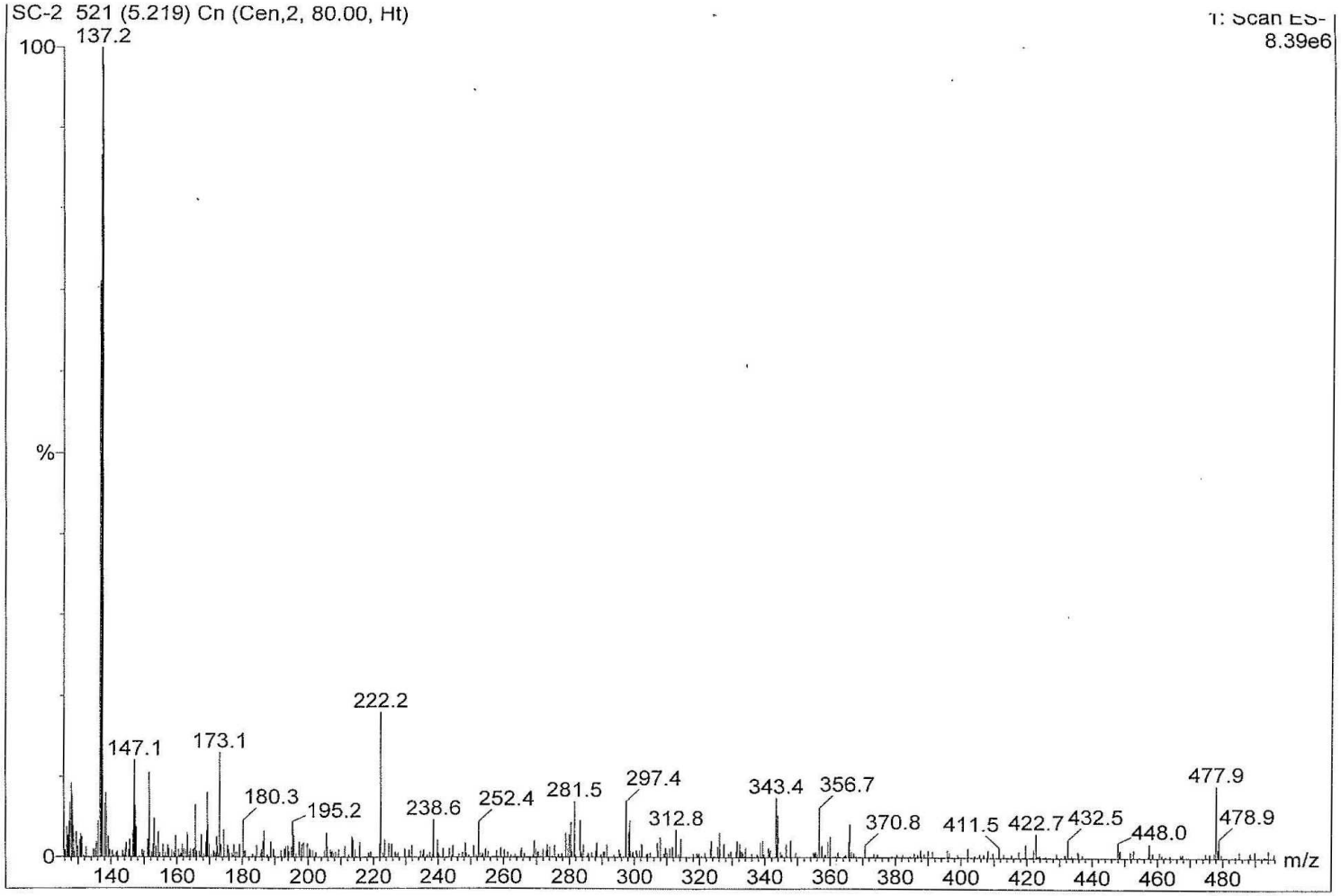


Figure 3.3. LC-MS spectrum of *p*-hydroxybenzoic acid detected in the Et_2O extract of *Silene compacta FISCHE*

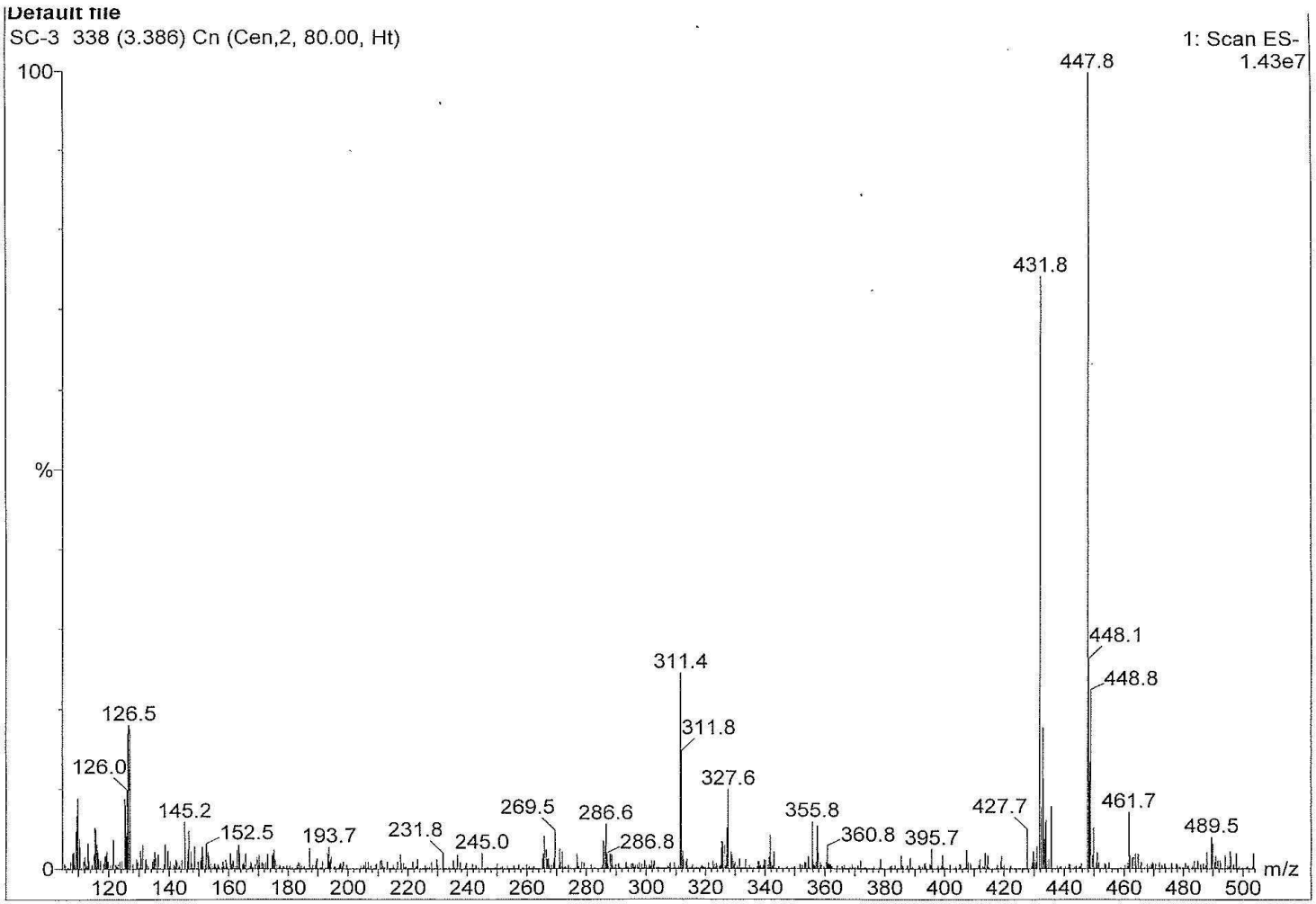


Figure 3.4. LC-MS spectrum of Quercetin-3-O-rhamnoside detected in the EtOAc extract of *Silene compacta FISCHE*

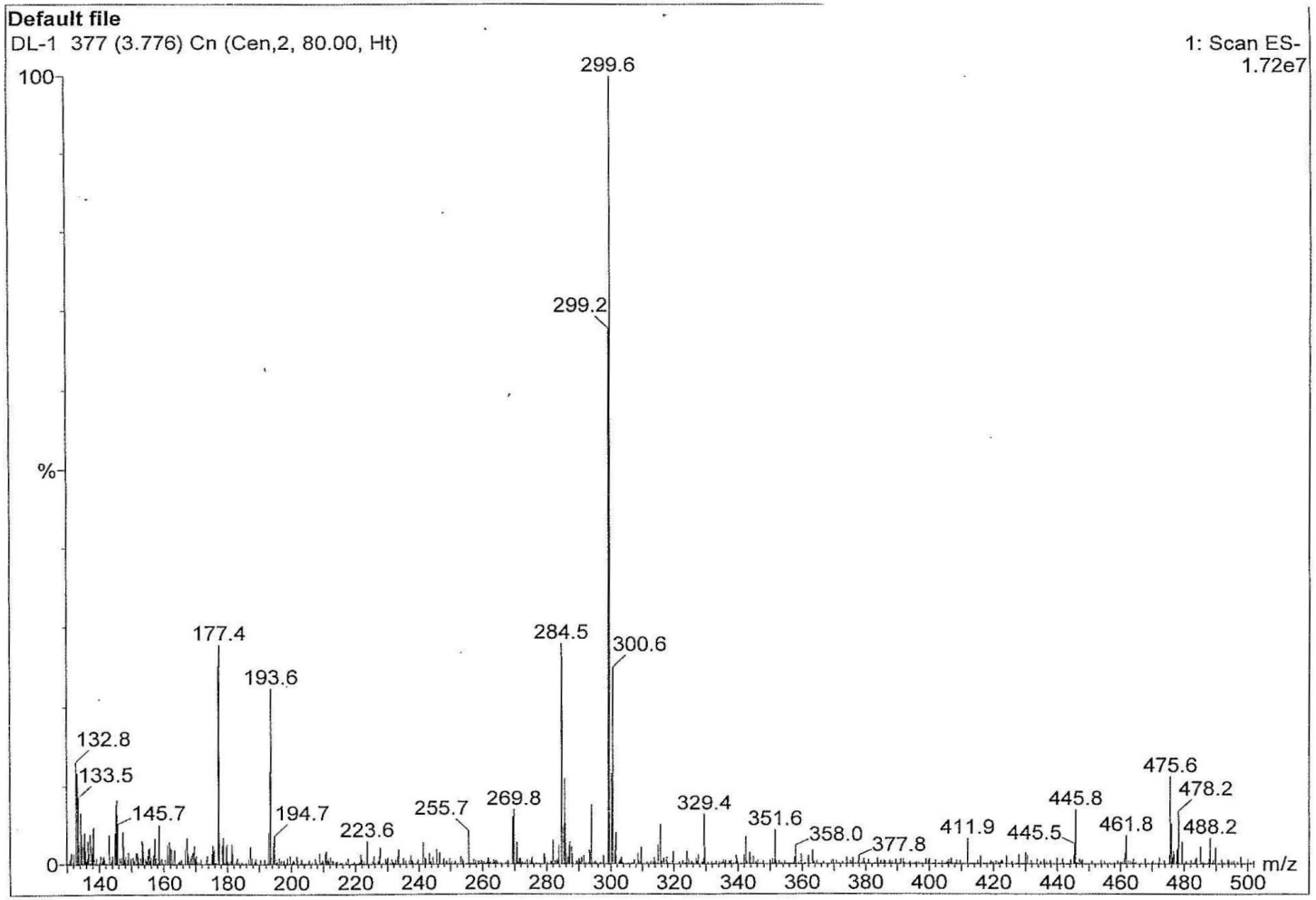


Figure 3.5. LC-MS spectrum of Quercetin detected in the $CHCl_3$ extract of *Digitalis lamarcki*

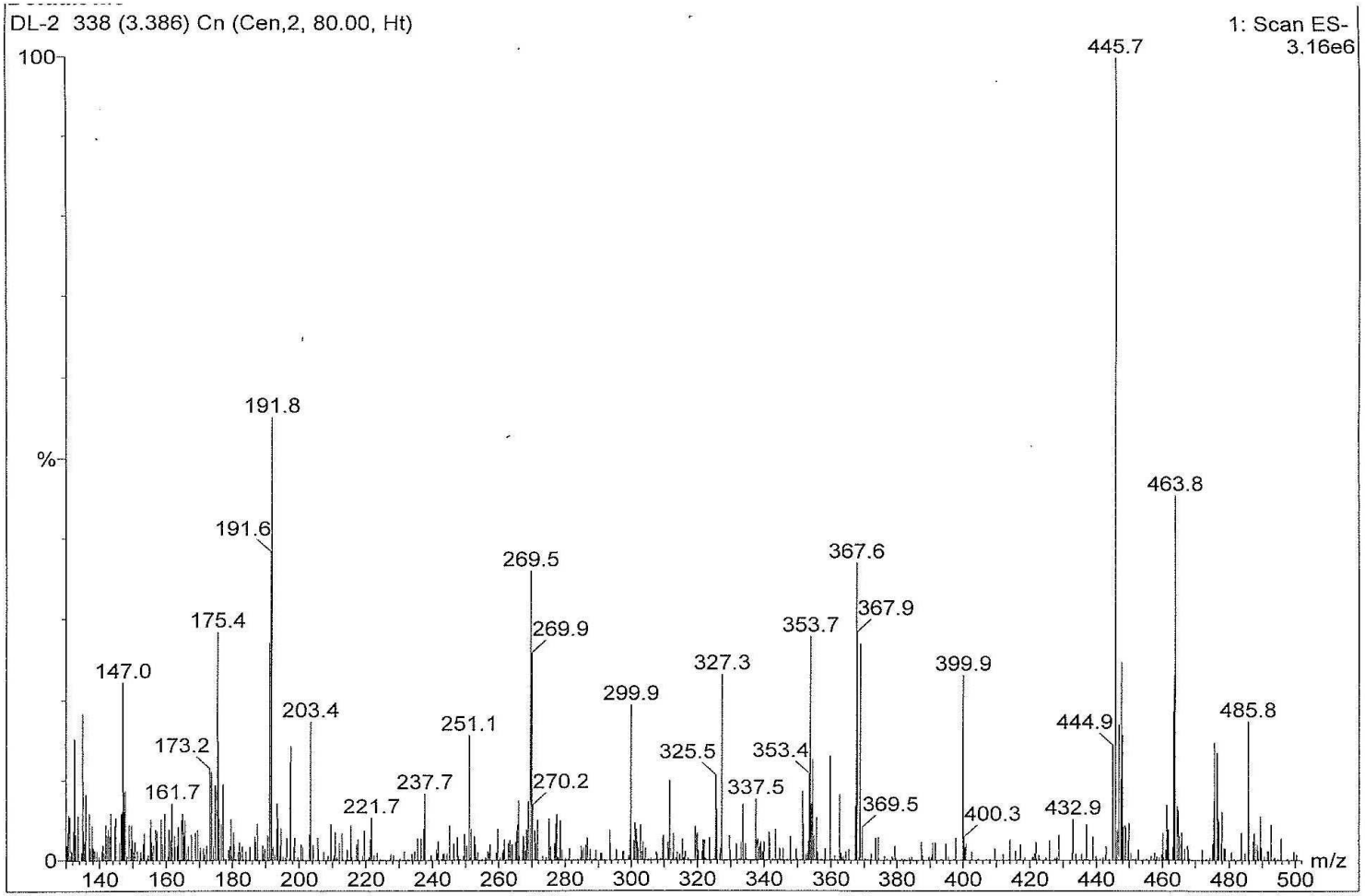


Figure 3.6. LC-MS spectrum of Myricetin-3-O-rhamnoside detected in the Et_2O extract of *Digitalis lamarcki*

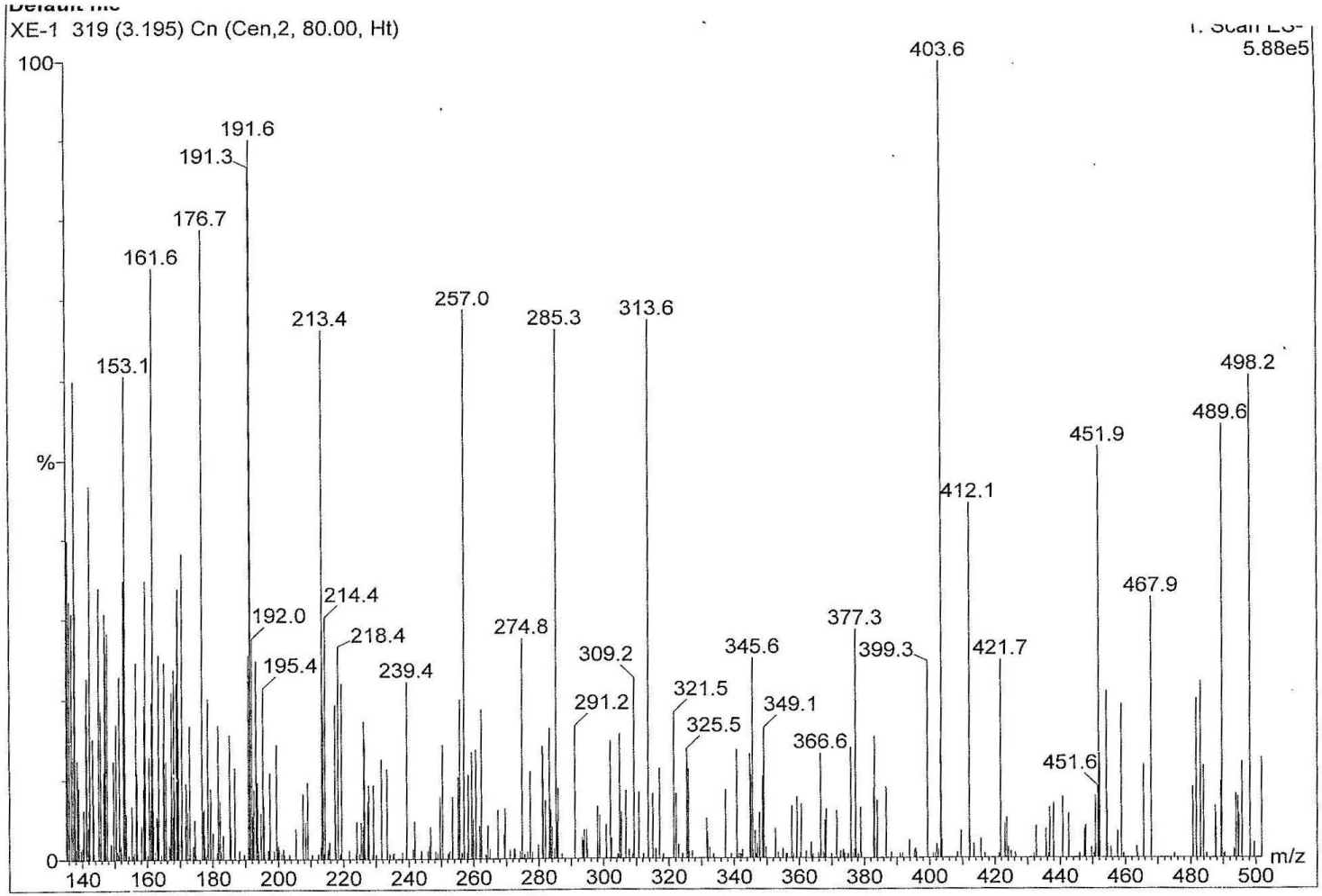


Figure 3.7. LC-MS spectrum of Ferulic detected in the $CHCl_3$ extract of *Xeranthemum annuum L.*

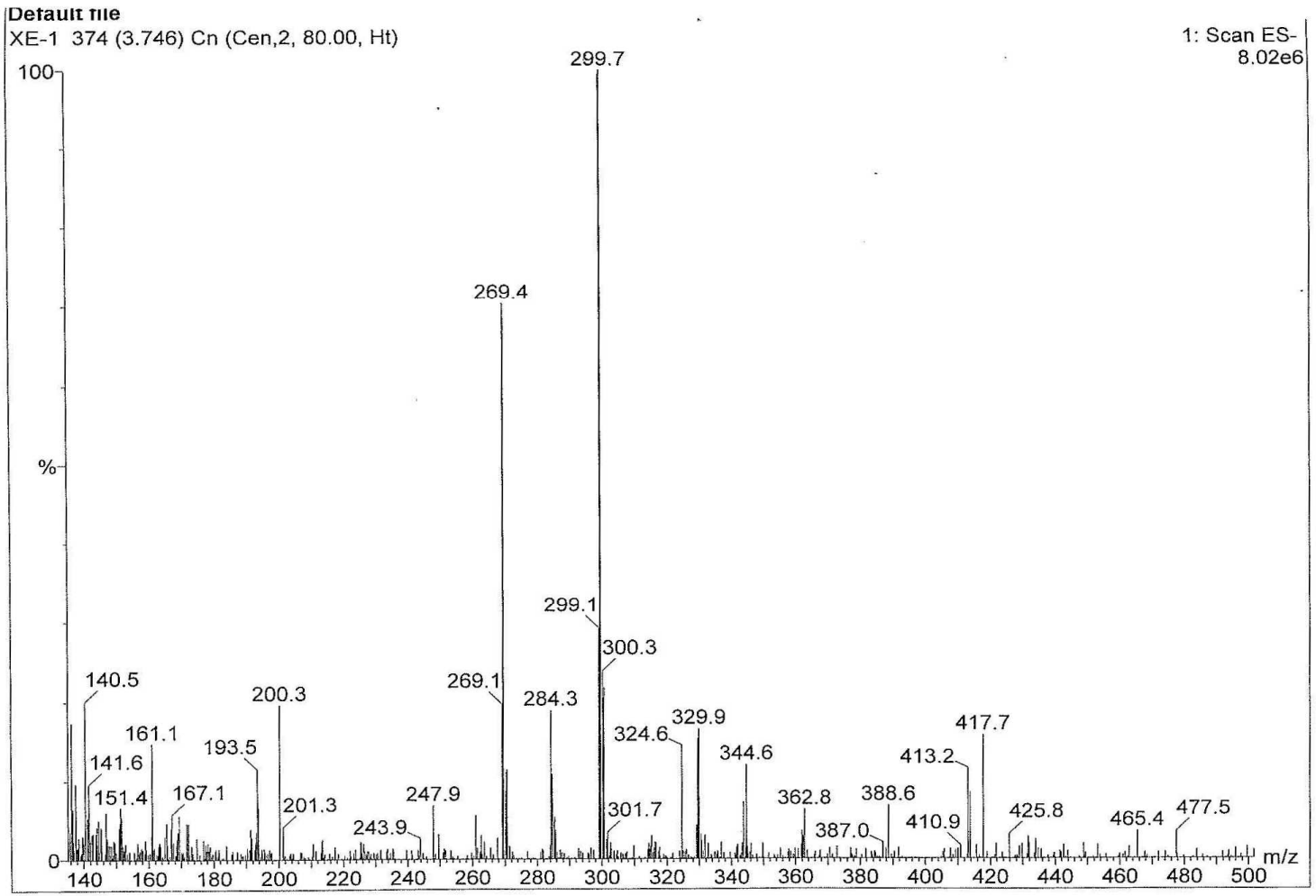


Figure 3.8. LC-MS spectrum of Galangin detected in the $CHCl_3$ extract of *Xeranthemum annuum L.*

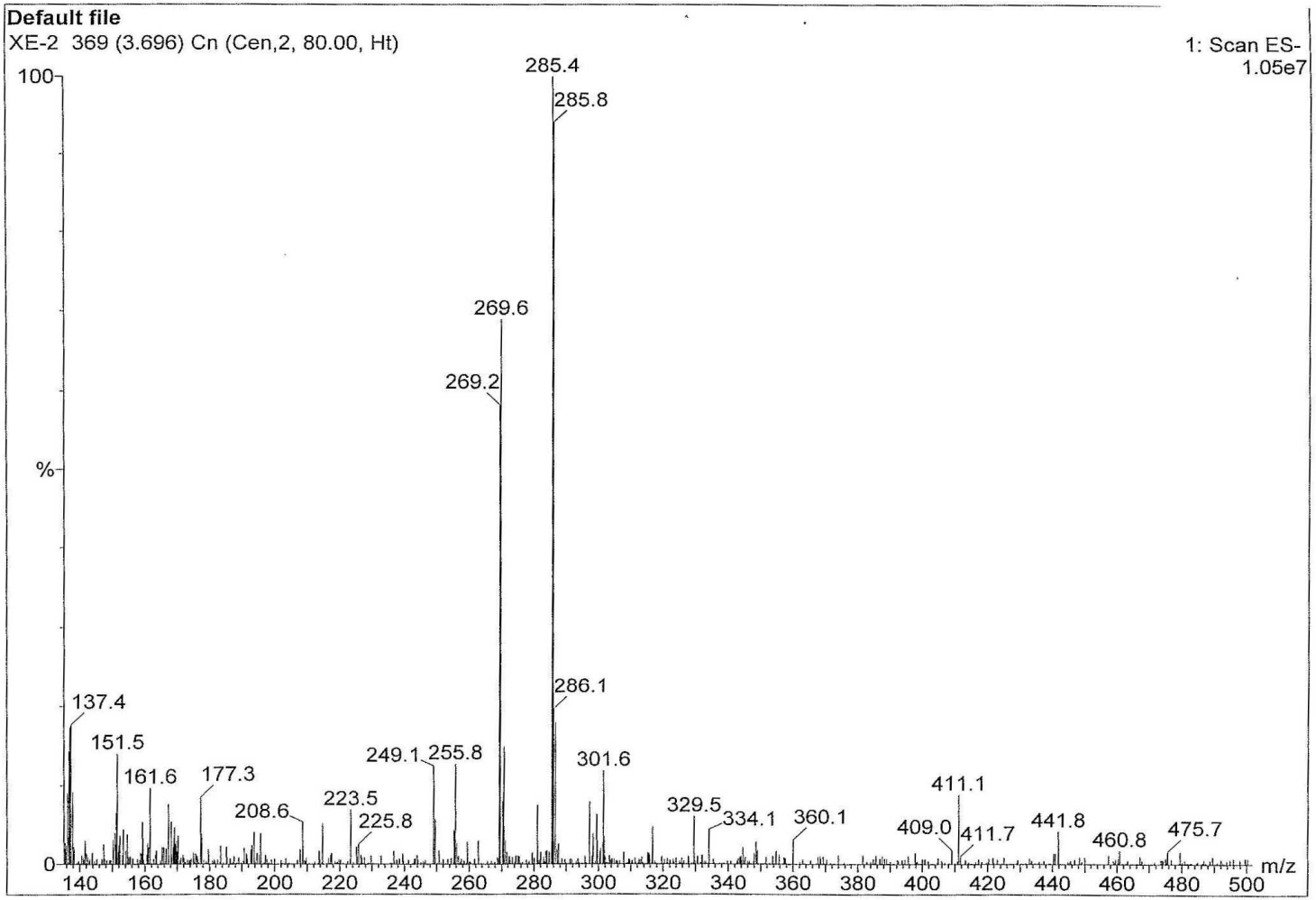


Figure 3.9. LC-MS spectrum of Kaempferol detected in the Et_2O extract of *Xeranthemum annuum L.*

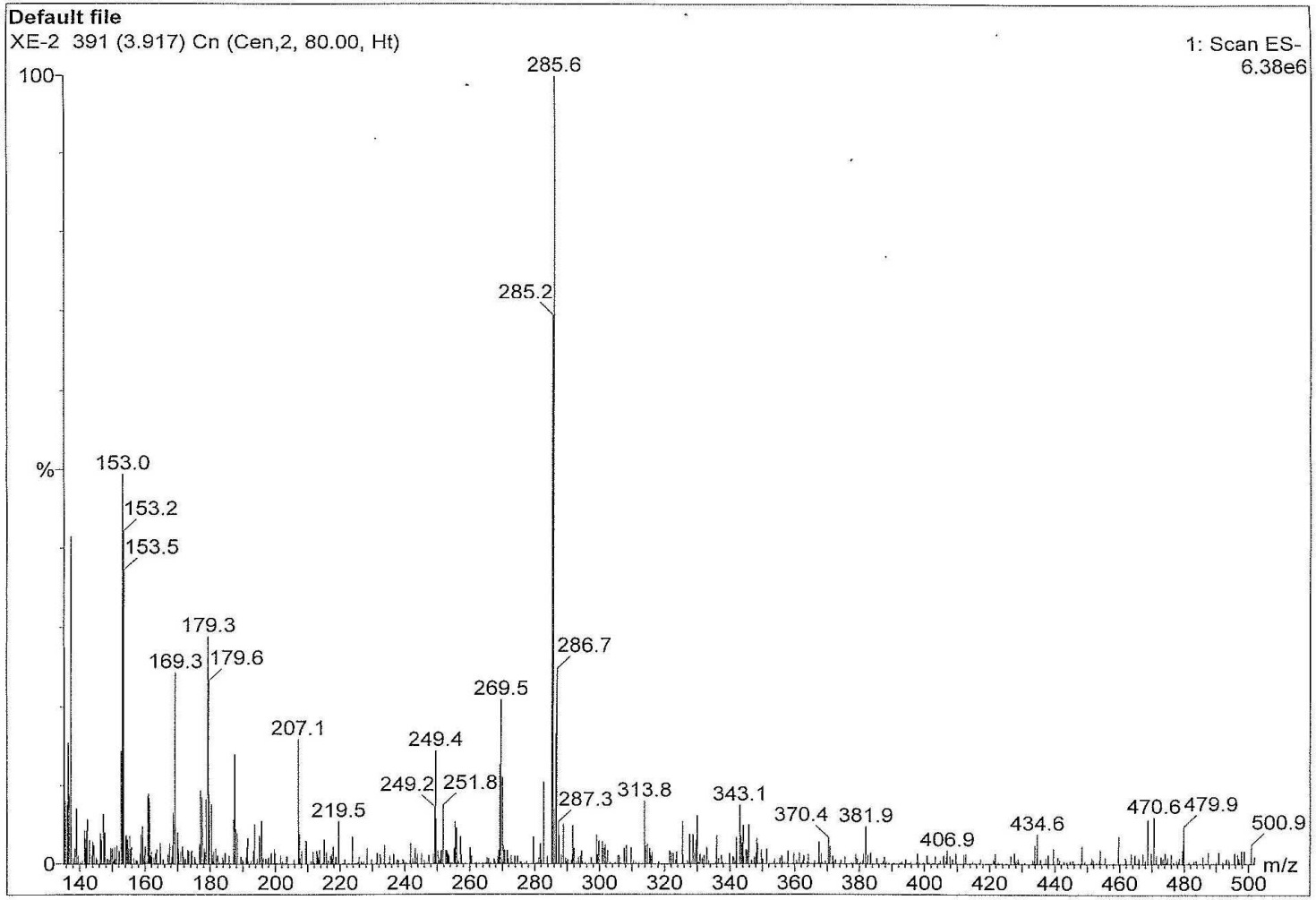


Figure 3.10. LC-MS spectrum of Protocatechuic acid detected in the Et_2O extract of *Xeranthemum annuum L.*

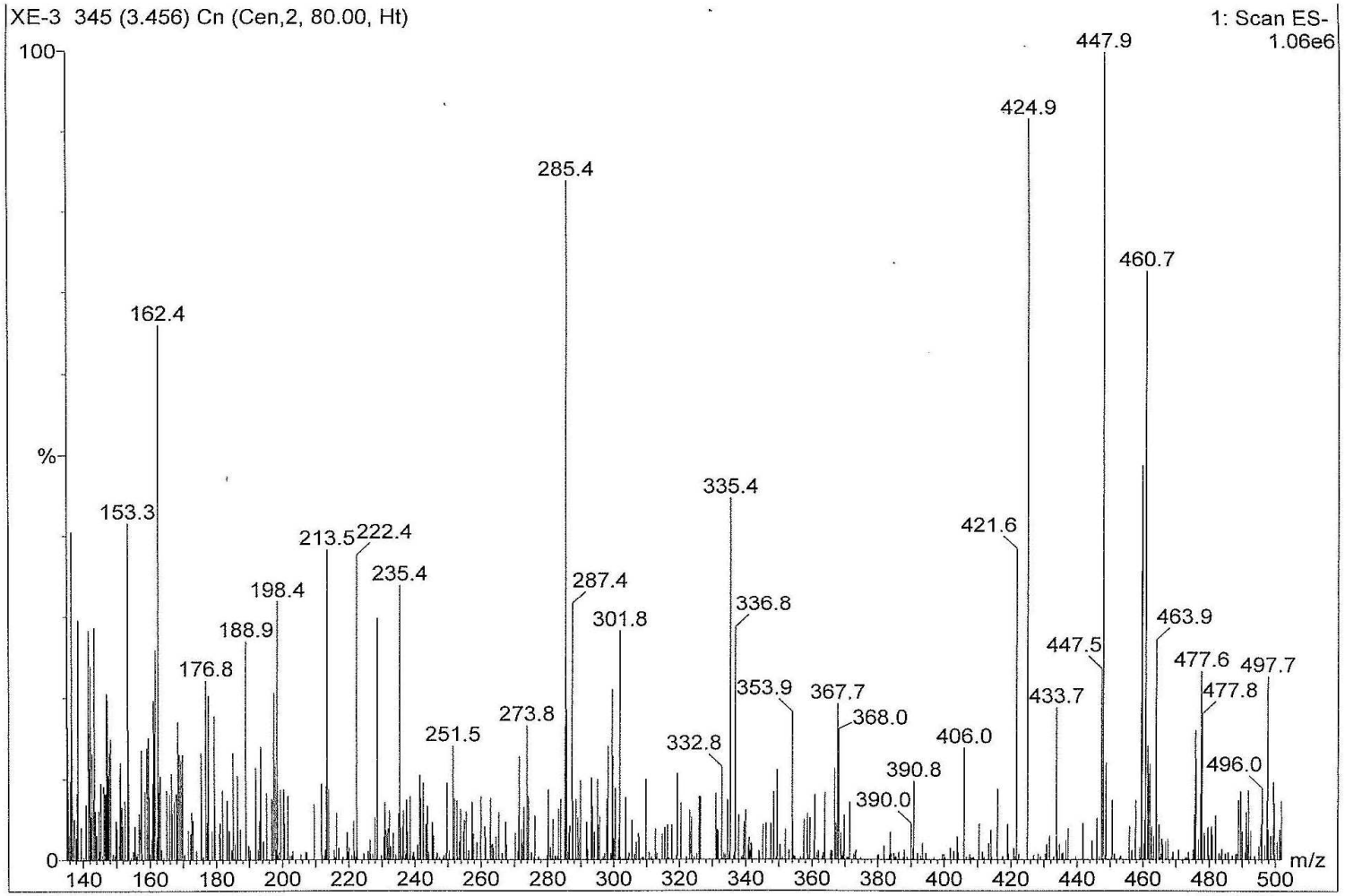


Figure 3.11. LC-MS spectrum of Luteolin detected in the EtOAc extract of *Xeranthemum annuum L.*

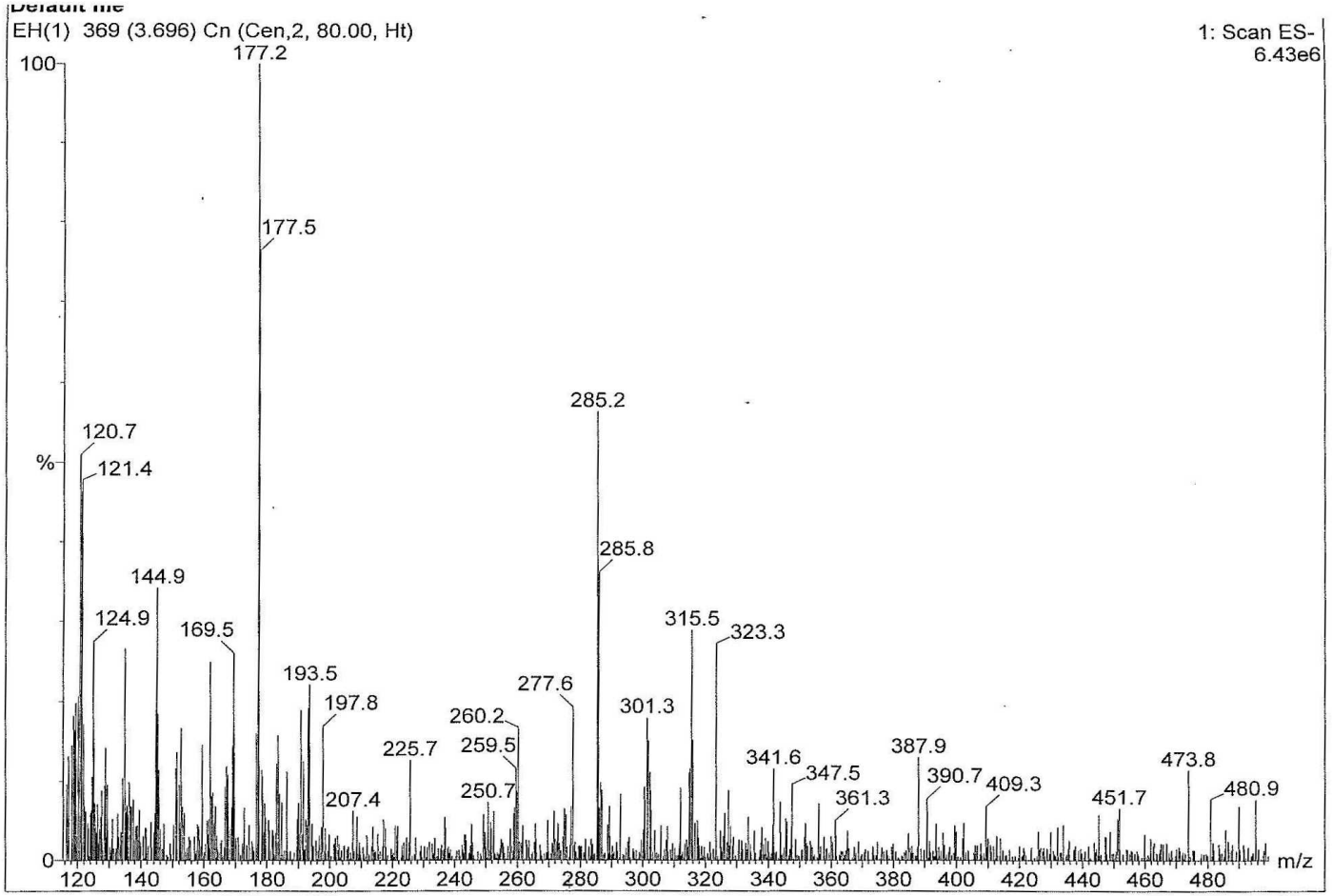


Figure 3.12. LC-MS spectrum of Caffeic acid detected in the $CHCl_3$ extract of *Epilobium hirsutum L.*

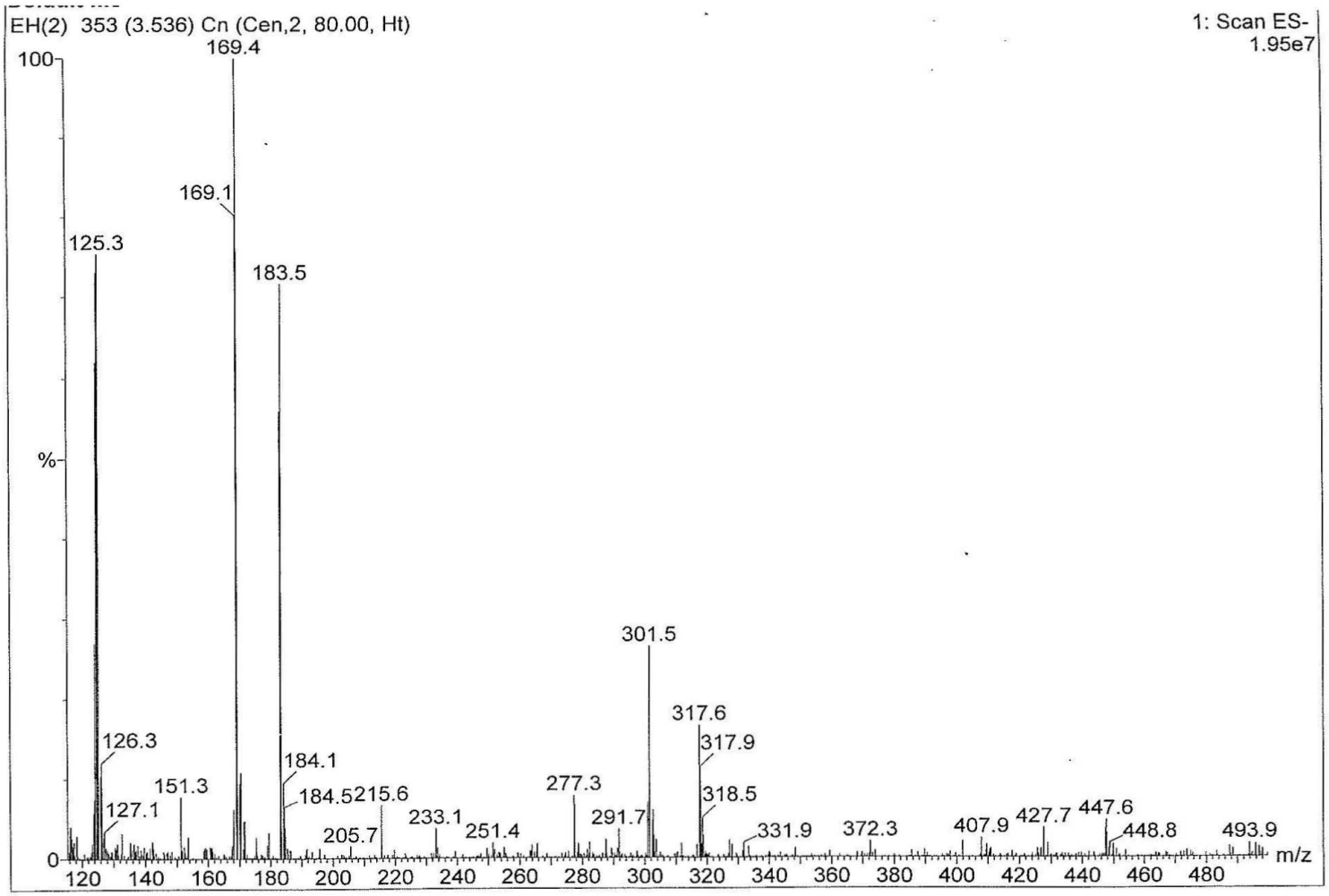


Figure 3.13. LC-MS spectrum of Gallic acid detected in the Et_2O extract of *Epilobium hirsutum L.*

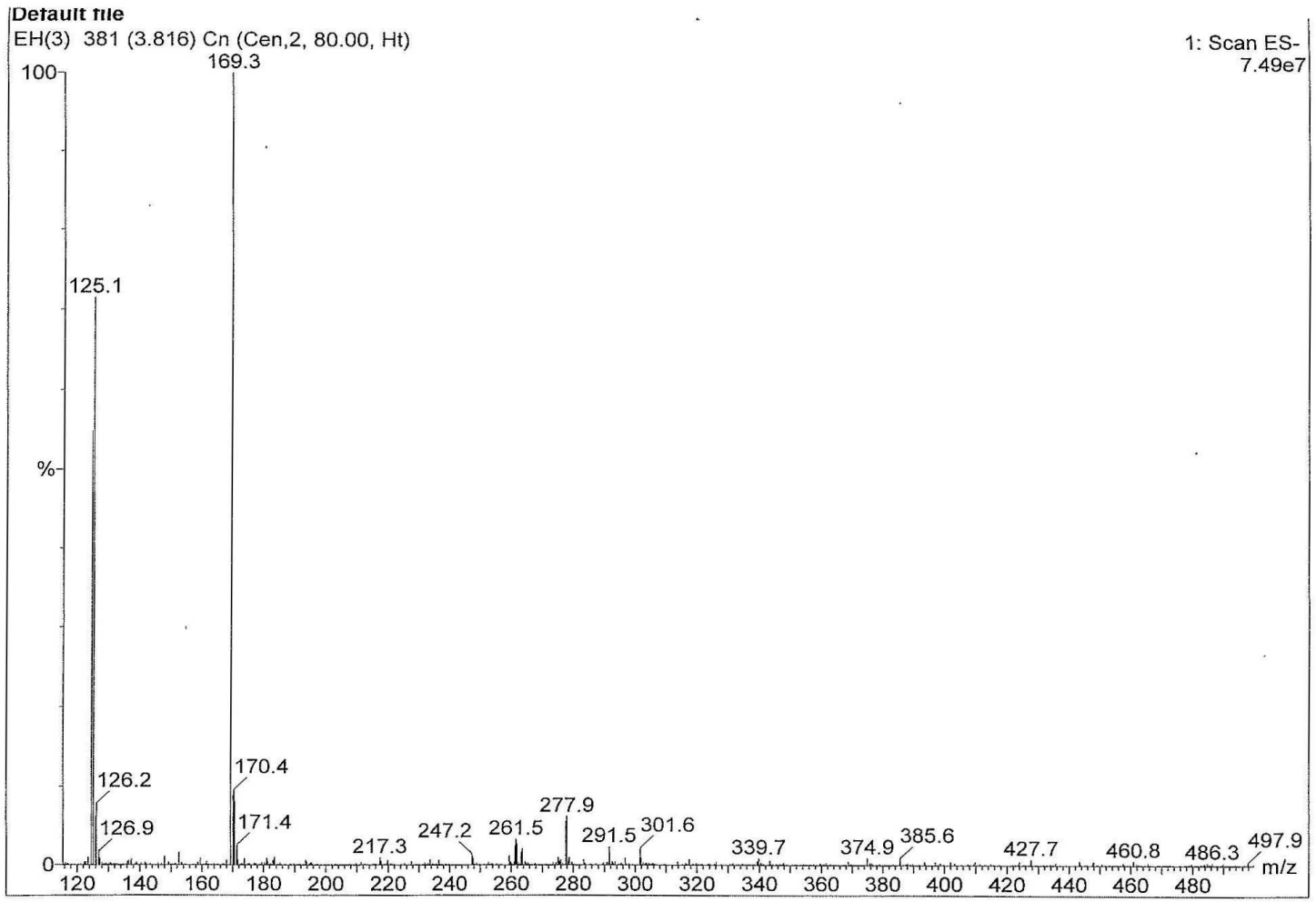


Figure 3.14. LC-MS spectrum of Gallic acid detected in the EtOAc extract of *Epilobium hirsutum L.*

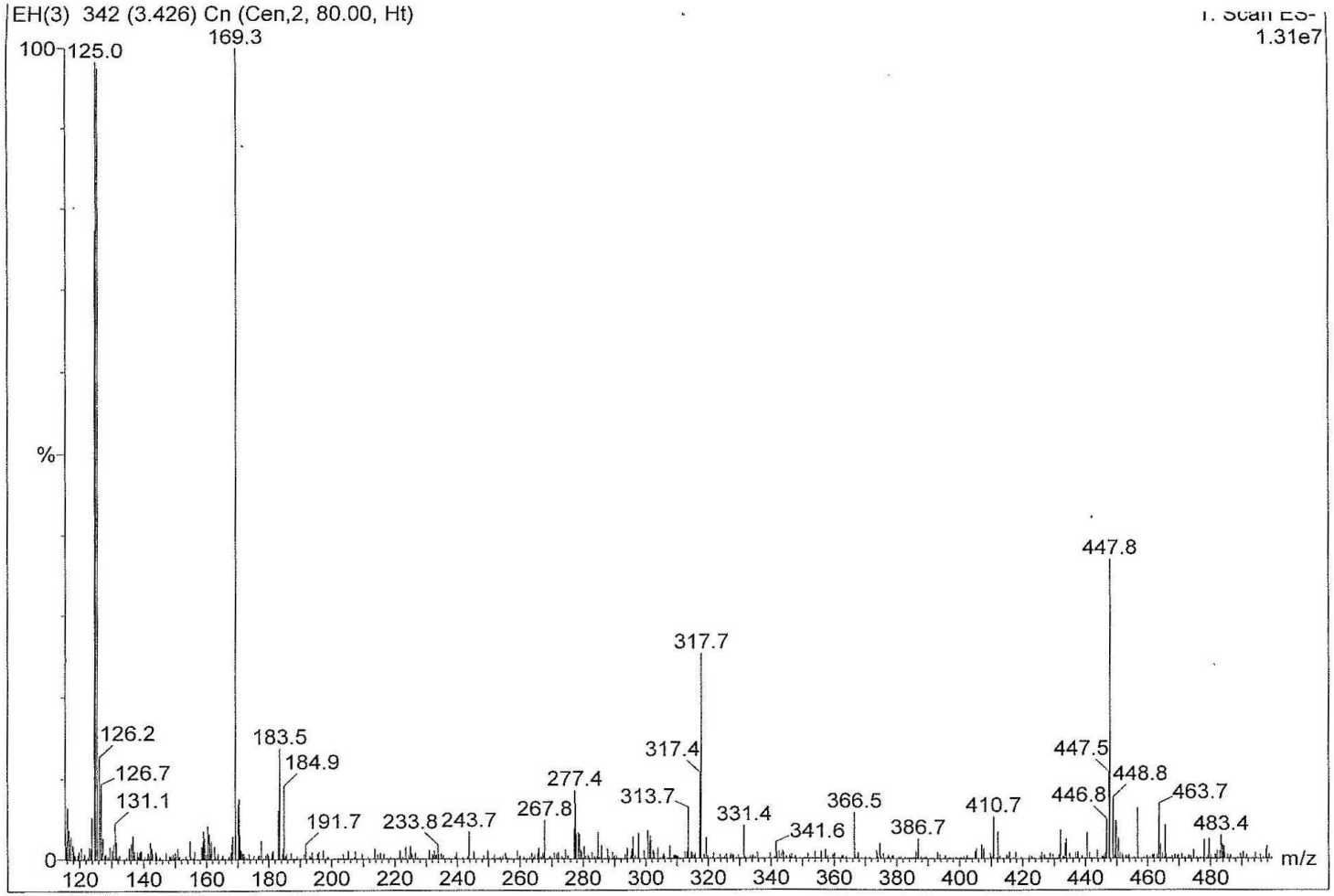


Figure 3.15. LC-MS spectrum of Quercetin-3-O-glucoside detected in the EtOAc extract of *Epilobium hirsutum L.*

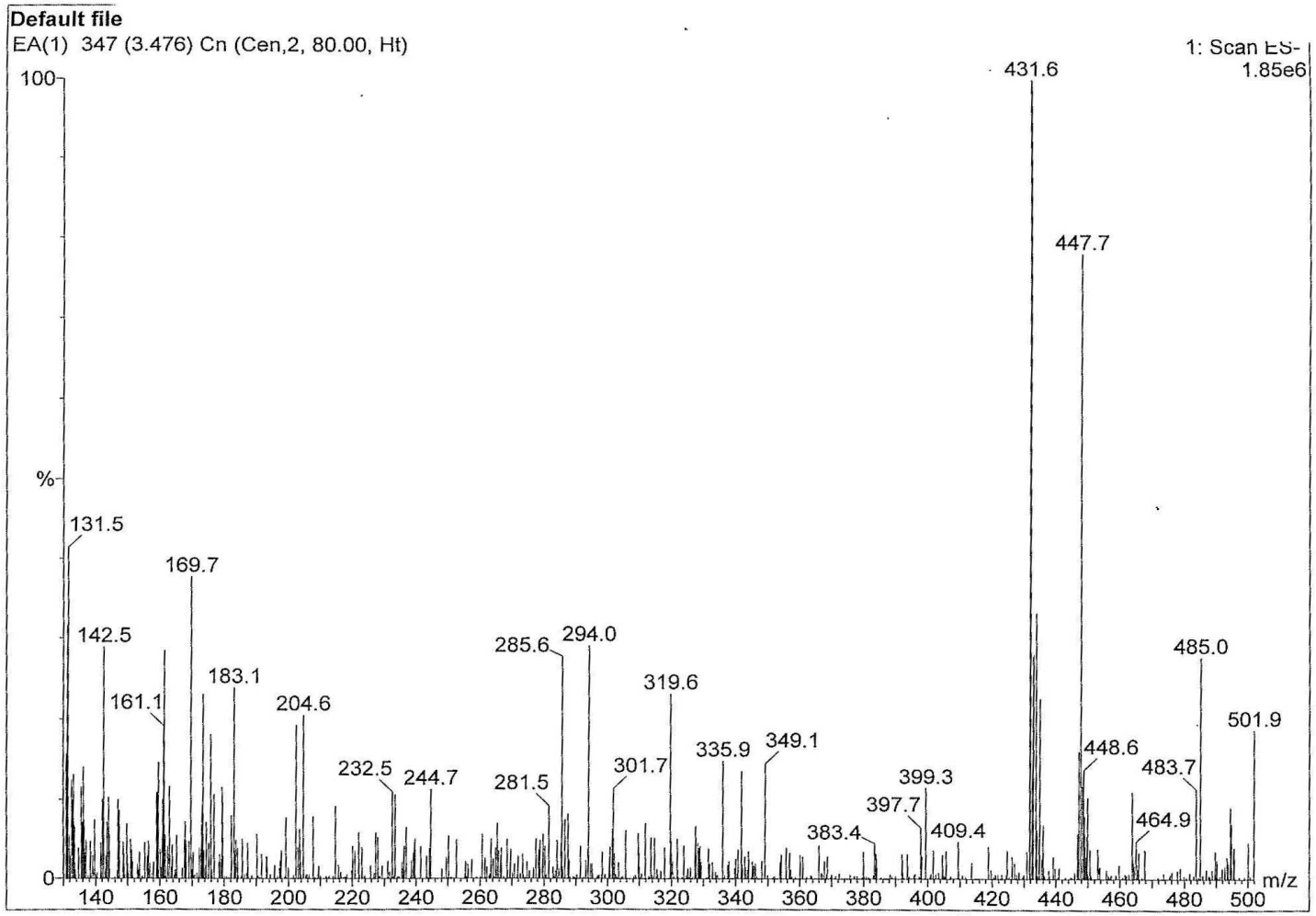


Figure 3.16. LC-MS spectrum of Apigenin-7-O-glucoside detected in the $CHCl_3$ extract of *Epilobium angustifolium L.*

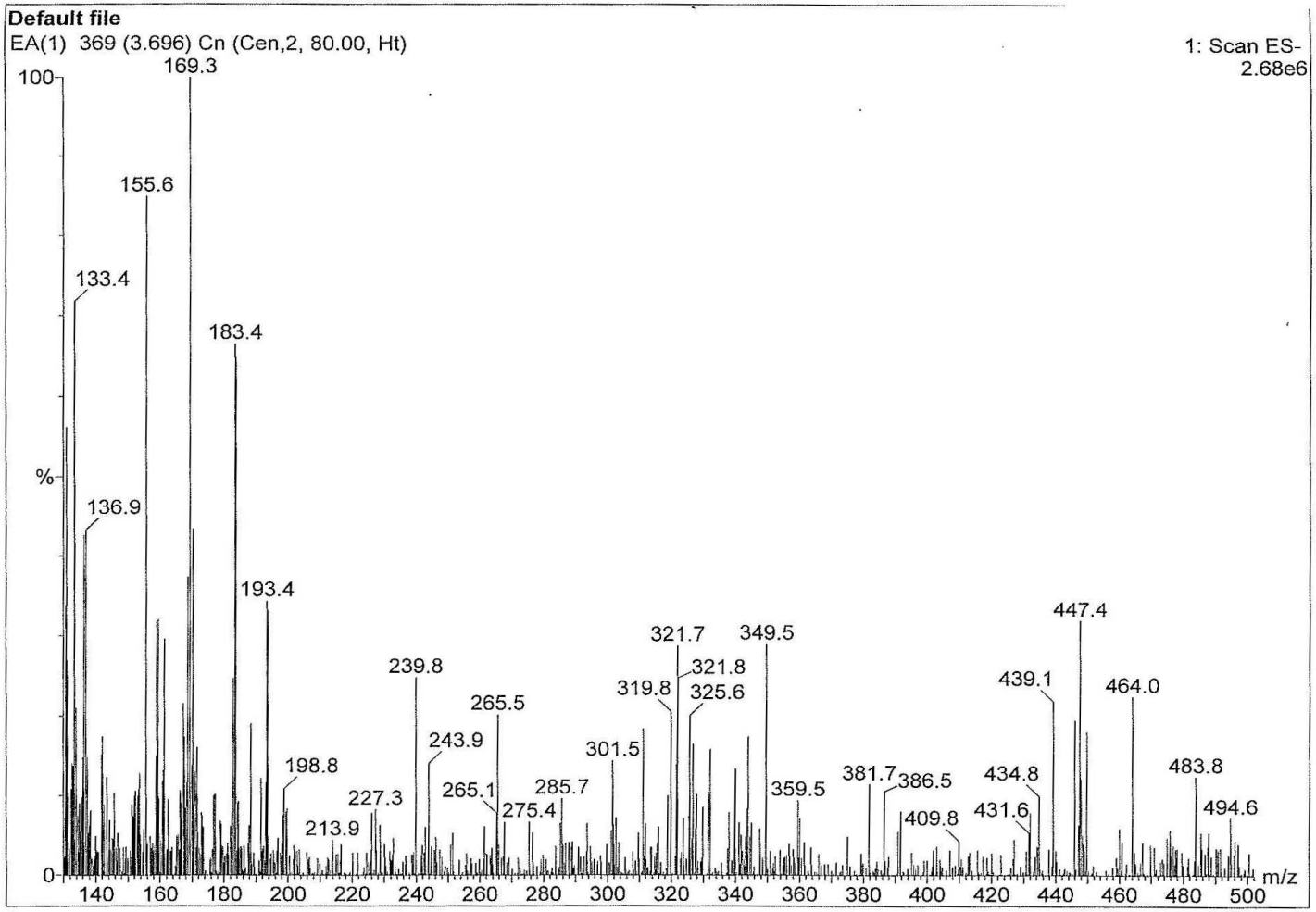


Figure 3.17. LC-MS spectrum of Gallic acid detected in the $CHCl_3$ extract of *Epilobium angustifolium L.*

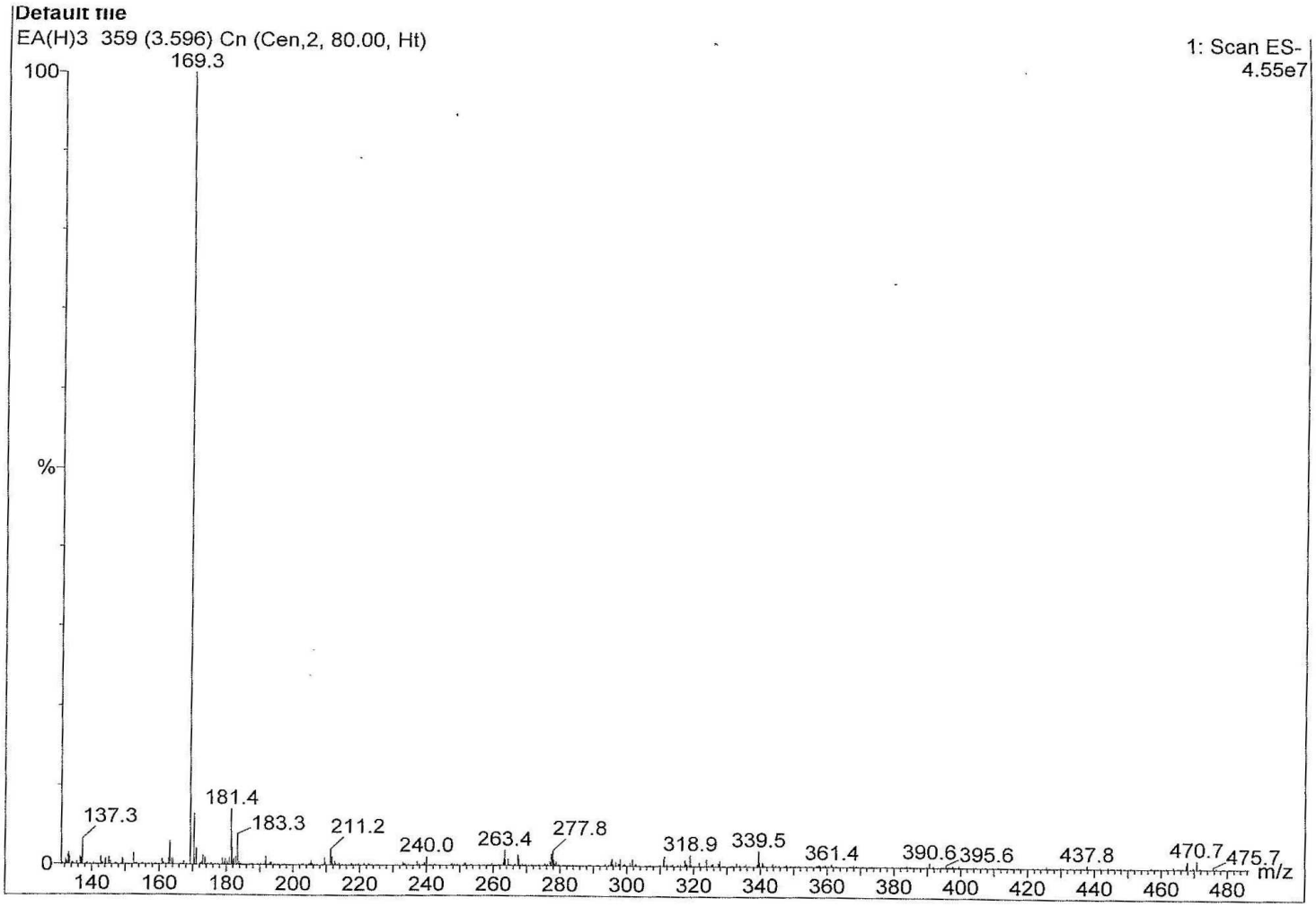


Figure 3.18. LC-MS spectrum of Vanilic acid detected in the EtOAc extract of *Epilobium angustifolium L.*

The quantitative analysis of the polyphenolic compounds in the plant extracts has been performed by High Performance Liquid Chromatography (HPLC). For this purpose, in order to increase the yield and to prevent the amount loss during fractionation process the dried and ground flowers have been extracted by a different method. Plant has been defatted by applying a 8-h Soxhlet extraction with petroleum benzine and then defatted plant was refluxed with methanol (70%) for 2h and crude methanolic extract has been further extracted with ethyl acetate for the isolation of polyphenolic compounds. In addition, after subjecting hydrolysis of the defatted plant with 1.2 M HCl an EtOAc extract was obtained. The amounts of polyphenolic compounds in each plant; for SC, DL, XA, EH, respectively, were shown in Tables 3.2-5. The calibration curves are presented in Figure 3.19-21. The HPLC chromatograms of the extracts were introduced in Figures 3.22 - 31, respectively.

Table 3.2. The quantity of phenolic compounds in *Silene compacta* FISCHE.

	BA DERIVATIVES mg/100g dry plant	**CA DERIVATIVES mg/100g dry plant**	**FLAVONOID DERIVATIVES mg/100g dry plant**
SC Extraction 1 (70% aqueous MeOH)	*p*- hydroxybenzoic acid[a] 1.88 ± 0.69 Vanilic acid[a] 1.08 ± 0.01	Ferulic acid[b] 7.05 ± 0.24 Other CAs(total)[b] 84.82 ± 1.15	Flavan-3-ol[a] 17.00 ± 0.10 Quercetin-3-O-rutinoside[c] 632.29 ± 96.97 Quercetin-3-O-glucoside[c] 137.23 ± 19.30 Other Flavonoids(total)[c] 19.86 ± 2.25
	Total 2.96 ±0.69	**Total 91.87 ± 1.17**	**Total 806.38 ± 98.90**
SC Extraction 2 (Hydrolysis with 1.2 M HCl)	*p*- hydroxybenzoic acid[a] 2.01 ± 0.19 Vanilic acid[a] 3.48 ± 0.16 Gallic acid[a] 0.81 ± 0.01 Protocatechuic acid[a] 2.94 ± 0.18	*trans*-cinnamic acid[a] 21.04 ± 4.44 Ferulic acid[b] 16.25 ± 2.15 Other CAs(total)[b] 162.84 ± 5.83	Flavan-3-ol[a] 508.76 ± 40.34 Quercetin-3-O-rutinoside[c] 318.82 ± 15.07 Quercetin-3-O-glucoside[c] 112.85 ± 4.86 Other Flavonoids(total)[c] 46.72 ± 1.72
	Total 9.24 ± 0.31	**Total 200.13 ± 7.64**	**Total 987.15 ± 43.37**

Notes: [a] → as Gallic Acid Equivalent , [b] → as Chlorogenic Acid Equivalent , [c] → as Quercetin Equivalent , BA (Benzoic acid), CA (Cinnamic acid), ±SD (Standard Deviation)

Table 3.3. The quantity of phenolic compounds in *Digitalis Lamarcki*

	BA DERIVATIVES mg/100g dry plant	**CA DERIVATIVES mg/100g dry plant**	**FLAVONOID DERIVATIVES mg/100g dry plant**
DL Extraction 1 (70% aqueous MeOH)	Protocatechuic acid[a] 3.11 ± 0.09 *p*- hydroxybenzoic acid[a] 4.53 ± 1.12 Vanilic acid[a] 1.86 ± 0.20	Chlorogenic acid[b] 25.89 ± 0.10 Caffeic acid[b] 13.36 ± 2.47 Ferulic acid[b] 27.81 ± 0.11 Other CAs(total)[b] 161.20 ± 2.06	Apigenin-7-O-glucoside[c] 20.12 ± 0.33 Apigenin[c] 40.66 ± 0.57 Other Flavonoids(total)[c] 111.26 ± 5.43
	Total 9.50 ± 1.14	**Total 228.26 ± 3.22**	**Total 172.04 ± 5.47**
DL Extraction 2 (Hydrolysis with 1.2 M HCl)	Protocatechuic acid[a] 3.08 ± 0.46 Vanilic acid[a] 4.81 ± 0.10 Gallic acid[a] 0.11 ± 0.00	Caffeic acid[b] 8.37 ± 0.54 Ferulic acid[b] 13.22 ± 2.20 Other CAs(total)[b] 39.00 ± 1.53	Flavan-3-ol(totatl)[a] 4.15 ± 0.08 Apigenin[c] 9.50 ± 0.29 Other Flavonoids(total)[c] 0.49 ± 0.01
	Total 8.00 ± 0.47	**Total 60.59 ± 2.73**	**Total 14.14 ± 0.30**

Notes: [a] → as Gallic Acid Equivalent , [b] → as Chlorogenic Acid Equivalent , [c] → as Quercetin Equivalent , BA (Benzoic acid), CA (Cinnamic acid), ±SD (Standard Deviation)

Table 3.4. The quantity of phenolic compounds in *Xeranthemum annuum L.*

	BA DERIVATIVES mg/100g dry plant	**CA DERIVATIVES mg/100g dry plant**	**FLAVONOID DERIVATIVES mg/100g dry plant**
XA Extraction 1 (70% aqueous MeOH)	Protocatechuic acid[a] 2.17 ± 0.22 Vanilic acid[a] 1.58 ± 0.08	Chlorogenic acid[b] 18.42 ± 0.21 Caffeic acid[b] 5.76 ± 1.93 Ferulic acid[b] 4.92 ± 0.67 Other CAs(total)[b] 67.37 ± 0.82	Flavan-3-ol(total)[a] 14.94 ± 13.90 Quercetin-3-O-glucoside [c] 45.89 ± 11.27 Apigenin-7-O-glucoside [c] 857.88 ± 6.93 Other Flavonoids(total)[c] 717.77 ± 50.14
	Total 3.75 ± 0.23	**Total 96.47 ± 2.21**	**Total 1636.48 ± 53.69**
XA Extraction 2 (Hydrolysis with 1.2 M HCl)	Vanilic acid[a] 2.49 ± 0.08	Caffeic acid [b] 2.55 ± 0.00 Ferulic acid [b] 1.77 ± 0.02 Other CAs(total)[b] 179.99 ± 2.31	Flavan-3-ol(total)[a] 56.42 ± 6.69 Quercetin-3-O-glucoside [c] 49.33 ± 0.76 Luteolin [c] 35.48 ± 3.10 Other Flavonoids(total)[c] 1933.74 ± 22.05
	Total 2.49 ± 0.08	**Total 184.31 ± 2.31**	**Total 2074.97 ± 23.26**

Notes: [a] → as Gallic Acid Equivalent , [b] → as Chlorogenic Acid Equivalent , [c] → as Quercetin Equivalent , BA (Benzoic acid), CA (Cinnamic acid), ±SD (Standard Deviation)

Table 3.5. The quantity of phenolic compounds in *Epilobium hirsutum* L.

Notes: [a] → as Gallic Acid Equivalent , [b] → as Chlorogenic Acid Equivalent , [c] → as Quercetin Equivalent , BA (Benzoic acid), CA (Cinnamic acid), ±SD (Standard Deviation)

	BA DERIVATIVES mg/100g dry plant	**CA DERIVATIVES mg/100g dry plant**	**FLAVONOID DERIVATIVES mg/100g dry plant**
EH Extraction 1 (70% aqueous MeOH)	Gallic acid[a] 14.33 ± 1.27 Vanilic acid[a] 2.94 ± 0.09	Chlorogenic acid[b] 31.59 ± 1.76 Caffeic acid[b] 8.14 ± 0.00 CA(total)[b] 14.54 ± 0.53	Quercetin-3-O-Glucoside[a] 17.72 ± 2.03 Flavan-3-ol(total)[a] 36.55 ± 1.74 Quercetin-3-O-glucoside[c] 451.81 ± 3.33 Quercetin-3-O-rutinoside[c] 231.41 ± 0.33 Other Flavonoids(total)[c] 1978.12 ± 148.06
	Total 17.27 ± 1.27	Total 54.27 ± 1.84	Total 2715.61 ± 148.12
EH Extraction 2 (Hydrolysis with 1.2 M HCl)	Gallic acid[a] 60.03 ± 4.87 Protocatecuic acid[a] 82.11 ± 21.15		Flavan-3-ol(total) [a] 14.37 ± 0.65 Other Flavonoids(total)[c] 1160.14 ± 24.29
	Total 142.14 ± 21.70	Total 0.00 ± 0.00	Total 1174.51 ± 24.30

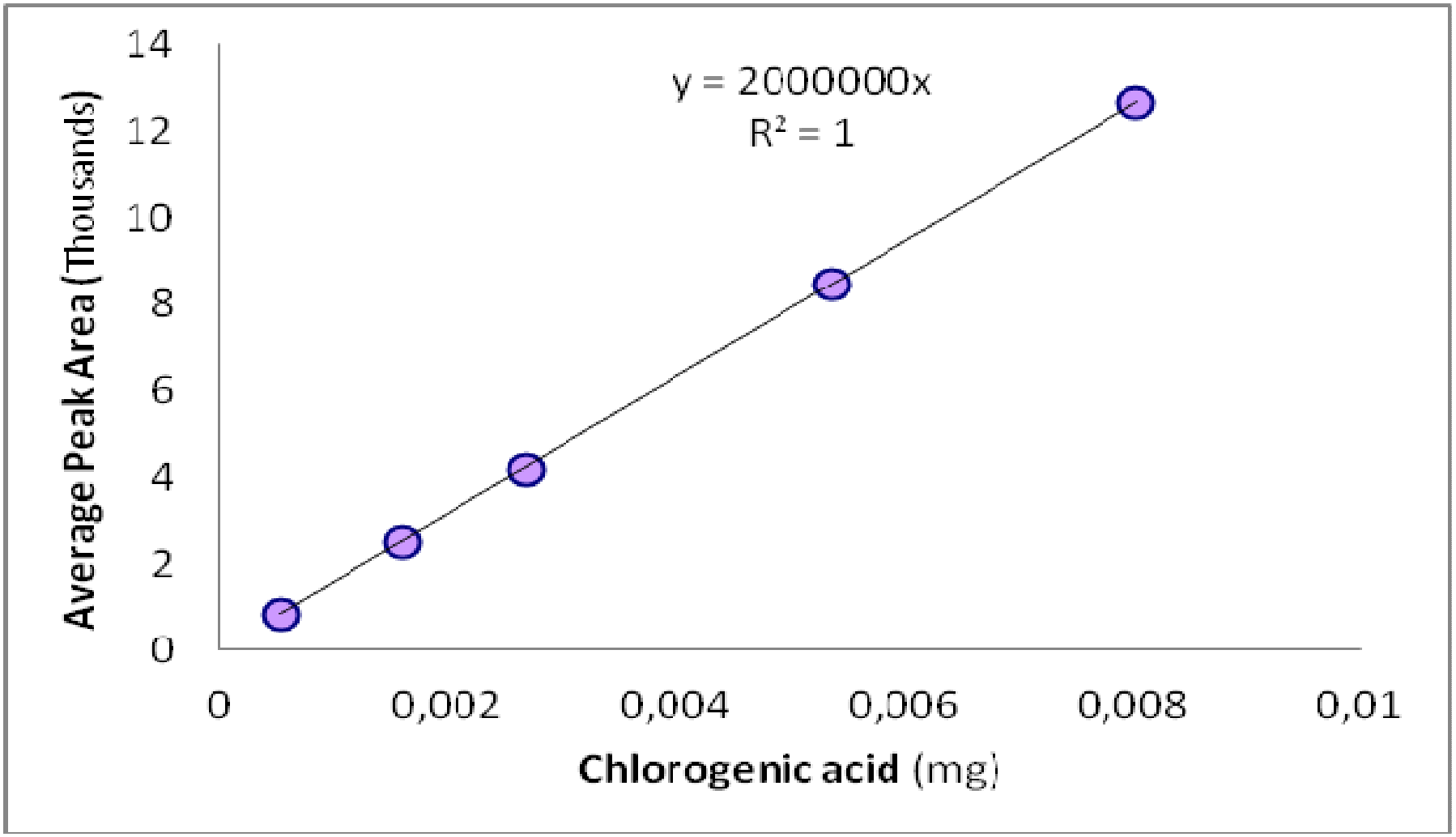


Figure 3.19. Calibration Curve of Chlorogenic Acid (mg)

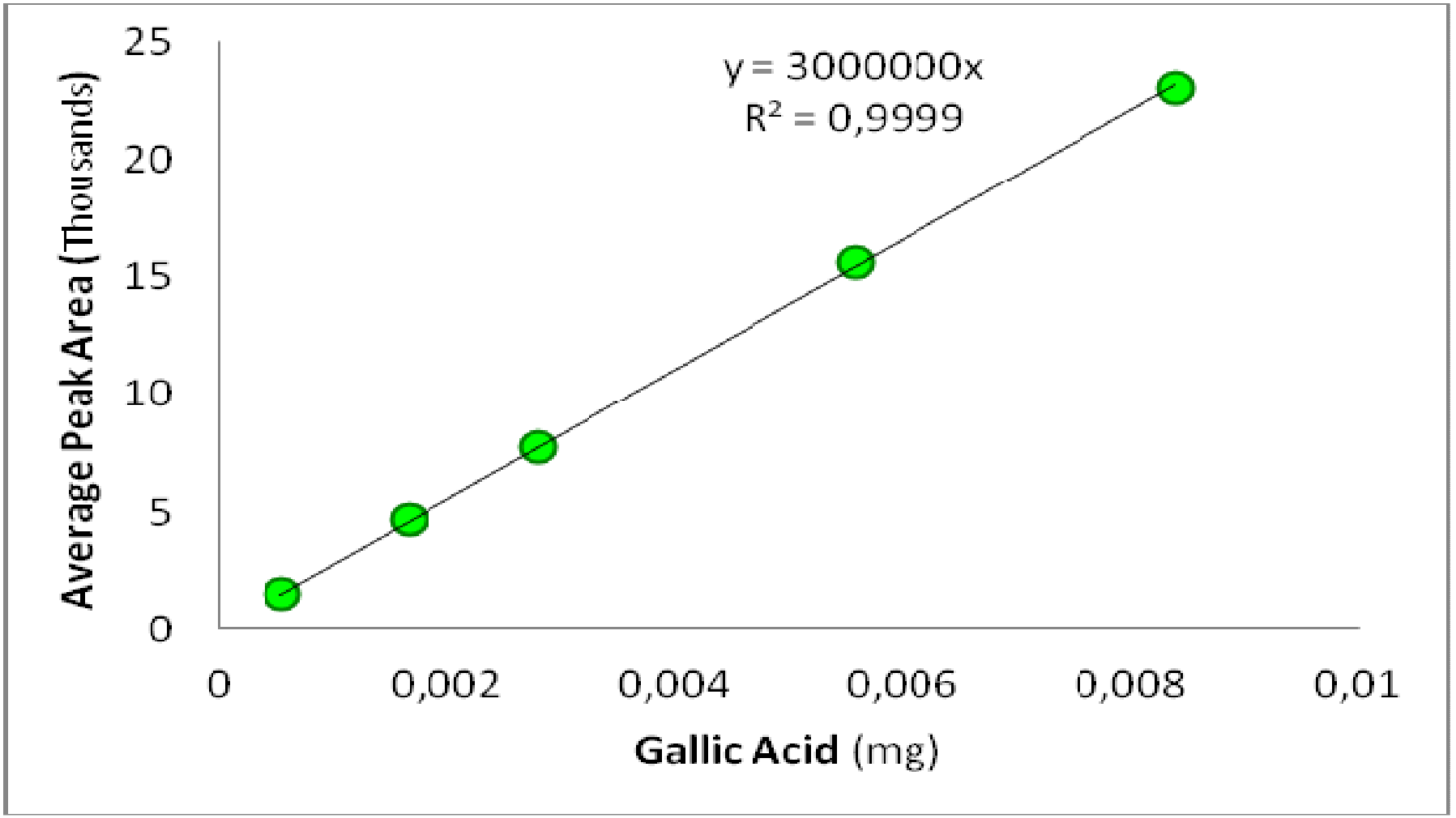


Figure 3.20. Calibration Curve of Gallic Acid (mg)

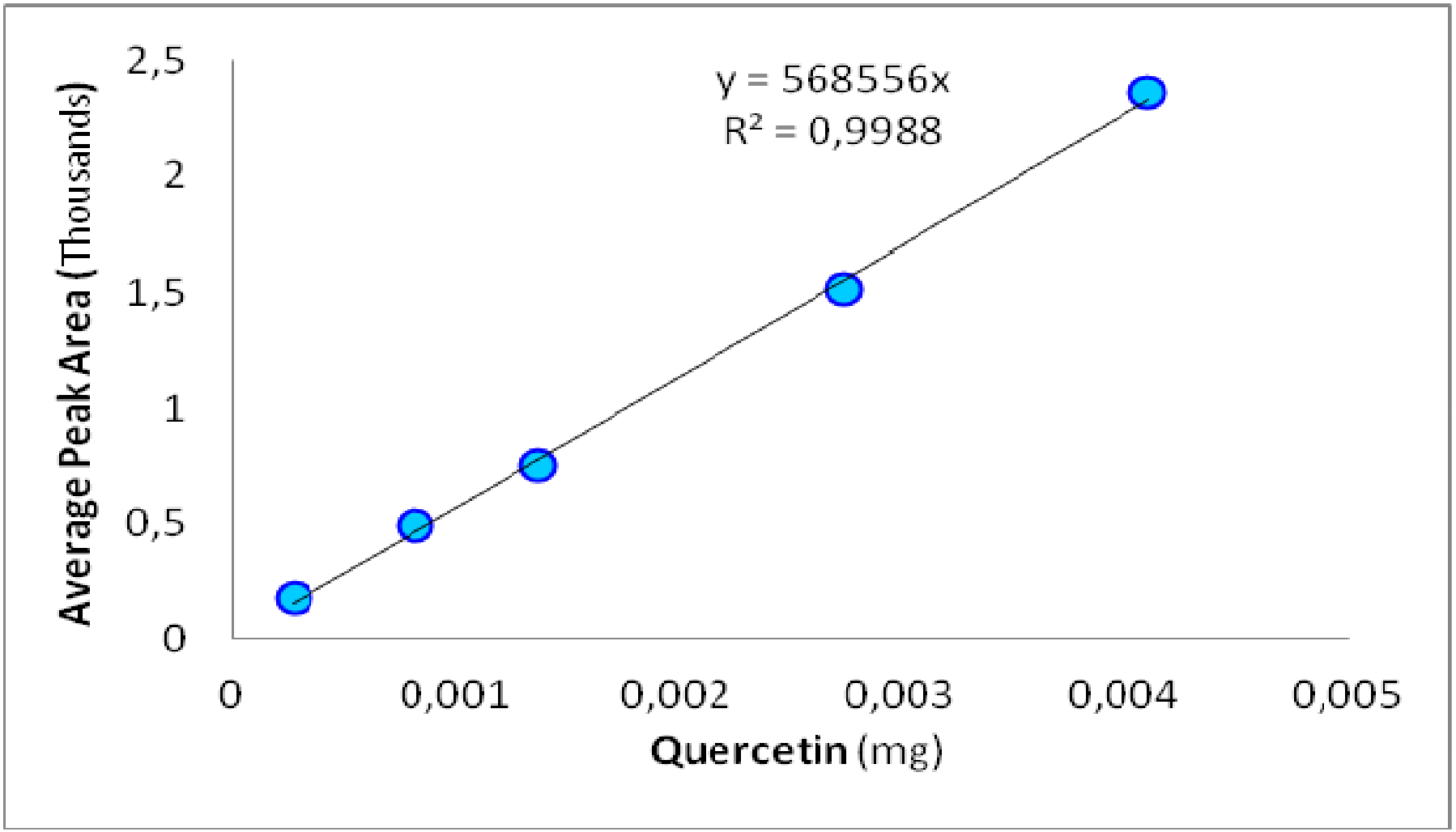


Figure 3.21. Calibration Curve of Quercetin (mg)

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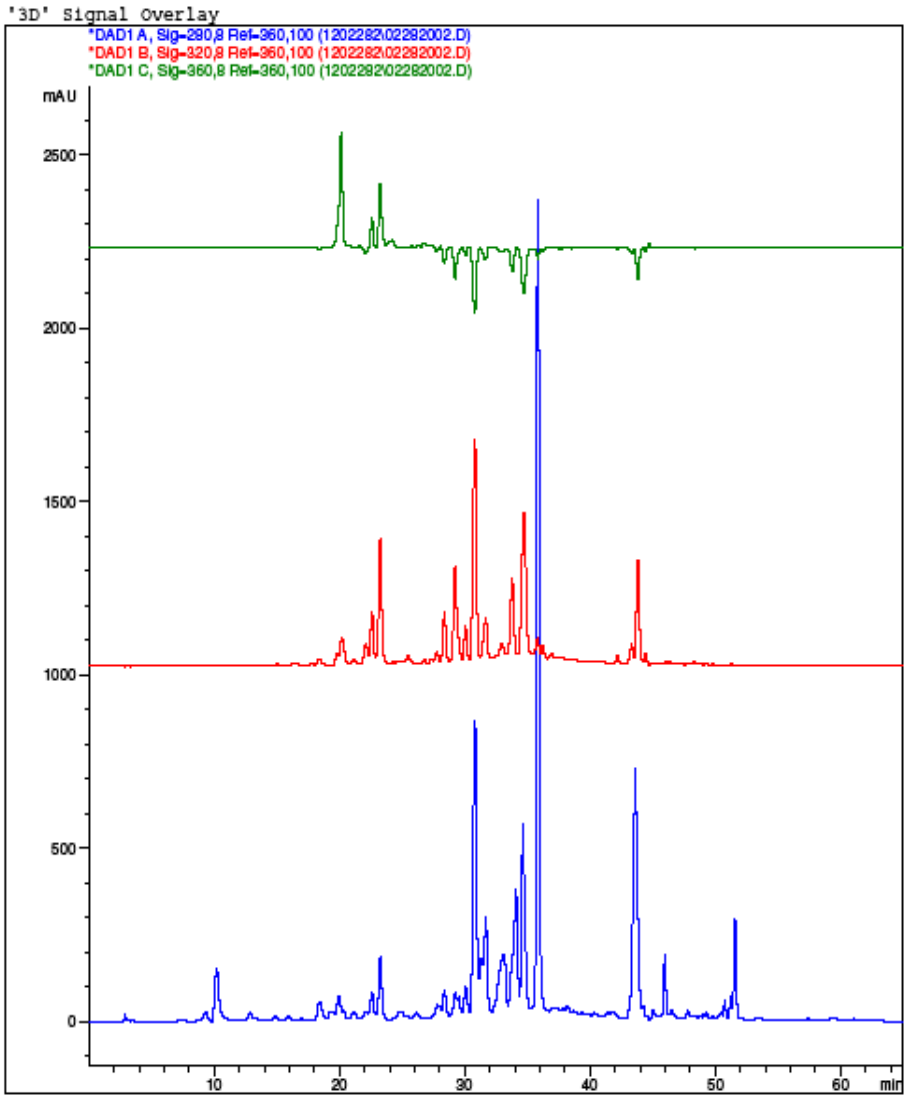

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Figure 3.22. HPLC Chromatogram of phenolic compounds in the EtOAc extract obtained from methanolic extraction (1) of *Silene compacta FISCHE*

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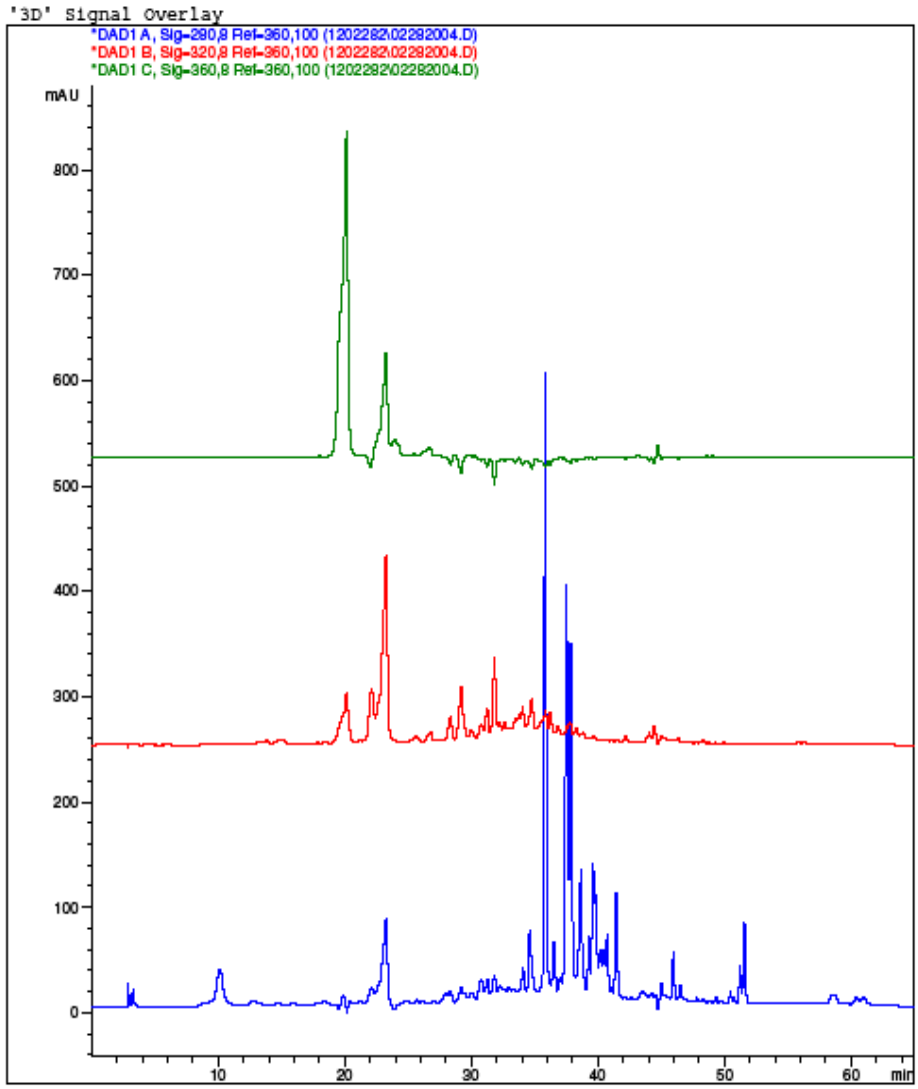

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Figure 3.23. HPLC Chromatogram of phenolic compounds in the EtOAc extract obtained from methanolic extraction (2) of *Silene compacta FISCHE*

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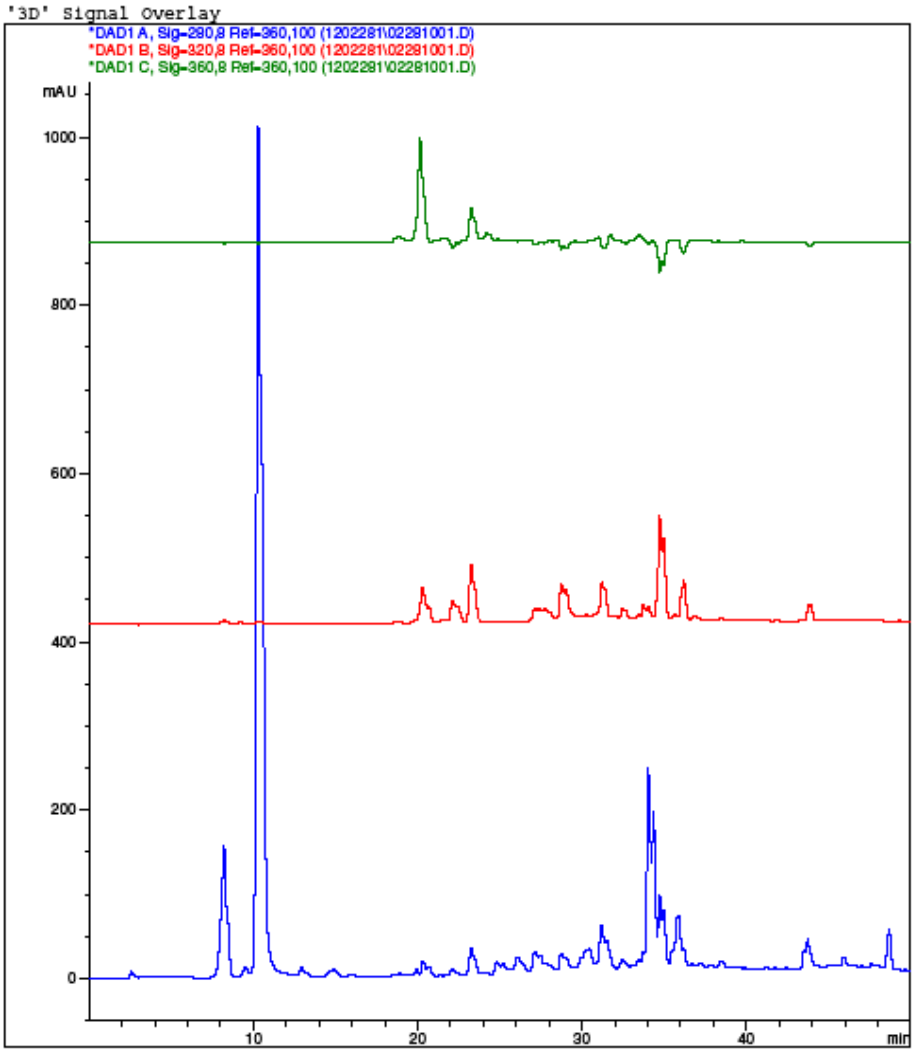

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Figure 3.24. HPLC Chromatogram of phenolic compounds in the EtOAc extract obtained from acidic hydrolysis of *Silene compacta FISCHE.*

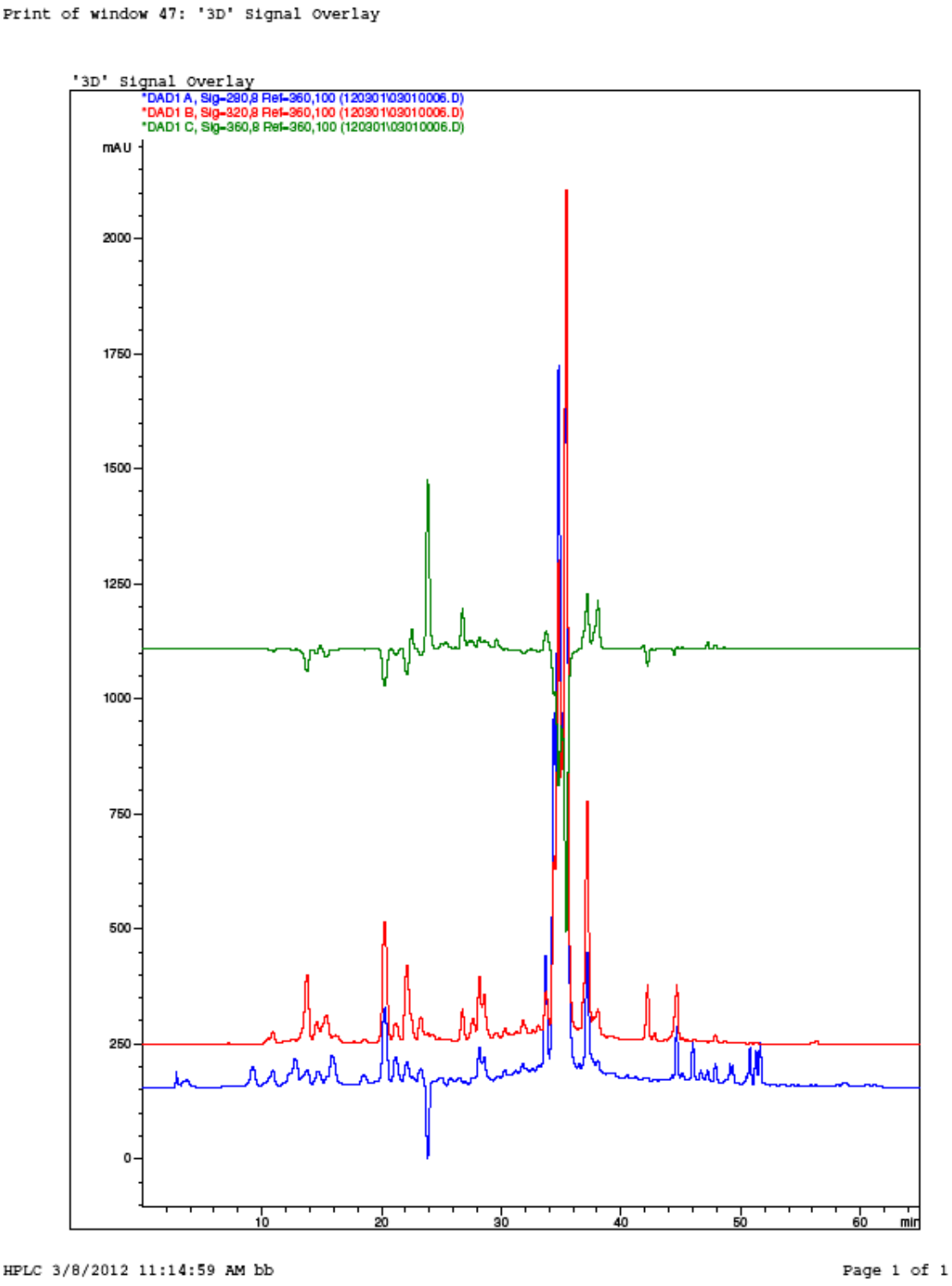

Figure 3.25. HPLC Chromatogram of phenolic compounds in the EtOAc extract obtained from methanolic extraction (1) of *Digitalis lamarcki*

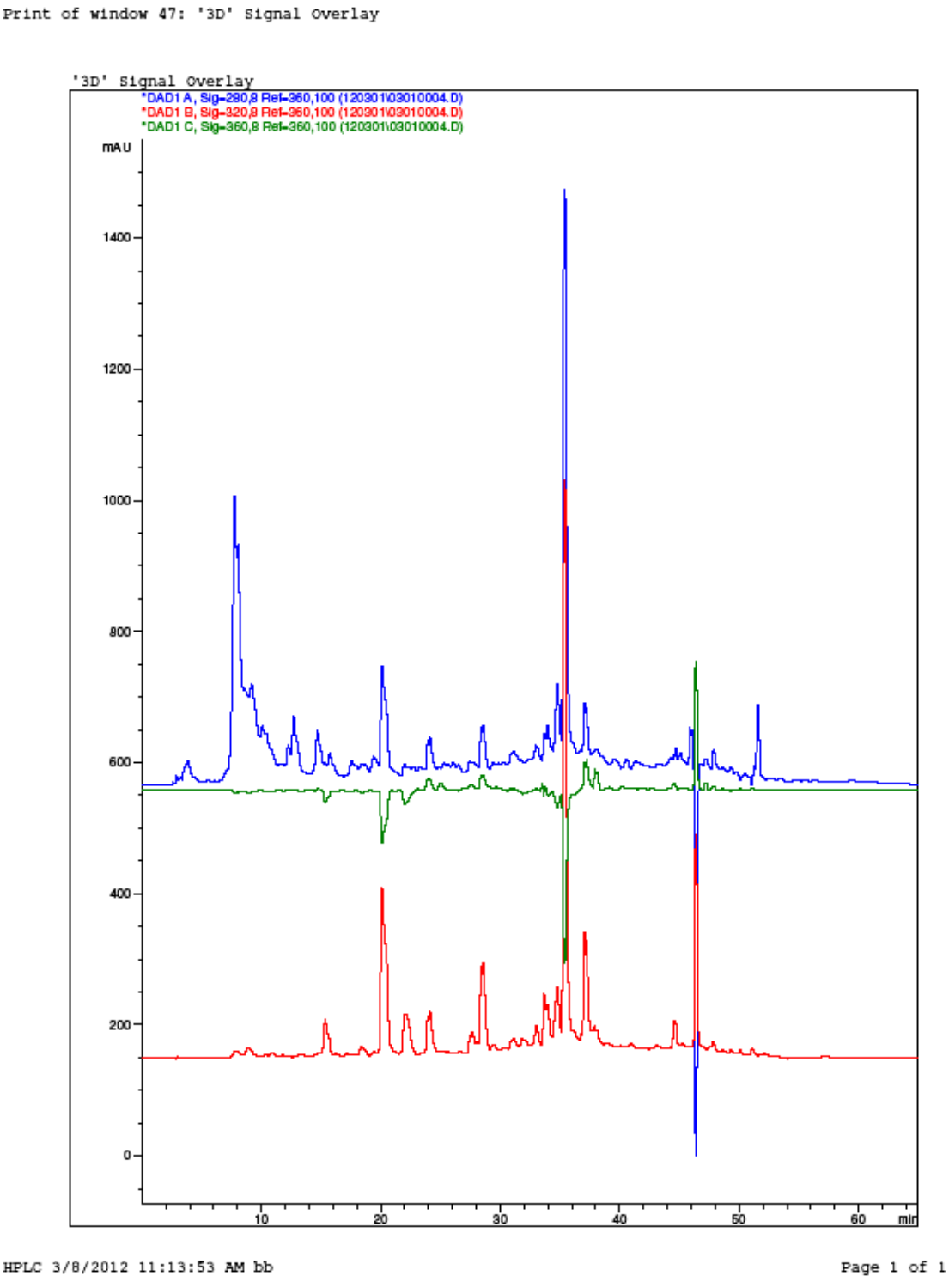

Figure 3.26. HPLC Chromatogram of phenolic compounds in the EtOAc extract obtained from methanolic extraction (2) of *Digitalis lamarcki*

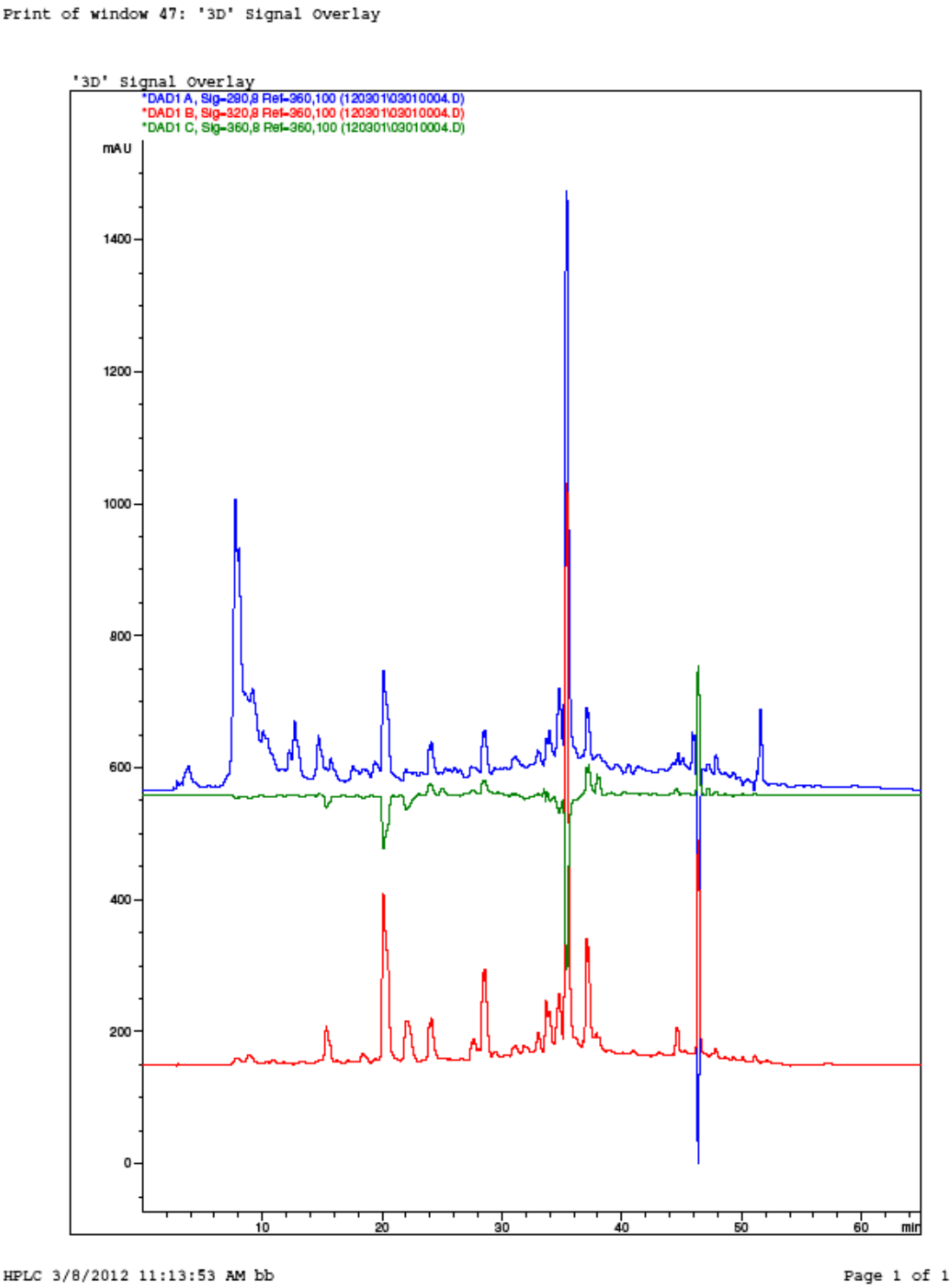

Figure 3.27. HPLC Chromatogram of phenolic compounds in the EtOAc extract obtained from acidic hydrolysis of *Digitalis lamarcki*

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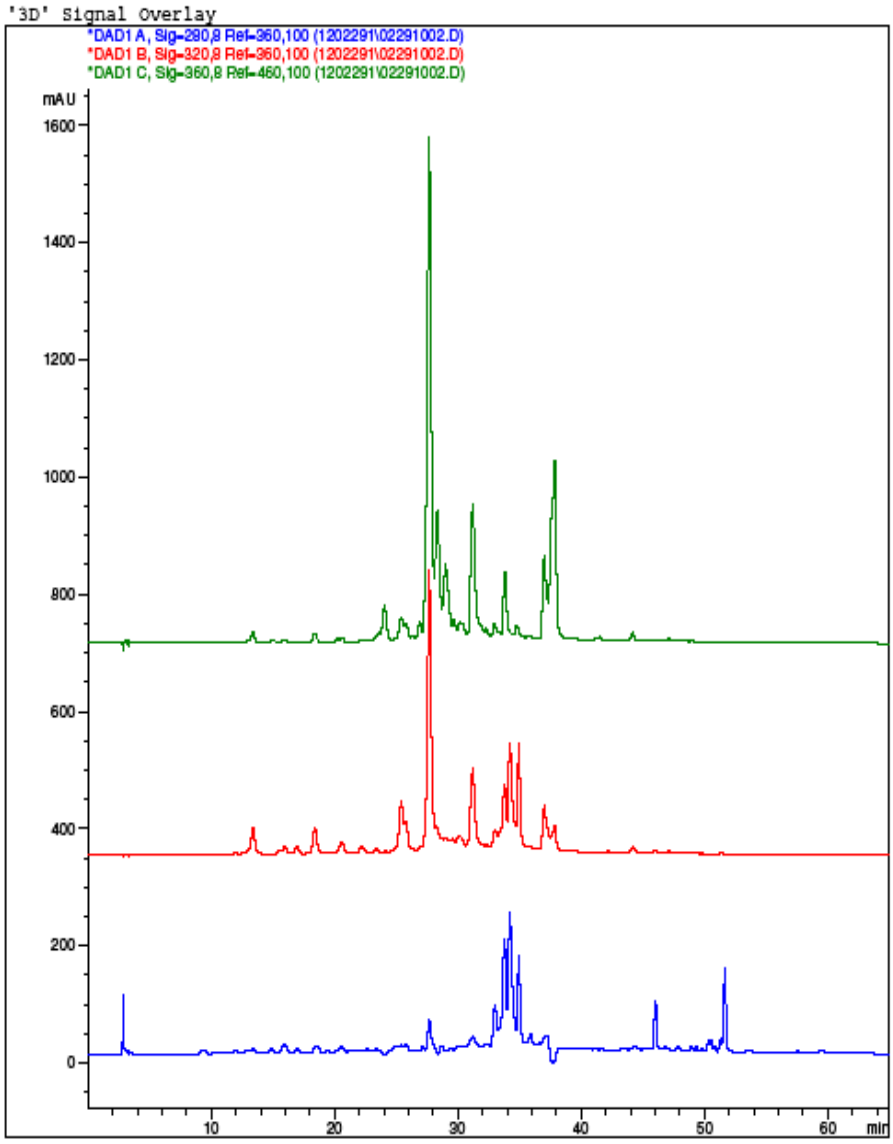

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Figure 3.28. HPLC Chromatogram of phenolic compounds in the EtOAc extract obtained from methanolic extraction of *Xeranthemum annuum L.*

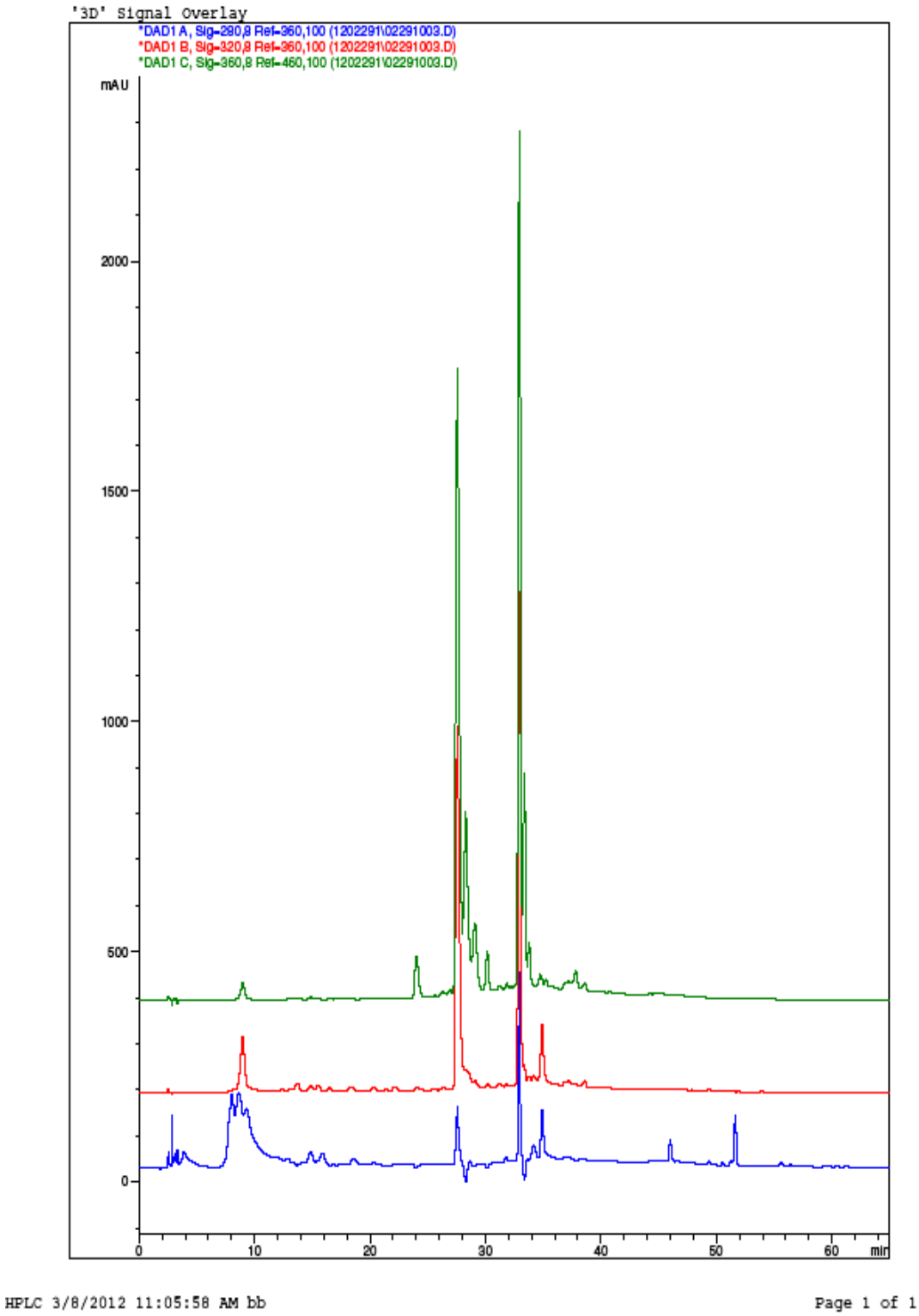

Figure 3.29. HPLC Chromatogram of phenolic compounds in the EtOAc extract obtained from acidic hydrolysis of *Xeranthemum annuum L.*

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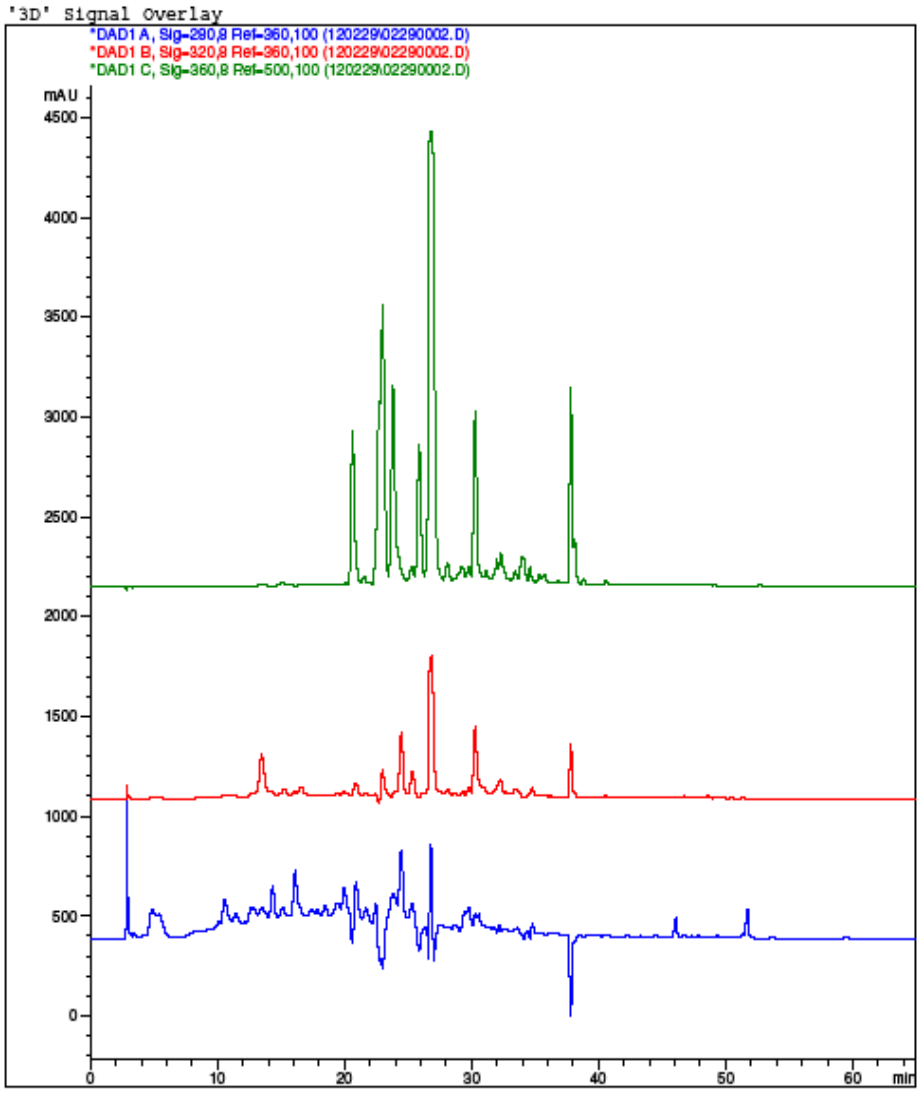

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Figure 3.30. HPLC Chromatogram of phenolic compounds in the EtOAc extract obtained from methanolic extraction of *Epilobium hirsutum L.*

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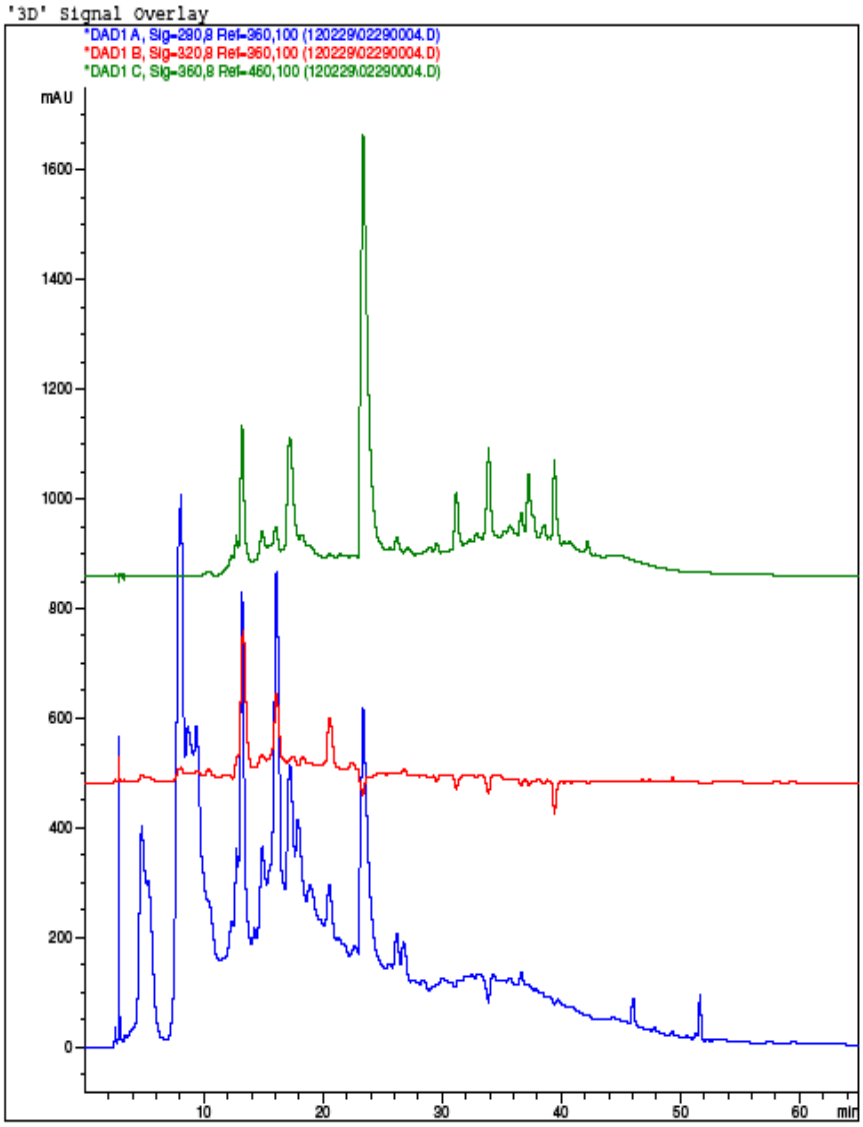

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Figure 3.31. HPLC Chromatogram of phenolic compounds in the EtOAc extract obtained from acidic hydrolysis of *Epilobium hirsutum L.*

Taking account of the LC-MS data; phenolic compounds detected in *Silene compacta FISCHE* (SC) are myricetin, gallic acid, kaempferol, *p*-hydroxybenzoic acid, and quercetin-3-O-rhamnoside. Phenolic compounds detected in *Digitalis lamarcki* (DL) are quercetin, caffeic acid, quercetin-3-O-glucoside, and myricetin-3-O-rhamnoside. Phenolic compounds detected in *Xeranthemum annuum L.* (XA) ferulic acid, quercetin-3-O-glucoside, galangin, kaempferol, quercetin, protocatechuic acid, myricetin-3-O-rhamnoside, and luteolin. Phenolic compounds detected in *Epilobium hirsutum L.* (EH) have been identified as kaempferol, quercetin, caffeic acid, gallic acid, quercetin-3-O-glucoside, and quercetin-3-O-rhamnoside. Phenolic compounds detected in *Epilobium angustifolium L.* (EA) were found as kaempferol-3-O-rhamnoside, quercetin-3-O-glucoside, apigenin-7-0-glucoside, gallic acid, quercetin-3-O-rhamnoside, and vanillic acid.

When compared the amounts given as mg/100 g dry plant in the following summarized table, the highest contents of benzoic acids were detected in *Epilobium hirsutum L.* as 159.41. But the highest contents of cinnamic acids were detected in *Silene compacta FISCHE* as 292. As for flavonoid content highest was *Epilobium hirsutum L.*.

Although total polyphenolic content was highest (4103,80 mg/100g dry plant) in the plant *Epilobium hirsutum L.,* for *Digitalis lamarcki* it has been found the lowest value (492,53 mg/100g dry plant) .

	SC		DL		XA		EH	
	70 % MeOH	Hydrolysis	70 % MeOH	Hydrolysis	70 % MeOH	Hydrolysis	70 % MeOH	Hydrolysis
Benzoic Acids (mg/100g dry plant)	2.96	9.24	9.50	8.00	3.75	2.49	17.27	142.14
Total	**12.20**		**17.50**		**6.24**		**159.41**	
Cinnamic Acids (mg/100g dry plant)	91.87	200.13	228.26	60.59	96.47	184.31	54.27	-
Total	**292**		**288.85**		**280.78**		**54.27**	
Flavonoids (mg/100g dry plant)	806.38	987.15	172.04	14.14	1636.48	2074.97	2715.61	1174.51
Total	**1793.53**		**186.18**		**3711.45**		**3890.12**	
Overall Total	**2097.73**		**492.53**		**3998.47**		**4103.80**	

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