IDENTIFICATION OF PHENOLIC COMPOUNDS IN ROOTS OF *RHEUM RIBES* L.

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ABSTRACT

DETERMINATION OF PHENOLIC AND FLAVONIOD COMPOUNDS IN
REHUM RIBES

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This study involves the investigation of antioxidant phenolic and flavonoid compounds in *Rheum ribes* L.

There is a continuous mounting pressure, in the scientific world, to discover new and potent antioxidants, to combat the free radicals. Among the antioxidant compounds, the phenolics and flavonoids comprise the largest class, and thus hold an important place in biochemical studies.

Rheum ribes belong to the family of Polygonaceae in the genus Rheum, which is consumed as a medicinal plant. Since this plant is a source of one of the most important crude drugs in the Middle East (Kashiwada et al., 1988), its antioxidative properties were also worthwhile to be investigated as well as its phenolic constituents. In this study, the crude extract of the roots of *Rheum ribes* subjected to solvent-solvent fractionation. and each of the fractions were then subjected to the DPPH , total phenolic content and total flavonoids tests. Ethyl acetate fraction exhibited the highest antioxidant capacity, total phenolics and flavonoids compounds. Further analysis by RP-HPLC showed that all fractions contained different number of phenolic compounds and gallic acid, p-coumaric acid and quercetin were present according to the co-injection in RP-HPLC in ethyl acetate extract.

Keywords: Antioxidants, *Rheum ribes L.* , DPPH, total flavonoid content, RP-HPLC

ÖZ

RHEUM RIBES L. FENOLİK BİLEŞENLERİNİN TANIMLANMASI

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Bu çalışmada *Rheum ribes* L. bitkisinin antioksidan fenolik bileşenleri incelenmektedir.

Bilim dünyasında, serbest radikallerle savaşmak için yeni ve etkin antioksidanlar bulmak üzerine, sürekli artan bir baskı vardır. Antioksidan bileşenler arasında, fenolikler en büyük sınıfı oluşturur ve bu nedenle biyokimyasal çalışmalarda önemli bir yer tutarlar. Fenolikler, bazı bitki familyalarında ikincil metabolitler olarak yüksek miktarlarda bulunurlar. Bu familyalardan biri olan Polygonaceae, pek çok türünde alkaloid ve fenolik bileşenler içermektedir .

Rheum ribes, Polygonaceae türlerinden biri olarak daha henüz fenolikleri ve antioksidan kapasiteleri açısından incelenmemiştir; bu nedenle bu çalışmada, bitkinin çiçekli bölümlerinin metanol özütü çıkartılarak fraksiyonlarına ayrılmış ve fraksiyonlara antioksidan kapasite tayin metodları uygulanmıştır.

DPPH testi antioksidan kapasitenin araştırılmasında, total fenol miktarı tayini ise hangi fraksiyonda en fazla fenolik içerik olduğunu bulmak için kullanılmıştır. Bununla birlikte, total flavonoid miktar testi, en fazla flavonoid miktarını içeren fraksiyonun tespiti için yapılmıştır. Fraksiyonlar RP-HPLC metodu kullanılarak incelenmiş, bunun sonucunda etil asetat fraksiyonunda diğer maddelerin yanında gallik asit, p-kumarik asit ve kuersetin'in varlığı RP-HPLC kromatogramı ile gösterilmiştir.

Anahtar kelimeler: Antioksidanlar, *Rheum ribes L.*, DPPH, total flavonoid, RP-HPLC

To my beloved family

ACKNOWLEDGEMENTS

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CHAPTER 1

INTRODUCTION

There are more than six hundred thousand plant species in this world which are present over the land and under the water. Still many are waiting to be characterized and counted. Scientists predict that of all the known species of plant two-third have medicinal use in one way or the other. Many of the plants have antioxidant properties. This refers to their being active and combatting the oxidative stress. We as animals encounter oxidative stress during cellular respiration and also we as humans encounter oxidative stress due to intake of many oxidative toxins through various routes in the body. In such a picture, intake of antioxidant compounds through natural sources is useful in preventing such diseases to occur for example; cancer, alzheimer, cardiovascular and inflammatory diseases. This explains the reason of recent researches trying to focus on finding natural sources of potent antioxidants. For such a reason, complete and through study about any plant species, the chemicals present in it, is very important. (Govaerts, 2001; Schippmann et al., 2002 ; Batugal et al, 2004).

One of the most important groups of plant's secondary compounds is the phenol containing compounds. These are often referred to as the phenolic compounds. The phenolic compounds are formed in the plant as by products generally and are required many times by plants to combat the oxidative stress faced by them. Still most of the time, these phenolic compounds are useful for us when we intake them with the plant itself. Major sources of phenolic compounds are fruits and vegetables, especially when eaten raw. Other valuable sources of antioxidants which are also natural sources are the by-products produced by agriculture industries and processing food industries. Antioxidants from natural sources have been proved to be very effective health improvements, while those from artificial compounds from man-made resources had lowered their value because of their side effects. However,

natural antioxidants present in edible oils and phenolic rich extracts of some plants have been shown to be effective at least as much as the synthetic ones. Therefore, the main focus today is not in synthesizing new antioxidants, rather it is in finding new ways of extracting antioxidants from natural plant sources and characterizing them.

Recently, there is a great awakening towards the "Green wave" theory, which refers to the return of human beings to the "Mother Nature" to fulfill all of its necessities. This is keeping the fact under consideration that this step leads to lesser side effects or sometimes even no side effects at all. Also this step helps in conservation of biodiversity and protection of ecosystem while preserving the human race itself (Gijetenbeek, 1999). This makes it necessary, therefore, to divert our focus to chemically identifying the plants which have been used in folk medicine and then isolate and identify their bioactive compounds. Next to be followed is the testing of that bioactive compound in the animal studies and if found useful, estimation of the dosage and their side effects if any. This can be said without doubt that many species in the plant kingdom are waiting to be discovered chemically and brought to use for human health. (Phillipson, 2003).

There are many Turkish plants, especially from the herbs group which have been used traditionally to alleviate the symptoms of many diseases. The most common effects explained by Turkish folk medicines are to be antispasmodic, anticancer and effective against skin diseases. Plants from the Rheum genus are found in some parts of Turkey and are also used in folk medicine. *Rheum ribes* L. also belongs to this genus, is found in Turkey, and has undergone pharmacological research. It has been used as food in some parts of the Turkey as well as Iran. The cooked food is a rich source of polyphenols. This study, therefore, is aiming to identify the antioxidant components in this particular species.

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1.1. Botanical Information

1.1.1. Classification of *Rheum ribes* L.

Species: *R. ribes*
Genus: Rheum
Family: Polygonaceae
Order: Caryophyllales
Class: Magnoliopsida
Phylum: Tracheophyta
Kingdom: Plantae

1.1.2. The Family of Polygonaceae

It is a family of flowering plants and has a total of 1200 known species in it. These known species are further classified into 50 genera (Antoine Laurent, 1789). Eriogonum is the largest genus and has 240 species under its flag, while next to be followed is Rumex with 200 species, then Coccoloba having120 species, Persicaria having 100 species, Calligonum possessing 80 species and finally our genus of interest Rheum genus which includes 60 species in it. (Craig C, 2005). Species of this family are spread worldwide but are more concentrated in the North Temperate Zone.

The word polygonaceae is derived from Greek language where poly refers to as many and goni means joint or knee. The plants in this family have swollen nodes. The name polygonaceae is derived from one of the genus under it which is called Polygonum while the word polygonaceae refers to as the smartweed or knotweed family. (Antoine Laurent, 1789).

The plants included in the family Polygonaceae are herbs and have swollen nodes. Some trees and shrubs are also present in this family. The arrangement of leaves in this family is alternate while itself the leaves are simple. Stipule is present and is fused in the form of a sheath. Such a structure of fused stipule is also called ochrea. Not all species have this ochrea structure and those in which ochrea is absent have, for identification involucrate flowers. The leaves of Polygonaceae are simple, and arranged alternately on the stems. In this genus the flowers are small and have both

the sexes in it. They are actinomorphic and the number of sepals varies from three to six. When the fruits begin to form, the sepals become thick and grow in size covering the fruit in partial. Corolla is often absent while some species have brightly colored sepals. Superior ovary, one to three styles and one stigma make the gynoecium while three to eight stamens are man[king the androecium (Samuel B. Jones & Arlene E., 1979; Walter S. Judd et al., 2008; Armen L. Takhtajan, 2009).

1.1.3. Rheum Genus

Rheum or commonly known as Rhubarb, this genus is the most known genus of family polygonaceae. All the species in this included in this genus are perennial. Some of the examples of this genus are, Rheum rhaponticum L., Rheum palmatum L., Rheum officinale Baill, L., Rheum emodi L., and *Rheum ribes* L. (Wang et al., 2005). Most of the species included in this genus are consumed as food or for their medicinal uses. The medicinal use may be due to the presence of chemical compound anthracene or its derivatives present in it. Because of the medicinal uses of these epecies, the chemical constituents present in many of the species of the rheum genus have been isolated and chemically characterized (Ye et al., 2007; Han et al., 2008). Some of those chemicals belong to the clasees of phenols, stillbenes and essential oils (Zhang Chengzhong et al.,2005; Xiang Lan et al.,2055; Zhao Jun et al.,2002). Some of the folk medicinal uses of the plants of this species are antidiabetic (Choi SZ et al., 2005), antispasmodic (Yuan X Gong et al., 2005) and purgative (Xia Zhou et al., 2006).

1.1.4. *Rheum ribes* L.

Rheum ribes L. (Figure 1.9) is a stout herb and its life cycle is perennial. It is generally not cultivated and thus is considered wild. It is edible and is eaten in the form of stew. It grows in sub-tropical and temperate regions like western Asia (Sindhu R., Kumar A., Arora S. 2010) but main part of cultivation are Iran, Turkey, Lebanon and some parts of northern Iraq (Shokravi & Agha Nasiri 1997. In Turkey this is the only species of Rheum genus which is found (Cullen, 1966) and has names of ''Işkın, uşgun or uçgun '' by local community. Other names of this species are "Rewas, Rivas and Ribas" by kurdish, persian and arab local people respectively

(Cullen, 1966). The stem of this species used to make the stew while the leaves and even sometimes the petioles are eaten raw as salads in the local area (Andıç et al., 2009).

Figure 1.1. *Rheum ribes* L.

1.1.5. *Rheum ribes* L. medicinal use

The use of *Rheum ribes* L. is not only as a food source but in folk medicine it has long been used to alleviate some disease symptoms. It was considered as one of the most important source of crude drugs in the middle east (Kashiwada et al., 1988). In Iran there are many sources to prove that it was used as antipsoriatic and also for its laxative effects (Shokravi & Agha Nasiri, 1997). Another use of this species is as anti-diarrhea and as an antiemetic. These effects are obtained by making a stew of young shoots and petioles but sometimes are eaten raw. Also the juice of some parts of the plants is good for treating measles, hemorrhoids and small (Baytop, 1999). In eastern parts of Turkey, especially in Bitilis, the stems and petioles of Rheun ribes species are used as appetizers. Also the roots, raw and cooked, are used for the treatment of hypertention, diabetes, obesity, ulcer and as an antihelmintic (Abu-Irmaileh & Afifi, 2003; Tabata et al., 1994), (Abu-Irmaileh & Afifi, 2003), (Abu-

Irmaileh & Afifi, 2003), (Tabata et al., 1994), (Tabata et al., 1994), (Tabata et al., 1994).

One striking benefit seen in this species is the antimicrobial effect against some gram negative bacteria and Herpes virus. This effect is seen to be present in the roots, stems and leaves of the species grown in Iran (Bazzas et.al, 2005) (Bonjar, 2004).

1.1.6. The Phenolic Constitutes of *Rheum ribes* L.

There are some chemical studies done on R. ribes in literature to investigate its antioxidant activity and also to analysis its phenolic constitutes. Flavonoids, stilbenoids and anthraquinones are the major phenolic constituents of *Rheum ribes* to provide a potential source of antioxidants (Uyar et al., 2014).

In one study 3 anthraquinones (chrysophanol, physcion and emodin), and 5 flavonoids (quercetin, 5-desoxyquercetin, quercetin 3-0-rhamnoside, quercetin 3-0-galactoside and quercetin 3-0-rutinoside) were isolated from the shoots of *Rheum ribes*, collected from Hakkari in Turkey (Tosun & Akyu˙z-Kızılay, 2003).

The roots of the plant collected from Erzincan, and 6 anthraquinones (chrysophanol, physcion, rhein, aloeemodin, physcion-8-O-glucoside, and aloeemodin-8-O-glucoside), one stilbenoids (rhaponticin) and sennoside A have been isolated (Mericli & Tuzlacı, 1990; Tuzlaci & Mericli, 1992).

The antioxidant activity of chloroform and methanol extract of roots and stems of R. ribes L. were studied by the means of different antioxidant tests (Ozturk et al., 2007)

The antioxidant activity of ethyl acetate extract of roots and young shoots of R. ribes L. were studied along with cytotoxic and apoptotic effects against human promyelotic leukemia (HL-60) cells (Uyar, Coruh and İscan, 2014)

Total antioxidant activity of ether, ethanol and water extracts of different parts of isgin (*Rheum ribes*) was investigated using thiocyanate method for antioxidant activity (Yildirim, 2007)

1.2. Radicals and their biological effects

1.2.1. Free radials

Free radicals can be described as the molecules/atoms with unpaired electrons. They are formed in the cells as secondary products of typical oxidation reactions. Most of the free radicals are produced during cellular aerobic respiration which is called Reactive Oxygen Species (ROS) (Gutteridge and Halliwell, 2000). In the simplest definition, ROS are oxygen derived chemically active molecules (Fridovich, 1999; Betteridge, 2000; Halliwell, 1999). In the cells, free radicals and ROS attack to numerous biomolecules and start a sequence of reactions. These chain reactions do not stop until the elimination of free radicals or reacting with an antioxidant molecule.

1.2.2. Biological effects of radicals

There are several beneficial effects of ROS in biological systems. They are useful in intracellular signaling and redox regulation. Nitric oxide (NO) is found to be a signaling molecule (Furchgott, 1995; Palmer, 1987) and it regulates transcription factor activities and other determinants of gene expression (Bogdan, 2001). Hydrogen peroxide and superoxide show similar intracellular functions (Kamata, 1999; Finkel, 1998). Moreover, ROS also take part in intracellular signal transduction as secondary messengers for several cytokines, growth factors, hormones, and neurotransmitters (Thannickal and Fanburg, 2000).

Paradoxically, radicals have many deleterious effects. They oxidize key constituents of cell eternally injuring them. They oxidize lipids, proteins, DNA and other unsaturated fatty acids (Halliwell and Gutteridge, 1989). Hydroxyl radical is the most reactive among all the radicals generated in the body. It is capable of reacting with any molecule in the living cell (Halliwell, 1989). ROS are found to be mutagenic. They damage deoxyribo nucleic acid (DNA) mainly by the reaction with •OH radicals, chemically modifying them by cleavage of DNA; DNA Protein cross links or by oxidation of purines etc., leading to structural changes (Marnett, 2000; Mates, 1999). Structural changes in DNA will lead to mutations and cytotoxic effects (Diplock, 1991; Lonsdale, 1986), which, in turn may lead to cancer and other diseases. This may be the reason for the high incidence of cancer in people who are

wide-open to oxidative tension (Marnett, 2000; Mates, 1999).ROS cause lipid peroxidation. Lipids form an important part of the cell and many foods. The unsaturated sites of polyunsaturated fatty acids are simply attacked by free radicals. Low density lipoproteins (LDL) are oxidized to form atherosclerotic plaques, which are responsible for the development of cardiovascular disease (Halliwell, 1993; Frei, 1999). Lipids are degraded on reaction with oxygen, a process known as autoxidation. The process involves three stages 1) initiation, 2) propagation, and 3) termination reactions. Free radicals also initiate oxidation of lipids in food systems and this leads to the development of rancidity, protein damage, and oxidation of pigments causing a loss of sensory properties, nutritive value, and shelf life of food products (Madhavi, 1996).

1.3. Antioxidants

Antioxidant-meaning against oxidation-is the term used to describe any species, which possesses the ability to neutralize the free radicals, before the latter reacts with any cell structure or molecule. A variety of antioxidants occur in human bodies, which work in perfect co-ordination with each other to protect the body, in general, against the damaging effects of free radicals. These antioxidants are either formed by the body or are obtained exogenously from various foods. Antioxidants also can be categorized into two classes of natural or synthetic antioxidants. Those which are extracted from plants are natural antioxidants, and those which are prepared synthetically in the laboratory are called synthetic antioxidants. In figure 1.2 a brief overview of their types is given.
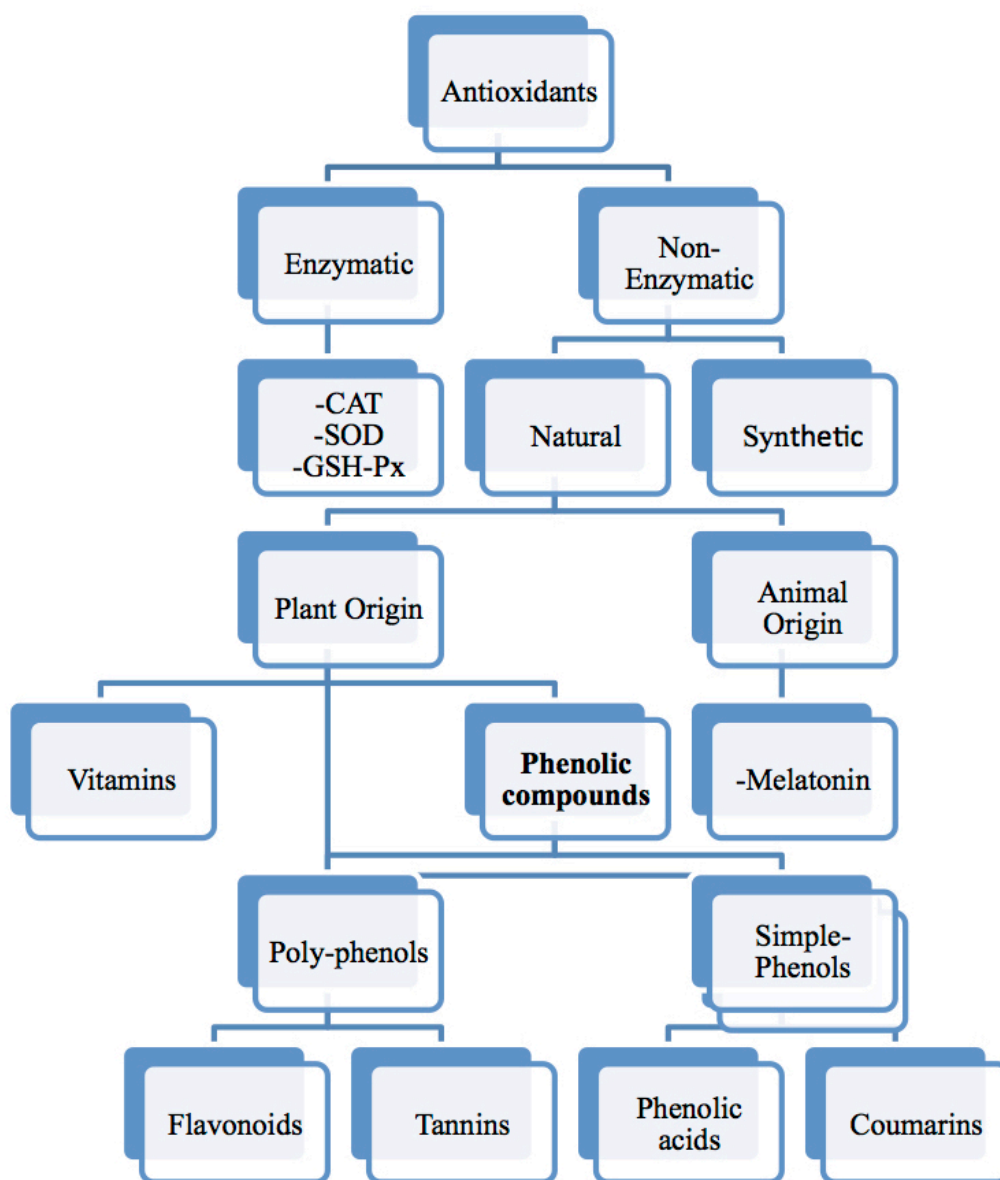
Figure 1.2. Overview of Classification of Antioxidants Adapted from (Halliwell,1996)

Antioxidants are compounds that show reducing activity. They protect the components of cells and biomolecules from oxidation by scavenging or donating an electron / hydrogen atom to free radicals / reactive oxygen species (ROS) such as superoxide, hydroxyl, and peroxy radicals. Antioxidants have numerous important functions in cells and they have many useful effects. For example, they can be used to prevent degenerative, cardiovascular and neurogical diseases, cataracts and

oxidative stress dysfunctions (Stahelin, 1989; Riemersma, 1991; Ames, 1993; Riemersma, 1994; Mackerras, 1995; Halliwell, 1996; Schwartz, 1996). Vitamin E, a natural antioxidant shows anti-carcinogenic properties because it prevents lipid oxidation and scavenges radicals (Gaby and Machlin, 1991).

The importance of antioxidants in prevention of diseases and as promoters of good health is widely recognized and studied. The demand for functional foods that are supplemented with antioxidants is increasing each year as more and more people are realizing the importance of a diet rich in antioxidants in prevention of diseases. They are now being considered as an important class among nutra-ceuticals. The important function of antioxidants in foods is to increase their shelf-life by preventing lipid peroxidation, thereby keeping them fresh for a long time. They can be incorporated (with or without chemical modification) into food delivery systems, such as dairy products, and other food products.

Antioxidant photochemical in foods especially in vegetables, fruits, and grains are found to have human disease prevention abilities, and may improve food quality (Yu et.al 2002). Endogenous antioxidants, such as glutathione present in living cells, alone cannot completely prevent the damaging effects of free radicals (Simic, 1988). There is a need for exogenous antioxidants (e.g. antioxidants from food) that are widely available from food.

Study of the antioxidant nature of fruits, vegetables and plant products helps the chemical industry choose such plants that have high antioxidant capacity. The research will provide important information regarding its antioxidants nature. Due to the various benefits of antioxidants present in foods, fruits, grains and extract, it was decided to study the antioxidant nature of R. ribes root extract.

1.4. Phenolic Compounds in plants

Phenolic compounds represent a large group of molecules with a variety of functions in plant growth, development and defense. Phenolic compounds include signaling molecules, pigments and flavors that can attract or repel, and also compounds that can protect the plant against insects, fungi, bacteria, and viruses. Phenolic compounds are secondary metabolites that are derivatives of the pentose phosphate, shikimate, and phenylpropanoid pathways in plants (Randhir, Lin, & Shetty, 2004). These compounds, one of the most widely occurring groups of phytochemicals, are of considerable physiological and morphological importance in plants. These compounds play an important role in growth and reproduction, providing protection against pathogens and predators (Bravo, 1998), besides contributing towards the colour and sensory characteristics of fruits and vegetables (Alasalvar e.al, 2001).

Structurally, phenolic compounds comprise an aromatic ring, bearing one or more hydroxyl substituents, and range from simple phenolic molecules to highly polymerized compounds (Bravo, 1998). Despite this structural diversity, the group of compounds are often referred to as 'polyphenols'. Most naturally occurring phenolic compounds are present as conjugates with mono and polysaccharides, linked to one or more of the phenolic groups, and may also occur as functional derivatives such as esters and methyl esters (Harborne, 1989; Harborne, Baxter, & Moss, 1999; Shahidi & Naczk, 1995). Though such structural diversity results in the wide range of phenolic compounds that occur in nature, phenolic compounds can basically be categorised into several classes as shown in Table 1.2 (Harborne, 1989; Harborne et al., 1999). The structure of polyphenols varies from simple phenols to highly polymerized compounds according to the basic Carbon skeleton (Waterman, 1994). Of these, *phenolic acids*, *flavonoids* and *tannins* are regarded as the main dietary phenolic compounds (King & Young, 1999).

Table 1.1. Classification of phenolic compounds in plants

Basic skeleton	Class	
C_6	Simple phenols, Benzoquinones	Phenol, cathecol, hydroquinone
C_6-C_1	Hydroxybenzoic acids	Gallic acid, vanillic acid
C_6-C_2	Acetophenones Phenyl acetic acids	Annphenone, p-Hydroxyphenylacetic acid
C_6-C_3	Hydroxycinnamic acids, Phenylpropanoids (Coumarins, Isocoumarins, Chromones)	Caffeic acid, ferulic acid
C_6-C_4	Naphthoquinones	Juglone
$C_6-C_1-C_6$	Xanthonoids	Mangostin, mangiferin
$C_6-C_2-C_6$	Stilbenoids, Anthraquinones	Resveratrol, Emodin
$C_6-C_3-C_6$	Flavonoids, Isoflavonoids	Apigenin, luteolin Quercetin, myricetin Hesperitin, naringenin Daidzein, genistein
$(C_6-C_3)_2$	Lignans, Neolignans	Sesamin
$(C_6-C_3-C_6)_2$	Biflavonoids	Agathisflavone
$(C_6-C_3)_n,$	Lignins	Sesamin
$(C_6-C_3-C_6)_n$	Condensed tannins (Flavolans or Proanthocyanidins)	Raspberry ellagitannin, Tannic acid

1.4.1. Phenolic acids

Phenolic acids are the aromatic secondary metabolites, which are widely spread in plant kingdom. The term "phenolic acids" represents phenols having with one carboxylic acid. Nevertheless, when describing plant metabolites, it refers to a different group of organic acids. Those naturally occurring phenolic acids have two distinct fundamental carbon structures: the hydroxycinnamic and hydroxybenzoic (Figure1.2). Hydroxybenzoic acids include gallic, p-hydroxybenzoic, vanillic and syringic acids, which in common have the C6–C1 structure. Hydroxycinnamic acids, on the other hand, are aromatic compounds with a three-carbon side chain (C6–C3), with caffeic, ferulic, p-coumaric and cinnamic acids being the most common (Bravo, 1998).
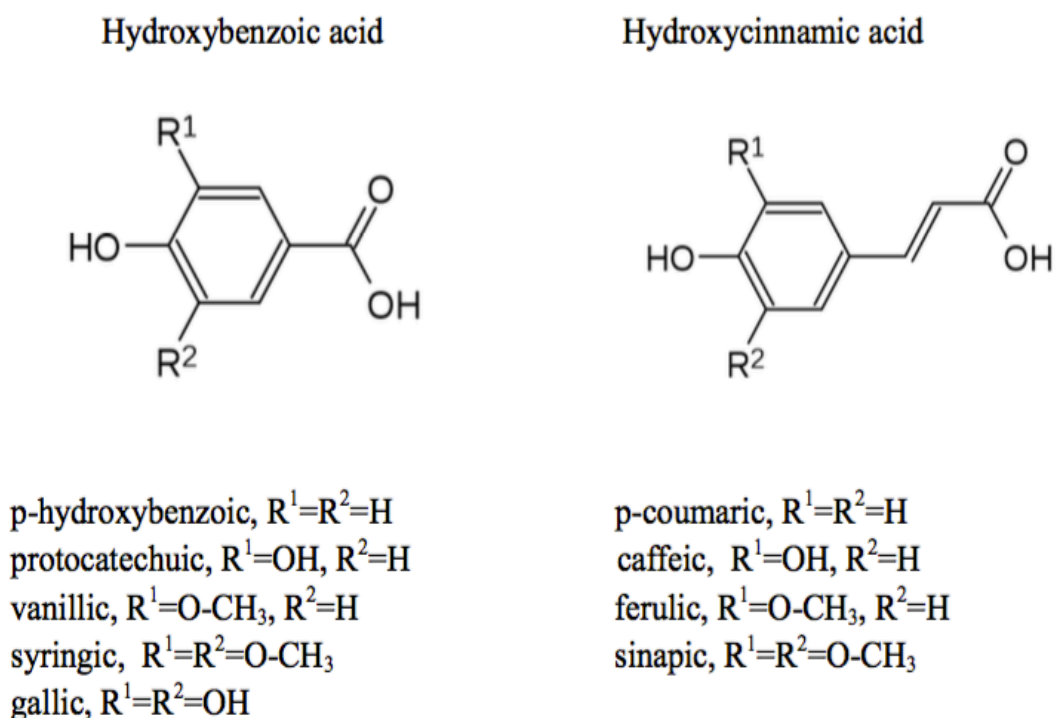
Figure 1.3. Basic structure of (a) hydroxybenzoic and (b) hydroxycinnamic acid derivatives

Hydroxycinnamic and their derivatives have significant functions in plants secondary metabolisms and they are found in different plant species. (Molgaard, 1988; Macheix, 1990; Bengoechea, 1995). Hydroxycinnamic acids are largely found in bound form rather than in free form. They usually occur in conjugated forms, which are the esters of hydroxyacids such as quinic, shikimic and tartaric acid, and their sugar derivatives. p-coumaric, caffeic, ferulic and sinapic acids are the main hydroxycinnamic acids types present in fruits. Caffeic acid is the most widespread hydroxycinnamic acid in many fruits which constitutes more than 75% of all hydroxycinnamic acids. It has been found in plums, apples, apricots, blueberries and tomatoes (Kono et.al, 1995).

Hydroxybenzoic acids are the main constituents of tannins and lignins. Moreover, they can be also present in the form of sugar derivatives (Schuster and Hemann, 1985). The amount of hydroxybenzoic acid present in plants consumed in diet is generally low. Gallic acid is one of the most common hydroxybenzoic acid derivatives. The dimeric condensation product and associated dimeric lactones of the gallic acid known as ellagic acid, is commonly found in plants. Recently, the researchers have focused on ellagic acid due to its potential anti-carcinogenic and anti-oxidative properties. (Meyer et al., 1998)

1.4.2. Flavonoids

Flavonoids constitute the largest group of plant phenolics, accounting for over half of the eight thousand naturally occurring phenolic compounds (Harborne et al., 1999). Flavonoids are part of polyphenolic compounds and commonly found in vegetables, nuts and fruits (Hollman, 1997; Heim, 2002; Clifford, 2000). According to Middleton stated that humans should consume approximately 1 g of mixed flavonoids each day (Middleton, 1984). Flavonoids are low molecular weight compounds, consisting of fifteen carbon atoms, arranged in a C6–C3–C6 configuration. Essentially the structure consists of two aromatic rings A and B, joined by a 3-carbon bridge, usually in the form of a heterocyclic ring, C (Figure 1.3).
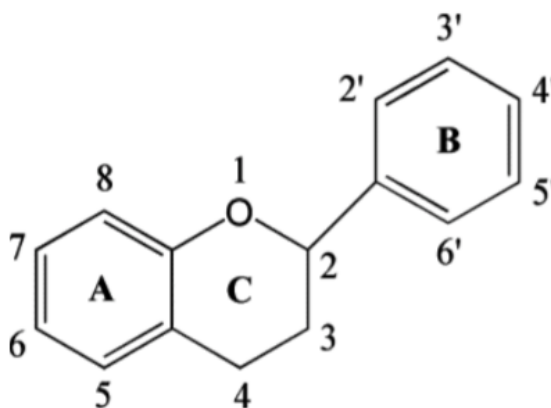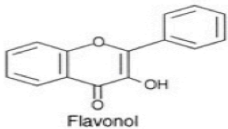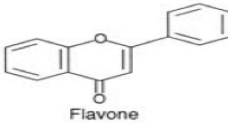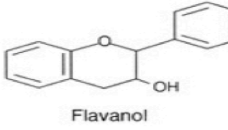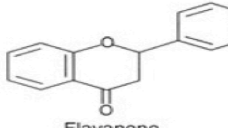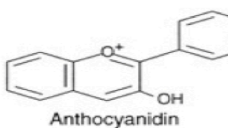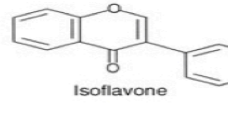
Figure 1.4. Basic structure of flavonoid (phenylbenzopyrone structure)

Variations in substitution patterns to ring C result in the major flavonoid classes, i.e., flavonols, flavones, flavanones, flavanols, isoflavones, flavanonols, and anthocyanidins (Hollman & Katan, 1999), of which flavones and flavonols are the most widely occurring and structurally diverse (Harborne et al., 1999). Substitutions to rings A and B give rise to the different compounds within each class of flavonoids (Pietta, 2000). These substitutions may include oxygenation, alkylation, glycosylation, acylation, and sulfation (Bohm, 1998; Hollman & Katan, 1999).

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Table 1.2. Common dietary and subclasses of Flavonoids (Pokorny, 1991)

Flavonoid subclass	Dietary Flavonoids	Food source Some common	Structure
Flavonol	Quercetin Kaempherol Myricetin	Yellow onion, kale, broccoli, apples, berries, teas	Flavonol
Flavone	Apigenin Luteolin	Parsley,thyme,celery,hot peppers	Flavone
Flavanol	Catechin, Epicatechin derivatives, Proantho-cyanidins Theaflavins	Catechins;tea(greeen), chocolate, grapes, berries, Proanthocyanidins;chocolate, berries, red wine, red grapes	Flavanol
Flavanone	Hesperetin, Naringenin	Citrus fruits and juices e.g., orange, lemons, grapefruits	Flavanone
Anthocyanidin	Cyanidin, Delphinidin, Malvidin, Pelargonidin	Red blue and purple berries, red and purple grapes, red wine	Anthocyanidin
Isoflavone	Daidzein, Genistein,	Soy beans, soy foods and legumes	Isoflavone

1.5. Scope of this study

The purpose of this study was to identify the phenolic constituents of *Rheum ribes L.* by using solvent-solvent fractionation method and RP-HPLC. Since this plant has a wide use in folk medicine as a functional food, its antioxidative properties were also worthwhile to be investigated as well as its phenolic constituents.

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CHAPTER 2

MATERIAL AND METHODS

2.1. Materials

2.1.1. Chemicals

Acetonitrile, ethanol, chloroform, , hexane and methanol all of them were made available by Merck (Darmstadt, Germany) and were chromatography grade. These all chemicals were used in, extraction, fractionation and HPLC. Ultra pure water gained by Millipore system (>1 M ohm.cm) was also used. Sodium carbonate (Na_2CO_3) and 2,2-diphenyl-1-picrylhydrazyl also known as DPPH were bought from St. Louis MO, USA and Folin-Ciocalteu reagent was taken from Merck (Darmstadt, Germany). Reference compounds such as, myricetin, luteolin, caffeic acid, quercetin, quercitrin, caffeic acid, apigenin, hesperetin, catechin, esculin and etc, were purchased from Sigma-Aldrich (St. Louis MO, USA).

2.1.2. Plant material

Rheum ribes roots were collected from Tatvan, Alacabük Mountain, northwest slopes during spring of 2014 by Assoc. Prof. Dr. Fevzi Özgökçe from Biology Department of University of Yüzüncü Yıl , Van, Turkey , the samples of the plant were deposited in the herbarium. *Rheum ribes* roots were air-dried on filter-papers under shade for 15 days at room temperature (RT). Dry roots were powdered by Waring (model 32BL80) commercial blender at a high speed for at least 2 minutes and stored in dry, dark and tempered conditions (RT) until use.

2.1.3. Instruments

To obtain all the spectrophotometer result (determination of total phenol and total flavonoid and DPPH tests) Carry 5-Bio UV-VIS spectrophotometer from (Varian) was used. A list of instruments, which were used in other experiments, is mentioned below.

Disposable filter (0.45 μm and 0.22 μm) (Millipore Corporation), weighing Balance (Precisa XC 220A Swiss made, OPTIC ivymen System rotary incubator, Heidolph Laborota 4000 rotary evaporator, ultrasonic bath (Bandelin Sonorex RK100H), warning commercial belender (model 32BL80), FINPIPETTE micropipette, Whatman filter paper, VELP SCIENTIFICA vibrator.

HPLC High performance liquid chromatography having the system of WATERS 2475 Multi Fluorescence Detector, WATERS 2996 Photodiode Array Detector and WATERS Delta 600 HPLC was used along with the column of C18. 5 μm. 4.6*150 mm. Hamilton injector (710 Nr), and 0.20 μm filter (Sartorius Minisart RC 4) were used for making injections in HPLC.

2.2. Methods

2.2.1. Extraction

Dried roots of *Rheum ribes L.* were blended on high speed for 3 minutes to gain a 1-3 mm particle size by the help of warning commercial blender and then 50 g were weighed. In order to obtain the root extract, the powdered roots (50 g) were taken in a dark glass bottle and keeping the ratio of 1:10 w/v, 500 mL of methanol was added to it. This was then incubated at a temperature of 25 ºC with a continuous rotation of 180 rpm. After 24 hours the extract was filtered by filter paper (Whattman), and the filtrate was then kept at 4 ºC in refrigerator. The root residue on the filter paper was again transferred to the same dark bottle and 500 mL of methanol was again added to the same roots and the same procedure was performed from the incubation to the filtration. Filtered extracts were mixed and brought to complete dryness by the help of rotary evaporator. Then in order to do the yield calculations, dried crude extract was weighed.

2.2.3. Solvent-solvent fractionation

Initially the crude extract was taken in a round bottom flask and 500 mL mixture of methanol and water with ratio of 70:30 was added to it. The flask was shaken to completely dissolve the extract in aqueous methanol. This solution was then added to a separatory funnel. Right after this 500 mL of n-hexane (equal volume with the methanol/water mixture) was added to the funnel and shaken vigorously in hand for 30 seconds and then the separating funnel was kept in a steady state in order for separation of two layers. Organic layer i.e. the n-hexane layer was collected and transferred to round bottom flask and sent for evaporation with rotary evaporator to get dry. This dried n-hexane extract was kept at 4 ºC until analysis stage. The procedure was repeated as many times as required, every time with similar volume of n-hexane till the n-hexane layer obtained after vigorous shaking was clear. The aqueous methanol layer, which was present at the bottom of the separating funnel, was also collected. This layer will be called aqueous layer further in this thesis for convenience. Aqueous layer shaken with chloroform and ethyl acetate, respectively, followed this. All the solvent layers were separated and then dried by evaporation. The extract obtained after drying was weighed. This gives us the yield. The procedures are summarized in Figure 2.1.
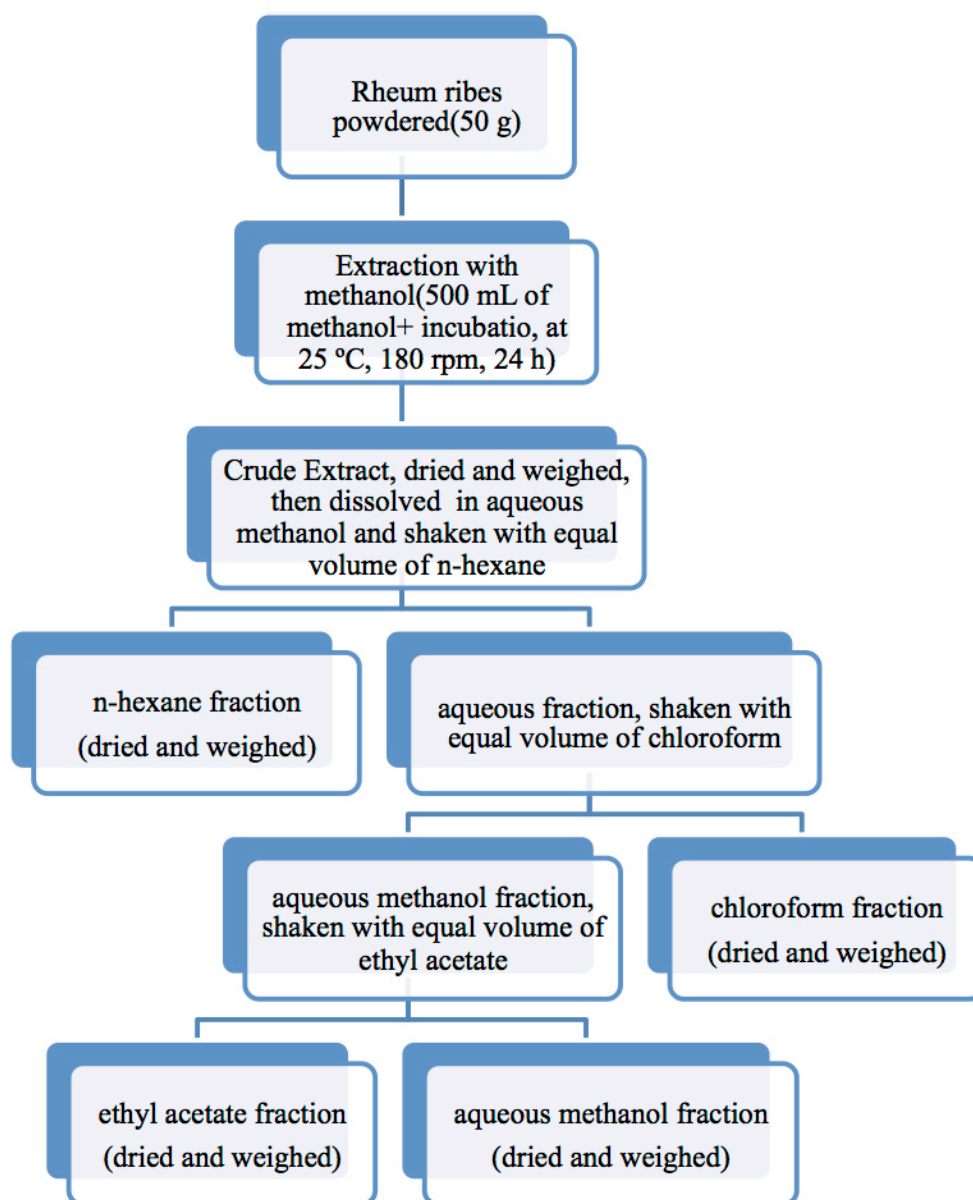
Figure 2.1. Scheme for extraction and fractionation of *R. ribes* roots.

2.2.4. Radical Scavenging Ability by DPPH Method

It is one of the easiest and quickest ways to determine the antioxidant capacity of samples in the lab. DPPH is a stable and comparatively less reactive free radical. 2,2-diphenyl-1-picryhydrazyl is its formal name. This method is generally used to establish the ability to act as a scavenger of free radical or the ability to donate hydrogen ion of a given sample.

2,2-diphenyl-1-picryhydrazyl, is commonly used in the laboratory studies of plant biochemistry to analyze the antioxidant capacity of the given sample. A single electron present on the third nitrogen atom gives the molecule its free radical nature. Also this unpaired electron gives a characteristic absorption to the molecule, which is recorded the highest at 517 nm in the visible region wavelength (Pyrzynska and Pekal, 2013). When this free radical is dissolved in methanol, the resulting solution is purple in color. If an antioxidant is added to this purple solution, the free radical is scavenged or stabilized in turn changing the color of the solution from purple to dull yellow. A general view of the reaction between an antioxidant and DPPH is demonstrated in the equation $DPPH^{.} + R\text{-}H \rightarrow + R^{.} + DPPH\text{-}H$

With a slight modification to the method mentioned by Blois et al (1958), the test was performed, as mentioned by Çoruh et al (2007). The solution of DPPH was prepared in methanol and the final concentration of the solution was 0.05 mg/mL. This final solution when checked for absorption at 517 nm, showed to have absorption of 1.38 - 1.40, approximately. Fresh DPPH solution was prepared for each day's experiments. Next, solutions of sample extracts as well as standard in methanol were prepared at different known concentrations. Eppendorf tubes were taken and 1400 μL of the freshly prepared DPPH solution was added to them. This was followed by addition of 100 μL of sample or standard of different concentrations to the eppendorf tubes. The tubes were shaken well by vortex mix and kept in the dark for fifteen minutes. Then the solution of the eppendorf tubes was transferred to a cuvette one after the other and its absorption was noted at 517 nm. The reading when subtracted from the original absorption of the DPPH solution alone and divided by the same value and multiplied by 100 gave us the percentage radical scavenging activity of each sample extract and standard at different concentrations. A graph was then plotted taking the concentration as independent variable and % RSA as dependent variable. The standard used in this test was Quercetin.

Formula for Radical Scavenging Activity

Percentage Radical scavenging Activity (% RSA) = $\{(X_0\text{-}X_1)/X_0\}$ x 100;

X_0 = absorption of control (methanol + DPPH); also called blank

X_1 = absorption of sample (Sample solution in methanol + DPPH)

2.2.5. Determination of Total Phenol Content

This is also a colorimetric test and it involves Folin-Ciocalteu Reagent. Other names for this reagent are Folin-Phenol Reagent, Folin-Denis Reagent. This test is also known as Gallic Acid Equivalence (GAE) test because gallic acid is used in it. It is cost-effective, easy and gives quick and accurate information about the given sample's quantity of total phenol. The chemistry of this test is that when a reaction takes place between Folin-Ciocalteu reagent and polyphenols at a higher pH (pH~ 10), a blue colored complex is produced as shown in Figure 2.2, which is the change in color following the incubation for half an hour in the dark; where towards the left is control solution and concentration of sample increases as we proceed to right in the figure. Higher pH is obtained by adding sodium carbonate (Na_2CO_3). This complex has the highest absorption at 750 nm. At higher pH the phenols present in the solution have the ability to liberate the hydrogen and become a cation. These cations, called phenolate ions, then are attracted to Molybdenum (VI), which is present in the Folin-Ciocalteu reagent, and form a complex with the metal. This can be summarized in the equation below (Sánchez-Rangel et al., 2013).

Phenolic compound $+ Na_2CO_3 \rightarrow$ Phenolate ion$^-$ $+ H^+$ (pH~10)

Phenolate ion$^-$ $+$ Mo (IV) $\rightarrow$ Complex of Mo $+$ Phenolic compound (blue colored)
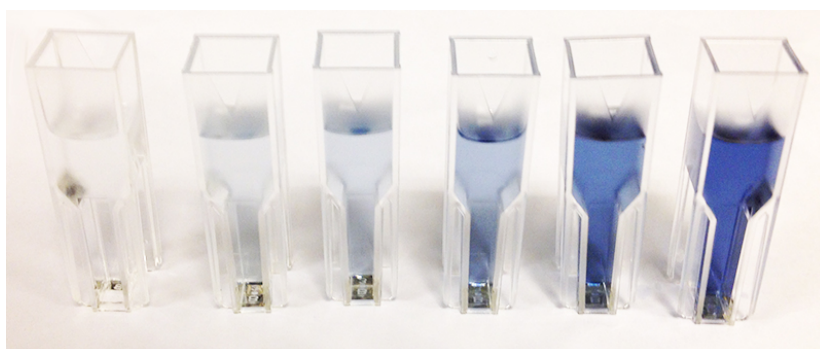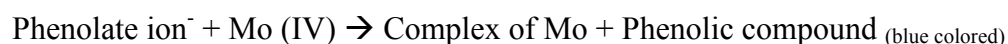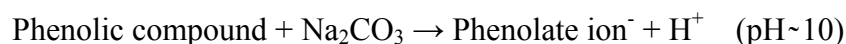

Figure 2.2. Result of Total Phenol Content test.

The total phenol content of the crude extract and the fractions was determined using the procedure employed by Singleton and Rassi, (1965). Gallic acid was used as the standard for comparison and its different concentrations were prepared in ethanol.

Similarly solutions of different concentrations of crude extract and fraction extracts were prepare in ethanol. Other solutions that were prepared were; 50 % (v/v) of Folin-Ciocalteu Reagent and 2 % (w/v) of sodium carbonate in distilled water. A test tube was taken and to it 100 µL of any sample or standard was added. This was followed by addition of Folin-Ciocalteu Reagent 100 µL and the solution in the test tube was mixed by vortex mixing. Now 2000 µL of the above prepared sodium carbonate was added and again the mixing was done. The set of testubes were then left in the dark for thirty minutes. Following this the absorption was measured of the solution in test tubes by using spectrophotometer. Also the absorption of the control was measure. The control contains plain ethanol 100 µL along with other reagents but no sample in it. After this the absorption of the control was subtracted from that of the sample/standard. From the calculations, plotting of gallic acid calibration graph was done using concentration as the independent variable and absorbance as the dependent variable. By use of this calibration curve graph, total phenol, in terms of Gallic Acid Equivalent, was calculated for all the samples, i.e. the crude extract and the fractions' extract. This tells us the amount in micrograms of GAE per milligram of the sample. This experiment was performed twice, each time in triplicates, i.e. having three readings.

2.2.6. Determining the Total Flavonoid Content

This test is also a colorimetric as well as a stoichiometric test. Here the amount of flavonoids present in the given plant sample are determined. (Bakar, 2009). The chemistry this test involves is that when sodium nitrite is present along with aluminum chloride, the latter reacts with the flavonoids present in the given plant sample and form a complex which has a red colour. Therefore, the red color intensity is directly proportional to the amount of flavonoids present in the sample or standard solution. This red color complex has highest absorption at 510 nm wavelength and thus stoichiometrically can be calculated using a spectrophotometer (Bakar, 2009).

The method employed was similar to that mentioned in Zhishen et al. (1997)except some modifications. Solutions were prepared in various concentrations for sample and the standard. In this experiment rutin was used as the standard. The following solutions were prepared; 5 % (w/v) sodium nitrite in distilled water, 10 % (w/v)

aluminum chloride in distilled water and 1 Molar solution of sodium hydroxide in distilled water. In a test tube 0.2 mL of the sample or standard was taken and 0.075 mL of sodium nitrite was added. The test-tube was incubated for five minutes in dark at room temperature. This was followed by addition of 0.15 mL of aluminum chloride and again the test tube was incubated at room temperature for another six minutes. This is when the complex formation occurs. Finally the reaction is stopped by the addition of 0.5 mL of sodium hydroxide and the total volume of the solution was made to 3 mL by adding distilled water. The absorbance of the resolution solution was determined immediately at a wavelength of 510 nm. Thorough mixing was done after each step by vortex mixing. The absorbance of the control was subtracted from that of the sample. The control had all the solutions except the sample. Finally a graph was plotted for the standard used here, that is, rutin; with concentration as independent variable and absorbance as dependent variable. A straight line was obtained and the slope of it was calculated. Followed by this was the calculation of total flavonoid content in each sample in terms of rutin equivalent. This tells the microgram of rutin equivalent present in each gram of the sample.

2.2.7. Analytical HPLC Analysis

The technique used here for HPLC analysis was Reverse Phase-HPLC (RP-HPLC). This technique implies that the mobile phase is more hydrophilic while the stationary phase is hydrophobic. Thus the column is packed with carbon, C-18 molecules which are hydrophobic in nature and will attract and retain the hydrophobic compounds from the sample. They will only elute when the mobile phase is changed to hydrophobic. The mobile phase employed here had a gradient flow which started with being more hydrophilic and gradually became hydrophobic in nature. Thus the separation of compounds was done according to their solubility property. This technique was used for the given samples because the compounds under observation are phenolic compounds and these vary from each other according to their nature of being very hydrophilic like phenolic acids to being almost completely hydrophobic like coumarin.

Therefore, each of the fractionated extract as well as the crude extract were subjected to the Reverse Phase HPLC. The unit used was Multi-Solvent delivery system was

the unit used and it had fluorescence as well as photodiode array detector attached to it. The range of wavelength between which the measurement was done was from 210-800 nm.

Elution program was optimized as a slight modification to the one described by Çoruh et al (2014). The Mobile phase was a mixture of two components one containing ultra filtered distilled water while the other was a solution of acetonitrile, methanol and acetic acid had a gradient flow, Table 2.1, and consisted of a mixture of two entities, (A) contained pure water distilled and ultra filtered, (B) contained a solution of methanol, acetonitrile and 2 % (v/v) acetic acid in water in a ratio of 2:2:1. This is shown in the table 2.1.

Table 2.1. RP-HPLC gradient flow of mobile phase

Time (minutes)	Percentage of A	Percentage of B
0	99	1
10	99	1
11	85	15
50	65	35
74	0	100

The rate of injection flow through the column was constant for each injection and was fixed as 1.3 mL/min. The column was re-equilibrated by solution B after each injection. Twenty minutes were allowed for re-equilibration of the column. This whole test was performed at room temperature, i.e. 25 °C. Each time the injection volume was kept constant at 15 µL and prior to injecting into the HPLC apparatus the solution was filtered using a 22 µm filter. The dimension of the C18 column used was 5µm, 4.6 x150mm column suitable for Reverse Phase Chromatography.

CHAPTER 3

RESULTS AND DISCUSSION

3.1. Extraction

The 50 g powder of *Rheum ribes* was transferred to a dark bottle containing methanol as explained in section 2.2.1. All the steps mentioned in section 2.2.2 were performed and the filtrates were dried and weighed using a very precise weighing balance. The total yield was 5.7 g, out of which 0.7 g was stored for further tests and the remaining 5.0 g was used for further fractionations.

3.2. Fractionation

A total of 5.0 g of dried crude extract was subjected to fractionation as explained under section 2.2.3 and shown in Figure 3.1 with the yield in g. Table 3.1 gives the yield of the fractions. As the Table 3.1 shows aqueous methanol dissolved most of the crude extract while the least of the compounds were dissolved in chloroform. All the calculations in Table 3.1 are a mean of three separate extractions and fractionation experiment.

Table 3.1. Yield of each of the fractions

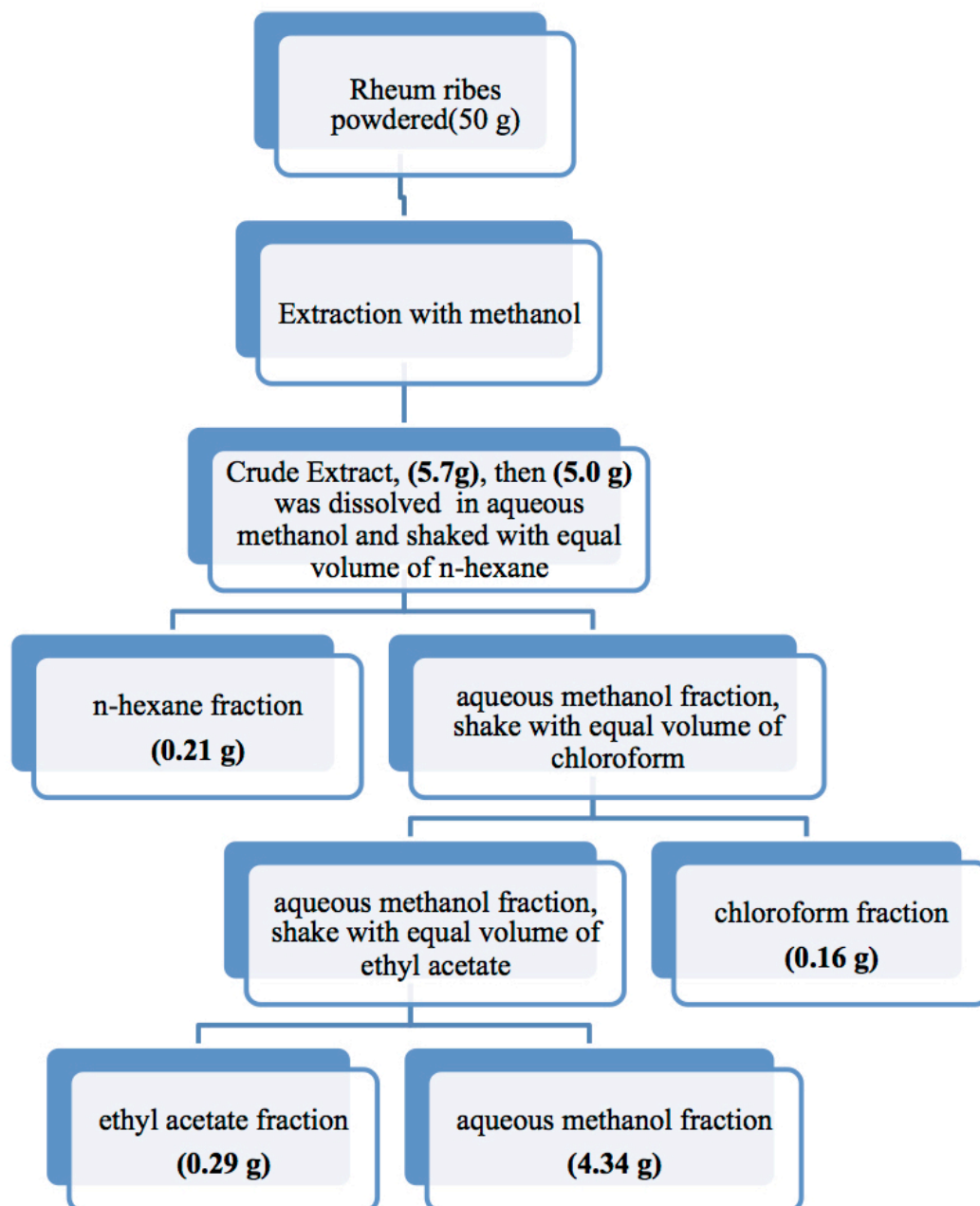| Name of Fraction	Yield (gram)	Yield (%)
n-Hexane	0.21	4.2
Chloroform	0.16	3.16
Ethyl Acetate	0.29	5.8
Aqueous	4.34	86.84

Figure 3.1. Flow chart for the extraction and fractionations of the methanol extract from the root of *Rheum Ribes*

3.3 Determination of the Antioxidant Capacity of the sample Extracts

Any plant material which is said to have phenolic compounds does show some antioxidant activity. Thus it is necessary at the first step to measure this antioxidant activity to get an idea about which solution to focus more on when performing HPLC. The antioxidant capacity of all the fractions separately and the whole extract, thus, has to be estimated at this step. If any fraction does not show significant antioxidant activity, it will not be investigated by RP-HPLC. DPPH is the most convenient and widely used test for estimation of antioxidant capacity so it was used here.

3.3.1 DPPH Method for Radical Scavenging

The method of this experiment is explained in the section 2.2.4 and was performed accordingly. A graph was plotted using the results of this test , with the percentage of Radical Scavenging Activity (RSA) as dependent variable versus the concentration of the samples as independent variable, and the effective concentration (EC_{50}) was also calculated. The EC_{50} of any sample can be explained as the amount of sample needed to achieve half of its maximal effect. The EC50 value gives us an idea about the strength of the sample. Any sample having lower EC50 value will have higher antioxidant activity. This EC50 value can was calculated by the graph of %RSA versus the concentration of the samples and is shown in Table 3.2.
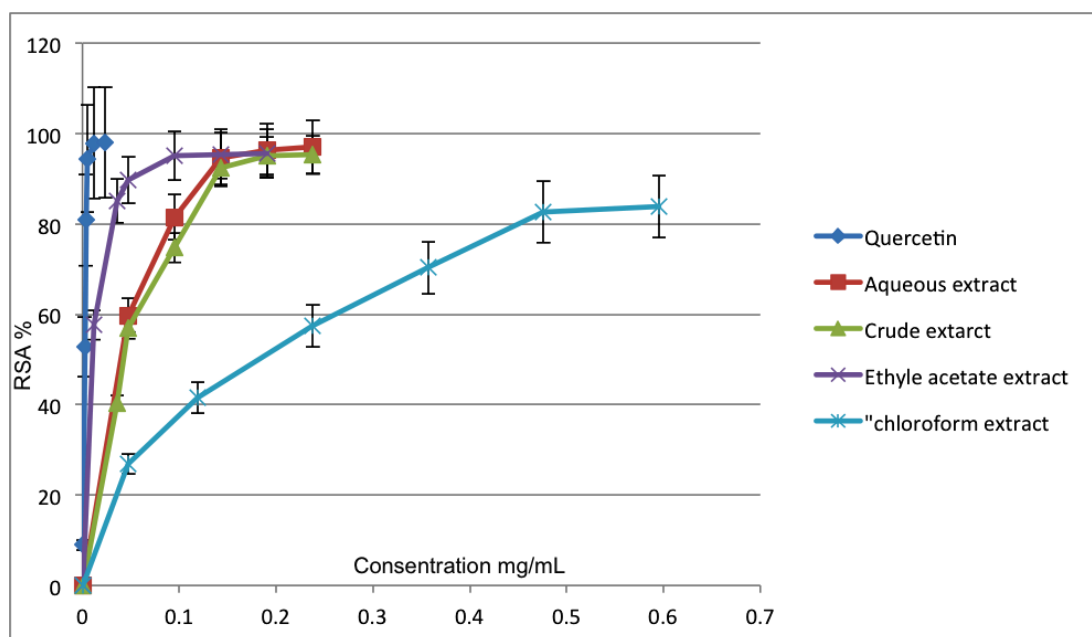
Figure 3.2. DPPH radical scavenging activity of quercetin and 4 extract

N= 6 triple of two sets of replicates

The purpose of this experiment was to analyze the strength of each fractionated extract as well as the crude extract. The standard used in this experiment was pure quercetin and all the samples extracts were compared with quercetin. In the Figure 3.2 it is seen that the first curve is of standard quercetin. It reaches 100% of its activity at very low concentration because it is a pure compound. Among all the samples' curve, ethyl acetate curve is the nearest to the quercetin curve. This is interpreted as ethyl acetate fraction having the highest amount of antioxidant activity among all the sample extracts. Therefore, this ethyl acetate fraction will definitely have highest amount of antioxidant compounds which are thought to be phenol in nature. Next to ethyl acetate curve is present the curves of crude and aqueous extract respectively with minor difference to each other and they both reach 100 % of their activity at a higher concentration value as compared to that of ethyl acetate fraction. Thus the deduction from this graph and table 3.1, which shows that the yield of ethyl acetate fraction was as little as 5.8% is that most of the phenol antioxidants from crude extract were dissolved in ethyl acetate while lesser of it dissolved in aqueous fraction. On the other hand the light blue curve belongs to chloroform fraction, which doesn't achieve its maximal effect of 100% RSA even at higher

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concentrations. Thus chloroform extract has the least antioxidant compounds in it and antioxidant activity.

Table 3.2. Radical scavenging activities of the extracts and quercetin.

Sample	DPPH EC_{50} (µg/mL)*± SD	(%) RSA (Concentration)
Crude extract	70.08 ± 0.053	96.99 ± 0.49 (5 mg/mL)
N-hexane	ND	ND
Chloroform	221.68 ± 0.082	83.77 ± 0.82 (12.5 mg/mL)
Ethyl acetate	34.34 ± 0.042	95.63 ± 0.13 (4 mg/mL)
Aqueous	74.38 ± 0.023	95.29 ± 0.18 (5 mg/mL)
Quercetin	8.15 ± 0.125	97.97 ± 0.06 (0.5 mg/mL)

ND: Not determined.

* Mean of two independent experiments in triplicates.

It is seen in the Table 3.2 that except one extract i.e. the n-hexane extract, all of the other extracts showed some antioxidant activity. Ethyl acetate fraction had the highest antioxidant activity and the least EC_{50} value (34.336 ± 0.04167 µg/mL) while the chloroform fraction had the highest EC_{50} value of 221.677 ± 0.08194 µg/mL. In addition, the antioxidant activity of all the fractions in the decreasing order is given as ethyl acetate > crude extract > aqueous > chloroform with the respective EC_{50} values of 34.336 > 70.085 > 74.383 > 221.677 µg/mL. Thus, the EC_{50} values calculated here had confirmed our initial implication obtained from the graph in Figure 3.2.

Quercetin, a pure flavonoid compound, was used as a standard for the positive control of DPPH radical scavenging activity revealed a 98% effectiveness with an EC_{50} value of 8.146 µg/mL, in this study.

In literature there are some studies published regarding antioxidant capacity of *Rheum Ribes*. Here are some studies on DPPH activity of Roots of *Rheum Ribes*. For

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example, in the study of Ozturk et al (2007), methanol and chloroform extract of the roots respectively showed 60.60 ± 0.86% and 50.87 ± 0.30% DPPH radical scavenging activity at 100 μg/mL concentration. In the study by Pembegul Uyar (2015) ethyl acetate root extract had EC_{50} of 10.92 ± 0.21 μg/ml and 96.67% scavenging potential.

3.4. Determination of Total Phenol Content

Folin-Ciocalteu also known as Folin reagent was used to determine the total phenol content of all the fractionated extracts as well as crude extract. This test is quite sensitive and gives quick results. It is a simple and easy to perform experiment. Phosphomolybdic acid and phosphotungstic acid together make the Folin reagent. This mixture of phosphomolybdic and phosphotungstic acids when reacts with phenolate ion, forms a colored chelate complex. This complex absorbs light which reaches maximum absorption at 750nm. The phenolate ion is formed when phenol liberates the hydrogen ion in basic environment.

Gallic acid is a phenol compound and was used as a standard in this experiment due to its high solubility in Folin reagent. All the sample extracts were compared to gallic acid. Therefore, first of all the calibration curve of gallic acid was plotted in which absorbance of gallic acid was the dependent variable and concentration of gallic acid was the independent variable. This is shown in Figure 3.3. The slope of the graph was obtained to calculate total phenol contents of the samples.
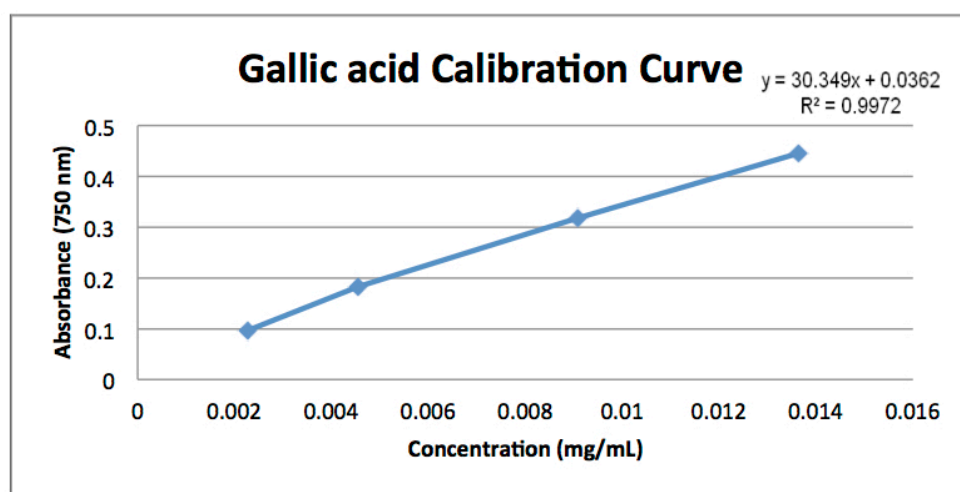
Figure 3.3. Gallic acid calibration curve, concentration versus absorbance

In the Figure 3.3 the calibration curve of gallic acid can be seen. The resultant is a straight line and the slope of this line, called the linear regression is measured by the equation of linear regression $y = mx + b$ where m is the slope and b is the intercept. The result was $y= 30.349x + 0.0362$. By using this equation the phenolic concentration of all the fractions and the crude extract were calculated in terms of Gallic Acid Equivalent. The result is expressed in µg/mg. Table 3.3 shows the total phenol content as GAE.

The results of this test related with the DPPH test. The highest GAE was that of ethyl acetate fraction as seen in Table 3.3, 55.92 GAE µg/mg. This tells that each milligram of the ethyl acetate extract contains or is equivalent to 55.92 µg of gallic acid. Ethyl acetate fraction has the highest amount of phenols in it with a significant difference as compared to other extracts. However, the phenolic content of n-hexane was the least, amounting to only 3.83 GAE µg/mg of the n-hexane extract. The highest amount of phenolic content indicates that it can be a better source of phenolic compounds.

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Table 3.3. Total Phenolics content of *Rheum ribes* extracts

Fraction/standard	Total Phenol GAE (µg/mg) ± SD
Crude extract	10.21 ± 0.001
n-hexane	3.83 ± 0.000
chloroform	7.14 ± 0.005
ethyl acetate	55.92± 0.004
aqueous	9.22 ± 0.001

SD: Standard Deviation

ND: Not Determined

GAE: Gallic Acid Equivalent

N= 6 (Set of triplicates repeated separately two times)

In literature there are some studies on phenolic content of *Rheum ribes* roots. For example in the study of Ozturk et al (2007), Chloroform and methanol extract of roots showed phenolic content of 48.66 ± 1.23 µg/mg and 25.91 ± 1.09 µg/mg respectively. Another study (Uyar, 2015) showed ethyl acetate root extract containing 207.22 ± 6.96 µg/mg.

3.5. Determination of Total Flavonoid Content

This test also involves the formation of colored complexes. Thus it is a colorimetric test and the absorption of these complexes can be measured at a wavelength where these complexes show highest absorption, i.e. 510 nm. Flavonoids are a group included in phenolic compounds and are important because they show high antioxidant activity. Therefore, measuring the flavonoid content of sample gives an idea of the strength of free radical scavenging activity of plant

The standard used in this test was rutin. Rutin is a flavonoid and all the sample extracts were compared against it. First the absorption of rutin itself was calculated at a wavelength of 510 nm for its different concentration and a calibration curve was plotted, Figure 3.4. The absorbance is at y-axis while the concentration being the independent variable, is at x-axis.
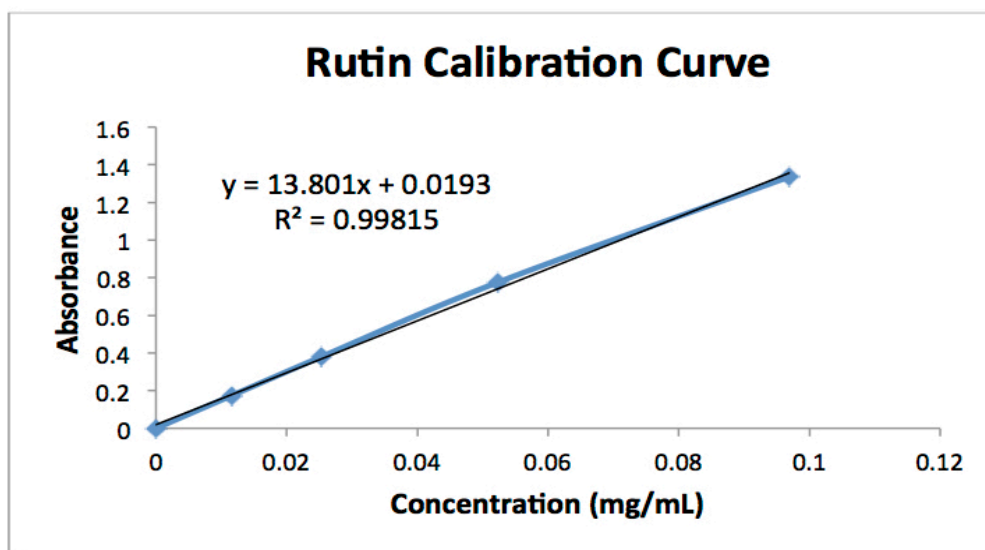
Figure 3.4. Rutin standard calibration curve at 510 nm (Two independent experiments in triplicates were performed).

This graph shows a straight line which tells that absorbance of rutin is directly proportional to its concentration. The slope of this straight line is determined by the equation of linear regression, i.e. $y = mx + b$. In this equation m is the slope of the curve and b is its intercept. The result obtained was $y = 13.801x + 0.0193$, and R^2 was calculated as 0.9982. This equation was then used to calculate the flavonoid content of all the sample extracts and expressed in terms of Rutin equivalent µg/mg. The results of all the calculations of total flavonoid content experiment are shown in Table 3.4. When Table 3.4 was studied, it was deduced that ethyl acetate fraction has the highest concentration of flavonoids in it. Thus this result if correlated with the previous two tests, helps us in forming a conclusion that, most of the phenolic contents present in ethyl acetate fraction are from the flavonoid family. Also it is seen that although chloroform extract did not show a good DPPH scavenging activity and its total phenolic contents were far less than aqueous fraction, the total flavonoids content in chloroform is much closer to that of aqueous fraction. This indicates that the phenols dissolved in the chloroform fraction are mostly flavonoids. Thus it can be concluded that flavonoids are generally more soluble in less polar solvent.

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Table 3.4. *Rheum ribes* total flavonoids content of extracts

Extracts	Total flavonoid content RE (µg/g) ± SD
Crude extract	159.70 ± 0.78
n-hexane	ND
Chloroform	123.16 ± 0.34
Ethyl acetate	645.92 ± 0.42
Aqueous	143.56 ± 0.72

SD: Standard Deviation

ND: Not Determined

RE: Rutin Equivalent

N=6 (Triplicate sets repeated twice)

The highest amount of rutin equivalent of flavonoid content was present in ethyl acetate fraction and the order of increasing of this rutin equivalent of flavonoid content was chloroform < aqueous < crude extract < ethyl acetate as shown in the Table 3.4.

In the literature there are some studies reporting total flavonoid content of *Rheum ribes* roots. Uyar, 2015 shows ethyl acetate root extract 50.49 ± 2.03 mg of catechin/g of sample (rutin is used in our study as standard). Another study (Ozturk, 2007) showed chloroform extract to have flavonoid content of 145.59 ± 0.22 µg of quercetin/mg of sample while methanol extract contained 16.23 ± 0.47 µg of quercetin/mg of the sample.

3.6. Analytical High Pressure Liquid Chromatography (HPLC) analysis

Reverse phase, analytical HPLC was performed to characterize the type of compounds present in methanol extract of *Rheum ribes*. Except the n-hexane fraction, all the other fraction extracts as well as the crude extract was subjected to reverse phase HPLC analysis. The reason of not analyzing n-hexane extract was that it did not show any results in all the three above mentioned tests and, therefore, it did not have any antioxidant in significant amount. The reverse phase HPLC not only gives an information of the number of compounds present in each sample but also

provides an idea about the chemical nature of the compound. When any sample is injected in HPLC the compounds present in the sample interact with the mobile phase and the stationary phase. This interaction between a compound and stationary phase if weak, the compound will elute first from the column and vice versa. Each compound has an absorption spectrum which is noted by the florescence detector attached to HPLC. This absorption spectrum is characteristic of that compound and helps in its identification.
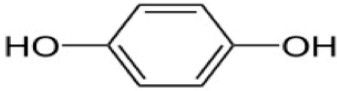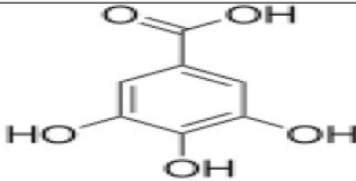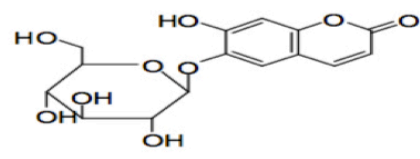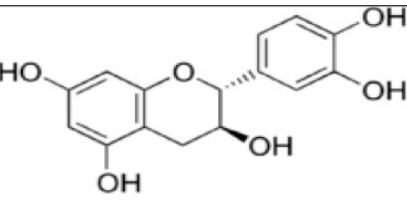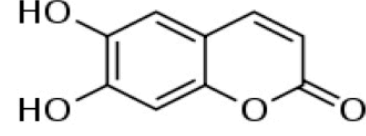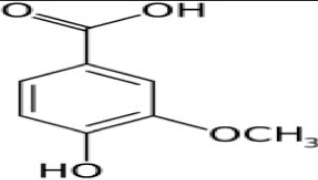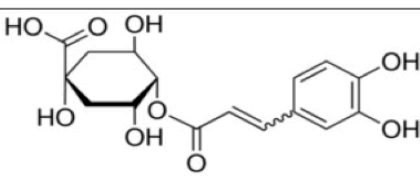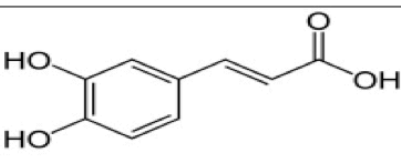
Firstly optimization of all the conditions for *Rheum ribes* extracts was done, i.e. the temperature, pressure, composition of mobile phase and flow rate was determined for good separation of maximum of the compounds present in the sample. Then the known phenolic compounds called standards were applied to reverse phase HPLC, first alone and then in mixture of six standard compounds, to get an idea and form a summary, Table 2.1, of their elution from the column at that particular conditions
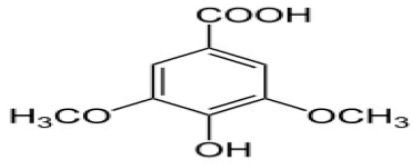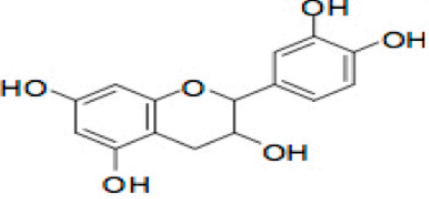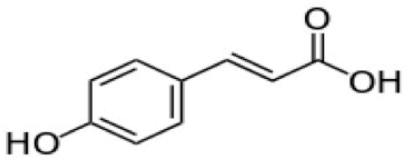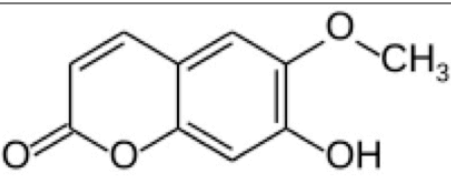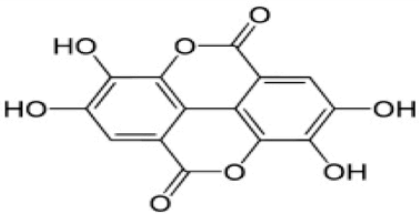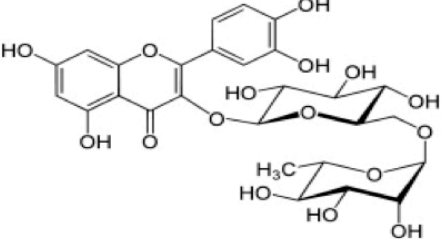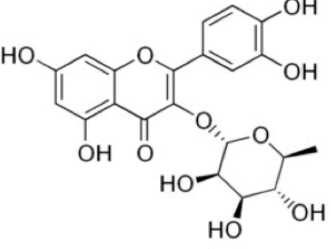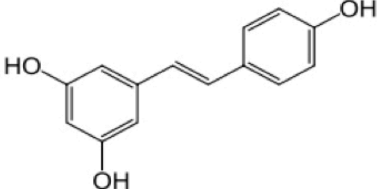
3.6.1. Phenolic standards optimization
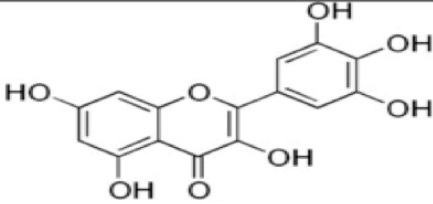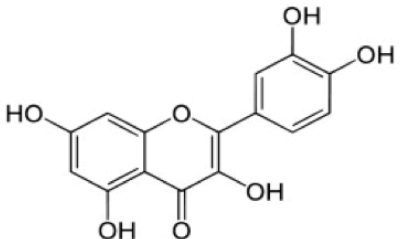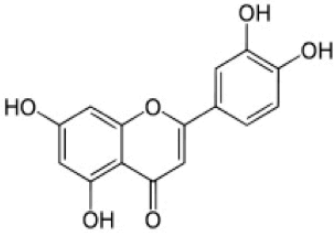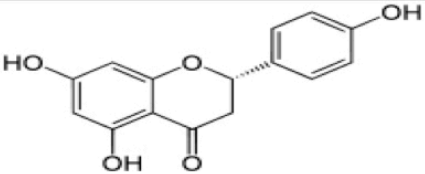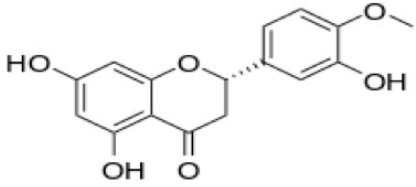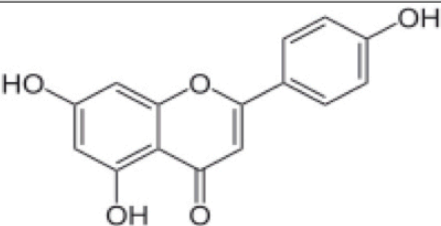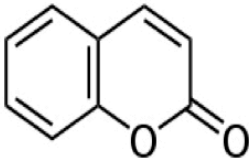
All 23 phenolic standards, which were available in our laboratory, were also run on RP-HPLC separately and in mixtures to optimize their retention time on our mobile phase. Keeping the conditions same, the standard i.e. known phenolic compounds were injected in HPLC to find out their retention time when injected alone.

The volume of each injection of all the sample and standards was 15 microliter and the solution of standard compounds was prepared by dissolving 1 mg of each standard separately in methanol. As it is shown in the Table 3.5, retention times of each of the standards were first noted when they were injected to the column separately (alone). Next the standards were mixed and three separate mixtures were made, each containing almost six compounds. These mixtures were then subjected to reverse phase HPLC analysis separately to analyze the change in their retention times when they are present in a mixture. Each of the standards retention time when injected in a mixture, their structures and their molecular weight is also shown in Table 3.5.

Table 3.5. Retention time of Phenolic compounds in RP-HPLC

Name	Structures	Molecular weight	Retention Time (alone)	Retention Time (mixture)
Hydroquinone		110.11	5.967	6.311
Gallic acid		170.12	10.263	9.876
Esculin		340.282	19.746	19.560
Catechin		290.27	21.741	21.376
Esculetin		178.14	21.566	22.243
Vanillic acid		168.14	22.705	22.214
Chlorogenic acid		354.31	23.030	22.377
Caffeic acid		180.16	23.760	23.396

Syringic acid		198.17	24.411	23.836
Epicatechin		290.27	26.90	24.895
P-Coumaric acid		164.16	30.563	29.643
Scopoletin		192.16	31.581	30.979
Ellagic acid		302.197	42.20	41.712
Rutin		610.52	41.137	41.847
Quercitrin		448.38	49.40	48.987
Resveratrol		228.24	49.54	49.609

Myricetin		318.34	50.943	50.854
Quercetin		302.236	59.980	59.077
Luteolin		286.24	60.539	60.190
Naringenin		272.257	62.089	61.838
Hesperetin		302.28	63.025	63.018
Apigenin		270.24	63.492	63.347
Coumarin		146.14	69.888	69.890
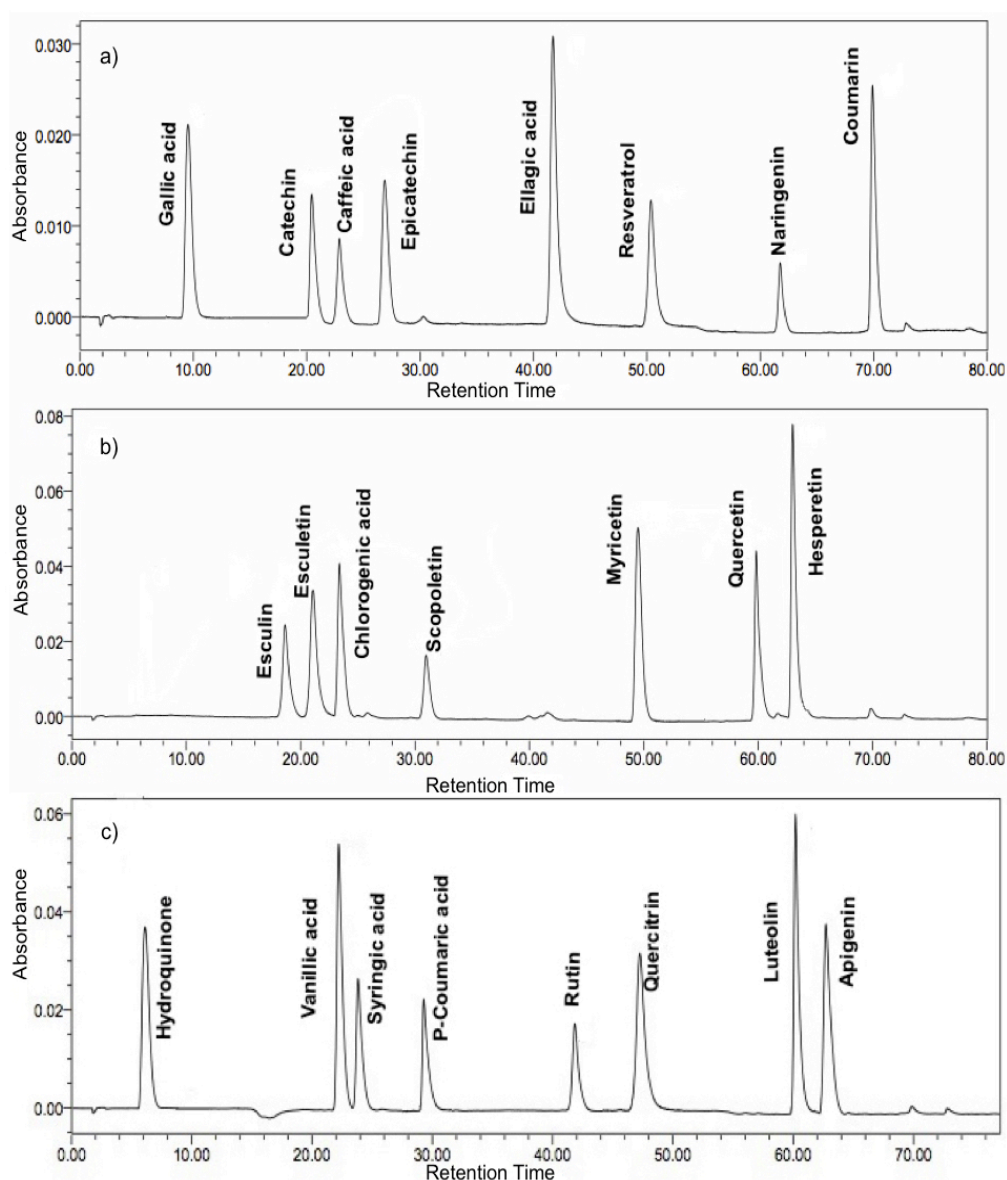
Figure 3.5. Chromatogram of RP-HPLC analysis of 23 phenolic standard compounds divided to three mixtures, using photo diode array detector at 254 nm

Figure 3.5 illustrates chromatograms of three mixtures of phenolic standards at 254 nm. The Figure 3.5 helps deducing that phenolic acids that are eluted in the mobile phase given in methods 2.2.7. The phenolic acids eluted between tenth and thirty fifth minutes. Hence, the gradient of the mobile phase used for the given period of time was more hydrophilic and thus dissolved the phenolic acid groups and letting them leave the column initially. The flavonoids are less hydrophilic and show some

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attractions to the stationary phase. They are eluted only if the mobile phase becomes more hydrophobic which happens after the thirty-five minutes of the injection flow. If the molecular weight of the two compounds is similar, then that compound will have greater hydrophilicity whichever has higher number of hydrophilic substituents attached to it. Such a compound will have a shorter retention time and will elute first in a reverse phase column. Examples of this phenomenon are the differences of retention time between scopoletin and esculetin and gallic acid and syringic acid.

Another point of consideration is that the column's pore size does not effect the retention time. For example, if we compare the retention time of esculin and esculetin, esculin elutes before esculetin, although the size of esculin is much bigger than the latter. This can be explained that because esculin has a sugar molecule attached to it, which increases the hydrophilicity of the molecule to great extent eluting it first. On the other hand esculetin contains only two hydroxyl groups and due to less hydrophilic nature elutes well after the former. Both esculin and esculetin has a basic skeleton of benzene ring which is fused with a pyrone ring. Esculin is larger in size owing to a glucose molecule attached to it, but this does not play any role in determining the retention time of the molecule.

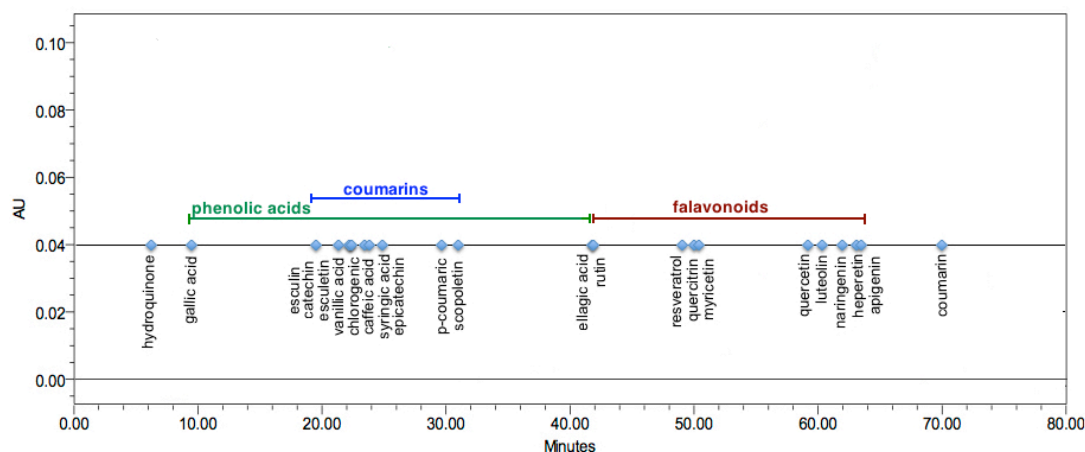
Figure 3.6. Representation of all standard molecules in one chromatogram

All the phenolic compounds if mixed and that solution is injected in reverse phase HPLC with all the same conditions, the resultant chromatogram will be like shown in Figure 3.6 where elution of compounds show some overlap in elution with no definite borders. Phenolic acids are the first to elute followed by coumarin

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derivatives and finally the flavonoids are eluted between 9th and 41st, 19th and 31st and 41st and 63rd minute respectively.

In literature elution profile similar to that of the one shown in Figure 3.6 has been exhibited formerly by Nuutila et al.2002; Mattila et al., 2002; Nicoletti el al, 2008; Proestos et al, 2006; Burin et al, 2011;. used catechin, p-coumaric acid, epicatechin, gallic acid, scopoletin apigenin and quercetin and also showed similar profile where acids eluted first followed by flavonoids and then coumarin.

3.6.2. UV-Visible spectrum of phenolic standards

All phenolic compounds have a typical UV absorbance spectra and this particular spectra is characteristic of that compound under the given conditions. There is a red shift observed in the spectra. This suggests that delocalized π electrons are present in conjugation, for example a vinyl group attached to a benzene ring.

In that sense, the flavonoids generally exhibit two major absorption bands in the UV-Vis spectrum. One is the longer wavelength absorbance in the region of 320−385 nm and is called Band I, while the other is the shorter wavelength absorbance, in the region of 250−285 and is referred to as Band II. The Band I represent the absorption due to B-ring of the flavonoids, while the Band II represents the A-ring and substituents attached to it, Figure 3.7. The nature of C-ring also influences the absorption wavelength. Thus any chromatogram of phenolic compounds could be visualized at different wavelengths from 250 to 400 nm. The wavelength to compare the phenolic UV-VIS spectra should be selected according to the peaks obtained in chromatogram of the phenolic mixtures that should be clearly resolvable and the UV-VIS spectra of each phenolic compound should show a typical reference to the absorption spectrum of Band I and II (Anderson, 2006).
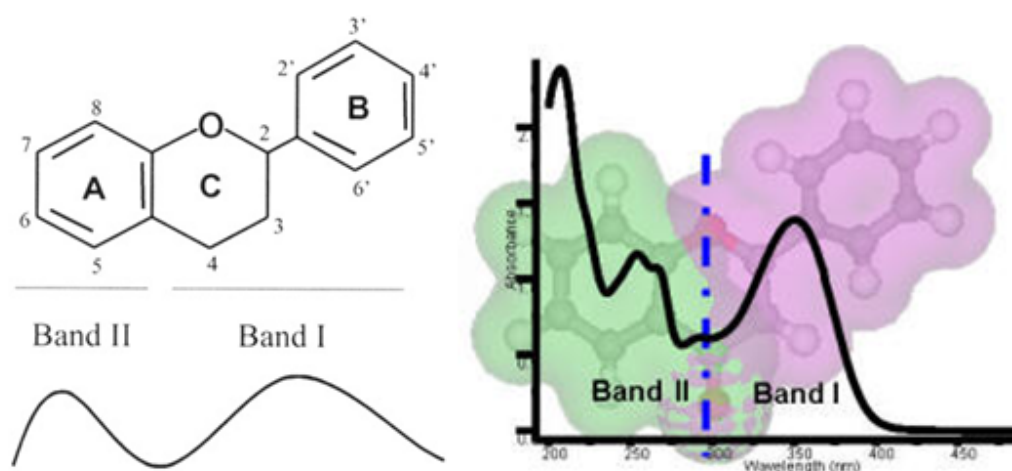
Figure 3.7. Basic structure of flavonoid with absorption band (Anderson, 2006)

UV-Visble spectra of the phenolic standards mentioned in Table 3.5 is shown in Figure 3.8 to Figure 3.11. These are taken by photo diode array detector that can measure absorption of multiple wavelengths from 210-800 nm. It is evident from Figures 3.8 to 3.11 that all the phenolic standards show a characteristic phenolic nature of two bands, I and II. The band I corresponds to the π to $\pi *$ transition of the electron while the band II corresponds to π to n transition of the electron after the absorbance of energy.

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Figure 3.8. UV-VIS spectra of phenolic standards using photo diode array detector, scanned between 210-800 nm.

Figure 3.9. UV-VIS spectra of phenolic standards using photo diode array detector, scanned between 210-800 nm.

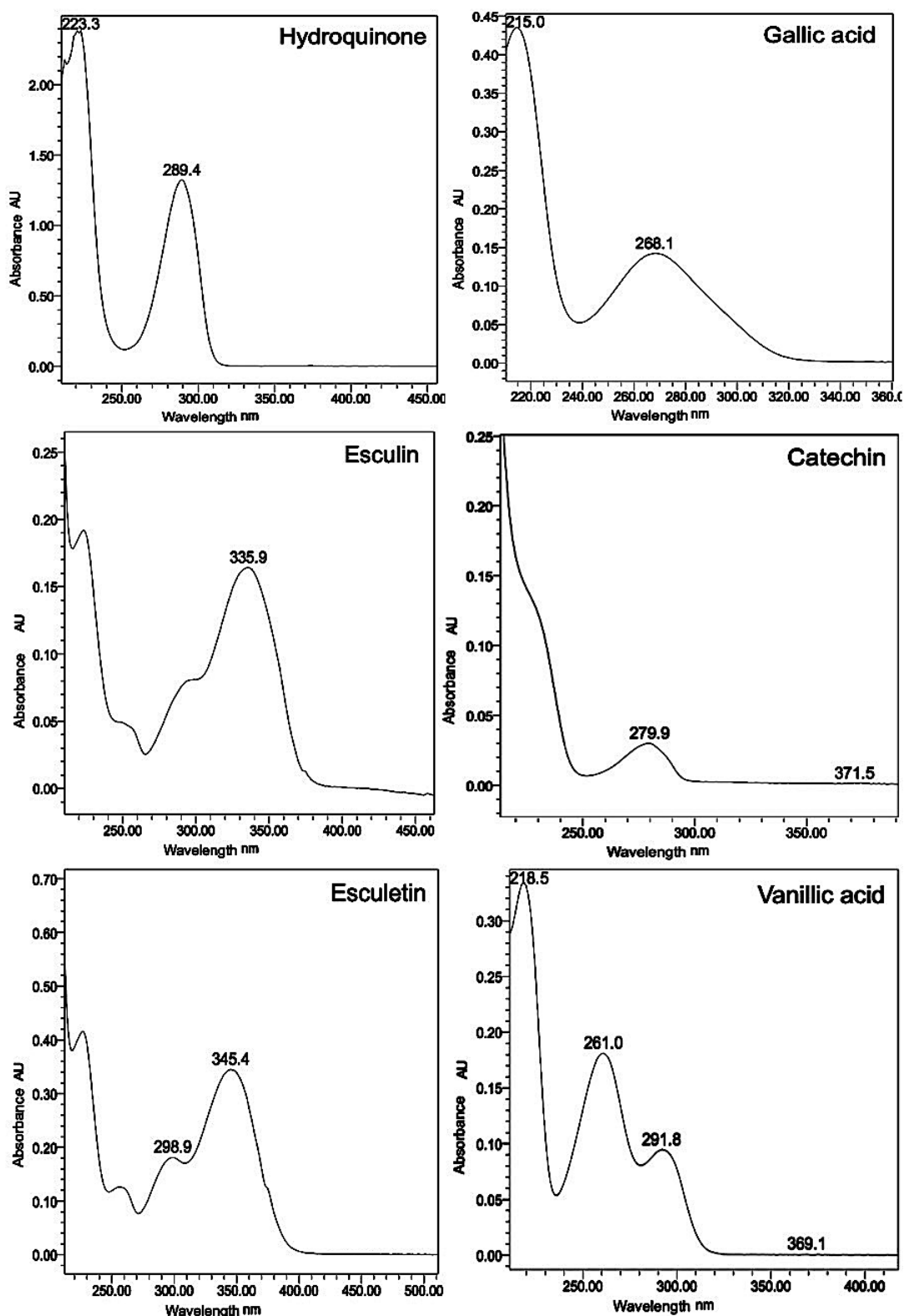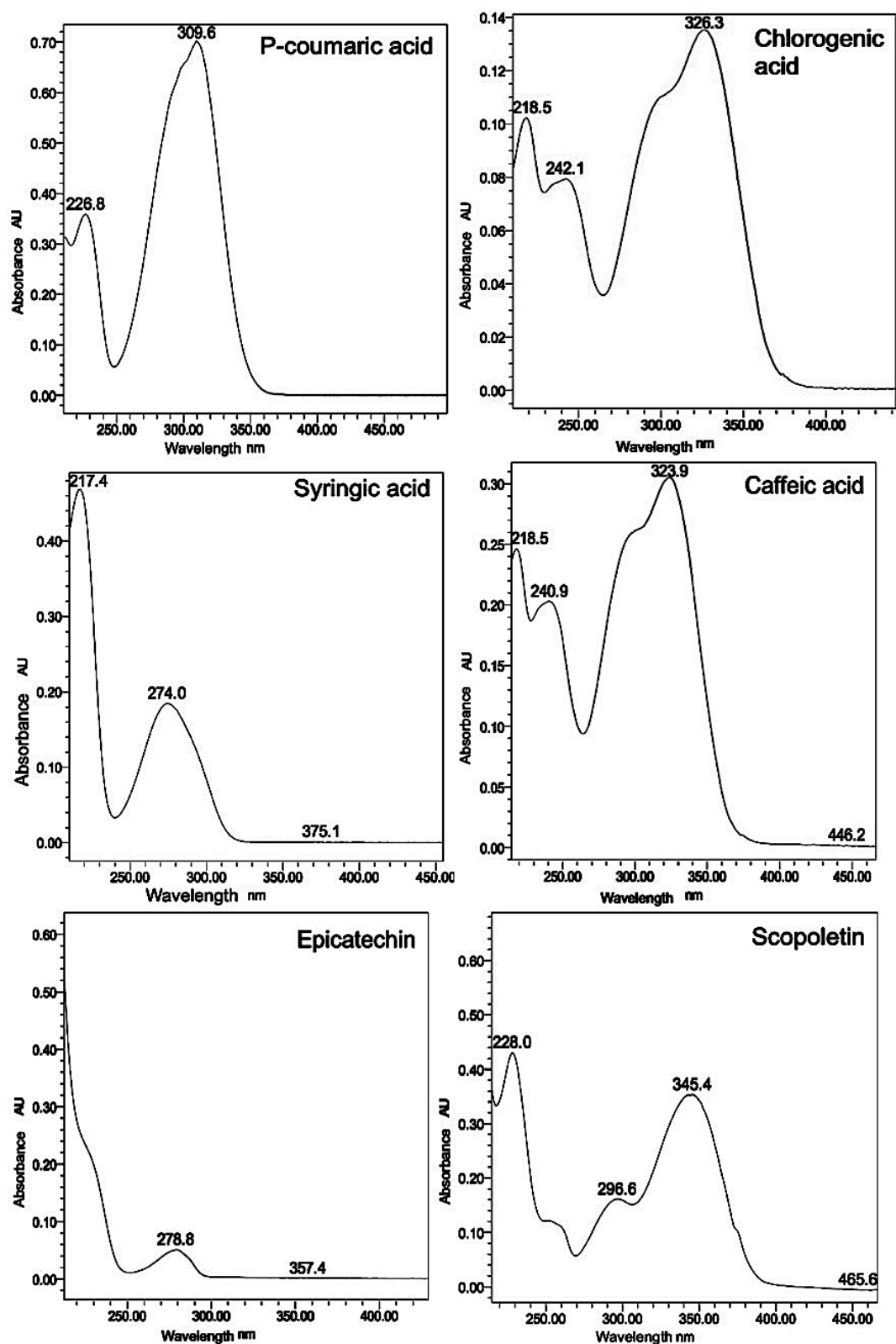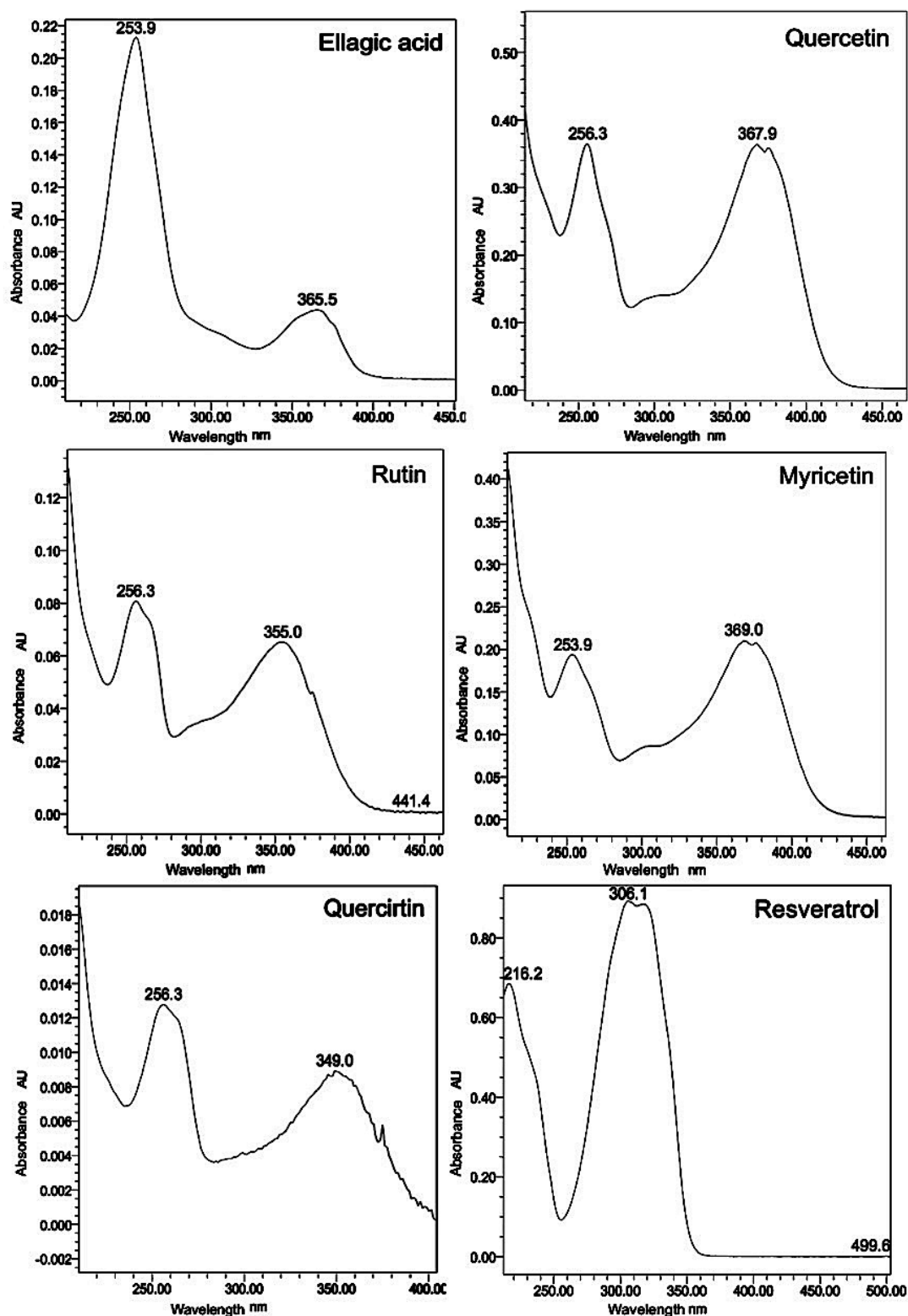Figure 3.10. UV-VIS spectra of phenolic standards using photo diode array detector, scanned between 210-800 nm.
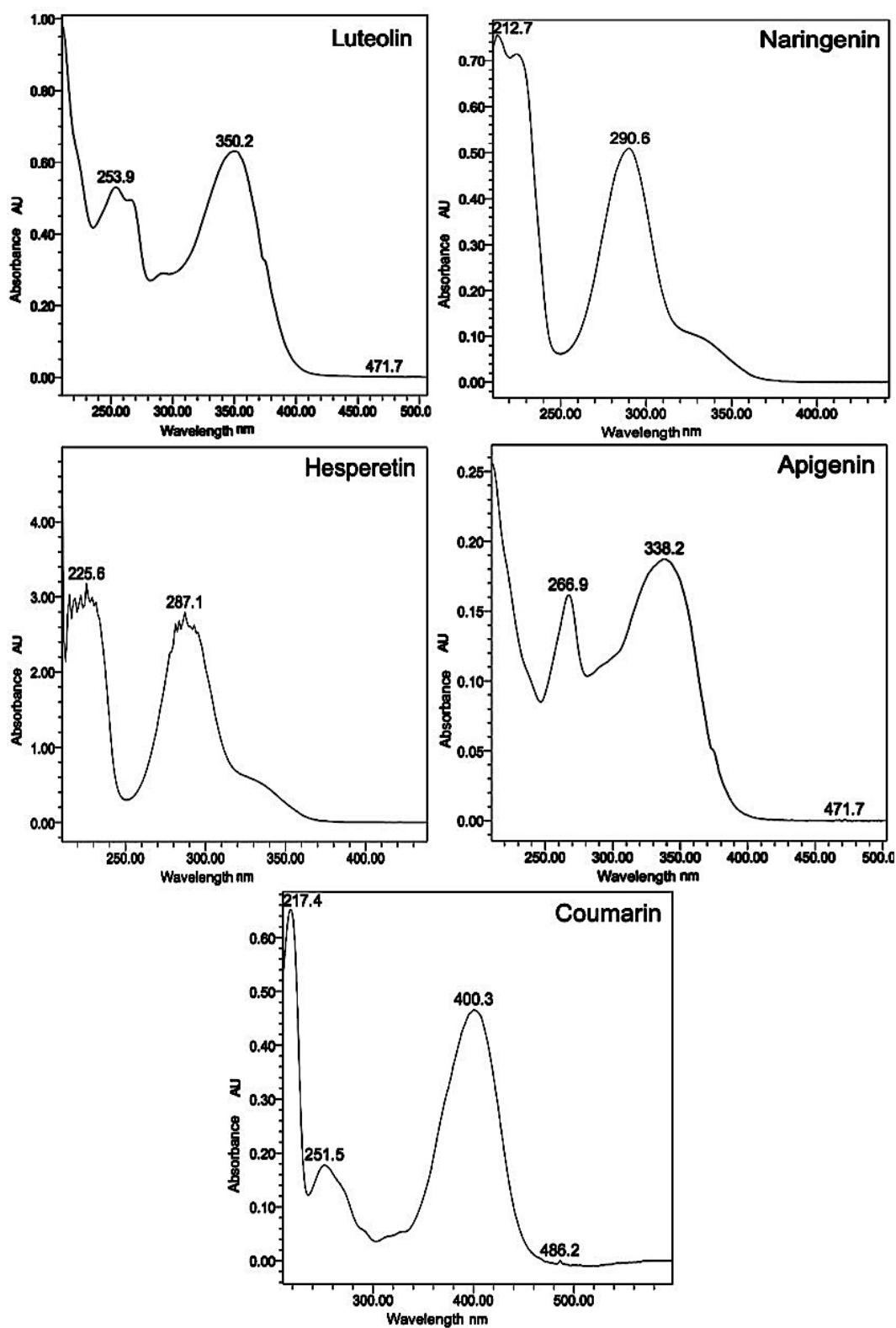
Figure 3.11. UV-VIS spectrums of phenolic standards using photo diode array detector, scanned between 210-800 nm.

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Many of the standards used here were also reported in literature, for their UV-Vis spectra. The UV-Vis spectrum of these phenolic compounds obtained from literature are shown in appendix A for comparison.

3.7. Extract fractions RP-HPLC application

Fractions in different concentrations were injected in reverse phase-HPLC and the chromatograms obtained were studied at following wavelengths of 254, 280, 300, 320, 340 and 360 nm to choose the best wavelengths and best concentration for analyzing the peaks as well as comparing with the standard chromatograms Figure 3.5 to analyze which compound may be present in the fractions.

3.7.1. Crude extract RP-HPLC analysis

The first extract to be run on RP-HPLC was crude extract. A solution was prepared containing 10 mg/mL of crude extract in methanol. This was then filtered using a 0.22 µm filter. Next, 15 µL of it was injected in HPLC. The chromatogram obtained was analyzed at wavelengths of 360, 340, 320, 300, 280 and 254 nm (Figure A-1 and A-2). As it is seen from the Figures in Appendix , the absorbance unit is less at 254 nm (0.02 AU) wavelength. Therefore, higher concentrations i.e. 50 mg/mL of crude extract in methanol was prepared to have better results.
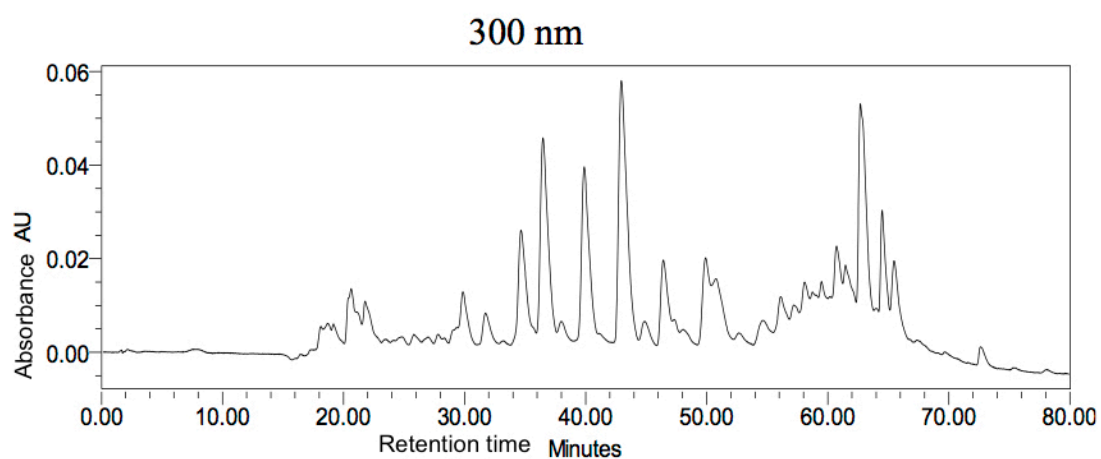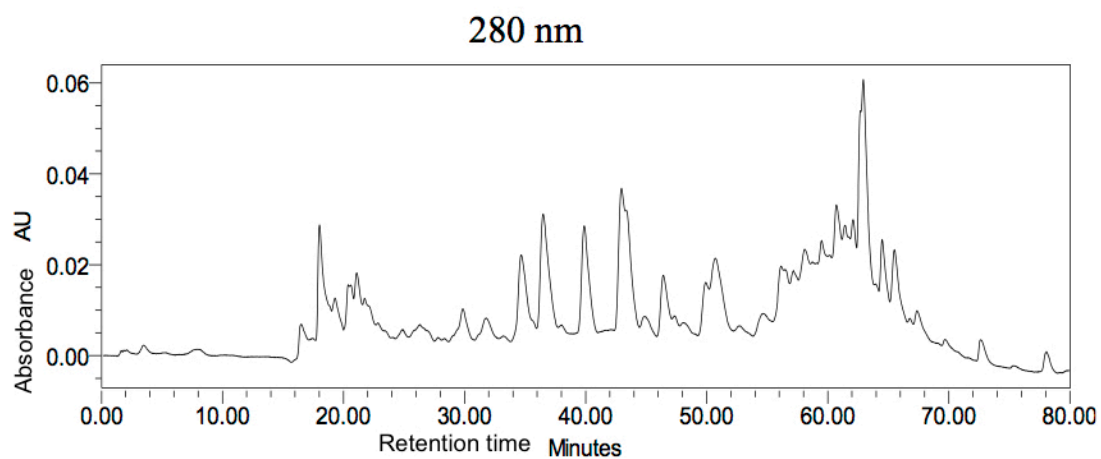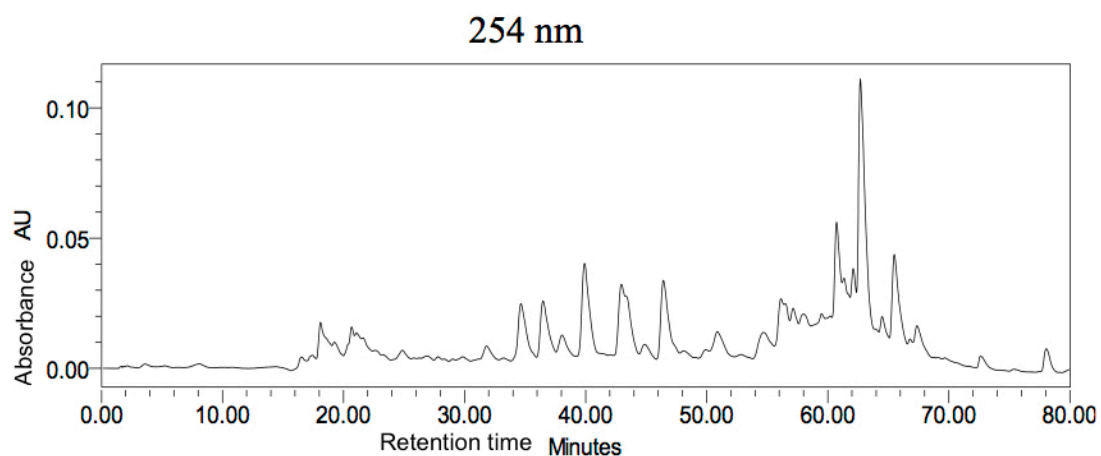
254 nm

280 nm

300 nm

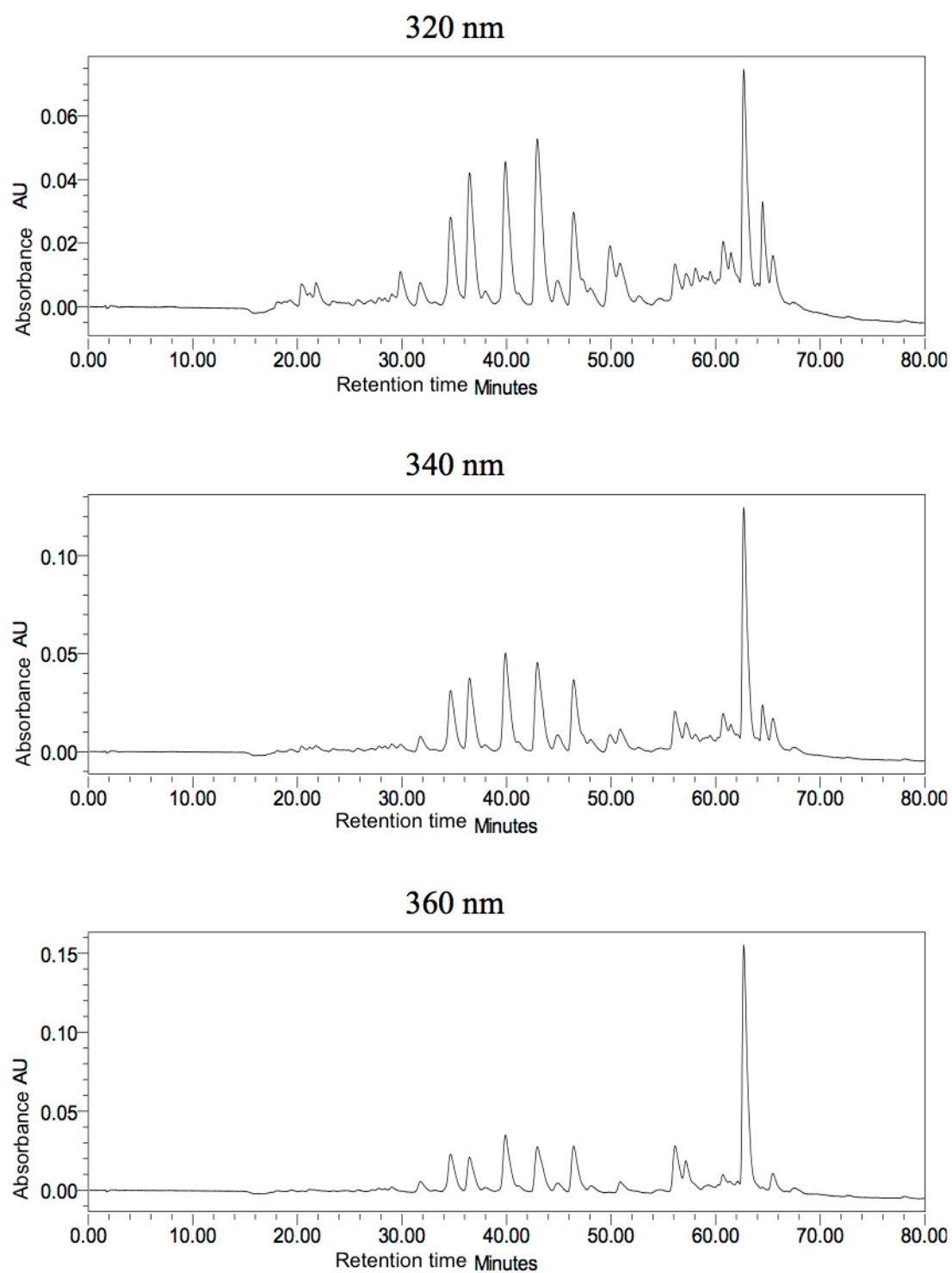Figure 3.12. Crude extract 50 mg/mL chromatograms, with RP-HPLC, at wavelengths of 254, 280, 300 nm

Figure 3.13. Crude extract 50 mg/mL chromatograms, with RP-HPLC, at wavelengths of 320, 340, 360 nm

Figure 3.12 and 3.13 show the resulting chromatogram in wavelengths of 254, 280, 300, 320, 340 and 360 nm for crude extract 50 mg/mL (each injection were done 3 times). Determination of the phenolic constituents started with the extraction process for the crude extract preparation. Crude extract was first analyzed by the RP-HPLC. There were too many peaks, and as it is obvious from the Figure 3.12 and 3.13 the resolution was quite low due to the intervention of the excessive number of compounds dissolved in pure methanol.

The interference of this chromatogram was not possible as many compound peaks were present and many peaks did not have a sharp baseline. Therefore, further study with this chromatogram was not suitable. This chromatogram showed that the plant root under investigation consisted of various phenol compounds with varying characteristics of hydrophilicity and hydrophobicity. Therefore, it was thought better to continue our identification of compounds with the fractionated extracts as each fraction had a specific group of compounds concentrated in it due to their solubility in that particular solvent.

3.7.2. Ethyl acetate extract RP-HPLC analysis

The first fractionated extract to be analyzed was ethyl acetate extract because from literature it is clear that this extract dissolves higher amount of the phenol compounds (Coruh et al, 2014; Uyar, 2015, Ozdogan, 2014). Firstly, a solution of 1mg of ethyl acetate extract was prepared in methanol. Prior to injection it was filtered. A 15 µL was taken in the HPLC syringe and injected in reverse phase-HPLC. The chromatogram obtained was studied at wavelengths of 254, 280, 300, 320, 340 and 360 nm (Figure A-3 and A-4). As it is seen in the Figures in the appendix not only the unit of absorbance is less at 245 nm (0.015 AU), but also the noise is high and it is difficult to differentiate between noise and significant peaks. Therefore, solutions of 10 and 20 mg/mL of ethyl acetate were prepared in methanol to minimize the noise and analyze the peaks clearly.
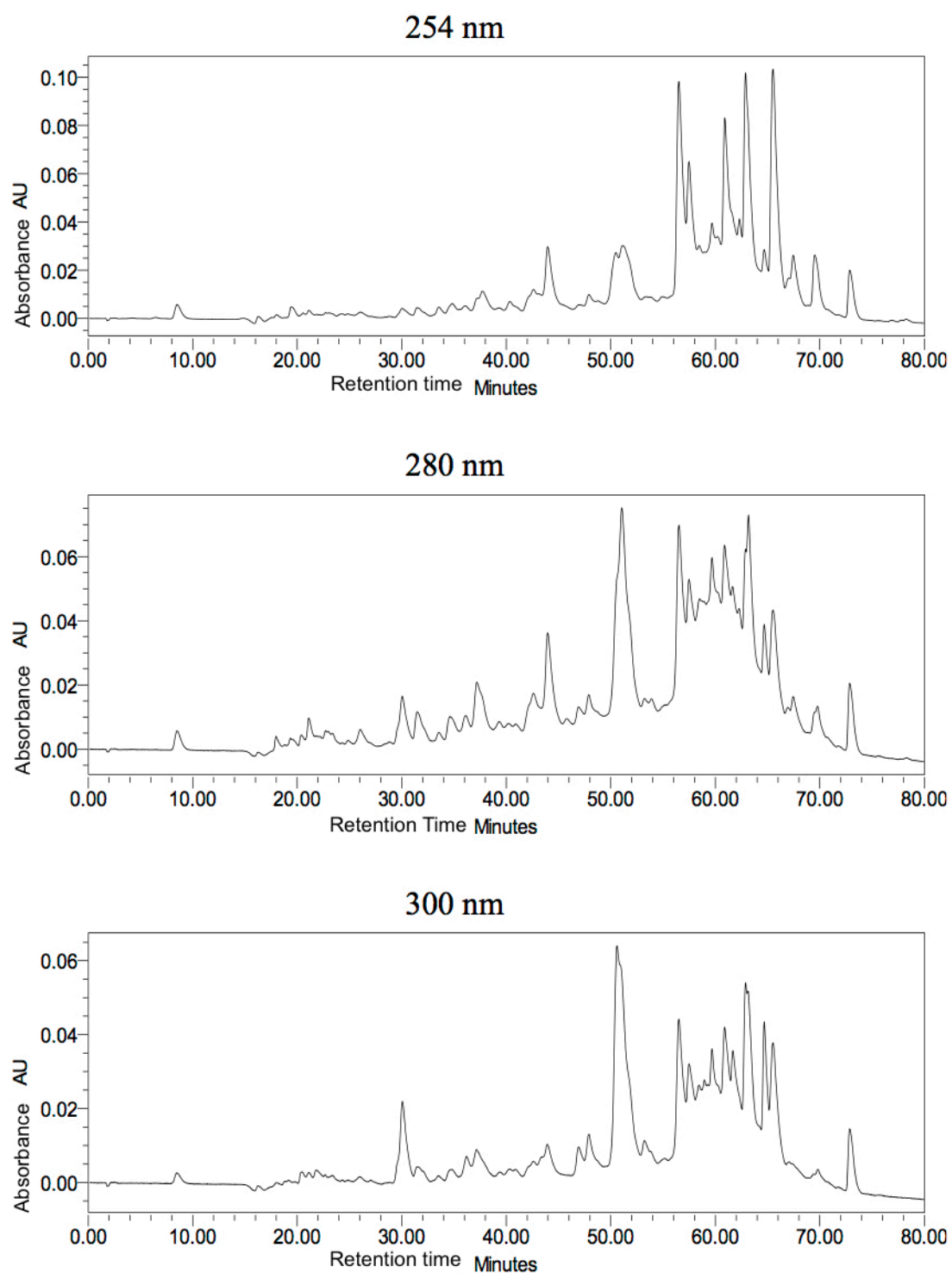
Figure 3.14. Ethyl acetate extract 10 mg/mL chromatograms, with RP-HPLC, at wavelength of 254, 280,300 nm
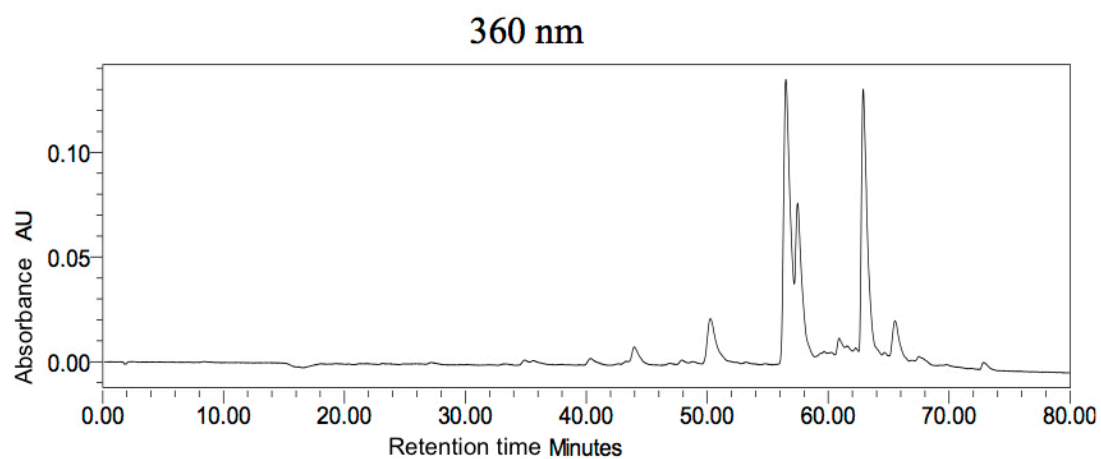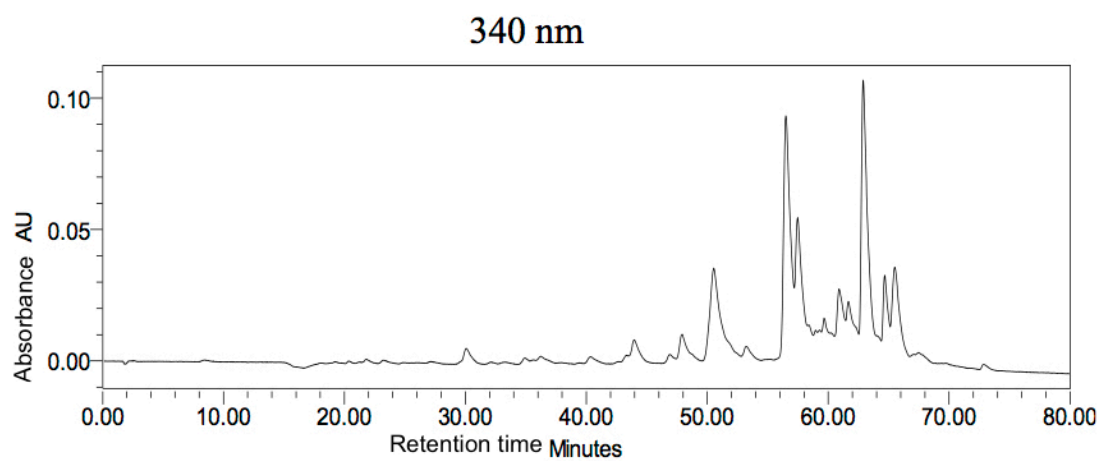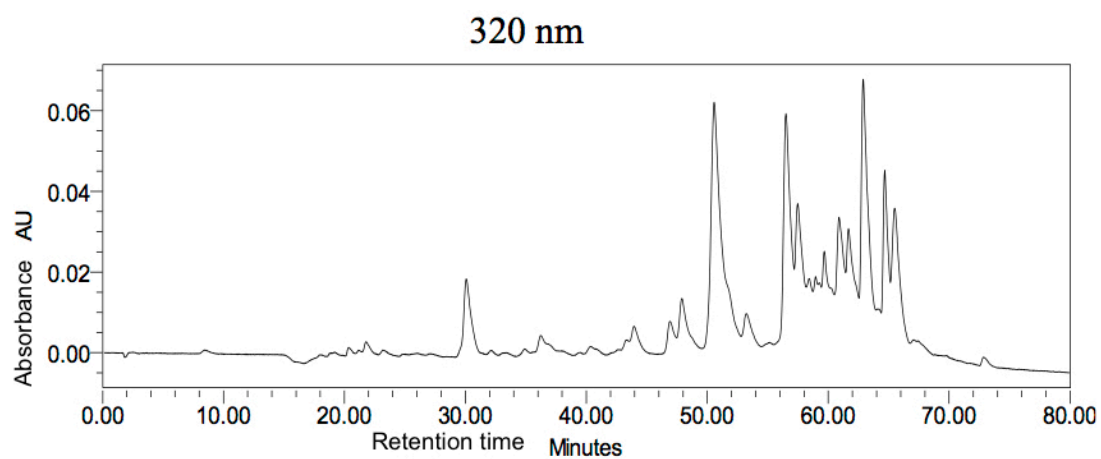
Figure 3.15. Ethyl acetate extract 10 mg/mL chromatograms, with RP-HPLC, at wavelength of 320, 340, 360 nm

Figure A-5 and A-6 shows the chromatogram of ethyl acetate extract at 20mg/mL while Figures 3.14 and 3.15 show the chromatograms of ethyl acetate extract at 10 mg/mL in wavelengths of 254, 280, 300, 320, 340 and 360 nm (each injection were done 3 times). As it is seen in Figure 3.14 and 3,15 the peaks are sharp and well defined with least noise level.

3.7.2.1. Ethyl acetate extract peak identification

Of all the chromatograms observed for ethyl acetate extract (Figure 3.14 and 3.15) chromatogram of ethyl acetate extract 10 mg/mL of methanol at 254 nm and 320 nm are the best chromatograms for investigating and defining the peaks. Because peaks were better resolved at these wavelengths i.e. more considerable, clear and sharp peaks are present. In Figure 3.16 the significant peaks are numbered.
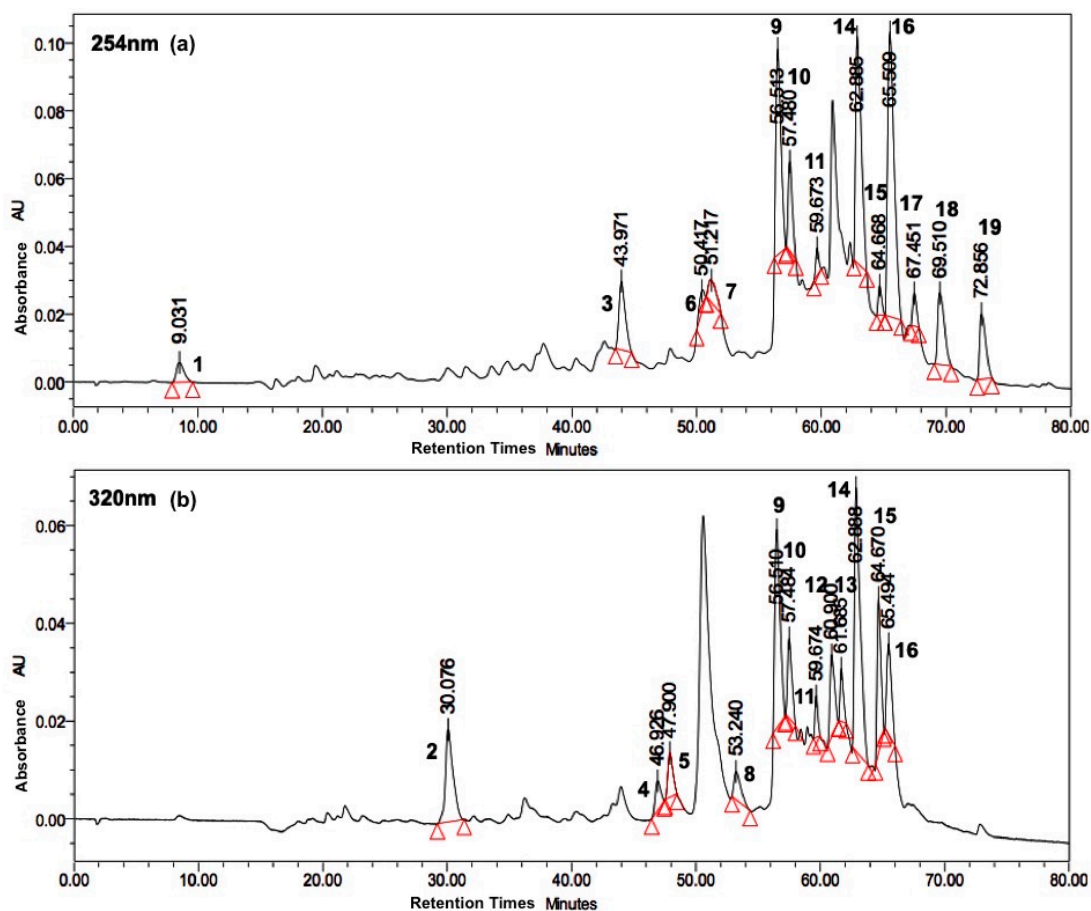
Figure 3.16. (a) and (b), Chromatograms of ethyl acetate extract (10 mg/ml) at 254nm and 320 nm.

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There are 19 sharp, clear and significant peaks observed in these two chromatograms (Figure 3.16 (a) and (b)), and all are numbered. Some peaks are visible clearly at one of the wavelengths such as, peak 1 and 2, the former appearing at 254 nm while the latter is observed at 320 nm. On the other hand some peaks are visible in both wavelengths such as peak 9 and 10. Also a closer view of the chromatograms of Figure 3.16 are shown in Figure 3.17 (a) and (b). Some of the overlaps of peaks in Figure 3.16 are cleared off when seen in closer view and thus can be analyzed clearly.

It can be seen in the Figure 3.17 (a), there is a peak eluting at 60.909 minutes in chromatogram taken at 320 nm. But as it is shown the peak is not sharp and it has curves, which means this is a complex peak, and that 2 or more compounds are eluting at the same time. When this peak was analyzed at different wavelength, it was separated into two peaks, which is seen in the Figure 3.17 (b), and they are numbered as Peak 12 and 13, eluting at 60,900 and 61.685 minutes. Similarly it is seen in the Figure 3.17 (b) there is a significantly large peak present at 50.574 minutes chromatogram taken at 320 nm. But as it is shown the peak is not sharp and it has curves, which indicates that this is a complex peak, and that 2 or more compounds maybe eluting at the same time. Therefore this peak was analyzed at different wavelength, and at 254 nm the peak was separated into two peaks corresponding to two separate compounds, which is seen in the Figure 3.17 (a), and they are numbered as peak 6 and 7, eluting at 50.443 and 51.217 minutes.
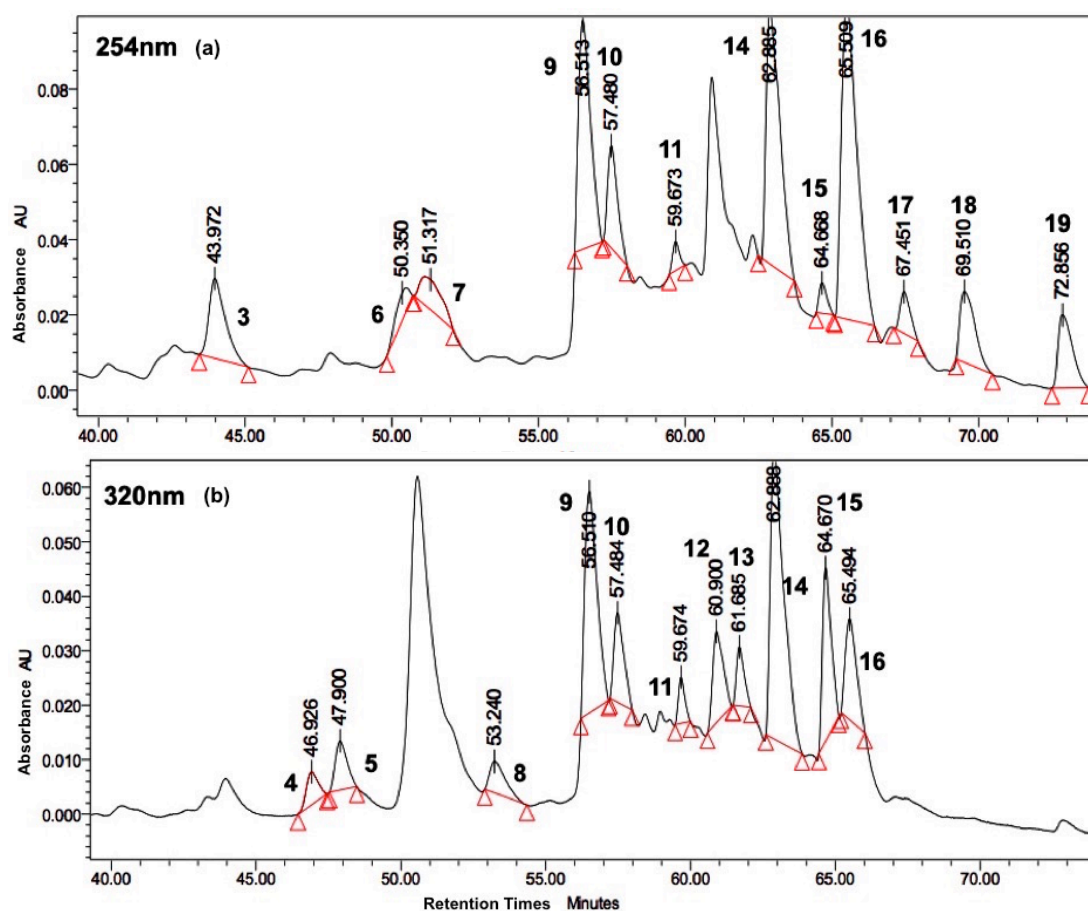
Figure 3.17. (a) and (b), Chromatograms of ethyl acetate extract 10 mg/ml at 254nm and 320 nm enlarged view from 40 to 70 minutes.

3.7.2.2. UV-VIS spectra of the 19 peaks of Ethyl acetate extract

After marking the peaks and analyzing their elution time, a thorough study of the UV-Vis spectra of those peaks were done to confirm whether the compounds corresponding to those peaks were phenolics and thus whether they contained the characteristic bands of phenolic compounds. From Figures 3.18, 3,19 and 3,20 were seen that all the peaks marked here (except peak 19) were significant in terms of S/N ratio and had a UV-Vis spectral characteristics of the phenolic compounds i.e. containing the two bands of I and II as explained under section 3.6.2.
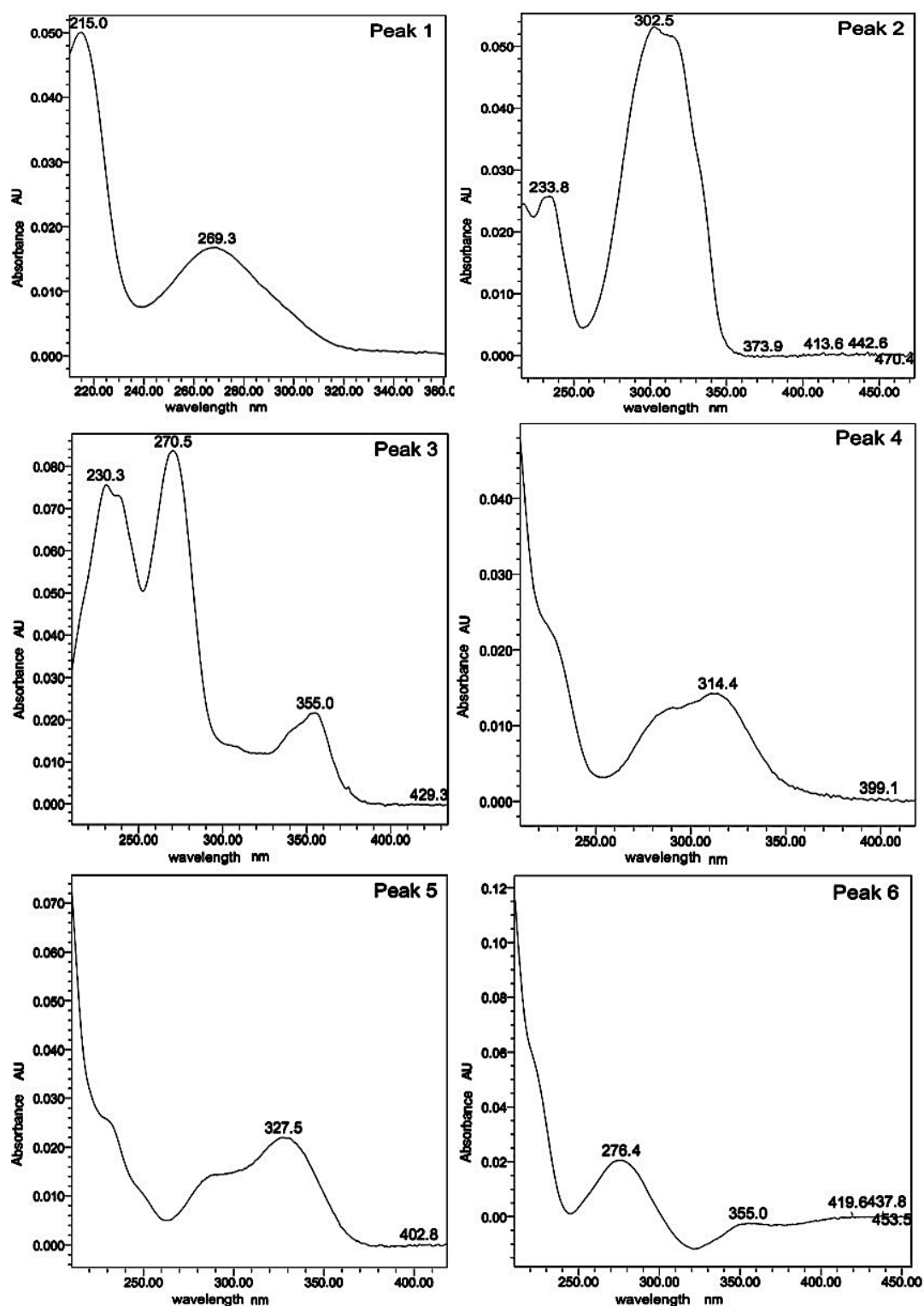
3.18. UV-VIS spectrums of ethyl acetate extract peaks using photo diode array detector, scanned between 210-800 nm.
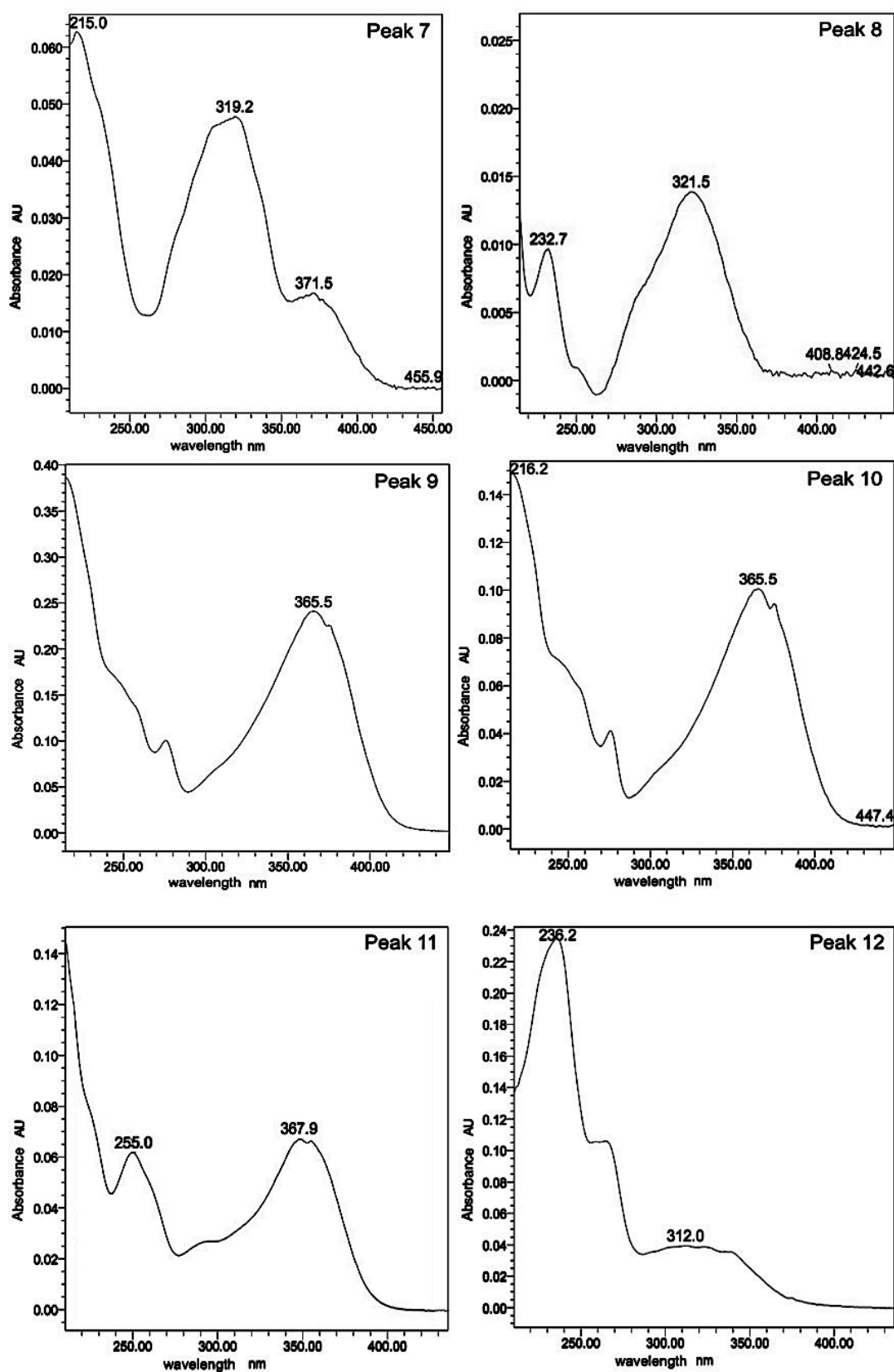
3.19. UV-VIS spectrums of ethyl acetate extract peaks using photo diode array detector, scanned between 210-800 nm.
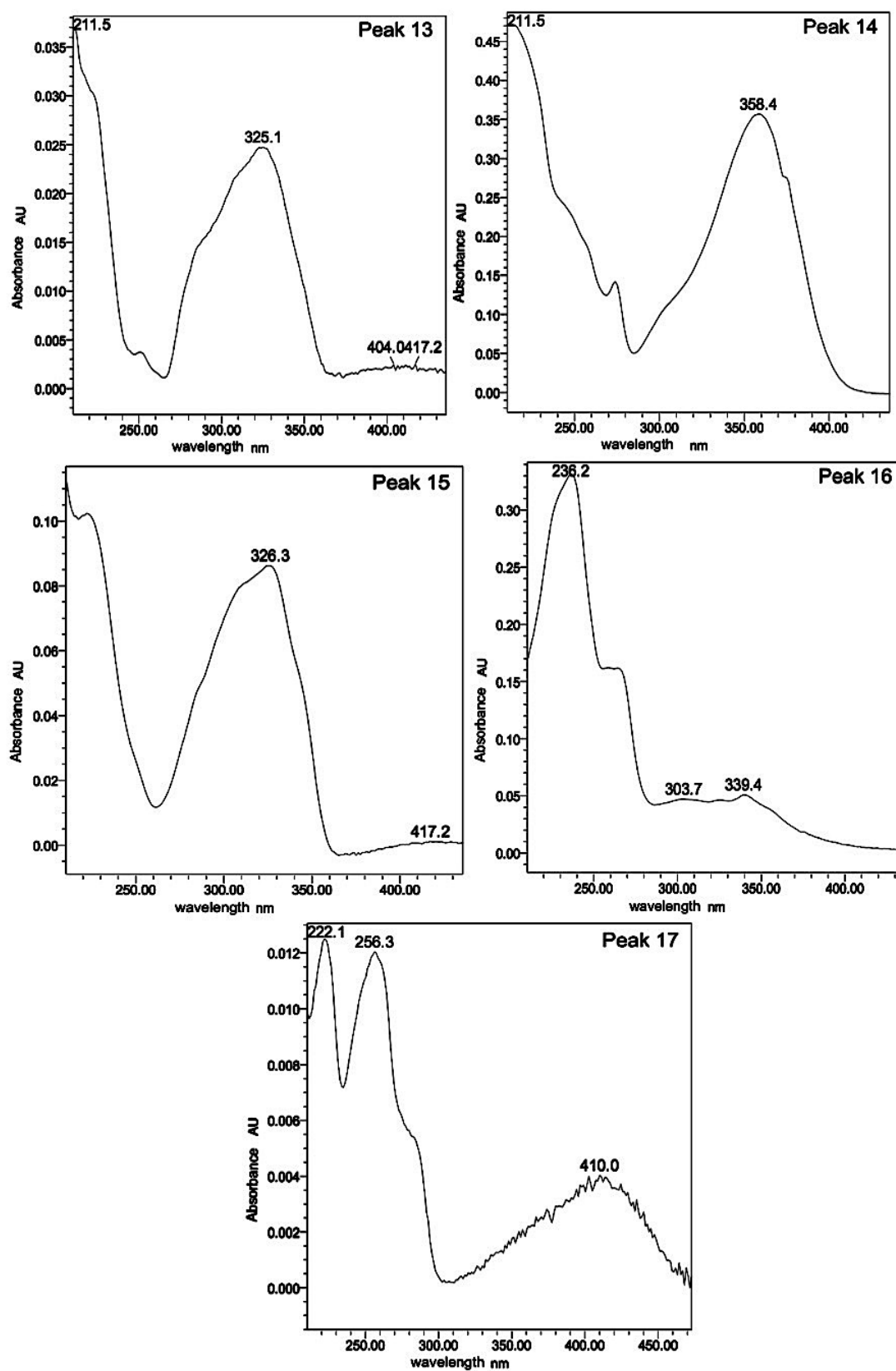
3.20. UV-VIS spectrums of ethyl acetate extract peaks using photo diode array detector, scanned between 210-800 nm.

3.21. UV-VIS spectrums of ethyl acetate extract peaks using photo diode array detector, scanned between 210-800 nm.

3.7.2.3. Co-injections of ethyl acetate extract

The chromatograms in Figure 3.16 (a) and (b) were compared with the chromatograms of mixture of phenolic standards (Figure 3.5) and it was assumed that due to elution at similar timings, some of the phenolic compounds may be present in the ethyl acetate fraction. Therefore, to confirm their presence, co-injections were done with each standard separately. A co-injection cons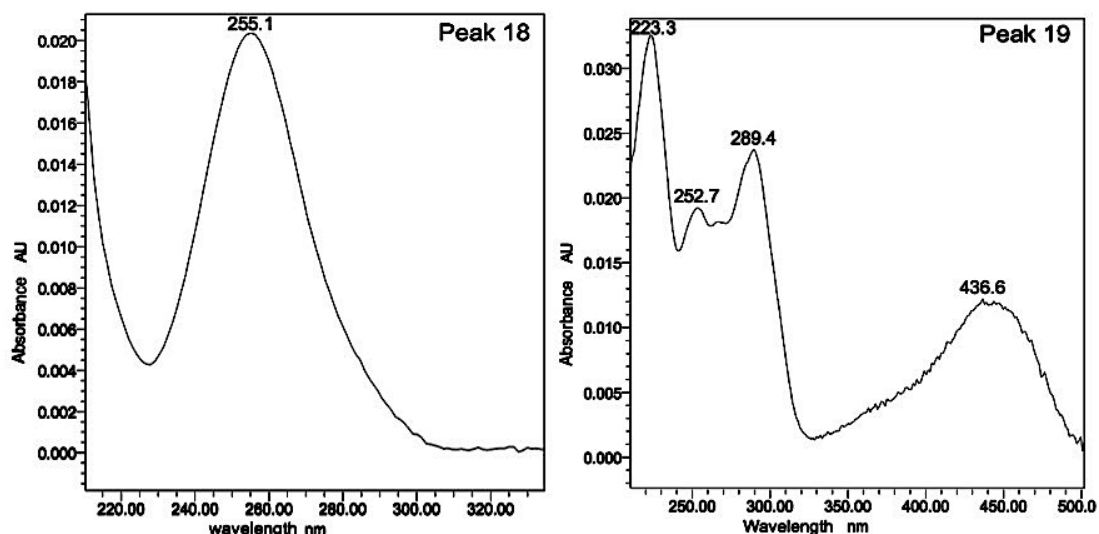ists of ethyl acetate fraction in same concentration along with a specific amount of the standard added. The concentrations of standard solutions are 1 mg/mL, but the amount of this standard solution added to the extract to make co-injections is dependent on the absorbance extinction coefficient of the standard which varies from compound to compound.

The standard co-injections may increase any one of the peaks, indicating that it may be present in the given fraction or elute separately in the chromatogram, indicating that it is completely absent in the given fraction.

The first significant peak is present at 9.031 minutes, which can be observed only at 254 nm, after comparing with the chromatograms of mixture of phenolic standards in Figure 3.5, it is assumed that gallic acid may be present due to very close retention time to this peak, so a solution of ethyl acetate extract along with gallic acid were

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prepared and run on the RP-HPLC. In Figure 3.22 the chromatogram of co-injection of gallic acid with ethyl acetate fraction is shown. As is evident from the dotted part in Figure 3.22, the first peak is increased in length while all the other peaks remain same, therefore, it is concluded that gallic acid may be present in this fraction. And also comparing the Figure 3.8 (UV-Visible spectrum of gallic acid) and Figure 3.18 (UV-Visible spectrum of peak 1), both are showing same UV-Visible spectrum, which means, this assumption (presence of gallic acid in ethyl acetate extract) is likely to be correct.
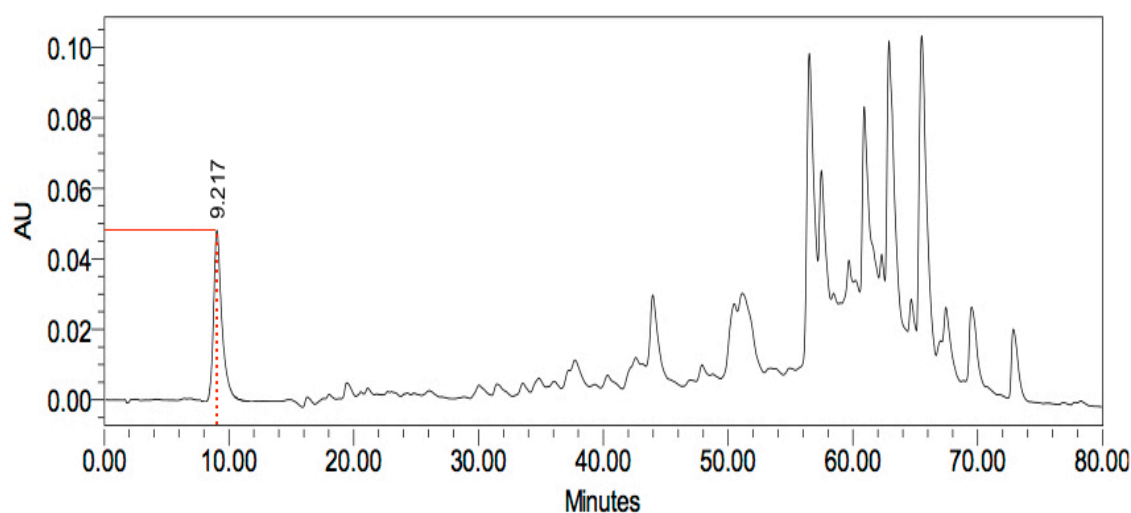
Figure 3.22. Chromatogram of co-injection of gallic acid with ethyl acetate fraction

The second significant peak is present at 30.076 minutes, which is visible at 320nm chromatograph. After comparing with the chromatograms of mixture of phenolic standards, it is assumed that scopoletin or p-coumaric acid may be present due to very close elution time to this peak. In Figure 3.23 the chromatogram of co-injection of scopoletin with ethyl acetate fraction is shown. From the marked part it is seen that scopoletin is not present in the ethyl acetate fraction because it elutes out as a separate peak in the chromatogram, and peak 2 also did not increase in length when compared to Figure 3.16 (b).
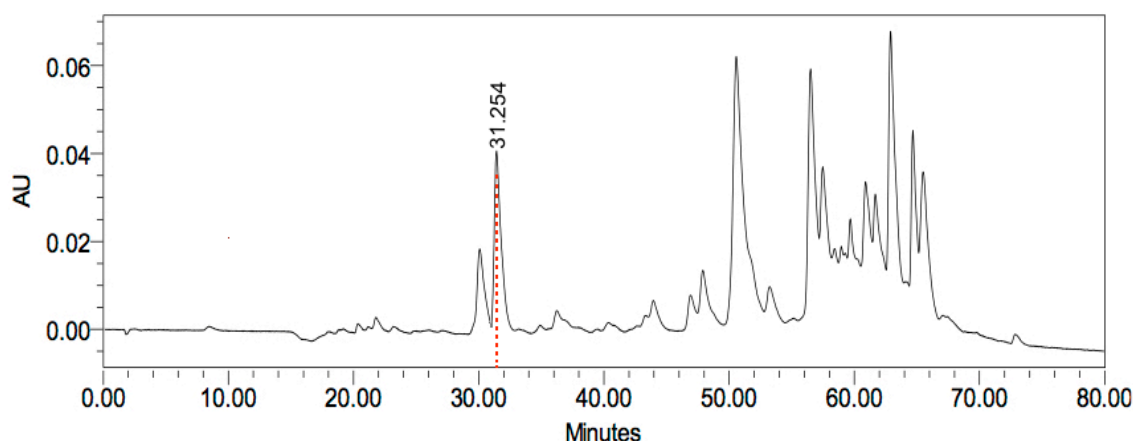
Figure 3.23. Chromatogram of co-injection of scopoletin 1 mg/mL with ethyl acetate fraction 10 mg/mL

Next co-injection done was ethyl acetate extract with p-coumaric acid standard. The resultant chromatogram is shown in Figure 3.24. As it is shown from the marked part in the figure, peak 2 is increased in length while all the other peaks remain same, therefore, it is assumed that p-coumaric may be present in this fraction. Also, comparing the Figure 3.9 (UV-Visible spectrum of p-coumaric acid) and Figure 3.18 (UV-Visible spectrum of peak 2), they are almost the same with a slight difference which is a shift towards shorter wavelength of both the bands I and II in UV-Vis spectra of peak 2. This suggests that P-coumaric acid might be present in our ethyl acetate extract and this shift may be due to a slight change in the acidity of the solution of ethyl acetate extract.

Figure 3.24. Chromatogram of co-injection of p-coumaric acid with ethyl acetate fraction.

Third peak elutes at 43.971 minutes and it is visible in chromatogram taken at 254 nm. After comparing chromatogram of ethyl acetate Figure 3.16 (a) with the chromatogram of phenolic standards in Figure 3.5, it is seen that no phenolic standard elutes with a close retention time to peak 3. But this peak can be referred to a compound which maybe a phenolic acid or its derivatives according to Figure 3.6.
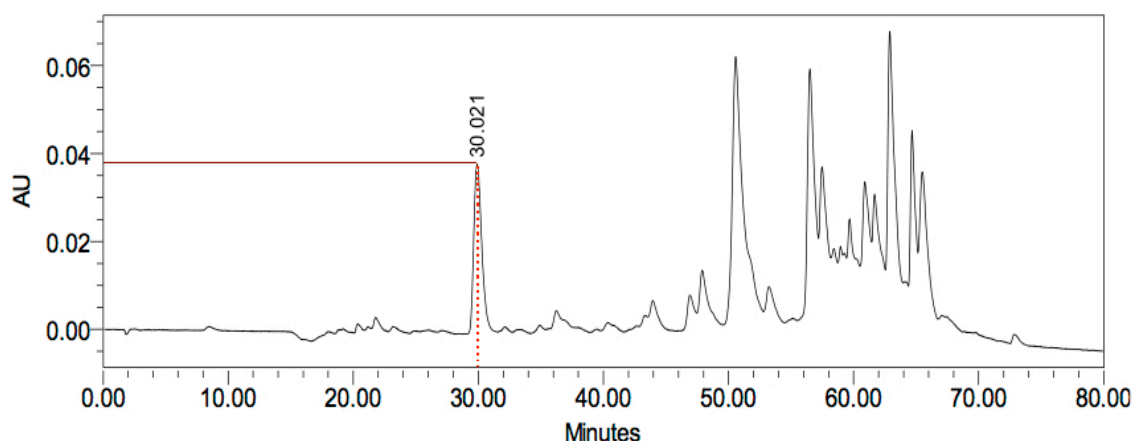
Now we consider peak 4, 5 and 8 (Figure 3.16 (b)), three peaks are visible at 320 nm and they elute at 46.926, 47.900 and 53.240 minutes respectively. According to their UV-Vis spectrum (Figure 3.18 and 3.19), all three peaks refer to compounds phenolic in nature, having the characteristic bands, I and II. After comparing Figure 3.16 (b) with the chromatograms of mixture of phenolic standards (Figure 3.5), it is seen that we have no phenolic standard compound that elutes with a close retention time to peak 4, 5 and 8 to be applied for co-injections. But from the Figure 3.6, it is assumed that peak 4 and 5 refers to phenolic acids or its derivatives as they elute much earlier in the chromatogram and peak 8 may be a flavonoid compound due to its delayed elution time in the chromatogram.

Moving further, in Figure 3.16 (b) a significantly large peak at 50.57 minutes is shown. This peak resolves into two clearly separated peaks when the chromatogram is observed at 254 nm. These two peaks are labeled as peak 6 and 7, eluting at 50.443

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and 51.217 minutes respectively. After comparing the chromatogram of ethyl acetate (Figure 3.16 (b)) with the chromatograms of mixture of phenolic standards (Figure 3.5), it was assumed that resveratrol, myrcetin or quercitrin may be present due to very close retention time to peak 6 and 7.

As it is shown in the marked part in the Figure 3.25, peak 7 is increased in length while all the other peaks remain same, therefore, it is assumed that myrcetin may be present in the ethyl acetate fraction. But comparing the Figure 3.10 (UV-Visible spectrum of myricetin) and Figure 3.19 (UV-Visible spectrum of peak 7), the bands I and II of both the figures achieved their maximum absorbance at different wavelengths. Therefore, it was confirmed that myricetin was absent in our ethyl acetate extract.
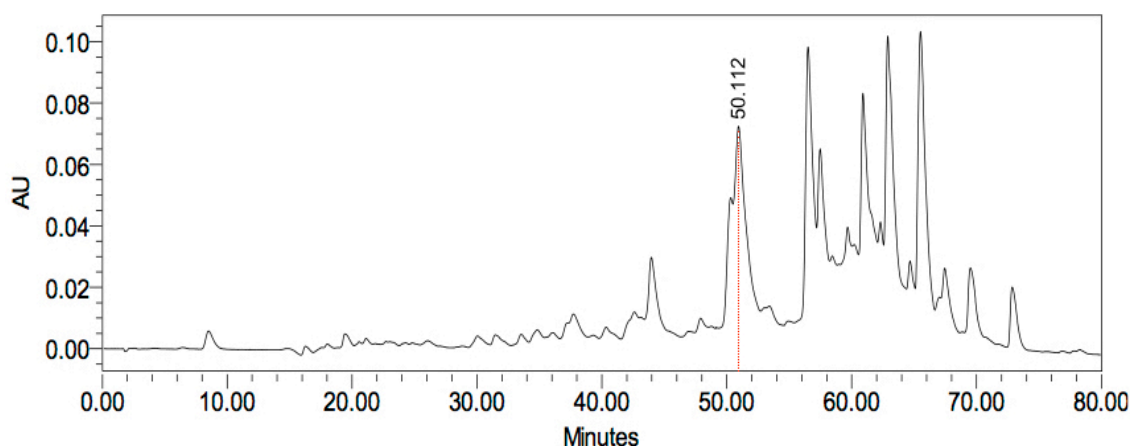
Figure 3.25. Chromatogram of co-injection of myrcetin with ethyl acetate fraction

The next two co-injections done were ethyl acetate with quercitrin and resveratrol standard. The resultant chromatograms are shown in Figure 3.26 (a) and (b). From the marked peaks in the Figure 3.26 (a) and (b), it is seen that quercitrin and resveratrol are not present in the ethyl acetate fraction, because both of them are eluted as separate peaks in the chromatograms and all the others peaks remain the same.

Although the exact compound could not be confirmed but the conclusion can be made that peaks 6 and 7 are flavonoids and that peak 7 may have structural similarities with myricetin.

Figure 3.26. Chromatogram of co-injection of a) quercitrin and b) resveratrol with ethyle acetate solution of

In Figure 3.16 (a) and (b) peak 9 and 10 which are shown which are eluting at 56.510 and 57.580 minutes. Both peaks are visible, sharp and significant in both wa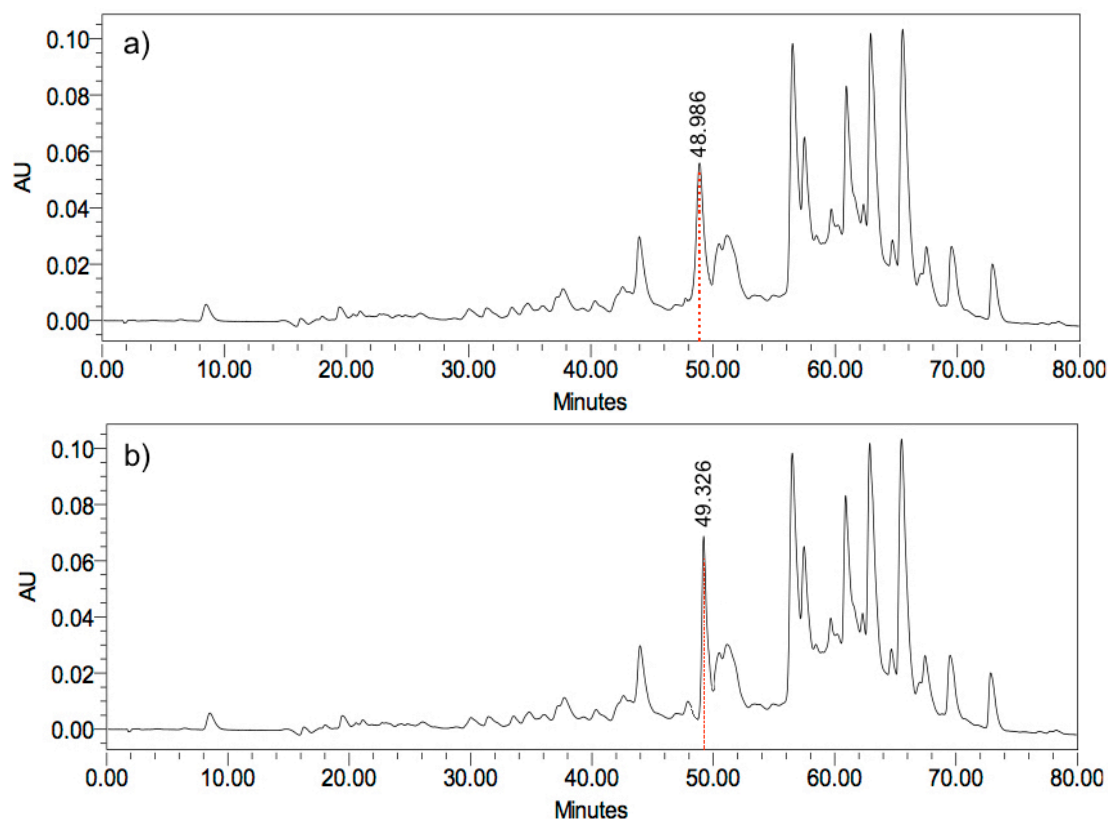velengths i.e. 254 nm and 320 nm. After comparing with the chromatograms of ethyl acetate extract in Figure 3.16 (a) and (b) with the chromatograms of phenolic standards (Figure 3.5), it is seen that there is no phenolic standard present with a close retention time to peak 9 and 10 to apply co-injections. But these peaks may refer to compounds having structure similar to flavonoids as they are eluting later in the chromatogram and therefore show a solubility in hydrophobic media.

Peak 11 elutes at 59.674 minutes, and it is visible at both wavelengths of 245 and 320 nm Figure 3.16 (a) and (b), but it is more sharp and significant at 320nm. According to its UV-Vis spectrum, peak 11 refer to compounds phenolic in nature,

having the characteristic bands, I and II . After comparing with the chromatograms of mixture of phenolic standards (Figure 3.5), it is assumed that quercetin may be present in our ethyl extract due to very close retention time of quercetin compound to this peak. In Figure 3.27 (enlarged view in Figure B-1) co-injection of ethyl acetate with quercetin is shown in both wavelengths of 254 and 320 nm. As it is seen from the marked part, peak 11 is increased in length while other peaks remain the same, therefore it is assumed that quercetin may be present in this fraction. Comparing the Figure 3.10 (UV-Vis spectrum of quercetin) and Figure 3.19 (UV-Vis spectrum of peak 11), the bands I and II of both the figures achieved their maximum absorbance at same wavelengths. Therefore, it was confirmed that quercetin was present in our ethyl acetate extract.
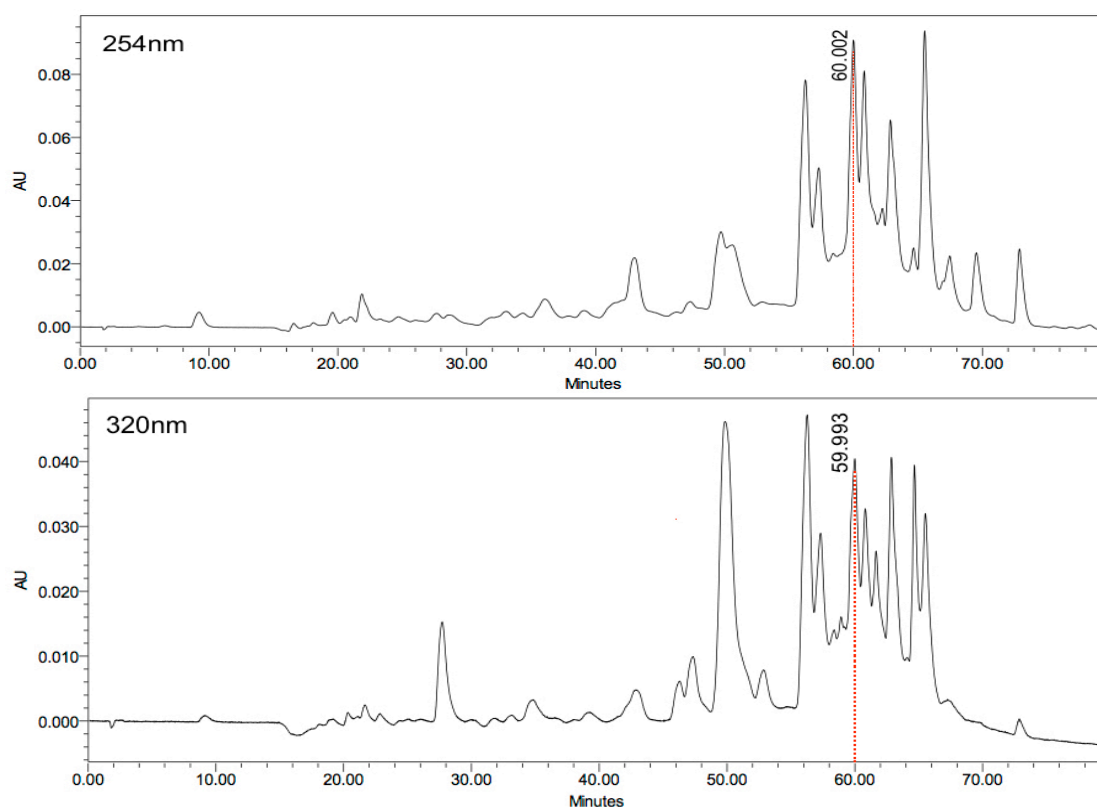

Figure 3.27. Chromatogram of co-injection of quercetin with ethyl acetate fraction

Moving on, it is seen in the Figure 3.16 (a) a peak is eluting at 60.909 minutes. But the peak is not sharp and it has curves, which means this is a complex peak, i.e. two or more compounds are eluting at the same time. When this peak was analyzed at

different wavelengths, it was separated into two peaks at 320 nm, which are labeled as Peak 12 and 13, eluting at 60,900 and 61.685 minutes (Figure 3.16 (b)). Both of these peaks refer to compounds phenolic in nature, having the characteristic bands, I and II according to their UV-Visble spectra (Figure 3.19 and 3.20). After comparing the chromatogram of ethyl acetate extract, Figure 3.16 (b), with the chromatograms of mixture of phenolic standards (Figure 3.5) it was assumed that luteolin or naringenin may be present in the ethyl acetate extract due to very close retention time to peak 12 and 13. Therefore, respective co-injections were prepared and applied on RP-HPLC, and the resultant chromatograms were analyzed.

In Figure 3.28 (enlarged view in Figure B-2) co-injection of ethyl acetate extract and luteolin is shown. As it is seen from the dotted part, peak 12 is increased in length while other peaks remain the same. Therefore, it was assumed peak 12 corresponds to the phenol compound luteolin and that luteolin may be present in ethyl acetate extract. But comparing the Figure 3.11 (UV-Visible spectrum of luteolin) and Figure 3.28 (UV-Visible spectrum of peak 12), the bands I and II of both the figures achieved their maximum absorbance at different wavelengths. Therefore, it was confirmed that luteolin was absent in our ethyl acetate extract. But it can be concluded that peak 12 may be structurally similar with luteolin and also it refers to flavonoids according to Figure 3.6
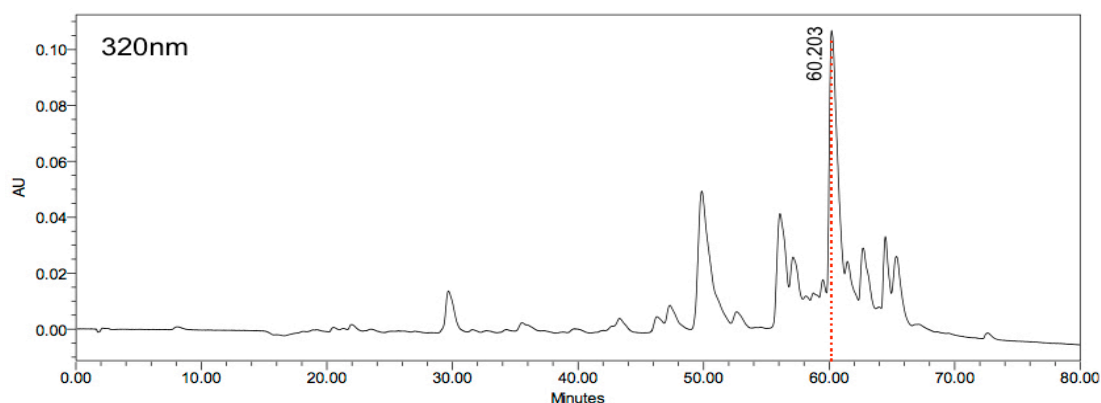

Figure 3.28. Chromatogram of co-injection of luteolin with ethyl acetate extract

Chromatogram of Naringenin co-injection with ethyl acetate extract is shown in Figure 3.29 (enlarged view in Figure B-3). As it is obvious from the marked part, peak 13 is increased in length while other peaks remain the same. Therefore it was assumed that Naringenin may be present in the ethyl acetate extract. But comparing the Figure 3.11 (UV-Visible spectrum of naringenin) and Figure 3.20 (UV-Visible spectrum of peak 13), huge difference was observed in bands I and II wavelength, which means; there is a no possibility for presence of naringenin in ethyl acetate fraction. But it can be concluded that peak 13 maybe structurally similar with naringenin, and also it refers to flavonoid group of phenol compounds according to Figure 3.6.
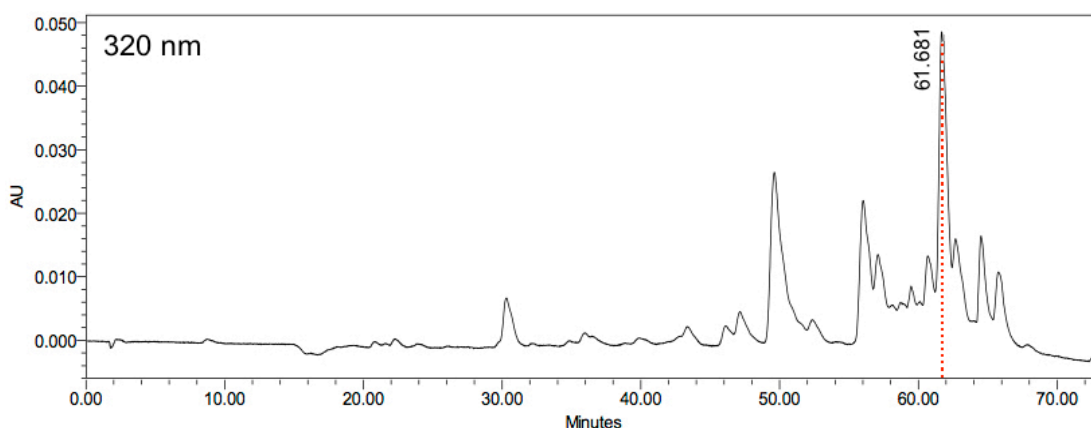

Figure 3.29. Chromatogram of co-injection of naringenin with ethyl acetate extract

As it is seen in Figure 3.16 (a) and (b), peak 14 is marked and it elutes at 62.88 minutes and also it is visible in both wavelengths of 254 and 320 nm. After comparing the chromatogram of ethyl acetate extract, Figure 3.16 (a) and (b) with chromatograms of mixture of phenolic standards (Figure. 3.5), it is assumed that hesperetin or apigenin may be present in the ethyl acetate extract due to very close retention time of theirs to peak 14, therefore respective co-injections were prepared and applied. Chromatogram of co-injection of hesperetin with ethyl acetate extract is shown in Figure 3.30 (a) and apigenin with ethyl acetate extract is shown in figure 3.30 (b). As it is obvious from the marked area, peak 14 is increased in length in both co-injections while other peaks remain the same. Therefore it was assumed that

hesperetin or apigenin, may be present in the ethyl acetate extract. But comparing Figure 3.11 (UV-Visible spectra of hesperetin and apigenin) with Figure 3.20 (UV-Visble spectrum of peak 14), it is seen that the UV-Visble spectra of both phenolic standards (Figure 3.11) are different from the UV-Visible spectrum of peak 14. So it is concluded that both of the compound are not present in the ethyl extract. Looking at the structures of both apigenin and hesperetin Figure 3.5, and keeping in view that they increased peak 14, a conclusion can be made that the compound eluting as peak 14 has the same basic structure of apigenin and hesperetin and may differ from them with regards to only a single substituent.
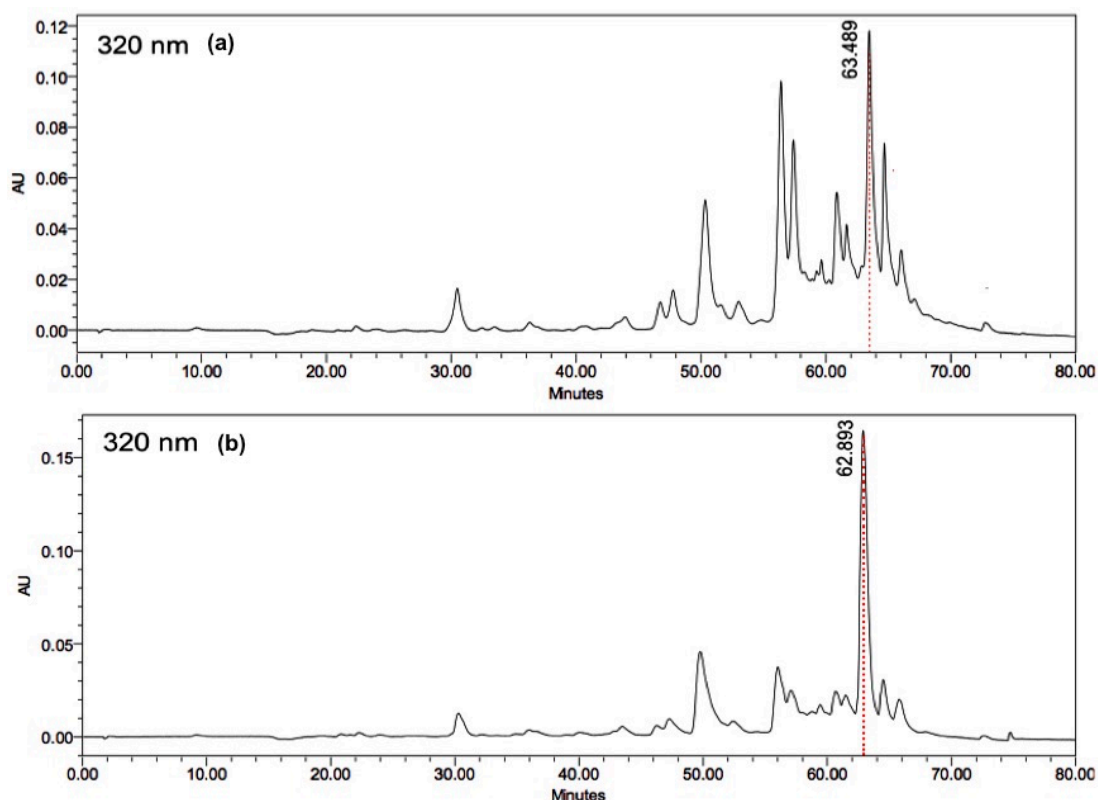

Figure 3.30. Chromatogram of co-injection of a) hesperetin b) apigenin with ethyl acetate extract

In Figure 3.16 (a) and (b) peak 15 and 16 is shown, eluting at 64.688 and 65.509 minutes respectively. Considering their UV-Visible spectra (Figure 3.20), they are meaningful peaks i.e. their absorption bands refer to any phenolic compound. After comparing with the chromatograms of mixture of phenolic standards, it is seen that

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there is no phenolic standard with close retention time to peak 15 and 16 to apply for co-injection.

Peak 17 elutes at 67.448 minutes which is only visible at 254 nm wavelength (Figure 3.16 (a)). According to its UV-Vis spectra absorption bands (figure 3.11), peak 17 is not a meaningful peak as the there is no absorption band characteristic of phenol compounds. Peak 18 is numbered in Figure 3.16 (a), eluting at 69.510 minutes. This peak is a meaningful peak as a phenolic compound according to its UV-Visible spectrum (Figure 3.21). After comparing the chromatogram of ethyl acetate extract with chromatograms of mixture of phenolic standards (Figure 3.5), it is assumed that coumarin may be present in the ethyl acetate extract due to very close retention time to peak 18. Co-injection of coumarin with ethyl acetate fraction is shown in Figure 3.31. As it is seen peak 18 is increased in length while other peaks remain the same. Comparing UV-Visible spectrum of coumarin (Figure 3.11) with UV-Visble spectrum of peak 18 (Figure 3.21), huge difference is seen between the bands of both the compounds. Therefore there is no possibility of presence of coumarin in the ethyl acetate extract. This peak has a compound which may be containing the nucleus of coumarin with some hydrophobic substituent.
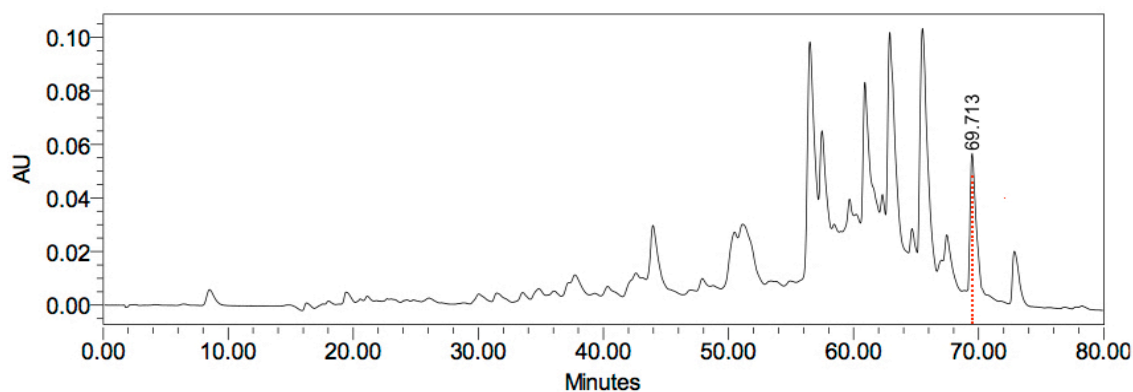

Figure 3.31. Chromatogram of co-injection of coumarin with ethyl acetate fraction

As a last peak, peak 19th is numbered in Figure 3.16 (a) and it elutes at 69.713 minutes. According to its UV-Visible spectrum (Figure 3.21) of peak 19, it seems that the band I and II were visible however its UV-Vis spectra very different than

that of a phenolic spectral evidence and by looking at its retention time, this compound may not be one of the phenolic compounds. Moreover, perhaps two or more compounds which may be are structurally similar eluting at the same time.

The ethyl acetate extract of *Rheum ribes* obtained by fractionation process was found to contain the highest number of phenol compounds as is evident with the number of peaks in the chromatogram. three of the compounds were confirmed to be gallic acid, p-coumaric acid and quercetin. Most of the phenol compounds concentrated in ethyl acetate extract lie in the flavonoid group according to the Figure 3.6.

3.7.3. Aqueous extract RP-HPLC analysis

Next aqueous extract was analyzed, and similarly the first injection was started with higher concentration as antioxidant test showed lesser concentration of phenolic contents in this extract. Solutions of 10 mg/mL prepared in methanol 15 µL of it was filtered and run on RP-HPLC. The resulting chromatogram was analyzed at wavelengths of 254, 280, 300, 320, 340 and 360 nm (Figure A-11 and A-12). As it is seen in the Figure 3.26 the unit of absorbance is very less, 0.015 U at 245 nm and also the noise is high and it is difficult to differentiate between noise and significant peaks. Therefore, solutions of 20 mg/mL of aqueous extract were prepared in methanol to have better results. Figure 3.32 and 3.33 show the resulting chromatogram at wavelengths of 254, 280, 300, 320, 340 and 360 nm for aqueous extract 20 mg/mL. In the Figure 3.28 absorbance is 0.03 AU at 254 nm for 20 mg/mL of aqueous extract. (each injection were done 3 times)
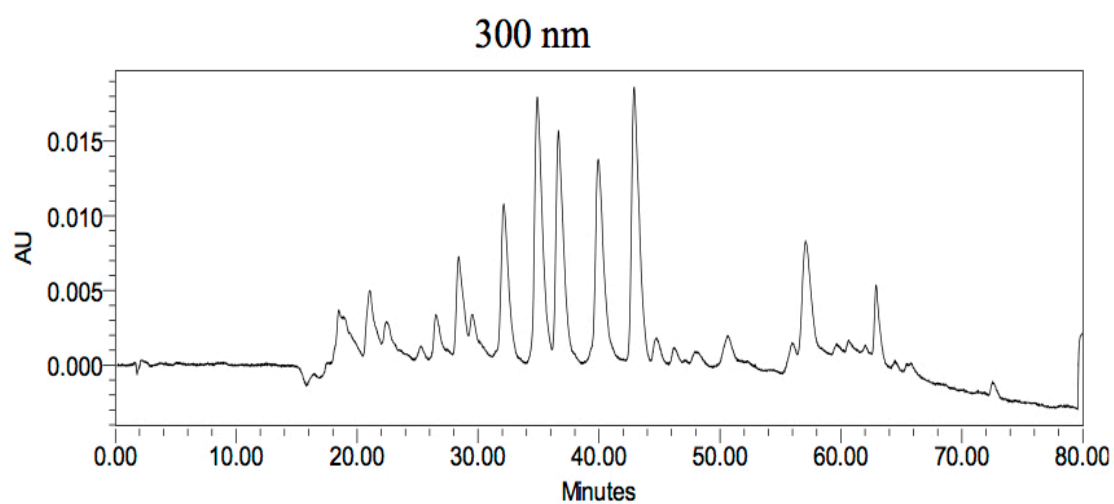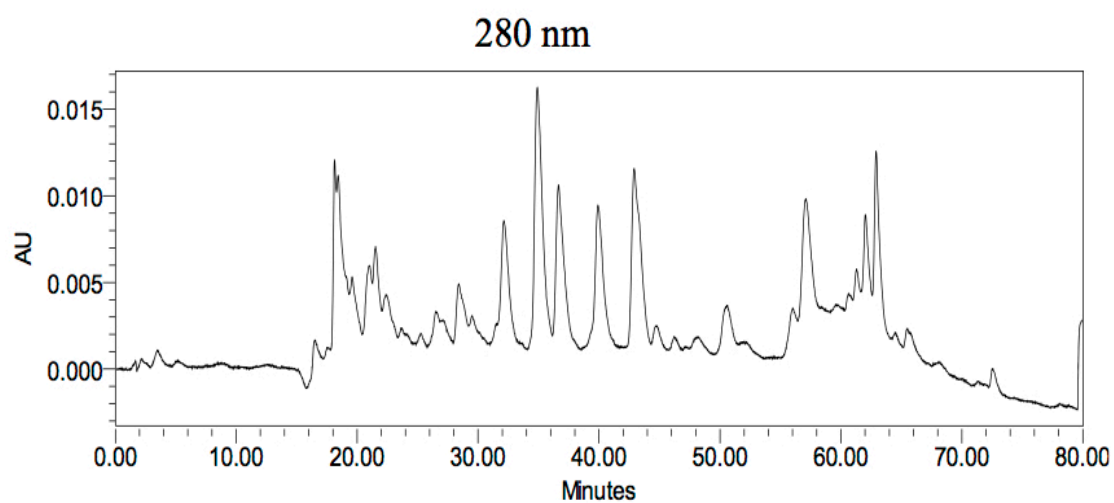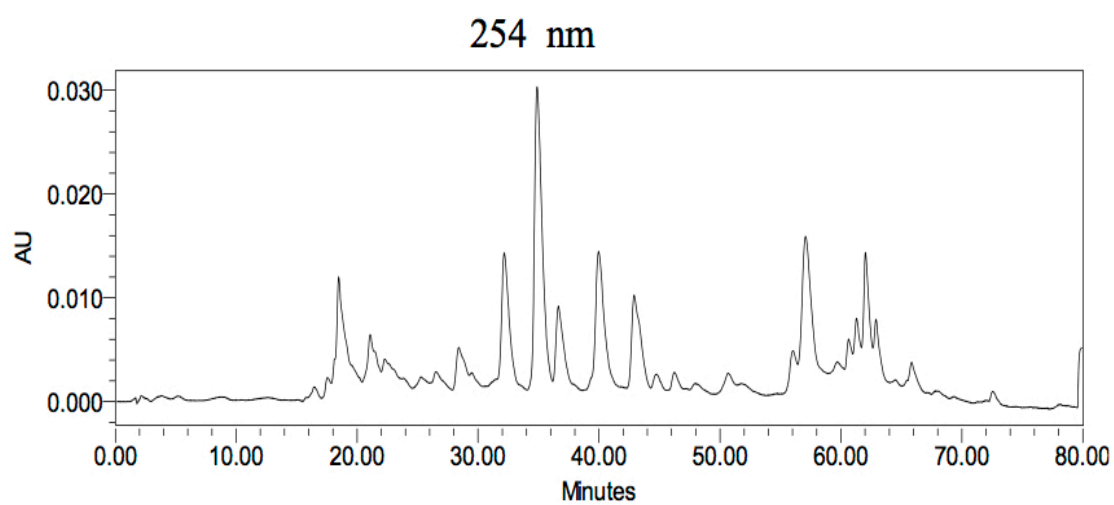
Figure 3.32. Aqueous extract 20 mg/mL chromatograms, with RP-HPLC, at wavelengths of 254, 280, 300 nm.
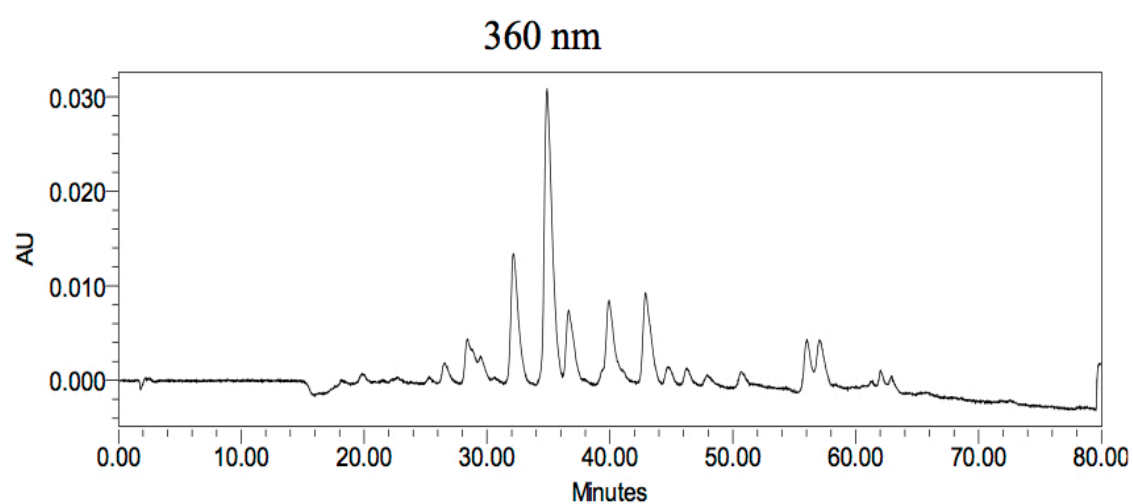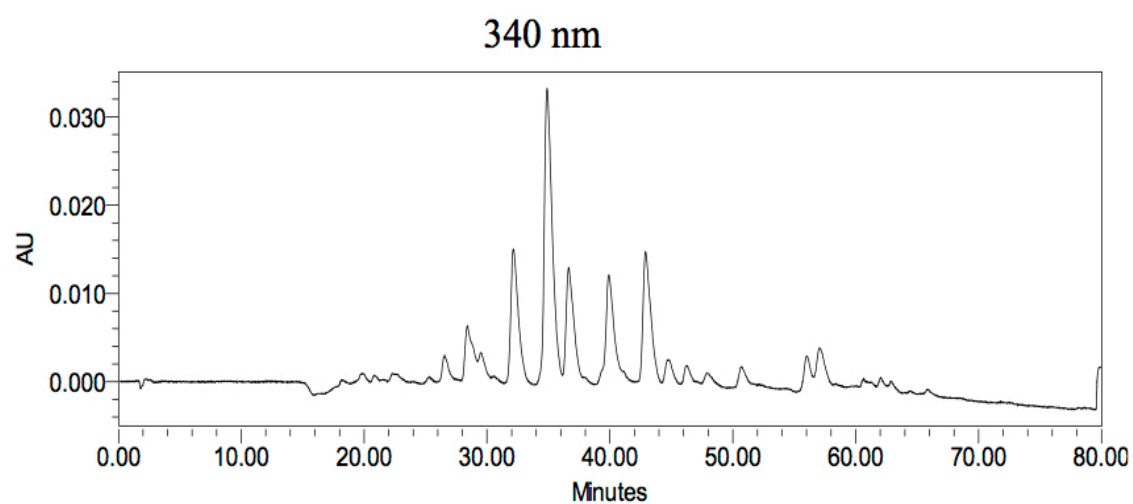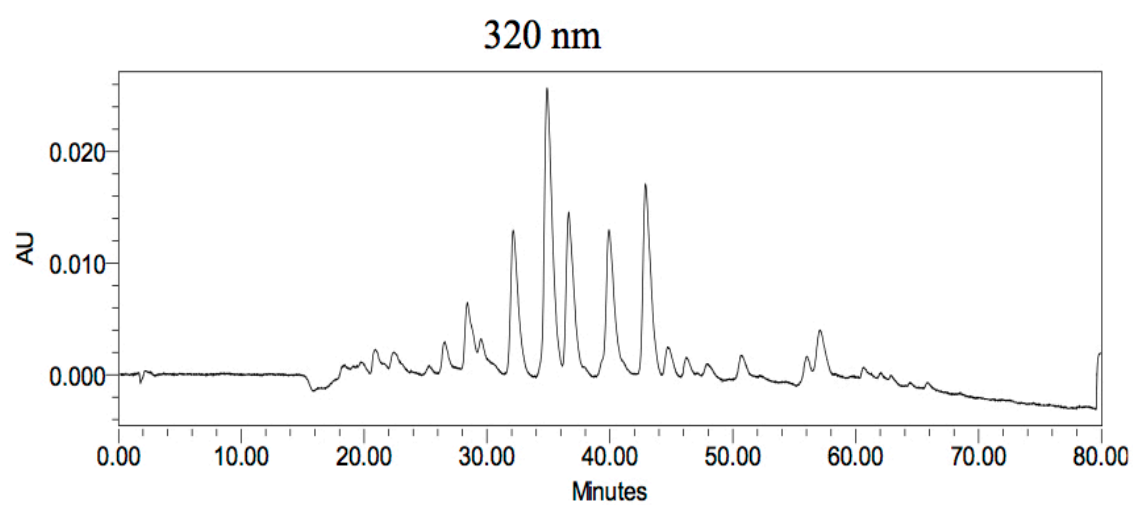
73

Figure 3.33. Aqueous extract 20 mg/mL chromatograms, with RP-HPLC, at wavelengths of 320, 340, 360 nm.

3.7.3.1 Aqueous extract peak identification

Of all the wavelengths observed for aqueous extract (from Figure 3.31 and 3.32), chromatogram of aqueous 20 mg/mL at 300 nm was the best chromatogram for investigating and defining the peaks.
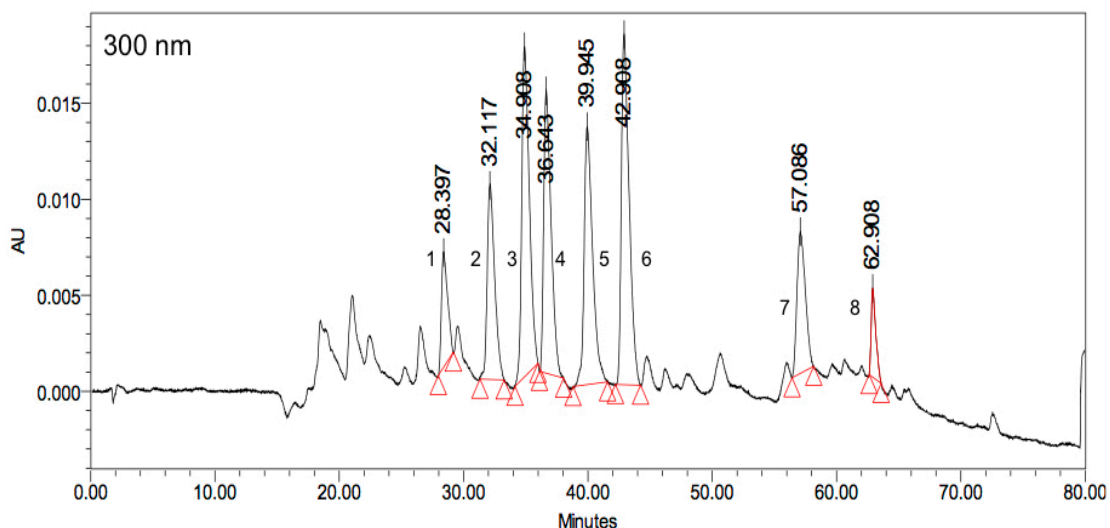
Figure 3.34. Chromatogram of aqueous extract 20 mg/mL at 300 nm with marked significant peaks

Because peaks were better resolved at this chromatogram as well as they were sharp, clear and significant peaks. Figure 3.34 is highlighting all the significant peaks.

As it is shown in the figure 3.33, 8 peaks (numbered) eluted at 28.397, 32.117, 34.908, 36.945, 42.908, 57.068 and 62.908 minutes respectively.

Figure 3.35. UV-VIS spectrums of aquoues extract peaks 1, 2, 3, 4, 5 and 6

Figure 3.36. UV-VIS spectrums of aqueous extract peaks 7 and 8
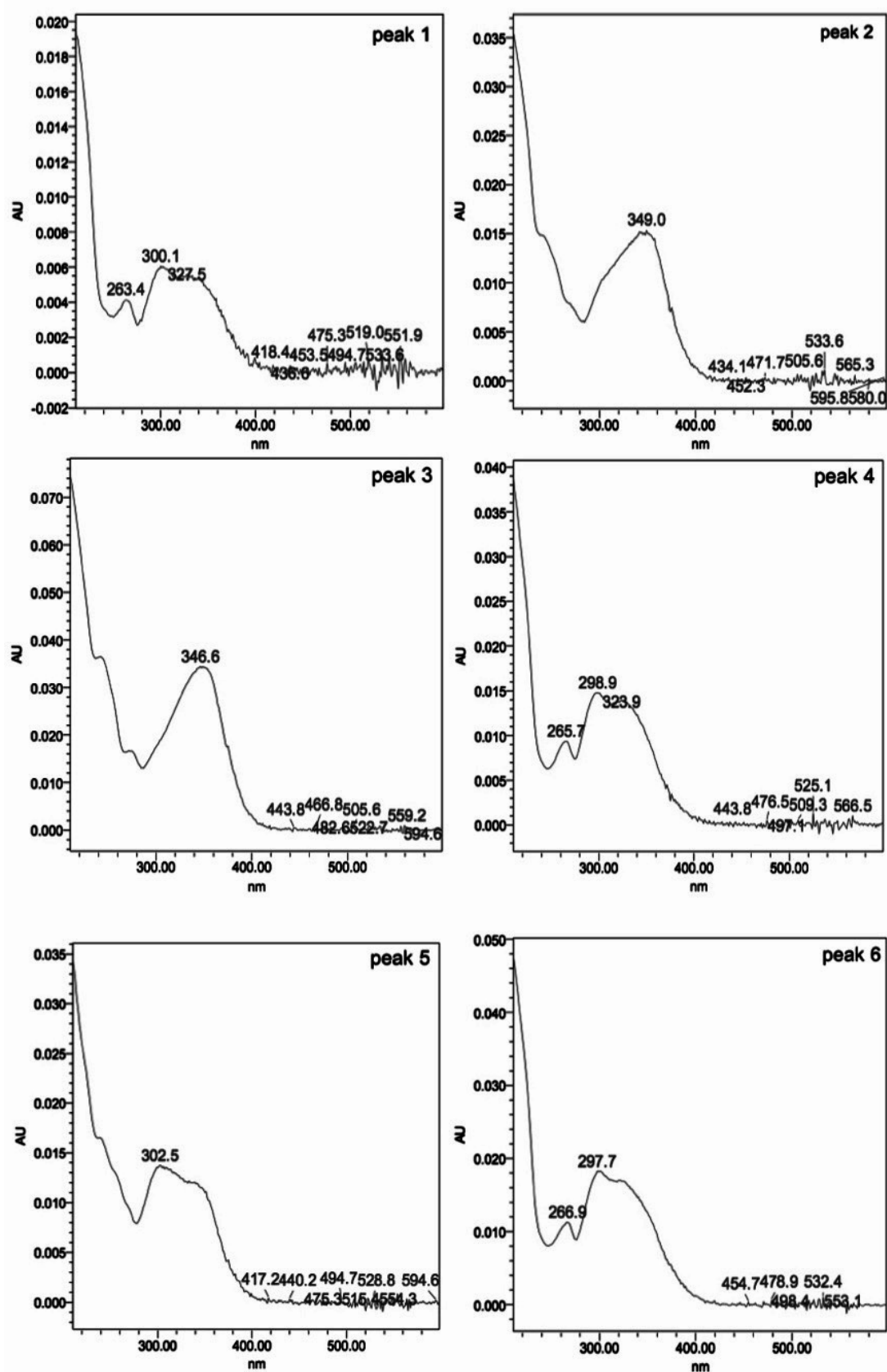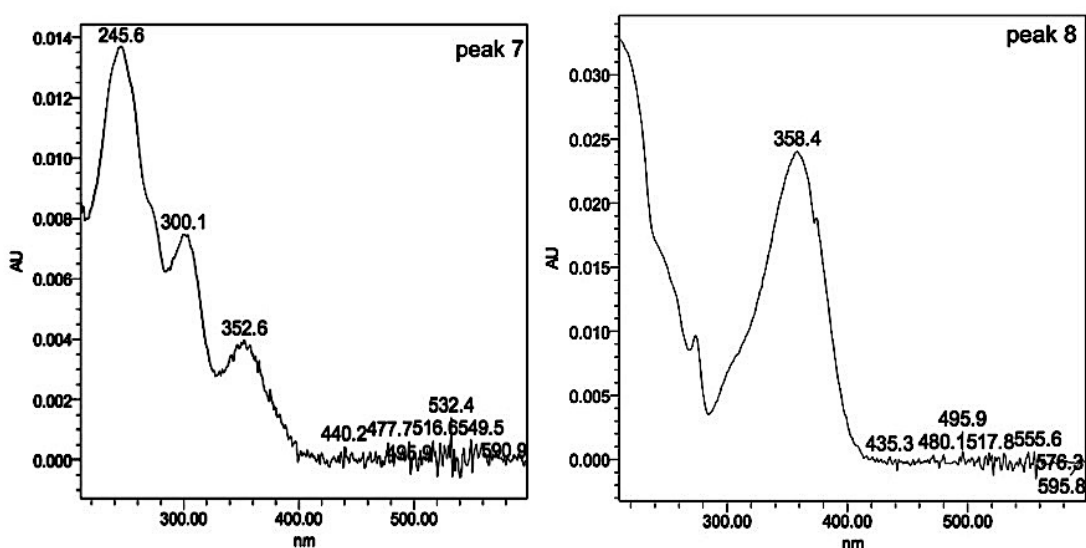
According to Figure 3.35 and 3.36 (UV-Visble spectra of all the 8 peaks separately), all peaks (except peak 7) were significant in terms of S/N ratio and had a UV-Vis spectral characteristics of the phenolic compounds i.e. containing the two bands of I and II as explained under section 3.6.2.

What is important here is that according to UV-Visble spectrum of peak 8 in aqueous extract (Figure 3.35) and UV-Visble spectrum of peak 14 in ethyl acetate extract (Figure 3.20), it is assumed that these two peaks belong to a same compound, because band I and II reaches their maximal absorbance at same wavelength in UV-Visible spectra of both peaks.

3.7.3.2. Aqueous extract co-injections

After comparing the chromatogram of aqueous extract Figure 3.33 with the chromatogram of phenolic standards Figure 3.5, it is assumed that scopoletin and p-coumaric acid (due to close retention time to peak 1 and peak 2), ellagic acid and rutin (due to close retention time to peak 5 and 6), hesperetin and apigenin (due to close retention time to peak 8), may be present in the aqueous extract. But co-injections with hesperetin and apigenin was already performed with ethyl acetate extract, therefore were not repeated again. So scopoletin, p-coumaric acid, ellagic acid and rutin were co-injected with aqueous extract. Solution of co-injections were

prepared and applied on RP-HPLC the resultant chromatograms were shown in Figures 3.37, 3.38, 3.39 and 3.40.

The two first co-injections to be done were p-coumaric acid with aqueous extract (Figure 3.36), and scopoletin with aqueous extract (Figure 3.37). As it is seen from the figures both p-coumaric acid and scopoletin eluted as separate peaks, and also peak 1 and peak 2 did not increase in length and remained same. Therefore there is no possibility that p-coumaric and scopoletin are present in aqueous extract.
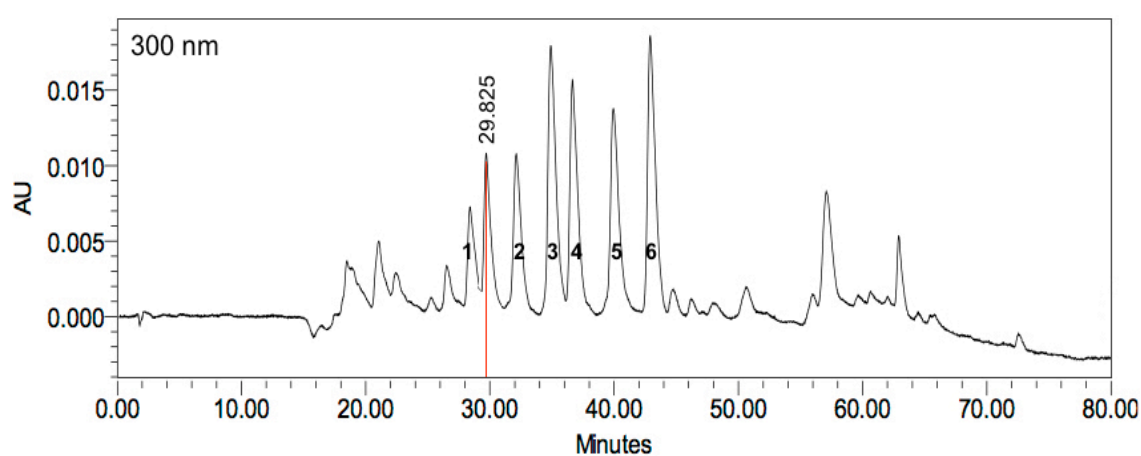
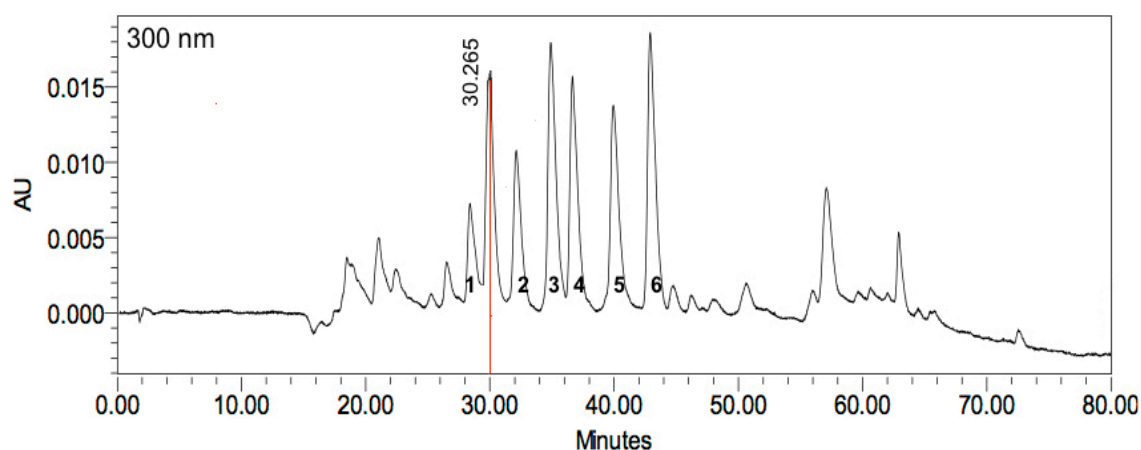Figure 3.37. Chromatogram of co-injection of p-coumaric acid with aqueous extract

Figure 3.38. Chromatogram of co-injection of scopoletin with aqueous extract

The next two co-injections to be done were ellagic acid with aqueous extract (Figure 3.39), and rutin with aqueous extract (Figure 3.40).

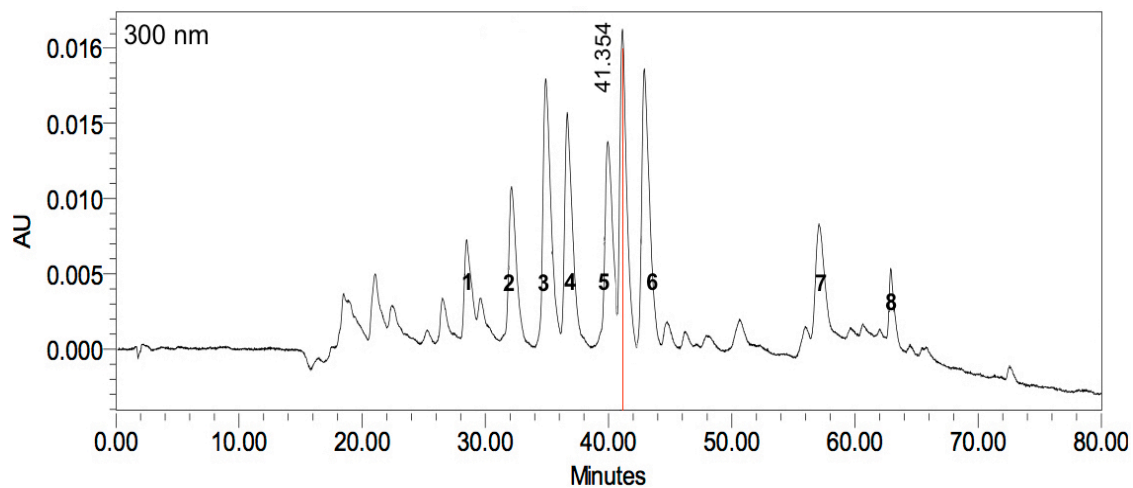Figure 3.39. Chromatogram of injection of ellagic acid with aqueous extract

As it is seen in the figures both ellagic acid and rutin eluted as separate peaks, and also peak 5 and peak 6 did not increased in length and remained same. Therefore it is concluded that ellagic acid and rutin are not present in aqueous extract.
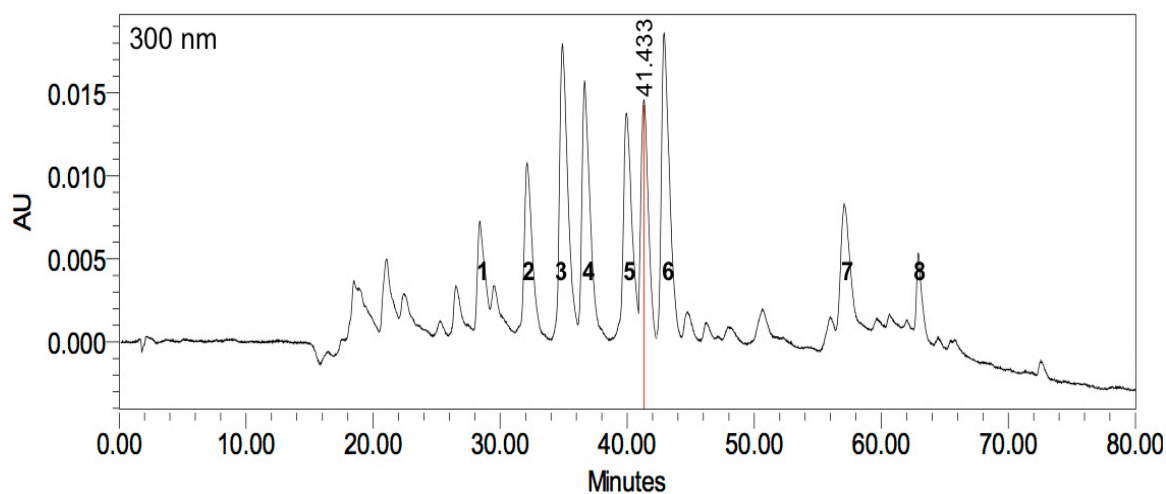

Figure 3.40. Chromatogram of co-injection of rutin with aqueous extract

Keeping in mind the elution time of the peaks of aqueous extract Figure 3.33 and the Figure 3.6 and also taking into consideration the hydrophilic nature of the aqueous extract, it can be easily concluded that all the compounds eluting as clear separate peaks in Figure 3.33 belong to the phenolic acid group of the phenol compounds.

3.7.4. Chloroform extract RP-HPLC analysis

Then chloroform extract was analyzed. Solution of 10 mg/ml was prepared in methanol, higher concentration was used in the beginning rather than 1mg/mL because, chloroform extract showed less quantity of phenolic compounds in the antioxidant tests. A 15 µL of it was filtered and run on RP-HPLC. The resulting chromatogram was analyzed at wavelengths of 254, 280, 300, 320, 340 and 360 nm (Figure A-7 and A-8).

As it is seen in the Figures in appendix , not only the unit absorbance is so less at 245 nm (0.08 AU) but also the noise is high and it is difficult to differentiate between noise and significant peaks. Therefore, solutions of 20 mg/mL (Figure 3.41 and 3.42) and 30 mg/mL (Figure A-9 and A-10) were prepared in methanol to decrease the noise effects. The resulting chromatogram at wavelengths of 254, 280, 300, 320, 340 and 360 nm were observed. From the Figure 3.41 absorbance unit is 0.15 AU at 254 nm for 20 mg/mL of chloroform extract and 0.3 AU at 254 nm for the 30 mg/mL in Figure A-9.
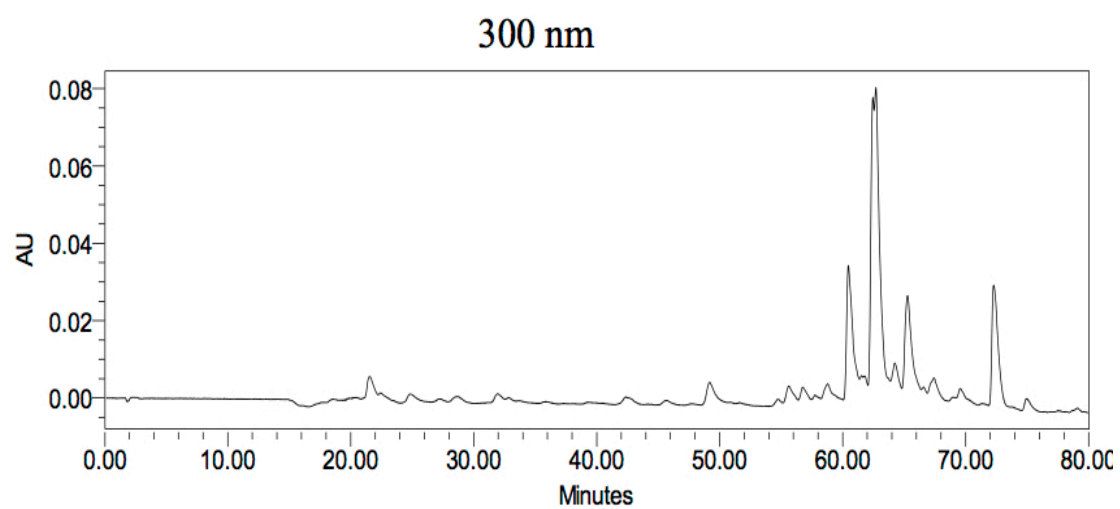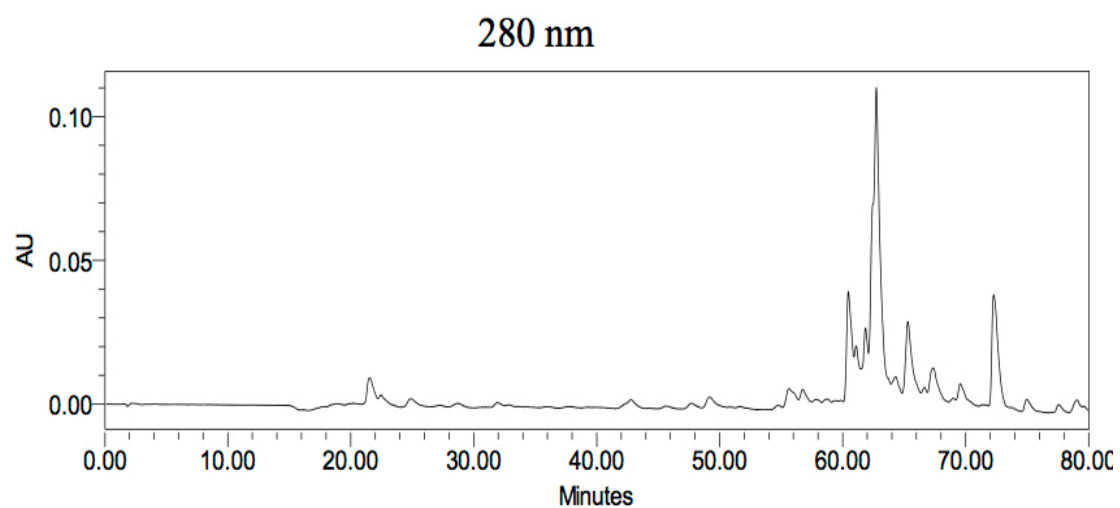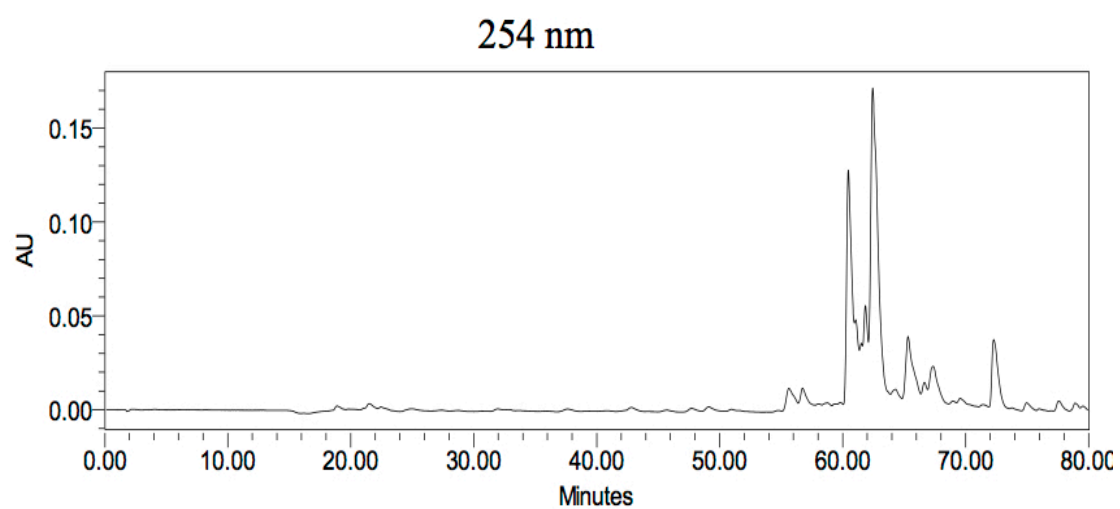
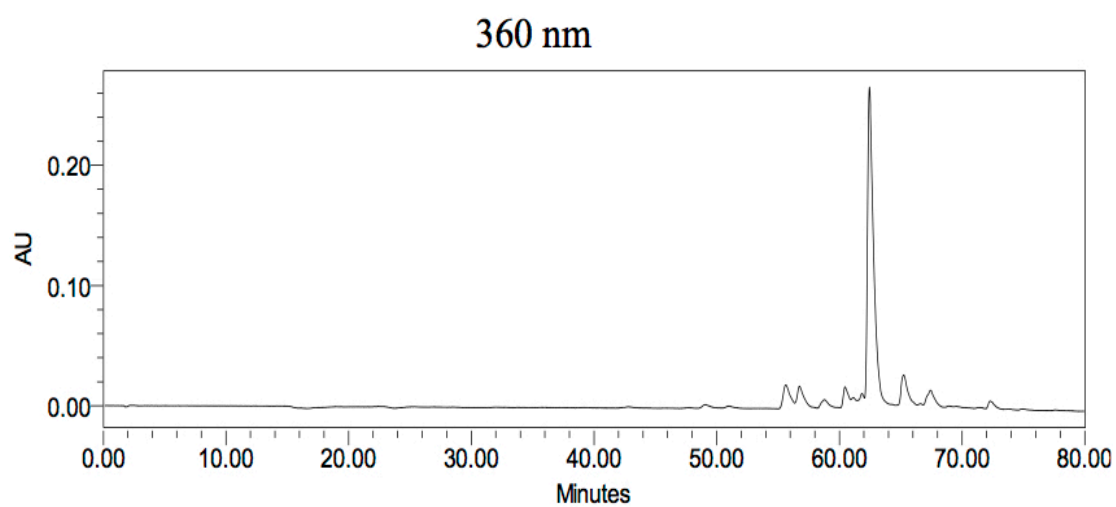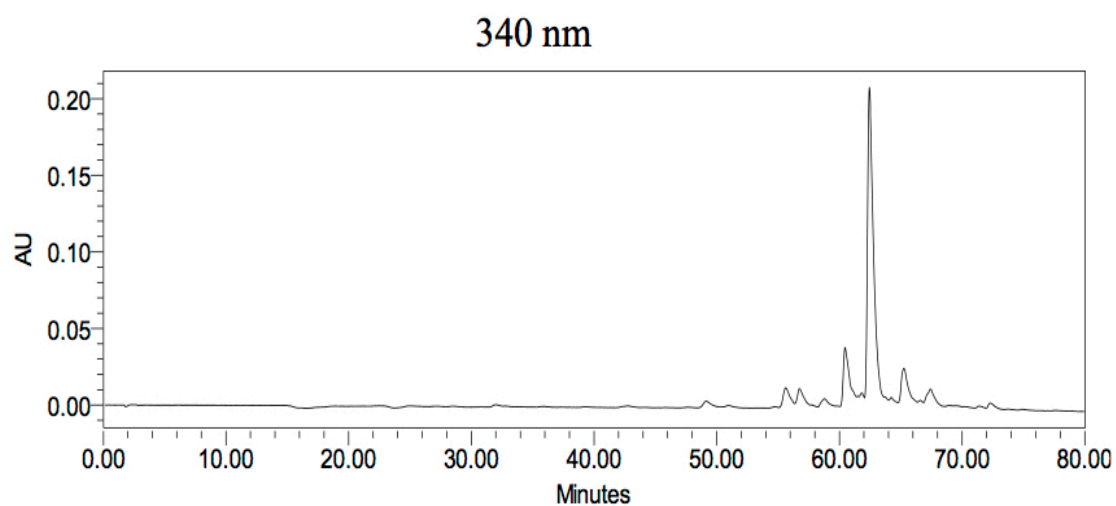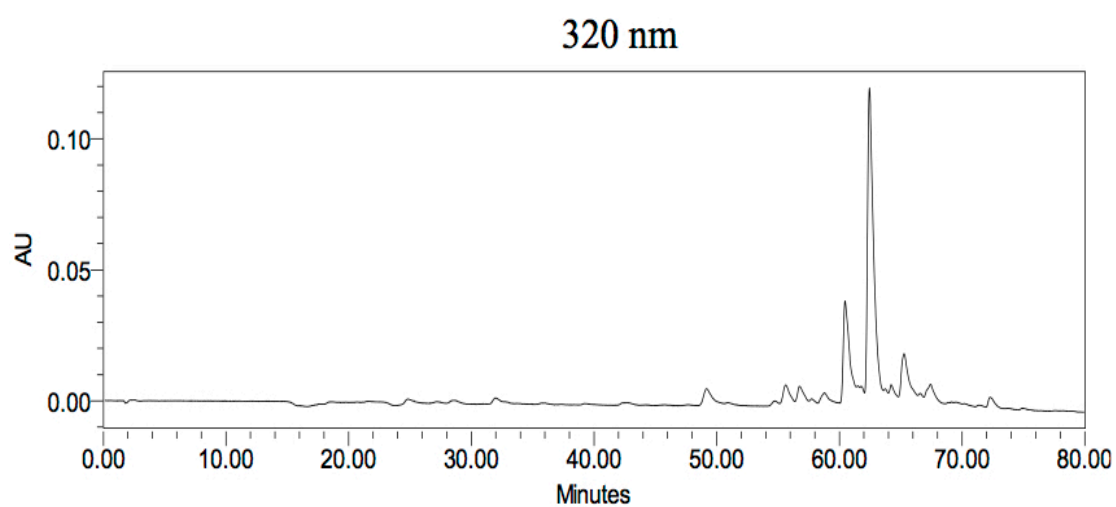Figure 3.41. Chloroform extract 20 mg/mL chromatograms, with RP-HPLC, at wavelength of 254, 280, 300 nm

Figure 3.42. Chloroform extract 20 mg/mL chromatograms, with RP-HPLC at
wavelength of 320, 340, 360 nm.

3.7.4.1. Chloroform extract peak identification

Next chlorofrom extract fraction was analyzed. Of all chromatograms observed for chloroform extract (Figure 3.41 and 3.42), chromatogram of chloroform extract 20 mg/mL at 254 nm wavelength was the best one to select for investigating and defining the peaks. Because peaks were better resolved at this chromatogram. significant and visible peaks were numbered in Figure 3.43.
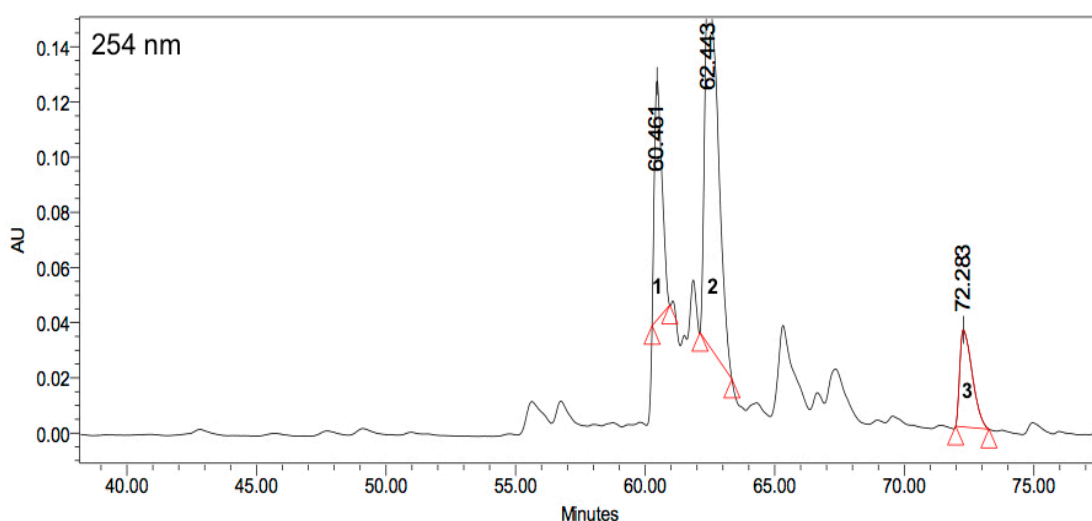
Figure 3.43. Chromatogram of chloroform extract 20 mg/mL at 300 nm

As it is shown from the chromatogram of chlorofrom (Figure 3.43), only 3 significant peaks are present which are numbered. These peaks are eluting at 60.461, 62.443 and 72.283 minutes. According to Figure 3.44, Peak 1 refer to compounds phenolic in nature, having a UV-Vis spectral characteristics of the phenolic compounds i.e. containing the two bands of I and II as explained under section 3.6.2. UV-Vis sepctrum of peak 2 (Figure 3.44) is same with peak UV-Vis spectrum of peak 14 in ethyl acetate (Figure 3.11), therefore these two peaks are reffreing to a same compound which has shown its solubility for both solvents. Similarly peak 3 in chloroform extract and peak 19 (Figure 3.20) in ethyl acetate exctract can be considered as same compound because of same wavelenght of bands I and II.

Figure 3.44. UV-Visble spectrums of chloroform extract peaks 1,2 and 3

3.7.4.2. Chloroform extract co-injections

After comparing the chromatogram of chloroform extract (Figure 3.43) with the chromatogram of phenolic standards (Figure 3.6), it is assumed that quercetin or luteolin (due to the close retention time to peak 1), hesperetin or apigenin (due to the close retention time to peak 2), may be present in the chloroform extract. Co-injections with hesperetin and apigenin were done already with ethyl acetate due to close retention time to peak 14. Only quercetin and luteolin were co-injected with chloroform extract. Solution of co-injections were prepared and applied on RP-HPLC, and the resultant chromatograms are shown in Figure 3.45 and 3.46.

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The first co-injection to be done was quercetin acid with chloroform extract (Figure 3.45), This compound was assumed to be present because of the close retention time to peak 1. But as it is seen from the figure 3.45 quercetin eluted as a separate peak at 59.957 minutes, and peak 1 remains same, therefore there is no possibility that quercetin present in chloroform extract.
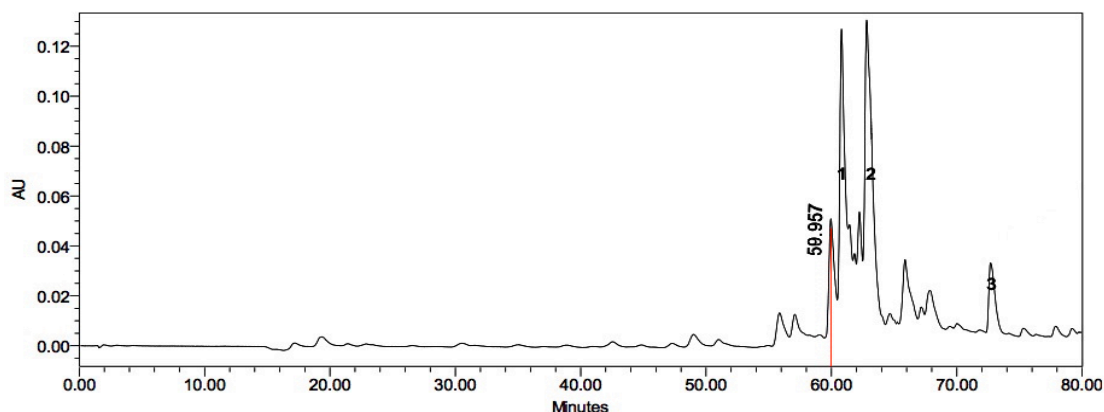
Figure 3.45. Chromatogram of co-injection of quercetin with chloroform extract

The next co-injection was luteolin with chloroform extract (Figure 3.46). Peak 1 increased in length while other peaks remained the same as before. Therefore it is assumed that luteolin may be present in chloroform extract.
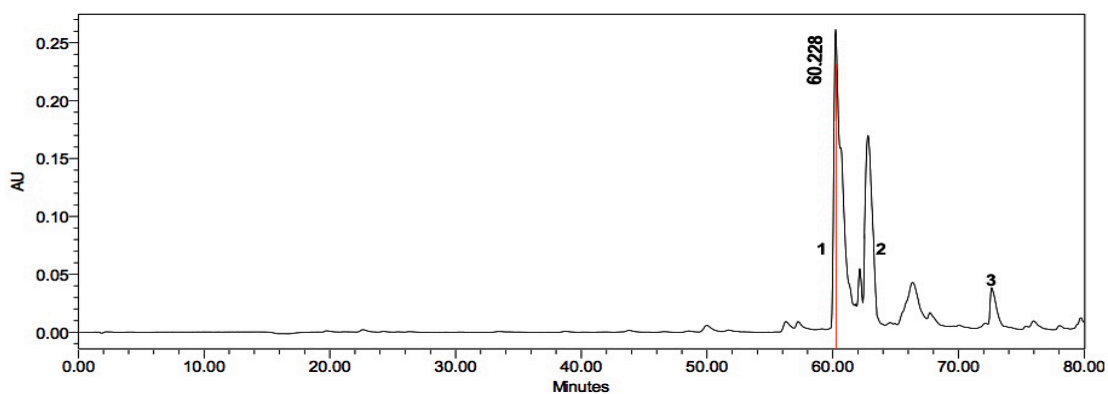
Figure 3.46. Chromatogram of co-injection luteolin with chloroform extract

Comparing the Figure 3.11 (UV-Visible spectrum of luteolin) and Figure 3.44 (UV-Visible spectrum of peak 1), there is a considerable difference between wavelengths of band I and II which means, there is no possibility for the presence of luteolin in the chloroform extract.

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CHAPTER 4

CONCLUSION

Many of the rheum species have been used in medicine for various diseases because of their medical properties and therefore they were highly inspected by the researches. Correspondingly, in this study the *Rheum ribes* L. of polygonaceae family was investigated for its phenolic constituents and their anti-oxidative properties.

Since the crude extract of the root sample of the *Rheum ribes* showed antioxidative properties, solvent-solvent fractionation process was employed in order to distinguish its antioxidant components. The fractions obtained were then tested individually for their antioxidant capacities by DPPH test. Ethyl acetate fraction exhibited the highest antioxidant capacity indicating many compounds, highly antioxidant, concentrated in ethyl acetate fraction. Besides, determination of total phenol and total flavonoid contents of each fractionated extract also has supported the presence of highly effective compounds concentrated in the ethyl acetate fraction.

Further analysis by RP-HPLC showed that all fractions contained different number of phenolic compounds, were evident by peaks in the chromatograms. These chromatograms of the fractions were compared with that of the standard phenolic compounds and co-injections were performed with those standards which were eluting correspondingly. Of all the co-injections of standard phenolic compounds available in this study, gallic acid, p-coumaric acid and quercetin were present according to the co-injection in RP-HPLC in ethyl acetate extract.

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A. RP-HPLC chromatograms of fractions of *Rheum ribes* L.
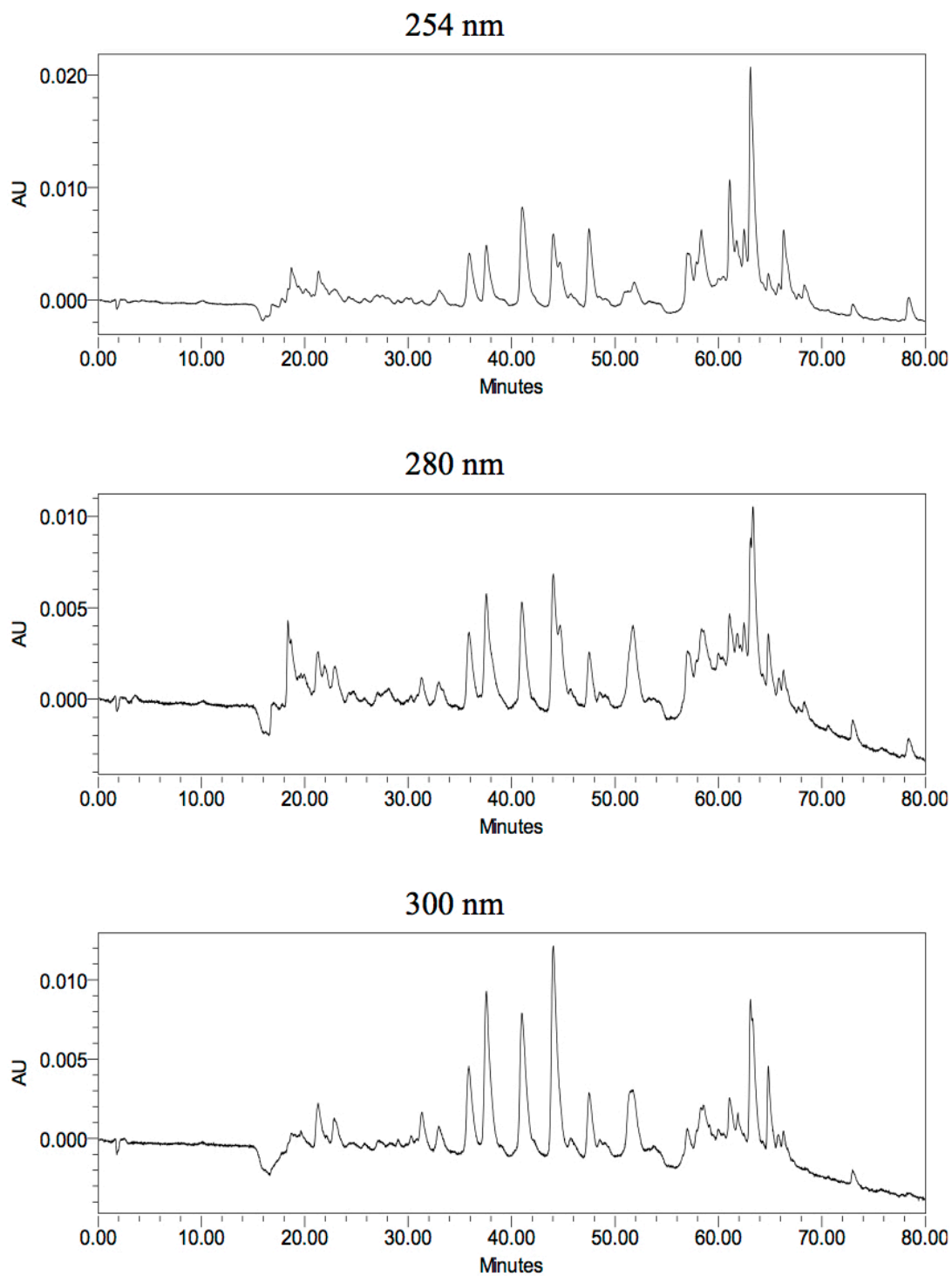
Figure A-1. Crude extract 10 mg/mL chromatograms, with RP-HPLC, at wavelengths of 254, 280, 300 nm
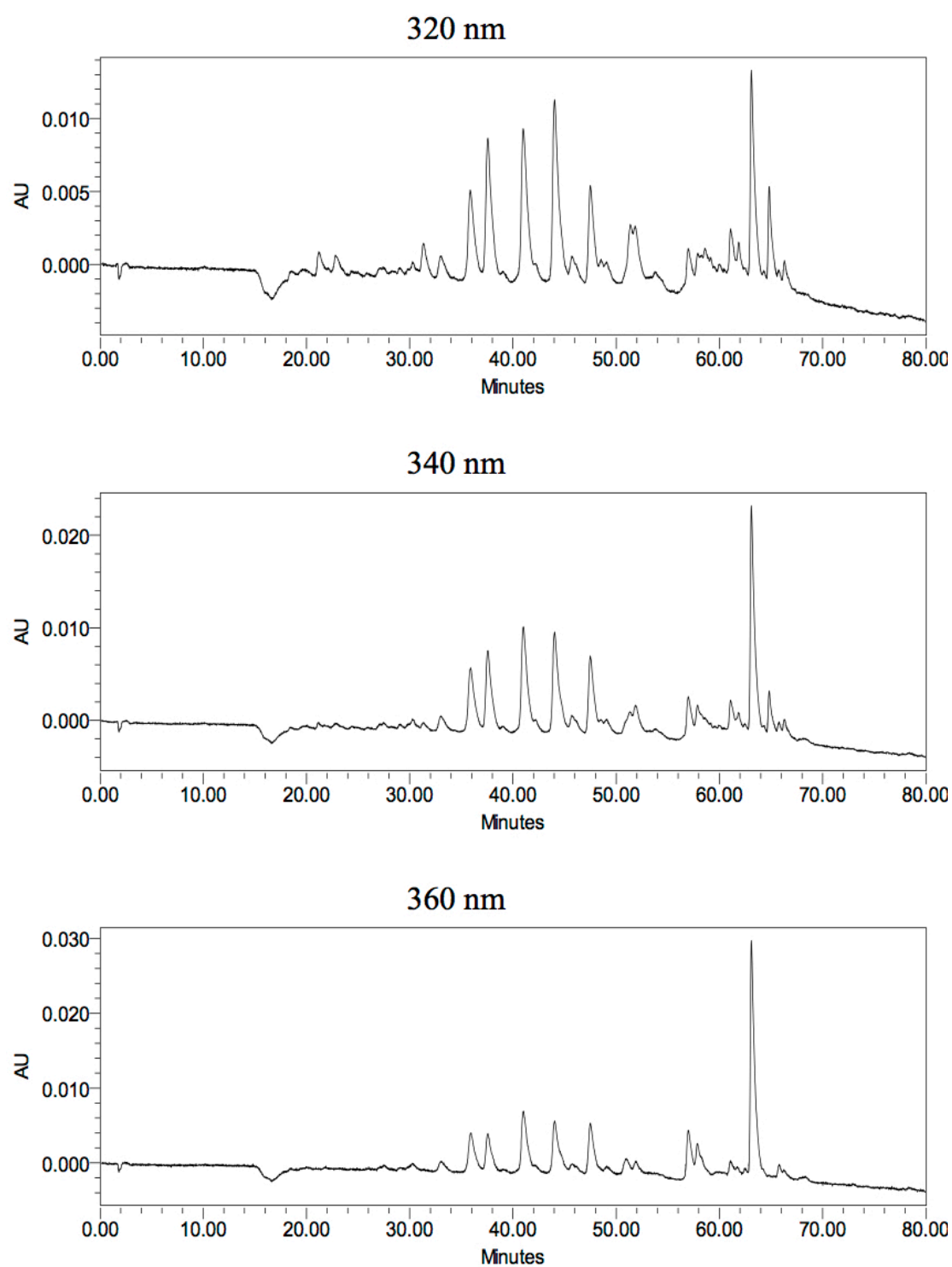
Figure A-2. Crude extract 50 mg/mL chromatograms, with RP-HPLC, at
wavelengths of 320, 340, 360 nm
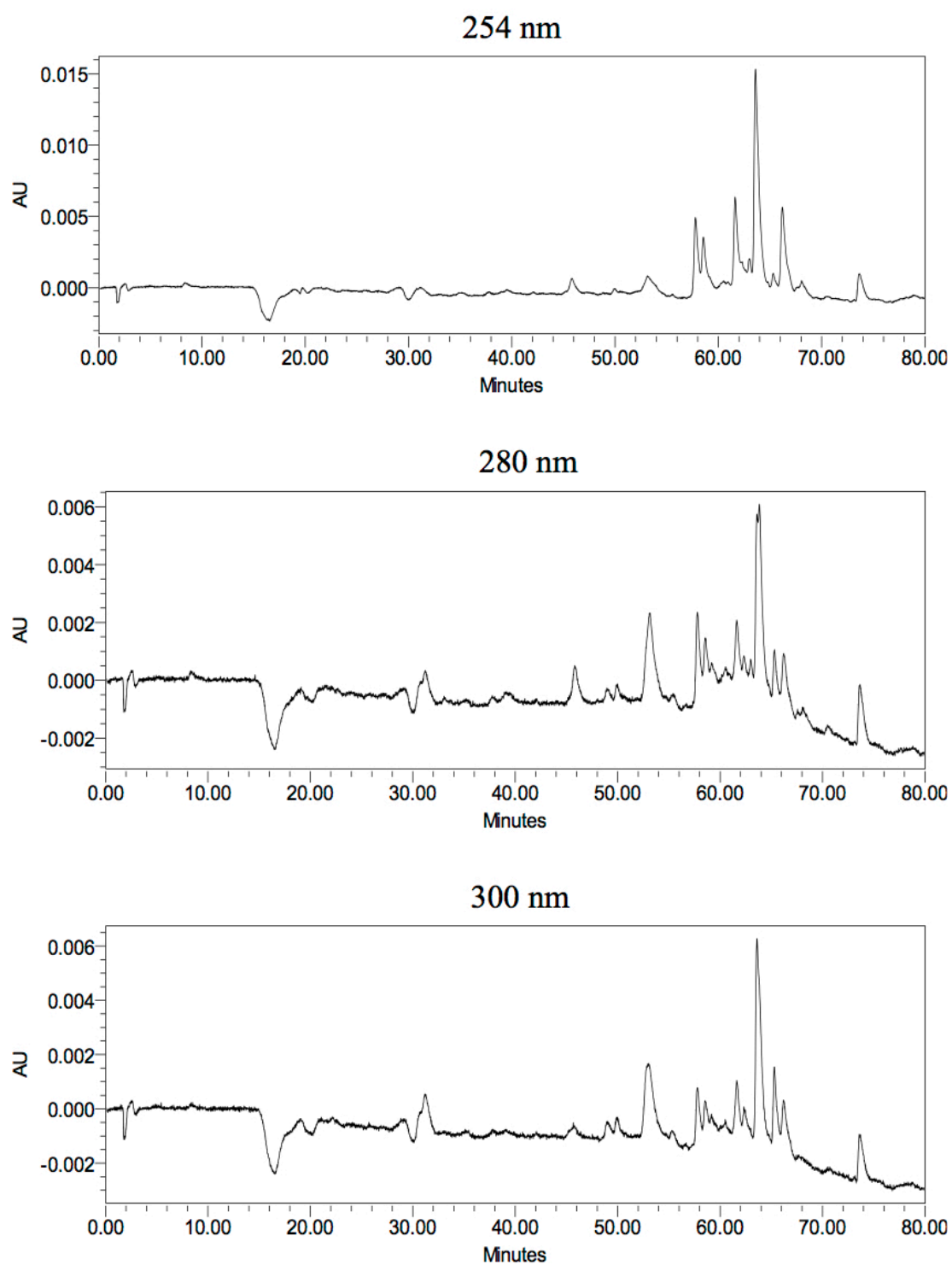
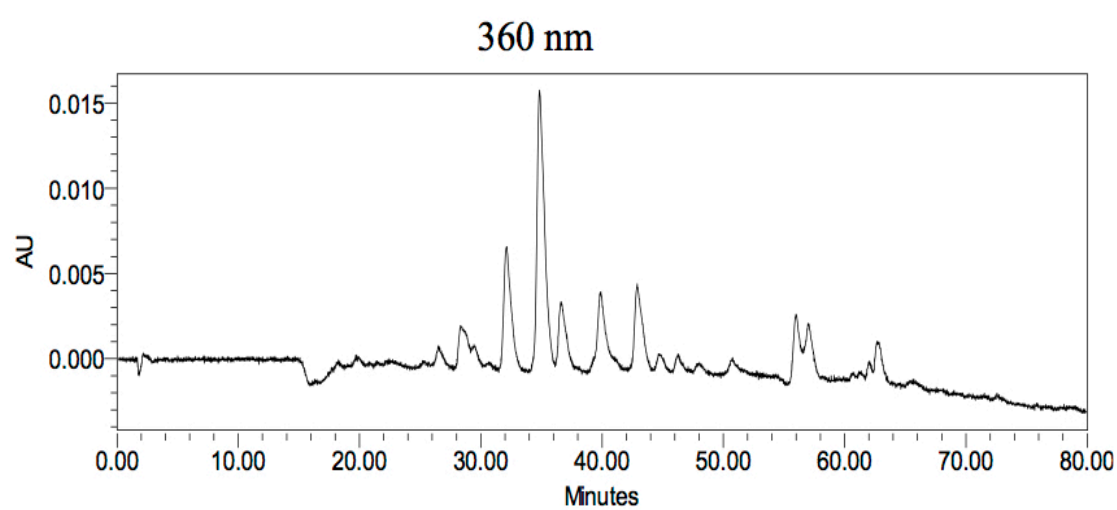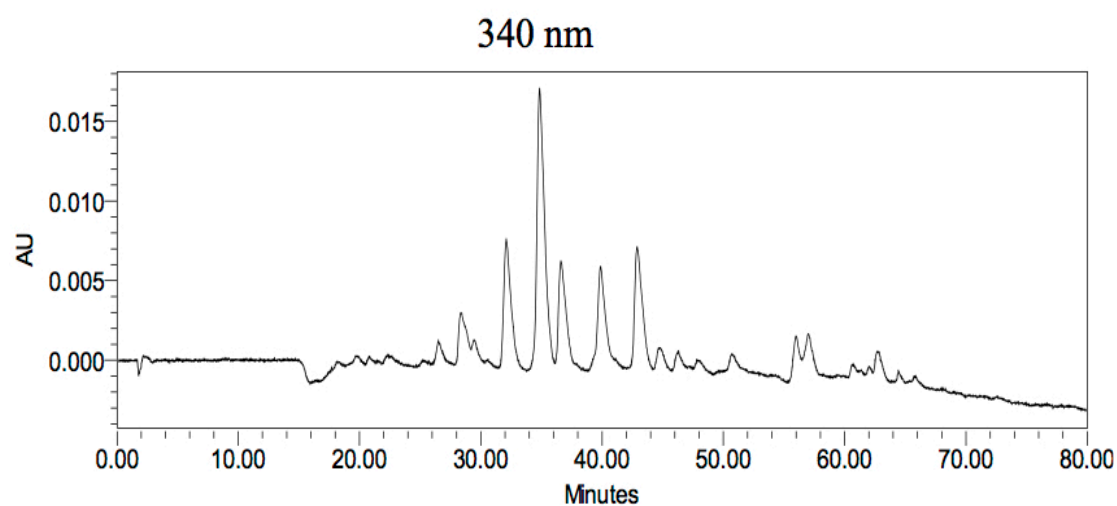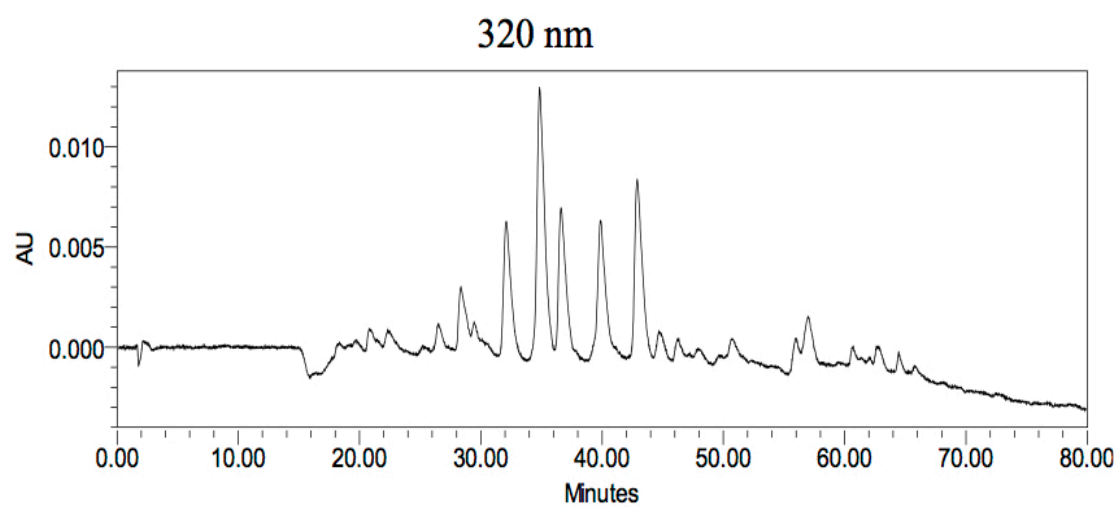Figure A-3. Ethyl acetate 1 mg/mL chromatograms, with RP-HPLC, at wavelengths of 254, 280, 300 nm
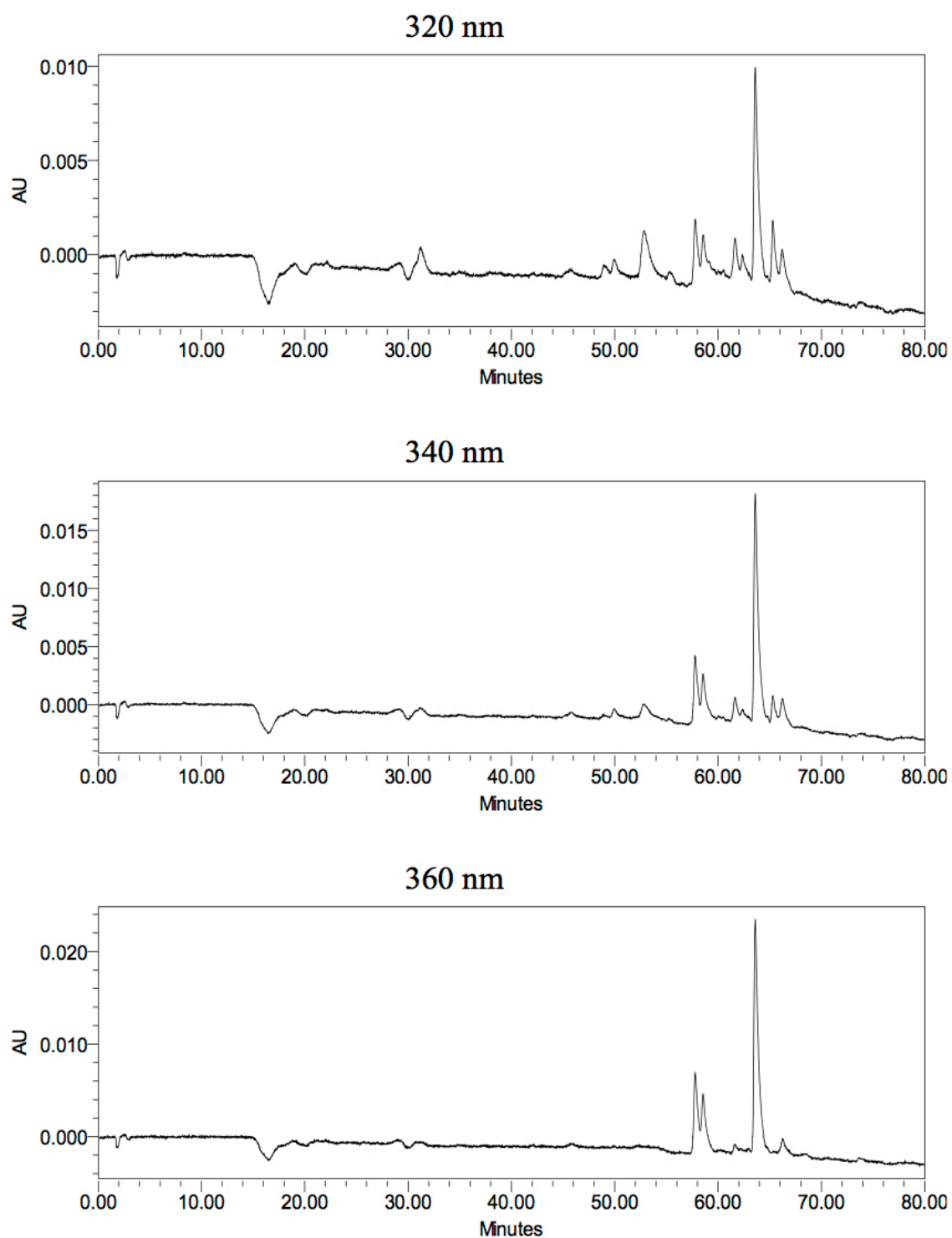
Figure A-4. Ethyl acetate 1 mg/mL chromatograms, with RP-HPLC, at wavelengths of 320, 340, 360 nm
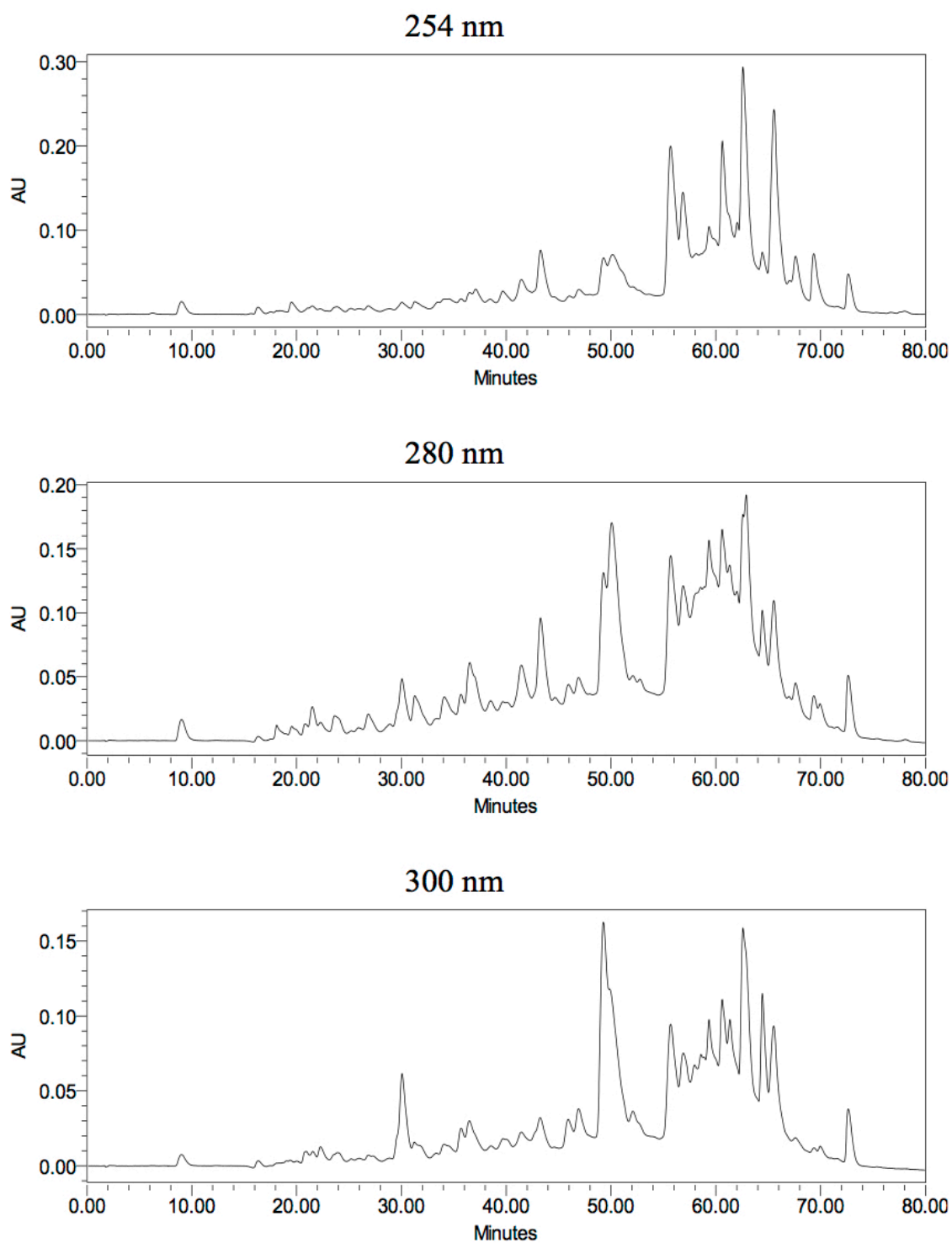
Figure A-5. Ethyl acetate 20 mg/mL chromatograms, with RP-HPLC, at wavelengths of 254, 280, 300 nm
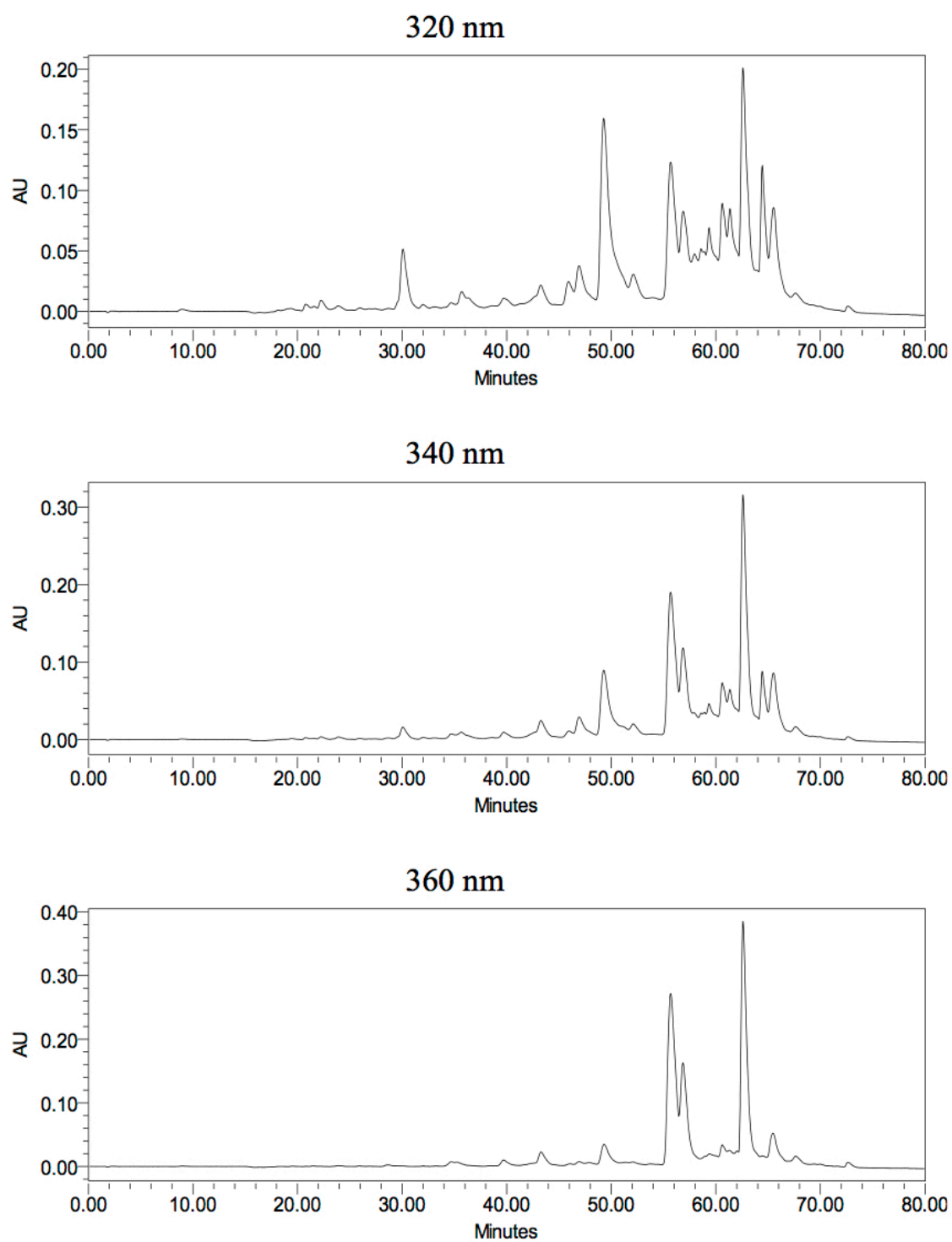
Figure A-6. ethyl acetate 20 mg/mL chromatograms, with RP-HPLC, at wavelengths
of 320, 340, 360 nm
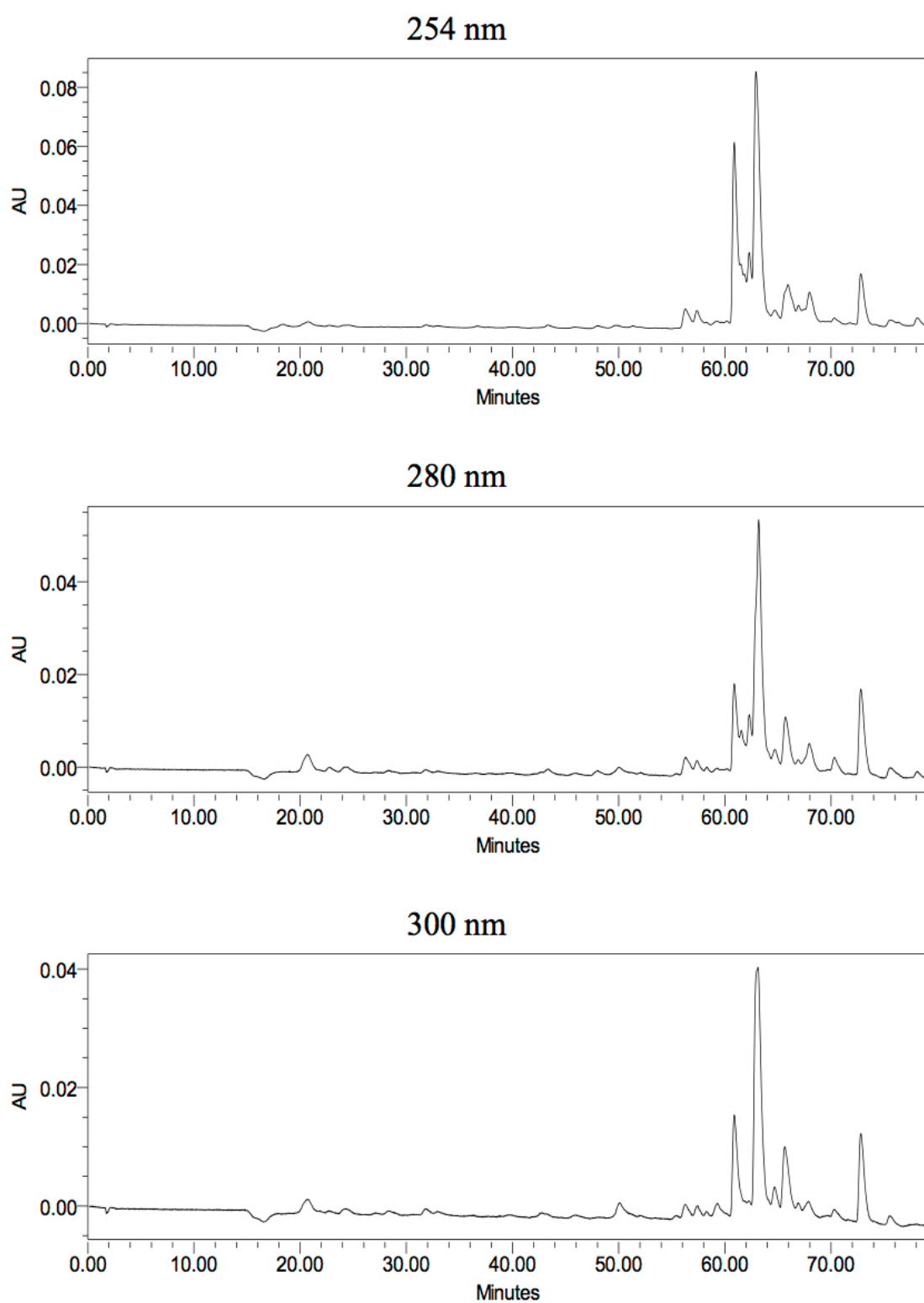
Figure A-7. Chloroform extract 10 mg/mL chromatograms, with RP-HPLC, at
different wavelength of 254,280,300
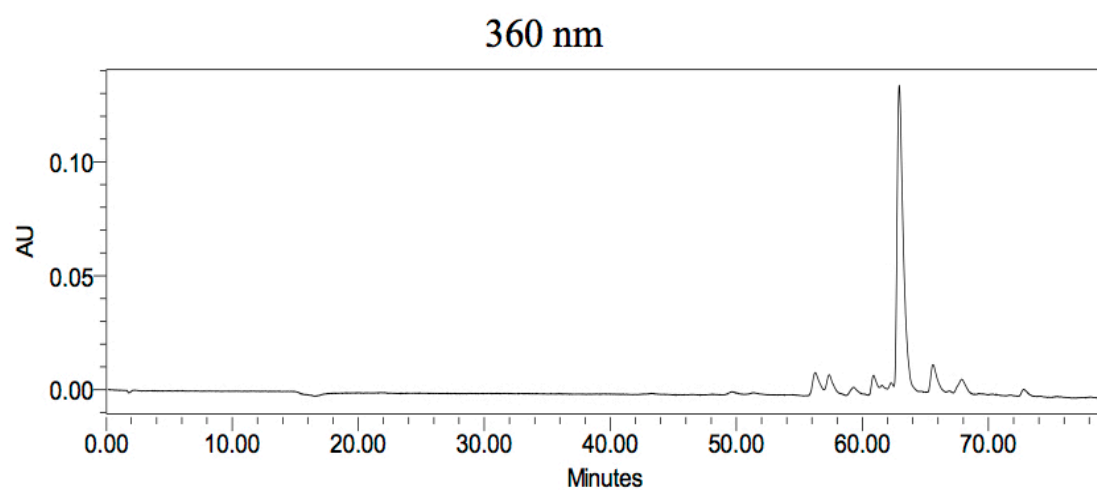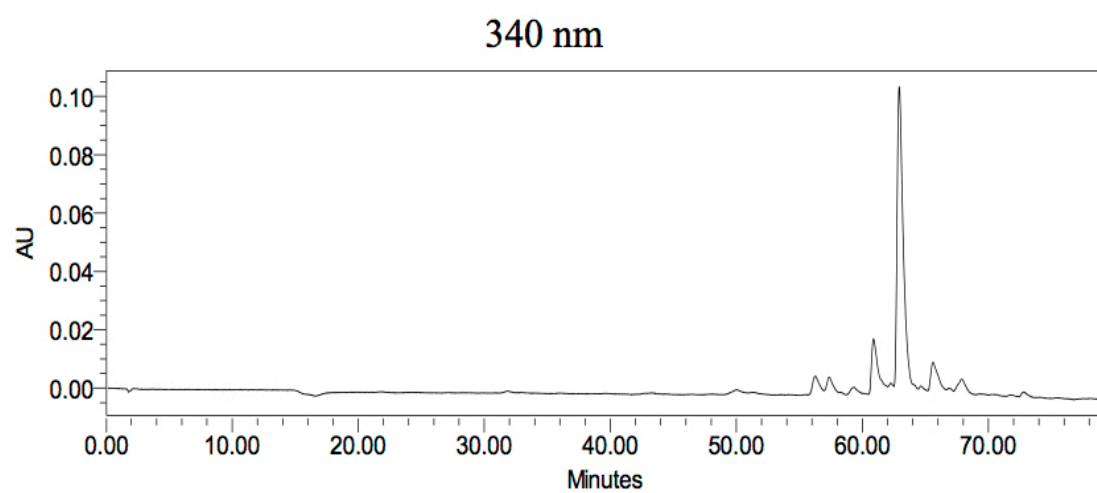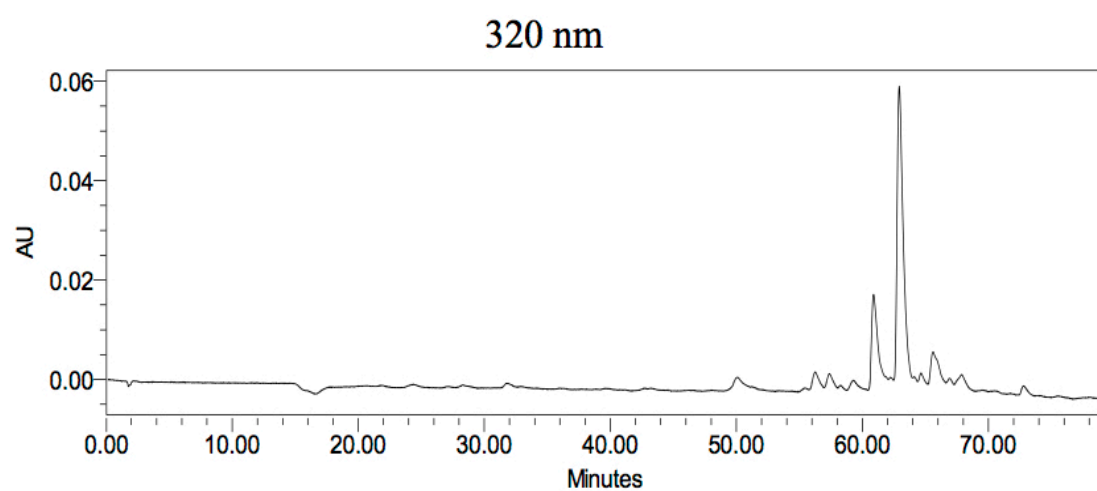
Figure A-8. Chloroform extract 10 mg/mL chromatograms, with RP-HPLC, at different wavelength of 320,340,360
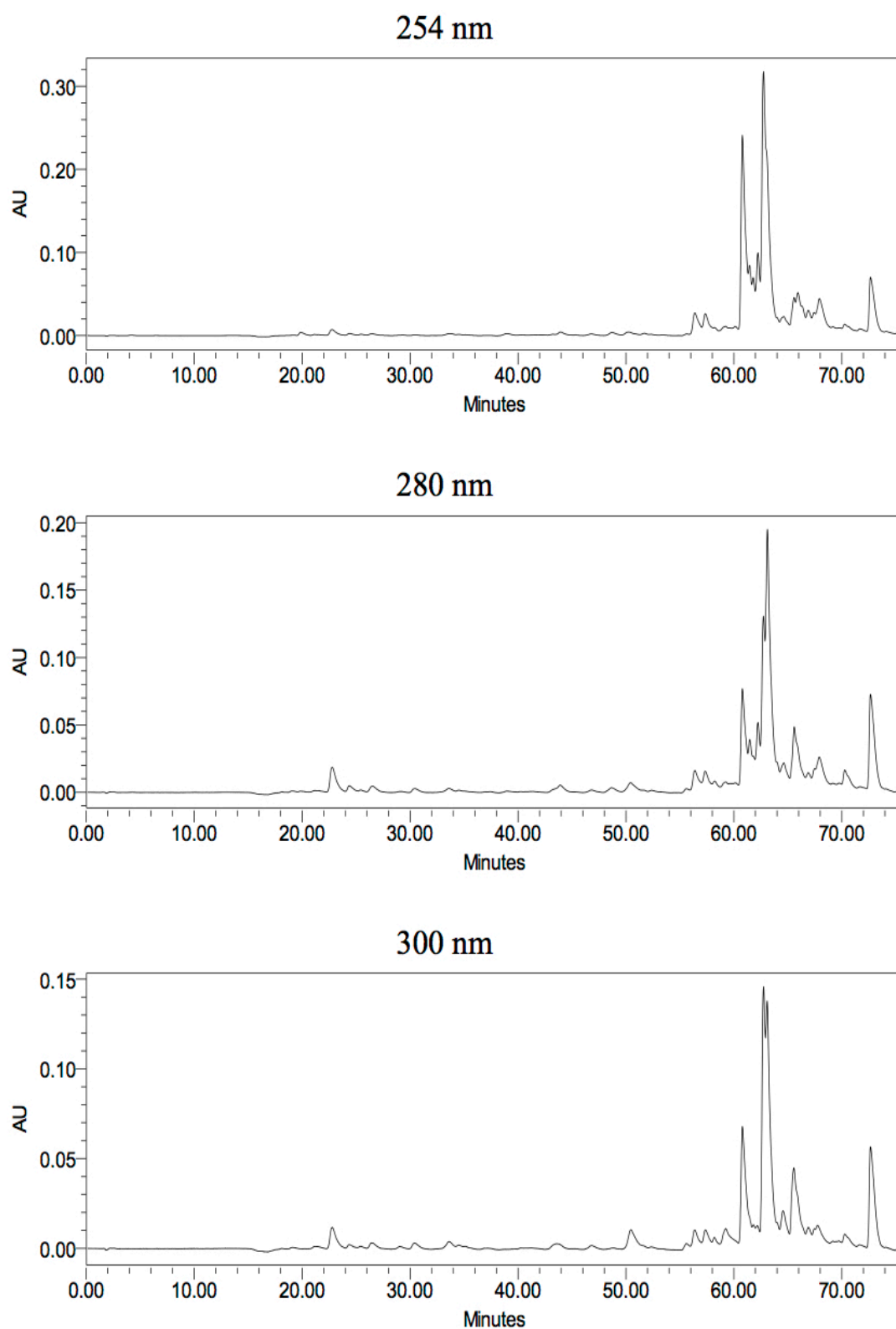
Figure A-9. Chloroform extract 30 mg/mL chromatograms, with RP-HPLC, at different wavelength of 254,280,300
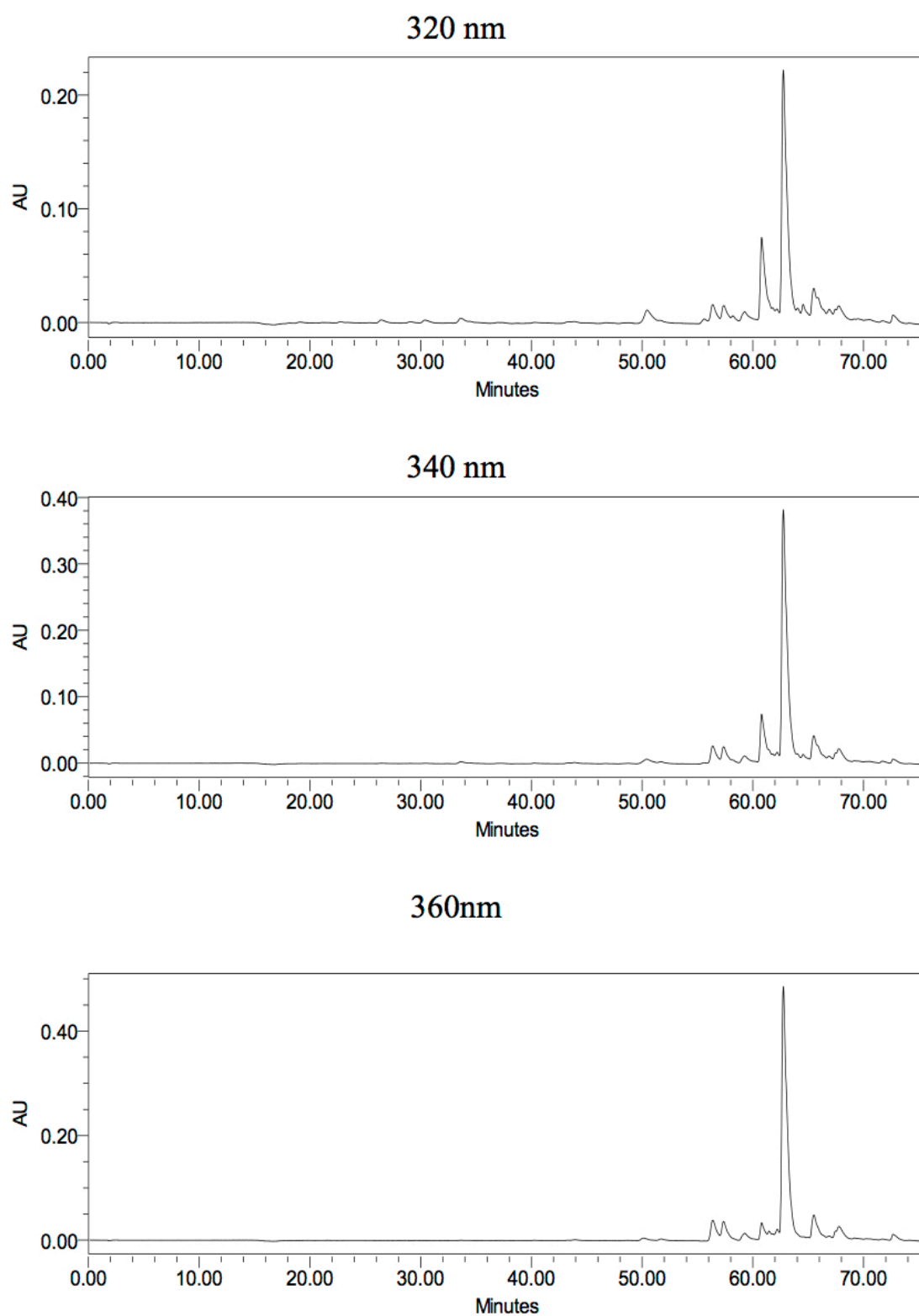
Figure A-10. Chloroform extract 30 mg/mL chromatograms, with RP-HPLC, at different wavelength of 320,340,360
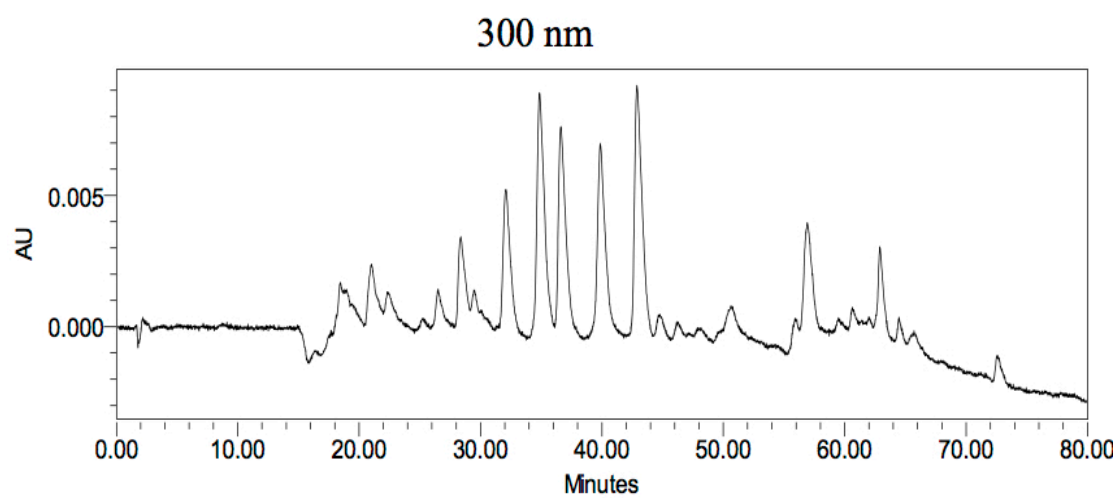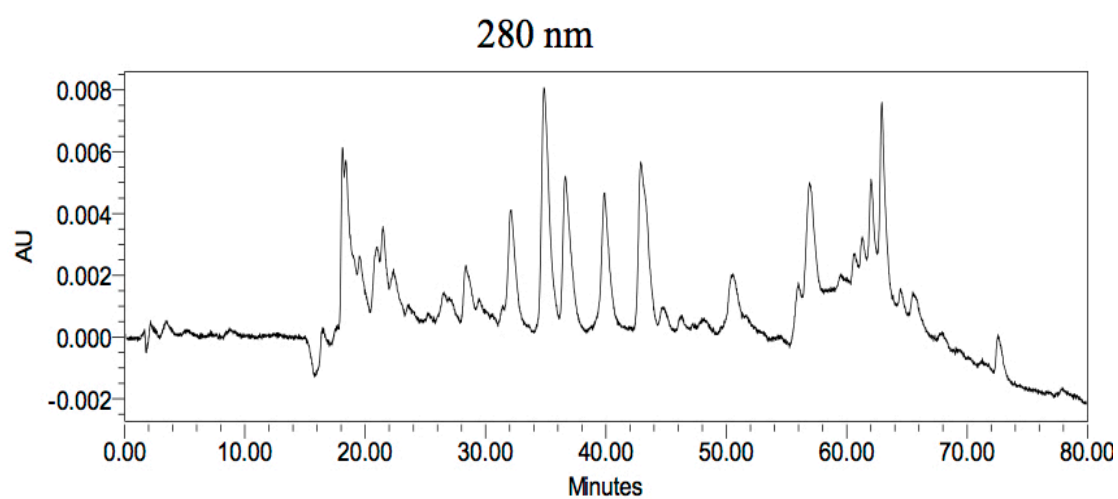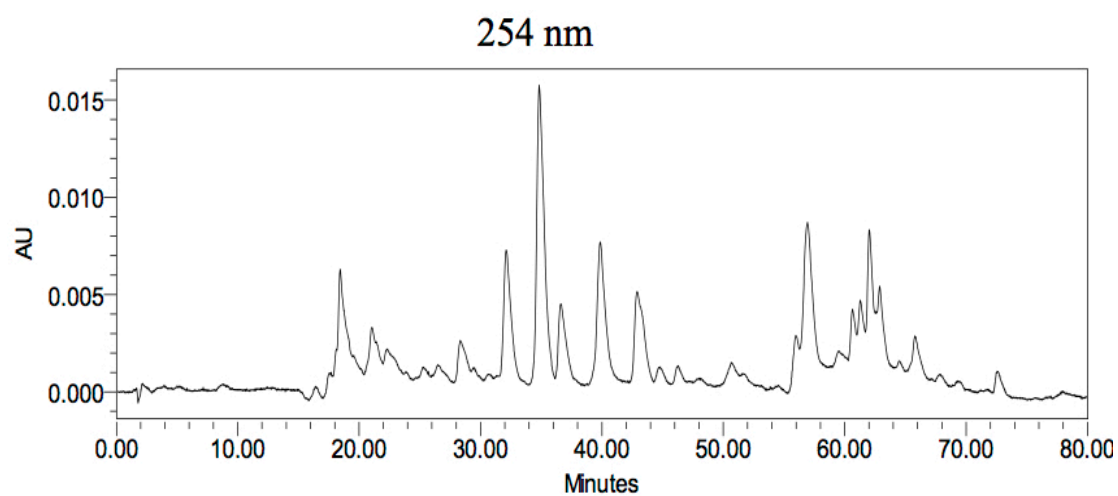
Figure A-11. Aqueous extract 10 mg/mL chromatograms, with RP-HPLC, at

different wavelength of 254,280,300

Figure A-12. Aqueous extract 10 mg/mL chromatograms, with RP-HPLC, at
different wavelength of 320,340,360

B. Chromatograms of co-injections of ethyl acetate
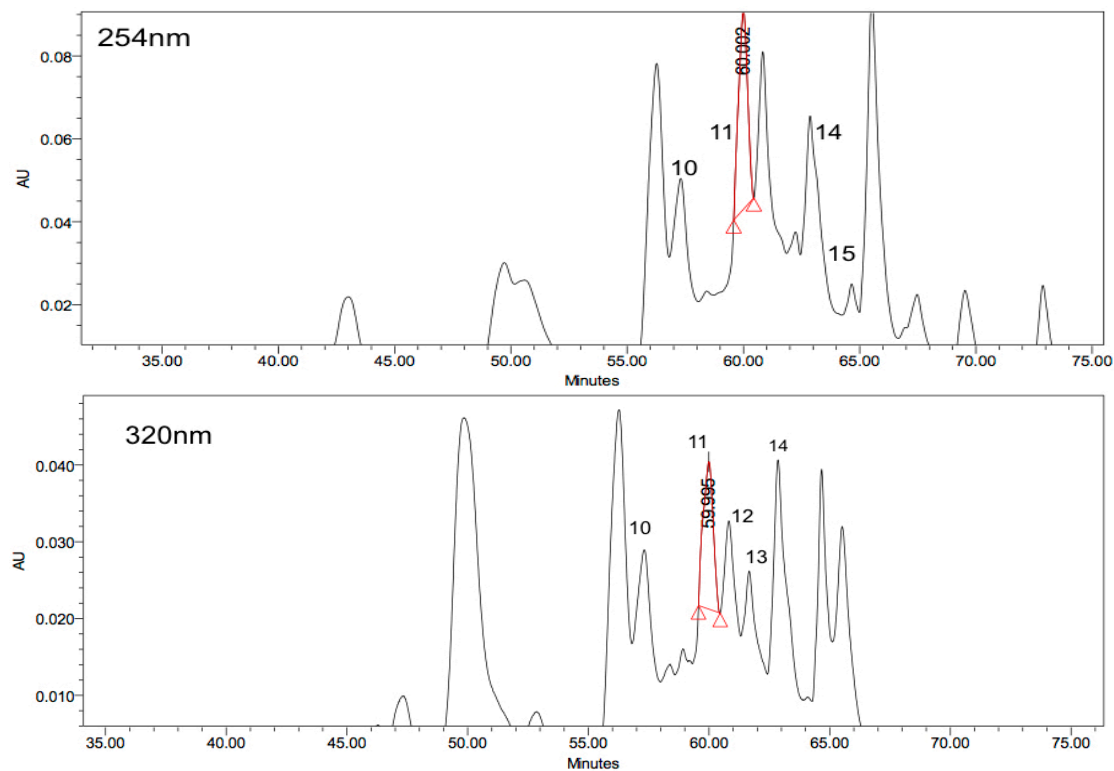
Figure B-1. Chromatogram of co-injection of quercetin with ethyl acetate fraction in a enlarge view
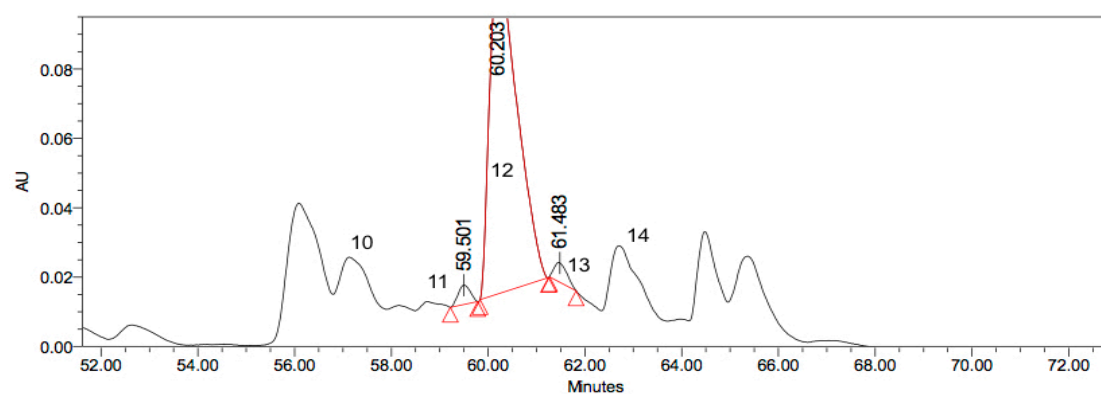
Figure B-2. Chromatogram of co-injection of luteolin with ethyl acetate extract in a enlarge view
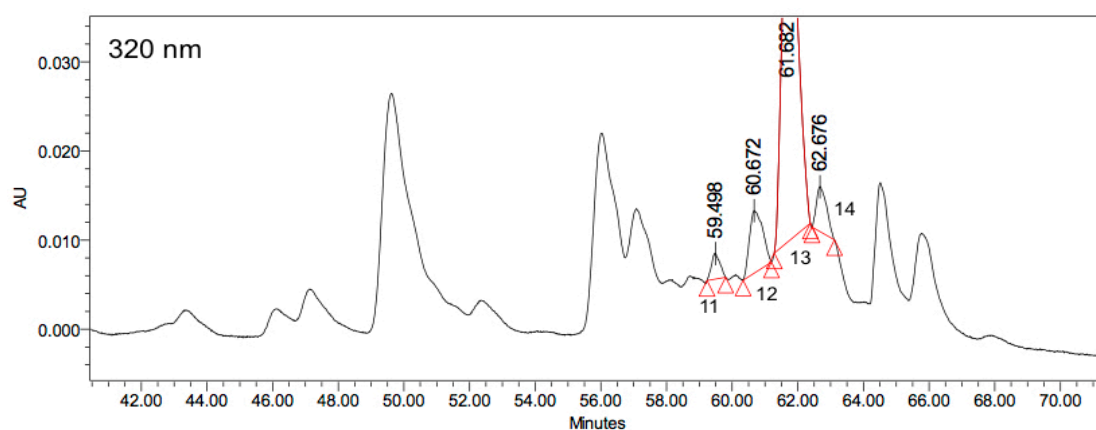
Figure B-3. Chromatogram of co-injection of naringenin with ethyl acetate extract in a enlarge view
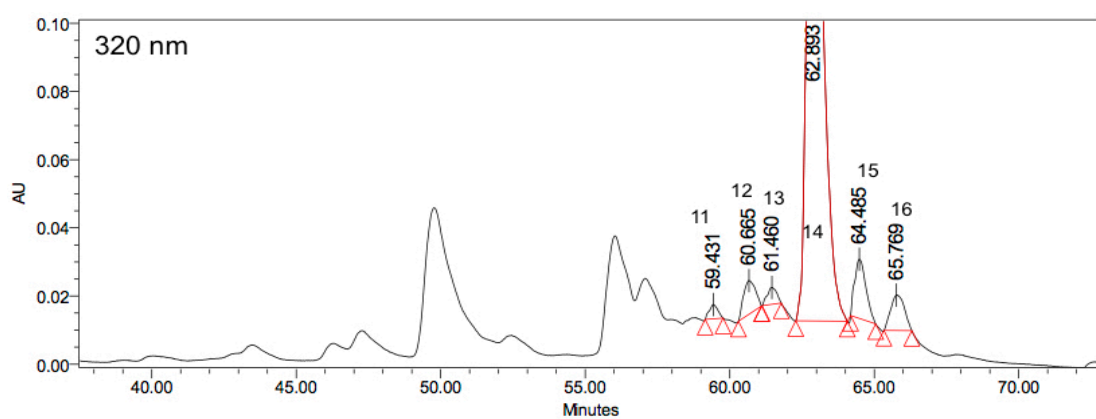
Figure B-4. Chromatogram of co-injection of hesperetin with ethyl acetate in a enlarge
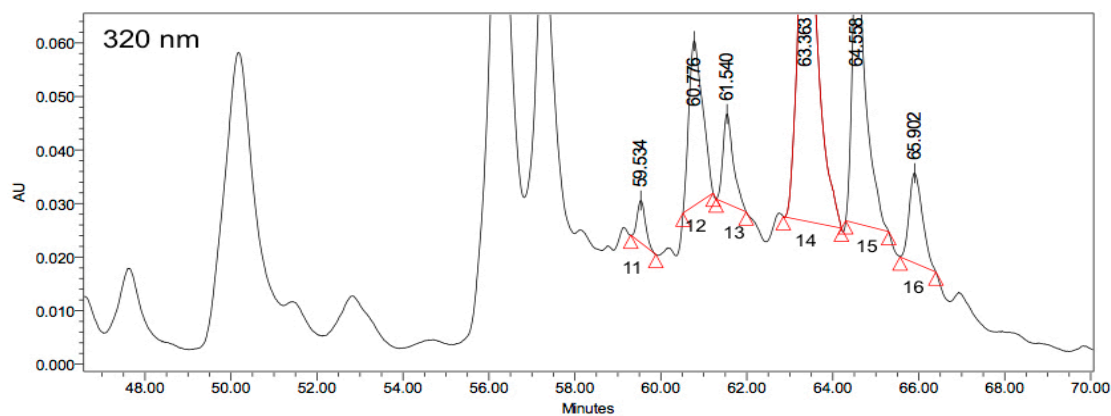
Figure B-5. Chromatogram of co-injection of apigenin with ethyl acetate in a enlarge

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APPENDIX C

C. UV-Visible spectrums of some phenolic standards from literatures
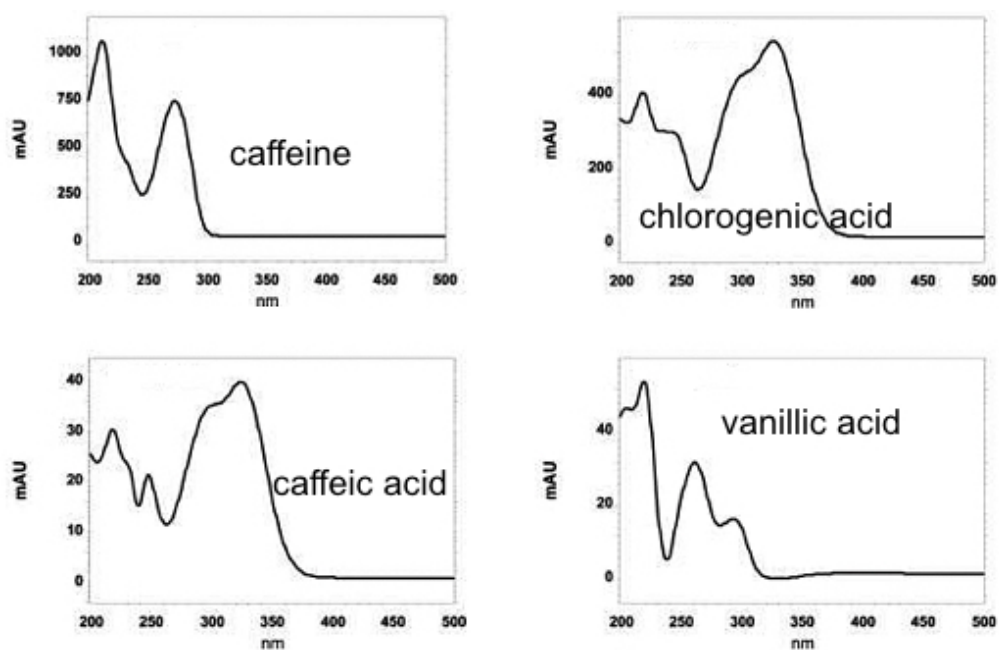
Figure C-1. UV-Vis spectrum of caffeine, Chlorogenic acid, caffeic acid and vanillic acid (Ion Trandafir, Violeta Nour, Mira Elena Ionica, 2013)
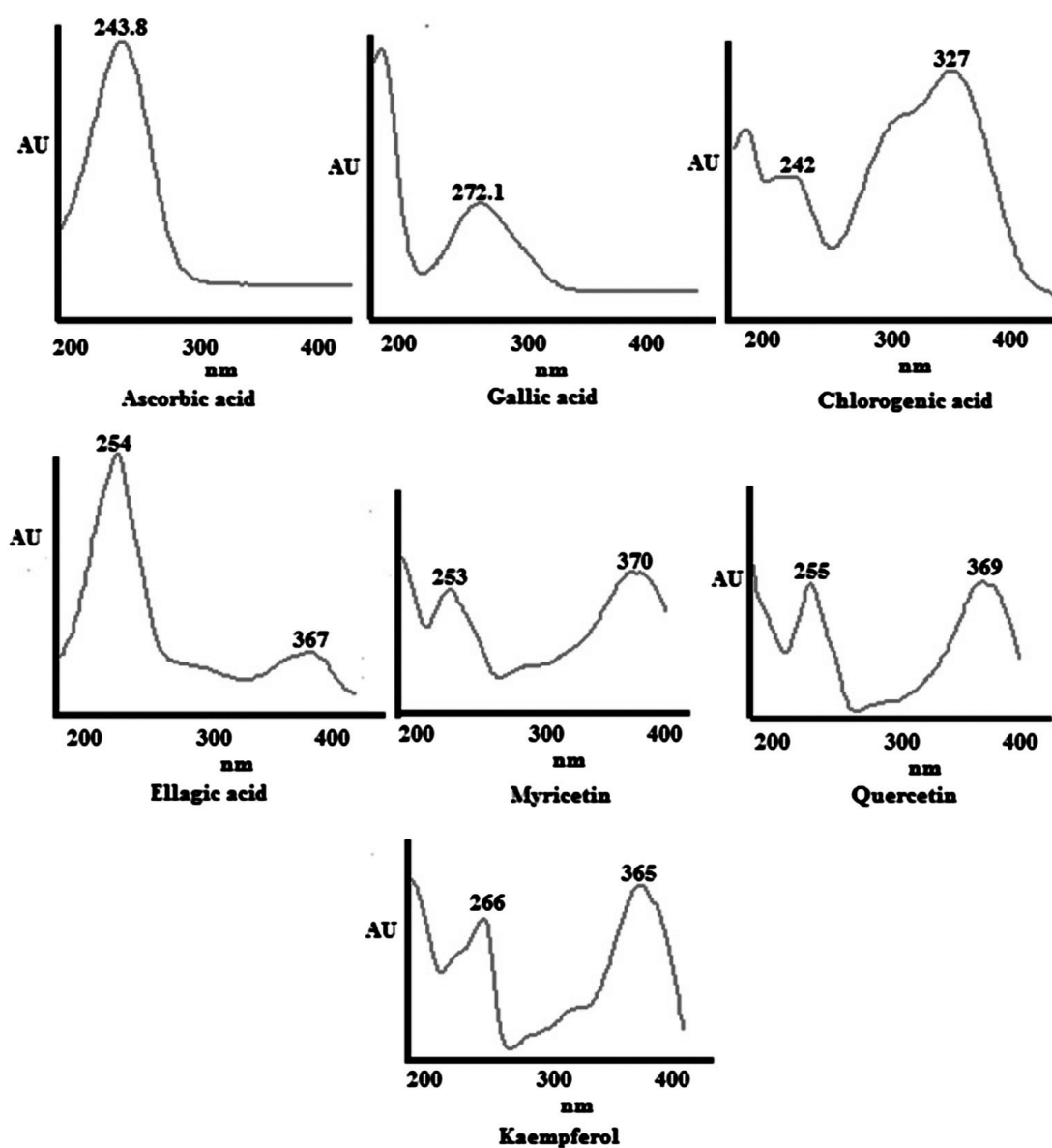
Figure C-2. UV-Vis spectrum of ascorbic acid, galic acid, chlorogenic acid, ellagic acid, myricetin, quercetin and kaempferol (Vasudha Bansal, Anupma Sharma & C. Ghanshyam and M. L. Singla, 2014)
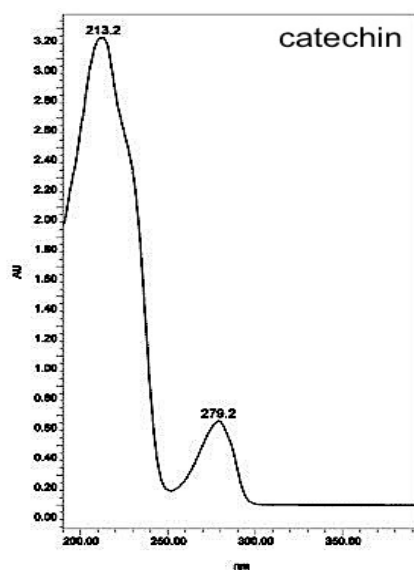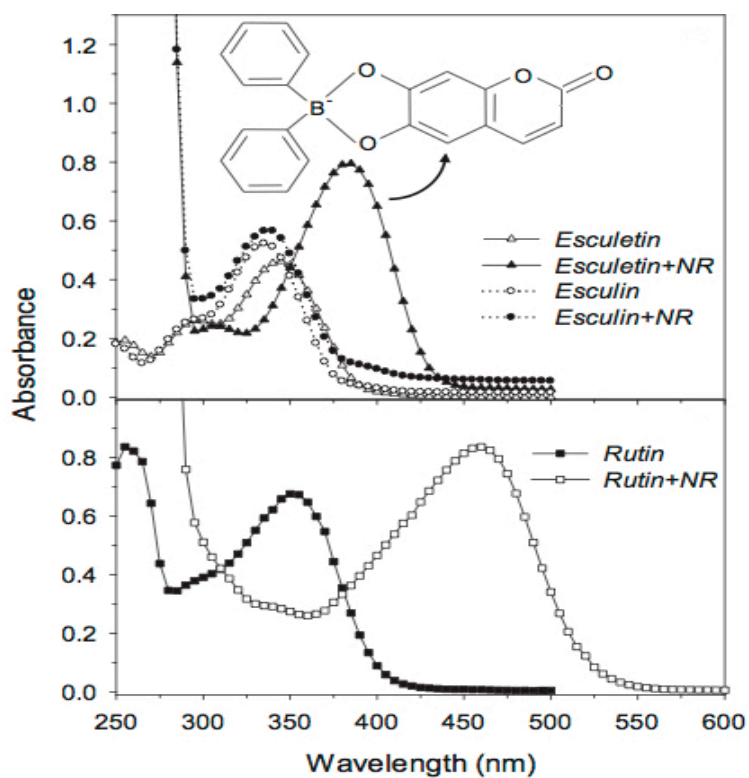
Figure C-3. UV-Vis spectra of catechin (Daniel P.M. Bonrez et.al, 2004)

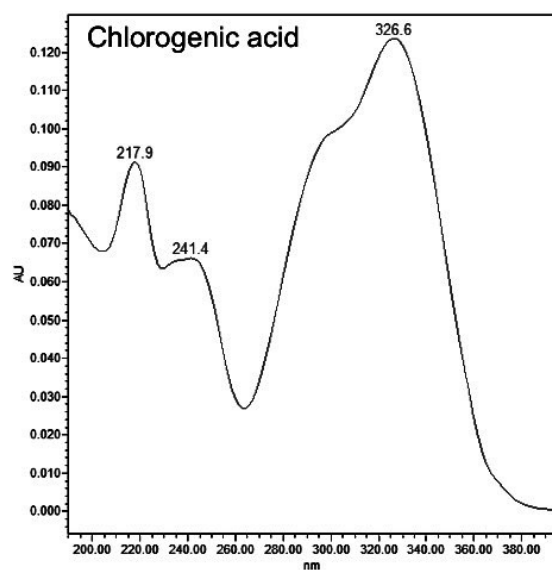Figure C-4. UV-Vis spectrum of esculin, esculetin and rutin (Tattini et.al, 2014)

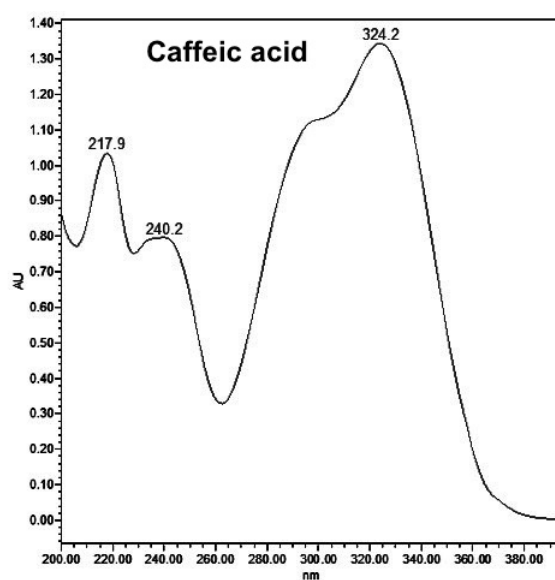Figure C-5. UV-Vis spectra of chlorogenic acid (Abebe Belay and
A.V.Gholap,2009)

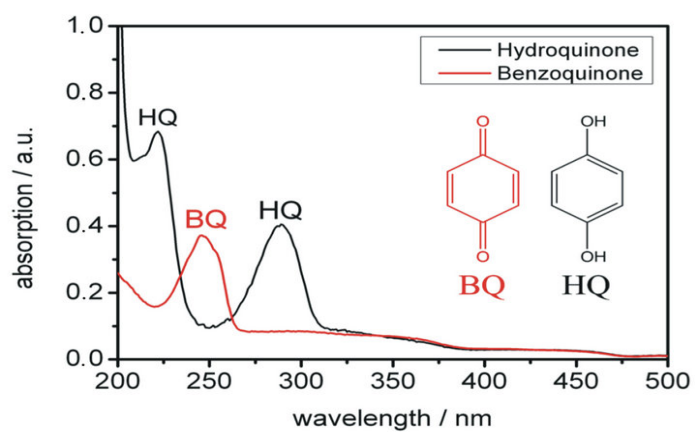Figure C-6. UV-Vis spectra of caffeic acid (Spangol et.al, 2015)

Figure C-7. UV-Vis spectrum of hydroquinone and benzoquinone (Thorsten Wilke, Michael Schneider, Karl Kleinermanns, 2013)
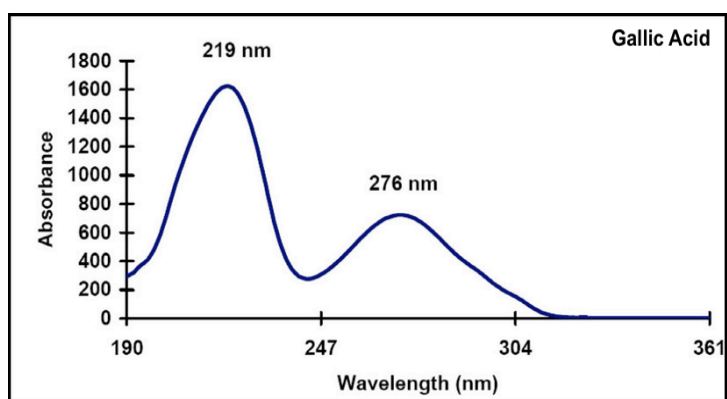

Figure C-8. UV-Vis spectra of gallic acid from (Joonhee Lee, 2004)