ROSA HECKELIANA (ROSACEA) ROOTS EXTRACT: BIOACTIVITY GUIDED FRACTIONATION AND CYTOTOXICITY AGAINST BREAST CANCER CELL LINES

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ABSTRACT

ROSA HECKELIANA (ROSACEA) ROOTS EXTRACT: BIOACTIVITY GUIDED FRACTIONATION AND CYTOTOXICITY AGAINST BREAST CANCER CELL LINES

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The genus Rosa, including about 100 species, is found to have a wide distribution throughout Europe, Asia, the Middle East and North America. *Rosa heckeliana* being a member of Rosa genus has been frequently used in folk medicine. Medicinal plants used in folk medicine are found to reveal their therapeutic effect due to their high content of phenolic compounds. These phenolic compounds contained in Rosacea families are located in their fruits and roots, most abundantly.

This study was designed to determine the anti-oxidative and anti-proliferative properties of the roots of *R. heckeliana.* Roots of the plant were extracted crudely in methanol. The polyphenolic compounds of the crude extract were concentrated and separated by using bioactivity guided solvent-solvent fractionation process. All the fractions and the crude extract were subjected to antioxidant activity assays. Eventually, ethyl acetate fraction, among the others, has been found to expose the highest antioxidant properties. Therefore, the ethyl acetate fraction was further carried out for quantitative and qualitative analyses to reveal its phenolic components.

Main phenolic compounds in the ethyl acetate fraction were purified by simple chromatographic methods using “Silica gel 60” and “Sephadex LH-20”. There were three major compounds isolated, they were also identified and characterized using NMR (Carbon and Proton) and HR-MS techniques. These three compounds found in the ethyl acetate fraction, in major amounts, were namely catechin, caffeic acid and ellagic acid. In the same fraction, there were also other phenolic compounds present in minor amounts, they were only subjected to identification through spectroscopic and chromatographic methods (RP-HPLC, UV-Vis spectroscopy). Identifiable compounds were epicatechin, p-coumaric acid, scopoletin and quercetin, and their structures were also confirmed by HR-MS technique.

Additionally, the anti-proliferative properties of the roots of *Rosa heckeliana* were examined by using XTT method. Crude extract, ethyl acetate fraction, and the isolated major phenolic components as well as matching standard compounds were all compared for their cytotoxicity on breast cancer cell lines; MCF-7 and MDA-231. The isolated major phenolic compounds were demonstrating moderate cytotoxicity, while the crude extract and ethyl acetate fraction verified only minimal cytotoxicity.

Keywords: *Rosa heckeliana*, antioxidants, RP-HPLC, cytotoxic effects.

ÖZ

GÜL KÖK ÖZÜTLERİNİN BİYOAKTİVİTE REHBERLİ FRAKSİYONLANMASI VE MEME KANSERİ HÜCRE HATLARINA KARŞI SİTOTOKSİSİTESİ

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Avrupa, Asya, Orta Doğu ve Güney Amerika'da geniş bir yayılım gösteren Gülgiller, bünyesinde yaklaşık 100 tür barındırmaktadır. Bu cinsin üyelerinden olan *Rosa heckeliana,* halk arasında bitkisel ilaç olarak sık sık kullanılmaktadır. Halk arasında bitkisel ilaç olarak kullanılan tıbbi bitkiler, yüksek miktarda fenolik bileşik ihtiva ettiklerinden dolayı terapötik etki göstermektedirler. Rosacea familyasında bulunan bu fenolik bileşiklerin meyve ve köklerde bol miktarda bulunduğu tespit edilmiştir.

Bu çalışma, *R. heckeliana* köklerinin antioksidatif ve antiproliferatif özelliklerini araştırmak için tasarlanmıştır. Bitkinin kökleri ham olarak metanolde özüt haline getirildi. Ham özütün fenolik bileşikleri, bioaktivite rehberli sıvı sıvı fraksiyonlama işlemi kullanılarak yoğunlaştırıldı ve ayrıldı. Tüm fraksiyonlar ve ham özüt antioksidan aktivite denemesine tabi tutuldu. Sonuç olarak, etil aseat fraksiyonu diğerlerine göre en yüksek antioksidan özellik sergiledi. Bu nedenle, etil aseat fraksiyonunun fenolik bileşiklerinin ortaya çıkarılması için ileri kantitatif ve kalitatif analizleri yapıldı.

Etil asetat fraksiyonu içerisindeki büyük fenolik bileşikler, basit kromatografi metodları "Silica gel 60" ve "Sephadex LH-20" kullanılarak saflaştırıldı. NMR (Karbon ve Proton) ve HR-MS teknikleri kullanılarak tanımlanan ve karakterize edilen üç büyük bileşik izole edildi. Kateşin, kafeik asit ve ellagik asit olarak adlandırılan bu üç bileşik büyük miktarlarda etil asetat fraksiyonunda bulundu. Aynı fraksiyonda, küçük miktarlardaki diğer fenolik bileşikler sadece spektroskopik ve kromatografik metotlar (RP-HPLC, UV-Vis spectroscopy) kullanılarak tanımlamaya tabi tutuldu. Epikateşin, p-kumarik asit, skopoletin ve kersetin tanımlanabilen bileşiklerdi ve yapıları da HR-MS tekniği ile doğrulandı.

Bunlara ek olarak, *R.heckeliana'ın* köklerinin antiproliferatif özellikleri XTT metodu kullanılarak incelendi. Ham özüt, etil asetat fraksiyonu, ve izole edilen büyük fenolik bileşiklerin antiproliferatif aktiviteleri, MCF-7 ve MDA-MB-231 meme kanser hücre hatlarında uygun standart bileşiklerle karşılaştırılmıştır. Ham özüt ve etil asetat fraksiyonu sadece minimal sitotoksisite gösterirken, izole edilen büyük fenolik bileşikler ılımlı sitotoksisite göstermiştir.

Anahtar kelimeler: *Rosa heckeliana*, antioksidanlar, RP-HPLC, sitotoksisite

To my beloved wife and daughter

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LIST OF ABBREVIATIONS

mg	Milligram
mL	Milliliter
µL	Microliter
mm	Millimeter
nm	Nanometer
ABTS	2,2-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid)
CE	Catechin Equivalent
DPPH	2,2-diphenyl-1-picrylhydrazyl
GAE	Gallic acid equivalent
DMSO	Dimethyl sulfoxide
EC50	Effective Concentration
ED50	Effective Dose
ER	Estrogen Receptor
FCS	Fetal Calf Serum
HPLC-DAD	Photo diode array dedector
HPLC-FU	Fluoresence dedector
HR-MS	High Resolution Mass Spectroscopy
MCF-7	Michigan Cancer Foundation-7
MDA-MB-231	Monroe Dunaway Anderson-Metastatic Breast
NMR	Nuclear magnetic resonance spectroscopy:
PBS	Phosphate Buffered Saline
RP-HPLC	Reverse Phase-High Pressure Liquid Chromatography
RSA	Radical scavenging activity
ROS	Reactive Oxygen Species
SD	Standard deviation:
TEAC	Trolox Equivalent Antioxidant Capacity
TLC	Thin Layer Chromatography
UV-Vis	Ultra violet visible spectroscopy
XTT	2,3-bis[2-methoxy-4-nitro-5-sulfopheny]-2H-tetrazolium-5-carboxyanilide innersalt

CHAPTER 1

INTRODUCTION

Since the beginning of civilizations, in every culture, plants have been used for medical treatments. Plants have been primary source of medicine in the traditional healthcare system around the globe, until recently and even currently in most of the developing countries. The approach to characterization and isolation of active ingredients from plants started in the late 19th century. Consequently, chemical substances isolated are currently used as important drug as such as their derivative(s) today. Cragg and his co-workers stated that 39% of newly approved drugs were obtained from natural sources (Cragg, 1997). Parallel to synthetic drug demand, the global natural products market is growing exponentially.

The number of higher plant species is estimated at 422.000 on this planet (Bramwell, 2002; Govaert, 2001). Of these, only around 6 % have been screened for biological activity and of which only 15% are reported to be phytochemically characterized (Verpoorte, 2000). The investigations on therapeutic applications of plants have led to discovery several clinically applicable drugs (digoxin, digitoxin, morphine, etc.). Elucidation of the structure of active principles paved the way for synthesis and derivization for compounds with higher efficacy and lower adverse effect (metformin, nabilone, oxycodon, etc.) (Fabricant, 2001; Sidambaram, 2011). As science develops, scientists are interested in utilizing only the potential phytocomponents. Hence scientists started isolation, identification and purification of chemical and natural compounds that are mainly responsible for pharmacological action (Fabricant, 2001).

Plant-derived natural products have been used for many purposes such as for food, dietary, cleaning, personal care, cosmetic and perfumery. In addition to that, plants have important impact on human health with their antioxidant, anti-inflammatory, antimicrobial, anticancer and anti-mutagenic properties. They are used in medicine to support physical, mental and spiritual health as well as to treat specific conditions and illnesses.

Traditional Turkish medicinal herbs have been used in the treatment of different diseases for centuries. The genus Rosa contains some of these traditional Turkish medicinal herbs. The present study proposes to evaluate the *R. heckeliana* root extract for its photochemical constituents and bioactive potentials.

1.1. Botanical Information

1.1.1. Classification of *Rosa heckeliana* Tratt.

Division: Phylum Magnoliophyta
Class: Magnoliopsida
Subclass: Rosidae
Order: Rosales
Family: Rosaceae
Genus: Rosa L.
Species: *Rosa heckeliana* Tratt

1.1.2. Family of Rosaceae

The family of Rosaceae is a complex and family of blossoming plants including in 95 genera about 3000 species (Judd, 1999; Mabberley, 1987). Initially divided into four subfamilies according to fruit type, the Rosoideae (Fragaria, Rubus), Prunoideae (Prunus), Maloideae (Malus, Pyrus) and Spiraeoideae (Spirea) have since been reclassified following phylogenetic analysis by Potter et al., (2007) into three subfamilies: Dryadoideae, Rosoideae (Fragaria, Potentilla, Rosa, Rubus), and Spiraeoideae (Maloideae, Prunoideae).

The members of Rosecea family can be trees, shrubs or herbaceous. Some members of this family are climbing plants. Most of the species of this family are deciduous, however, some of them are evergreen. They are distributed worldwide, but mostly concentrated in the northern hemisphere.

The leaves show an alternate form of growth on the stem. The leaves are rarely pinnately compound and they generally have stipules. But some plants are rarely reported to lack stipules.

The flowers of these plants are usually hermaphrodite or unisexual, actinomorphic, perigynous or epigynous. The symmetry of the flowers can be heat originated generally. They are rarely zygomorph.

There are 4 or 5 sepals which are gamosepalous. The petals are either free or they do not exist at all. They also consist of 4 or 5 pieces. The stamens are singular or multiple and they can form a circle or multiple circles.

Gynoceiums usually have singular combined pistils or multiple pistils, which are circulary or spirally located. The fruit is achene(s), follicles, drupe or forming a pome. Ovary is either located above or below and it consists of 1 to 5 locuses or carpels. The ovules can vary in amount ranging from one to higher numbers (Davis, 1965-2000, vol.4).

1.1.3. Rosa L.

The genus Rosa, one of the members of this herbs consist of approximately 100 species that found a wide distribution throughout Europe, Asia, the Middle East and North America. Turkey is native to nearly 25 percent of all Rosa species in the world. Our country is the natural habitat of 24 Rosa L. Species, which can be categorised to 4 sub species, 2 varities, 2 endemic species and 13 hybrid species. (Özçelik, 2010). In previous studies on garden roses, 42 species in 13 sections of 2 sub-genus have been reported. Rosa species are usually decidious plants, but there some evergreen species too. All the species are thorny shrubs. The leaves show an alternate form of growth. They are inparipinnat and they have stipuls. The flowers of these species are either singular or korimboz. Epicalyx absent; sepals 5, all equal or the 3 outer lobed and the 2 inner entire. Petals 5, yellow, white or pink to red. Stamens , styles and carpels numerous. Hypantium urceolate, becoming colured and fleshy when ripe; styles free or connate in a column, protruding through the orifice of the disc, stigmas usually forming a distinct head (Mandenova, 1970; Nilsson, 1972)

1.1.4. Rosa species of growing in Turkey

1. *Rosa sempervirens* L.
2. *Rosa phoenicia* Bois
3. *Rosa arvensis* Huds
4. *Rosa pisiformis* (Christ) D.Sosn
5. *Rosa beggeriana* Schrenk
6. *Rosa foetida* J. Herrm
7. *Rosa hemishphaerica* J.Herrm
8. *Rosa pimpinellifolia* L.
9. *Rosa elymaitica* Boiss Hauskn
10. *Rosa gallica* L.
11. *Rosa villosa* L.
12. *Rosa hirtissima* Lonacz.
13. *Rosa tomentosa* Smith
14. *Rosa jundzilli* Besser
15. *Rosa micrantha* Sm.
16. *Rosa agrestis* Savi.
17. *Rosa pulverulentha* Bieb.
18. *Rosa sicula* Tratt.
19. *Rosa horrida* Fischer
20. *Rosa iberica* Stev.
21. *Rosa montana* chaix.
22. *Rosa canina* L.
23. *Rosa dumalis* Bechst
24. *Rosa heckeliana* Tratt (Özçelik, 2010)

1.1.5. *Rosa heckeliana* Tratt.

Rosa heckeliana tratt (Figure 1.1) as one members of this genus is frequently used in folk medicine. Rosa is locally known as ‘kuşburnu’ and found especially in Hakkari, Bitlis, Agrı, Bingöl, Van, and it has been cultivated in many areas of Armenia and Northern Iran now. It is a small plant in 0.1–0.6 m high. Petals are from pale to deep pink, but rarely in white color. The species appear as brushes spread over rocks, usually on limestone, slopes and near the woody areas, about at the height of 1300–2900 m in East Anatolia (Nilsson, 1997)

Figure 1.1: *Rosa heckeliana* tratt

1.1.6. Distribution of the Taxon over Turkey

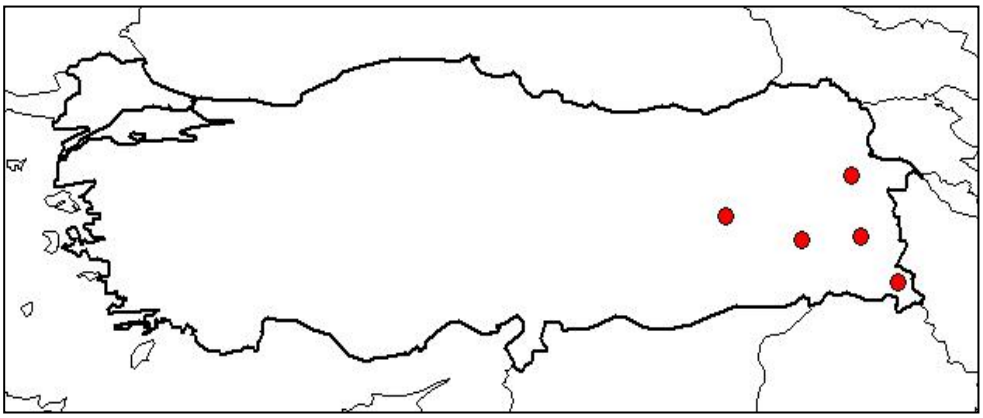


Figure 1.2: Distribution of *R. heckeliana* Tratt in Turkey.

Based on provinces: Hakkari, Bitlis, Ağrı, Bingöl, Van.
East Anatolia, B8 Bingöl: nr. Boğlan 1370 m, Kotschy 540! B9 Ağrı: d.Suluçem (Musun), S. End of Balik G., 2300 m, D47264! Bitlis: Süphan Da., above Adilcevaz, 2900 m, D.24704! Van: Çuk gediği pass between Hoşap and Başkale, 2600 m Birand karamanoğlu 486! C9 Hakkari: Cilo Da., between Diz deresi and Cilo Y., 2440 m, D 24247 (Davis, 1965-2000, vol. 4).

1.1.7. The phenolic constitiuents of *Rosa heckeliana*

R. heckeliana root extracts have not yet been investigated for their chemical studies in literature. However, there are some studies carried out on Rosa species other than *R. heckeliana.*

1.1.8. The phenolic constitiuents of Rosa species

Many species of Rosa are quite rich in phenolic compounds (Yoshida, 1993; Hvattum, 2002). For instance, as Harborne (1967) stated, flowers of Rosa species and its cultivars were found to contain 4'-glucosides, 3-rhamnosides, 3-sophorosides and 3-glucosides of kaempferol and quercetin.

Rosa pulverulenta: The fruits of this species have rich ascorbic acid and mineral content (Kurt, 1983).

Velioglu et al., in 1991 reported that in *R. damascena, R. persica* species cyanidin 3,5-diglucoside was the main anthocyanin derivative as well as kaempferol and quercetin glucosides, galactoside, arabinosides, and rhamnosides.

Biolley and his co-workers (1992, 1994) studied the anthocyanins and the flavonols of rose cultivars by chemometric methods in order to identify the cultivars and determine the metabolic mechanisms of flavonoids.

Sumere et al., in 1993 reported that the Rosa petals included many of ellagic acid, kaempferol, cyanadin and quercetin derivatives.

Mikanigi et al. (1995) analyzed the petals of 120 Rosa taxa for flavonol and anthocyanin contents. 19 flavonols and 6 anthocyanins were found: six kaempferol glycosides, six quercetin glycosides, two cyanidin glycosides, two peonidin glycosides, seven unidentified flavonols and two unidentified anthocyanins.

Rose hips of *Rosa canina* were reported as rich sources of vitamin C, a well-known antioxidant (Hemila, 1992).

The chemical composition (Hodisan, 1997) of *R. canina* fruits has already been studied and Osmianski et al. (1986) demonstrated the presence of phenolics and particularly proanthocyanidins and catechin.

Flower extracts of *Rosa rugosa* were analyzed for its phenolic constituents and found to include gallic acid and its derivatives and hydrolysable tannins (in the leaves and petals), catechin derivatives (roots), flavonoids (leaves), 2-phenoxychromones (leaves), monoterpenes (floral parts, leaves), sesquiterpenes (leaves) and triterpenes (leaves and roots) (Ng, 2004; Ng, 2005; Hashidoko, 1996).

Rosa persica flower extracts were found to have polyphenolic antioxidant activity with strong free radical scavenging capacity (Jassbi, 2003).

Nowak et al., 2005 published that the R. dumalis, R, rubiginosa and R. rugosa contain high polyunsaturated fatty acid especially linoleic and linolenic acid.

Nowak et al., 2007 published that the following taxons were examined. R. *canina* L. var *canina*, R. *canina* L. var *corymbifera* (Borkh.) Boulenger, R. *canina* L. var. *puberula* R. Keller, R. *canina* L. var *dumalis* Baker, R. *vosagiaca* Desportes, R. *subcanina* (H. Christ) Dalla Torre et Sarnath, R. *coriifolia* Fri., R. *caryophyllacea* Besser pro parte, R. *subcollina* (H. Christ) Dalla Torre et Sarnath, R. tomentosa Sm., R. *rubiginosa* L., R. *villosa* L., R. *inodora* Fri., R. *agrestis* Savi, R. *jundzilli* Besser, R. *pendulina* L., R. *rugosa* Thunb., R. *gallica* L. and investigated leaves of some rose species. They reported that rose leaves contain large amounts of phenolic compounds especially quercetin, kaempherol and ellagic acid.

R. bourboniana, R. *brunonii* and R. *damascena* fresh flowers extracts were determined to contain 15 polyphenols compounds (Kumar, 2008 and 2009).

1.1.9. Ethno botanical uses of Rosa species

Rosa species have a broad habitation in Turkey and quite a number of them are important in folk medicine. They can be used as diuretics, expectorants and tonics and for the treatment of heartburn, diarrhea and nephritis (Kultur, 1998).

There are some studies carried out on Rosa species. For instance, fruits of *R. canina* L. have prophylactic and therapeutic activities against inflammatory complaints including soreness, rheumatism, gout and lumbago. Moreover, these Rosa species can be used for diseases with fever, for colds and influenza (Orhan, 2007; Wenzig, 2008; Fecka, 2009).

The popular usage of tea plant such as R. *canina* is used against obesity (Everest, 2005). Rosehips from R.*canina* are useful to heal the stomach pain, diarrhea, hemorrhoids, stomach aches, and kidney stones (Sezik, 2001, Tuzlacı, 2001, Yeşilada, 1995, Yeşilada, 1999).

R. damascena also has some other benefits such as cooling, soothing, astringent, anti-inflammatory effects (Ody, 1995) and antioxidant, antibacterial hepatoprotective effects (Ozkan, 2004; Achuthan, 2003). *R. damescana* rose oil had an anticonvulsant effect and could reduce frequency of seizures in children who were resistant to anti-epileptic drugs (Ashrafzadeh et al., 2007). *R. damascena* has beneficial effects on brain function and has potential applications for the treatment of dementia (Kheirabadi, 2008). An additional recent study suggested that rose oil and a main component of the rose fragrance, phenyl ethyl alcohol, significantly inhibits acetylcholine esterase and butyrylcholine esterase (Senol, 2011).

In Maltese folk medicine, Petals of *R. gallica* used in rose-water has been used as an astringent, rinse, on inflamed eyes and skin. The tea produced from the leaves have laxative properties (http://eatt1.tripod.com/amp7.html).

Rosa alba Linn: The flowers of this species have soothing properties in fevers and in palpitation of heart. Also, leaves have the activity to abort the pregnancy. A pharmaceutical preparation from white rose extract enriched by other ingredients is used to treat liver disorders (Kumar, 2009).

Rosa rugosa flower extracts were proved to intensify the antioxidant enzymatic activity and to decrease lipid peroxidation due to gallic acid derivative contents (Ng, 2004 and 2005).

Rosa persica flower extracts were found to hold polyphenolic compounds as good scavenger of DPPH radical (Jassbi, 2003).

Rosa pulverulenta: Fruits of this species are rich in ascorbic acid and minerals, and they can be used in jam, marmalade, fruit juice making (Kurt, 1983).

Rosa montana: The fruit of this species is very suitable for making jam, marmalade and fruit juice (Kurt, 1983).

Rosa blanda has many medicinal uses. The roots, when ground up and boiled, make a paste that alleviates skin irritation. A wash for irritated eyes and a treatment of headache or lower back pain are also made from the roots. The skin of the rose hips is used to aid with stomach pain and digestion problems. The dried flowers are a suppressor of heartburn (Moerman et al).

The people of ancient times used *Rosa nutkana* for nutrition, remedy and for construction materials. The fruits were consumed as medication for the children with diarrhea. The leaves were also prepared as dressing for the bee stings (http://www.nwplants.com/business/catalog/ros_nut.html)

Rosa indica: Buds and petals are used for the removal of gall bladder and kidney stone and flowers are use against asthma (http://www.iccs.edu/folkmed/P51.php)

1.2. Phenolic compounds in plant

Phenolic compounds are generally classified as "secondary" metabolites which are obtained from the pathways of phenylpropanoid in plants (Randhir, 2004).

The phenolic compound family is huge and comprises a complex group of compounds (more than 8000 identified compounds) (Bravo, 1998). They all contain a phenol group (an aromatic ring having at least one hydroxyl substituent) (Croteau, 2000).These compounds are well-known virtually in all plant foods, often at high level.

These compounds have significant physiological and morphological roles in plants and they are one of the most common phytochemicals in nature. They are found in most of the fresh fruits and vegetables as simple compounds or they are found in bark, flowers, roots and leaves of the many plants as complex compounds. For instance, anthocyanins are a group of polyphenols which are liable for the shade of several fruits, vegetables, and flowers. There are several important classes of phenolics which are listed in Table 1.1. The structure of polyphenols varies from simple phenols to highly polymerized compounds according to the basic Carbon skeleton (Waterman, 1994).

Table 1.1: Grouping of phenolic compounds

Basic skeleton	Class	Examples
C6	Simple phenols	Phenol, cresol
C6-C1	Hydroxybenzoic acids	Gallic acid, vanillic acid
	Condensed tannins	Gallotannins, ellagitannins
C6 -C2	Acetophenones	Annphenone
	Phenyl acetic acids	p-Hydroxyphenylacetic acid
C6 -C3	Hydroxycinnamic acids	Caffeic acid, ferulic acid
	Phenylpropenes	Eugenol, myristicin
	Coumarins, isocoumarins	Umbilliferone, scopoletin
	Chromones	Eugenin
C6 -C4	Naphthoquinones	Juglone
C6 -C1 -C6	Xanthones	Mangostin, mangiferin
C6 -C2 -C6	Stilbenes	Resveratrol
	Anthraquinones	Emodin
C6 -C3 -C6	Chalcones	Phloridzin, arbutin
	Dihydrochalcones	Phloretin
	Aurones	Sulferetol
	Flavones	Apigenin, luteolin
	Flavonols	Quercetin, myricetin
	Dihydroflavonol	Taxifolin
	Flavanones	Hesperitin, naringenin
	Flavanol	(epi)Catechin
	Flavandiol	(+)-Leucocyanidin
	Anthocyanidins	Cyanidin, pelargonidin
	Isoflavonoids	Daidzein, genistein
(C6-C3-C6)2	Biflavonoids	Agathisflavone
(C6-C3-C6)n	Proanthocyanidins	Procyanidins
(C6-C3)2	Lignans, neolignans	Sesamin
(C6-C3)n	Lignins	

Sources: Hijova (2006), Bravo (1998), Singh (1997), Tomás-Barberán (2000b), Villemin, (1998).

1.2.1. Flavonoids

Flavonoids are part of polyphenolic compounds and commonly found in vegetables, nuts and fruits (Hollman, 1997; Heim, 2002; Clifford, 2000a). According to Middleton stated that humans should consume approximately 1 g of mixed flavonoids each day (Middleton, 1984). Flavonoids are water soluble molecules consisted of two aromatic rings (each aromatic ring with at least one hydroxyl group) containing 15 carbon atoms. These aromatic rings are connected through a three-carbon "link" that may be part of a six-member heterocyclic pyran ring in Figure 1.3 (Merken, 2000).

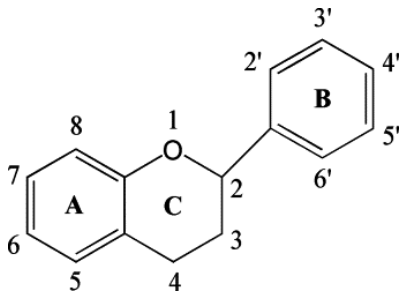
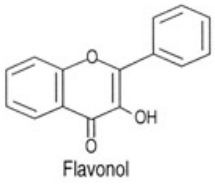
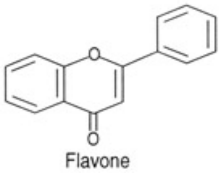
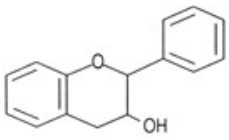
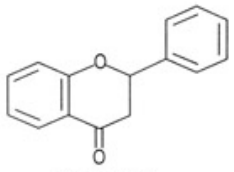
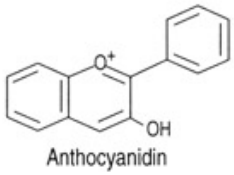
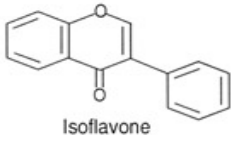


Figure 1.3: Basic structure of flavonoid (phenylbenzopyrone structure)

There are six major subgroups of flavonoids which are flavanol, flavanone, flavone, flavonol, anthocyanins and isoflavonoids. The subclasses of flavonoids and typical food sources are illustrated in Table 1.2.

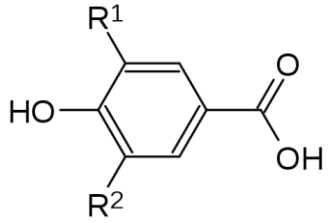
Table 1.2: Common dietary and subclasses of Flavonoids

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

1.2.2. Phenolic acids

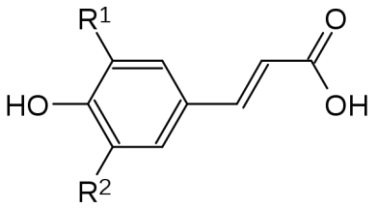
Phenolic acids are the aromatic secondary metabolites which are widely spread in plant kingdom. The term “phenolic acids” represents phenols having with one carboxylic acid. Nevertheless, when describing plant metabolites, it refers to a different group of organic acids. Those naturally occurring phenolic acids have two distinct fundamental carbon structures: the the hydroxycinnamic and hydroxybenzoic (Figure 1.4).

Hydroxybenzoic acid



p-hydroxybenzoic, $R^1=R^2=H$
protocatechuic, $R^1=OH$, $R^2=H$
vanillic, $R^1=O\text{-}CH_3$, $R^2=H$
syringic, $R^1=R^2=O\text{-}CH_3$
gallic, $R^1=R^2=OH$

Hydroxycinnamic acid



p-coumaric, $R^1=R^2=H$
caffeic, $R^1=OH$, $R^2=H$
ferulic, $R^1=O\text{-}CH_3$, $R^2=H$
sinapic, $R^1=R^2=O\text{-}CH_3$

Figure 1.4: 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, 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)

1.2.3. Tannins

Tannins are the other important group of phenolics which have relatively high molecular weight. They can be subdivided into two major groups called hydrolysable and condensed tannins (Porter, 1989). The hydrolysable tannins are the esters of gallic acid (gallo- and ellagi-tannins), and the condensed tannins (also known as proanthocyanidins) are the polyhydroxyflavan-3-ol monomers (Porter, 1989).

1.2.4. Lignans

The basic units of lignans (Figure 1.5), the C6-C3 units (two propyl benzene), are formed by the phenylpropanoid pathway starting from phenylalanine. Also lignin and flavonoids are derived from this pathway.

The major sources of lignans are flaxseed, sesame seeds, rye, nuts, grains, vegetables and tea in the human diet. Plants lignans also act as important biological actions such as antiviral, anticancer, anti-inflammatory, antioxidant, antimicrobial and immunosuppressive (Saleem, 2005; Cos, 2008; Pan, 2009; Yousefzadi, 2010)

The lignans are the major subclasses of estrogen-like chemicals called phytoestrogens. The other subclasses are isoflavones and coumestans.

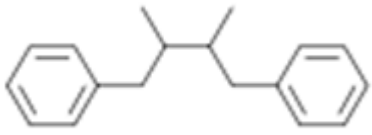


Figure 1.5: Basic chemical structures of lignan

1.2.5. Coumarins

Coumarin (1,2-benzopyrone) is the building block of coumarin derivatives which are made of fused benzene and apyrone rings (Keating, 1997). The coumarins can be generally classified as follows;

Simple: These are the hydroxylated, alkoxylated and alkylated derivatives of the parent compound
Furanocoumarins: These compounds contained a a five-membered furan ring
Pyranocoumarins: These compounds consist of a six-membered ring
Pyrone-substituted: These compounds substituted in the pyrone ring

1.3. Functions of phenolic compounds in plant

Phenolic compounds are significant for plant physiology, morphology (colour, mechanical support), growth (nutrient uptake, protein synthesis, enzyme activity, photosynthesis), reproduction (attracting birds and insects for pollination) and ecologic functions contained defense against invading organism, and other stress factors (Stalikas, 2007; Schijlen, 2004; Heim, 2002; Parr & Bolwell, 2000). Furthermore, phenolic compounds were also useful for taxonomic reasons (Bravo, 1998).

1.4. Phenolic compounds in foods

Phenolic compounds are found in almost every plant-derived food. The main sources are fruits, vegetables, cereals, legumes, and nuts (Tura, 2002; Bravo, 1998). The main sources of the different phenolic classes;

Hydroxy-benzoic acids: Berries, grapes, oranges, kiwi, apples, peaches, grapefruit, pears, cherries, potatoes, cereals, olives, vegetables, wine, propolis (Robbins, 2003; Tomas-Barberan, 2000a).

Hydroxy-cinnamic acids: Apples, pears, cherries, plums, peaches, apricots, blueberries, white grapes, kiwi, tomatoes, coffee, white wine, cider, citrus juices, potatoes, olives, vegetables, cereal, brans (Robbins, 2003; Clifford, 2000b).

Proanthocyanidins: Grapes, cherries, apples, pears, blueberries, raspberries, cider, red wine, beer, cocoa bean, cereals (Santos-Buelga, 2000).

Tannin-like compounds: Tea and wine (Santos-Buelga & Scalbert, 2000).

Ellagic acid and ellagitannins: Raspberries, strawberries, blackberries, nuts (Clifford & Scalbert, 2000).

Flavonoids previously shown in Table 1.1

However, the best dietary source also depends on cultivation practices and on the precise dietary composition (Tura, 2002).

1.5. Phenolic compounds and human health

In general, a diet rich in fruit and vegetables has been linked to various favorable properties on human health, such as decreasing the risk of coronary heart disease (Dauchet, 2006), cancer (Zhang, 2009), hypertension (Mignone, 2009), diabetes, and inflammatory processes (Zafra-Stone, 2007). The constituents responsible for these protective effects include some vitamins (e.g. A, C, E, and folate), minerals (e.g. potassium, zinc, and selenium), carotenes, dietary fiber and phenolic compounds (Anderson, 2009; Mignone, 2009; Muhammad, 2006; Dillard, 2000). Phenolic compounds are considered to be antioxidants, scavengers of free radicals, metal chelators, anti-mutagens, and signaling agents (Dillard, 2000; Bravo, 1998). There is a wealth of epidemiological evidence on the impact of diet and lifestyle on the risk of developing chronic diseases, but in the case of fruit and vegetable consumption the results are sometimes inconsistent (Huxley, 2009). Although, negative effects of phenolic compounds have not been shown in the literature, some of them can be harmful for human health when they are consumed in large amounts. The best-known problems related of phenolic compounds are the ability to precipitate proteins, formation of complexes with polysaccharides, ability to influence lipid metabolism and interfering with the bioavailability of metal ions (Bravo, 1998).

1.6. Radicals and their biological effects

1.6.1. Free radicals

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; Halliwell, 1996). 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.

1.6.2. Types of Reactive Oxygen Species and their generation

The major reactive oxygen species present in the cells are superoxide, hydrogen peroxide, hydroxyl radical, and nitric oxide. Superoxide anions are formed by an electron addition to the molecular oxygen. It is not as reactive as other ROS. It is formed with the respiratory chain in an electron-rich environment in the vicinity of inner mitochondrial membrane. Hydrogen peroxide (H_2O_2) is produced by enzymes such as superoxide dismutase (SOD), NADPH-oxidase, glucose oxidase, and xanthine oxidase (Jonas, 2001). The hydroxyl radical is very reactive compared with other radicals. It is formed from hydrogen peroxide in a reaction known as Fenton reaction that is catalyzed by metal ions (Fe2+ or Cu2+) (Halliwell, 1999; Halliwell, 1987). Nitric oxide (NO) does not react readily with biomolecules. It is synthesized from L-arginine by the enzyme NO synthase (NOS) (Andrew, 1999; Beck, 1999; Bredt, 1999).

1.7. 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; Patel, 2000). Moreover, ROS also take part in intracellular signal transduction as secondary messengers for several cytokines, growth factors, hormones, and neurotransmitters (Thannickal and Fanburg, 2000). Another vital role of radicals is to provide protection against infections. For instance, active phagocytes produce ROS which destroy bacteria entering the cells (Thomas, 1988).

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).

1.7.1. Importance of antioxidants

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 nutraceuticals. 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, 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. heckeliana* root extract.

1.8. Antioxidants classification based on their sources

Antioxidants can be classified into two classes as natural or synthetic antioxidants. Natural antioxidants are extracted from plant and animal sources. Synthetic antioxidants are prepared synthetically in the laboratory

1.8.1. Natural antioxidants

Natural antioxidants such as eugenol, vanillin, rosemary, and vitamins such as vitamin C and vitamin E can act as primary antioxidants (Rajalakshmi, 1996; Niki, 1997) and are efficient radical scavengers, other naturally occurring antioxidants such as thiols, sulfides, free amino groups of proteins, carotenoids act as secondary antioxidants. Chelating agents such as citric acid and phytic acid are also available naturally. The antioxidants present in cells such as superoxide dismutase, enzymes that metabolize reactive oxygen species, superoxide reductase that catalyzes direct reduction of superoxide, catalases that catalyze dismutation of hydrogen peroxide to water and molecular oxygen, glutathione-related systems, selenium compounds, lipoic acid, and ubiquinones are other examples of naturally occurring antioxidants (Table 1.3). Polyphenolic compounds are an important group of natural antioxidants. Phenols contain one aromatic ring with a minimum of one hydroxyl group. Polyphenols contain a minimum of two aromatic rings with a minimum of one hydroxyl group in each aromatic ring (Lazarus et al., 2001).

Table 1.3: Some natural antioxidants and their sources (Pokorny, 1991)

Natural antioxidants	**Sources**
Tocopherols,tocotrienols,sesamol, phospholipids, olive oil resins	Oils and oil seeds
Several lignin-derived compounds	Oats and rice bran
Ascorbic acid, hydroxycarboxylic acids, flavonoids, carotenoids	Fruits and vegetables
Phenolic compounds	Spices, herbs, tea, cocoa
Amino acids, dihydropyridines, Maillard reaction products.	Proteins and protein hydrolysates
Catechin, Epicatechin, Myricetin, Quercetin, Kaempferol	Teas

1.8.2. Synthetic antioxidants

Synthetic antioxidants are prepared synthetically in the laboratory. They are generally phenolic compounds. Therefore, the mechanism of their reaction with radicals is the same as that of phenolic antioxidant compounds, i.e. they act as chain breaking antioxidants and involve transfer of a hydrogen atom or an electron to radicals such as BHA and BHT.

Many synthetic antioxidants (butylated hydroxyanisole (BHA), propyl gallate, butylated hydroxytoluene (BHT), and tert-butyl hydroquinone) are approved to be safe and used as food additives to increase shelf life and prevent oxidative damages

1.9. Determination of phenolic compounds in plants

1.9.1. DPPH radical scavenging capacity assay

As DPPH assay is simple and highly sensitive compared to other methods, it has become widely used in natural antioxidant studies. Assay basically depends on stable organic nitrogen radical property of DPPH that gives a deep-purple color in ethanol. The colour fades upon reaction with phenolic compounds in the test solution (Huang, 2005). The reaction is monitored at 515 nm until it reaches the plateau. The percentage of DPPH• remaining in the solution is calculated and the reduction is proportional to the concentration and strength of antioxidants (Sánchez-Moreno, 1998; Brand, 1995).

The concentration of antioxidants that decreases the initial DPPH• concentration by 50% is defined as EC_{50} and the time needed to reach the plateau at that concentration (Prior, 2005). The drawbacks are mainly due to the reactivity of the DPPH• radical, which may react slowly or not at all with some antioxidants, and possible interference with compounds that present UV-Vis absorption maxima around 515 nm, leading to underestimations (Huang, 2005).

1.9.2. (ABTS•+) radical cation decolourisation assay

After the reaction between ABTS and potassium persulphate, the blue/green ABTS•+ is formed and the absorbance was adjusted to 0.7 ± 0.02 at 734 nm by diluting in ethanol. The reduction of the radical when mixed with antioxidants is monitored by spectrophotometric readings after 1 min and 6 min and results are expressed as Trolox equivalents (Re, 1999). In biological systems, there are multiple free radicals and oxidant sources and antioxidants may respond in very different ways to them through single or multiple mechanisms (Prior, 2005). The assays are useful as a first step in the evaluation of interactions between food items, extracts or supplements and reactive oxygen species (ROS) that can be potentially harmful and in the search for possible sources of antioxidants for further applications in food products or health (Pérez-Jiménez, 2008; Espín, 2007; Prior, 2005).

1.9.3. Determination of Total Phenolics Quantification by using Spectrophotometric Methods

In order to quantify the plant phenolics, there are several spectrophotometric methods developed. Each assay bases on different principles and each are aimed to determine different structural groups present in phenolic compounds. In order to determine total phenolics content, the Folin–Ciocalteu assay (Tsao, 2003; Lapornik, 2005) is widely used. On the other hand, a colorimetric method based on the complexation of the phenolic compounds with Al (III) (Naczk, 2006) is used to determine total flavonoids content.

1.10. Cell based assay

1.10.1. Cancer cell proliferation studies of phenolic compounds

Antioxidant, anti-inflammatory and anticancer activities are some of the various photochemical bioactivities that take place in plants. According to some studies, extracts from natural products, such as fruits, vegetables and medicinal herbs, are more effective against cancer, compared to chemotherapy or recent hormonal treatments (Pezzuto, 1997; Wu, 2002). Thus, several plants have been examined whether they have new and effective antioxidant and anticancer capacities (Pietta, 1998; Kang, 1998; Swamy and Tan, 2000).

Extract of these natural products can interact with cellular pathways such as cell proliferation and apoptosis and finally may have effects on cell cytotoxicity and cell survival. This study was designed to investigate the anti-oxidative properties and cytotoxic effect of *R. heckeliana* root extract.

1.10.2. MCF-7 and MDA-MB-231 cell lines

MCF-7 is a breast cancer cell line isolated in 1970 from a 69-year-old Caucasian woman. As MCF-7 cells process estrogen, in the form of estradiol, via estrogen receptors in the cell cytoplasm, they are said to be estrogen receptor (ER) positive control cell line. With this property, MCF-7 cells have several ideal characteristics particular to the mammary epithelium

The MDA-MB-231 breast cancer cell line was obtained from a patient in 1973 at M. D. Anderson Cancer Center. Having epithelial-like morphology and lacking estrogen and progesterone receptor, the MDA-MB-231 cell line with as spindle shaped cells, has a highly invasive phenotype

In this study MCF-7 and MDA-MB-231 cell lines were used for the cytotoxicity studies.

1.10.3. Cytotoxicity

Cytotoxicity refers to properties which caused poisonous effects to cells by chemical substance, or by an immune cell. In cytotoxicity assays, drug-induced changes in structural integrity or in metabolic pathways, are measured which can be related to cell death, although in survival assays, ultimately metabolic perturbations that are measured depending on the cell recovery or cell death. There are many techniques such as XTT, MTT and MTS dye exclusion test, available to monitor the cytotoxic effects of compounds/extract of interest on the cell viability.

1.10.4. XTT assay principles

In the 1950's, the use of tetrazolium salts (such as XTT or MTT) were first rendered as a new technique. It was based on the fact that lives cells converts tetrazolium salts into colored formazan compounds. In determining the viability of cells the technique was found to be very helpful. Activity of mitochondrial enzymes which are inactivated shortly after cell death confirms the basis for this biochemical procedure. A colorimetric method based on the tetrazolium salt, XTT (sodium 3,3'-{1-[(phenylamino) carbonyl]-3,4-tetrazolium}- bis (4-methoxy-6-nitro) benzene sulfonic acid hydrate), was first manifested in 1988 by P.A. Scudiero.

The application of MTT caused the formation a non-soluble formazan compound which is required dissolving the dye for its measurement. However the application of XTT produces a soluble dye, it simplifies the procedure of measuring proliferation in a wide range of concentration. Therefore it is a perfect solution for finding the quantities about cells and determining their viability without requiring the use of radioactive isotopes.

1.11. Scope of thesis

This study was designed to investigate the anti-oxidative and cytotoxic properties of *R. heckeliana* root extracts. The primary objective of this study is to determine the antioxidant capacity using different methods, find out its phenolic and flavonoid content and to identify potential antioxidant compounds.

Isolation and identification of important bioactive phenolic compounds from the roots was carried out by utilizing the simple column chromatography (Silica gel 60 and Sephadex LH-20), RP-HPLC equipped with the photo diode array and fluorescence detectors and UV-Vis spectroscopy. Isolates then were identified and characterized using NMR (Carbon and Proton) and HR-MS techniques

Furthermore, the isolated compounds were examined for their anti-oxidative activity and for their cytotoxic effects on the MCF-7 and MDA-MB-231 cells by using XTT assay method.

CHAPTER 2

MATERIALS AND METHODS

2.1. Materials

2.1.1. Plant material

R. heckeliana were collected from a shrub, in the month of June or July, growing between the altitudes of 1300–2900 m, especially in Van region, in East Anatolia. After collecting plants, herbarium samples were pressed according to the herbarium rules just before losing morphology.

The plant materials were identified by assistant professor Fevzi Ozgokce, Deparment of Botany, Faculty of Biology, Yüzüncü Yıl University, Van, TURKEY. The voucher specimen has been deposited in Herbarium of Yüzüncü Yıl University (Voucher specimen NO. F5474).

2.1.2. MCF-7 cell lines

MCF-7, which constitutes the basic properties of a breast cancer cell line, was purchased from ATCC (American Type Culture Collection).

2.1.3. MDA-MB-231 cell lines

MDA-MB-231 is a breast cancer cell line was purchased from ATCC (American Type Culture Collection).

2.1.4. Chemicals and other materials

The extracts essential for our study, were prepared by using the chromatography grade methanol. And for the solvent system preparation, we used HPLC grade methanol, and acetonitrile. These two material groups were purchased from Merck (Darmstadt, Germany). In order to obtain ultra pure water, Milli–Q system was used. This system was produced by Milli–pore, Bedford, MA, USA.

For the fractionation process, chromatography grade petroleum ether, chloroform, ethyl acetate, and n-butanol were purchased from Merck (Darmstadt, Germany) and and magnesium sulfate was purchased from Sigma Chemical Company (St.Louis, MO, USA).

Disposable syringe filter (pore size: 0.22 µm and 0.45 µm Diameter: 33mm) was purchased from Millipore Corporation (Bedford, MA USA) and syringe filter (0.20 µm Minisart RC 4), from Sartorius (Gottingen AG, Germany)

Folin–Ciocalteu reagent, 1, 1-Diphenyl-2-picryl hydrazyl (DPPH) and the reference compounds such as gallic acid, hydroquione, ascorbic acid, esculin, catechin, caffeic acid, epicatechin, p-coumaric acid, scopoletin, ellagic acid, quercitrin, quercetin, apigenin, and hesperetin were all purchased from Sigma–Aldrich (St. Louis, MO, USA). Aluminium chloride ($AlCl_3$) was from Merck.

Column chromatographic separations were performed by using Silica Gel 60 with a particle size of 0.063–0.200 mm and thin layer chromatography (TLC) was performed by silica gel 60 F_{254} using 0.25 mm aluminium coated plates purchased from Merck. Sephadex LH-20 was bought from Pharmacia (Sweden).

Dimethyl sulphoxide (DMSO), Roswell Park Memorial Institute (RPMI 1640) medium 4-(2-hydroxyethyl)-1- piperazineethanesulfonic acid (HEPES) modified, Fetal-Calf Serum (Heat-inactivated) were purchased from Biochrom Ltd. (Cambridge, UK). L-glutamine, gentamicin, steril Dulbecco's phosphate buffered saline (sPBS), sodium 3,3'-{1-[(phenylamino)carbonyl]-3,4-tetrazolium}-bis(4-methoxy-6-nitro) benzene sulfonic acid hydrate (XTT), phenazine metho-sulfate (activator), were purchased from Biological Industries (Haemek, Israel). Tryphan Blue (Biological Industries). For cell culture studies, all the reagents and chemicals were cell culture grade.

2.1.5. Instruments

All the spectroscopic data were obtained by using Cary 50 Bio UV-Vis spectrophotometer (Varian). Other instruments used for the experiments are listed as followings: Bandelin Sonorex (ultrasonic bath); Optic Ivymen System (incubator); rotary evaporator (Heidolph Laborota 4000); lyophilizator (Heto-Holten Model Maxi-Dry Lyo); blender: Waring model 32BL80 (New Hartford, CT, USA). Hamilton injector (710 Nr), NMR (Bruker-Spectrospin 400 ultrashield) 100 MHz ^{13}C-NMR and 400 MHz ^{1}H-NMR) was used in Chemistry Department, METU. High resolution-mass spectrometry (electrospray), HR-MS (ESI) data were measured with Q-TOF mass spectrometer.

Equipment used for cell culture:
Laminar Flow: Class II Safety Cabinet (Teknomar) was used for anti-proliferative assays. Hemocytometer: Naubauer (Bright-line, Hausser Scientic, Horseam) (use for counting blood cells), phase-contrast microscopy: Olympus CKX 41, microscopy: Olympus CX 31, CO_2 incubator: Hera Cell 150 (Kendro).

ELISA, 96-well microplate reader (Bio-tek, Elx808, Germany) was used from Prof. Dr. Mesude İşcan Laboratory in Biological Sciences Department, METU.

2.2. Methods

2.2.1. Extraction procedure

Roots of *R. heckeliana* were grinded to obtain a 2 -4 mm particle size with Waring (model 32BL80) commercial blender at a high speed for at least 3 minutes. Then they were stored in dry, dark and tempered conditions (RT) until use. To obtain the root extracts, roots (120 g) were incubated in 1:10 ratio of methanol for 24 hours in oval-rotating incubator rotating at 180 rpm at 25 °C temperature. Then, mixtures were filtered through a double layered cheese cloth or a rough filter paper. This procedure was repeated 3 three times with the addition of methanol solvent each time as described in the literature (Coruh et al., 2007). Collected filtrates were brought to a complete dryness using a rotary evaporator (Heidolph Laborota 4000) under vacuum at 40 °C. Dried crude extract samples were weighed for the yield calculation.

2.2.2. Fractionation procedure

For the fractionation of crude extracts, the solvents were applied gradually, from non–polar to polar (Table 2.1). The fractionation method which was applied to obtain the crude extract is described by Naczk and Shahidi (2004).

Table 2.1: Physical constants of organic solvents were used in this study. They are listed according to their polarity index numbers, which are increasing gradually.

	Formula	**Formula Weight**	**Density**	**Boiling point**	**Polarity index**
P.ether	C_6H_{14}	86.18	0.77	40-60	0.0
Chloroform	$CHCl_3$	88.11	1.48	61.7	4.10
Ethyl acetate	$CH_3COOC_2H_5$	119.39	0.90	77.1	4.40
Methanol	CH_3OH	32.04	0.79	64.7	5.1
n-buhanol	C_4H_9OH	74.12	0.80	117.7	4.0*
Water	H_2O	18,02	1.0	100	9.0

*n-butanol was saturated with water at a ratio of 1:1 before use

At first, 20.12 g of crude methanol extract was dissolved in a 500 mL methanol/water mixture (70:30 v/v) and immediately after this dissolving process, 500 mL petroleum ether was added in a separator funnel. This mixture was shaken vigorously for some time and it was kept in a steady state until the organic and aqueous phase were completely separated. The aqueous phase sank to the bottom of the separator funnel. It was separated from the organic phase by draining. This aqueous phase was put in a beaker for further fractionation procedure. The remaining organic phase (petroleum ether) in the separator funnel was transferred into a rotary evaporator for evaporation. This separation procedure was repeated three times for the aqueous phase, enabling us to get a completely transparent pure organic phase.

The organic phase (petroleum ether) that was acquired from these separating operations was then evaporated to dryness and kept at +4 °C until analysis stage. Further steps of fractionation continued to separate aqueous phase and organic solvents, which were applied according to their increasing polarities. The secondary fractionation was carried out by mixing chloroform and aqueous phase. The other organic solvents were ethyl acetate and n–butanol subsequently. Each of these fractionated phases was evaporated to dryness and kept later at +4 °C. The yield of each fractionation step was determined in terms of (w/w) % per 20.12 g crude extract. The extraction and fractionation procedures are summarized in Figure 2.1.

***R.heckeliana* root (120g)**

Extraction with methanol

Filtered and concentrated

Crude extract dissolved in methanol-water (70:30, v/v) and mixed with petroleum ether and vigorous shaking.

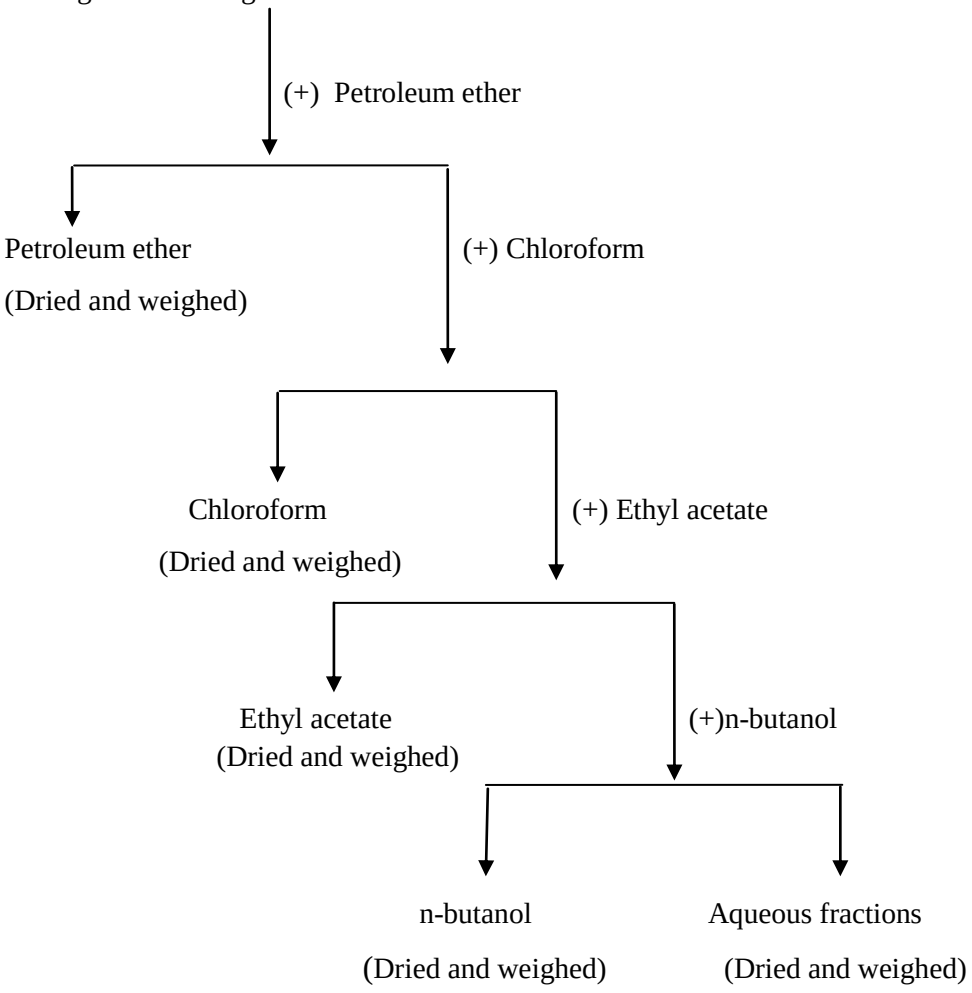


Figure 2.1: Sequential organic extractions and fractionation of *R.heckeliana* roots.

2.3. Determination of in vitro Antioxidant Capacity

2.3.1. Free radical scavenging capacity by DPPH method

DPPH radical scavenging activity method of Blois *et al*, (1958) was applied with some modifications as previously mentioned by Coruh *et al* (2007). In briefly, a 20 mg DPPH radical dissolved in 400 mL absolute ethanol to get around 1.3-1.4 unit of absorbance at 517 nm (0.05 mg/mL DPPH solution). DPPH radical (2,2-diphenyl-1-picrylhydrazyl radical) scavenging capacity of the crude extract and the purified isolates along with the standard reference compounds was determined by monitoring the decrease due to depletion of DPPH radical in the mixture after the 15 minutes of reaction time (Figure 2.2). The results were expressed as the radical scavenging capacity (RSA) in percent and calculated as

$$\text{Radical Scavenging Activity (RSA) (\%)} = [(A_0 - A_1)] / A_0 \times 100$$

where A_0 is the absorbance of DPPH alone in ethanol and A_1 is the absorbance of DPPH in ethanol in the presence of the samples. EC_{50} values are used to evaluate the antioxidant activity. EC_{50} values were calculated by constructing the plot of RSA (%) versus log (extract concentration).

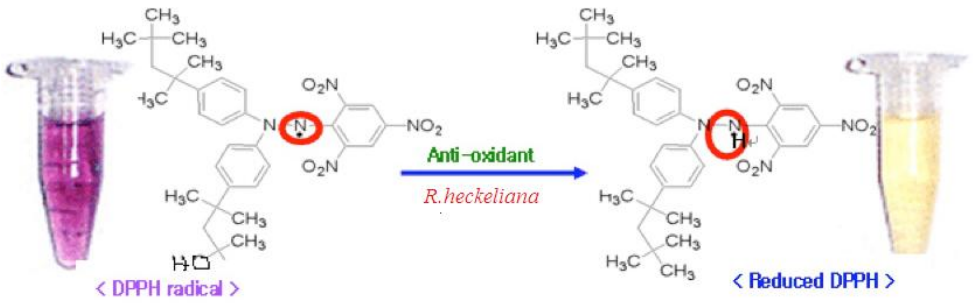


Figure 2.2: Schematic demonstration of the reduction of DPPH with chemical reaction (http://www.naturalsolution.co.kr/tech21e.html)

2.3.2. Free radical scavenging capacity by ABTS method

The antioxidant capacity was determined using the ABTS method, which is based on scavenging a radical cat-ion, and the ability of its antioxidant compounds to scavenge free radicals is expressed as trolox vitamin E analogue equivalent (Rice–Evans, 1997).

The generation of the ABTS radical involves the reaction between ABTS and potassium per-sulfate ($K_2S_2O_8$) which results in the direct production of a green $ABTS^{\cdot 1}$ chromospheres as shown in Figure 2.3. Addition of antioxidant reduces the generated radical, and the decrease of the absorbance is measures as 734 nm (0.7 ± 0.02 a.u). Chemical reaction stops end of the 5 minutes and the absorbance value is measured.

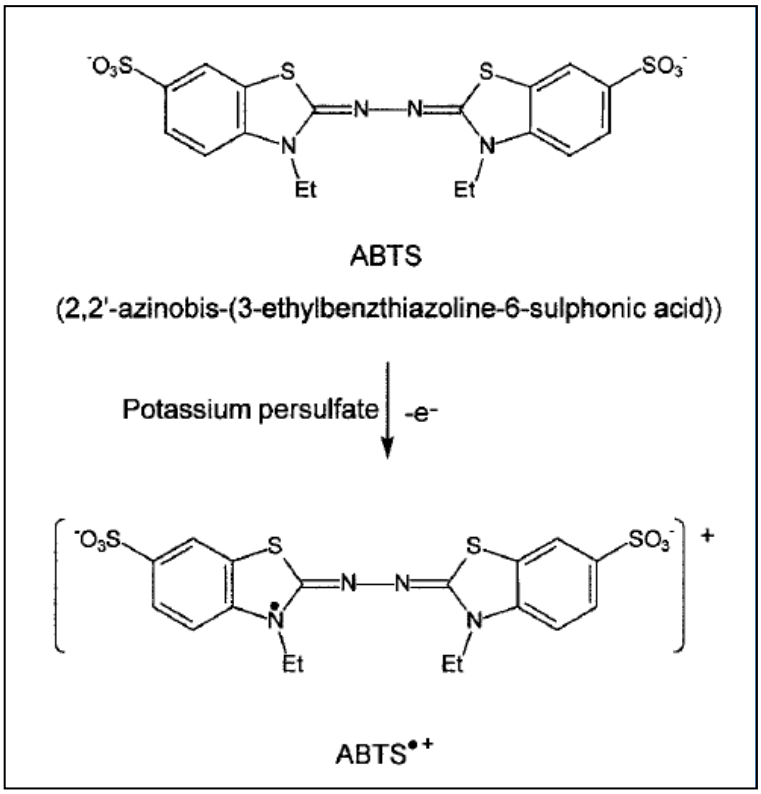


Figure 2.3: Formation of ABTS radical cat-ion (Pannala, 2001)

A solution of 7 mM ABTS and 2.45 mM potassium per-sulfate was prepared for the production of the ABTS radical cat-ion (ABTS•+). The solution was kept in a dark room for 16 hours at room temperature. 100 ml of 96 % ethanol (0.7 ± 0.02 a.u) was added before use to dilute the solution. The percentage of ABTS radical scavenging activity (RSA) was determined using the following formula;

$$\text{Radical Scavenging Activity (RSA) \%} = [(A_0-A_1)/A_0] \times 100$$

Where:

A_0 = the absorbance of ABTS solution which includes methanol
A_1= the absorbance of ABTS solution which contains Trolox as standard or extract at various concentrations.

The standard curve is constructed after radical scavenging activity is calculated. The radical scavenging activities of plant extracts as were subsequently calculated as equivalents of Trolox according to the standard curve formula;

$$y = mx + n$$

Where:

y = radical scavenging activity (%)
n = concentration

(x) solves for extract concentration as trolox equivalent. The experiments were repeated for three different experimental preparations and were done in duplicates.

2.3.3. Determination of total phenolic content

The Folin-Ciocalteu method was used to determine total phenolic content of the extract and fractions as proposed by Singleton and Rossi (1965). Gallic acid as standard in varying concentrations, crude extract and fractions were prepared in ethanol for the evaluation. Each of 100 µL of samples was added to a test tube filled with 100 µL of % 50 folin-ciocalteu reagent, was vortex mixed for a couple of seconds and mixed with 2000 µL of 2 % Na_2CO_3. The mixture was left for incubation in dark at room temperature for 30 minutes. At the end of the reaction (time), absorbance values at 750 nm were compared against blank, which contains only 100 µL pure ethanol without (the addition of) any sample. A curve of gallic acid concentration (prepared as 0.05, 0.1, 0.2, 0.3 mg/mL) versus absorbance curve was plotted. The amount of phenolic content of dry samples was measured expressed as gallic acid equivalent (GAE) value, with the help of a calibration curve plotted using the curve mentioned above. The experiments were carried out in duplicates), repeated three times independently.

2.3.4. Determination of total flavonoid content

The total flavonoid quantity in the sample extracts can be determined by making use of the reaction of sodium nitrite with aluminum chloride with flavanoids present, which generates the colored flavonoid-aliminum complex, as proposed by Bakar, 2009. Determining total flavonoid content is possible by monitoring this complex spectrophotometrically at 510 nm. The method used for the determination of total flavonoid quantity was a slightly modified version of that described by Zhishen et al. (1997). Solutions containing 0.2 ml of catechin in various concentrations, crude extract and all fractions were prepared and diluted. The same process was applied for samples and standard. 0.75 mL of 5% $NaNO_2$ was added. After incubating at room temperature for 5 minutes, 0.15 mL of 10 % $AlCl_3$ was added. At the end of 5 minutes, 0.5 mL 1 M NaOH, and adequate amount of water was added to complete the solution to 3 mL. After mixing the the solution well, measurement of absorbance was performed against blank solution at 510 nm immediately. Each extract solution was subjected to the same procedure. Graphs of absorbance at 510 nm against concentration were plotted to construct the calibration curves of the standard and extract. The catechin standard curve equation $y = mx + n$ was used to calculate total flavonoid content as catechin equivalent.

2.4. Column chromatography

2.4.1 Preliminary purification

Once a suitably polar plant extract is obtained, a preliminary cleanup is advantageous. Solvent partition may be applied to remove a large proportion of extraneous material for preliminary purification of samples which are to be separated by liquid chromatography (Hostettmann, 1997). Solvent partitioning depends on solubility of compounds in two different solvents (see fractionation process).

The fracionation of crude extracts in this study was achieved by using solvents, according to their polarity values, scaled from non–polar to polar. The method used for this fractionation was first described by Naczk and Shahidi (2004).

2.4.2. Thin Layer Chromatography (TLC)

Thin layer chromatography is commonly used to separate material because the method development and operation requires minimal time and cost investment (Ahuja, 2003). Separation of compounds is based on the competition of the solute and the mobile phase for binding places on the stationary phase. Detection is mainly performed using UV light at 350-365 and 250-260 nm (Stalikas, 2007).

TLC can be considered as a micro version of column chromatography. It is used to quickly determine purity of mixture number of compounds contained and the combination of eluent to separate the compounds. The solvent system for column chromatography can be efficiently selected by using TLC. On the other hand, TLC requires an absolute minimum of sample preparation (Andersen, 2006).

TLC analysis for ethyl acetate fraction and its sub-fractions screening were performed on silica gel 60 F_{254} (Merck, 0.2mm) plates using chloroform: methanol: ethyl acetate (3:0.75:1) as the mobile phase. The plates were visualized by spraying with sulphuric acid: methanol (5:95) spray solution followed by heating at 100 ºC.

2.4.3. Injection of extract/fraction solution

After the extract/fraction was completely solved in the required amount of the solvent/solvent system, it was applied to the column (Silica gel 60 and Sephadex LH-20) with a micropipette (5 mL). The valve of the column was opened to let the adsorbent absorb the extract/fraction. The elution was initiated by adding the solvent. This process was carried out slowly to avoid any disorders on the adsorbent's surface.

2.4.4. Silica gel 60 and Sephadex LH-20

Column chromatography is a method commonly used for purifying individual compounds that depends on polarities of stationary phase, eluents and compounds. This method is based on the interaction of polar compounds and silica, which causes separation of the molecules in the target extract/fraction. The separation depends on the degree of eluent's polarity. Retention factors were determined by thin layer chromatography. The same eluent system was subsequently used in the column chromatography. The amount and the number the compounds in the mixture were taken into account in choosing column size and type of silica (Skoog, 1997).

Silica gel 60: The required amount of silica gel 60 (Darmstadt, Germany) was obtained with a scale. A suspension was prepared with adequate amounts of solvent system. This mixture was transferred into a glass column (3.5-cm diameter and 110 cm height; 2-cm diameter and 60-cm height). After a required amount of the solvent system was flowed through the column, the adsorbent settled. Some solvent, 2-3 mm thick, was left on the adsorbent after a while. The obtained extract/fractions/sub-fractions which was solved in this process was applied to the column. We used silica gel column chromatography to acquire pure phenolic compounds in target fractions.

Sephadex LH-20: 10–50 g of sephadex LH-20 (Pharmacia, Sweden) was weighed and mixed with an adequate amount of methanol. The glass column (2-cm diameter and 60-cm height) was filled with the filling material and it was flowed through the column until the material settled well. This process was maintained until methanol solvent, 2-3 mm thick, was left on the adsorbent. A, 0.366 g sample of dried fraction 4 powder was dissolved in 3 mL methanol solvent and was introduced to the top of the column. The same solvent was used for elution.

In this study, the purification of the phenolic compounds root extract of *R. heckeliana* were done by using silica gel 60 and sephadex LH-20 column chromatography.

The ethyl acetate fraction (1.52 g) of roots extract of R. *heckeliana* was first subjected to column chromatographic separation on Silica gel 60 (70-230 mesh, particle size 0.063–0.200 mm) then loaded into open column using chloroform: methanol (9:1) 100%, chloroform: methanol (80:20), chloroform: methanol (70:30), chloroform: methanol (60:40), chloroform: methanol (50:50) and methanol 100% were used to elute the column with a gradient. Gradient elution with chloroform-methanol was applied and yielded 212 fractions which were pooled together according to their TLC. Nine main fractions (Fr.1-Fr.9) were collected and monitored by TLC using chloroform: ethyl acetate: methanol (3:1:0.75, v/v) as mobile phase. Mobile phase is determined by TLC analysis for silica gel. The flow rate was 1 mL/min. Samples were collected at the end of every 10 min (10 mL/tube). Collected fractions were freeze-dried after removing the organic solvent under vacuum (40 °C).

Fraction 2 (273 mg), fraction 4 (366 mg) and fraction 6 (131 mg) showed stronger antioxidant activity while other fractions did not show little or no antioxidant activity, hence these fractions (Fr 2, Fr 4 and Fr 6) were further chromatographed using sephadex LH-20 (Pharmacia, Sweden) and silica gel 60 (Darmstadt, Germany) for the isolation of phenolic compounds.

Fraction 2 (273 mg) was re-chromatographed on silica gel 60 column with chloroform: ethyl acetate: methanol (3:1:0.75, v/v) as mobile phase and gave 5 sub-fractions (Fr.2A-Fr.2E).). Sub-fraction Fr.2C (99 mg) was chromatographed on a silica gel 60 column (70–230 mesh) and eluted with chloroform: ethyl acetate: methanol (3:1:0.75, v:v).

Fraction 4 (366 mg) was submitted to column chromatography on sephadex LH-20, eluted with methanol 100 %. Four sub-fractions (Fr.4A-Fr.4D) were collected and checked by TLC using ethyl acetate: chloroform (4.5:0.5, v/v). Sub-fraction Fr.4B (137 mg) was chromatographed on a silica gel 60 column (70–230 mesh) and eluted with chloroform: ethyl acetate: methanol (3:1:0.75, v/v).

Fr.6 (131 mg) was applied to separation by a silica gel 60 (70-230 mesh), eluted with chloroform: ethyl acetate: methanol (3:1:0.75, v/v). The chemical structures of the isolated compounds from ethyl acetate fraction of *R. heckeliana* root extract were identified by UV-Vis, RP-HPLC, NMR (Carbon and Proton) and confirmed by HR-MS. All of the compounds were identified by comparing with commercial standards.

2.5. Spectroscopic techniques

2.5.1. Preparation of crude/fractions/standards for HPLC analysis

The crude/fractions/standards were prepared prior to HPLC analysis by re-suspending them in HPLC grade methanol. After preparing a stock solution for all samples, diluted solutions were prepared and filtered through a 0.20 μm syringe filter (Minisart RC 4 single use syringe filter, Sartorius). The filtered solution then was injected to the injection loop of HPLC instrument.

2.5.2. Analysis by UV-Vis spectroscopy

Phenolic compounds also commonly detected using UV-Visible spectroscopy. No single wavelength is ideal for phenolic compounds (flavonoids, phenolic acid, etc.) since they display absorbance maxima at distinctly different wavelength. The most commonly used wavelength for routine detection is 280 nm which represents a suitable compromise.

The absorption spectra of purified compounds and unknown peaks in methanol were recorded in 210-600 nm and their absorption maxima were determined.

2.5.3. Analytical HPLC analysis

RP-HPLC is the most popular technique for the separation of phenolic compounds both on analytical and preparative scales. Analytical RP-HPLC analysis was performed on a Waters multi-solvent delivery system equipped furnished with an online degasser unit, and equipped with photodiode array and fluorescence detectors, enabling the separation, identification and quantification of the phenolics from the methanol extract of *R. heckeliana* roots.

An optimal elution program that results in a good resolution was found on the following conditions for extract/fractions/standards.

Two mixtures made up the mobile phase: an aqueous solution (A), and a solution consisting of acetonitrile, methanol and 2% acetic acid in a ratio of 2:2.5:1 (B). The gradient profile was linear, starting with 99 % from 0 to 10 th min, 85 % at 11th min., 65 % at 50th min and reaching 0 % at 74th-85th minutes of solution A. The column was then washed with 100% solution B and a time period of 10 min was used for re-equilibration. All runs were done with the flow rate of 1.3 mL/min and the constant column temperature of 25 °C.

A reversed-phase Waters Symmetry Column was used for stationary phase: C18, 4.6 x 150 mm, 5μm (4.6 mm: column internal diameter; 150mm: column length; 5 μm: column dimension)

The diode array detector observed absorption spectra in the range of 210-800 nm. Chromatograms of extract, fractions and standards were obtained using the following maximal absorption wavelengths in nm: 254, 280, 320 and 360.

2.5.4. Nuclear Magnetic Resonance Spectroscopy (NMR)

Proton and carbon NMR are used to characterize the structure of column chromatography isolated photochemical purified from the ethyl acetate fraction. All the purified samples were dissolved in deuteriated dimethyl sulphoxide (DMSO-d_6). Nuclear magnetic resonance (NMR) spectrometer was used Bruker-Spectrospy 400 ultra shield (100 MHz for ^{13}C-NMR and 400 MHz for ^{1}H-NMR, respectively in chemistry lab, METU.

2.5.5. High Resolution Mass Spectrophotometer (HR-MS)

HR-MS analysis was performed to confirm the phenolic compounds (known/unknown) detected in RP-HPLC analyses. LC was performed with a Waters Acquity UPLC (Waters Corp.) and a Waters Acquity UPLC BEH Shield RP18 (1.7 μm, 1 x 100mm, Waters) analytical column (Waters Corp.). The mobile phase consisted of (A) water and (B) MeOH: ACN and water containing 2 % acetic acid in ratio of 2,5:2:1. The gradient was linear with 90 % from 0 to 30 th min, 40 % at 35th min. 20 % at 45th minutes of solution A. The flow rate was 0.06 mL/min. The injection volume was 2 μL. The raw data were acquired and processed with MASSLYNX.

2.6. Cell Culture

2.6.1. Cell lines and Growth Conditions

MCF-7, which is one of the most widely used a breast cancer cell line, were grown in medium RPMI-1640 with 10 % heat-inactivated fetal calf serum and 0.2 % gentamicin and incubated at 37 °C in a 95 % humidified atmosphere of 5 % CO_2.

MDA-MB-231, a breast cancer cell line, was grown in a 5% CO_2 humidified incubator at 37 °C, using a medium which consisted of RPMI-1640 cell culture medium with phenol red supplemented with 10% fetal calf serum and 0.2% gentamicin.

The RPMI-1640 medium was collected and replaced with fresh medium every 2-3 days for the purpose of maintaining a constant ratio of number of cells to amount of growth medium.

2.6.2. Cell Passaging

The cells were passaged at about 80-90 % confluence; at the point where 80-90 % of the flask was covered by monolayer cells. If the cells were passaged at a later confluency stage, the cells would overgrow, detach and eventually die. The medium was removed from the attached cells with the help of a pipette without distribution to the cells. For the purpose of washing off the medium adhering to the cells which were still attached to the flask, 1-2 ml of Dulbecco's phosphate buffered saline (PBS) was used. For detaching the cells from the flask, 1-2 ml trypsin/EDTA was added and left for a period of 2-5 minutes at 37 °C for incubation. Complete medium twice the amount of trypsin/EDTA was added, which stopped trypsinization, since the serum inside the complete medium has inactivatory effect on trypsin. Detached cells were subsequently pipetted up and down in order to achieve cell suspension and to separate cells from each other and to avoid clumping of cells.

2.6.3. Freezing and Thawing

Freezing of the cells was achieved through the use of a freezing medium which contained 10% DMSO in a complete growth medium supplemented with serum. Following the trypsinization, the cell suspension was subjected to centrifugation, due to which the cells were precipitated and the supernatant fluid was decanted. The cells were re-suspended in an adequate volume of freezing medium, taking into account the number of the cells, and were transferred into cryovials. The cryovials were placed in a box containing isopropyl alcohol, which facilitated gradual freezing of the cells, and were stored at the temperature of -80 °C for 24 hours. Subsequently, the cryovials were transferred into a tank which contained liquid nitrogen at about -196 °C.

In the thawing process of the cells, cryovials were taken from the liquid nitrogen tank and put into a water bath at 37 °C. Once the medium had fully liquefied, it was placed very gently into T-25 tissue culture flasks each containing each of which contained 10 ml of complete medium. Then, media were removed by centrifuging (100 g, RT, 5 min) and the cells were washed with 2 ml sPBS by centrifuging twice (100 g, RT, 5 min) to remove the DMSO and cell debris, derived from the freezing medium. Cell pellet were redissolved in fresh complete medium in T-75 flasks. The next day, trypsinization was performed to separate the attached cells, and the cells were passaged to T-75 tissue culture flasks containing 20 ml of medium.

2.6.4. Determination of growth curve

Cells from MCF-7 and MDA-MB-231 breast cancer cell lines were seeded in 6-well plats, at a density of 100000 cells/well, as indicated earlier. Different two wells from each cell line were trypsinized and harvested after 24 hour and the medium was changed for the cells continuing to grow at each time point. Cells were stained with 0.25 % (w/v) trypan blue (Biological Industries, Israel) and counted in a hemocytometer (neubauer) and the average number of 2 wells was used for the growth curve under light microscopy. For the duration of 240 hours, the cells were counted. A growth curve was plotted with number of cells ($x10^4$) / ml against time (hour).

2.7. Cytotoxicity

2.7.1. Preparation of crude extract, ethyl acetate fraction and isolated compounds stock solution

Crude extract, ethyl acetate fraction and isolated compounds, were used as cytotoxic agents in MCF-7 and MDA-MB-231 proliferation studies. 10 mg dry crude extract, ethyl acetate fraction and isolated compounds were dissolved in 5 mL 10 % DMSO prepared in RPMI-1640 medium without phenol red (2 mg/mL).

Preparation of working solutions:

- Dilute this solution (2 mg/mL) 1/5 in RPMI-1640 complete medium w/o phenol red (0.4 mg/mL= 400 µg/mL) **1X extract/fraction/isolated compounds**

- **1.34X crude extract/ethyl acetate fraction/isolated compounds:** Dilute 1X solution 1.34:1 in 2 % DMSO containing complete RPMI-1640 medium (0.3 mg/mL= 300 µg/mL)

- **2X crude extract/ethyl acetate fraction/isolated compounds:** Dilute 1X solution ½ in 2 % DMSO containing complete RPMI-1640 medium (0.2 mg/mL= 200 µg/mL)

- **4X crude extract/ethyl acetate fraction/isolated compounds:** Dilute 1X solution 1/4 in 2 % DMSO containing complete RPMI-1640 medium (0.1 mg/mL= 100 µg/mL)

- **10X crude extract/ethyl acetate fraction/isolated compounds:** Dilute 1X solution 1/10 in 2 % DMSO containing complete RPMI-1640 medium (0.04 mg/mL= 40 µg/mL)

After preparing the working solutions of crude extract, ethyl acetate fraction and isolated compounds solutions of varying concentrations (from 40 µg/ml to 400 µg/mL) were prepared by appropriately diluting the stock working solution in 2 % DMSO containing complete RPMI-1640 medium without phenol red. The working solutions prepared in DMSO were twice more concentrated and crude extract, ethyl acetate fraction and isolated molecules than that of treatment medium. Therefore, DMSO concentration was kept constant at 1%, and crude/fraction/isolated compounds concentrations were varied from 20 to 200 µg/ml in each well during experiment.

2.7.2. Cell viability (trypan blue dye exclusion assay)

Cell membrane integrity and direct counting of living and dead cells was evaluated by trypan blue dye exclusion method. This dye can not penetrate into living cells, but passes through the membranes of dead cells. Prior to use, stock trypan blue solution, 0.5 % (w/v), was diluted to 0.25 % (w/v) with sPBS.

MCF-7 and MDA-MB-231 cells were detached by trypsin/EDTA Cell monitoring becomes possible once the cells were separated and cell suspension is obtained. 20 µl of 0.25 % (w/v) trypan blue solution is added into a different eppendorf tube which contains homogenous mixture of MCF-7 and MDA-MB-231 cells suspension. Final solution in eppendorf tube was further mixed gently several times with pipetting up and down. Once the solution is well mixed, 10 µl of it was loaded in each of the two counting chambers of a Neubauer hemacytometer (Bright-line, Hausser Scientic, Horsham, PA). Finally, under a light microscope, viable and nonviable cell numbers were monitored.

2.7.3. Morphological evaluation

The MCF-7 and MDA-MB-231 cell lines normally grown in 24-well plates or incubated with the desired (over or under the ED_{50} value; 50, 100 and 150 µg/ml) crude extract/ethyl acetate fraction/isolated compounds concentration for the purpose of viability testing were photographed by using an inverted light microscope (Olympus CKX 41) for 24. Any morphological changes in the cells shape, level of adhesion and any other alterations were observed by photography.

2.7.4. XTT (2,3-bis-(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide) assay

The cytotoxic effects of crude extract, ethyl acetate fraction and isolated compounds in MCF-7 and MDA-MB-231 cells were investigated by using Cell Proliferation XTT Kit. Metabolically active cells are able to reduce the tetrazolium salt of XTT to orange colored compounds of formazan (Figure 2.4).

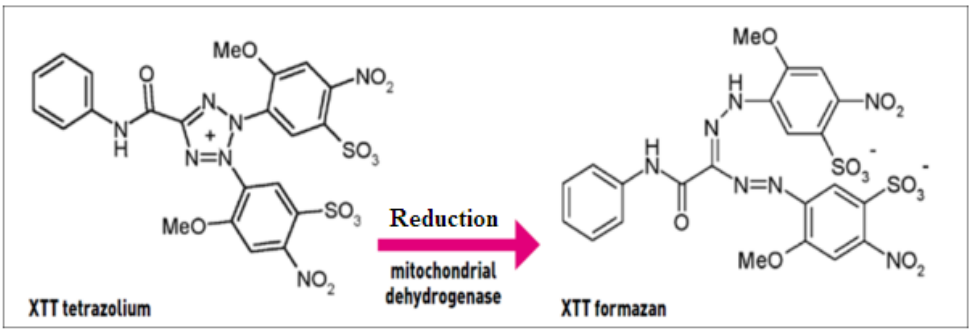


Figure 2.4: The reduction of XTT to form the colored formazan.

96 well plates were inoculated with MCF-7 and MDA-231 cells (about 10000 cells/well) and incubated for 24 hours in order to let them attach and grow. Subsequent to 24 hours incubation, medium was changed with addition of 50 µl fresh complete medium. The cells were treated with 50 µl of working solutions of extract/fraction/isolated compounds in order to reach final extract/fraction/isolated compounds concentrations; 0, 20, 50, 100 150, 200 µg/mL, and DMSO concentration of 1%, and left for 24 hours incubation. Two control wells were used. One of the control wells contained only growth medium in which cells were grown (100 µl) and the other one contained only 2% DMSO (final 1% well conc.) in growth medium which was used as the solvent of extract/fraction/isolated compounds. Figure 2.5 represents the 96 well plate of XTT assay.

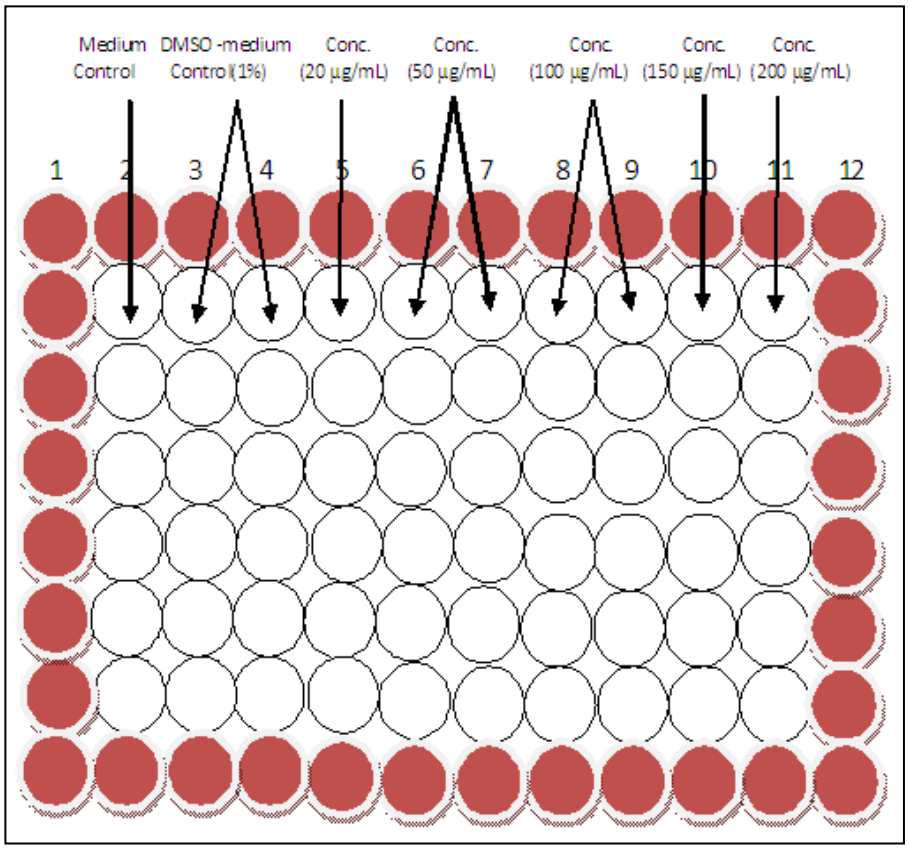


Figure 2.5: 96 well plate representation of XTT assay.

B 2-11 and C 2-11, no cells, complete medium control, 100 µL
B 3 and 4 and C 3 and 4 no cells, 1% DMSO medium control, prepared as 50 µL 2% DMSO medium + 50 µL complete medium
B 5 to G5, 20 µg/mL extract/fraction/isolated compounds, prepared as 50 µl 40 µg/mL extract/fraction/isolated compounds + 50 µl complete medium
B 6 to G6 and B7 to G7, 50 µg/mL extract/fraction/isolated compounds, prepared as 50 µL 100 µg/mL extract/fraction/isolated compounds + 50 µL complete medium
B 8 to G 8 and B 9 to G 9, 100 µg/mL extract/fraction/isolated compounds, prepared as 50 µL 200 µg/mL extract/fraction/isolated compounds + 50 µL complete medium
B 10 to G 10, 150 µg/mL extract/fraction/isolated compounds, prepared as 50 µL 300 µg/ml extract/fraction/isolated compounds + 50 µL complete medium
B 11 to G 11, 200 µg/mL extract/fraction/isolated compounds, prepared as 50 µL 400 µg/mL extract/fraction/isolated compounds + 50 µL complete medium,
B, C, D, E, F and G wells contain MCF-7/MDA-MB-231 cells.


Empty wells

As soon as the reaction time was completed, 100 µL of phenazine metho-sulfate is added to 5 mL of XTT reagent prior to use, and 50 µL of this XTT solution is added to the cells which are already cultivated in 96 -well plate for 24 hours. The 96-well plate is then incubated for 20 hours in CO_2 incubator at 37 ^{0}C. Following the incubation, the absorbance was read at 415 nm with ELISA reader.

A large number of samples can be tested easily and rapidly with a multi-well plate reader. Percent proliferation effect of the extract/fraction/isolated compounds concentration is given in the following:

$$\text{Cell viability (\%)} = \frac{\text{(average OD with cells of X conc.)- (average OD without cells of X conc.)}}{\text{(average OD with cells of DMSO control)- (average OD without cells of DMSO control)}}$$

X: crude extract or ethyl acetate fraction or isolated compounds
OD: Optical density
DMSO: Dimethyl sulfoxide

2.8. Statistical analysis

All values expressed as mean ± standard error of the mean by Microsoft Excel. The data were statistically analyzed by one-way ANOVA tests and $P < 0.05$ is the value where the difference is considered as statistically significant.

CHAPTER 3

RESULTS AND DISCUSSION

3.1. Extraction of *R. heckeliana* L.

Dry powder samples of *R. heckeliana* roots (120 g) were extracted with 1200 mL of methanol (1:10) (wt/v) in the rotating-incubator (180 rpm) at 25 °C for 24 hours. The extract was concentrated and dried through evaporation that was described in "Materials and Methods". Yield of the crude extract is calculated in percentage. One hundred twenty grams of root sample produced about 16.8 % of extract in yield. Voucher number is F5474.

3.2. Fractionation of crude extract

A 20.0 g of dried crude extract was dissolved in 500 mL of methanol-water mixture (70:30, v/v) to ensure the solubility of hydrophilic (phenolic) components. Whole volume of the mixture was transferred into a separatory funnel with the same volume of petroleum ether for fractionation. Through the fractionation process the organic and aqueous phase separation was achieved in the separatory funnel. The organic phase was decanted of the funnel and evaporated each time. Same procedure was repeated by the next organic solvent specifically selected for their increasing polarity (chloroform, ethyl acetate, and n-butanol). At the end of the final step of the fractionation, remaining aqueous phase was collected and was lyophilized as the aqueous fraction. All of these fractionation processes were repeated 3 times. In Figure 3.1, the subsequent fractionations and their yields are schemed**.**

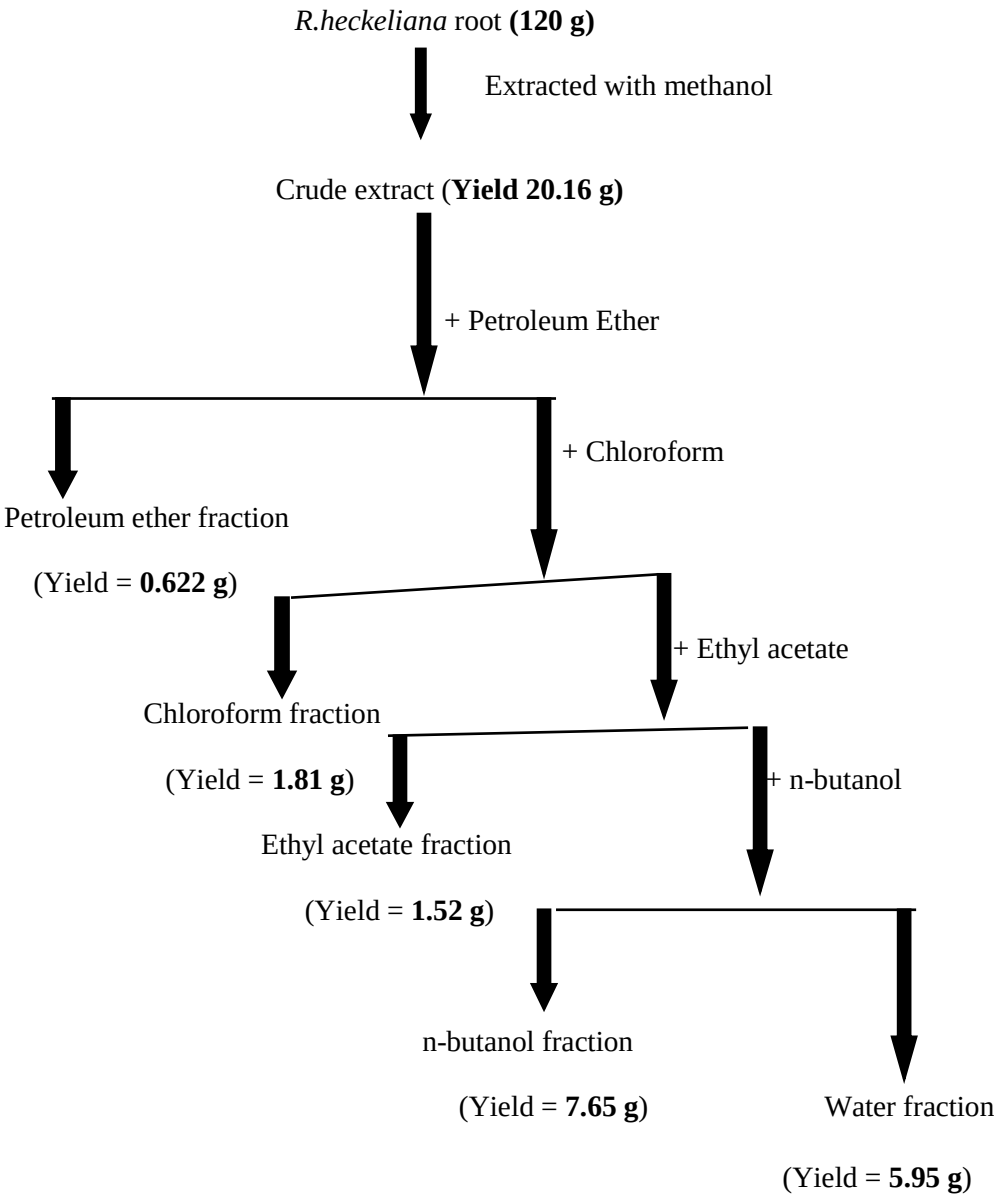


Figure 3.1: Solvent-solvent fractionation scheme of *R. heckeliana* roots crude methanol extract using different solvents of increasing polarity sequentially.

Plant crude extracts, usually containing large amounts of carbohydrates and/or lipoidal materials, may comprise only low concentrations of the phenolic compounds. Therefore, the phenolic compounds of the plant extracts must be enriched before the analysis for the phenolic compositions. The liquid-liquid partitioning (fractioning) strategy based on the solvent polarity has been successfully used. Also, this approach by facilitating a bioactivity guided fractionation may be useful to understand concentrate the active components of the plant extracts.

Separation of bioactive compounds present in crude extract can eliminate undesirable chemicals and help identifying the most important phenolic compounds (Faustino, 2010). In general, elimination of lipoidal materials can be achieved by washing the crude extract with non-polar solvents such as hexane (Ramirez-Coronel, 2004), dichloromethane (Neergheen, 2006), or chloroform (Zhang, 2008).

The percent yields of the sequential fractionation processes recorded in range of 3-38% for the roots of *R. heckeliana* which are given in Table 3.1.

Table 3.1: Yields of the fractionation steps

Solvents used in Fractionation	**Yield (%)**
petroleum ether	3.08
chloroform	8.98
ethyl acetate	7.38
n-butanol	37.95
aqueous	29.51

3.3. Determination of Antioxidant Capacity

Plant extracts with phenolic constituents may also involve of compounds with antioxidant activity. It is important to determine the antioxidant capacity of the plant extracts by means of bioactivity guiding of each extraction/fractionation steps. Antioxidant capacities of the extracts are generally examined by different methods in *vitro/vivo*. For the present study a few in *vitro* methods were selected for their sensitivity, reproducibility and simplicity. These methods are;

- DPPH radical scavenging capacity
- ABTS radical scavenging capacity

3.3.1. Free radical scavenging capacity by DPPH

The DPPH method is extensively used to determine antioxidant capacity of purified phenolic compounds as well as natural plant extracts and foods (Koleva, 2002). It is popular since it needs only a simple UV-Vis spectrophotometer to perform.

The results were expressed as the radical scavenging activity (RSA) in percent and explain in section 2.3.1. Free radical scavenging capacities of the extract/fraction/standard were expressed in the effective concentration at 50 percent (EC_{50}), the lower the EC_{50} value the higher the antioxidant capacity. The EC_{50} values are calculated from the plot of RSA (%) versus concentration (µg/mL) of the extract as displayed in Figure 3.2.

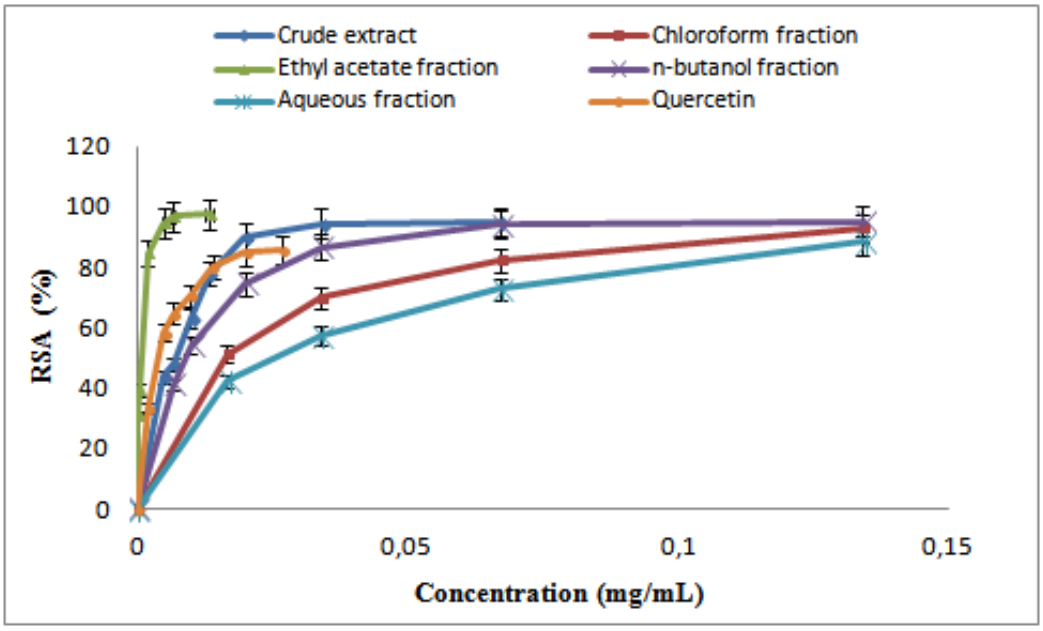


Figure 3.2: DPPH radical scavenging depending on extract concentration, monitored at 517 nm. Each point is the mean of double measurements from three different sets of experiments (n=6).

In this study, the free radical scavenging capacity of the crude extract and the fractions of *R.heckeliana* were estimated by DPPH assay. As shown in Table 3.2, most of the samples of crude extract and fractions showed antioxidant capacity against the DPPH radical, except petroleum ether fraction. Among them the lowest EC_{50} value, was observed for ethyl acetate fraction with an EC_{50} value of 2.78 ± 0.018 µg/mL, which indicates that this fraction had highest radical scavenging activity for DPPH radical. On the other hand, the lowest radical scavenging capacity was in chloroform fraction with an EC_{50} value of 82.64 ± 0.0422 µg/mL.

In addition, the EC_{50} values of the ethyl acetate fraction, crude extract, n-butanol, aqueous, and chloroform fraction were 2.78 > 12.83 > 25.31 > 60.80 > 82.64 µg/ml, respectively. In literature, among the Rosa species, metanolic petal extract of *R. damescana* revealed DPPH radical scavenging activity of EC_{50} value of 2.24 ± 0.98 µg/mL (Yassa, 2009).

Quercetin in flavonol structure, used as a phenolic standard, gave an EC_{50} value of 8.097 ± 0.172 µg/mL for the DPPH radical scavenging. It is used as a strong positive control in the DPPH assays.

Table 3.2: DPPH radical scavenging comparisons

Samples	**DPPH EC_{50} (µg/mL) ± SD***
crude extract	12.83 ± 0.21
petroleum ether	ND
chloroform	82.64 ± 0.42
ethyl acetate	2.78 ± 0.01
n-butanol	25.31 ± 0.02
aqueous	60.8 ± 1.24
quercetin	8.097 ± 0.172

DPPH EC_{50}: Effective concentration of crude extract/fractions/standard required for scavenging 50% of DPPH radical
*Mean of three independent experiments in duplicates
ND: Not determined.

In literature there was no other study published regarding antioxidant capacity of *R. heckeliana* from Rosacea family, however there were reports related to other species of Rosa.

For example, respective DPPH scavenging capacities for some of the Rosa species including *R. burunonii*, *R. bourboniana* and *R. damescana* were determined as EC_{50} values of 35.2 ± 0.69, 25.0 ± 0.71 and 21.4 ± 0.52 µg/mL, in the study by Kumar et al, (2009). In another study by Ozsoy and co-workers DPPH scavenging for the leaves and flowers of *R. horrida,* prepared as ethanolic extracts, were obtained in EC_{50} values of 0.62 ± 0.031 and 0.61 ± 0.035 mg/mL (Ozsoy, 2013). Another Rosa species investigated was flowers of *R. chinensis*, with an EC_{50} value of 21.3 µg/mL (Cai, 2005). In addition, *R. agretis* (leaves), collected from Turkey, was estimated for the DPPH scavenging capacity with an EC_{50} of 47.43 µg/mL (Bitis, 2010).

In the present study, the fractionation process was applied for concentration and separation purpose of the phenolic components from the crude extract as well as determination of these compounds. Although, in literature there were no fractionation steps were considered, in this study after the bioactivity guided fractionation, ethyl acetate fraction due to its increasing solvent polarity revealed the highest DPPH radical scavenging capacity.

3.3.2. Free radical scavenging capacity by ABTS

ABTS assay is another method widely used for determination of antioxidant capacity in biological samples. One of the advantages of this method is its easy applicability and other is its high precision for the determination of total antioxidant capacity in plant extracts. Likewise, in principle, ABTS assay is quite similar to the assay of DPPH scavenging.

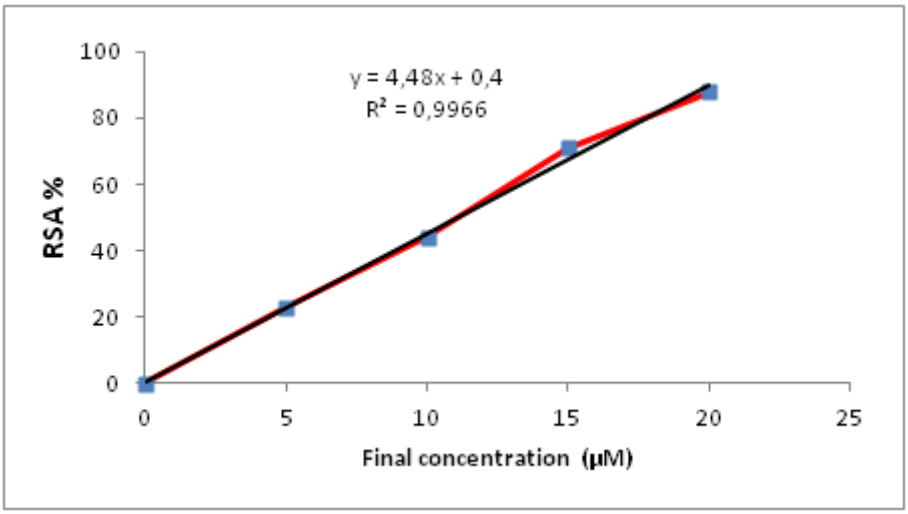


Figure 3.3: Radical scavenging activity in percent versus final concentration

The radical scavenging potentials of the *R. heckeliana* crude extract and the fractions were expressed according to with ABTS method as previously described in the section 2.2. Trolox is used as a standard in the ABTS assays, the concentration of trolox is plotted against radical scavenging activity (RSA) (%) as shown in Figure 3.3. The linear regression equation determined from Figure 3.3 was used in order to calculate values of the trolox equivalent antioxidant concentration (TEAC) for the samples of extracts. For this calculation, the slopes of samples were divided to the slope of standard trolox in order to and the TEAC values of each sample were obtained in trolox equivalent (µmol/g) of extract. Related results were listed in the Table 3.3.

Table 3.3: Trolox equivalent antioxidant capacities (TEAC) of crude extract, fractions and standard

extract/fractions/standard	**TEAC value (µM Trolox ± SD)**
crude methanol extract	49.82 ± 0.95
petroleum ether fraction	ND
chloroform fraction	9.64 ± 0.17
ethyl acetate fraction	586 ± 6.64
n-butanol fraction	32.1 ± 0.58
aqueous fraction	16.0 ± 0.39
quercetin	NA

TEAC value: Radical scavenging activity µM equivalents of trolox/g of crude extract and fractions.
ND: Not determined
NA: Not applicable
SD: Standard deviation

In ABTS assays the TEAC values were separately calculated for the crude extract and fractions of *R. heckeliana* roots and obtained between 9.64 ± 0.17 to 586 ± 6.64 µM, as tabulated in Table 3.3. Among the investigated fractions, ethyl acetate fraction revealed the highest TEAC value indicating that ethyl acetate fraction is enriched by the compounds with the highest radical scavenging capacity. Also, ethyl acetate fraction has resulted in the highest TEAC values as measured in DPPH method.

These results were found quite compatible with the radical scavenging results obtained by the DPPH method. Likewise, in literature this is merely the study of ABTS scavenging capacity in details of fractionation with respect to *R. heckeliana.*

However, ABTS scavenging capacities of different Rosa species were reported in trolox equivalents (TEAC) of *R. chinensis* (flowers) as 235.8 ± 16.7 mM/100g, of *R. sempervirens* extracts in the range of 1.1-10 ± 0.1 µg/mL and that of *R. horrida* leaves and flowers extracts as 1.47 ± 0.102 and 1.44 ± 0.110 mg/mL, respectively (Cai, 2005, Ghazghazi, 2012 and Ozsoy, 2013).

3.3.3. Determination of total phenolic content

Total phenolic content of *R. heckeliana* extracts/fractions was determined by the method of Singleton and Rossi (1965), as previously defined in the section 2.3.3. This procedure is a simple, fast, precise and quite sensitive as an assay also known as the Folin-Ciocalteu method in literature. The Folin-Ciocalteu method is based on the oxidation of phenolic groups using Folin reagent which is a mixture of phosphomolybdic and phosphotungstic acids. Oxidation of phenolics by the Folin reagent produces a green-blue complex which shows an absorbance maximum of 750 nm.

Gallic acid is usually used as the phenolic standard in the assays for its being easily soluble in the Folin reagent. Using a UV-Vis instrument a spectrometric standard calibration curve is prepared for the absorbance maximum (750 nm) of gallic acid against concentration of it. The phenolic content of the extract samples is determined in terms of gallic acid equivalent value (GAE), obtained from an equation is motivated by the linear regression of the calibration curve plotted for gallic acid. The plotted calibration curve is displayed in Figure 3.4. The linear regression equation of that curve is y = 2.4718x - 0.0637 with an R^2 = 0.9988.

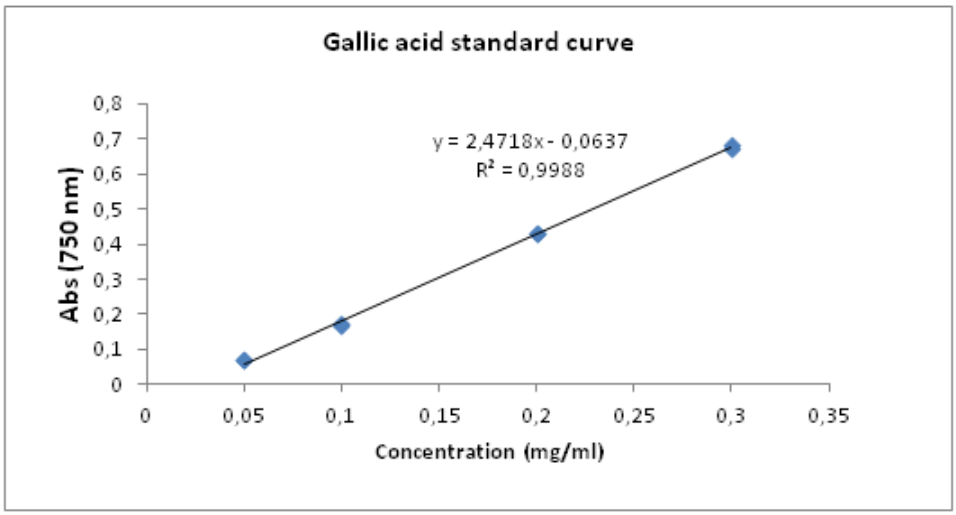


Figure 3.4: Gallic acid standard curve. Experiments were performed three independent experiments in duplicates.

The results were intended as the equivalents of gallic acid (GAE) in µg/ mg for each of the extract samples and are shown in the Table 3.4.

Table 3.4: Total phenol contents of *R.heckeliana* in gallic acid equivalents (GAE)

extract/fractions/standard	**Total Phenol GAE (µg/mg) ± SD**
crude methanol extract	0.568 ± 0.14
petroleum ether fraction	NA
chloroform fraction	0.215 ± 0.0047
ethyl acetate fraction	8,8 ± 0.19
n-butanol fraction	0.73 ± 0.018
aqueous fraction	0.294 ± 0.006
quercetin	NA

TP GAE: Total phenolic contents in equivalents of gallic acid (µg/mg) of the plant extract
NA: Not applicable
SD: Standard deviation

Ethyl acetate fraction, with a value of 8.8 GAE µg/mg extract, of root presented the highest total phenolic content, among the others, it was more significantly different than the crude extract, chloroform fraction, n-butanol fraction and aqueous phase. However the phenolic content of aqueous fraction, with a value of 0.294 GAE µg/mg extract, of root was statistically lower than all. The highest phenolic content (8.8 GAE µg/mg) values indicate the significance of ethyl acetate fraction as a source of natural phenolic compounds.

No study reported previously about total phenolic content of *R. heckeliana* root extract, but in literature different rosa species were studied for their total phenol content. Sezai et al. reported total phenolic content of *R. canina* fruits, *R. dumalis* subsp. *boissieri* fruits, *R. dumalis* subsp. *antalyensis* fruits, *R. villosa* fruits, *R. pisiformis* fruits and *R. pulverulenta* fruits as gallic acid equivalents and results were 96 mg/g, 84 mg/g, 85 mg/g, 73 mg/g, 79 mg/g and 94 mg/g, respectively.

Another research group reported phenol content of *R.burunonii* flowers, *R. bourboniana* flowers, and *R. damescana* flowers as gallic acid equivalents and results were 25.4 ± 0.16 g/100 g, 17.8 ± 0.21 g/100 g and 14.5 ± 0.14 g/100 g, respectively (Kumar, 2007). In addition, total phenolic content of different rosa extracts were stated as gallic acid equivalent by Fattahi (2012), Bitis (2010), Cai (2005), Abdel-Hameed (2012), Ghazghazi (2012), and Ozsoy (2013). Results were gallic acid equivalent; *R. pimpinellifolia* fruits extract 225.65 ± 2.50 mg/100g, *R. agretis* leaves extract 489.76 mg/g, *R. chinensis* flowers extract 18.9 ± 0.13 g/100g, *R. damascena trigintipetala* Dieck flowers extract

53.25 ± 5.92 mg/g, *R. sempervirens* extract 1.2-7.5 ± 0.1 mg/mL, and R. *horrid* leaves and flowers extracts 152.0±0.69 and 159.3±1.76 mg gallic/g of extract, respectively.

3.3.4. Determination of total flavonoid content

Phenolic substances have been shown to be responsible for the antioxidant activity of plant materials. Total flavonoid contents can be determined in the sample extracts/fractions by reaction with sodium nitrite, followed by the development of colored flavonoid-aluminum complex formation using aluminum chloride in alkaline condition which can be monitored spectrophotometrically at maximum wavelength of 510 nm (Abu Bakar, 2009).

The total flavonoid content was expressed as catechin (CE) equivalents in microgram per milligram dry material of crude extract and fractions. The calibration curve of catechin to determine flavonoid content was shown in Figure 3.5. Equation of the catechin curve results y = 2.729x + 0.001 with R^2 = 0.9914.

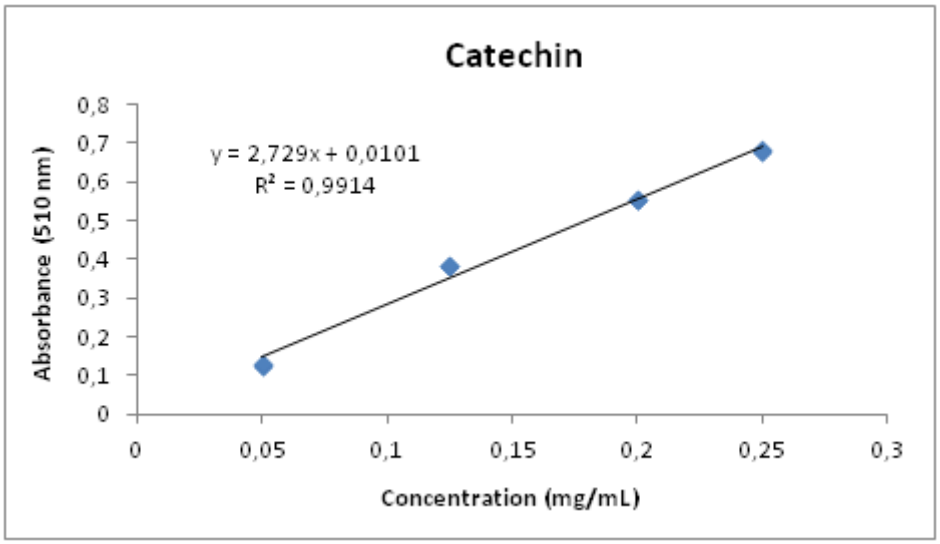


Figure 3.5: Catechin standard curve. Experiments were performed three independent experiments in duplicates.

Total flavonoid content of crude extract and its fraction was given in Table 3.5. Significantly highest results were found in the order ethyl acetate fraction > crude extract > n-butanol fraction > aqueous fraction > chloroform fraction for total flavonoids content. The ethyl acetate fraction was found to be having the maximum content of total flavonoids content (4.58 ± 0.018 µg CE/mg of extract). The chloroform fraction had the lowest content of total flavonoids content (0.097± 0.001 µg CE/mg of extract).

Table 3.5: Total phenolic content of crude extract and fractions

Extract/fractions/standard	**Total flavonoid CE (µg/g) ± SD**
crude methanol extract	0.184 ± 0.0047
petroleum ether fraction	ND
chloroform fraction	0.097 ± 0.028
ethyl acetate fraction	4,58 ± 0.125
n-butanol fraction	0.208 ± 0.0061
aqueous fraction	0.161 ± 0.0044
quercetin	NA

TF CE: Total flavonoid contents µg equivalents of catechin/mg of plant extract
NA: Not applicable
ND: Not determined
SD: Standard deviation

There is no study in the literature related to application of total flavonoid content for this species, but there are studies on other Rosa species. For example, total flavonoid content of different Rosa extracts was stated as quercetin equivalent by Fattahi (2012), Montazeri (2011) and as rutin equivalent by Abdel-Hameed (2012) and Ghazghazi (2012) and as catechin equivalent by Bitis (2010) and Ozsoy (2013). Results were quercetin, rutin and catechin equivalent; *R. canina* fruits 0.41± 0.02 mg quercetin/100 g of extract, *R. canina* fruits 23.6 ± 4.2 mg quercetin/g of extract and *R. damascena trigintipetala* Dieck flowers 31.27 ± 1.88 mg rutin/g of extract, *R. sempervirens* extract 0.08-0.37± 0.0 mg rutin/g of extract and *R. agretis* leaves 265.01 ± 30.33 mg catechin/g of extract, and *R horrida* leaves and flowers extracts of 67.64±0.78 and 68.25±0.93 mg catechin/g of extract, respectively.

As in the case of earlier results, in comparison of crude extract and fractions, ethyl acetate fraction have revealed highest antioxidant effect, total phenolic content and total flavonoid content.

There were good relationship among the antioxidant capacity measured by ABTS and DPPH method and also there were good relationship between antioxidant activities and total phenolic, total flavonoid content. This indicated that the phenolic compounds which might be responsible for the scavenging activity in *R. heckeliana* root extract is phenolic acid and flavonoid constituents. Also, it can be stated that scavenging effect of extracts/ fractions is not limited to phenolics and flavonoid compounds. The activity may also come from the presence of other antioxidant secondary metabolites in the extracts/fractions such as volatile oils, carotenoids, and vitamins (Javanmardi, 2003). Finally, ethyl acetate fraction of *R. heckeliana* is a natural source of antioxidants and can be used in functional foods and nutraceuticals.

From the Table 3.6, summary of the antioxidant capacity of crude extract and fractions from *R. heckeliana*, total phenol and total flavonoid content can be compared. Approximately one two of total phenol is found to be flavonoid.

Table 3.6: Comparison of antioxidant capacity including DPPH EC_{50} (µg/mL) and $TEAC_{ABTS}$ (µM Trolox) results for crude extract, fractions and standard quercetin (positive control). Comparison of total phenol and total flavonoid contents results for all fraction and crude extract.

Fraction/Standard	**DPPH EC_{50} (µg/mL)**	**$TEAC_{ABTS}$ (µM Trolox)**	**Total Phenol GAE (µg/mg)**	**Total Flavonoid CE (µg/mg)**
Crude Extract	12.83 ± 0.21	49.82 ± 0.95	0.568 ± 0.14	0.184 ± 0.0047
Petroleum eter	> 250	ND	NA	NA
Chloroform	82.64 ± 0.42	9,64 ± 0.17	0.215 ± 0.0047	0.097 ± 0.028
Ethyl acetate	2.78 ± 0.01	586 ± 6.64	8.8 ± 0.19	4.58 ± 0.125
Butanol	25.31 ± 0.02	32.1 ± 0.58	0.73 ± 0.018	0.208 ± 0.0061
Aqueous	60.8 ± 1.24	16.0 ± 0.39	0.294 ± 0.006	0.161 ± 0.0044
Quercetin	8.097 ± 0.172	NA	NA	NA

ND: Not determined, NA: not applicable,
EC50: Effective concentration of extract/fraction and standard
Milligrams of total phenolic content permiligrams of extract as gallic acid equivalents (GAE)
Micromoles trolox per mg of extracts
Total flavonoid content is expressed as catechin equivalents (CE; µg catechin/mg of extract).

The antioxidant activity of *R. heckeliana* root extract and its phenolic content have not been analyzed before. This is the first study regarding these aspects. Previous research shows the potential of different Rosa species as a medicine. But results in this research indicate its potential as an antioxidant source. The medicinal properties of *R. heckeliana* root extract may be due to the presence of antioxidants.

As a result, among extract and fractions evaluated, the ethyl acetate fraction in this study was selected for further purification and isolation of the antioxidant compounds.

3.4. Isolation of phenolics by Silica gel 60 and Sephadex LH-20

A number of different separation/isolation techniques such as column chromatography, sephadex chromatography, flash chromatography, TLC and HPLC, are used to the isolation of bioactive compounds from plant material. Silica gel 60 (Darmstadt, Germany), sephadex LH-20 (Pharmacia, Sweden) and TLC (Darmstadt, Germany) were chosen for isolation part in this study. For the reasons explained earlier, further isolation and purification were performed on ethyl acetate fraction from *R. heckeliana* using silica gel 60 and sephadex LH-20 to identify the marker compound(s) responsible for antioxidant capacities.

Before starting the isolation and purification procedure, all the fractions were analyzed by using RP-HPLC chromatograms. Figure 3.6 shows the HPLC chromatogram of ethyl acetate fraction before further isolation and purification. HPLC chromatograms of other fractions are shown in Appendix A (Optimization of separation using RP-HPLC for extract/fraction detailed in materials and methods).

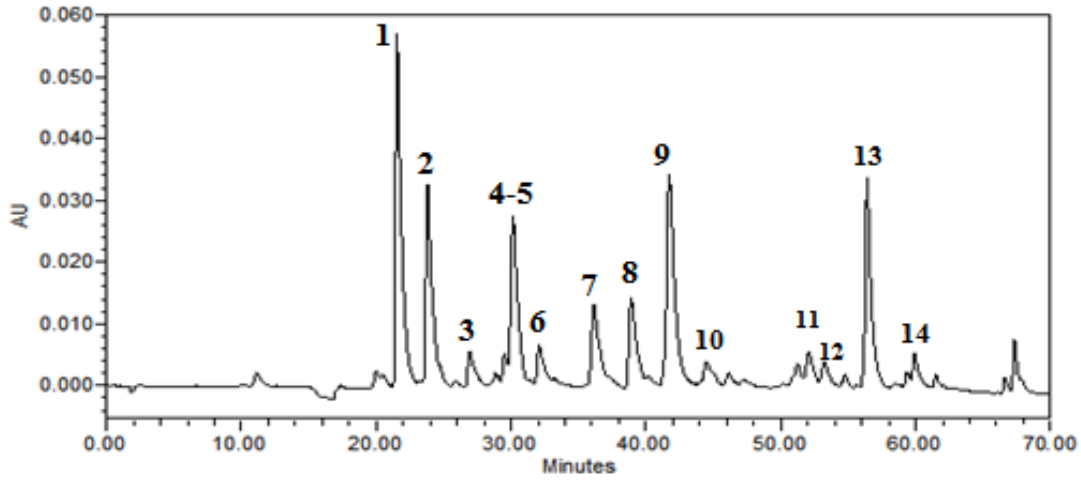


Figure 3.6: RP-HPLC chromatogram of ethyl acetate fraction of crude methanol extract from *R. heckeliana* roots at 280 nm.

The HPLC profile in Figure 3.6 exhibits 14 peaks, representing 14 compounds contained in the ethyl acetate fraction at 280 nm. Compounds correspond to peak 1, peak 2, peak 9 and peak 13 exist in high concentrations while compounds correspond to other peaks were present in lower quantities. The aim of this isolation part is to isolate four major peaks according to the HPLC chromatogram of ethyl acetate fraction.

The main aim of this research is to isolate and purify four major peaks (1, 2, 9 and 13) using silica gel 60 (Darmstadt, Germany), sephadex LH-20 column chromatography (Pharmacia, Sweden), and TLC.

Silica gel 60 (70-230 mesh, particle size 0.063–0.200 mm) column chromatography was first used to isolate and purify chemical constituents of ethyl acetate fraction from *R. heckeliana* root extract in this study. Gradient elution with chloroform-methanol was applied and yielded 212 fractions which were pooled together according to their TLC. This let to 9 main fractions (Fr1-Fr9) were collected and monitored by TLC using chloroform: methanol: ethyl acetate (3:0.75:1). Mobile phase is determined by TLC analysis for silica gel. The suitable mobile phase was found to be chloroform: methanol: ethyl acetate (3:0.75:1). These fractions (Fr1-Fr9) were also analyzed by using RP-HPLC as shown in Figure 3.7.

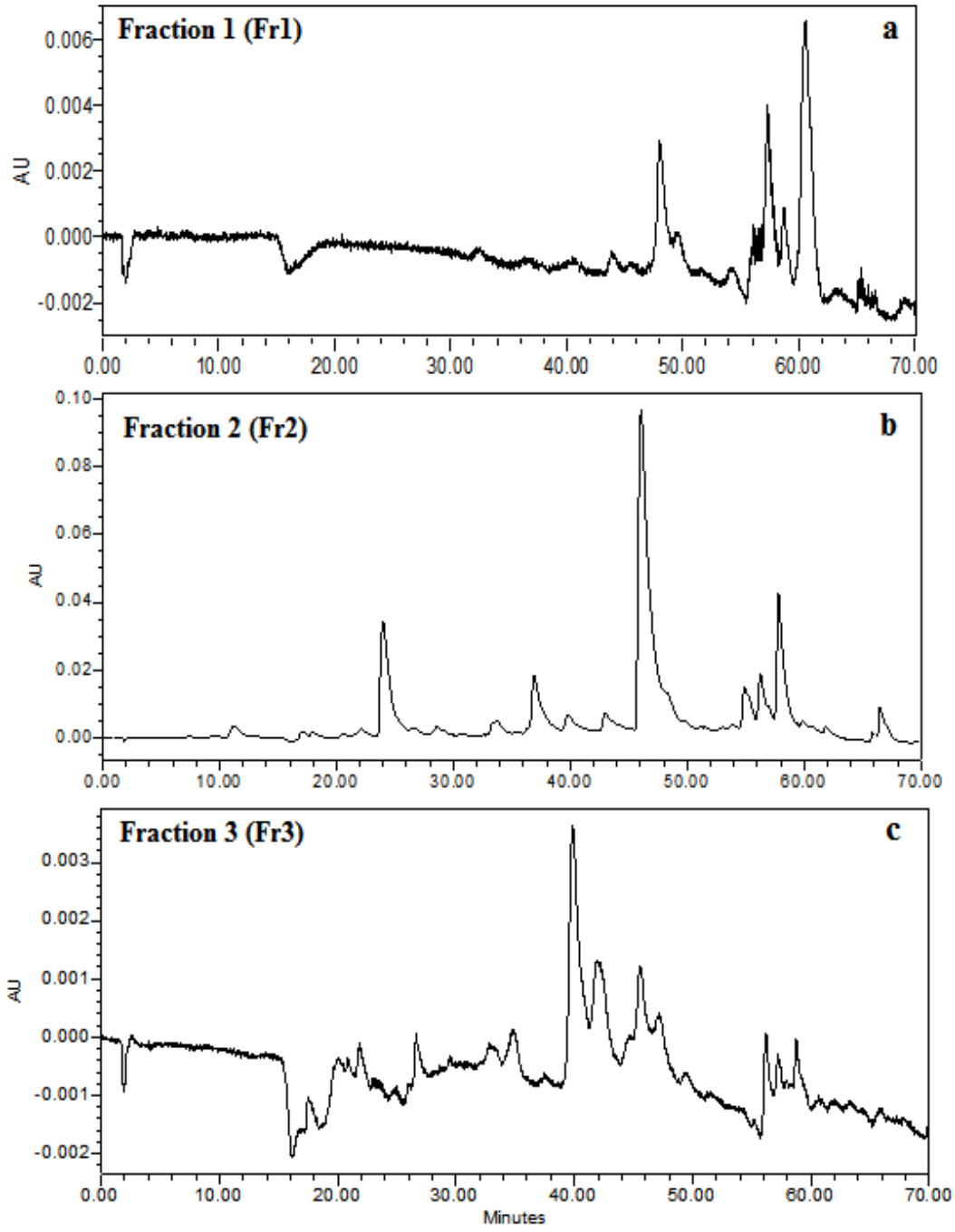


Figure 3.7: RP-HPLC chromatograms of Fraction 1 (Fr1) to Fraction 3 (Fr3) of ethyl acetate fraction. Signal was detected at 280 nm.

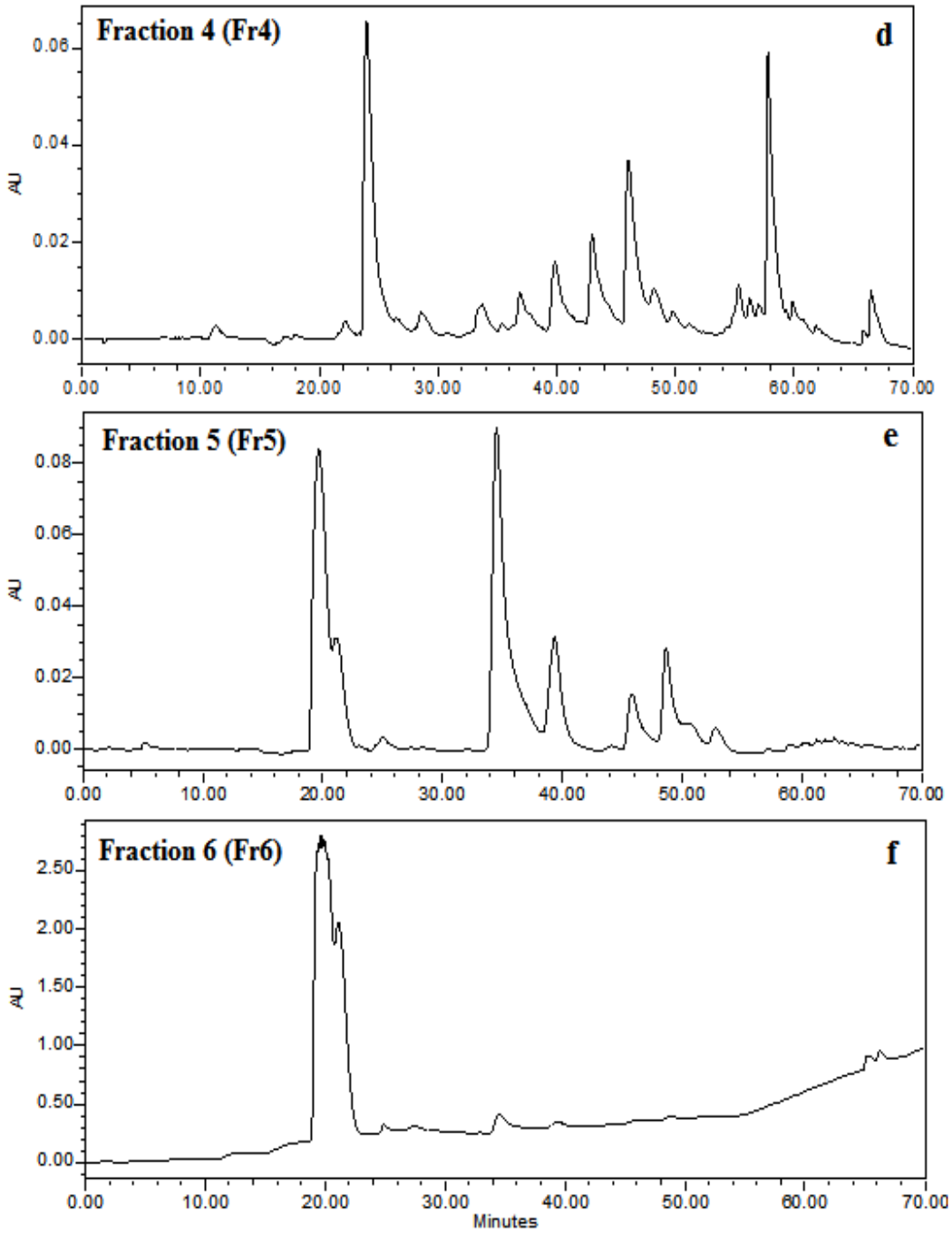


Figure 3.7: RP-HPLC chromatograms of Fraction 4 (Fr4) to Fraction 6 (Fr6) of ethyl acetate fraction. Signal was detected at 280 nm.

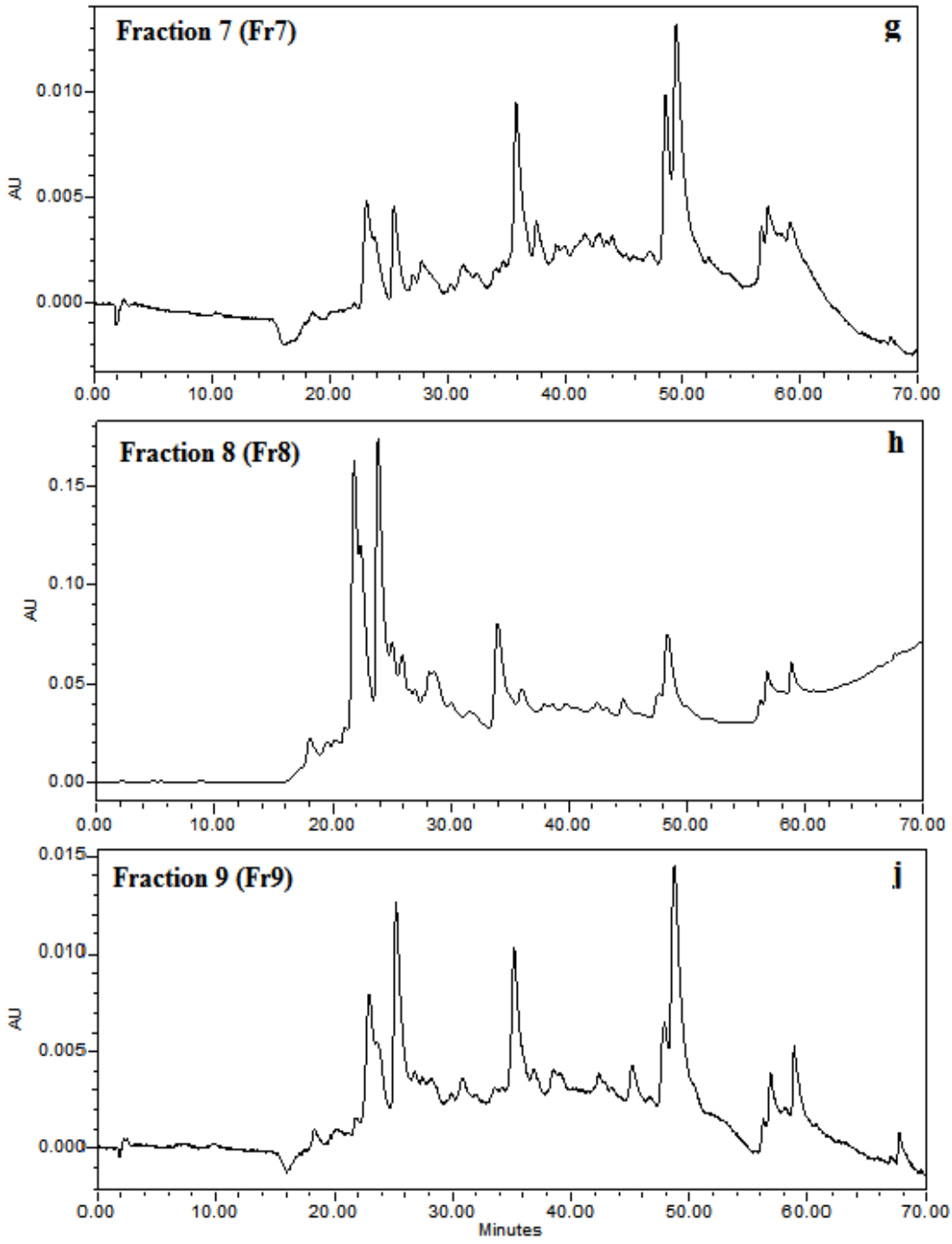


Figure 3.7: RP-HPLC chromatograms of Fraction 7 (Fr7) to Fraction 9 (Fr9) of ethyl acetate fraction. Signal was detected at 280 nm.

Fraction 2 (Fr2-273 mg), fraction 4 (Fr4-366 mg) and fraction 6 (Fr6-131 mg) showed stronger antioxidant activity while other fractions did not show little or no antioxidant activity, hence these fractions were further chromatographed using sephadex LH-20 and silica gel for the isolation of bioactive compounds (in detailed experimental section).

At the end of a series of column chromatography, fraction Fr 2 yielded pure compound 1 (85 mg). Fraction Fr 4 yielded pure compound 2 (178 mg) and Fr 6 yielded pure compounds 3 (72 mg). Figure 3.8 schematically represent the extraction and fractionation for isolation of *R.heckeliana* root extract (chromatographic separation detailed in section 2.4.4).

Three of them isolated from ethyl acetate fraction were of high purity with RP-HPLC chromatograms given in the Appendix B. The compound 1(peak 9), 2 (peak 1) and 3 (peak 2) were identified as catechin, caffeic and ellagic acid, respectively. Other major compound (peak 13) further improvements in isolation could be achieved by using different gradient programming.

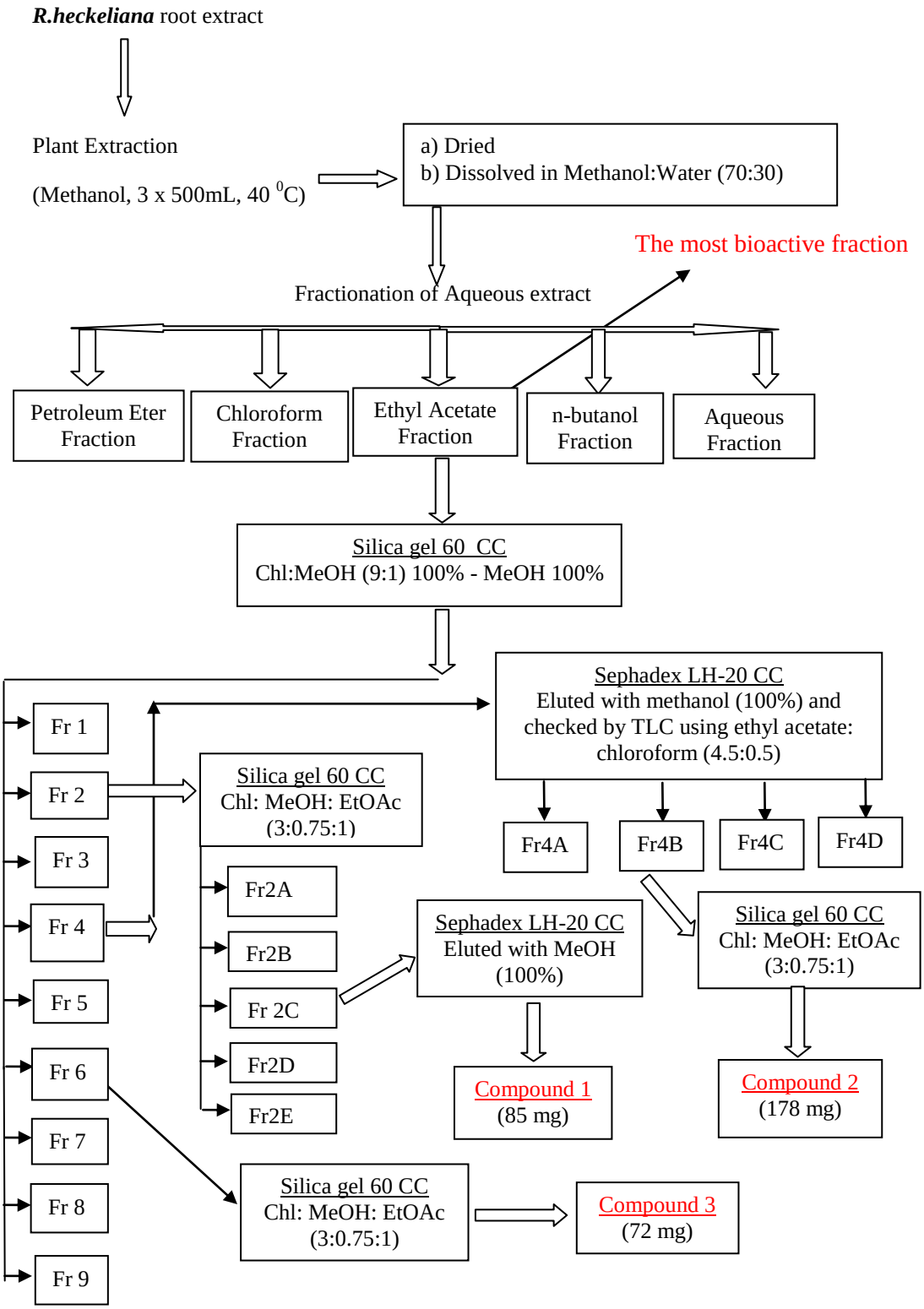


Figure 3.8: Schematic diagram representing the isolation of bioactive compounds from ethyl acetate fraction of *R. heckeliana*.

The chemical structures of the isolated compounds of ethyl acetate fraction from *R.heckeliana* were identified using NMR spectroscopy. Proton (^{1}H NMR) and carbon (^{13}C NMR) spectra given in the Appendix C were compared with those reported in literature.

Compound 1 was obtained as a brown amorphous powder, UV λ max 253, 364 nm (methanol). ^{1}H NMR (400 MHz, DMSO-d_6): ^{1}H-NMR spectra showed peaks at δ: 7.46 (2H, s, H-1 and H-6).

^{13}C-NMR (DMSO-d_6): Carbon atoms showed peaks at δ: 159.4 (C-5 and C-10), 148.4 (C-3 and C-8), 140.9 (C-2 and C-7), 109.9 (C-4 and C-9).

The above spectral data indicated that the compound 1 was ellagic acid. Ellagic acid is a natural phenol antioxidant found in numerous fruits, vegetables and nuts (Seeram, 2004). The highest levels of ellagic acid are found in Rosaceae family like blackberries, cranberries, pomegranates, raspberries, strawberries, and peach (Amakura, 2000). For example, the health-promoting potential of pomegranate juice is attributed to a high content of polyphenols, including ellagic acid (Clifford, 2000).

Ellagic acid has been previously isolated from *C. sylvestris* aqueous extract (family of Flacourtiaceae) (Da Silva, 2008). It has been isolated from methanolic extract of *N. lappaceum* peels (family of Sapindaceae) (Thitilertdecha, 2010) and the fruit of *Phyllenthus emblica* (Phyllanthaceae) (Luo et al, 2009), and the roots of *Decalepis hamiltonii* (family of Apocynaceae) (Srivastava, 2007) and many other plants.

Ellagic acid was studied in the 1960s, mainly for its effects on blood clotting, its hemostatic activity and its effects in whitening of the skin (Zhang, 2010). The biological activities of ellagic acid have been studied extensively. Ellagic acid is assumed to possess antioxidant, anti-mutagenic, anti-inflammatory, and cardioprotective activities (Priyadarsini, 2002; Giedrius-Miliauskas, 2004). The hepatoprotective property of ellagic acid has been reported both in *vitro* and in *vivo* (Singh, 1999a and 1999b)

Compound 2 was obtained as a yellowish powder, UV λ max 276 nm (methanol). ^{1}H NMR (400 MHz, DMSO-d_6): ^{1}H-NMR spectra showed peaks at δ: 6.84 (1H, d, J = 2.0 Hz, H-2'), 6.77 (1H, dd, J = 8.1 Hz, H-5'), 6.71 (1H, dd, J = 2.0, 8.1 Hz, H-6'), 5.92 (1H, d, J = 2.4 Hz, H-8), 5.85 (1H, d, J = 2.4 Hz, H- 6), 4.56 (1H, d, J = 8.0 Hz, H-2), 3.97 (1H, ddd, J = 8.0, 8.0, 4.8 Hz, H-3), 2.85 (1H, dd, J = 4.8, 16.0 Hz, H-4), 2.50 (1H, dd, J = 8.0, 16.0 Hz, H-4).

^{13}C-NMR (100 MHz, DMSO-d_6): Carbon atoms showed peaks at δ 156.4 (C-9), 156.1 (C-5), 155.3 (C-7), 144.8 (C-3'), 144.8 (C-4'), 130.5 (C-1'), 118.4 (C- 6'), 115.0 (C-5'), 114.5 (C-2'), 99.0 (C-10), 95.06 (C-6), 93.8 (C-8), 83.0 (C-2), 66.3 (C-3), 27.8 (C-4).

Compound 2 was identified as catechin. Catechin belongs to the group of flavan-3-ols (or simply flavanols), part of the chemical family of flavonoids. It is a plant secondary metabolite, a type of natural phenol and antioxidant. Good sources of catechins are peach, apricot, apples, and berries (in Rosaceae family). Catechin has been previously isolated from roots of *Rosa rugosa* (Young, 1987a). It has been isolated from roots of *Sanguisorba officinalis* (family of Rosaceae) (Zhang, 2012), *Acacia catechu* (family of Fabaceae) (Azad et al, 2001), *Camellia sinensis* (family of Theaceae) (Vuong, 2010) and many others plants.

It has been reported that catechin posses numerous important biological activities, such as antimutagenic activity, antitumor activity and antioxidant properties (Pannala, 2001; Mendoza, 2006; Kondo, 1999). Due to catechin's antioxidant properties, it exhibits protective effects against diseases involving oxidative stress such as cancers (Jankun, 1997; Garbisa, 1999) cardiovascular diseases (De Lorgeril, 1999; Stein, 1999) and neurodegenerative diseases (Smith, 1996). It is known that catechin is an antioxidant which affects plasma antioxidant biomarkers and energy metabolism (Williamson, 2005).

Compound 3 was obtained as a yellow powder, UV λ max 325 nm (methanol). ^{1}H NMR (400 MHz, DMSO-d_6): ^{1}H-NMR spectra showed peaks at $_{\delta}$: 7.42 (d, 1H, J=15.8 Hz), 7.02 (d, 1H, J=2.1 Hz), 6.95 (dd, 1H, J=2.1, 8.1 Hz), 6.75 (d, 1H, J=8.1 Hz), 6.17 (d, 1H, J=15.8 Hz)
^{13}C-NMR (100 MHz, DMSO-d_6): Carbon atoms showed peaks at δ: 167.9, 148.08, 145.5, 144.55, 125.6, 121.1, 115.05, 115.05, and 114.54. Thus, compound 2 was identified as caffeic acid.

Compound 3 was identified as caffeic acid. Caffeic acid is one of the most common phenolic acids, naturally occurring organic compound, found in plant based foods such as fruits, grapes, olives, spinach, sweet potatoes, grains, coffee, dietary supplements and Chinese medicinal herbs (Larson, 1988; Rice-Evans, 1996; Jiang, 2005).

Caffeic acid has been previously isolated from flowers of *Delphinium formosum* (family of Ranunculaceae) (Durust, 2001). It has been isolated from leaves of *Perilla frutescens* (family of Lamiaceae) (Park, 2010), *Origanum vulgare* (family of Lamiaceae) (Koukoulitsa, 2007), *Eupatorium adenophorum* (family of Asteraceae) (Wei, 2010) and many other plants.

The reported biological properties of caffeic acid and its derivatives exhibits a wide range of biological activities including antibacterial, antiviral, anti-inflammatory, anti-carcinogenic antioxidant and antiproliferative properties (Fernandez, 1998; Olthof, 2001; Tanaka, 1993; Vieira, 1998; Fiuza, 2004; Son, 2002). It shows increase of γ-GCS activity and GSH level (Park, 2010). Caffeic acid is known to have an antidiabetic effect in streptozotocin-induced diabetic rats (Hsu, 2000; Cheng, 2003; Okutan, 2005)

All the isolated compounds, known in the literature, were isolated for the first time from *R.* h*eckeliana*. The chemical structures of the phenolic compounds isolated from *R.heckeliana* are shown in Figure 3.9.

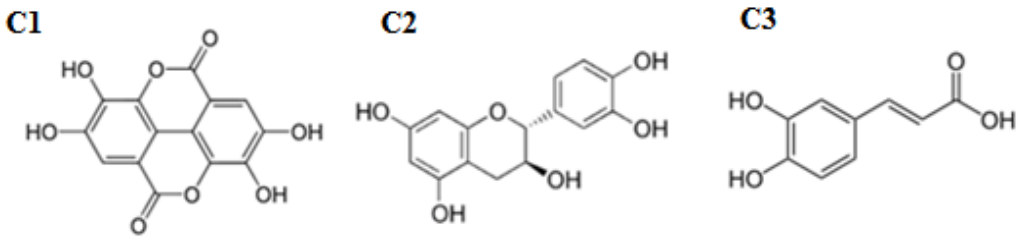


Figure 3.9: Chemical structures of the isolated compounds (C1, C2, and C3) of ethyl acetate fraction from *R. heckeliana* roots.

Besides, further identify and approval of chemical structure of all isolated compounds checked by UV-vis spectroscopy and High Resolution-Mass Spectroscopy (HR-MS), respectively.

Compound 1, 2 and 3 were also identified from the UV-vis spectra in methanol. Results from the UV-Vis spectrophotometric analysis show that for compound 1 (ellagic acid), 2 (catechin) and 3 (caffeic acid) as shown in Figure 3.10. They exhibit UV-Vis absorption maxima at 254, 364 nm for ellagic acid, about 280 nm for catechin and about 240, 324 nm for caffeic acid, respectively.

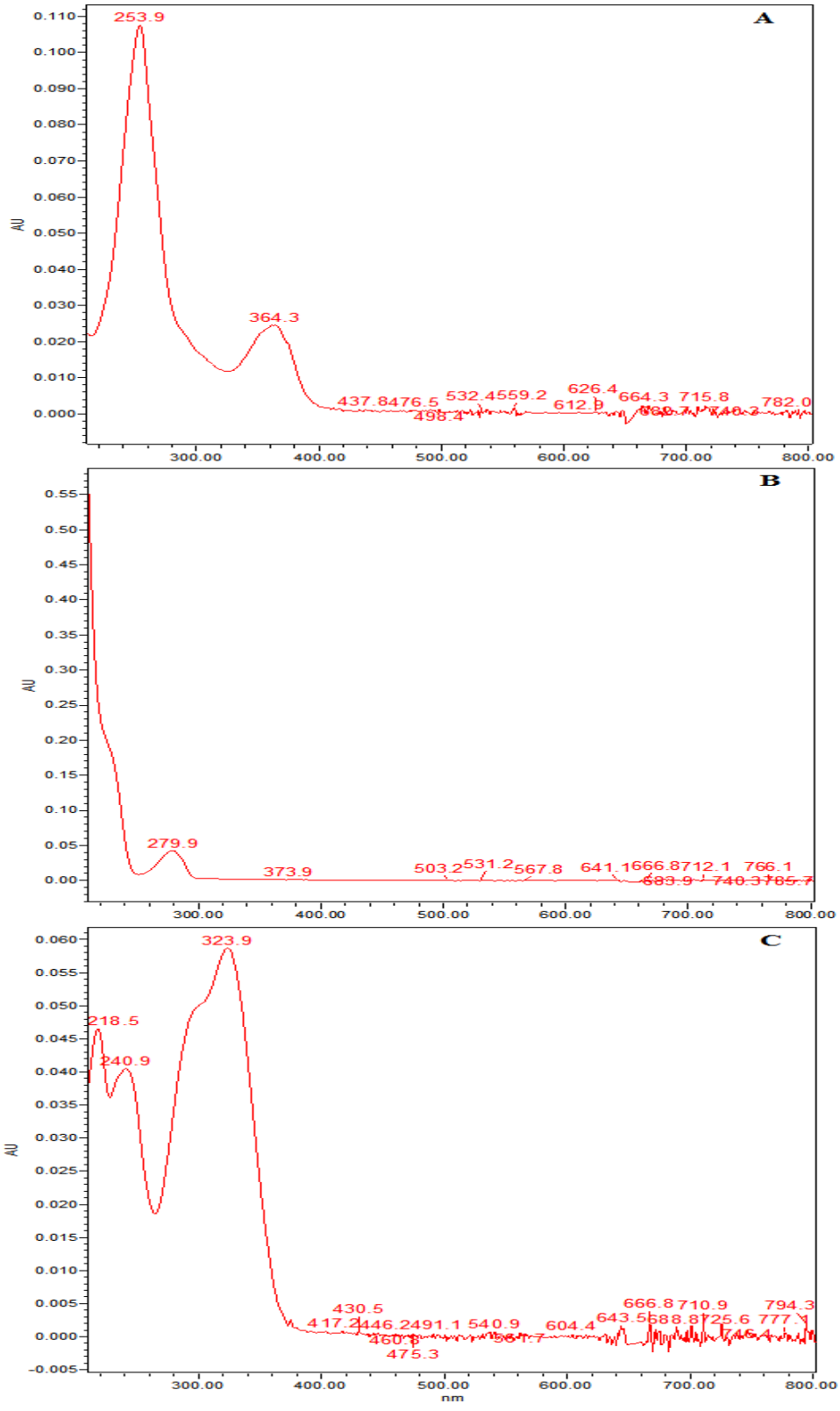


Figure 3.10: UV-Visible spectra of A) compound 1 (ellagic acid), B) compound 2 (catechin) and C) compound 3 (caffeic acid) recorded in the range of 210-800 nm.

High Resolution-Mass Spectrophotometry (HR-MS) analysis confirmed the chemical structures of the isolated compounds.

The HR-MS analysis of compound 1 gave a sodiated molecular ion peak at m/z 303.0141 $[M-H]^+$. The molecular formula of compound 1 was determined to be $C_{14}H_6O_8$ by HR-MS (m/z 303.0141) $[M-H]^+$ (calcd for $C_{14}H_6O_8$, 303.0127). Mass spectrum (Figure 3.11) exhibited the $[M+H^+]$ peaks at m/z=302

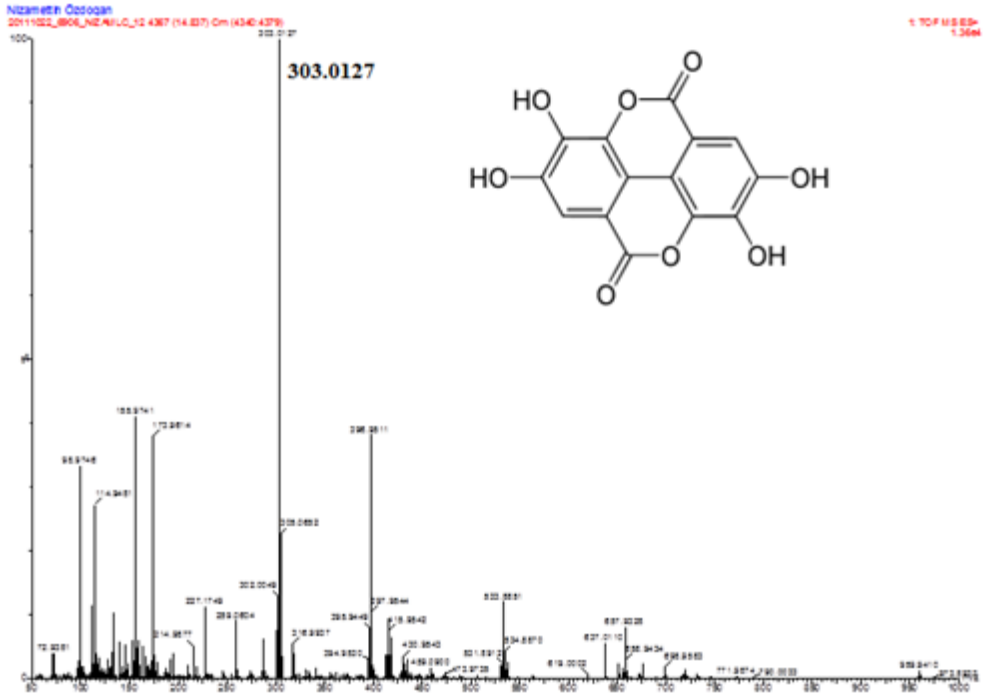


Figure 3.11: HR-MS spectrum of compound 1 (ellagic acid).

The HR-MS analysis of compound 2 gave a sodiated molecular ion peak at m/z 291.0869 $[M-H]^+$. The molecular formula of compound 2 was determined to be $C_{15}H_{14}O_6$ by HR-MS (m/z 291.0869) $[M-H]^+$ (calculated for $C_{15}H_{14}O_6$, 291.0869). The MS spectrum (Figure 3.12) showed the $[M+H^+]$ peaks at m/z = 291.

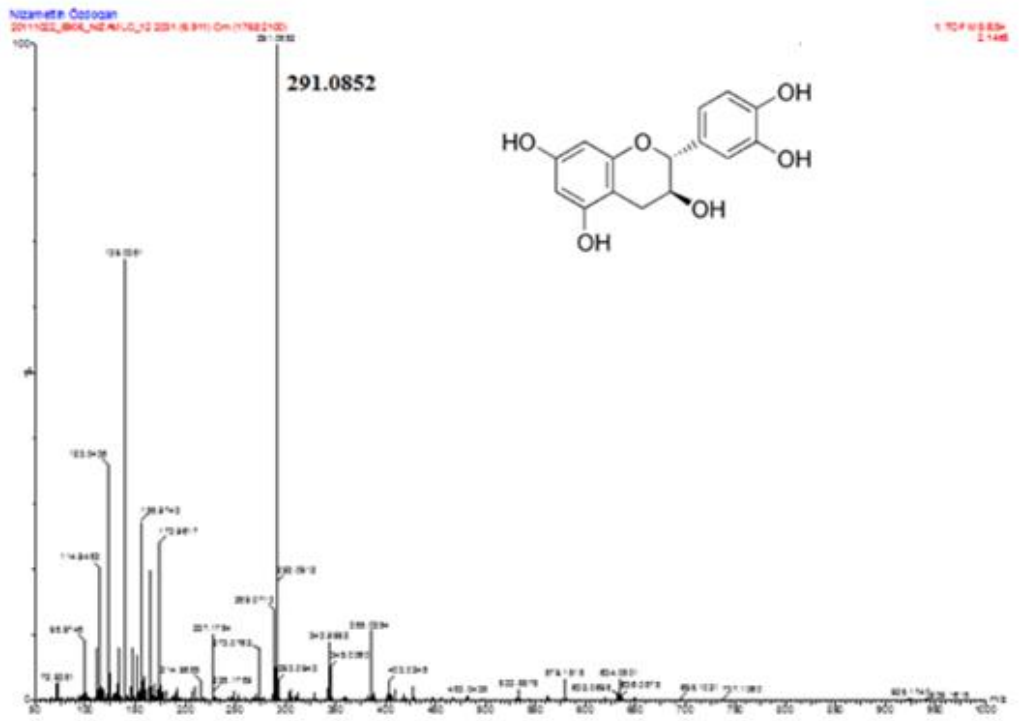


Figure 3.12: HR-MS spectrum of compound 2 (catechin)

HR-MS analysis of compound 3 gave a sodiated molecular ion peak at m/z 181.0501 [M-H]$^+$.The molecular formula of compound 3 was determined to be $C_9H_8O_4$ by HR-MS (m/z 181.0501) [M-H]$^+$ (calculatedd for $C_9H_8O_4$, 181.0511). Mass spectrum (Figure 3.13) exhibited the [M+H$^+$] peaks at m/z=180.

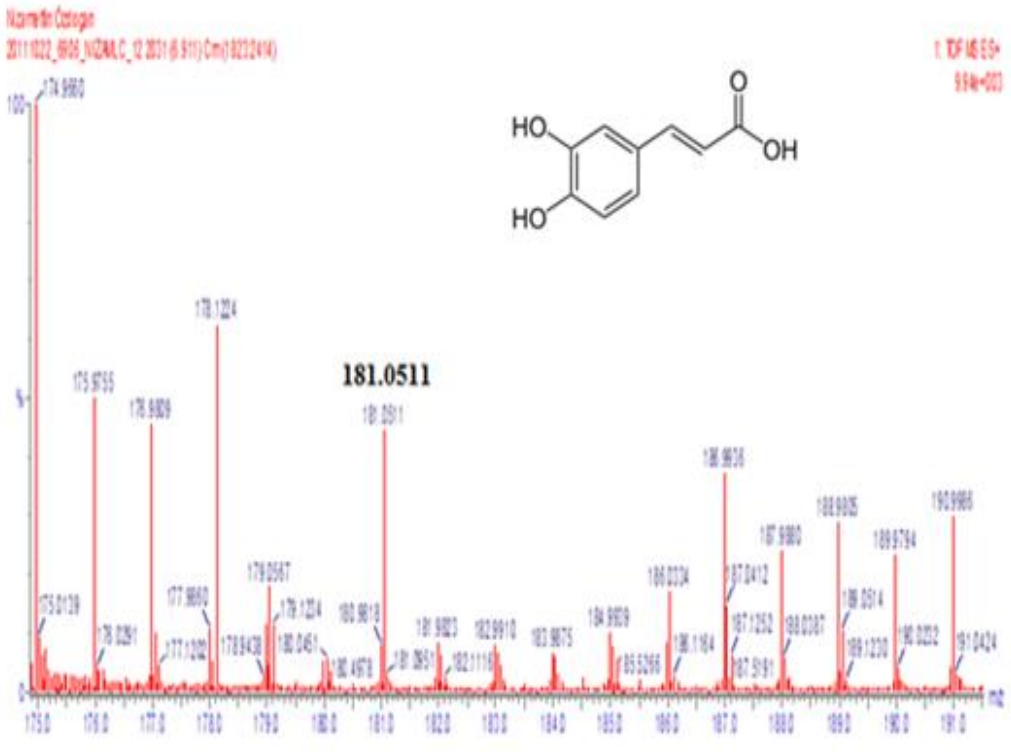


Figure 3.13: HR-MS spectrum of compound 3 (caffeic acid).

Phenolic compounds are usually isolated as mixtures from several plants and then tested as antioxidant compounds. The isolation (silica gel, sephadex, poliamid, etc.) and identification (liquid chromatograhy and mass spectrophotmetry) of each compounds as well as correlation their structures to the observed activity are often difficult, due to the similarities of their structures and polarities (Tsimogiannis, 2007).

After isolation procedure, as the other minor (peaks 3, 4, 5, 6, 7, 8, 10, 11, 12, and 14) phenolics compounds in the ethyl acetate fraction were attempted and identified using RP-HPLC (equipped with photodiode array and fluorescence detectors) by comparing their retention times with those of available phenolic standards. Afterwards, to confirm the RP-HPLC results of ethyl acetate fraction High Resolution Mass Spectroscopy (HR-MS) experiment was performed.

3.5. RP-HPLC analysis

3.5.1. Optimization of analytical conditions by RP-HPLC

Reverse-Phase High Performance Liquid Chromatography (RP-HPLC) is an extensively used multipurpose technique for the isolation of natural products (Cannell, 1998). In this study RP-HPLC was utilized for the purpose of identification of the bioactive compounds in the plant of interest.

For the mobile phase optimization of the chromatographic conditions different elution mixtures were tried including methanol, acetonitrile, water, acetic acid, and formic acid on both isocratic and gradient systems. Eventually, the best resolution of the phenolic compounds were accomplished by a gradient system, consist of two different solvents as given in section 2.5.3

Figure 3.14 demonstrates the different chromatograms of HPLC analysis of 15 references standards using RP-HPLC photodiode array detector at 254 nm, 280 nm, 320 nm and 360 nm. In this study, 280 nm was chosen as monitoring wavelengths according to absorption maxima of all selected standards. The literature (Escarpa, 1998; Markham, 1998) indicated that the optimal detection of phenolic and flavonoid standards was at 280 and 320 nm.

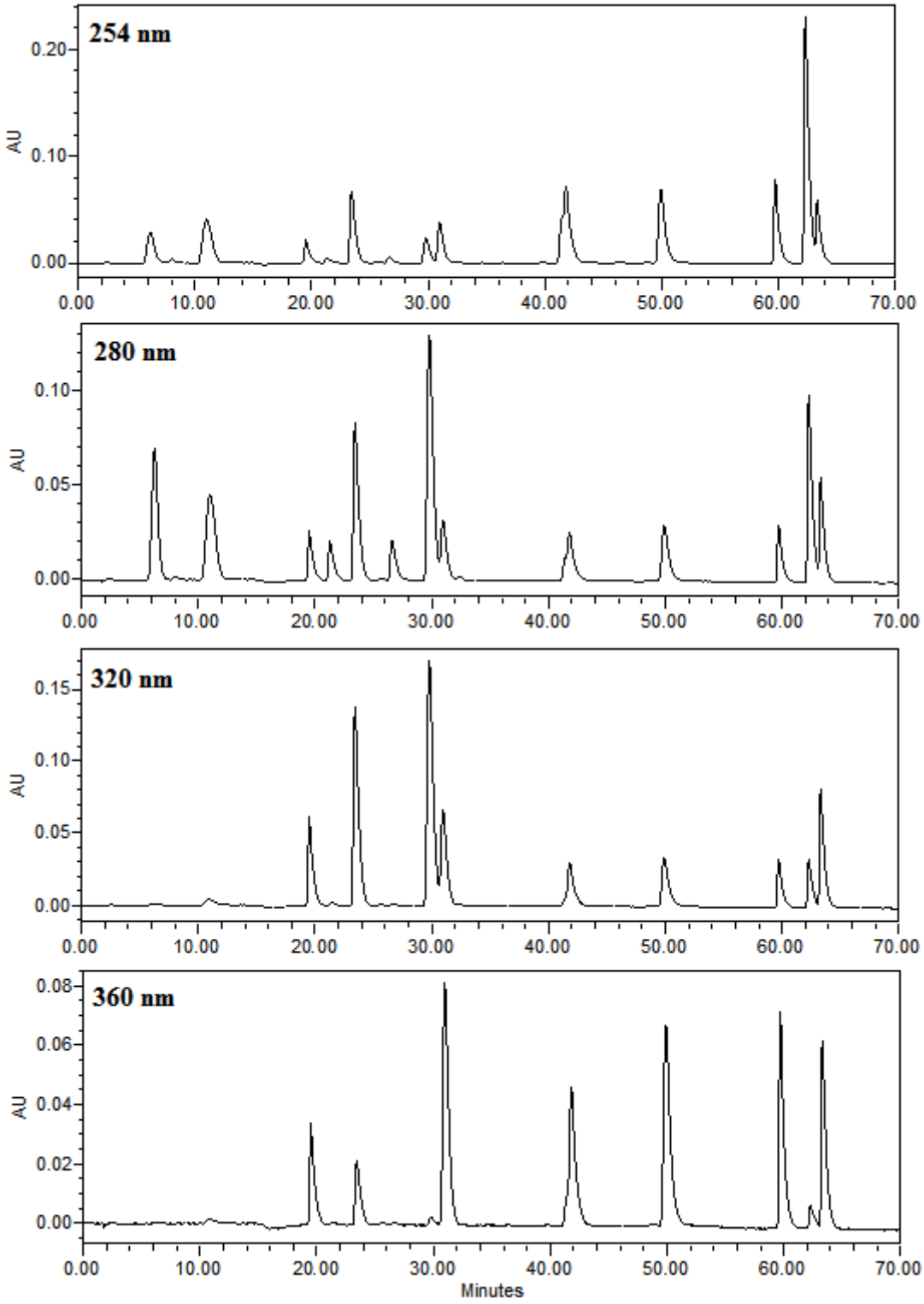


Figure 3.14: Different chromatograms of HPLC analysis of 15 phenolic standard using RP-HPLC photodiode array detectors at 254 nm, 280 nm, 320 nm and 360 nm.

Good resolution was obtained for all 15 references standard used that enable to prepare standard chromatogram. The RP-HPLC-DAD chromatogram of the standard solution mixture at 280 nm is seen in Figure 3.15. Gallic acid and hyroquinone, p-coumaric acid and scopoletin, rutin and ellagic acid, apigenin and hesperetin were found to be eluted closely together, but the differences in their maximum absorption spectra by comparing their chromatograms recorded at 270 nm, for gallic acid and 290 nm for hyroquinone, 310 nm for p-coumaric acid and 345 nm for scopoletin, and 354 nm for rutin and 254 nm for ellagic acid, and 325 nm, for apigenin, and 288 nm, for hesperetin, respectively.

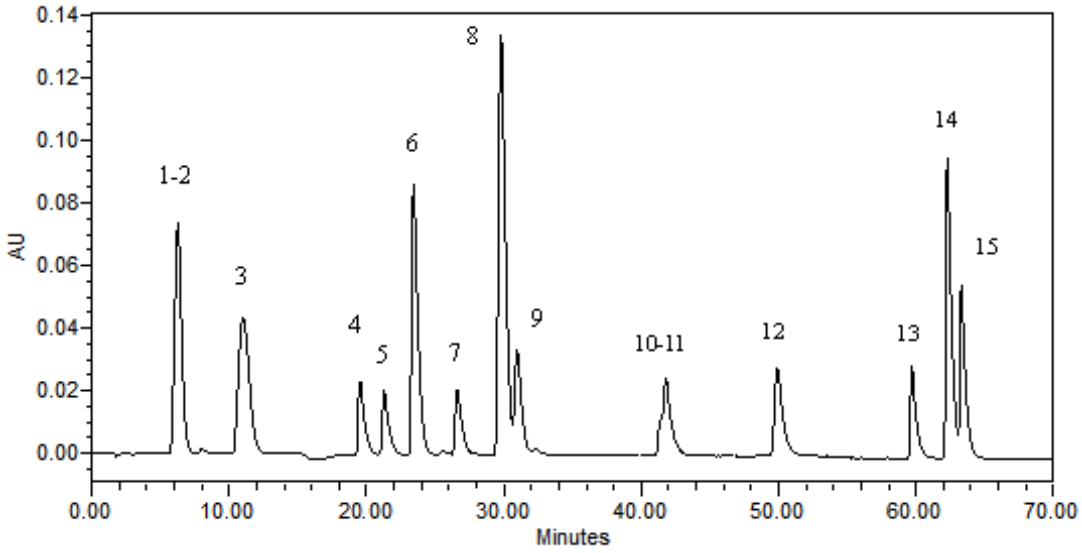


Figure 3.15: Chromatogram of RP-HPLC analysis of 15 phenolic standard compounds using photo diode array detector at 254 nm, 280 nm, 320 nm, and 360 nm. Peaks are indicated as follows: 1-2) gallic acid and hyroquinone; 3) ascorbic acid; 4) esculetin; 5) catechin; 6) caffeic acid; 7) epicatechin; 8) p-coumaric acid; 9) scopoletin; 10-11) rutin and ellagic acid; 12) quercitrin; 13) quercetin; 14) apigenin; 15) hesperetin.

The results (Figure 3.15) show general profile usually seen in reverse-phase (RP) system where most of the phenolic acids were eluted in the first 35 minute of the run, whereas the flavonoids were eluted after 35 minute mainly because of their higher hydrophobicity.

A similar order of the elution profile has also been demonstrated by Escarpa et al., 2000; Mattila et al., 2002; Nuutila et al., 2002; Proestos et al, 2006; Burin et al, 2011; Nicoletti el al, 2008; Plazonić et al, 2008; Irakli et al, 2012. The elution profile is similar to that reported by Abad-García et al. (2007), where gallic acid, catechin, caffeic acid, epicatechin, p-coumaric acid, scopoletin, ellagic acid, quercetin and apigenin were eluted in the same order.

RP-HPLC analysis of individual standards is depicted in Table 3.7. Each standard was first injected individually to determine the exact retention time and chrornatographic characteristic (λ_{max}, and absorbance value) followed by the analysis of the standard mixture (dissolved in methanol to obtain 5 mg/mL stock solutions). The retention time of references standard were ranged from 6.41 min (gallic acid) to 63.5 min (hesperetin).

Table 3.7: Retention time and relative standard deviation of phenolic standards

Phenolic standards	**Retention time (min)**	**RSD (%)**	**Phenolic compounds**	**Retention time (min)**	**RSD (%)**
gallic acid	6.41	0.61	scopoletin	31.3	0.36
hyroquinone	7.35	0.64	rutin	41.7	0.30
ascorbic acid	10.65	0.54	ellagic acid	42.2	0.28
esculetin	19.69	0.32	quercitrin	50.4	0.19
catechin	21.57	0.47	quercetin	59.9	0.21
caffeic acid	23.74	0.47	apigenin	62.5	0.18
epicatechin	26.90	0.54	hesperetin	63.5	0.15
p-coumaric acid	30.10	0.48			

3.5.2. Identification of phenolic constituents by RP- HPLC

In order to separate and identified the phenolic substances that are defined (three isolated compounds: catechin, caffeic and ellagic acid) or not defined in ethyl acetate fraction of *R.heckeliana* root extract reverse phase-high performance liquid chromatography (RP-HPLC) equipped with both photodiode array (PDA or DAD) and fluorescent detector was performed.

A PDA detector allows recording of the UV-vis spectra of each chromatographic peak. Each chromatographic peak may then be attributed to a polyphenol subclass, since each subclass exhibit characteristic UV-VIS spectra (De Rijke, 2006; Stalikas, 2007; Abad-Garcia, 2009a). UV-PDA detection furthermore supplies information on the purity of all peaks and offers the best means of quantifying phenolic compounds in routine applications (Harnly, 2007; Stalikas, 2007).

Qualitative analyses of phenolic substances were based on comparing retention time and UV-vis λ_{max} data obtained for ethyl acetate fraction and reference phenolic standards. Comparison of spectral characteristic (scans from 210 to 800 nm) of ethyl acetate fraction and standard components provided confirmation on the presence of phenolics.

After isolation, several phenolic compounds from ethyl acetate fraction were identifed by RP-HPLC. The compounds were identified by comparing their retention times with those

of phenolic standards. Figure 3.16 shows quite well resolved RP-HPLC chromatogram obtained from ethyl acetate fraction of the roots of *R.heckeliana* extract and monitored at 254 nm, 280 nm, 320 nm and 360 nm under the same analytical conditions. The analytical conditions for RP-HPLC used here are described in experimental section. In our study, 280 nm was chosen as monitoring wavelengths according to absorption maxima.

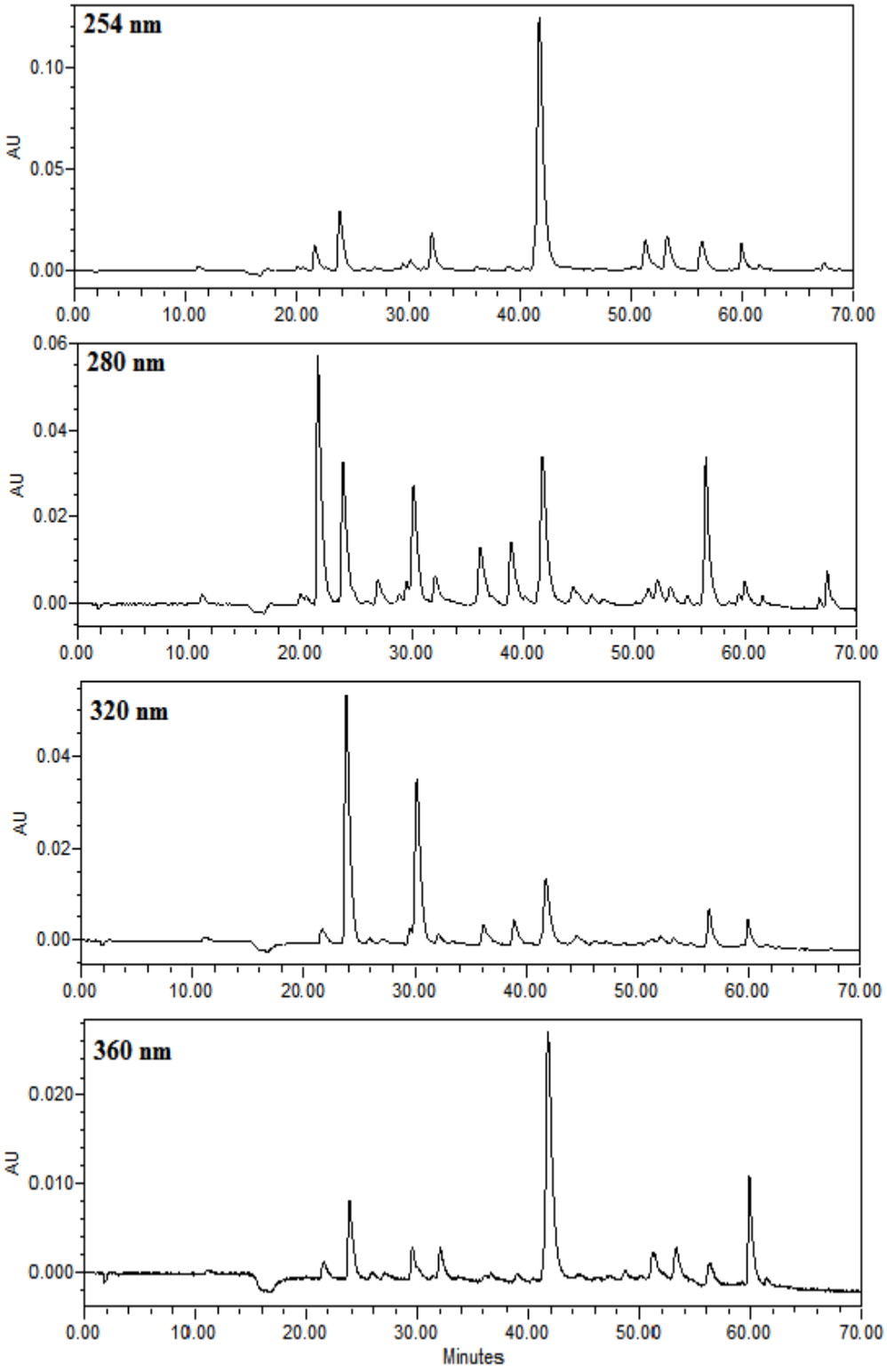


Figure 3.16: RP-HPLC chromatograms of ethyl acetate fraction of different wavelengths (254, 280, 320 and 360 nm).

In addition, Figure 3.17 shows the chromatogram of ethyl acetate fraction (A) and references phenolic standards (B). The RP-HPLC chromatogram in Figure 3.17 (A) exhibits 14 peaks, representing 14 compounds contained in the ethyl acetate fraction. Compounds correspond to peaks 1, 2, 9, and 13 exist in high concentrations while compounds correspond to peaks 3, 4, 6, 7, 8, 10, 11, 12 and 14 were present in lower quantities. Peaks 1, 2 and 9 were previously isolated from ethyl acetate fraction using silica gel 60, sephadex LH-20 column chromatography and TLC in this study as shown in section 3.4.

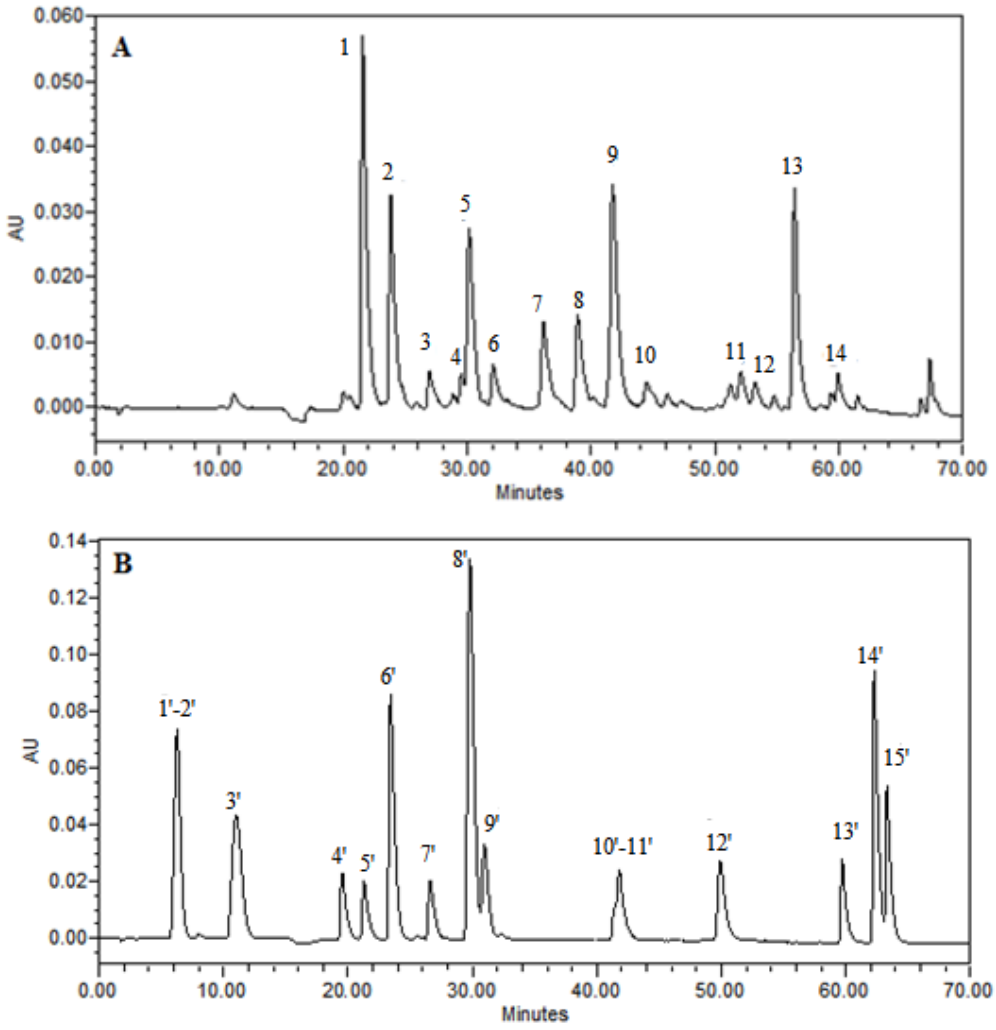


Figure 3.17: A) The chromatogram of phenolic constituents of the ethyl acetate fraction. Peaks are indicated as follows; 1) catechin (isolated compound); 2) caffeic acid (isolated compound); 3) epicatechin; 4) p-coumaric acid; 5) scopoletin; 9) ellagic acid (isolated compound); 14) quercetin and some other phenolic compounds. B) Chromatogram of 15 references phenolic standards. Peaks are indicated as follows: 1'-2') gallic acid and hyroquinone; 3') ascorbic acid; 4') esculetin; 5') catechin; 6') caffeic acid; 7') epicatechin; 8') p-coumaric acid; 9') scopoletin; 10'-11') rutin and ellagic acid; 12') quercitrin; 13') quercetin; 14') apigenin; 15') hesperetin. Separation conditions are the same as those in Figure 3.15 (A). Signal was detected at 280 nm.

The RP-HPLC separations allowed identifying 4 phenolic compounds (flavonoids, phenolic acid etc) except for 3 previously isolated compounds (peaks 1, 2 and 9). The phenolic compounds were identified by comparing their retention times with those of phenolic references standards.

Peak 3 with a retention time of 26.9 min was identified as epicatechin. It has been reported that epicatechin shows a wide range of biological properties like antioxidant, cardio protective agents, antiradical, anti-proliferative effect (Lakopini, 2008; Damianaki, 2000).

Peak 4 with a retention time of 30.10 min was identified as p-coumaric acid. P-coumaric acid is a hydroxycinnamic acid, an organic compound that is a hydroxy derivative of cinnamic acid. It plays key role as an antioxidant and an anti-inflammatory compound in fruits, vegetables, cereals, tea and wine (Rice-Evans, 1997; Svobodova, 2003). Recent studies indicate that p-coumaric acid acts as an antimelanogenic agent in cultured human melanocytes (Lamy, 2010; Seo, 2010). P-coumaric acid decreases angiogenic activities of endothelial cells in vitro (Kong, 2013).

Peak 5 with a retention time of 31.3 min was identified as scopoletin. Scopoletin or 6-methoxy-7-hydroxylcoumarin is a phenolic coumarin that has been isolated from many plants, including a number of medicinal plants. It has antioxidant, anti-inflammatory, antibacterial, antifungial, hepaprotective (Garcia, 1995; Kang, 1998; Shaw, 2003; Mohamed, 2005; Moon, 2007).

Peak 14 with a retention time of 59.9 min was identified as quercetin. Quercetin is a flavonoid (flavonol) widely distributed in nature. It is found in fruits, vegetables, leaves and grains. Quercetin is considered to be a strong antioxidant due to its ability to scavenge free radicals and bind transition metal ions. These properties of quercetin allow it to inhibit lipid peroxidation (Hollman, 1997; Sakanashi, 2008). Quercetin does not only stop the propagation of lipid peroxidation, but also increases glutathione (GSH) levels (Ansari, 2009). Quercetin can reduce inflammation by scavenging free radicals (Boots, 2008). The compound has also been demonstrated to display the antiviral, antibacterial, anticarcinogenic, anti-inflammatory, anti-proliferative effects (Walle, 2004; Braganhol, 2006; Marchand, 2002) and inhibit to the growth of several types of human cancer cell lines (Castillo, 1989; Lamson, 2000). Quercetin has a broad range of biological, pharmacological and medical applications (Duthie, 2000; Harborne and Williams, 2000; Jakubowicz-Gil, 2005).

R. heckeliana root extracts have not yet been investigated for identification and characterization of phenolic compounds such as flavonoids, phenolic acid and anthocyanins by reverse-phase high performance liquid chromatography (RP-HPLC) technique in literature but there are many studies about the other species of Rosa. Roses are a rich source of phenolic compounds (Yoshida, 1993; Hvattum, 2002).

Few reports are available on the characterization of phenolic compounds such as anthocyanins and flavonoids, phenolic acid by (HPLC) techniques on different rosa species and genotype including *R. damascena, R. persica* (Mikanagi, 1995 and 2000; Hutsebaut, 1993; Sumere, 1991; Velioglu and Mazza, 1991; Jassbi, 2003; Nowak, 2005). They reported that the main compounds of quercetin, kaempferol and their glycosides were usually observed in all the Rosa species.

According to Kumar et al. in fresh flowers of *R. bourboniana, R. brunonii* and *R. damascena* was determined to contain 10 polyphenols, namely gallic acid, catechin, epicatechin, rutin, m-coumaric acid, quercitrin, myricetin, quercetin and its derivatives apigenin, kaempferol and its derivatives, flavonol glucosides and galloyl tannin (Kumar, 2008 and 2009).

Nowak et al., 2007 published that the following taxons were examined. *R. canina* L. var. *canina, R. canina* L. var. corymbifera (Borkh.) Boulenger, *R. canina* L. var. puberula R. Keller, *R. canina* L. var dumalis Baker, *R. vosagiaca* Desportes, *R. subcanina* (H. Christ) Dalla Torre et Sarnath, *R. coriifolia* Fri., *R. caryophyllacea* Besser pro parte, *R. subcollina* (H. Christ) Dalla Torre et Sarnath, *R. tomentosa* Sm., *R. rubiginosa* L., *R. villosa* L., *R. inodora* Fri., *R. agrestis* Savi, *R. jundzilli* Besser, *R. pendulina* L., *R. rugosa* Thunb., *R. gallica* L. and investigated leaves of some rose species. They reported that rose leaves contain large amounts of phenolic compounds especially quercetin, kaempherol and ellagic acid.

Isolation and identification of ethyl acetate fraction of *R. heckeliana* root extract is similar to that reported by Sumere, 1993; Hvattum, 2002; Kumar 2009, where ellagic acid, catechin, epicatechin, and quercetin were determined by means of repeated column chromatography and RP-HPLC. Different from other studies detected scopoletin, p-coumaric acid, caffeic acid in our study.

Four phenolic compounds (correspond to peaks 3, 4, 5 and 14) identified by RP-HPLC with photo diode array detector from ethyl acetate fraction.

As the minor phenolics exist in the ethyl acetate fraction, peaks 6, 7, 8, 10, 11, 12 and 13 were not identified because the standards were unavailable for these seven compounds. Further improvements in identification could be achieved by using UV-vis spectra. UV-Vis spectra of flavonoids are particularly informative, providing considerable strucrural information that can distinguish the type of phenol and the oxidation pattern (Andersen, 2006).

Flavonoids (e.g., flavones, flavonol, flavanones...) typically exhibit two major absorption bands in the visible region: Band I in the 320–385 nm region representing the B-ring absorption and Band II in the 250–285 nm range representing A-ring absorption (Figure 3.18)

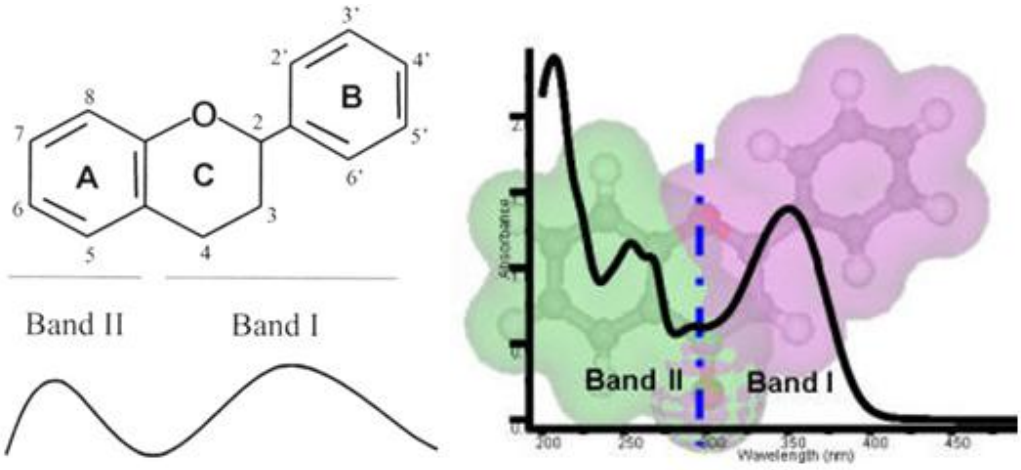


Figure 3.18: Basic flavonoid structure

In this study, for some other unknown (peaks 6, 7, 8, 10, 11, 12 and 13) phenolic compounds, the values of λ_{max} ranged between 350–375 and 254–268 nm for peaks in bands I and II, respectively, indicating that the flavonols in ethyl acetate extract. Results from the UV-vis spectroscopic analyses show that the peak for 10, there are two different main λ_{max} at 250.4 and 376.3 nm, which suggests that peaks 10, 11, 12, and 13 could be flavonols. Peaks 10, 11, 12 and 13 exhibited the typical UV-vis spectra of **flavonols** (Figure 3.19).

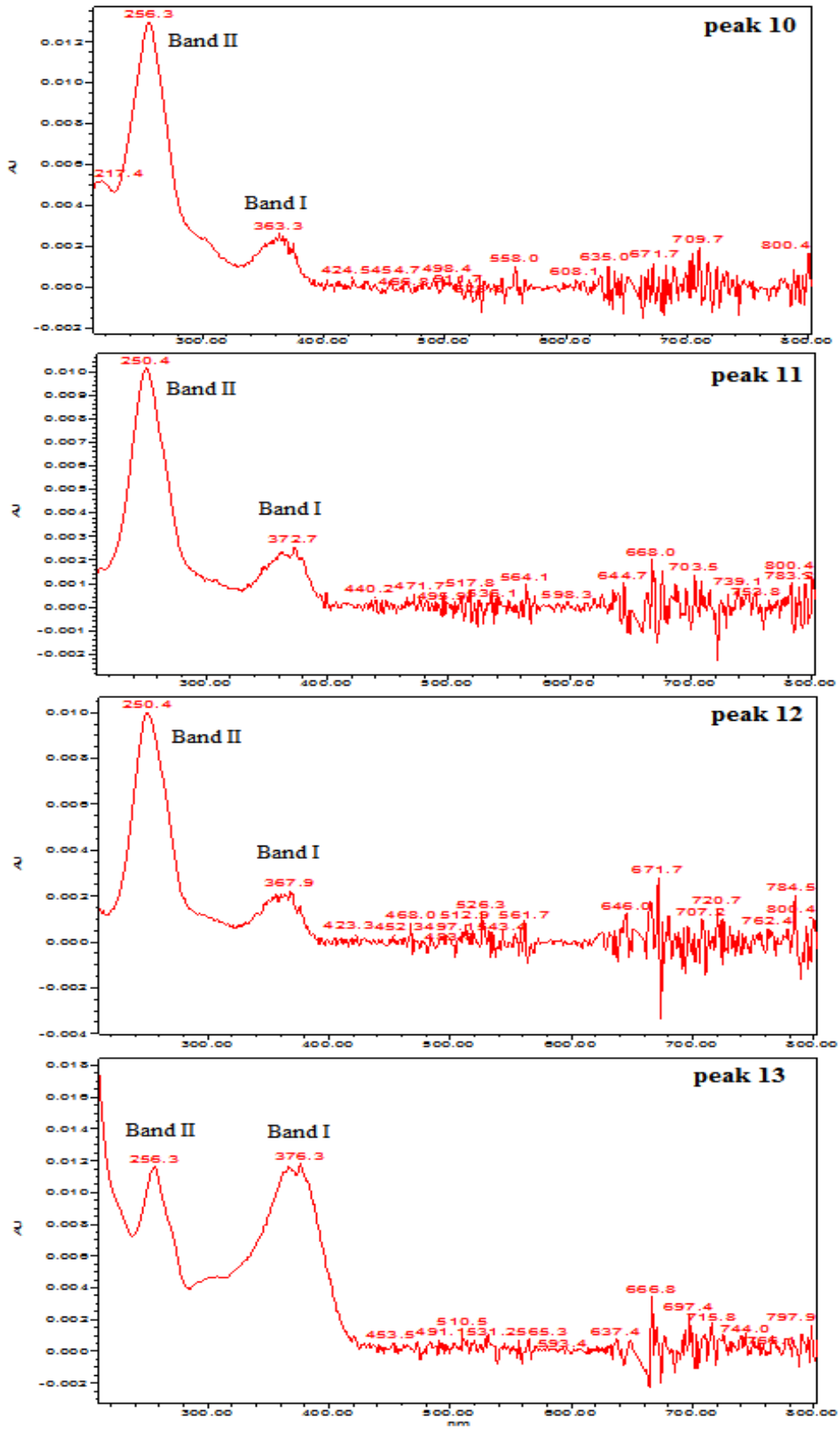


Figure 3.19: UV-Vis spectra of peaks 10, 11, 12 and 13 recorded between 210-800 nm.

The remaining peaks (6, 7 and 8) were found to be associated with simple phenolic compounds such as flavanone glycoside. Under reversed-phase chromatographic conditions, peaks 6, 7 and 8 exhibited UV-vis maxima around 290 nm. The spectra of the flavanones were very characteristic with an intense band (Band II) near 290 nm which was shifted to lower wavelengths by glycosidation. These spectral features of the flavanones can be attributed to the saturated heterocyclic C-ring and the resulting lack of conjugation between the A- and B-rings. Peaks 6, 7 and 8 have the maximum absorbance λ_{max} about at 290 nm, which suggests that peaks 6, 7 and 8 could be flavanones. Peaks 6, 7 and 8 exhibited the typical UV-vis spectra of **flavanones** (Figure 3.20).

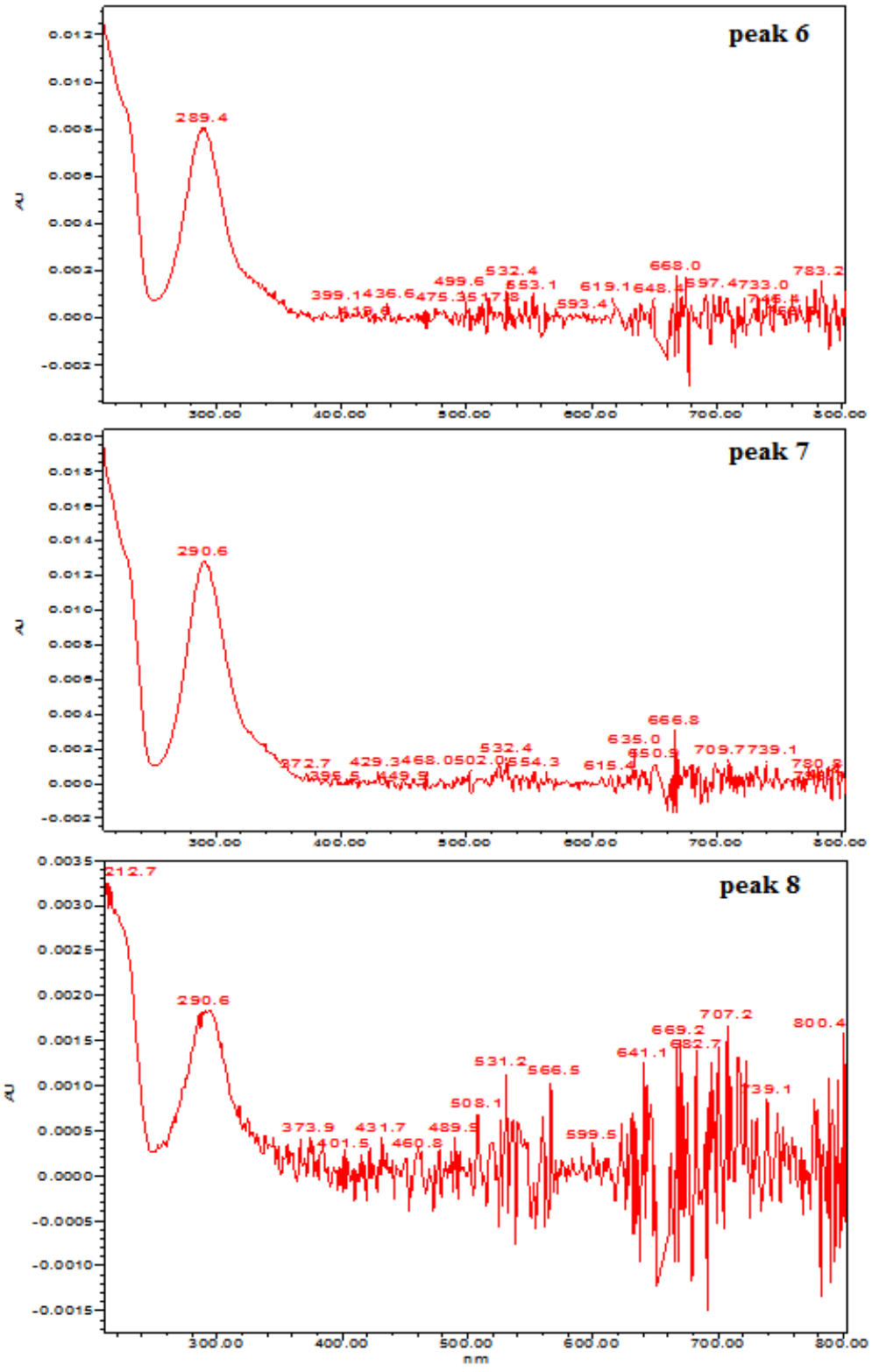


Figure 3.20: UV-Vis spectra of peaks 6, 7 and 8 recorded between 210-800 nm.

In addition to, catechin, epicatechin and scopoletin display emissive characters as presented in the Figure 3.21. Figure 3.21 demonstrates the chromatograms of RP-HPLC analysis of ethyl acetate fraction by means of fluorescence detector. As it was clearly noticeable from the Figure 3.21 of elution profile catechin appears to display higher quantum yield of emission compared to that of epicatechin and scopoletin. As it is known, the molecules with emissive properties encountered seldom in nature and are quite important for the spectral information regarding their environment. In other words, these compounds (fluorophores) are sensitive to their environment. P*er se*, a change in the solvent viscosity or polarity around a fluorophore would present itself as a change in the intensity and/or as a spectral shift between the excitation and emission wavelengths (Lippert, 1957). Spectral shifts are also important for interpreting the changes taking place around the fluorophores upon binding to the biologically important macromolecules (McClure, 1966; Lakowicz, 1980). Catechin, epicatechin and scopoletin exhibited important spectroscopic features with the emission maxima of 360 nm, 360 nm and 468 nm, respectively.

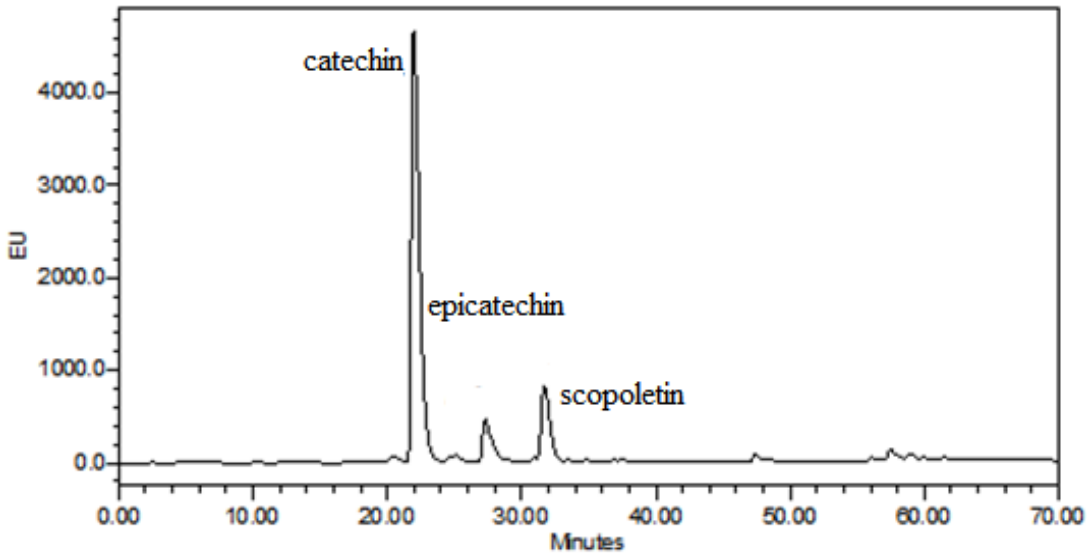


Figure 3.21: Fluoresence chromatogram of catechin, epicatechin and scopoletin. Excitation and emission wavelengths: 278 and 360 nm for the catechin and epicatechin. Excitation wavelentgh 366 nm, emission wavelentgh 468 nm for scopoletin

To confirm the RP-HPLC results of ethyl acetate fraction High Resolution Mass Spectroscopy (HRMS) experiment was performed.

3.5.3. Confirmation by HR-MS

HR-MS analysis is suitable for rapid and accurate identification of molecular weights of organic compounds. Here the HR-MS spectra revealed virtually the exact mass and molecular composition of the phenolic compounds of our interest. It was also employed for the confirmation of the chemical identity of the phenolic compounds recognized by RP-HPLC. The confirmation procedure was aided by comparing with the phenolic standards. Through the HR-MS analysis roots of *R. heckeliana* fractionated by ethyl acetate was found to contain the highest amount of flavonoids, phenolic acids and coumarin, the results are displayed in Figure 3.22.

Epicatechin: HR-MS analysis of epicatechin gave a sodiated molecular ion peak at m/z 443.0990 $[M-H]^+$ in Figure 3.20. The molecular formula of epicatechin was determined to be $C_{22}H_{18}O_{10}$ by HR-MS (m/z 443.0990) $[M-H]^+$ (calculated for $C_{22}H_{18}O_{10}$, 443.0978).

*p-coumaric acid***:** HR-MS analysis of p-coumaric acid gave a sodiated molecular ion peak at m/z 165.0544 $[M-H]^+$ in Figure 3.20. The molecular formula of p-coumaric acid was determined to be $C_9H_8O_3$ by HR-MS (m/z 165.0544) $[M-H]^+$ (calculated for $C_9H_8O_3$, 165.0552).

*Scopoletin***:** HR-MS analysis of scopoletin gave a sodiated molecular ion peak at m/z 193.0482 $[M-H]^+$ in Figure 3.20. The molecular formula of scopoletin was determined to be $C_{10}H_8O_4$ by HR-MS (m/z 193.0482) $[M-H]^+$ (calculated for $C_{10}H_8O_4$, 193.0501).

*Quercetin***:** The HR-MS analysis of compound quercetin gave a sodiated molecular ion peak at m/z 303.0503 $[M-H]^+$ in Figure 3.20. The molecular formula of quercetin was determined to be $C_{15}H_{10}O_7$ by HR-MS (m/z 303.0503) $[M-H]^+$ (calculated for $C_{15}H_{10}O_7$, 303.0505).

In literature however, a variety of Rosa species except *R. heckeliana* were reported for their phenolic constituents by using LC-MS. According to Cai et al., 2005 the flowers of *R. chinensis* contained 36 known and unknown phenolics, including tannins, flavonols, and anthocyanins. Kumar et al., 2009 reported that quercetin, kaempferol and their glycosides as the main phenolic constitiuents of flowers of *R. damascena, R. bourboniana and R.brunonii* which were evaluated by the method of UPLC-ESI-QTOF-MS.

Some of the results described for other Rosa species were partly similar to the findings of our study (Hvattum, 2002; Cai, 2005; Kumar, 2008; Ozcan, 2012). Furthermore, mostly found in the leaves, roots and flowers of the genus Rosa were gallotannins and ellagitannins (Okuda, 1992; Mikanagi, 1995; Hashidoko, 1996). Catechin derivatives and proanthocyanidins were also detected in the roots and leaves of *R. rugosa* and *R. persica*, however in lesser amounts in the flowers (Hashidoko, 1996; Mikanagi, 2000; Ng et al., 2004; Jassbi, 2003).

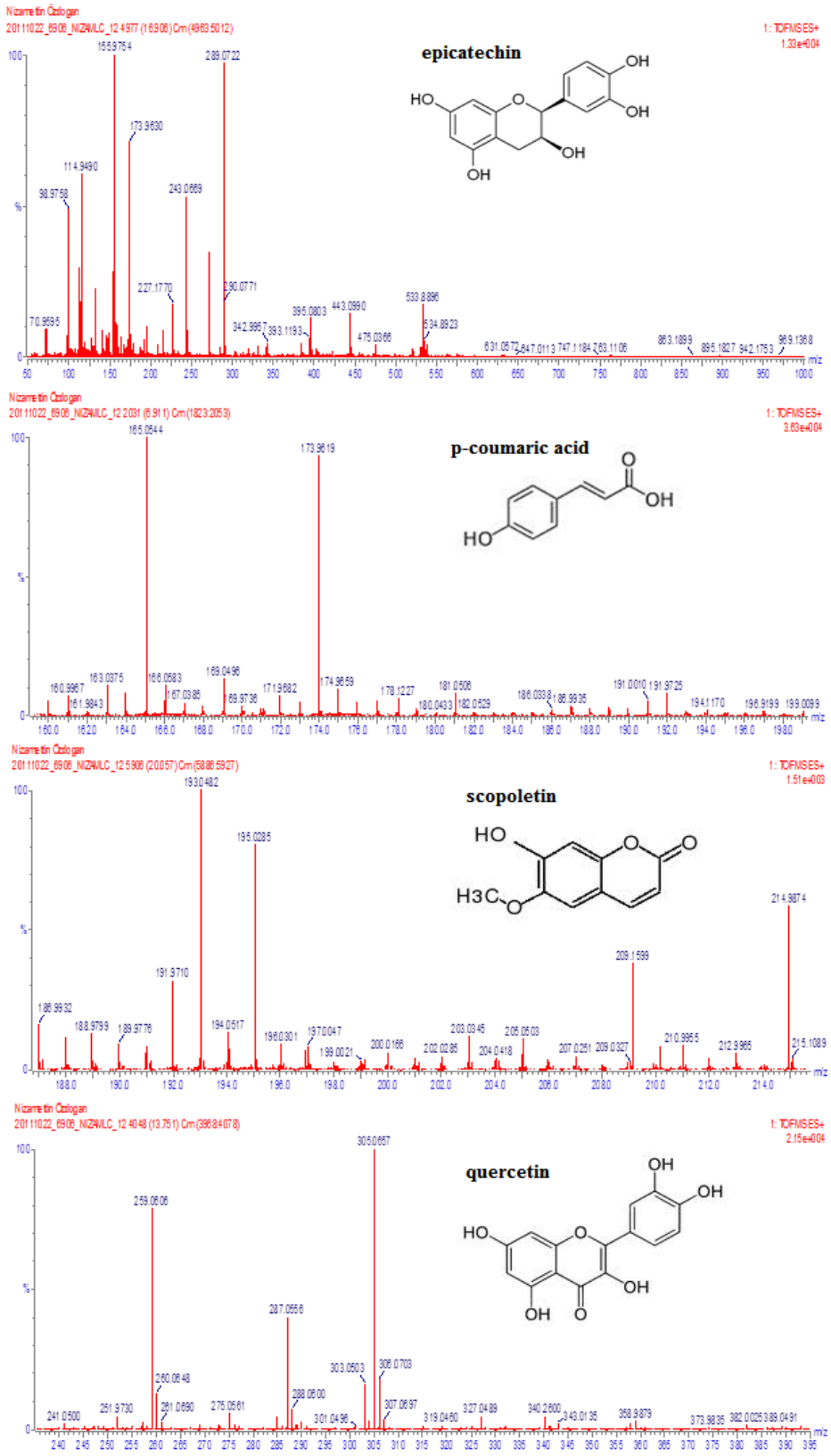


Figure 3.22: HR-MS spectra of epicatechin, p-coumaric, scopoletin and quercetin.

3.5.4. Quantitation of identified phenolic compounds in *R. heckeliana*

In order to quantitate the amounts of the basic phenolic compounds identified in the ethyl acetate fraction of the *R. heckeliana* root extract which was necessary to utilize analytical RP-HPLC method.

First, various known concentrations of the corresponding phenolic standards were used for the calibration curve preparation by means of analytic RP-HPLC. Then the previously identified phenolic constituents of the *R. heckeliana* fraction were able to quantify by the peak areas using "process method" of the Waters Empower Software.

Utilizing the peak areas, quantities of the identified phenolics including catechin, caffeic acid, epicatechin, p-coumaric acid, scopoletin, ellagic acid and quercetin were calculated as 1.763 mg, 0.098 mg, 0.0276 mg, 0.0597 mg, 0.228 mg, 0.334 mg and 0.1101 mg, out of 10mg of ethyl acetate extract, respectively.

Standard curves were prepared for all the phenolic standards by plotting the peak area of each against the concentration (mg/mL). All the standards samples were measured in duplicates of the following concentrations mentioned on three consecutive days.

Catechin was observed as the major compound of flavanols present in the ethyl acetate fraction of root extracts of *R.heckeliana* which embraces important biological activities. This suggests *R. heckeliana* roots can be considered as a potential flavan-3-ol (also known as flavanols) source. Recently, catechin has drawn more attention because of its antioxidant and anti-carcinogenic properties.

This study reveals the first report on the isolation and identification of these phenolic constituents (catechin, caffeic acid, epicatechin, p-coumaric acid, scopoletin, ellagic acid and quercetin) from the roots of *R. heckeliana*. Although the same compounds have been previously recognized in the other rosa species and other plants.

To briefly summarize, our recent study showed that the ethyl acetate fraction of *R. heckeliana* root extracts contain a high phenolic phytochemicals and displays high antioxidant properties compared to other fractions. While some plant extracts were found to display antioxidant properties as well as high potentials of cytotoxicity on a wide range of cancer cell lines (Boivin, 2009; Ju, 2004). Therefore, it is suggested that the highly antioxidant crude extract, ethyl acetate fraction, as well as the three isolated compounds: catechin, caffeic acid, ellagic acid should also demonstrate cytotoxic effects on the selected cancer cell lines. The objective of this present study was to determine the cytotoxic effect of the extract/fractions/isolated compounds/standards against MCF-7 and MDA-MB-231 breast cancer cell lines.

3.6. Cell culture

3.6.1. Viability of MCF-7 and MDA-MB-231 cells

Over the last decade, MCF–7 and MDA–MB–231 cell lines have been subjects of a model system, developed for the study of phytochemical effects on biological systems. When a cell line was used, the growth and inoculation condition had to be optimized in order to get optimal results.

It is essential that the viability of cells be determined so that the required suitability of medium composition and the other environmental conditions can be ascertained. All cells are sensitive to their environments and the other physical and chemical conditions. Cancer cells too have some kind of environmental sensitivity, even though they are resistant to death. And also nutrient supplies should be under control with respect to the cell division periods. To enable maximum growth for the cells, nutrition supplies and physiological conditions have to be optimized, so that the cell viability reaches higher than 90% for the cells of interest.

An RPMI 1640 is a commonly used source of growth medium for the mentioned cell lines. In order to obtain a complete medium L – glutamine and pyruvate were added into RPMI medium for vitality of the cells. High levels of protein and growth factors can be provided by the addition of fetal calf serum. It is crucial to keep all the growth media from contamination as sterile as possible by the addition of the antibiotics such as gentamycin. However the concentration of the antibiotics should be limited for their harmfulness. These conditions can be provided by the UV sterilization in laminar flow, filtered sterilization of all solutions, and autoclaving of all glass wares at 120^0 C for 20 minutes.

The well-being of the cells was regularly monitored under the inverted microscope not less than every 24 hours. In order to plot the growth curves of cell viability, a 24-hour adjustment period needs to be observed before assembling of the data. In an actual growth (incubation) study, a reliable data can only be attained by eliminating physical and chemical environmental effects. This at least 24 hours of incubation period is to follow the adjustment and endurance of the cells to their new environmental conditions. After the first 24 hours period of incubation, as the first observation, cells were counted, and this was recorded as 0 time point. Further sampling was repeated for every 24 hours of interval during the following 240 hours of incubation. A hemo-cytometer was utilized for the cell viability tests using a dye exclusion technique with trypan blue (Figure 3.23 a). A 20 μL of the cell suspension from culture flask was examined under light microscope. These suspensions are taken from the culture flasks under aseptic conditions.

The cells that were healthy appear round and shiny and do not include trypan blue dye in their cytoplasm. This was due to their active cell membrane transport systems that could pump the dye out of the cell (Figure 3. 23 a). Contrary to those of healthy cells, the dye was confined in the cytoplasm of the dead cells their due to the failing active transport system (Figure 3.23 b and c). The maximum number of cells and a growth over 90 % of the cell viability were attained for for MCF–7 and MDA–MB–231 cells in optimal physical and chemical conditions. The optimum growth time was determined as 96 hours, to get maximum cell number and over 90% viability, based on the growth curves of MCF-7 and MDA–MB–231 cells (Figure 3. 24) using the well-established medium. The viability of MCF-7 and MDA-MB-231 cells were appeared to decline after 96 hours, this was not only for the lack of nourishment but also because of the accumulating toxic waste materials of the cells, as well as the pH change in the medium.

In this study, it is demonstrated that the cells have to be sub–cultured within no more than every 3 days, as the data table of cell viability for MCF–7 and MDA–MB–231 supports. This treatment can provide the maintenance of their robustnes. Different treatment can lead to some changes on their membrane that can be observed under inverted microscope. This can have lethal effects due to changes in their shape. The data obtained from the growth graphs indicates a steady state in the growth curver after 168 hours.

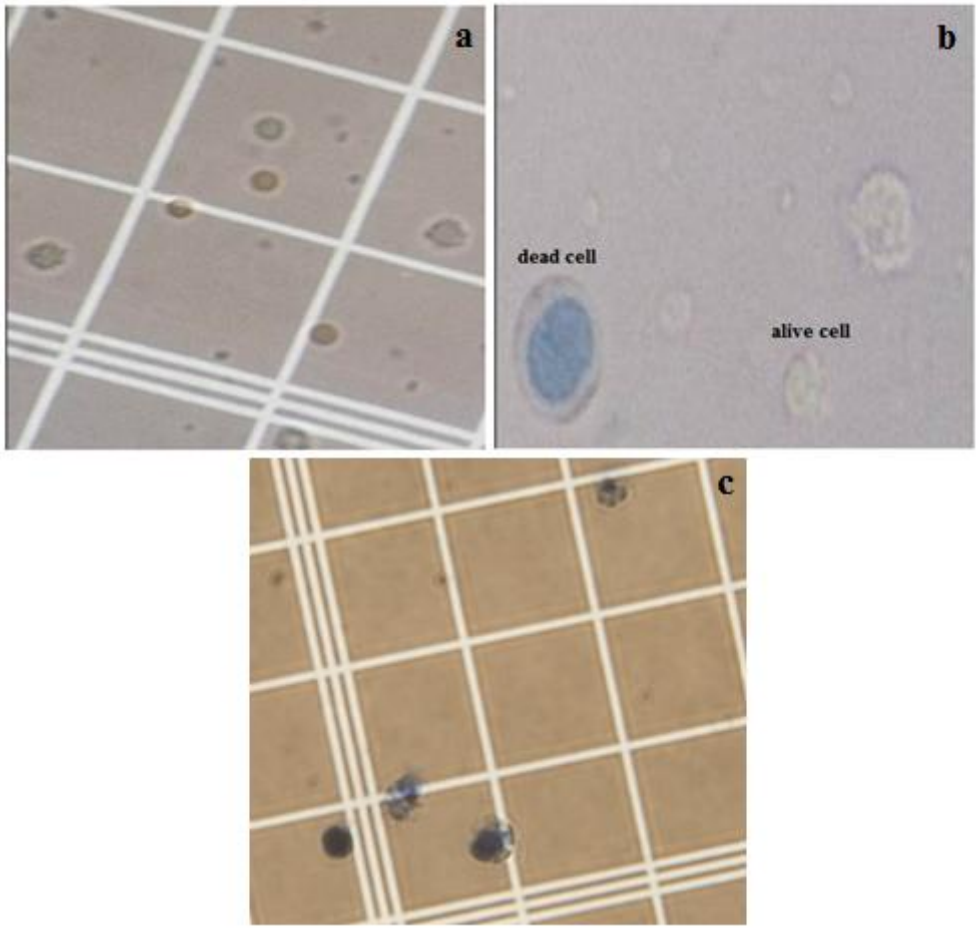


Figure 3.23: a) the healthy MCF-7 cells, b) the alive and dead MCF-7 cells, c) the dead MCF-7 cells were side by side at 400X magnification.

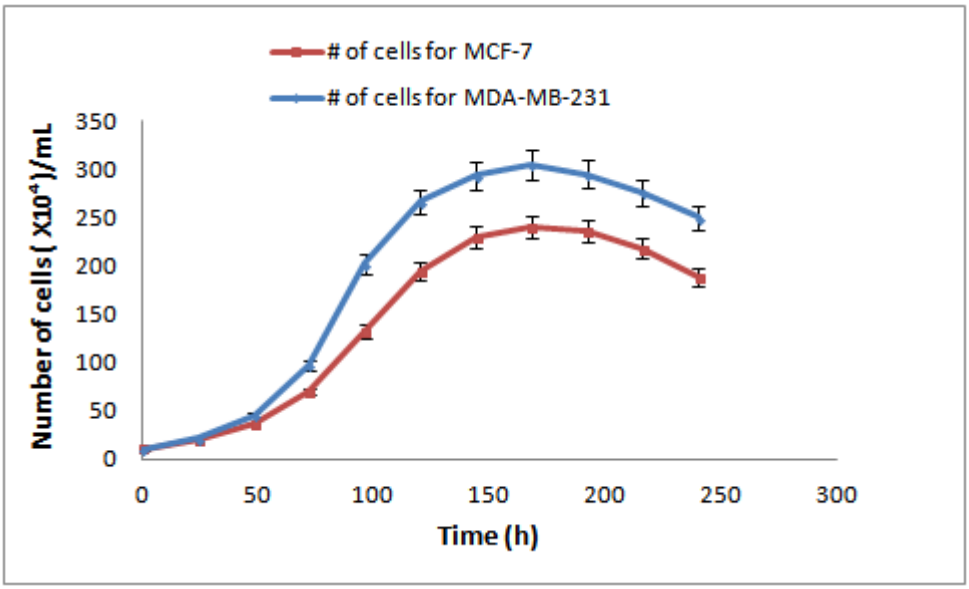


Figure 3.24: The growth curve of MCF-7 and MDA-MB-231 cells in 240 hour by trypan blue counting..

MCF-7 and MDA-MB-231 cells were seeded at a density of about 100,000 cells/well. The wells were prepared in duplicates for each time point and were incubated at 37˚C in 5% CO_2. Two wells from each cell line were harvested and counted at each time point and the rest of wells allowed continuing growing. The numbers of cells were plotted against the growth duration.

3.7. Cytotoxicity of extract/fraction/isolated compounds in MCF and MDA-MB-231 Cells

3.7.1. Evaluation on morphological changes by light microscopy

For the investigation of cytotoxic effects of extract, fraction, isolated compounds including catechin, caffeic acid, ellagic acid, and standards morphological alteration of MCF-7 and MDA-MB-231 cells lines were combined with commonly accepted biochemical methods under phase contrast microscope.

The effects of extract, ethyl acetate fraction, three isolated: catechin, caffeic acid, ellagic acid and standard compounds on morphological changes and viability in MCF-7 and MDA-MB-231 cells treated for 24 h were analyzed under light inverted microscope (magnification, 400X). MCF-7 and MDA-MB-231cells (2 x 10^5 cells/ml; 1 ml/well; 24-well plate) were either left untreated or treated with 50, 100 and 150 µg/ml crude extract, ethyl acetate fraction and three isolated compounds for 24 h.

The microscopic observations revealed the extract, fraction and isolated compounds to be having outstanding effect on treated MCF-7 cells compared to treated MDA-MB-231 cells and untreated cells (these cells were regular in shape and size, had eccentric nucleus and a relatively small piece of cytoplasm).

The number of dead cells increased correspondingly with concentration increase of the crude extract, fraction, and isolated compounds treatment in regard to observation in MCF-7 and MDA-MB-231 cells were illustrated (Fig 3.25-3.28) comparing with control and DMSO (final concentration is 1% within each well) treated cells.

3.7.1.1. MCF-7 cell lines

The proliferation rate of MCF-7 cells maintained in normal media was lower than MDA-MB-231, as about 80-90 % confluency was obtained after 72 hours of growth (Figure 3.24). Apparent changes in cell morphology and deformity of cells from the culture surface were observed after 24 hour of treatment with different extract/fraction/isolated compounds concentrations.

At low concentration, enlargement of the cells by crude extract and ethyl acetate fraction was not observed significantly for 24 h in microscopic analysis. On the other hand, decreased cell growth by isolated compounds including catechin, caffeic acid and ellagic acid was observed significantly for 24 h in microscopic analysis.

Control or DMSO treated cells were round in normal shape and size (Figure 3.25a and Figure 3.25 b for MCF-7). At 24 hours after treatment of MCF-7 cells with 150 μg/ml concentration of crude extracts showed a weaker activity (Figure 3.25 d). MCF was a decrease in the number of cells. On the other hand, at 150 μg/ml concentration of ethyl acetate fraction the level of adhesion was reduced and some of the cells were rounded (Figure 3.25 f).

Isolated compounds including catechin, caffeic acid and ellagic acid showed noticeable morphological alterations with stronger adhesion level at 50 and 100 μg/ml concentrations. At 50 μg/ml concentration of catechin their growth was inhibited and slight cell shrinkage was observed (Figure 3.26 a). On the other hand MCF-7 cells to round and deformity of some cells at 100 μg/ml concentration of catechin (Figure 3.26 b).

Exposure of MCF-7 cells to those isolated compounds including caffeic and ellagic acid at 50 μg/ml concentrations for 24 h led to shrinkage and shape formation (Fig 3.26 c and Fig 3.26 e) which were observed by light inverted microscope (400X).

At 100 μg/ml concentration, there was an increase in the number of dead cells too much for ellagic acid and caffeic acid for 24 h in microscopic analysis (Figure 3.26 d and Figure 3.26 f).

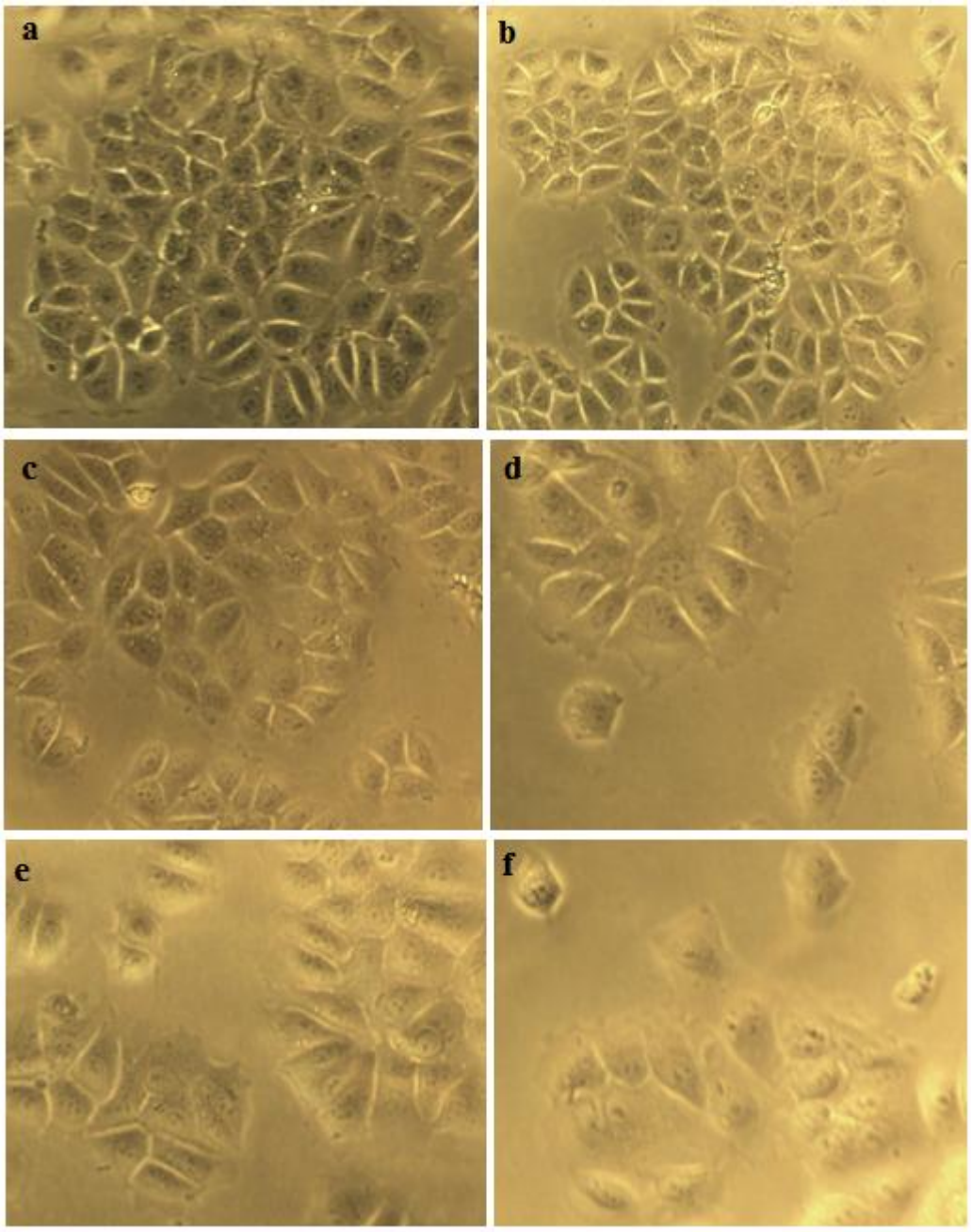


Figure 3.25: Morphological alterations of the MCF-7 cells after treatment at various concentrations of crude extract and ethyl acetate fraction a) control, b) 1 % DMSO control, c) crude extract: 100 µg/mL and d) 150 µg/mL, e) ethyl acetate fraction: 100 µg/mL and f) 150 µg/mL for 24 hours.

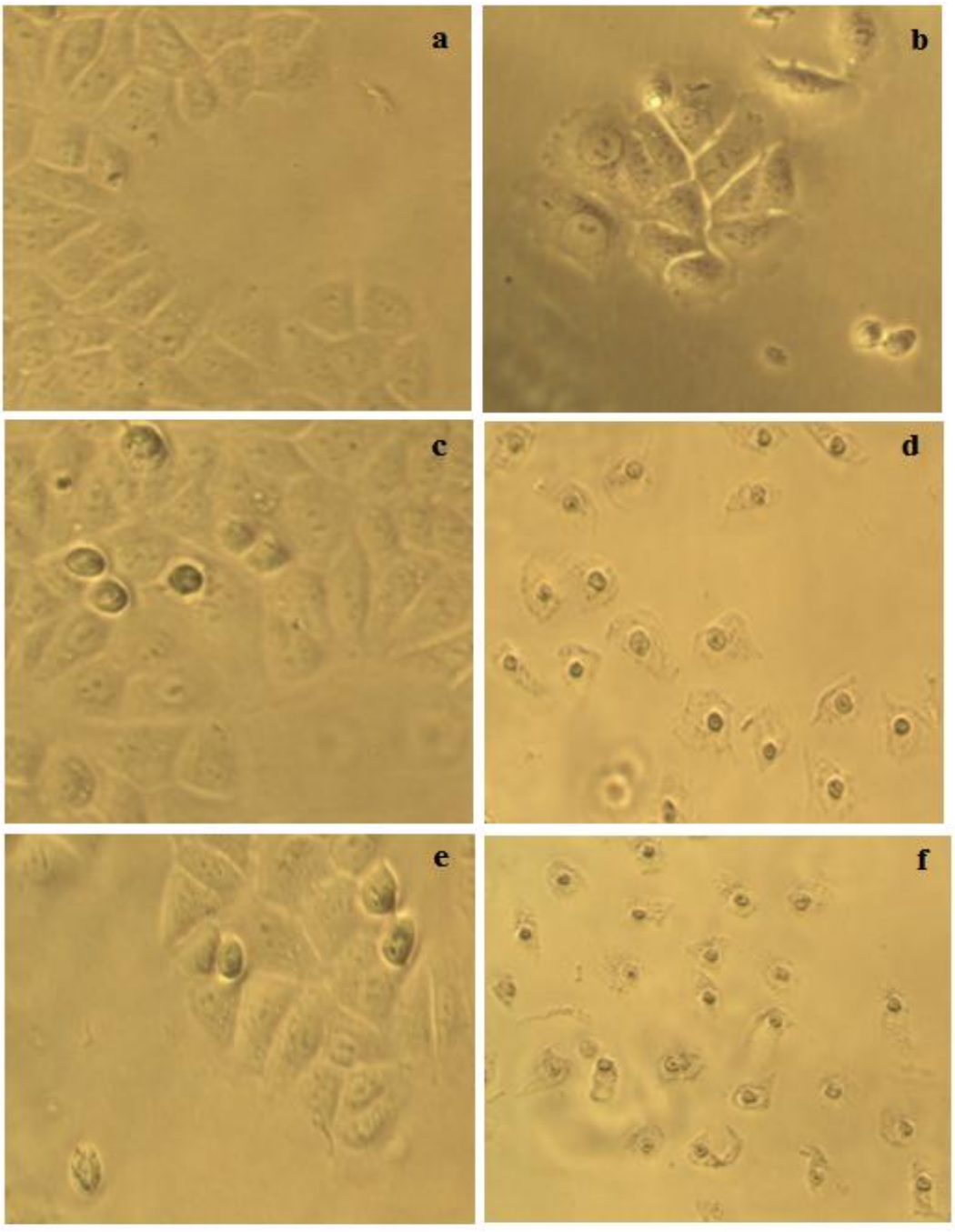


Figure 3.26:Morphological alterations of the MCF-7 cells after treatment at various concentrations of three isolated compounds a) isolated catechin at 50 µg/mL and 100 µg/mL, c) isolated caffeic acid: 50 µg/mL and d) 100 µg/mL, e) isolated ellagic acid: 50 µg/mL and f) 100 µg/mL for 24 hours.

3.7.1.2. MDA-MB-231 cell lines

The normally maintained MDA-MB-231 cells were proliferating with high rate, and formed a monolayer growth with no less than 90% confluency within 72 hours.

At 24 hours after treatment of MDA-MB-231 cells with 100 μg/ml crude and ethyl acetate fraction extract some of cells were detached and showed morphological changes with weaker adhesion level (Figure 3.27 c and Figure 3.27 e). Treatment of MDA-MB-231 cells with crude extract and ethyl acetate fraction at 150 μg/ml for 24 hours resulted in reduced density of the monolayer, and some of the cells were round and detached (Figure 3.27 d and Figure 3.27 f).

MDA-MB-231 treated with 50 μg/ml concentration of isolate catechin showed for 24 hour morphological alterations with weaker adhesion level (Figure 3.28 a). At highest concentration (100 μg/mL) the level of adhesion was reduced and nearly half of cells were rounded and showed a noticeable decrease in confluency (Figure 3.28 b).

24 hours after treatment of MDA-MB-231 cells with isolate caffeic acid (50 μg/ml) the cells began to round up and the level of adhesion was influenced (Figure 3.28 c). Higher concentrations (100μg/ml) had more pronounced effects and more than half of cells were rounded up (Figure 3.28 d).

When MDA-MB-231 cells were treated with 50μg/mL isolate ellagic acid for 24 hour about half of the cells were dead as evident by cellular rounding up and floating.

The dose-dependent isolate ellagic acid effect was observed in the degree of confluency (Figure 3.28 e). When MDA-MB-231 cells were treated with 100 μg/ml concentration of isolate ellagic acid a number of cells were dead as evident by cellular rounding up, floating and cell debris for 24 h (Figure 3.28 f).

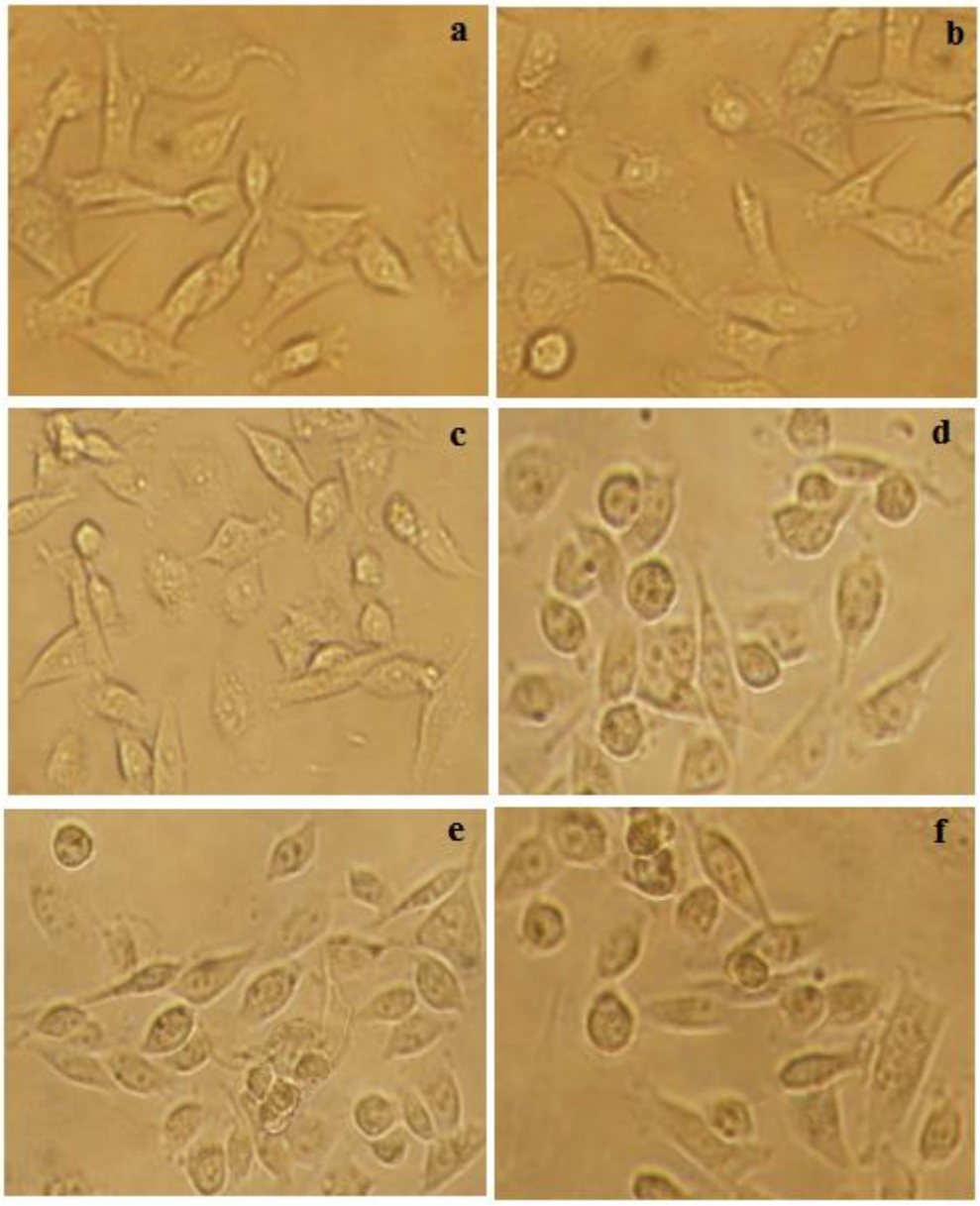


Figure 3.27:Morphological changes of the MDA-MB-231 cells after treatment at different concentrations of crude extract and ethyl acetate fraction a) control, b) 1% DMSO control, c) crude extract: 100 µg/mL and d) 150 µg/mL, e) ethyl acetate fraction: 100 µg/mL and f) 150 µg/mL for 24 hours.

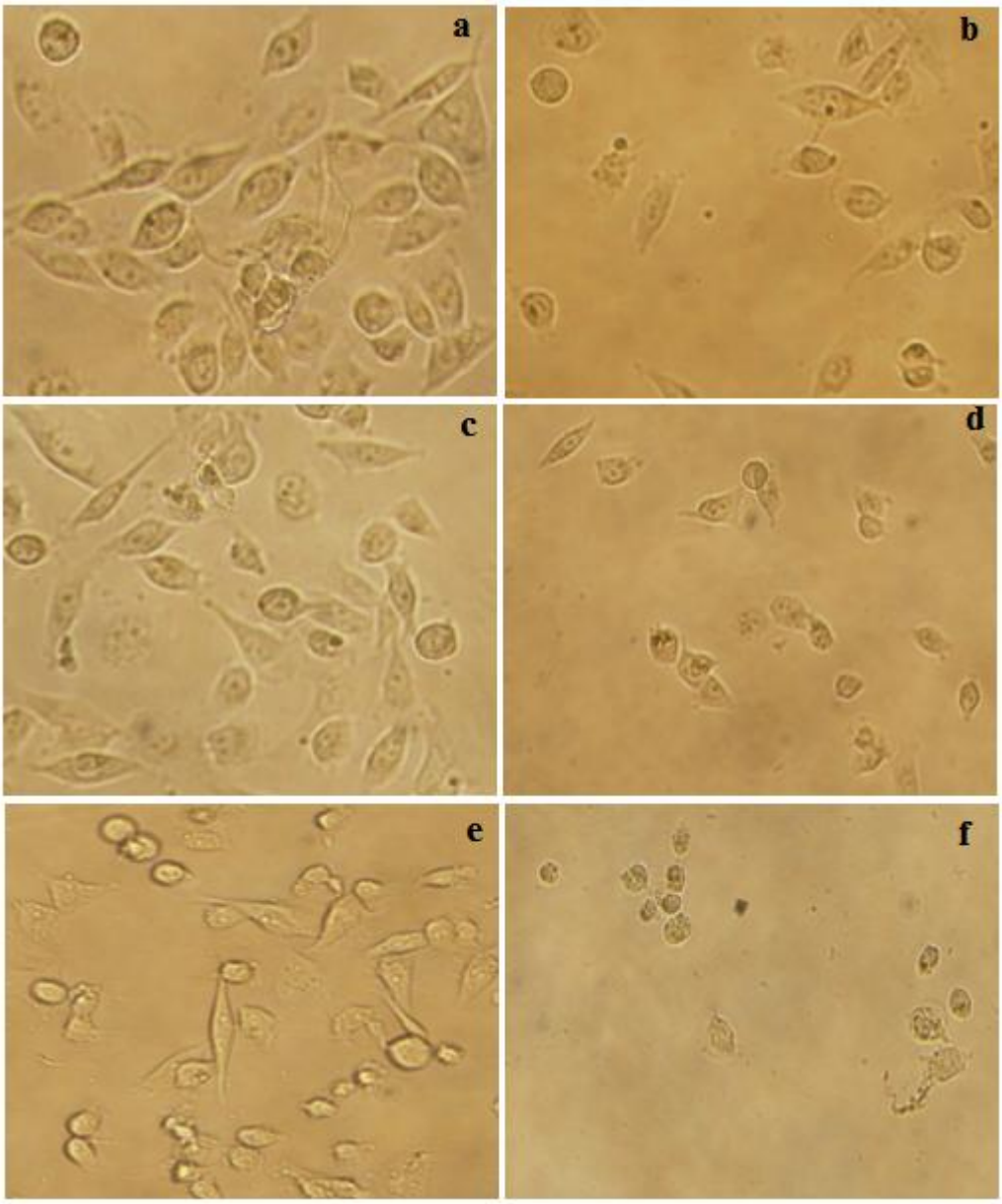


Figure 3.28: Morphological changes of the MCF-7 cells after treatment at different concentrations of three isolated compounds a) isolated catechin at 50 µg/mL and 100 µg/mL, c) isolated caffeic acid: 50 µg/mL and d) 100 µg/mL, e) isolated ellagic acid: 50 µg/mL and f) 100 µg/mL for 24 hours.

3.7.2. XTT cell viability method

Cell viability of two human breast cancer cell lines, metabolically active MCF-7 and MDA-MB-231, was determined with a rapid colorimetric assay using (2,3-Bis-(2-Methoxy-4-Nitro-5-Sulfophenyl)-2H-Tetrazolium-5-Carboxanilide) (XTT).

XTT a tetrazolium salt is pale yellow in its oxidized form. Mitochondrial enzymes of the metabolically active cells were able to reduce the tetrazolium salt which reveals an orange colored XTT formazan product. Those changes in the absorbance (415 nm) are easily monitored by micro plate reader. Principally, this assay is a measure of live and metabolically active cells.

Crude extract, fraction, isolated compounds and standards were dissolved in 2 % DMSO. DMSO works as a vehicle that it increases the permeability of cells and facilitates the entrance of phenolic compounds in cells. Final DMSO concentration in all wells was 1%.

Average of quadruple absorbance measurements at 415 nm of the wells treated with given concentration of extracts/samples was calculated for 24 hours. Cell viability examinations acquired from XTT method were changed into percent of cell viability by setting the control set as 100% cell viability (in the presence of 1% DMSO).

There were concerns about the usage of XTT technique in herbal extracts. It was indicated taht cytotoxicity tests with tetrazolium salts give false-negative results since extracts reduce tetrazoliun salts also in the absance of living cells. In addition to this, different antioxidant molecules containing free thiol groups were found to be able to reduce tetrazolium salts.

In order to prevent these interferences, control wells without cells were included in the XTT measurements and absorbance of average of blank wells (cells without extract) were subtracted from samples wells to observe direct action of the extracts/samples. In brief, to eliminate the effect of plant on absorption, the cultured cells without extract treatment were used.

3.7.2.1. MCF-7 cancer cell lines

MCF-7 breast cancer cell line, malignant adenocarcinoma in a pleural effusion, is one of the most commonly used in vitro breast cancer models. The majority of this cell line is derived from more aggressive and metastatic tumors, rather than the primary lesion. MCF-7 cells are useful for in vitro studies because the cell line retained several ideal characteristic particular to the mammary epithelium including the ability to process estradiol via cytoplasmic estrogen receptors.

Cell viability was measured at 24 h after treatment with crude extract, ethyl acetate fraction, and isolated compounds including catechin, caffeic acid, ellagic acid and standards on the growth of MCF-7 cancer cell lines at different concentrations of 0-200 μg/mL dose dependent manner.

Between the two extract studied here, crude extract and ethyl acetate fraction extract exhibited significant cytotoxic activity against the MCF-7 cancer cell lines in a dose dependent manner. Cytotoxic activity of crude extract also showed a trend similar to that of ethyl acetate fraction extract but, the rate of cell death in the range of 50-150 μg/mL concentration was significantly less than that of ethyl acetate fraction extract. Therefore even at 150 μg/mL maximum cytotoxicity observed for crude extract was about 65 % (Figure 3.29 A). On the other hand, cytotoxic effect of ethyl acetate fraction extract was found to be more because, it inhibited up to 75% cell growth at 100 μg/mL concentration as shown in Figure 3.29 B.

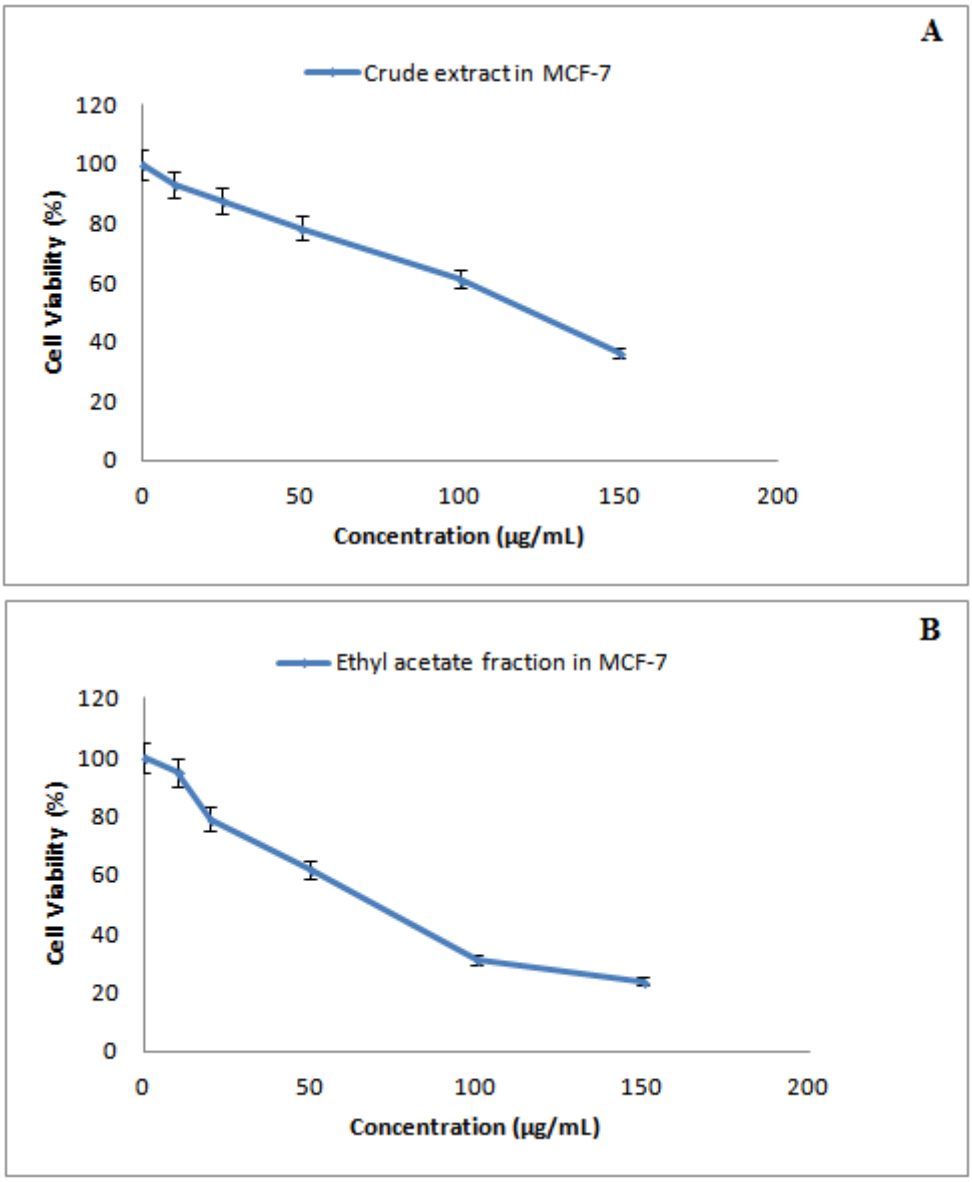


Figure 3.29: Analysis of cell viability for MCF-7 human breast cancer cell lines after treatment of different concentrations 0, 10, 25, 50, 100, and 150µg/mL of crude extract (A) and ethyl acetate fraction (B).

We also studied cytotoxic activity of an isolated compounds including catechin, caffeic and ellagic acid and commercial standards under the same conditions simultenaously like that extracts. Figure 3.30 A-F shows dose dependent cytotoxic activity of isolated compounds and standards on MCF-7 cancer cell line.

Among the three isolated compounds and standards studied here, cytotoxic activity of catechin, caffeic acid and ellagic acid was found to be higher than that of extracts.

Cytotoxic effect of isolate and standard catechin against MCF-7 cells studied at different concentration showed more than 80 % cell death at 100 μg/mL and 100 % cell death at 150μg/mL. Figure 3.30A and Figure 3.30 D shows the concentration dependent cytotoxic activity of isolate and standard catechin.

Dose dependent cytotoxic activity of isolate and standard caffeic acid on MCF-7 cancer cell lines is shown in Figure 3.30 B and Figure 3.30 E. More than 90 % cytotoxicity was observed at 100μg/mL and further reached about 100 % cytotoxicity at 150μg/mL.

Cytotoxic effect of isolate and standard ellagic acid studied at various concentration (0, 10, 25, 50 and 100 μg/mL) showed more than 90 % cell death above 100 μg/mL and 100 % cell death at 150 μg/mL (Figure 3.30 C and Figure 3.30 F).

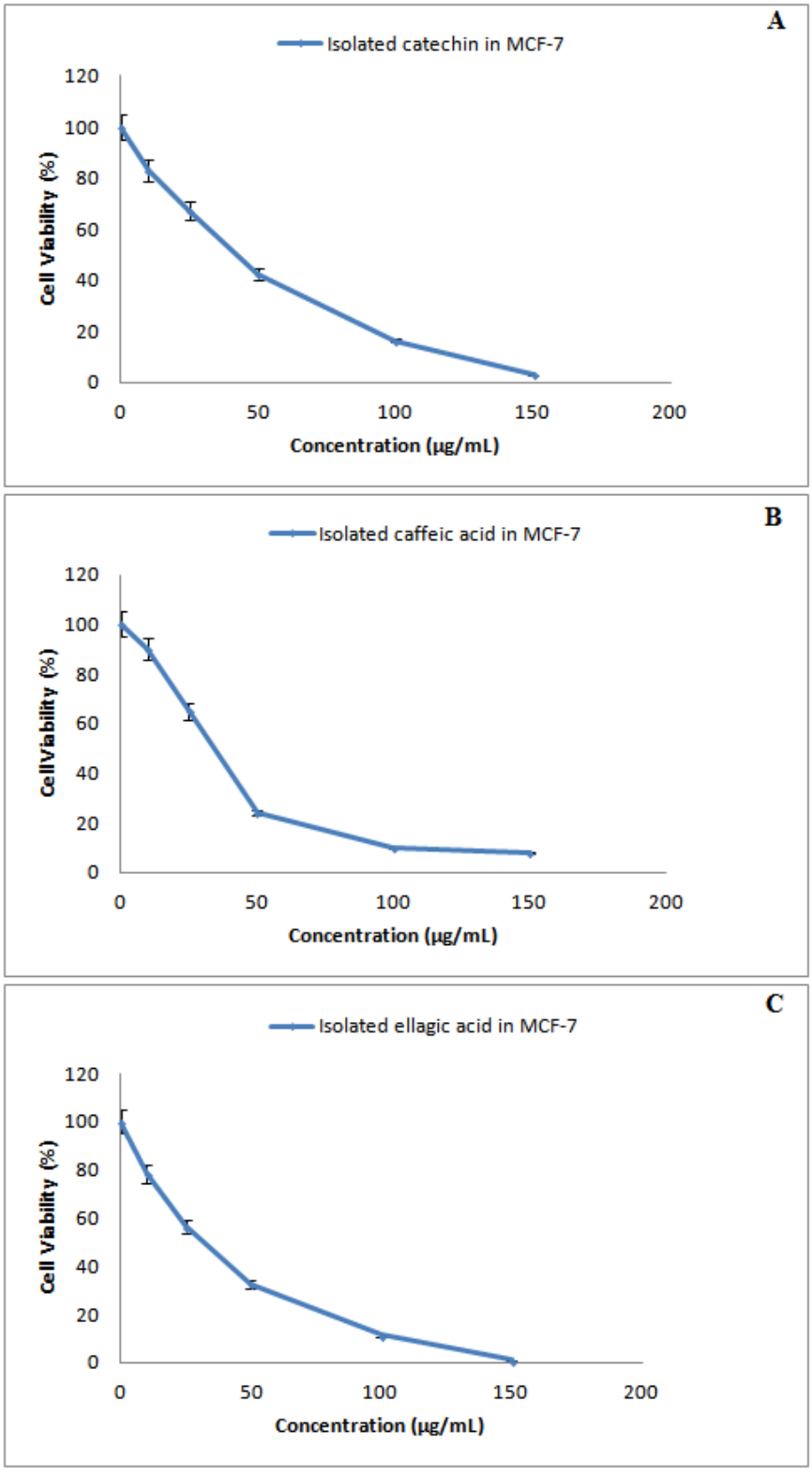


Figure 3.30: Dose dependent cytotoxic activity of isolated compounds including catechin (A), caffeic acid (B) and ellagic acid (C) against MCF-7 breast adenocarcinoma cell line at different concentrations for 24 hour. Each point is the mean of quadruple measurements from three different experiments (n=3)

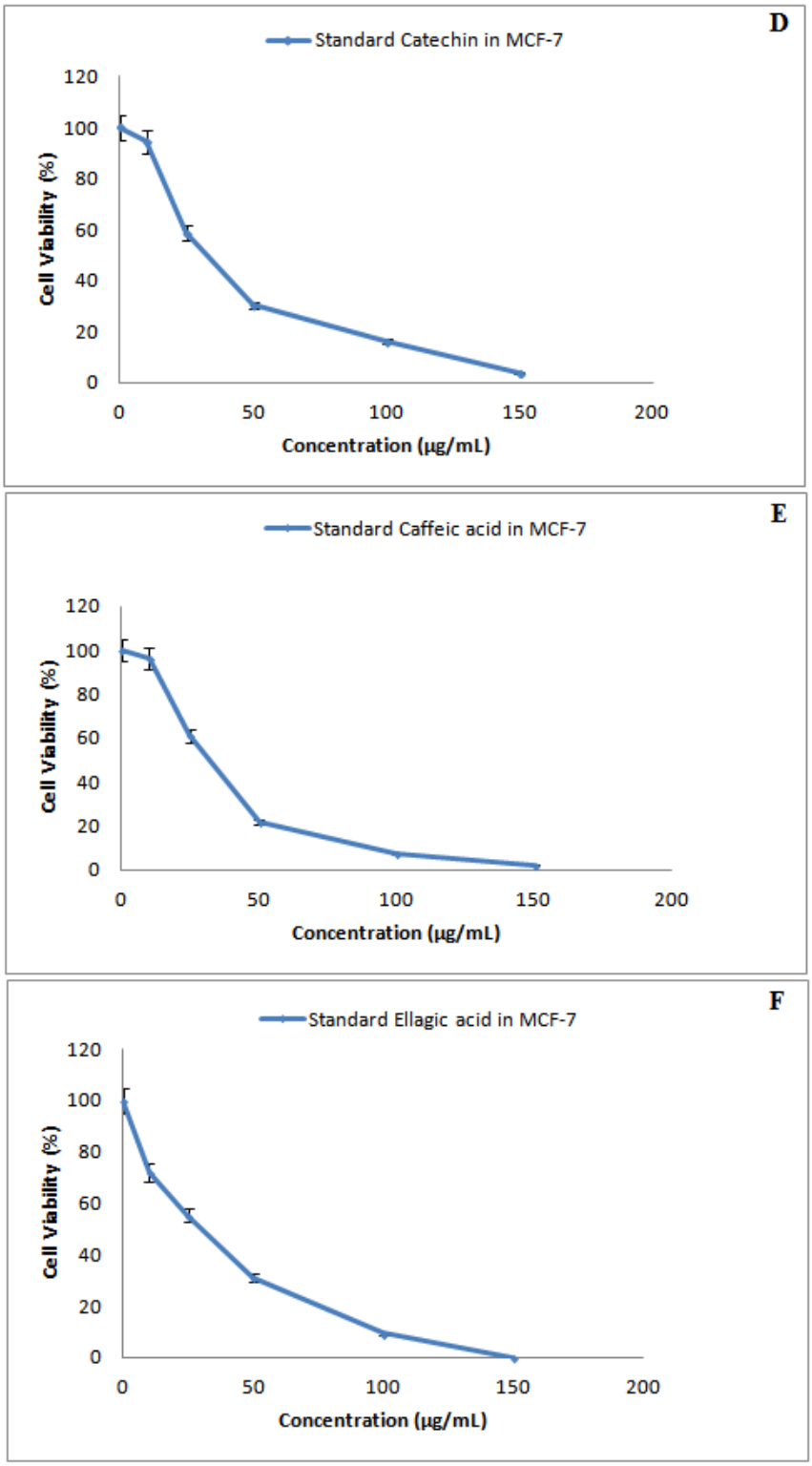


Figure 3.30: Dose dependent cytotoxic activity of phenolic standards catechin (D), caffeic acid (E) and ellagic acid (F) against MCF-7 breast adenocarcinoma cell line at different concentrations for 24 hour. Each point is the mean of quadruple measurements from three different experiments (n=3)

To simplify the analysis and to find out the relative position of extracts/samples in the standard's cytotoxic activity scale, ED_{50} values were derived from the dose response curves of extracts/samples and standards. The cytotoxic effect or antiproliferative activities of the extracts/samples expressed as the 50 percent effective dose (ED_{50}), lower the ED_{50} value means higher the antiproliferative activity.

ED_{50} values, concentration of crude extract, ethyl acetate fraction, isolated compounds and standards required to decraese cell viability by 50 %, were calculated in Table 3.8. Table 3.8 shows the ED_{50} values of extracts, isolated compounds and commercial standards derived from the dose dependent cytotoxic activity graph against MCF-7 cells.

Table 3.8: Comparison of ED_{50} values of extracts, isolated compounds and standards against MCF-7 breast cancer cell line for cytotoxic capacity. Experimental values are given as means ± standard deviation

Paramaters	**MCF-7**
extracts/isolated compounds/standards	**ED_{50} (µg/mL)**
crude extract	112.90 ±11.38
ethyl acetate fraction	81.60 ± 4.99
isolated catechin	58.48 ± 3.19
isolated caffeic acid	55.39 ± 2.20
isolated ellagic acid	50.76 ± 2.77
standard catechin	56.58 ± 3.85
standard caffeic acid	53.66 ± 3.25
standard ellagic acid	48.20 ± 2.56

ED_{50} values that were the *R.heckeliana* crude extract concentrations at which 50% of cells are viable were calculated as 112.90 ± 11.38 µg/ml for 24 hour. ED_{50} values were calculated as 81.60 ± 0.99 µg/ml after ethyl acetate fraction treatment for 24 hour. Ethyl acetate fraction was about one half times more toxic than crude extract according to the ED_{50} values. These results were statistically significant among extract for 24 h.

The highest cytotoxicity was exerted by isolated ellagic acid (ED 50 value: 50.76 ± 0.77 µg/mL) and isolated caffeic acid (ED 50 value: 56.58 ± 1.85 µg/mL) followed by catechin. ED_{50} value of catechin (58.48 ± 2.19 µg/mL) was higher than that of extracts (crude extracts, 112.90 ±11.38 µg/mL; ethyl acetate fraction, 81.60 ± 0.99 µg/mL). The highest cytotoxic effect was found in ethyl acetate fraction extract which was close to the ED_{50} value of a pure reference standard (catechin) and therefore significant **(Table 3.8)** These results were not statistically significant for 24 h among isolated compounds. Also, ED_{50} values of the isolated compounds by comparing with commercial standards, there is no statistically significant difference.

3.7.2.2. MDA-MB-231 cancer cell lines

The potential cytotoxic activity of crude extract, ethyl acetate fraction, isolated compounds and standards was investigated, determining their effects on the cell viability of an estrogen receptor-negative breast cancer cell line, MDA-MB-231. Figure 3.31 shows the cytotoxic effect of crude extract and ethyl acetate fraction extract in a dose-dependent manner on MDA-MB-231 cells.

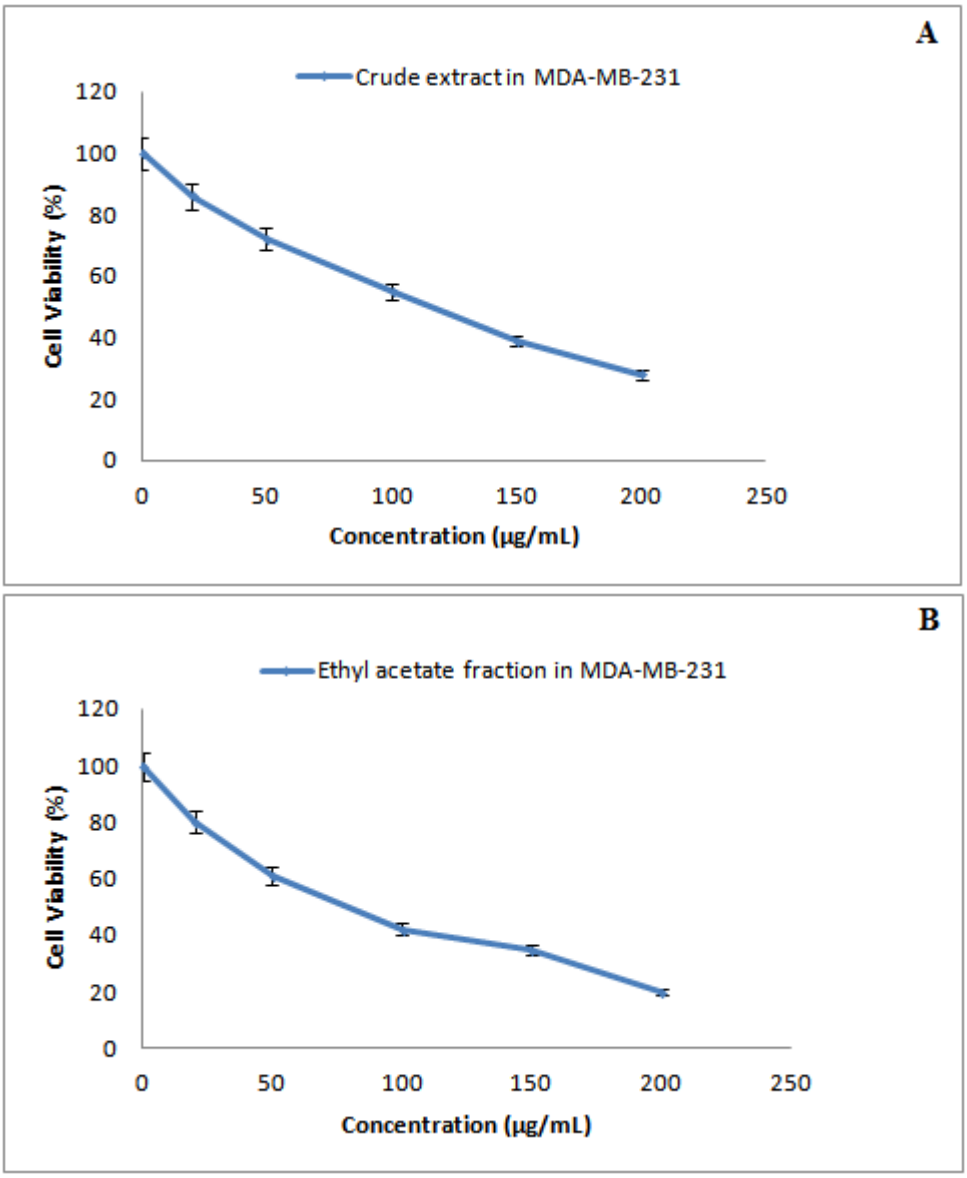


Figure 3.31: Analysis of cell viability for MDA-MB-231 breast cancer cell lines after treatment of different concentration 25, 50, 100, 150 and 200µg/mL of crude extract (A) and ethyl acetate fraction (B).

The cells treated with 25, 50 and 100 µg/mL of crude extract did not show significant cytotoxicity. However, at 150 and 200 µg/mL of concentration was found to be directly proportional to the concentration. More than 70 % cytotoxicity was observed at 200µg/mL (Figure 3.31A).

In the early stages of the percent viability of MDA-MB-231cell, a low concentration (25µg/mL) of the ethyl acetate fraction extract seems to have an insignificantly low effect. However, when ethyl acetate concentration is reached 200µg/mL, a sharp decrease in the cytotoxic effect is observed as shown Figure 3.31B.

Among the three isolated compounds and standards, cytotoxic activity of catechin, caffeic acid and ellagic acid was found to be higher than that of crude and ethyl acetate fraction.

The cytotoxic effect of isolate and standard catechin determined on breast carcinoma (MDA-MB-231) cell lines. Cells were exposed to increasing concentrations (0, 10, 25, 50, 100 and 150 µg/mL) of the extract for 24 hour. At low concentration of isolate and standard catechin showed an insignificant cytotoxic effect on cell viability of the cells but, if high concentration of catechin exhibited more than 90 % cell death at 100 µg/mL and 100 % cell death at 150 µg/mL (Figure 3.32 A and Figure 3.32 D).

Figure 3.32B and Figure 3.32 E show the dose dependent cell viability by isolate and standard caffeic acid. The MDA-MB-231 cells treated with 10 µg/mL of caffeic acid did not show significant cytotoxic activity but, in the presence of increasing concentration, cytotoxic effect was observed proportional concentration.

Dose dependent cytotoxic activity of isolate and standard ellagic acid on MDA-MB-231 breast cancer cell lines is shown in Figure 3.32C and Figure 3.32 F. More than 90 % cytotoxicity was observed at 75µg/mL and further reached about 100 % cytotoxicity at 100µg/mL.

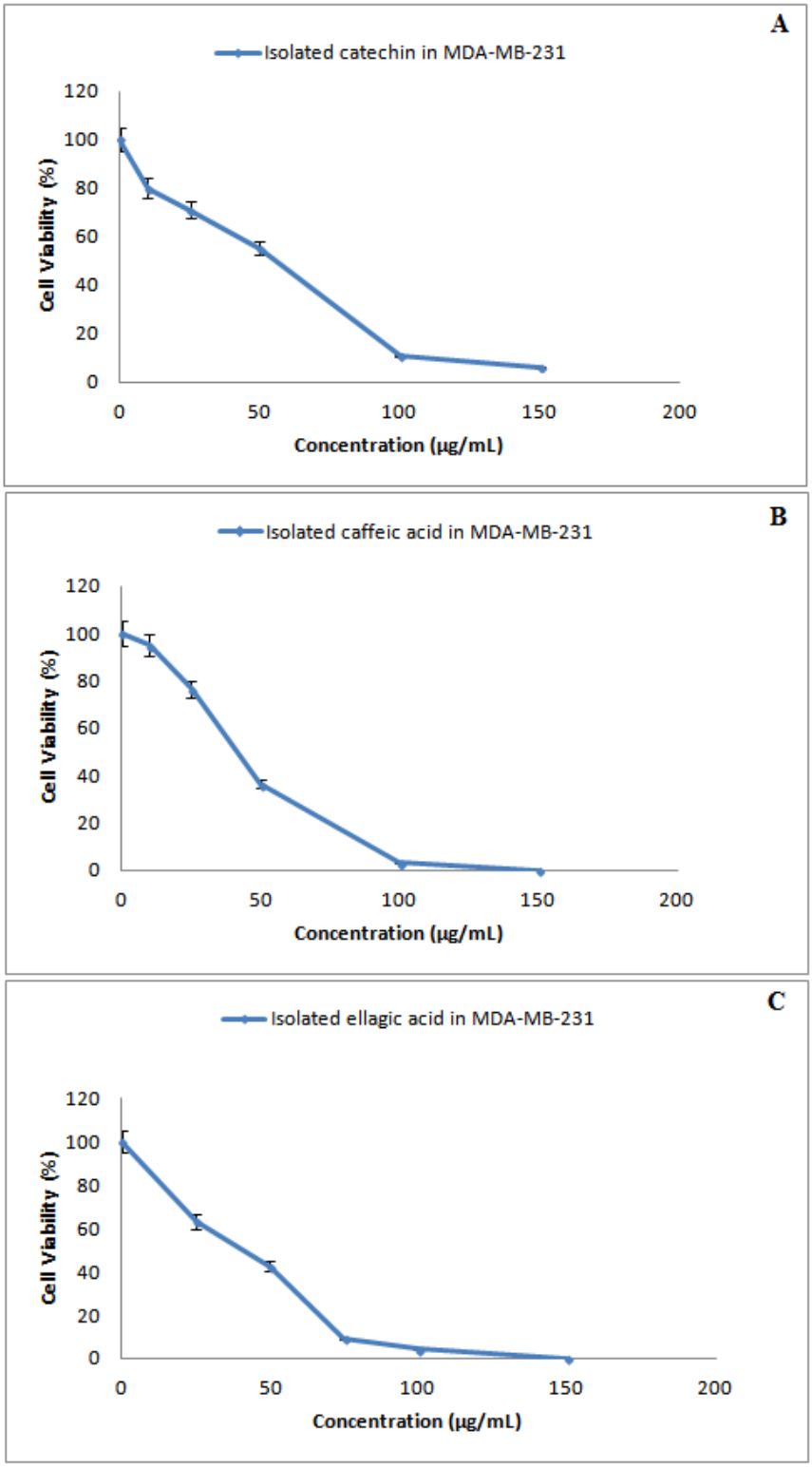


Figure 3.32: Dose dependent cytotoxic activity of isolated compounds including catechin (A), caffeic acid (B) and ellagic acid (C) against MDA-MB-231 adenocarcinoma cell line at different concentrations, for 24 hour. Each point is the mean of quadruple measurements from three different experiments (n=3).

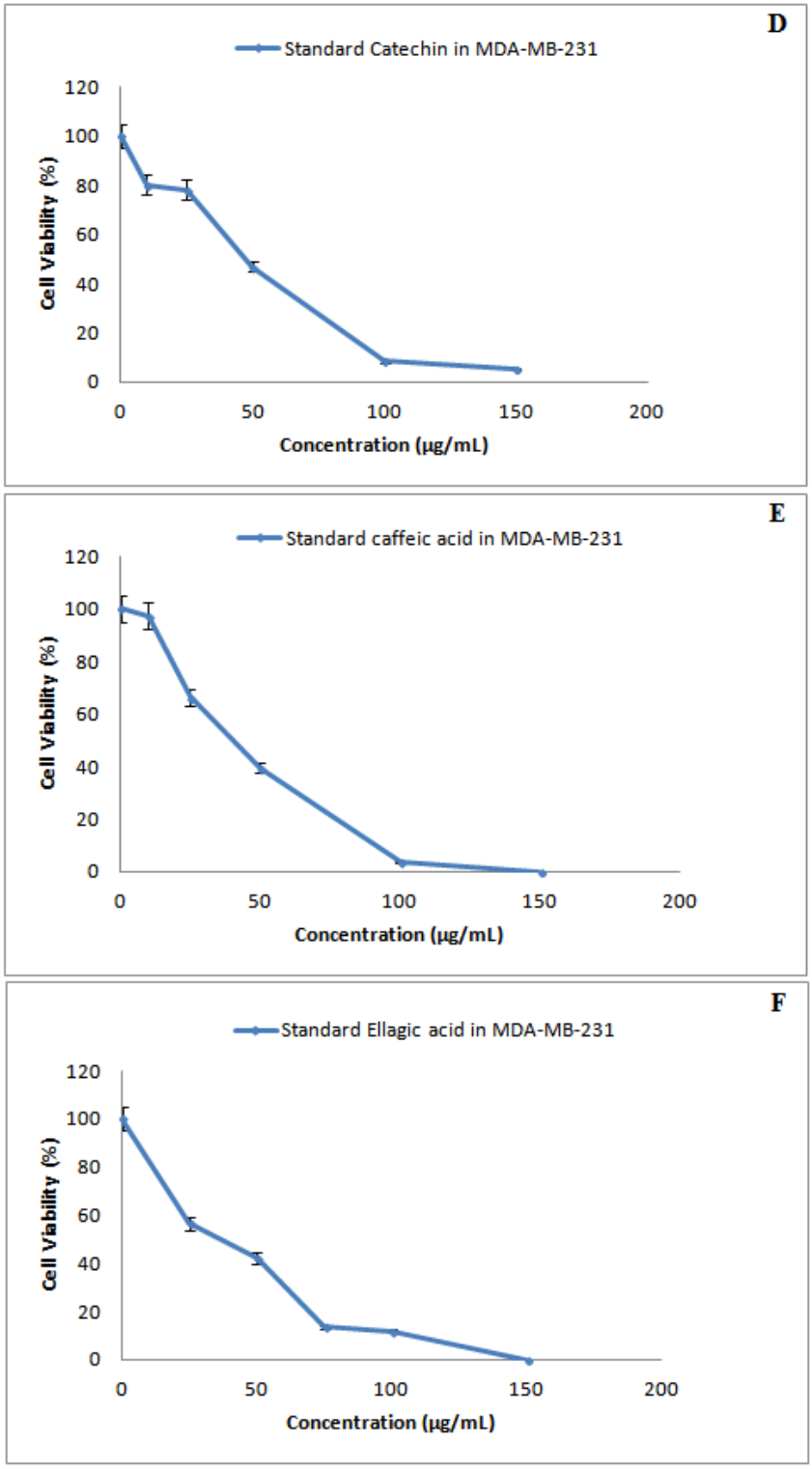


Figure 3.32: Dose dependent cytotoxic activity of phenolic standards catechin (D), caffeic acid (E) and ellagic acid (F) against MDA-MB-231 adenocarcinoma cell line at different concentrations, for 24 hour. Each point is the mean of quadruple measurements from three different experiments (n=3).

Cytotoxic effects of crude and ethyl acetate fraction extracts were calculated with the ED_{50} value of 119.46 and 96.68 µg/mL, respectively, in the presence of increasing concentrations between 0-200 µg/mL as shown in Table 3.9. The crude extract was found to display weak cytotoxicity toward the MDA-MB-231 human breast cancer cell line. According to the effective dose (ED_{50}) results, ethyl acetate fraction extract has shown more potential for cytotoxic effect than crude extract for 24 h.

Among the three isolated compounds, significantly highest results were found in the order ellagic acid > caffeic acid > catechin for cytotoxic activity. The ED_{50} values of the isolated compounds including ellagic acid, caffeic acid and catechin were 50.21 ± 0.53 > 58.26 ± 0.20 > 63.49 ± 2.17 µg/mL, respectively. The ellagic acid was found to be having the maximum cytotoxic effect (50.21 ± 0.53 µg/mL), on the other hand, the catechin had the lowest cytotoxic effect (63.49 ± 2.17 µg/mL). ED_{50} values of the compounds isolated from *R. heckeliana* were more active than extracts. ED_{50} results were not statistically significant among isolates. Besides, ED_{50} values of the isolated compounds by comparing with commercial standards, there is no statistically significant difference

Between extracts, ethyl acetate fraction extracts showed more cytotoxic effect than crude extract. ED_{50} value of a pure isolate catechin (63.49µg/mL) exhibited more cytotoxic than half ethyl acetate fraction extract. These results were not statistically significant according to the ED_{50} values. Comparison of ED_{50} values of extracts, isolated compounds and standards for cytotoxic capacities were presented in the Table 3.9.

Table 3.9: Comparison of ED_{50} values of extracts, isolated compounds and standards against MDA-MB-231 breast cancer cell line for cytotoxic activity. Experimental values are given as means ± standard deviation

Paramaters	**MDA-MB-231**
extracts/isolated compounds/standards	**ED_{50} (µg/mL)**
crude extract	119.46 ± 5.88
ethyl acetate fraction	96.68 ± 4.25
isolated catechin	63.49 ± 3.17
isolated caffeic acid	58.26 ± 3.20
isolated ellagic acid	50.21 ± 2.53
standard catechin	60.35 ± 3.98
standard caffeic acid	57.29 ± 2.01
standard ellagic acid	49.85 ± 2.13

It was reported Friedman et al. (2007) that catechins caused much higher cytotoxic effect on MCF-7 breast cancer cells for 48 hour. Cytotoxicity tests were completed using MTT at different concentration 50, 100, 200, and 400μg/mL. The catechins revealed high activity (percent cell death) against MCF-7 cells: catechin, (400 μg/mL=1.38 μmol/mL) IC_{50} value of 19.6 ± 1.1 μmol/mL. Another study was published Alshatwi et al (2010) that the inhibitory effects of catechin hydrate on MCF-7 cell lines were weaker. The cytotoxic effect of between at 0-160 μg/mL on MCF-7 cells was examined using the Cell Titer Bluer viability assay for 24 hour. IC_{50} of catechin hydrate was achieved at 127.62μg/mL.

Viveros-Valdez et al (2010) published that caffeic acid had the highest antiproliferative activity in the range of 5–50 μg/mL with an IC_{50} value of 10.7 ± 0.1μg/mL in MCF for 48 hours. Cell proliferation was observed by WST-1 assay. Indap et al (2006) showed that the MCF-7 breast cancer cells were more sensitive to caffeic acid. Percent cell viability of caffeic acid was obtained at 50 μg/mL concentration of 18 ± 2.65 μg/mL.

In 2009 Loizzo et al exhibited that ellagic acid shown good anti-proliferative activity against MCF-7 cells with IC_{50} value of >50 μg/mL for 48 hour. The protein-staining Sulforhodamine B (SRB) assay was used for following the cell proliferation. Another study was reported by Losso et al (2004) revealed that ellagic acid, in the concentration range of 1–100 μmol/L, showed strong anti-proliferative activity against MCF-7 cell for 48 hour. IC_{50} value of ellagic acid was accomplished at 72 μmol/L using adenosine triphosphate (ATP) bioluminescence as marker of cell viability. Other study, in MCF-7 cells, the anti-proliferative effect was observed only at high concentrations (more than 250 μM). Cells were exposed to different concentrations of ellagic acid for 24 hr. Cell proliferation was evaluated by MTT assay in this study (Kim, 2009)

Sartippour et al. (2001) published that catechin in the presence of increasing concentration (0-40 μg/mL) did not affect the viability of MDA-MB-231 cancer cells for 24 hour. Percent cell proliferation was obtained at 40 μg/mL concentration about 30%.

In the literature, caffeic acid exhibited an anti-proliferative effect against MDA-MB-231 cell lines at 50 μM concentration for 72 hour. Cell density is decreased. Cell viability was evaluated by the MTT colorimetric assay (Gomes, 2003). On the other hand, caffeic acid has no anti-proliferative effects on MCF-7 and MDA-MB-231 human breast cancer cells at a between 25-75 μM concentration for 24, 72 and 168 hours, respectively. Cell proliferation was accessed by the sulforhodamine B (SRB) colorimetric assay (Serafilm, 2011)

In 2009, it was reported about the dose dependent anti-proliferative effect of ellagic acid against MDA-MB-231 cells. MTT assay was applied on the cells after 24hr treatment of variable concentrations of ellagic acid. At the treatment of 250 μM concentration of ellagic acid cell viability dropped approximately 30 percent (Kim, 2009)

In cellular studies, cytotoxicity tests are very frequently examined. As cytotoxicity is an activity term that is associated with anticancer activity. The main benefit of cytotoxicity assays is that all probable mechanisms of cellular proliferation can be monitored all at once. Thus, the search for new anti-cancer agents had been often focused on plant extracts with cytotoxic effect on one or two cell lines in the past. The approach has been fruitful and led to the discovery of paclitaxel, among many other compounds

Many crude fruit extracts have been shown to possess the ability to inhibit the proliferation of cancer cells [e.g. from oranges (Camarda, 2007); fig fruit (Wang, 2008) and cranberry (Neto, 2008)]. Phytochemicals such as anthocyanins, phenolic acids, carotenoids and flavonoids might be responsible for the anti-proliferative properties.

The results of the present study demonstrate that a crude extract of the root of *R. heckeliana* displayed weak anticancer activity by preventing the proliferation of MCF-7 and MDA-MB-231 cancer cell lines. On the other hand, ethyl acetate fraction extract showed more cytotoxic effect than crude extract. This cytotoxic response was not dependent on the estrogen receptor, as related results were obtained in cells lines positive (MCF-7) and negative (MDA-MB-231) this receptor.

In the present study, the inhibition on both of the breast-cancer cell lines can be partly associated by the presence of phenolic phytochemicals in the extracts. Alternatively, particular phenolic phytochemicals may assert their effect in additive, cooperative and/or antagonistic manners with other compounds exerting the anti-proliferative activity (Yang, 2009).

Ethyl acetate fraction extract has been demonstrated to comprise a high total phenolic and flavonoid content and also displayed high antioxidant properties, compared to the phenolic standards.

Liu (2004) has suggested that the cancer chemo-preventive properties possessed by fruits and vegetables are the outcome of additive and synergistic actions between the complexes mixtures of phytochemicals present in whole foods. For example, pomegranate juice has been shown to display more potent anti-proliferative activity in different human colon cancer cell lines when compared to its isolated bioactive compounds such as punicalagin and ellagic acid (Seeram, 2005); suggesting synergistic actions of phytochemical components present in the crude extract.

In RP-HPLC and HR-MS sections, it was demonstrated that catechin, p-coumaric acid, caffeic acid, epicatechin, scopoletin, ellagic acid, and quercetin were identified and confirmed as the key polyphenol phytochemicals present in the ethyl acetate fraction extract and that these compounds might contribute to the cytotoxicity activity against breast cancer cell lines. Many of these are known to inhibit the growth of breast cancer cells (Indap, 2006; Kim, 2009; Serafilm, 2011), and thus it is expected that at least part of the cytotoxic effects of the extract can be attributed to these phenolic compounds.

In addition, comparable extracts of a related Rosa species, *R. canina* water and ethanol extract, have been demonstrated to contain a ellagic acid, in addition to other phenolics (quercetin and kaempherol), which has cytotoxic effects (Nowak, 2006; Robertson, 2008).

Another study reported that *R. canina* extracts in preventing cell proliferation of selected human glioblastoma cell lines. Each of the glioblastoma cell lines treated with rosehip extracts (1 mg/mL - 25 ng/mL) demonstrated a significant decrease in cell proliferation (Cagle, 2012). Other study, *R. damescana* receptacles extract at 100 μg/mL concentration displayed weak antiproliferative activity (IC_{50} value of 160 ± 7.46 μg/mL) against MCF-7 cell for 24 hour (Talib, 2010)

Plants and natural products play an important role in medicine and offer important models for the development of new medicines (Cragg, 1998). They introduce a valuable source of compounds with a diversity of biological activities and chemical structures. Many anti-cancer agents, available today, have been rendered from natural sources; directly as pure natural compounds, or as semi-synthetic correspondents (Pezzuto, 1997; Schwartsmann, 2000; Lee, 1999).

In this work, the proliferation and morphological characteristics of two cell lines (MCF-7 and MDA-MB-231) were studied in response to treatment with extracts (crude and ethyl acetete fraction), isolated compounds including catechin, caffeic acid, ellagic acid and phenolic standards.

In cellular proliferation activity, theoretically, any reduction in the number of metabolically active proliferating cells might mean that the proliferation pathway itself was halted (cell cycle arrest), or that a fraction of the cells went through a death pathway. Therefore, a morphological change was necessary to determine which of that option may play a role in this study. Therefore, the morphological evaluation was used to determine the plant extract-mediated cell death. The morphological alterations were performed with the over and under the ED_{50} concentration cell-extract combinations as in the proliferation assay. If we consider the results of the proliferation and morphological changes assay in parallel, then we would be able to say whether reduction of the number of the metabolically active cell has resulted from death of part of them or due to any other reason. In this study a combination of the two different assays was performed in parallel for crude extract/ethyl acetate fraction and each cell line

The findings of this study indicated that the proliferation activity of MCF-7 and MDA-MB-231 breast adenocarcinoma cells were inhibited by all of the extracts (crude and ethyl acetete fraction), isolated compounds including catechin, caffeic acid, ellagic acid and phenolic stadards. A dose response effect was obtained with all of these extracts, but the degree of this effect varied from one extract to another. The results indicated that isolated ellagic acid is the most potent compounds, even at low concentrations with ED_{50} value of about 50 µg/mL followed by caffeic acid with ED_{50} value of about 55 µg/mL and catechin ED_{50} value of about 60 µg/mL for both cell lines.

On the other hand, crude extract of *R. heckeliana* did not reach the same degrees of proliferation inhibition obtained by both ethyl acetate fraction and isolated compounds even at the highest concentration. Crude extract has a less/weak potent cytotoxic effect against MCF-7 and MDA-MB-231 cells.

Under the chosen experimental conditions, the extracts (crude and ethyl acetete fraction) did not entirely prevent cell proliferation. In the literature, there is no information about the *R. heckeliana* extract(s).

According to the norms established by the U.S. National Cancer Institute (NCI), these compounds with ED_{50} < 30 µg/mL, 30 µg/mL <ED_{50} < 100 µg/mL and ED_{50} > 100 µg/mL are categorized as active, moderately active and inactive (Suffness et al., 1990).

As a result of these findings, ethyl acetate fraction extract exhibited the highest antioxidant capacity although showed a moderate cytotoxicity with an ED_{50}< 100 µg/mL that fall within the NCI criteria. Crude extracts however revealed some cytotoxicity; those were referred as inactive according to NCI criterion for 24 h.

CHAPTER 4

CONCLUSIONS

Many of the Rosa species have been used in medicine for various diseases because of their curative properties and therefore they were highly inspected by the researches. Correspondingly, in this study the *Rosa heckeliana* of Rosacea family was investigated for the first time for its phenolic constituents and their anti-oxidative and cytotoxic properties.

Determination of the phenolic constituents started with the extraction process for the crude extract preparation. Crude extract was first analyzed by the RP-HPLC. However there were too many peaks and therefore the resolution was quite low due to the intervention of the excessive amounts of compounds dissolved in methanol. The complex interference of those compounds in different polarities needs to be attenuated by the solvent-solvent fractionation along with the bioactivity guidance. The most bioactive fraction was obtained as the ethyl acetate fraction of *R. heckeliana,* indicating the collection of a concentrated amount of phenolics present. This phenolic rich fraction was considered for further separation and isolation processes.

Isolation of the bioactive compounds, found in ethyl acetate fraction, give rise to identification of the three major phenolic compounds namely, catechin, caffeic acid and ellagic acid. Other minor amount of phenolics namely, epicatechin, p-coumaric acid, scopoletin and quercetin were too recognized in ethyl acetate fraction by RP-HPLC analysis. Besides, the chemical structures of those compounds identified were also confirmed by the use of HR-MS technique.The quantitative amounts of catechin, caffeic acid, epicatechin, p-coumaric acid, scopoletin, ellagic acid and quercetin were calculated as 17.6%, 1%, 0.3%, 0.6%, 2.3%, 3.3% and 1.1% in the crude extract. Ultimately, the dominant compound present in the roots of *R.heckeliana* was identified as catechin.

Finally, cytotoxic studies were carried out by using MCF-7 and MDA-MB-231 breast cancer cells. The crude extract, ethyl acetate fraction, isolated compounds and also their commercially available matching standards, in various concentrations were all examined and the results were expressed as effective dose in fifty percent (ED_{50}). Accordingly, the isolated ellagic acid, caffeic acid and catechin, were validated as the most potent agents among the others with the ED_{50} values of 50-65μg/mL. Subsequently, the ethyl acetate fraction presented moderately effective cytotoxicity (ED_{50}< 100 μg/mL) that fall within the NCI criteria. However, the crude extract exhibited some cytotoxicity (ED_{50} > 100 μg/mL) on both of the cell lines; nevertheless that was substantiated as inactive according to NCI criterion for 24 h.

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APPENDIX A

A. RP-HPLC chromatograms of all fractions of *R. heckeliana*

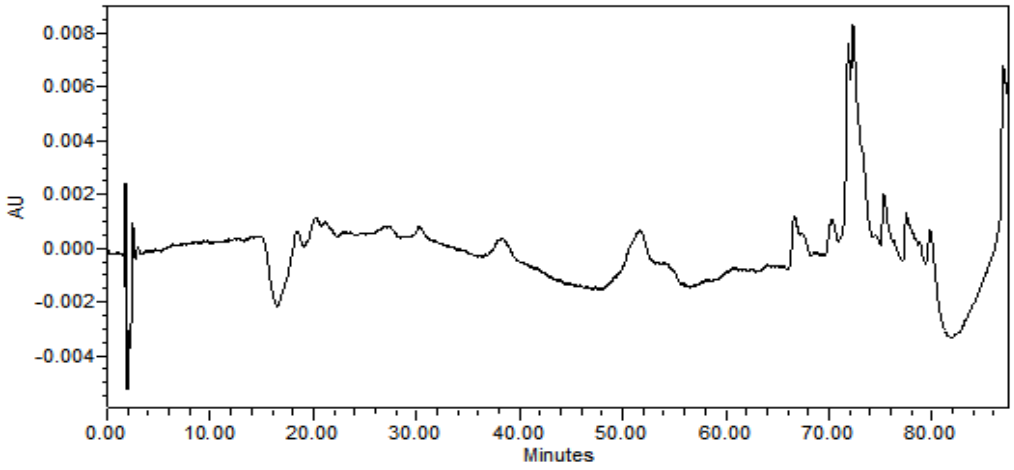

Figure A-1: RP-HPLC chromatogram of petroleum ether fraction of *R. heckeliana*

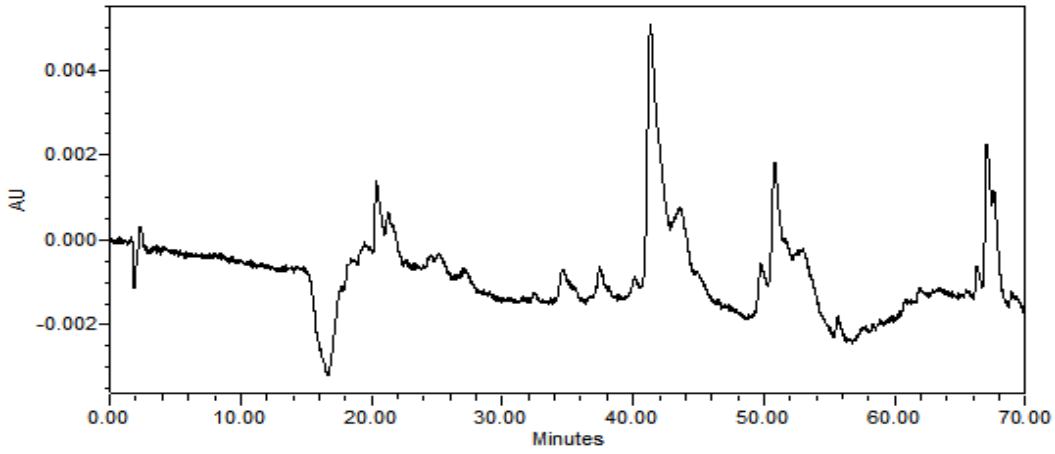

Figure A-2: RP-HPLC chromatogram of chloroform fraction of *R. heckeliana*

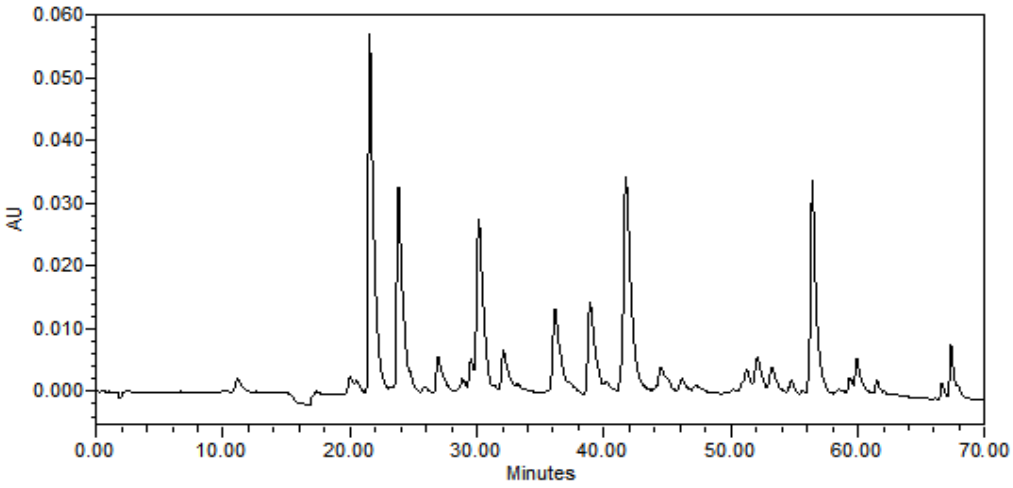

Figure A-3: RP-HPLC chromatogram of ethyl acetate fraction of *R. heckeliana*

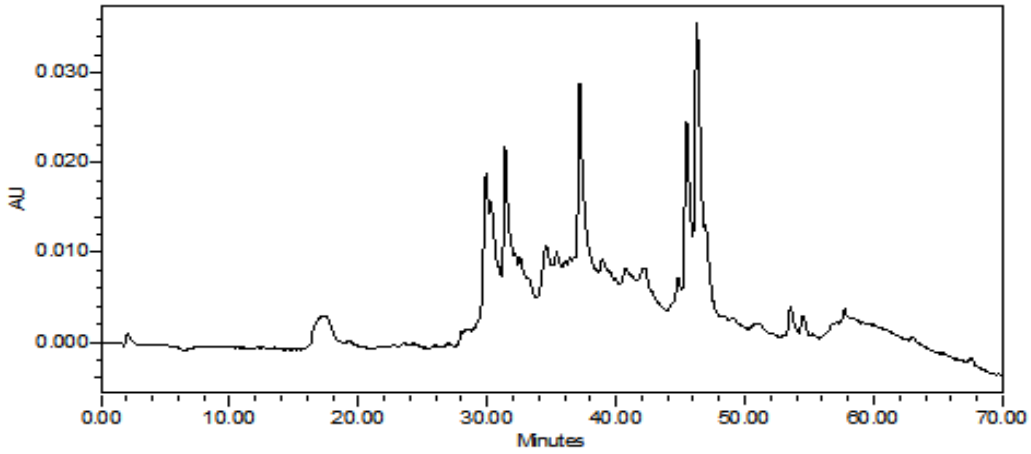

Figure A-4: RP-HPLC chromatogram of n-butanol fraction of *R. heckeliana*

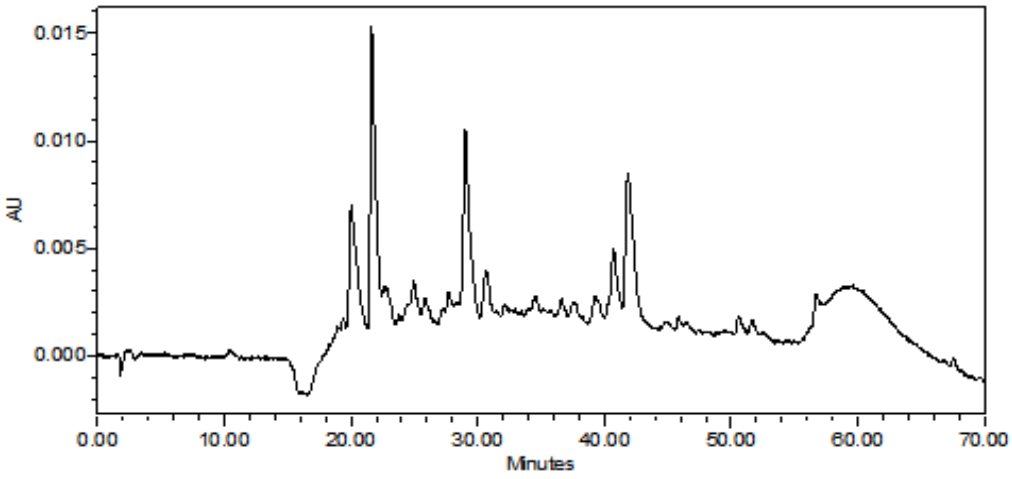

Figure A-5: RP-HPLC chromatogram of aqueous fraction of *R. heckeliana*

APPENDIX B

B. RP-HPLC chromatograms of isolated compounds

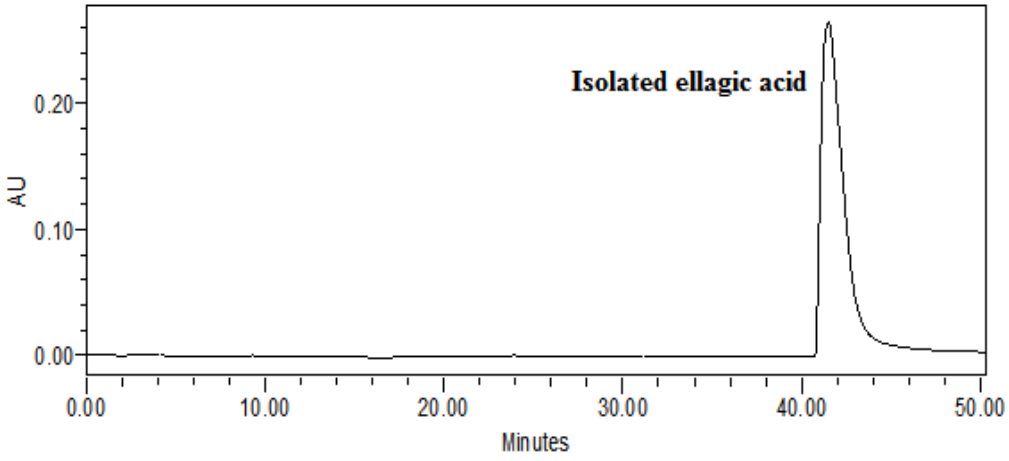

Figure B-1: RP-HPLC chromatogram of compound 1 (isolated ellagic acid) at 280 nm.

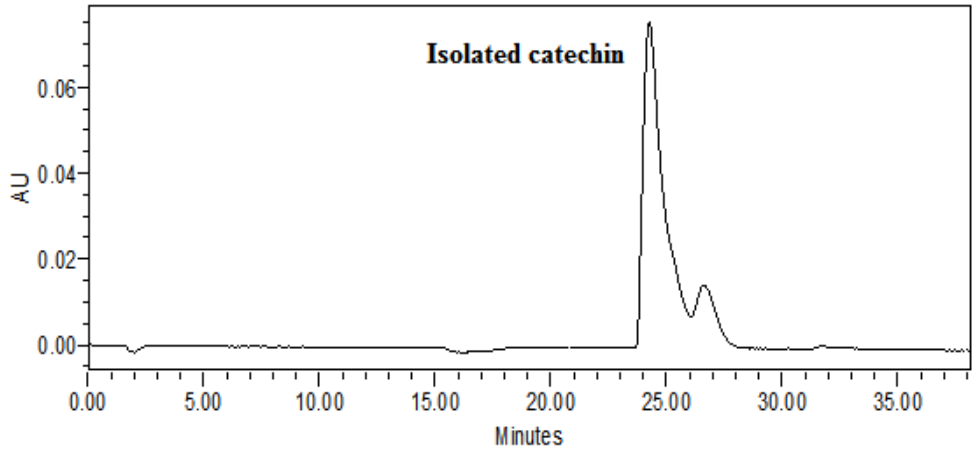

Figure B-2: RP-HPLC chromatogram of compound 2 (isolated catechin) at 280 nm.

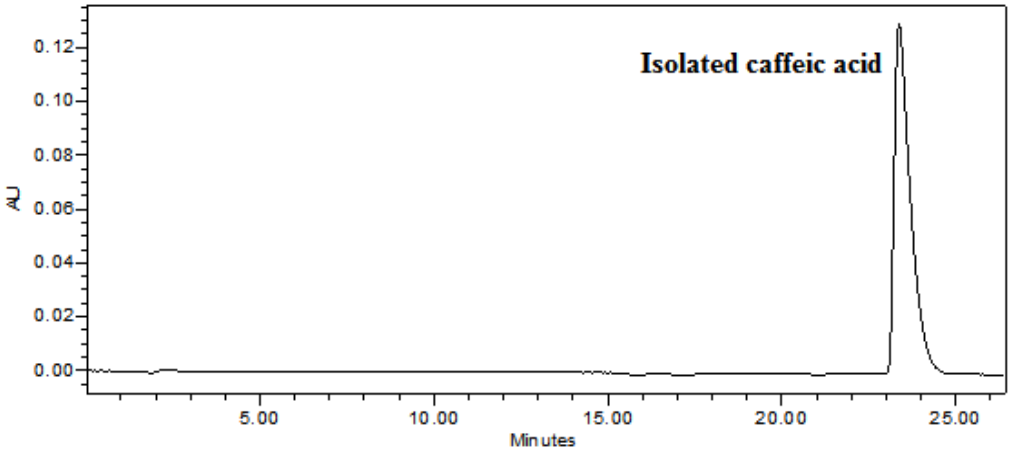

Figure B-3: RP-HPLC chromatogram of compound 3 (isolated caffeic acid) at 280 nm.

APPENDIX C

C. Results of proton and carbon NMR for isolated compounds

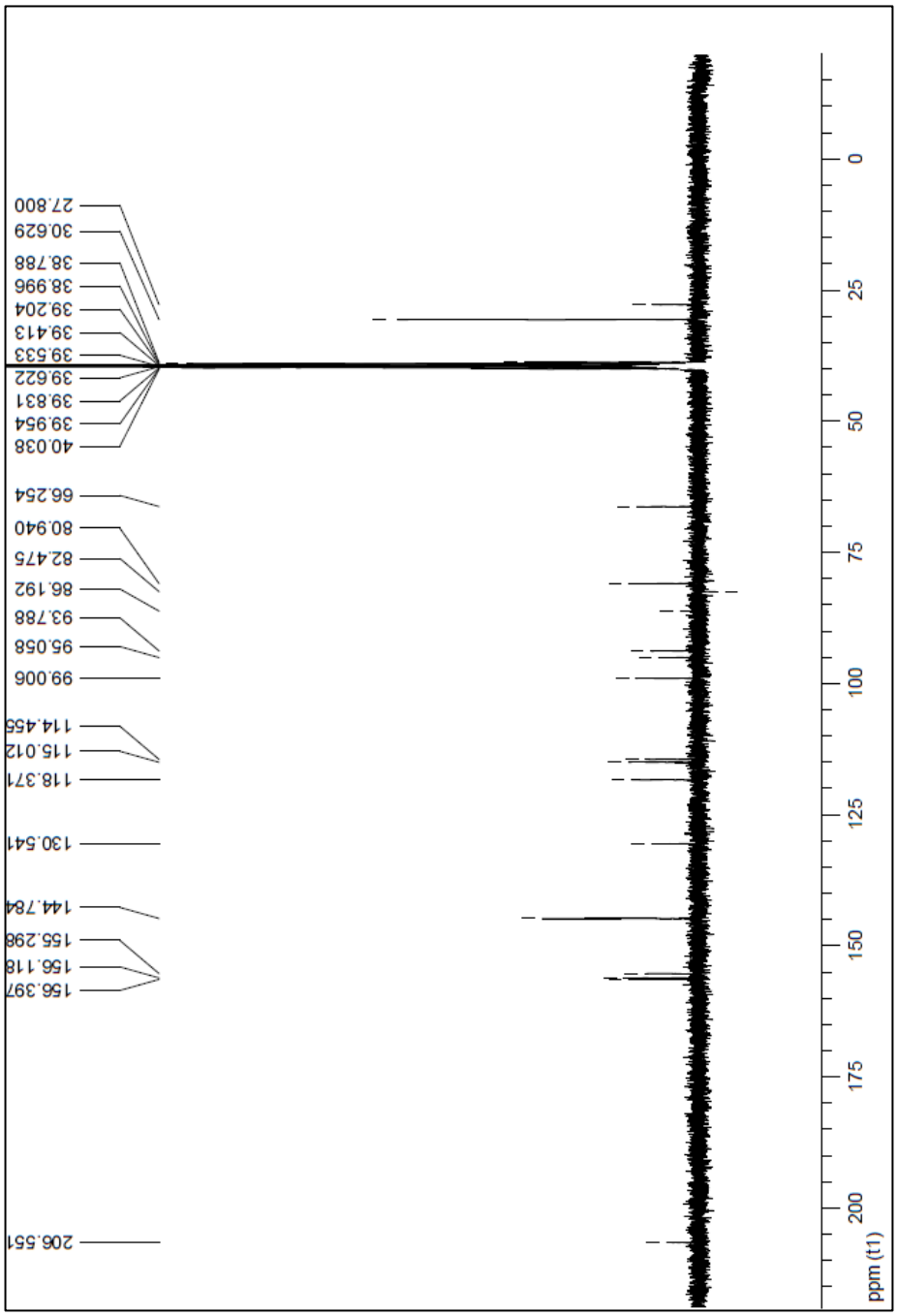

Figure C-1: Carbon NMR of isolated compound 2 (catechin)

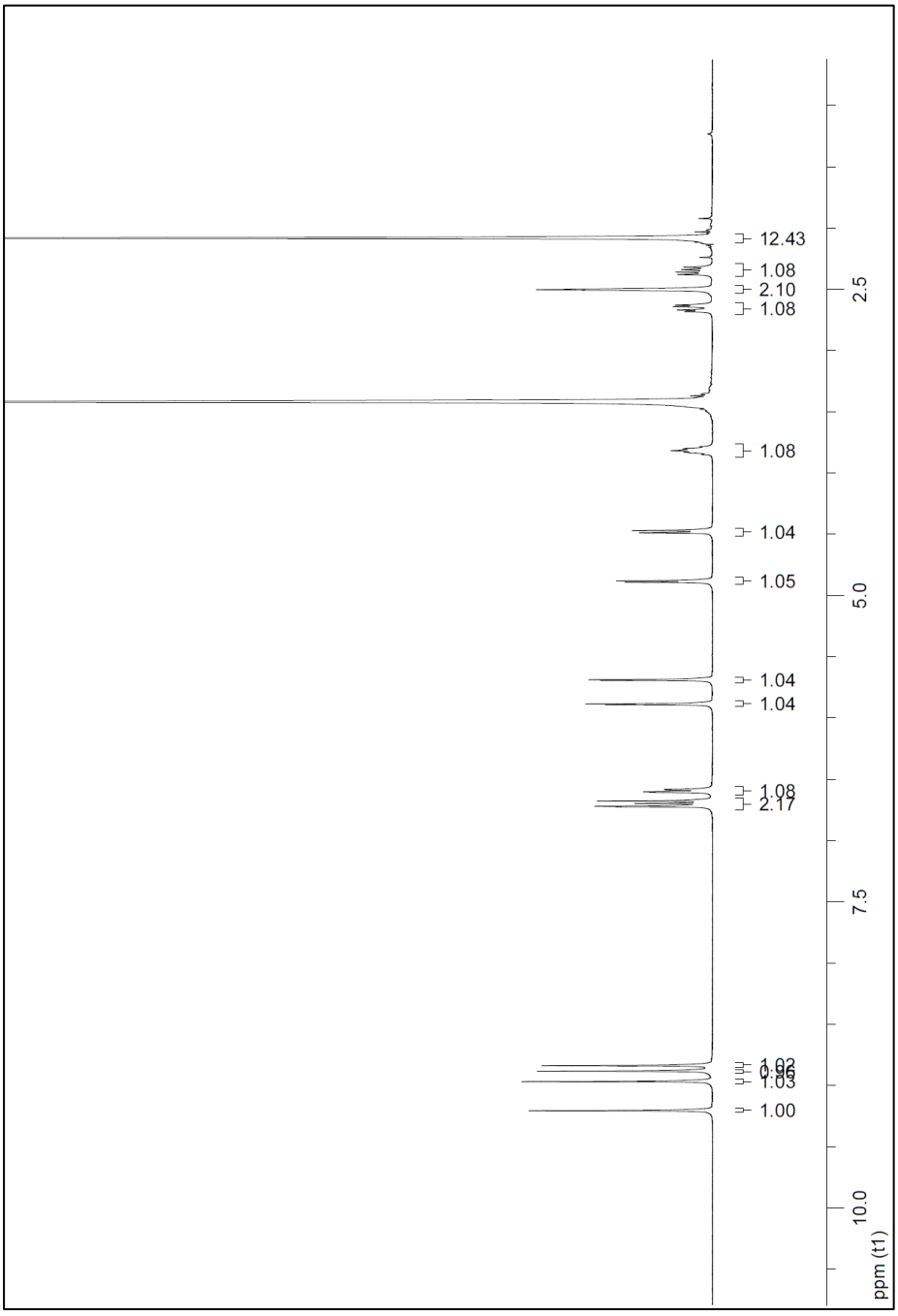

Figure C-2: Proton NMR of isolated compound 2 (catechin)

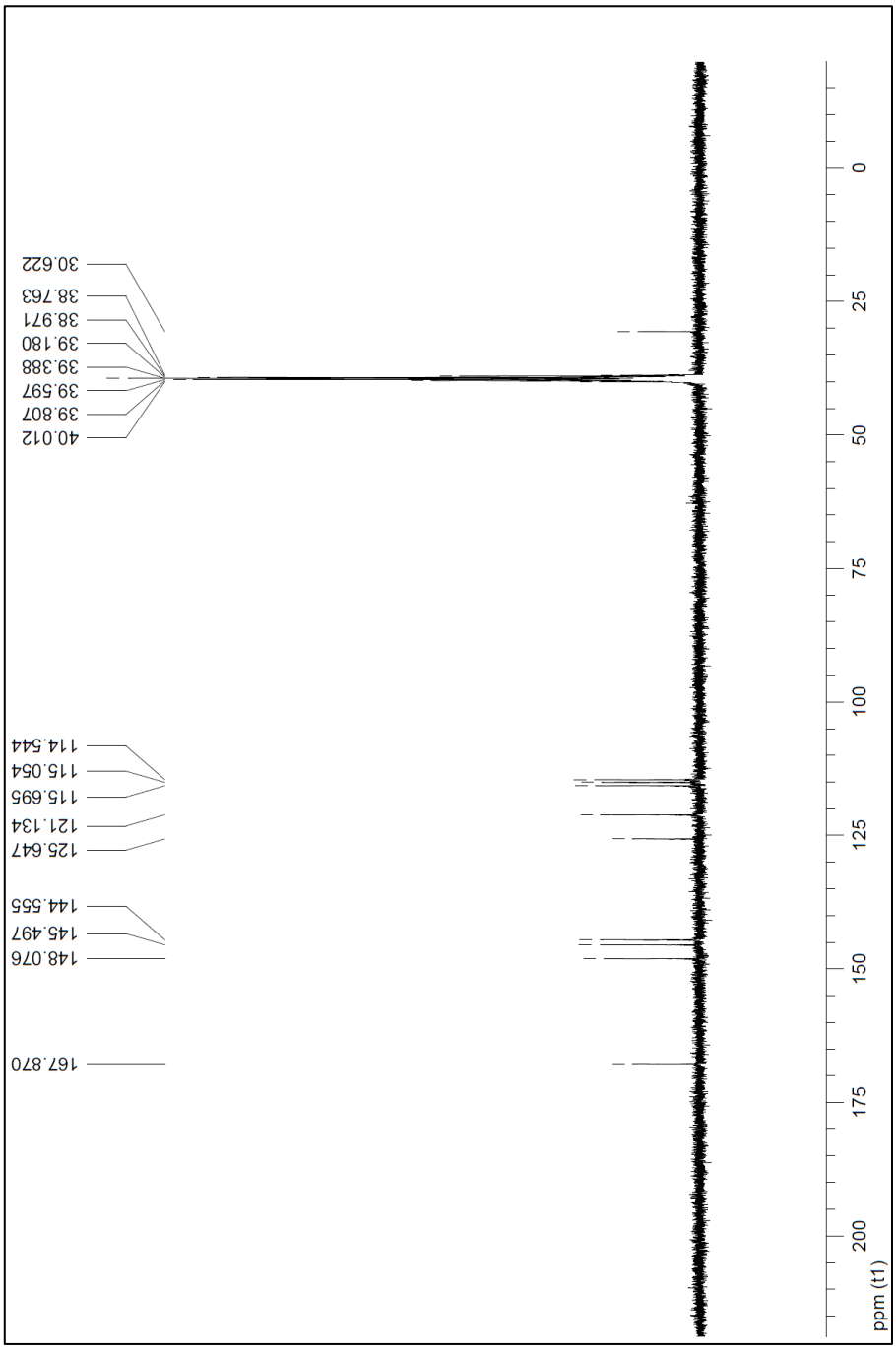

Figure C-3: Carbon NMR of isolated compound 3 (caffeic acid)

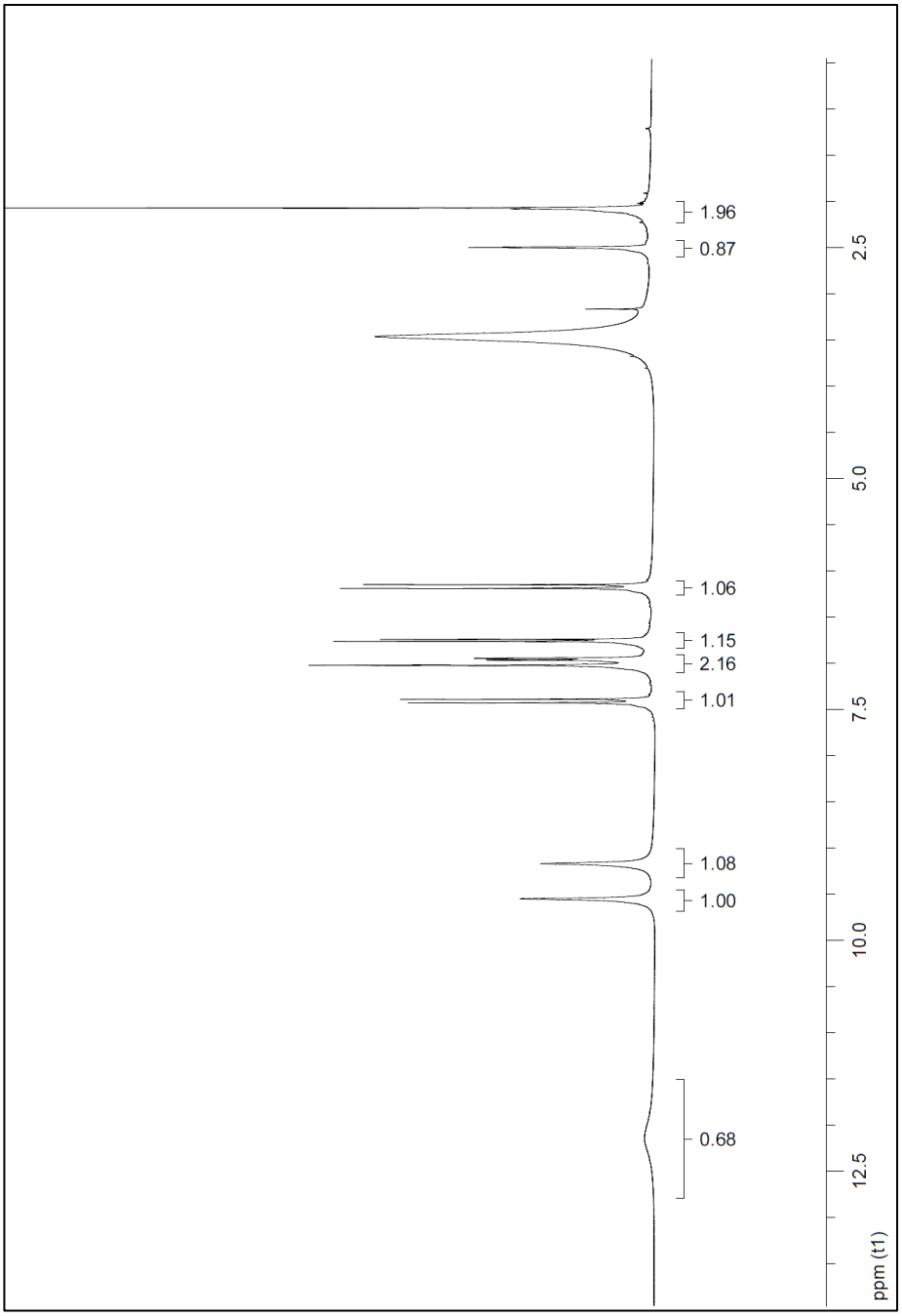

Figure C-4: Proton NMR of isolated compound 3 (caffeic acid)

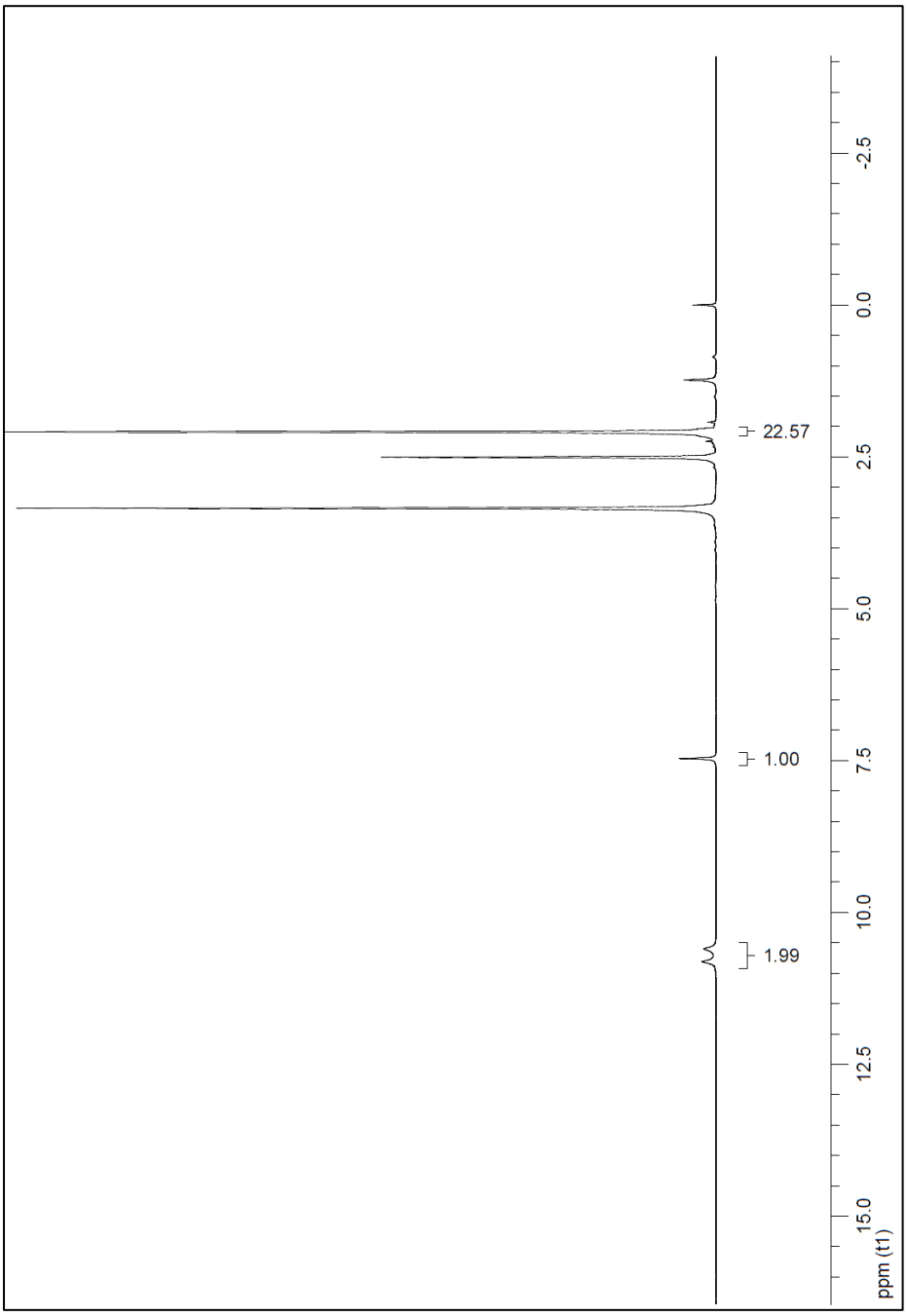

Figure C-5: Proton NMR of isolated compound 1 (ellagic acid)

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Investigation of antioxidant activity: Medicinal plant extracts and isolated bioactive compounds
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Spectroscopy: Fluorescence, Luminescence, UV-VIS
Animal cell culture

PUBLICATIONS

Papers and Abstracts Published in Conferences

1) Coruh Nursen, Ozdogan Nizamettin. Fluorescent Coumarins from the Bark Extract of Turkish *Aesculus hippocastanum.* Journal of Liquid Chromatography & Related Technologies (Doi:10.1080/10826076.2013.789803).

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9) N. Ozdogan, N. Coruh. Identification of Phenolic Constituents from Turkish A. hippocastanum by RP-HPLC. 38th FEBS congress Mechanisms in Biology, July, 2013, RUSSIA.

10) N. Ozdogan, P. Uyar, N. Coruh, M. Iscan. A. hippocastanum'un İçindeki Kumarinlerin Sitotoksik Etkileri. XXV. National Biochemistry meeting, Izmir, TURKEY.