DETERMINATION OF PHENOLIC COMPOUNDS IN ACONITUM COCHLEARE WOROSCHIN

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ABSTRACT

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This study involves the investigation of antioxidant phenolic compounds in *Aconitum cochleare*.

There is a continuous mounting pressure, in the scientific world, to discover new and potent antioxidants, to combat the free radicals. Among the antioxidant compounds, the phenolics comprise the largest class, and thus hold an important place in biochemical studies. Phenolics are abundantly present in some plant families as secondary metabolites and Ranunculaceae is one of those families that contain alkaloids and phenolic compounds in many of its species.

Aconitum cochleare is one of the Ranunculaceae specie which was yet to be exploited for its phenolic components and its antioxidant capacity and thus in this study, the methanolic extract of the flowering parts of this plant was obtained and subjected to fractionation and each of the fractions were subjected to the methods for antioxidant capacity determination.

The DPPH test was used to estimate the antioxidant capacity, while determination of total phenol was done to confirm which fraction has the most phenolic constituents. More over total flavonoid contents were determined to find out which fraction has the most flavonoids. Further investigation about fractions was done by RP-HPLC, which showed the presence of esculin in the ethyl acetate fraction, as well as some other compounds, evident from the RP-HPLC chromatograms. For confirmation of the molecular weight of phenolic compounds, HR-MS was employed and by its result it was concluded that ethyl acetate fraction has the highest content of flavanol and flavonol glycoside.

Keywords: Antioxidants, *Aconitum cochleare*, DPPH, total flavonoid content, RP-HPLC

ÖZ

ACONITUM COCHLEARE WOROSCHIN FENOLİK BİLEŞENLERİNİN TANIMLANMASI

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

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

DPPH testi antioksidan kapasitenin araştırılmasında, total fenol miktarı tayini ise hangi fraksiyonda en fazla fenolik içerik olduğunu bulmak için kullanılmıştır. Bununla birlikle, total flavonoid miktar testi, en fazla flavonoid miktarını içeren fraksiyonun

tespiti için yapılmıştır. Fraksiyonlar RP-HPLC metodu kullanılarak incelenmiş, bunun sonucunda etil asetat fraksiyonunda diğer maddelerin yanında esculin'in varlığı RP-HPLC kromatogramı ile gösterilmiştir. Fenolik bileşenlerin moleküler ağırlıklarını ispatlama yöntemi olan HR-MS uygulanmasıyla da yine etil asetat fraksiyonunun en yüksek flavanol ve flavonol glikozitlerini içerdiği sonucuna varılmıştır.

Anahtar kelimeler: Antioksidanlar, *Aconitum cochleare*, DPPH, total flavonoid miktarı, RP-HPLC

To my beloved family

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First of all, my immeasurable thanks to **The Almighty** for making it all possible.

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LIST OF ABBREVIATIONS

Mm	Millimeter
Nm	Nanometer
mg	Milligram
mL	Milliliter
µL	Microliter
AD	Alzheimer's disease
8-OH-dG	8-hydroxy-deoxyguanosine
CAT	Catalase
DNA	Deoxyribonucleic acid
DPPH	2,2-diphenyl-1-picrylhydrazyl
GAE	Gallic Acid Equivalent
GSH-Px	Glutathione reductase
EC50	Effective Concentration at 50% of the maximum activity
HR-MS	High Resolution Mass Spectroscopy
ROS	Reactive Oxygen Specie
RNS	Reactive Nitrogen Specie
RP-HPLC	Reverse Phase-High Pressure Liquid Chromatography
SOD	Super oxide dismutase
UV-Vis	Ultraviolet-visible spectroscopy

CHAPTER 1

INTRODUCTION

In the past two decades extensive studies have been done on free radicals, about what are they, what is their significance, how are they produced, how do they react and what are the beneficial and harmful side effects of them. But recent years and expectedly in the future years most of the research is focused on finding new and potent antioxidants to combat the devastating effects of free radicals, given their continuously mounting quantity in the human body, owing to the lifestyle of today's era. Therefore, a complete understanding of the true nature of this specie is very important to step forward in this field.

Nature gives man uncountable valuable products and ask little in return i.e. to conserve. Therefore, it offers the best example of a Symbiotic relationship. With the awareness growing tremendously about the artificial drugs and medicine and tens of harmful effects associated with their one benefit, man in today's world have returned back to mother earth for help (Verma and Singh, 2008).

More than 400,000 species of plants are present on our earth (May, 1992; Govaerts, 2001; Schippmann et al., 2002) and only a fraction, 0.5% (Batugal et al, 2004), of it has yet been utilized in the best interest of human kind, i.e., as cure for some diseases. The recent "Green Wave" or "Returning to Mother Nature" phenomenon is getting stronger due to the well known fact that plant products have less side effects and are more friendly to the ecology, biodiversity preservation and human themselves (Gijetenbeek, 1999). Therefore, it is necessary to focus more on chemically identifying the plants, long being used for some diseases, and to purify the bioactive compounds, estimating their dose and extent of effects, as without any doubt the plant kingdom still hold numerous

hidden chemicals in many species waiting to be discovered and brought to use for the best thought healthy human life (Parveen and Shrivastava, 2012; Phillipson, 2003).

An overview of free radicals, their types, production and action as given below, is necessary to go through to have an inference in how antioxidants will combat them.

1.1. Free Radicals

These are defined as independently existing, highly unstable, molecules possessing unpaired electron(s) in the outer atomic orbital. They have the following characteristics:

- Possession of one or more unpaired electron which can either be donated or paired with the acceptance of another electron from any other atomic or molecular species. Thus they may act either as oxidant or reductant.
- Highly reactive, with reaction rates equalizing diffusion rates.
- May be neutral, positively charged or negatively charged. (Del-Maestro 1980; Halliwell, 1989; Halliwell, 1996).

1.1.1. Types of free radicals

Broadly the free radicals may be classified into two main types,

- Reactive Oxygen Species
- Reactive non-oxygen species.

1.1.1.1. Reactive Oxygen Species

These can be formed endogenously and exogenously. In the body the major sites of their production are the sites where they have the highest function and where oxygen utilization is the maximum. This includes mitochondria (energy production), peroxisomes (detoxification), and the immune system (combating diseases). The different types of ROS formed in the body are,

1.1.1.1.1. Superoxide Radical

It is denoted by $\mathbf{O_2^{\cdot}}$. When oxygen is reduced the first reactive free radical formed is superoxide. The electron accepted is localized on anti bonding orbital and thus superoxide also has a paramagnetic character and an Electron Spin Resonance profile.

1.1.1.1.2. Hydrogen Peroxide

It is denoted by $\mathbf{H_2O_2}$. The addition of another electron to superoxide will form peroxide O_2^{-2}, but in vivo it is almost always present in the form of H_2O_2 and is perhaps the only free radical produced in largest amount in the body. The major site of production of this radical is in mitochondria, the Electron Transport Chain.

1.1.1.1.3. Hydroxyl Radical

It is denoted by $\mathbf{OH^{\cdot}}$. When hydrogen peroxide is reduced as follows it produces hydroxyl radicals,

$$H_2O_2 + Fe^{2+} \longrightarrow OH^{\bullet} + OH^{-} + Fe^{3+}$$

$$H_2O \longrightarrow (H_2O^{+}, e^{-}) \longrightarrow H_3O^{+}, H^{\bullet}, OH^{\bullet}, e^{-}aq, H_2O_2, H_2$$

The above reaction may be catalyzed by UV radiation or heat.

1.1.1.1.4. Peroxide Radical

It is denoted by $\mathbf{ROO^{\cdot}}$. These are the radicals formed when H_2O_2 reacts with other larger molecules, e.g., lipids. These have dangerous consequences and are the most studied free radicals because they are most stable.

1.1.1.2. Reactive Non-Oxygen Free Radicals

The free radicals in which the lone electron is present on any atom other than oxygen atom come under this heading.

1.1.1.2.1. Nitric Oxide

It is a free radical present in atmosphere and also formed in the body. This two faced molecule at one end has beneficial effects of vasodilatation and neuro transmission and at the other end can produce reactive oxygen radicals and reactive peroxynitrates.

1.1.1.2.2. Thylil Radical

It is the least studied due to very short half life and little if any electron spin resonance profile. The simple thiols in our body are protective against free radicals as they are the favorite point of attack of free radicals, but the thylil radical formed, although short lived is toxic.

1.1.1.2.3. Nucleic Acid Radicals

The DNA molecule when once attacked by free radicals, itself forms different radicals, especially of nucleic acids. These can further damage the cell structures. However it has been found that any –SH group present in close proximity of DNA saves it from getting damaged, e.g. cysteine. It may be noted here that the actual radical formed here most of the time is **OH**$^{\cdot}$. This is present as a substituent on the aromatic ring of the DNA bases.

1.1.1.2.4. Aromatic Radicals

Many aromatic compounds may enter the human body as pollutants and then undergo metabolism producing aromatic radicals like radicals of aniline, phenol, PABA etc which are very toxic. For example, phenol when converted to phenolic radical has the ability to combine with hemoglobin forming methemoglobin causing methemoglobinemia. (Valko et al., 2007; Gutteridge and Halliwell, 2000; Pham-Huy et al., 2008; Dröge, 2002)

1.1.3. Methods to measure free radicals

Free radicals can be measured directly or indirectly. Measurement of free radicals is necessary for two reasons.

- To assess the extent of damage/adverse affects.
- To plan the dose and type of antioxidants/curing therapy.

Different methods employed to measure free radicals are; (Slater, 1984; Pryor, Vol. 6; Knowles et al., 1967).

1.1.3.1. Spectrophotometry

B. Chance and J Marry were successful in analyzing the kinetics and mechanism of action of several antioxidants enzymes such as catalase (CAT) and peroxidase using this method where specialized spectrophotometers with excellent resolving power and capability to record high speed spectral changes were employed. Although it is one of the oldest method and has certain limitations like only those compounds with high molar absorption coefficient and those which react to form stable product can be analyzed, it is still widely employed for free radical analysis.

1.1.3.2. Chemiluminescence

It is an indirect way of measuring free radicals because it actually measures the effect of free radicals on surrounding environment. Some reactions particularly ROS generating reactions release high quantities of energy which excites the electrons. These electrons when relax to the ground state, they release the energy in the form of luminescence or non visible wavelength of light which is measured by this apparatus.

1.1.3.3. Electron Spin Resonance

As free radicals posses a single electron in the outer orbital, therefore its spin gives paramagnetic character to the molecule, so that under the influence of external magnetic field specific signals are produced which can be measured for quantitative as well as qualitative interpretation. Thus electron spin resonance (ESR) can be used to directly measure the free radicals, where the g factor is helpful in quantitative information

regarding magnetic moments of electron. This method although highly effective has limited use in biological system because tissues contain 75% of water and water being magnetic dipole, quenches ESR signals. Thus to estimate the amount of free radicals in a living system, all tissues are lyophilized and then free radical measurement is made at the temperature of liquid Nitrogen.

One of the successful results of ESR is estimation of supposed number of free radicals in cancer tissue.

1.1.4. Molecular damage caused by free Radicals

1.1.4.1. Lipid peroxidation

Lipids present at both cellular and sub-cellular organelles level are susceptible to damage by free radical. As with other free radical reactions, this is also a chain reaction in which along with hydro peroxides of lipids, several other toxic compounds are produced namely, malanoaldehyde, isoprostanes and alkanes which may act as secondary messengers, away from the site of production or may react directly with cell structure (DNA and protein) increasing the tissue damage. Thus lipid peroxidation has highly deleterious effects on the cell function.

1.1.4.2. Carbohydrates

Carbohydrates are susceptible to attack by the free radicals particularly **$OH^{\cdot}$** which randomly takes away the hydrogen from them resulting in chain breaking of essential molecules like hyaluronic acid and produce carbon centered radical. During rheumatoid arthritis many such oxy-radicals are present in the synovial fluids that surround the joint and this occurs upon activation of neutrophills causing inflammation. (Devasagayam et al., 2004)

1.1.4.3. DNA

The ability of every cell to perform normally depends directly or indirectly on DNA. This DNA if damaged leads to severe consequences and even death. The purines and pyrimidine bases in the DNA are susceptible to oxidative damage by ROS or RNS. Free

radicals, for example $OH^{\cdot}$, reacts with C_4-C_5 bond of pyrimidine very easily or get attached at position 8 of purines (Devasagayam et al., 2004) This results in 5-hydroxydeoxyuridine, 5-hydroxydeoxycytidine, thiamine glycol, uracil glycol, urea residue, hydantoin and 8-hydroxydeoxyguanosine (8-OH-dG), 8-hydroxydeoxyadenosine (8-OH-dA), and formamidopyrimidine respectively (Dizdaroglu et al., 2002). 8-OH-dG is considered a reliable marker in various cancers. Changes such as these in nucleotides of strands of DNA will result in mismatches and thus mutation (Marnett, 2000). Free radical may also react with DNA sugar and cause chain breakage but this is generally not seen in vitro. So the major damage implies to the reaction with Nitrogen basis of DNA (Beckman and Ames, 1997).

1.1.4.4. Proteins

Proteins are integral part of membrane and form enzymes. They are susceptible to oxidative stress damage by nitrosylation, carbonylation, disulfide bond formation and glutathionylation and the resultant is either the amino acids get oxidatively modified; cross linkages between proteins may occur if lipid peroxide are the reacting free radicals or free radical mediated protein cleavage may occur (Devasagayam et al., 2004). It is seen that amino acids containing sulfhydryl group, –SH, are more susceptible to free radical damage, example Methionine, cysteine, arginine and histidine because activated oxygen can extract hydrogen from –SH group and thylil radical will form which may react with another thylil radical to cross link forming S-S linkages in protein. Protein hydro peroxide when formed may also interact with transition metal forming a series of other radicals. Body cells although posses the mechanism of destroying and removing noxious elements including these oxidized proteins, still some might find their way to get accumulated in cell leading to various damages including increasing the chances of enzyme proteolysis (aging) affecting the activity of enzymes, receptors and membrane transport, signal transduction mechanism and heat stability. One example is lipofuscin, which is an aggregate formed by peroxidized lipids and proteins and has seen to be

accumulated in lysosomes of aged cells and brain cells of patients suffering with Alzheimer's disease (Davies, 1987, part I, II, III, IV)

1.1.5. Role of Free Radicals in different diseases

1.1.5.1. Neuro Degenerative diseases

The free radicals can cause the degeneration of neurons and the two most commonly observed results are persistent and slow loss in a man's motor and cognitive abilities. This negative effect on neuron is primary fact leading towards aging.

Different parts of the brain including hippocampus, brain stem and basal ganglia when examined histologically for the semi quantification of ferritin and brain iron in Parkinson's patients showed iron in Fe^{+3} form and ferritin to be increased in zona compacta region as compared to normal, suggesting their role either as initiators or progressors of the disease (Jellinger et al., 1990). Another study showed that transferrin was found in high amounts in globus pallidus of PD patients (Loeffler et al., 1995).

Alzheimer's disease (AD) is a condition involving a gradual destruction of neurons, starting with small and recurrent episodes of short term memory loss, but progresses to rather severe symptoms of language and direction impairment, and finally retiring from society and loss of small body function. This suggests that it involves slow and continuous destruction of neurons of the human body and once started it proceeds without any restriction. This idea suggested the involvement of free radicals in this disease, about three decades ago, when all other unsolved problems were attributed to this monster, the free radical. A study conducted on the brain testing of normal versus AD patients involving the assessment of trace elements concentration in the isolated mitochondria, microsomes and nuclei of the brain revealed that Hg and Br were in higher concentration in AD patients while Zn, Se, and Rb had been decreased as compared to the normal (Wenstrup et al., 1990; Ehmann et al., 1986, Cornett et al., 1998). Another study also involving comparison of AD and normal brain suggested that AD patients have also high concentration of Zn and Fe in various parts of the brain

while the protective measure against Hg, that is, Se was seen to be increased in AD, thus showing that Hg have achieved high level in AD suffering brain (Cornett et al., 1998). Thus from the above mentioned data Fe and Hg are the elements notorious for their ability to produce free radicals in vitro and in vivo.

Iron in the form of Fe^{+2} is known for the initiation of the reaction by donating its electron to oxygen and forming itself the stable Fe^{+3} .

$$O_2 + Fe^{+2} \rightarrow Fe^{+3} + O_2^{\cdot}$$

This $O_2^{\cdot}$ is known as superoxide radical and it is converted by an in vivo enzyme superoxide dismutase to a reactive compound hydrogen peroxide.

$$2H^{+} + O_2^{\cdot} + O_2^{\cdot} \rightarrow H_2O_2 + O_2$$

Although H_2O_2 is itself reactive specie, still it undergoes cleavage in the presence of excess/elevated Iron into super high reacting specie i.e. $OH^{\cdot}$. Owing to the Fe present there the reaction gets the name of Fenton.

$$Fe^{+2} + H_2O_2 \rightarrow Fe^{+3} + OH^{-} + OH^{\cdot}$$

The resulting radical has the power to destroy any biological organic compound getting in its way and our body does not possess any effective mechanism to combat this radical (Olinescu and Smith, 2002; Halliwell, 2007). The Fenton reaction, if no iron is present, can proceed in the following way also called Haber-Weiss reaction discovered in 1934.

$$H_2O_2 + O_2^{\cdot} \rightarrow O_2 + OH^{-} + OH^{\cdot}$$

The final product is same i.e. hydroxyl radical. Several studies have shown support in this hypothesis. Increased Ferritin in the hippocampus region of the brain of AD patients especially in the neuritic plague were observed, particularly important was the fact that microglial cells, which extensively proliferate had most of the Ferritin accumulation (Grundke et al., 1990). Another study comparing the AD patients with normal young and normal elderly showed Fe and transferrin in high amounts in the frontal cortex

(region of memory) and globus pallidus in AD patients with the decrease in transferrin/Iron ratio (Loeffler et al., 1995). One study showed that Iron level increase from 22% to 51% in different regions of the olfactory portion of brain in AD, when compared to normal control of same age (Samudralwar et al., 1995). Using the technique sensitive most for the detection of many elements at the same time, laser microprobe mass analysis (LAMMA) revealed increased amount of Aluminum, another trace element, in the tangles-neurofibrillary tangles-of the AD patients and no such finding was there in control (Good et al., 1992).

Also a greater concentration of protein in the oxidized form was seen to be accumulated in elderly normal humans as compared to young normal's, particularly in the occipital lobe and frontal lobe. (Smith et al., 1991) But in an age-matched study of elderly normal and elderly AD patients, an increase in the oxidized protein (up to 42% and 37%) was found in the hippocampus and inferior parietal lobe as compared to their own cerebellum, respectively, in AD patients, while in normal, the amount of oxidized protein was same in all the regions (Hensley et al., 1995). It is suggested that these oxidized proteins may be the product of free radicals and lead to cell death finally. As is expected from oxidation, i.e. the oxidative stress of free radicals, oxidation of lipids in the brain should be elevated in AD and so should the activity of antioxidant enzymes. This hypothesis was well enough proved by many studies. One such study revealed that TBARS (Thiobarbituric Acid Reactive Substances), levels were highly increased in the cortex, amygdala and hippocampus and so was the activity of the enzymes working against oxidation namely, GSH-Px, CAT, SOD and GSSR (Lovell et al., 1995) but decreased amounts of TBARS in cerebellum were reported as compared to same age normal controls. TBARS is used to calculate the amount of lipid peroxidation (Subbarao et al., 1994). Another study involved the calculation of malonaldehyde levels in brain's different regions and assessment of the activity of enzymes G6PD, CAT and SOD. This study too resulted in same conclusion of increased lipid peroxidation in certain region of the brain as compared to others (Balazs and Leon, 1994). Thus it suggests that certain regions/areas of the brain are more susceptible to the damage done by

neurodegeneration, which itself results from increase in oxidative stress, more than the body can handle, giving rise to the characteristics of AD. β-amyloid protein is seen to be accumulated in the specific neurons of the brain as plagues in the AD patients. This is the specific authentic marker for the confirmation of this disease. The basic mechanism by which and due to which is amyloid protein, consisting of almost 40-43 amino acids, forms the β-pleated sheets aggregates which are insoluble and cause the death of neuron, is unknown, but one thing is now to much extent accepted that these aggregates of β-amyloid protein play an important role in the initiation reaction or the induction of free-radicals, especially the hydroxyl radical, which further results in such reactions that lead to a slow and continuous process of neuronal death (Goodman and Mattson, 1994; Harris et al., 1995; Hensley et al., 1995).

1.1.5.2. Cancer

In a study involving patients with cystic fibrosis, there was increased evidence in favor of this hypothesis, "free radicals cause cancer". Patients suffering with cystic fibrosis disease have a compromised or damaged intestinal digestive activity, in their body, leading often always to the malabsorption of nutrients from the food. The more to suffer here are micronutrients i.e. the vitamins and trace elements (Castillo et al., 1981), which are required by the enzymes that work against free radicals. In such a case, body will be less viable to stand the oxidative stress produced by the normal body function Figure 1.1, let alone the extra free radical stress from the white blood cells i.e. the neutrophills and monocytes, produced while combating bacterial infection in lungs (Babior, 1984). Therefore, such patients owing to increased free radical concentrations in the body will have greater chances of malignancies to occur (Davis and Sawicka, 1985) because of higher probability that these free radicals when led astray, attack the DNA molecule (Biggs et al., 1986; Neglia et al., 1995). Thus, these patients should possess higher amount of damaged DNA product 8-hydroxydeoxyguanosine, a product excreted in urine (Shimoda et al., 1994) was the hypothesis made and proved in this study, along

with this statement too that these patients also have a less vitamin E and Se-trace element-in their blood (Salonen et al., 1984; Brown et al., 1995).

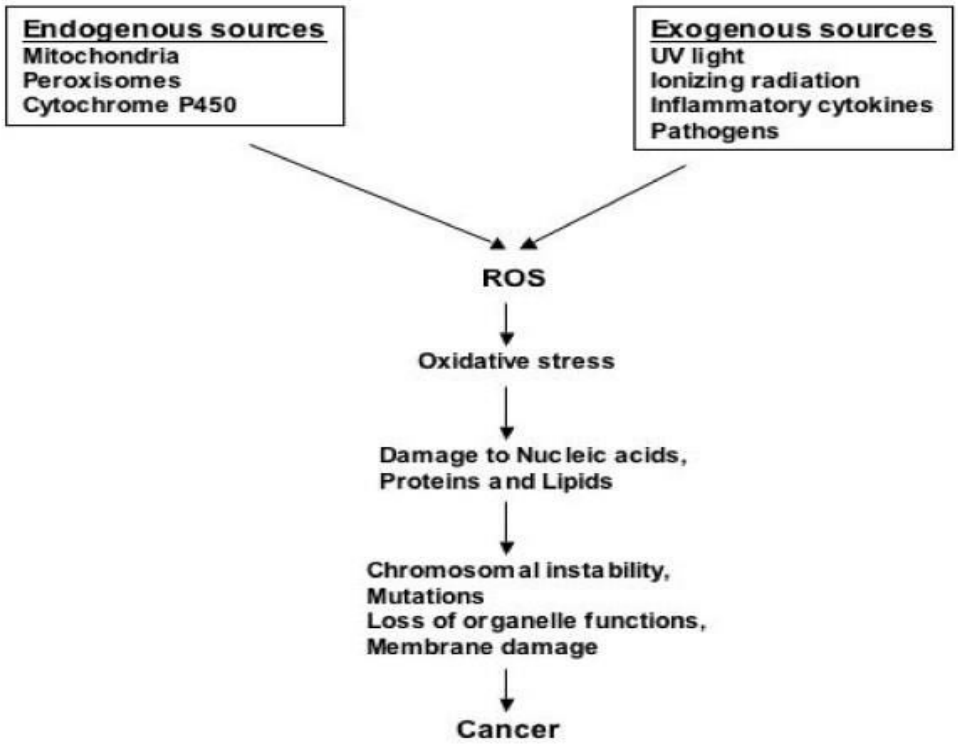
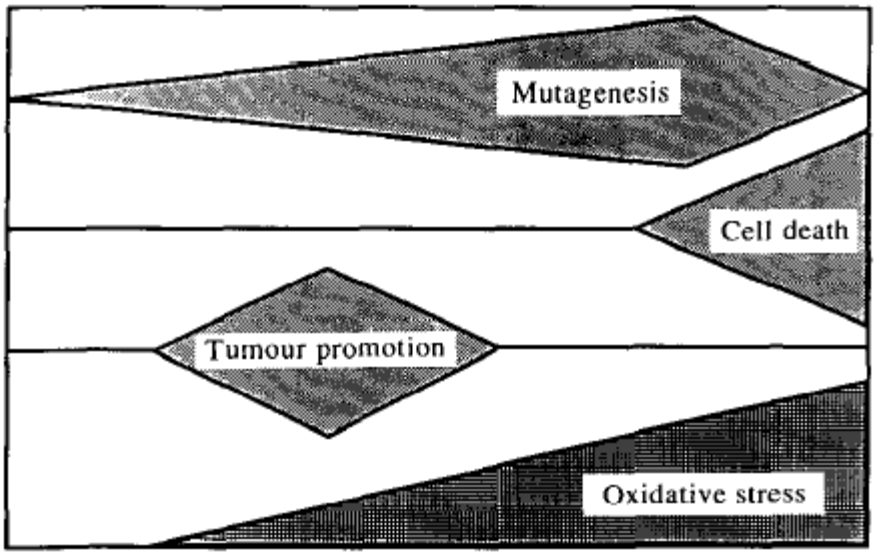


Figure 1.1: Schematic diagram of how ROS leads to cancer.

Cancer is a disease of continuous production of cells of a particular type at a higher speed. It is a universally accepted phenomenon that free radical especially the ROS are the causative agent of cancer, due to their damage to DNA (Ames, 1983). Patients having cancer are seen to be suffering from either those conditions/diseases which results in the production of ROS at a very high rate, or those diseases in which such reactions, which in a normal person, occur to combat these ROS, are compromised (Takeuchi and Morimoto, 1993; Brown et al., 1995), either by down regulation or destruction of the enzymes (Salonen et al., 1985). ROS led damage to the DNA of intermediate levels is necessary for tumor production, as damage below the threshold is

repaired by the cell itself and extreme damage of DNA leads to sudden cell death Figure 1.2, as such DNA do not support life reactions.



Adapted from "Free Radicals in Medicine"
Radu Olinescu, Terrance L. Smith

Figure 1.2: Graphical representation of degree of oxidative stress required for tumor production.

Although ROS are active enough to react with a nitrogen base or sugar backbone, the most common alterations seen are in purines, especially Guanine, while pyrimidines are less touched by ROS (Takeuchi and Morimoto, 1993; Higinbotham et al., 1992; Du et al., 1994; Lunec et al., 2002; Denissenko et al., 1996). One example of this is 8-OH-dG which when formed results in misreading at the time of replication causing initiation of tumor, where most probably, this mutation results in suppressing the good gene i.e. tumor suppressor gene or activating the monster, oncogene (Ames, 1993). Also the ROS, owing to their extremely high reactivity, can react and change all the base pairs but it is seen that most of cancers are initiated when the Guanine-Cytosine pair gets mutated (Retèl et al., 1993). The most common occurring mutations in human DNA is the replacement of a purine with a pyrimidine, commonly known as transversions, leading to disruption of the whole DNA structure, as DNA molecule is to some extent

tolerant to transition of purine with another purine and likewise but completely intolerant to the former (Harris and Hollstein, 1993). The induction of P_{53} tumor suppressor gene is the most common reason of cancer in human and it is caused by transversion of G → C (Hollstein et al., 1991; Brash et al., 1991).

Smoking causes release of many particles, which lead to formation of ROS, and these particles, called carcinogens (Rodgman et al., 2000), lead to the formation of the mutated purine 8-OH-dG, which is seen to be accumulated in the lungs of smokers (Das, 2003; Pfeifer et al., 2002; Shields, 2000). This leads in the final step to some diseases, like lung cancer, lung fibrosis, which owe to the ROS to initiate the reaction series (Zienolddiny et al., 2000). When people who were smoking were assessed, their lungs were found to contain, as compared to normal, 2-3 folds more amount of 8-OH-dG (Olinski et al., 1992). This 8-OH-dG also passes into the urine and thus is used as a marker for ROS initiated chronic diseases like, cancer, diabetes and atherosclerosis (Wu et al., 2004). People, who smoke, excrete nearly 10-fold more 8-OH-dG than the non-smokers (Fraga et al., 1996).

Another metastatic cancer, the breast cancer, is also believed to be caused by free radical. In a study where the cancerous tissue from the infected breast were compared with the normal one, from the point of view of DNA concentration, as high as 10-folds of the aforementioned entity i.e. 8-OH-dG, and other products of ROS + DNA reaction were observed in the diseased tissue, suggesting a role of ROS in onsetting and/or progression of this disease (Malins and Haimanot, 1991). Another study about the patients' treatment revealed that, breast cancer is metastatic in nature as most of the other ROS caused cancers are (Jaiyesimi et al., 1992). Also the extent of damage to the DNA done, by OH radical almost all the times lead to metastasis (Malins, 1996).

Liver Cancer is another cancer counted to be as the most common one in the whole wide world (El-Serag, 2002). It starts generally with the liver infection from a virus, the common one being hepatitis B virus and hepatitis C virus (Kountouras and Lygidakis, 2000). But sometimes, damage to liver can occur due to intake of toxin (Aflatoxin) with

food, and large amount of alcohol (El-Serag, 2002; Kountouras and Lygidakis, 2000). These result in the oxidation of enzymes, which if prevented, can prevent the chances of liver cancer (Kensler et al., 2002). A study was performed based on observation of the fact that more than half the populations exposed to aflatoxin B (AFB_1) and suffering from liver carcinoma have Guanine → Thymine transversions. It was clearly proved that the product formed when AFB_1 reacts with DNA is highly mutagenic having 6 times more power to exert transversion of Guanine in place of Thymine (Smela et al., 2002). Thus these viruses and toxins produce the oxidative stress, and body's own defense too, fighting against the viruses, produce the oxidative stress, in turn leading to mutations Figure 1.3, fulfilling the requirement of liver cancer (Waris and Siddiqui, 2003; Fecht and Befeler, 2004; McBride et al., 1991). As mentioned above, here too, the oxidative damage to DNA and presence of 8-OH-G is seen to be the common finding in patients with metastatic liver cancer (Schwarz et al., 2003; Ichiba et al., 2003) and thus can prove to be important in assessing the risk of developing hepatocellular carcinoma in future (Schwarz et al., 2003).

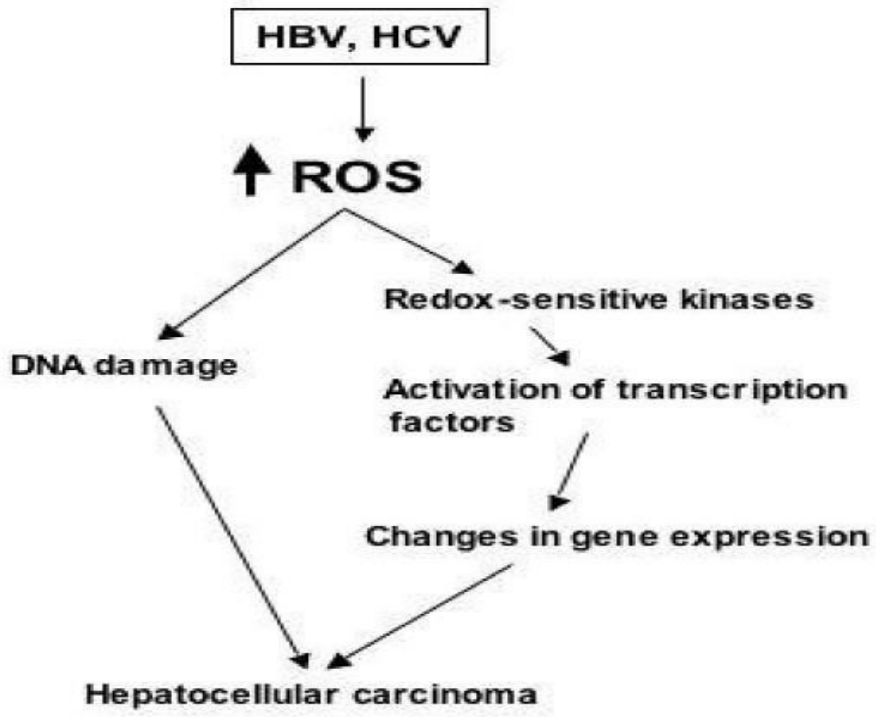


Figure 1.3: Flowchart of Hepatitis virus leading to liver carcinoma. (Waris and Ahsan, 2006)

1.1.5.3. Aging

Aging is the irreversible decrease in the biochemical and physiological performance of cells progressively (Harman, 1988). This process is slow at birth and up to middle age after which it accelerates at an exponential rate. Aging occurs due to loss of ability of cells to perform/function normally. As the cell performance and all the functions carried out by the cells depend on the DNA of the cell, thus it is widely accepted that aging is induced when the DNA is damaged (Timiras, 2007). Many logics have been put forward for the explanation of aging, including aging due to induction of autoimmune system, aging by exposure to pollutants, mutation leading to aging (Rockstein, 2012), etc, but the one which is in accordance with the modern knowledge and supports all the today's critiques is theory of free radical (Harman, 1988). This theory postulates that free

radicals produced in the body during normal metabolism, can cause oxidative damage of various molecular species in the cells, thus resulting in the alteration of normal function leading to tissue damage, necrosis and even cell death. Later a hypothesis was made that metabolic rate and life are inversely proportional (Harman, 1988). This was confirmed by several studies, one such study include Drosophila wild-type flies which were divided into 3 groups, the control group received the normal oxygen, the second group received H_2O_2 in excessive amount and the third group received along with H_2O_2 large doses of vitamin C. It was proved that the life span of flies living under higher doses of free radical environment had a mean of 8.39 days which is less than that of control; which was 14.3 days (Orr and Sohal, 1994). But this hypothesis has some contradiction with the living example of birds. These have high metabolic rate and long life than any mammal of the comparable body size. An explanation to this is that birds have fewer amounts of ROS produced in their body as compared to same size mammals. On the other hand, large mammals have long life span and they too show small amount of ROS production. Therefore, slight modification, making life span and ROS production directly proportional, can explain the theory better (Barja, 2004). This has been studied and complex-I is the point at which we can differentiate long-lived and short-lived specie depending upon the amount of ROS production (Herrero and Barja, 1997) which is low in long-life span species. Complex-I is of more importance because the ROS produced here can target the mitochondrial DNA easily, while ROS produced at complex-III is directed towards cytosol and mtDNA remains safe (St-Pierre et al., 2002). Thus this hypothesis, that short life span species should have large amount of markers showing the mtDNA damage (particularly 8-OH-G) as compared to long-life span species was tested and proved (Barja, 2000). This means that although damage to the nuclear DNA and mtDNA both can occur, and there are, present in cells, such enzymes, to mend these damages, still structural damage to mtDNA have significant consequences because it is more vulnerable, being situated near to the site of ROS production and have less enzymes nearby to mend it, once damaged (Barja, 2000).Another important point of consideration is the degree of double bonds or unsaturation present in the membrane

wall surrounding the cells as this is inversely proportional to aging (Pamplona et al., 2002). This has been supported by the fact that large mammals and birds both attributed the long life-span to less unsaturation in their phospholipids (Hulbert et al., 2002; Pamplona et al., 1999). Also it is experimentally proved that if fatty acid unsaturation is increased it leads to more damage to mtDNA which may be due to the oxidized lipid products which are highly reactive and can easily attack DNA guanine (Pamplona et al., 2004). Another hypothesis in favor of this study was that, if increase in ROS production by mitochondria at complex-I causes aging, than by decreasing calorie intake; this can be reversed or delayed. This hypothesis was proved when rats fed on low calorie diet, as shown in Figure 1.4, showed less production of ROS at complex-I in the mitochondria of liver cells (Gredilla et al., 2001). Another study showed that if calorie restriction is started in the middle of age, still it slows the process of aging in rodents (Weindruch and Walford, 1982).

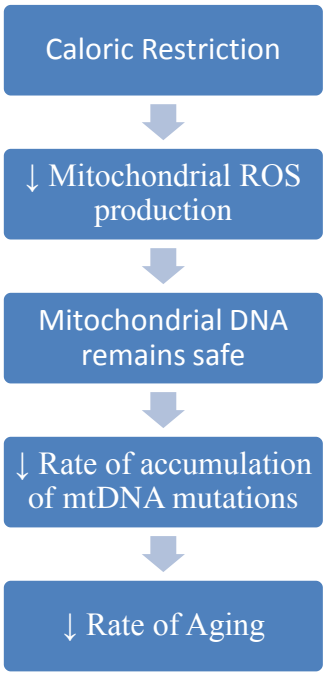


Figure 1.4: Summary of Aging due to ROS. (Barja, 2004)

1.1.5.4. Diabetes

In an attempt to demonstrate the pathway in early diabetes, it was found that some proteins which contain specific amino acids, that show high reactivity for glycation (Advanced Glycation End products), ended up in the formation of free radicals at a rate 50 times higher than the protein which were not attached to glucose, i.e. glycated. The radical generated in high amounts was superoxide which in turn oxidized the unsaturated lipids, thus proving that in vivo, such radical generation results in peroxidation of some important vulnerable unsaturated lipids like arachidonic acid, leading to atherogensis (Mullarkey et al., 1990). Another study in favor of this was done on rabbits aorta, and proved that high levels of blood glucose cause excessive ROS generation which lead the path to dysfunction of endothelial cell lining through activation of the COX enzymes, as seen in experimental rats (Pieper and Gross, 1988), and thus can be prevented by antioxidants (Tesfamariam and Cohen, 1992). The rate at which LDL is peroxidized is increased to many folds when it is provided a hyperglycemic environment at physiological pH in in-vitro conditions. This rate can be further increased if trace elements are added to the environment and can be slowed down if an element chelator, an antioxidant, is added (Hunt et al., 1990) indicating that diabetes leads to atherosclerosis through ROS production. It is also proved by study on rats that one of the long term complications of diabetes, renal complication, occurs due to ROS produced in kidney, increasing the stress of oxidation particularly in the medulla part. This increased ROS production was confirmed by checking the consumption and tension of oxygen (Palm et al., 2003; Ammon et al, 1986). It is widely thought that ROS stress leads to a cascade of events that finally result in diabetes (Ceriello, 2000). This was proved by a study on rats divided into two groups; having diabetes induced by two different drugs; streptozocin and alloxan. Both the drugs produce diabetes by action which differs from each other, but both mechanisms involve the ROS production (Lenzen, 2008) and the rats were saved from diabetes in the presence of ROS scavengers (Oberley, 1988;

Coskun et al., 2005). Also it is observed that simultaneous increase in ROS and decrease in plasma ROS scavengers, glutathione especially, prove drastic to the structure of the plasma membrane and function of pancreatic β-cells, overall having an effect of impaired insulin levels in the body (Paolisso and Giugliano, 1996). Also studies show that ROS of thiol type effect negatively on insulin secretion (Ammon et al., 1980) and action (Tiedge et al., 1997) and if protection is present to combat this thiol (glutathione and cysteine) the insulin secretion is improved (Ammon et al., 1980; Tiedge et al., 1997).

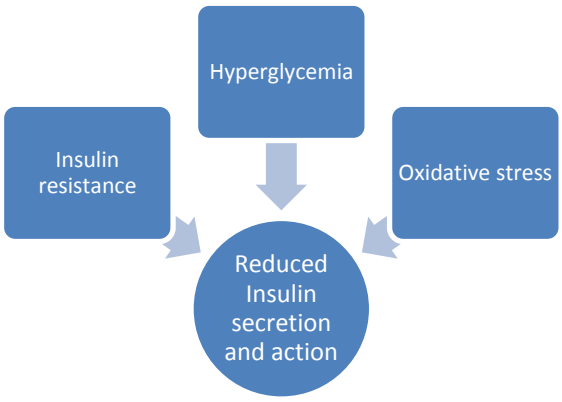


Figure 1.5: An insight of diabetic body. (Ceriello, 2000)

Also it is hypothesized that diabetes once started ROS help in leading towards complications Figure 1.5. When high levels of glucose circulate in the body, it is more vulnerable to oxidation, oxygen is then reduced and highly reactive superoxide and hydroxyl radicals are formed which accelerate advanced glycated end product formation, i.e., glycation of protein, lipid etc. These AGEs then lead to diabetes associated long term complications. (Ahmed, 2005)

1.1.5.5. Cardiovascular Disease

A study comparing patients suffering from congestive heart failure to controls i.e. completely healthy age-matched individuals, showed that the products of peroxidation of lipids i.e. malondialdehyde were notably higher in the CHF patients. A relation was

present between the amount of malondialdehyde plasma level in the patient and the ejection volume of the left ventricle, thus showing a relationship between ROS + lipid end products and disease complexity (Belch et al., 1991). Also there is an increase evidence that mitochondria of the myocytes in CHF patients is responsible for increased ROS production that eventually lead to abnormal contraction function of the myocytes and the damage to the structure of myocardium (Ide et al., 1999). Another study has provided insight that decrease in the Na^+ pump or Na^+/K^+ ATPase activity as a result of reperfusion and ischemia was reduced to a significant extent, in vitro, in the presence of oxygen radical scavengers like CAT, SOD, Vitamin E, histidine etc and simultaneously, the end products formed by lipid peroxidation, were reduced (Kim and Akera, 1987). It has long been thought that free radicals and oxidative stress is the reason in most of the cases of cardiovascular disease. This was said when experimentally it was seen that high concentration of ROS, particularly superoxide exposure to the septal cells of rabbits showed severe damage in the vascular endothelium. In the extracellular space there were evidences of cellular debris. If such a septal section was exposed to hydroxyl radical environment, the results were even more drastic. Mitochondria got swollen and large holes or vacuoles were formed (Burton et al., 1984). The effect of Xanthine oxidase, an ROS generator, on rats' hearts was observed as decrease in high energy phosphates and increase in peroxidized lipid products resulting in decreased muscular contraction of heart and vessels. Also if the exposure to Xanthine oxidase was prolonged, resting tension was increased and drastic changes in the structure of cells of the heart and blood vessels were observed (Gupta and Singal, 1989) along with cellular edema, and these results if short termed, could be easily directed backwards in the presence of CAT and SOD (Ytrehus et al., 1986). It is now thought that heart damage by increased catecholamines is mediated via the production of free radicals, as was demonstrated by experiments on rats' myocardium. Those who were fed vitamin E or Zn prior to injection of catecholamines, isoproterenol, showed less arrhythmias, swelling of mitochondria and loss of ATP and other high energy phosphates, as compared to those who were not. And simultaneous treatment with α-tocopherol decreased the intensity of

drastic effects, thus suggesting involvement of free radicals in the pathway leading to cardiovascular diseases (Singal et al., 1982; Singal et al., 1983). Therefore, long-time stress leading to high plasma levels of catecholamines proves harmful to cardiovascular system (Meerson, 1980).

It is seen that atherosclerotic plague is filled with cholesterol lesions coming from the LDL (Goldstein and Brown, 1977). Now the question is that how does LDL get deposited in these plagues. In an in vitro experiment it was noticed that, macrophages do not attract the normal LDL that circulates in the body (Brown and Goldstein, 1983). Therefore, for it to get attracted to the macrophages of the endothelial cells, LDL receptors need to get modified. This was seen in a study where LDL was exposed to higher concentrations of free radicals, the LDL peroxidation products were formed, along with other changes in LDL. Notable among these are its toxicity towards the fibroblasts, which was severely increased along with its mobility in electrophoresis. All these changes were not observed if ROS scavengers were added to the solution, suggesting role of ROS in modifying LDL (Morel et al., 1984). Also the density of LDL was increased after such an exposure and such LDL was rapidly taken up by macrophage to clean up gaining insight, that LDL receptors may have undergone a modification; most probably forming acetylated LDL (Henriksen et al., 1981). Also it was found that all the cells in circulatory system i.e. macrophages, cells of endothelial lining and cells of smooth muscles can cause oxidation in vitro (Esterbauer et al., 1992). This oxidation can be accelerated by the concurrent presence of transition metals in vitro and decelerated by metal chelators (Smith et al., 1992). Thus oxidized LDL is toxic not only in the sense that it is attracted by macrophages but it also helps in release of cytokines and forms the streak of foam cell lesion in the vascular wall (Aviram, 1993) and attract the monocytes, which can be stopped by high concentration of HDL (Navab et al., 1991; Quinn et al., 1987). To prevent this body has its own mechanism, the antioxidant which is lipid soluble, α-tocopherol, is present in the lipophilic part of LDL and saves it from oxidation until it is not completely used (Jessup et al., 1990; Esterbauer et al, 1991) suggesting that, diet less in such antioxidants is not good for a

healthy life. Also Ubiquinol-10 is seen to be effective in this reaction as ROS scavenger (Stocker et al., 1991; Maxwell and Lip, 1997).

Antioxidants

Anti oxidant-meaning against oxidation-is the term used to describe any specie which possesses the ability to neutralize the free radicals, before the latter reacts with any cell structure or molecule. A variety of antioxidants occur in human bodies which work in perfect co-ordination with each other to protect the body, in general, against the damaging effects of free radicals. These antioxidants are either formed by the body or are obtained exogenously from various foods. Till now all the antioxidants used are naturally occurring. Figure 1.6 gives a brief overview of their types.

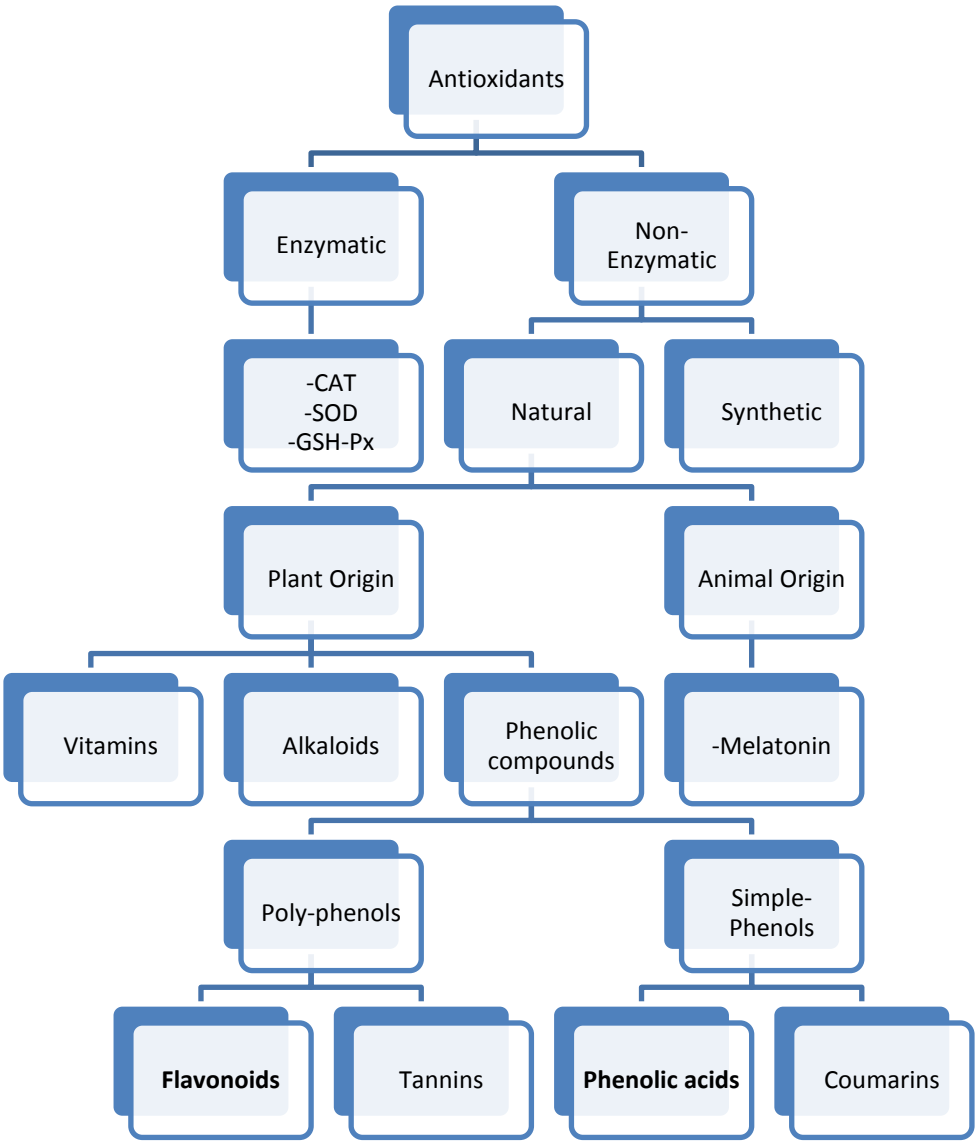


Fig1.6: Overview of Classification of Antioxidants Adapted from (Halliwell, 1996)

1.2.1. Phenolic acids

There are many types of phenolic acids present and they are generally classified on the basis of their structural similarities. Figure 1.7 is a Dendrogram of the commonly known phenolic acids. This Dendrogram is also based on the structural similarities between the phenolic acids.

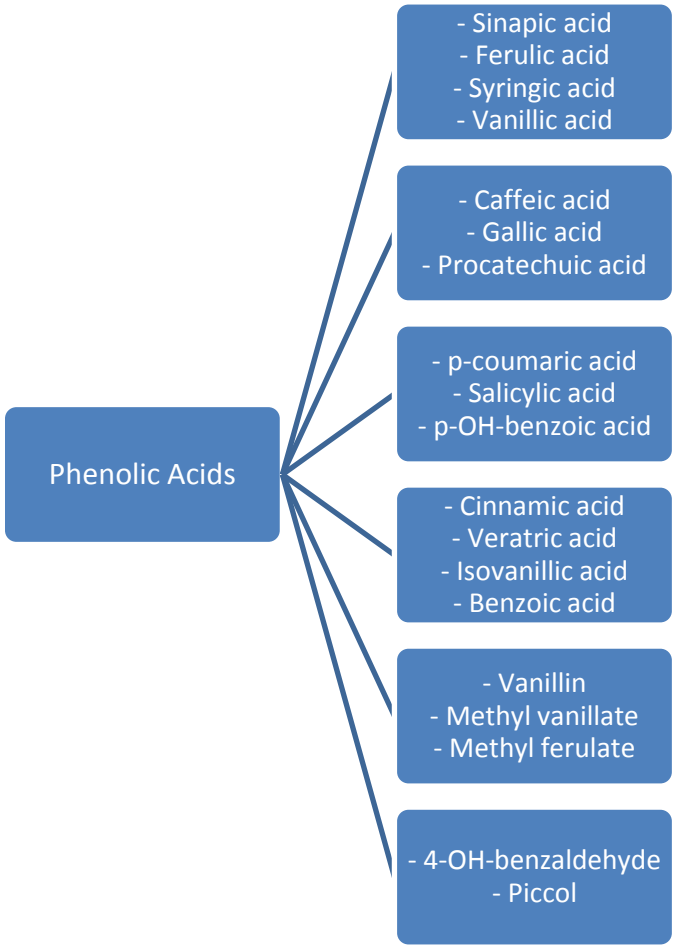


Figure 1.7: Dendrogram of the commonly known Phenolic Acids (Robbins, 2003)

1.2.1.1. Uses of Phenolic Acids:

Phenolic acids have found many uses since their discovery, long ago, and are being purified from the plants which posses them in higher concentrations, and are brought to use for a variety of medicinal purposes. Some of the uses of phenolic acids are mentioned below,

- p-coumaric acid and ferulic acid have strong antibacterial activity against *Bacteroides succinogenes*, *Ruminococcus flavefaciens* and *Ruminococcus albus* the bacteria present in the rumen of sheep. They suppress the gram –ve bacterial growth (Chesson et al., 1982).
- *Urtica dioica* showed high antibacterial activity when tested against *S. aureus*, *Listeria monocytogenes* and *B. cereus*, which is owed to the phenolic acids i.e. mainly high quantities of ferulic acid and low quantities of gallic acid and syringic acid in it (Proestos et al., 2006).
- Quantities as high as almost (41.6 mg/100g) of ferulic acid along with low quantities of other phenolic acids(11.2 mg/100 g caffeic acid etc.) are found in *Phytolaca americana* and exhibit strong antimicrobial properties when tested for *Listeria monocytogenes* (Proestos et al., 2006)
- Caffeic acid and ferulic acid have shown anti-tumor effects when applied topically on 12-O-tetradecanoylphorbol-13 acetate induced tumor in mouse skin (Huang et al., 1988).
- Ferulic acid has sufficient antioxidant activity for capturing the free radical hypochlorus acid and thus has potential to be used as additives in food (Scott et al., 1993).
- In a solution of vitamin C and vitamin E (good for photo-protection), on adding ferulic acid, following effects were observed, photo-protection was doubled and chances of apoptosis were inhibited, thus giving a new path to the protection of skin from aging and cancer (Lin et al., 2005).

- Caffeic acid and chlorogenic acid (ester of caffeic acid) have shown strong antioxidant property in ischemia-reperfusion injury protection in rat cells (Sato et al., 2011).
- Caffeic acid, when converted to its ester (phenylethyl ester, methyl ester, and phenylethyldimehyl ester), showed inhibition of mutagenicity induced in strains of *Salmonella typhimurium* and adenocarcinoma cells of human colon (Rao et al., 1992)
- Caffeic acid phenyl ethyl esters (CAPE) showed significantly increased activity against bacteria, viruses and mutation as compared to caffeic acid alone. Also the esters were seen to promote normal fibroblast cells in human respiratory tract while at the same time they inhibited the growth of damaged fibroblasts by virus (Noriaki et al., 2005).
- In one of the in vivo studies α-tocopherol, which is the body's own defense against free radicals, was spared from the stress in rats with orally administered caffeic acid and body's antioxidant action was doubled at a dose of 0.2-0.8% w/w in rats, as compared to the control (Nardini et al., 1997).
- Caffeic acid in its ester form as caffeic acid phenylethyl ester has seen to completely block the activation NF-κB; instead it activates cell death by activating the Fas-signalled apoptosis. This action depends on dose of CAPE as well as time (Natarajan et al., 1996; Watabe et al., 2004; Chiao et al., 1995; Huang et al., 1996).
- Rats when injected with subcutaneous Sodium selenite had cataract formation in eyes, which can be prevented by simultaneous injection of Caffeic acid Phenylethyl ester. Thus it has potential to be used clinically because there are no harmful effects reported on normal cells by CAPE (Doganay et al, 2002; Frenkel et al., 1993).
- Caffeic acid when compared to α-tocopherol, keeping BHA and BHT as standard in various in vitro tests of antioxidants, showed, caffeic Acid to be superior

antioxidant to the latter two artificial antioxidants and more closer, in activity, to the standards (Gülçin, 2006).

- When retinal cells are exposed to Electromagnetic radiations of 900 MHz (released by Mobiles), oxidative stress is induced resulting in NO and malondialdehyde production. CAPE when applied to such cells significantly reduced this stress by increasing SOD, GSH-Px and CAT activity and showed effects equal to melatonin when compared to it (Ozguner et al., 2006).
- Caffeic acid and its methylethyl ester has shown amazing effects in leukotrienes synthesis pathway. They inhibit the 5-lipoxygenase enzyme, thus halting the production of C4 and D4 leukotriene and do not interrupt the action of enzyme prostaglandin synthase. Thus they may prove clinically effective in numerous allergic diseases including asthma (Koshihara et al., 1984).
- Ferulic acid when administered to streptozocin induced diabetic rats showed an obvious and well pronounced increase in the antioxidant enzymes GSH-Px, SOD and CAT action and thus overall increase in the antioxidant power of the body to combat with the free radicals produced during diabetes. This potential effect decreases the adverse effects of diabetes to much extent on other parts of the body (Balasubashini et al., 2004).
- Alzheimer disease has pathogenesis related to β-Amyloid peptide, and is thought to be initiated by free radical oxidative stress. Therefore, ferulic acid when tested as long-term administration on rats showed some resistance to this oxidation and has a potential as a preventive care in Alzheimer disease (Yan et al., 2001; Kikuzaki et al., 2002).
- Cinnamic acid and its derivative, hydroxy-cinnamic acid is useful in decreasing the oxidation of human LDL (Chen and Ho, 1997)
- Various analogues of vanillic acid, when tested against infection of flaria caused by *Litomosoides carnii*, showed to be effective as possessing flaricidal activity (Varma et al., 1993).

- Vanillic acid and its derivatives, vanillin and ethyl vanillin are seen to have significant antibacterial activity against Coronobacler species, bacteria notorious to spoil food. Thus they find their way to be used as preservatives in food production and food storage (Yemiş et al., 2011).
- The hepatoprotective action of vanillic acid and syringic acid was seen by a study in which rats were induced liver injury by drugs, resulting in increase in their enzymes ALT and AST. These enzymes plasma level decreased rapidly and significantly when administered vanillic acid and Syringic acid. Also vanillic acid and syringic acid inhibited the release of cytokines TNF-α, IFN-γ and IL-6 (Itoh et al., 2009).
- In an in vivo study 12-0-(TPA)-induced tumor in mouse epidermis was applied topically with ellagic acid, gallic acid derivatives and tannic acid. These phenolic acids suppressed all the markers that promote skin tumor namely the Ornithine decarboxylase, production of H_2O_2 and synthesis of DNA, thus proving useful as anti tumor agent (Perchellet et al., 1992).
- In a comparative study of ascorbic acid and gallic acid, both acted as enhancing oxidation at low concentration, while the reducing ability increased when the concentration was increased (Yen et al., 2002).
- Gallic acid can also be used to detect the important safety cover upon us i.e. ozone. In this process ozone reacts with gallic acid, in the presence of eosin-Y and diethylene, glycol, where former act as sensitizer and latter as a modifier (Mikuska and Vecera, 1998).
- Esters of Gallic acid increased the bioavailability of the co-administered pharmaceutical drug by increasing its absorption, thus requiring lesser drugs to achieve a therapeutic goal. The suitable esters in this case were methyl octyl, lauryl and propylgallate (Wacher and Bent, 2001).
- Caffeic acid sinnapic acid and ferulic acid are used in the detection of proteins. They are used in the formation of matrices for the matrix assisted laser

desorption of protein and are much advantageous over the previously used nicotinic-matrices (Beavis and Chait, 1989).

- Gallic acid has shown significant and well pronounced protective effects on liver injury induced by high doses of paracetamol in rats, by reducing the marker enzyme levels in plasma. Thus it has a potential to be used in toxicology for immediate poisoning combat of paracetamol (Rasool et al., 2010).
- Gallic acid's anti-tumor effect when tested on different tumor cell lines, showed different results. In some, it immediately leads to cell death by fragmenting the DNA, while in others it leads to appearance of factors of apoptosis and shrinkage of cell, rather than cell death and in still others, it results only in opposing action to proliferation (Serrano et al., 1998).

1.2.2. Flavonoids

Anthocyanins were the first flavonoids to be discovered, because of their ability to impart color to the flowers. This word was first used in 1835 by Marquart. Of all the phenolic compounds known which number up to almost 8000 half of them are flavonoids, such is the importance of this group (Balasundram et al., 2006; Dey and Harborne, 1989). A simple classification of flavonoids based on their structure is given in Figure 1.8 along with their examples and food sources.

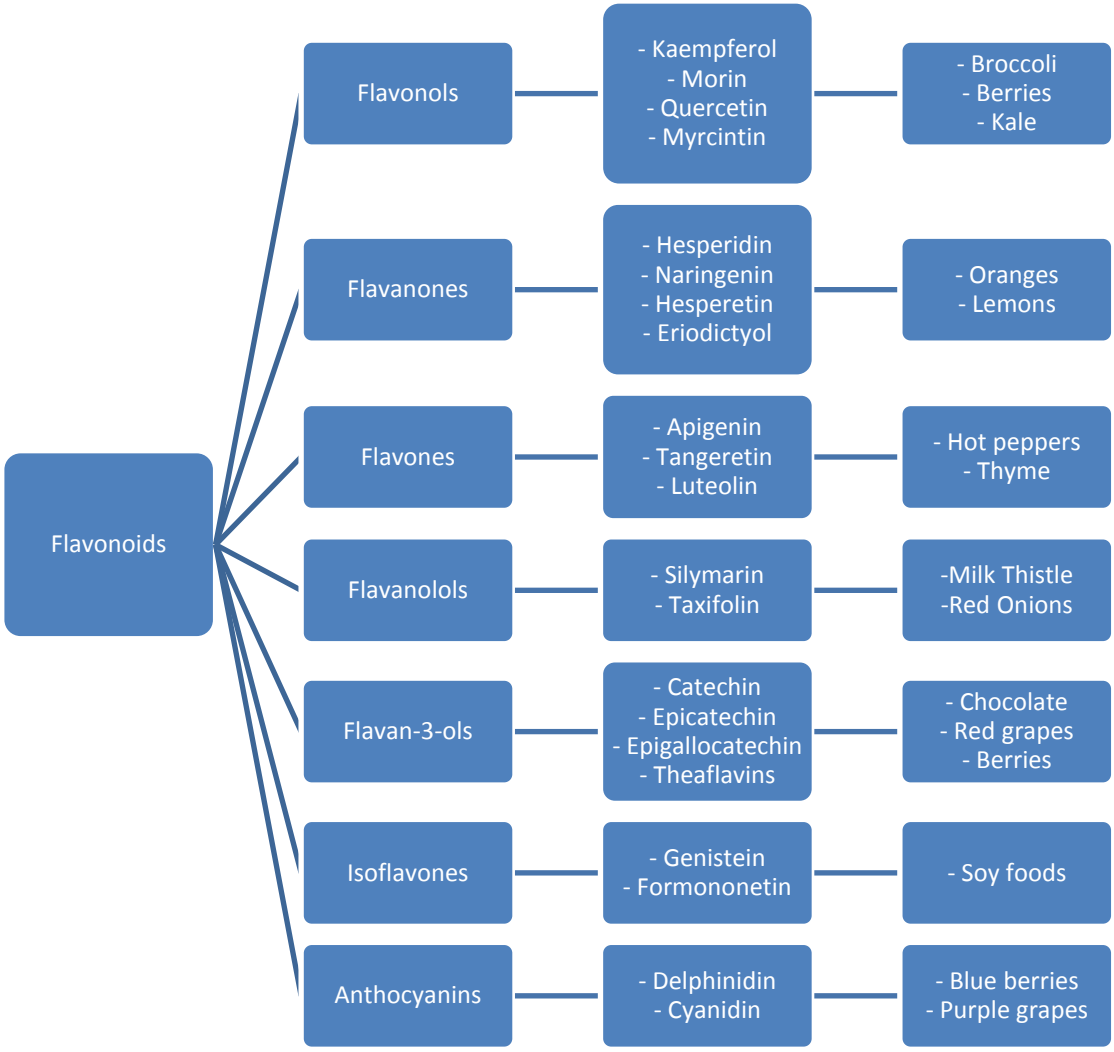


Figure 1.8: Classification of flavonoids with examples and food sources.

1.2.2.1. Uses of flavonoids

Flavonoids have many potential uses in severe diseases as mentioned below;

- Flavonoids from citrus family, hesperidin, hesperitin and naringenin are effective in treatment of fatty acid liver (Constantin et al., 2013)
- *Ammi majus L* and *Ammi visnaga*, Family, Apiaceae yield a flavonoid Khellin which has been used in asthma but its anti allergic properties as dermatologic product is also well known (Samuelson, 1992).
- Quercetin having many plant sources, *Solanum lucopersicum*, *Alium cepa*, *Glycyrrhiza glabra* etc. has highly potent anti-histamine properties and thus is used in dermatology (Amella et al., 1985).
- Isoflavone such as Genisten and Ipriflavone is found in *Soya hispida* and has known for osteoporosis prevention and other autoimmune diseases prevention (Barnes, 1998)
- Plants yielding Quercetin and Kaempferol have been proved to be effective as an anti-tumor agent, in vitro and in vivo (Zou et al., 2005).
- Derivatives of flavonoids, Isorhamnetin, Quercetin and Kaempferol, also show mild antiviral activity, in the extract of *Opuntia streptacantha* (Ahmad et al., 1996)).
- *Oryza sativa* contains naringenin which is an effective growth retardation causing agent in gramineous plants and this has helped a lot in learning about plant growth retarding mechanism (Deng, 2004).
- Naringenin has a strong antimicrobial effect against *S. aureus*, *S. epidermidis*, *Bacillus subtilis* and *Micrococcus luteus*. (Rauha et al, 2000)
- A strong antimicrobial agent Morin, is also a flavonoid from the sub class flavonol, and is effective against *S. aureus* strain. (Rauha et al, 2000)
- Flavones and Methyl gallate have antimicrobial activity equal to Bacitracin and Neomycin against Micrococcus luteus. (Rauha et al, 2000)

- 5-hydroxy-3,6,7,4'-tetramethoxy flavone, a flavonoid obtained from *A. nobilis* has antispasmodic activity when tested on rat duodenum (Karamenderes and Apaydin, 2003).
- Flavonoid fraction of *P. spinosa* also shows spasmolytic action in rat ileum. Therefore, this suggests they have anti acetylcholine activity as acetylcholine causes spasm in the intestine. (Sadraei et al., 2003)
- Another flavonoid (+) Catechin has shown anti acetylcholine activity and thus antispasmodic action in rat jejunum and also has bronchodilator effect; thus having a potential in asthma (Ghayur et al., 2007).
- Flavonoids also have gastro protective effects as was seen by injecting intraperitonially some flavonoids, Kaempferol, Rutin and Quercetin at a dose of 25-100 mg/kg (Izzo et al., 1991).
- When rats were treated with Naringenin for the protection against absolute ethanol, it showed an active cytoprotective effect at 400mg/kg which is its maximal dose, and the mechanism of action was independence of prostaglandin pathway (Martin et al., 1994).
- Another study involving the flavonoid extraction of seeds of plants, *Citrus paradisi* and Amaranthus genus showed high gastro protective activity (Zayachkivska et al., 2005).

1.3. Brief Description of Family Ranunculaceae

The family Ranunculaceae is one of the most investigated and studied family owing to its attractive flower pattern (Endress, 1995) and its genus Aconitum is one of the most specialized, when compared to other genus, in terms of evolution of floral characteristics (Endress, 1995). It has similarities to other genus when considering production of similar secondary compound e.g. Delphinium and Aconitum produce alkaloids and phenolic flavonoids. But Aconitum has distinct floral features (Brink, 1982).

The family Ranunculaceae has almost 60 genera with the top most being Ranunculus, with 600 species, Delphinium having 365 species, third in the row is Thalictrum with

330 species followed by Clematis, 325 species and then Aconitum containing a total of 300 species most of which are herbaceous. Commonly known as monkshood, owing to the shape of their flowers which is very similar to a hood, or wolf bane due to their deadly toxic chemical constituents used to hunt down and kill wolves in the past, they have been given botanical name of Aconitum (Srivastava et al., 2010). It comprises of almost 300 species of flowering plant which have certain such distinct characteristics that it has to be further divided into three sub genera; Aconitum, Lycoctonum (DC.) Peterm. and Gymnaconitum (Staph.) (Luo et al., 2005).

1.3.1. Aconitum Genus

This genus consists mainly of poisonous plants, owing to the presence of alkaloid aconite, which has cardiotoxic and neurotoxic effects. Its natural habitat is the northern hemisphere. In the Asia it is distributed widely in the northern Himalaya areas and is present in countries like Turkey, Pakistan, China, India, Nepal, Tibet etc with almost 10 species and a couple of varieties. Their species have numerous inter variation among them.

It generally grows at high altitude, some as high as 3,000 to 4,000 above sea level, in the rocky mountains where the soil is drained well and has good amount of moisture. All the species of Aconitum are perennial i.e. have a life cycle of two or more years (Srivastava et al., 2010). These plants have tuberous roots and irregular flowers hanging in loose clusters. The leaves are palmate, dark green, and are divided into 5 to 7 segments. Each segment has 3 lobes. The margin is coarse and stipule is absent (Singh et al., 2012).

1.3.2. *Aconitum cochleare* Woroschin

Aconitum cochleare W. (Figure 1.9) belongs to the genus aconitum of the family Ranunculaceae. It grows wildly in eastern parts of Turkey, where it is also called kurtboğan otu, in the local language. It is a herb and is perennial in life cycle. It has

purple colored flowers, which are irregular and are hanging in loose clusters. The leaves are palmate, with a coarse margin and no stipule is present.

Figure 1.9: *Aconitum cochleare* Woroschin

1.3.2.1. Taxonomy of *Aconitum cochleare* Woroschin.

Domain: Eukaryotae
Kingdom: Plantae
Subkingdom: Viridaeplantae
Phylum: Tracheophyta
Subphylum: Euphyllophytina
Infraphylum: Radiatopses
Class: Magnoliopsida
Subclass: Ranunculidae
Superorder: Ranunculanae
Order: Ranunculales
Family: Ranunculaceae
Subfamily: Trolliodeae
Genus: Aconitum
Specie: cochleare

1.3.3. Phenolic constituents in species of Aconitum

Many Aconitum species have been under investigation for a long time and have yielded numerous phenolic antioxidants in them. Some examples from the literature are given below, and for these reasons *Aconitum cochleare* was chosen for my study.

- *Aconitum ferox* root and *Aconitum heterophyllum* root has shown good results for the presence of phenolic compounds when tested by DPPH, ABTS and Folin-Ciocalteu tests (Surveswaran et al., 2007)
- *A.carmichaelii* Debx. lateral roots ethanolic extracts were found to contain phenol acids (p-coumaric acid and salicylic acid) along with other alkaloids (Zhang et al., 2010).
- *A. tanguticum* when subjected to different techniques of chromatography the whole plant yielded many antioxidants namely, syringic acid, vanillic acid, Kaempferol and Quercetin in the forms of various glycosides (Xu et al, 2013; Li et al., 2014)
- *A. carmichaelii* Debx. (Root) and *A. kusnezoffii* Reichb (Root) showed a 67.6 and 111.5 (µm Trolox/100g DW) and 149.6 and 219.4 of Trolox equivalent Antioxidant capacity (TEAC) by Methanol and aqueous extract respectively; 0.50 and 0.72 (g/100g) and 1.29 and 0.95 (g/100g) of total phenol content in methanol and aqueous extract respectively. While the major phenolic component in *A. carmichaelii* was identified as phenolic alkaloid (demethyl salsoline), the phenolic content in *A, kusnezoffii* was unidentified (Cai et al., 2004).
- Flavonols in the form of glycosides were seen to be present in *A. napellus* subsp. *Tauricum*, *A. paniculatum*, *A. napellus* subsp. *Neomonatum* and *A. vulparia* which are generally found in Italy and known as the Italian aconitum species. A total of 13 glycosides of flavonols namely quercetin and kaempferol were isolated from them. (Fico et al., 2000; Fico et al., 2001; Braca et al., 2003)

- The aerial parts of *A. chisanese* when extracted with methanol and fractionated, butanol fraction showed the presence of six new flavonoid glycosides of quercetin and kaempferol (Jeong et al., 1997)
- *A. naviculare* aerial parts also showed presence of kaempferol and quercetin as 3 new glycosides (Shrestha et al., 2006).
- Several flavonoids were extracted from *A. gymnandrum* by ultrasound assisted extraction with an extraction ratio of 1.278 %. (Zheng et al., 2014)
- *A. anthora* L. when extracted yielded a total of four flavonol glycosides of quercetin and kaempferol including 2 new and 2 old ones (Mariani et al., 2008).
- *A. jaluense for. Album* contains 3 flavonoids namely hyperoside and 2 glycosides of kaempferol (Whang et al., 1994).
- *A. burnatii* Gayer and *A. variegatum* L. when extracted with methanol, the aerial parts yielded six different glycosides of quercetin and kaempferol (Vitalini et al., 2010).
- *A. napellus* sp. *lusitanicum* yielded 2 quercetin glycosides when extracted with ethanol (Luis et al., 2006).
- *A. bicalense* has seen to contain flavonol glycosides i.e. kaempferol and quercetin in the form of 7-0-α-L-rhamnopyranosides, a glycoside of quercetin which is acylated (Zhapova et al., 1992).
- *A. tanguticum*, Tibetan specie when subjected to ethanol extraction, the whole plant yielded three glycosides of phenol antioxidants (Xu et al., 2013).

1.4. Scope of this study

The purpose of this study was to investigate *Aconitum cochleare* for its antioxidant properties due to phenolic constituents. Although this plant has been tested for alkaloids and those alkaloids show some antioxidant activities, the phenolic constituents have never been tested before and this was the aim here.

First the crude extract was tested for its antioxidant activity by DPPH and then its fractions were made and tested similarly by DPPH for antioxidant activity, Total Phenol and Total Flavonoid Content. The ethyl acetate extract showed good results in all the aforementioned tests and thus was further investigated by HPLC and HR-MS to identify the number of compounds present in meaningful concentration in it.

CHAPTER 2

MATERIAL AND METHODS

2.1. Materials

2.1.1. Chemicals

Methanol, ethanol, hexane, chloroform, acetonitrile all of them were chromatography grade and were made available by Merck (Darmstadt, Germany). These were used in fractionation, extraction, HPLC and HR-MS. Distilled water was used throughout the procedure and Millipore system (>1 M ohm.cm) was used to obtain it. 2,2-diphenyl-1-picrylhydrazyl (DPPH) and sodium carbonate (Na_2CO_3) were purchased from St. Louis MO, USA, while Folin-Ciocalteu reagent (FCR) from Merck (Darmstadt, Germany).

Compounds used as reference, i.e. gallic acid, esculin, apigenin, myrcetin, luteolin, catechin, scopoletin, caffeic acid and quercetin etc were bought from Sigma-Aldrich (St. Louis MO, USA).

2.1.2. Plant material

The medicinal plant, *Aconitum cochleare*, was collected in Van, a city of the eastern province of Turkey, and was dried by pressing according to the herbarium rules.

2.1.3. Instruments

Carry 5- Bio UV-VIS spectrophotometer (Varian) was used in obtaining all the spectroscopic results for DPPH, determination of total phenol and determination of total flavonoid tests.

For filtration purpose after extraction disposable syringe was used and disposable filter with pore size of 0.45 μm and 0.22 μm and a diameter of 33 mm were used which were purchased from Millipore Corporation (Bedford, MA USA).

Weighing Balance for all the weighing purposes was purchased from Precisa XC 220A (Swiss made).

Sonicator used for solution purposes at a given temperature was BANDELIN SONOREX RK100H purchased from BANDELIN electronic, Berlin, Germany.

OPTIC ivymen System rotary incubator was used for obtaining methanol extract.

For evaporation of the extract, Heidolph LABOROTA 4000 condenser was used.

HPLC apparatus consisted of; WATERS Delta 600 HPLC, WATERS 2475 Multi Fluorescence Detector, WATERS 2996 Photodiode Array Detector.

The syringe used for making injection in HPLC was HAMILTON-BONADUZ SCHWEIZ, MICROLITER #710, Switzerland.

Sartorius Minisart RC 4 single use filter of 0.20 μm was used to filter the sample before each injection in HPLC.

C18. 5 μm. 4.6*150 mm column suitable for Reverse Phase Chromatography was used in HPLC.

WATERS High Resolution-Mass Spectrophotometer from the Central Lab of Middle East Technical University was used for HR-MS procedure.

Other instruments used were, WARING COMMERCIAL BLENDER for breaking plant parts to smaller pieces, FINPIPETTE micropipette for precise volume measurement, Whatman filter paper for initial filtration and VELP SCIENTIFICA vibrator for vortex mixing.

2.2. Methods

2.2.1. Blending

The dried flowering part of *Aconitum cochleare* was broken to pieces 1-3 mm, with the help of blender, blending continuously for 3 minutes and weighed.

2.2.2. Extraction

Finely powdered, dried, sample of *Aconitum cochleare* was weighed and 40 g of it was transferred to a narrow mouthed dark glass bottle and 400 mL of methanol was added to it, ratio of 1:10 w/v. After securing the cap tightly, it was kept in rotating incubator for 24 hours, at a temperature of 25 ºC and 180 rpm. After 24 hours the sample was filtered using Whattman filter paper, the filtrate was preserved at 4 ºC in refrigerator, closed in a dark bottle. The procedure was repeated three times, reason, because the phenolic compounds present in the leaves take a little more time and volume of methanol to be extracted. All the filtrate obtained was collected; mixed and filtered using 0.45 µm filter. It was then dried in a rotary evaporator and the dried extract was weighed, to calculate yield.

2.2.3. Fractionation

The total extract, dried, (4g) was dissolved first in a mixture of 70:30 methanol and water; 400 mL The reason of making methanol aqueous was to dissolve the compounds having hydrophilic nature like phenolic acids, if any are present. Also it increases the solubility of phenolic compounds in methanol. This solution was then transferred to a separating funnel (1000 mL) and equal volume, that is, 400 mL of n-hexane was added to it and shaken vigorously and then allowed to settle. After the two layers settled, the n-

hexane layer was separated and the procedure was repeated 3 times, each time adding same volume of n-hexane. All the n-hexane separated was transferred to a round bottom flask and dried and weighed. The aqueous methanolic layer was subsequently shaken with chloroform, followed by ethyl acetate, in the same way. Each time, the volume of hydrophobic solvent added was same to the aqueous-methanol volume and each step was performed three times to ensure complete extraction of that specific fraction. Each separated layer was dried and weighed to calculate the yield. Finally the aqueous layer was also dried and weighed. This is summarized in Figure 2.1.

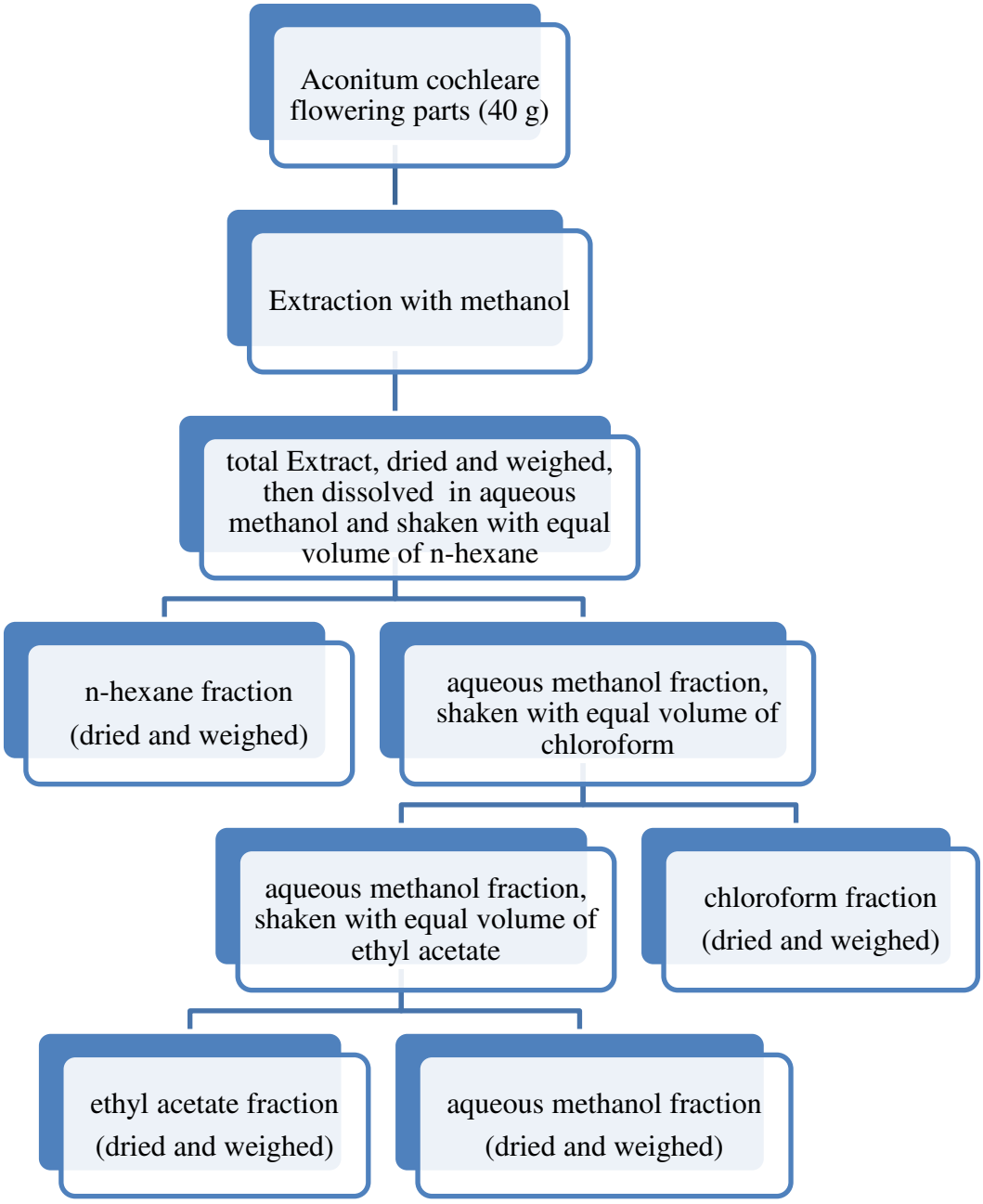


Figure 2.1: Scheme of extraction and fractionation of *A. cochleare* flowering parts.

2.2.4. DPPH Method for Radical Scavenging

2,2-diphenyl-1-picryhydrazyl, commonly known as DPPH, is a stable radical as compared to other free radicals. It is commonly used in the biochemistry of plant studies to determine the antioxidant activity of the given sample being one of the simplest and easiest methods to determine antioxidant capacity in-vitro. The molecule has a single electron on a Nitrogen atom at position 3 and due to this it gives high absorption which reaches its maximum when analyzed at 517 nm (Cuvelier, 1992; Pyrzynska and Pekal, 2013). The solution of this radical in methanol has a dark purple color. This purple color changes to light yellow as the free radical activity of DPPH is reduced by reaction with an antioxidant and also the absorption is reduced. This reduction in absorption is directly proportional to the number of electron paired and therefore, is used in stoichiometric calculations (Hogg, 1961)

The reaction is illustrated in the figure below

R-H + DPPH$^{\cdot}$ → DPPH-H + R$^{\cdot}$

A modification was done in the method mentioned in Blois et al (1958), as mentioned in Çoruh et al (2007), and was performed. DPPH solution with a concentration of 0.05 mg/mL was prepared by dissolving the free radical in methanol. The resultant solution had an absorption of approximately 1.38 - 1.40 at 517 nm. Different concentrations of the samples extracts and standard were also prepared in methanol. 100 µL of sample was added to 1400 µL of DPPH solution and shaken well, and kept in dark. The absorption was then noted after fifteen minutes and a graph was plotted with concentration on x-axis and RSA (%) on y-axis. Quercetin was used as standard for comparison.

The RSA was calculated by the following formula and is expressed in percentage.

Radical scavenging Activity (RSA) = $\{(A_0-A_1)/A_0\}$ x 100

Where;

A_0 is the absorbance of control (DPPH + methanol)

A_1 is the absorbance of sample (DPPH + Sample dissolved in methanol)

The control had 100 µL of methanol with 1400 µL of DPPH solution.

2.2.5. Determination of Total Phenol Content

It involves the usage of Folin-Ciocalteu Reagent. This reagent is also known by the name of Folin-Phenol Reagent, Folin-Denis Reagent, and Gallic Acid Equivalence (GAE) experiment. It is another colorimetric experiment generally used in laboratory because it is economic, quick, easy and accurate method for the determination of total phenol quantity in the given sample. This reaction involves the formation of a complex when polyphenols react with Folin-Ciocalteu reagent. This complex also called chromophore has blue color Figure 2.2 and shows strong absorption at 750 nm (Blainski, 2013).

The Folin-Denis Reagent was used to determine protein content while Folin-Ciocalteu Reagent is used to determine the total Phenol content of sample, where the latter being superior in terms of reproducibility and sensitivity as compared to the former. The basic chemistry of the reaction involved in this method is that, at higher pH (pH~10), which is achieved by the addition of sodium carbonate (Na_2CO_3), phenolic compounds lose a hydrogen/proton to form a phenolate ion. These phenolate ions then form a complex with Molybdenum (VI) in the Folin-Ciocalteu reagent.

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

Phenolate ion$^-$ + Mo (IV) → Complex of Mo + Phenolic compound $_{(blue\ colored)}$ (Sánchez-Rangel et al., 2013)

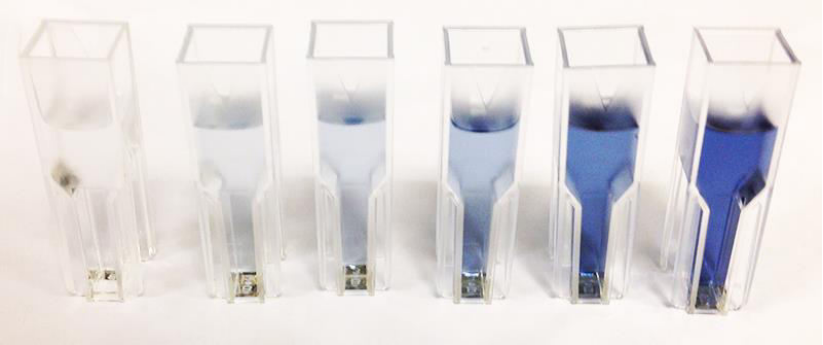


Figure 2.2: Color change after half hour of incubation in dark; left most is the control, proceeding to right, the sample concentration increases.

The method as described in Singleton and Rassi, (1965) was used to determine the total phenolic content of the plant extract and its fractions too. The standard used was gallic acid. Various concentrations of the gallic acid and sample in ethanol were prepared. Also sodium carbonate solution of 2 % (w/v) was prepared in distilled water and 50 % Folin-Ciocalteu Reagent was also prepared in distilled water. 100 µL of the sample was added to test tube followed by 100 µL of Folin-Ciocalteu Reagent and vortex mixed. This was followed by addition of 2000 µL of sodium carbonate and after vortex mixing; it was left in dark for a total of 30 minutes. After this, their absorption was measured and the absorption of control was subtracted from the sample. The control contained only 100 µL ethanol instead of sample, rest of the things were same. A graph was plotted for the standard i.e., gallic acid with concentration on x-axis and absorbance on y-axis. Using this curve as the calibration curve, the amount of total phenol were measured in sample and expressed as Gallic Acid Equivalent, i.e. each milligram of sample contains how many micrograms of total phenol in terms of GAE. The experiment was performed in triplicates, two times.

2.2.6. Determination of Total Flavonoid Content

The flavonoids present in the plant can be tested by a stoichiometric test, which is also a colorimetric test as described by Bakar. This test involves the reaction of aluminium chloride with flavonoids in the presence of sodium nitrite resulting in the formation of a complex having red color. The intensity of red color depends upon the amount of complex formed which in turn depends on the amount of flavonoids present in the sample or standard solution. This can be determined spectrophotometrically at a wavelength of 510 nm (Bakar, 2009).

The procedure as mentioned in Zhishen et al. (1997) was used with a slight modification. Different concentrations of the samples as well as standard were prepared. Rutin was used as a standard here. 0.2 mL of the sample or standard was taken in a test tube and 0.075 mL of 5 % (w/v) sodium nitrite was added to it. This was then incubated at room temperature for five minutes. Then 0.15 mL of 10 % (w/v) aluminium chloride was added to it and again the test tube was incubated at room temperature for six minutes. Finally 0.5 mL of sodium hydroxide was added to it, to stop the reaction, and the total solution was made up to 3 mL by adding water and the absorbance was noted immediately at 510 nm against the blank. Each step was followed by vortex mixing of the solution in the test tube.

This same procedure was applied to entire samples as well as the standard. A graph of concentration versus absorbance was plotted for the standard, rutin. The slope of the graph was calculated to give the value of y by the equation, $y = mx + n$. This equation was then used to calculate the rutin equivalent in μg present per gram of the sample.

2.2.7. Analytical HPLC Analysis

The HPLC technique employed here was Reverse Phase-HPLC (RP-HPLC). RP implies that the stationary phase was made up of hydrophobic molecules and thus tends to attract the compounds of hydrophobic nature. Therefore, the result is that hydrophilic compounds will elute first and hydrophobic compounds elute only when the nature of mobile phase changes to more hydrophobic/organic than the stationary phase.

This technique is one of the best available for the separation analysis of phenolic compounds, as phenolic compounds are diverse in nature and vary from very hydrophilic (phenolic acids) to very hydrophobic(coumarins and flavonoid).

For identifying the number of compounds present in the sample, total extract as well as each of the fractions of total extract underwent analytical RP-HPLC. The unit used was Multi-Solvent delivery system of Waters which was attached to fluorescence detector and photodiode array. It also had a degasser unit to remove air bubbles. This system efficiently separated the phenolic compounds present in different fractions of methanolic extract of *A. cochleare*.

The program that was set for the elution was optimized as used in Çoruh et al (2014) with a slight modification. The mobile phase had a gradient flow, Table 2.1, and consisted of a mixture of two entities, (A) contained pure water distilled and ultra filtered, (B) contained a solution of methanol, acetonitrile and 2% v/v acetic acid in water, in a ratio of 2:2:1. The mobile phase had a gradient flow as shown in table 2.1.

Table: 2.1: Gradient flow of mobile phase in RP-HPLC.

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

The flow rate for each injection was fixed to 1.3 mL/min. After each flow, the column was washed using solution B only and then re-equilibrated for 20 minutes. All procedures were carried out at constant temperature of the column at 25 ºC.

The column used was suitable for Reverse Phase purchased from Waters.

The range of absorption spectra in which the diode array detector observed the results was 210-800 nm. While all the chromatograms were observed at different wavelengths of 254, 280, 320 and 360 nm.

2.2.7. High Resolution-Mass Spectroscopy

It involved Ultra Pressure Liquid Chromatography (UPLC) and Mass Spectroscopy done simultaneously. UPLC included the usage of water (A) and methanol: acetonitrile and acetic acid in water at a concentration of 2% (v/v) (B), all three present in a ratio of 2:2:1 as the mobile phase. The flow of (A) and (B) was gradient proceeding from more polar to less polar, as shown in Table 2.2.

Table 2.2: Gradient flow of mobile phase in LC-MS

Time (minutes)	Percentage of A	Percentage of B
0-30	90	10
30-40	40	60
40-50	20	80

The rate of flow was set at 0.06 mL/min while the volume of injection in one injection was 2 µL. Waters Acquity UPLC purchased from (Waters corp.) present in Central Lab of METU was used to perform the liquid chromatography by using an analytical column (1 x 10 mm, 1.7 µm) from Waters Acquity UPLC, (BEH Shield). This procedure was done to estimate the number of unknown phenolic compounds present in ethyl acetate extract of the plant, in significant concentration, along with their molecular weight. A single direct injection of the fraction was analyzed at different voltages to estimate the molecular weights of compounds present therein.

CHAPTER 3

RESULTS AND DISCUSSION

3.1. Extraction

The finely powdered flowering parts of *Aconitum cochleare*, 40 g, were taken in a dark bottle and methanol was added as mentioned in section 2.2.1. All the process under section 2.2.2 was done and the filtrate was evaporated to complete dryness and weighed. The yield was 4.17 g. Out of this; 0.17 g was kept for further testing while 4 g were further fractionated.

3.2. Fractionation

A total of 4 g of dried total extract was taken and the procedure was carried out as mentioned under section 2.2.3 and shown in Figure 3.1, for fractionation and all the fractions were evaporated until completely dry and then weighed. The yield of each fraction is given in Table 3.1. Most of the total extract dissolved in the aqueous methanol fraction and the least of it total extract dissolved in ethyl acetate fraction.

Table 3.1: Percentage yield of each fractionation step

Name of Fraction	Yield in grams	Percentage yield
n-Hexane	0.317	7.92
Chloroform	0.296	7.40
Ethyl Acetate	0.062	1.55
Aqueous	3.325	83.12

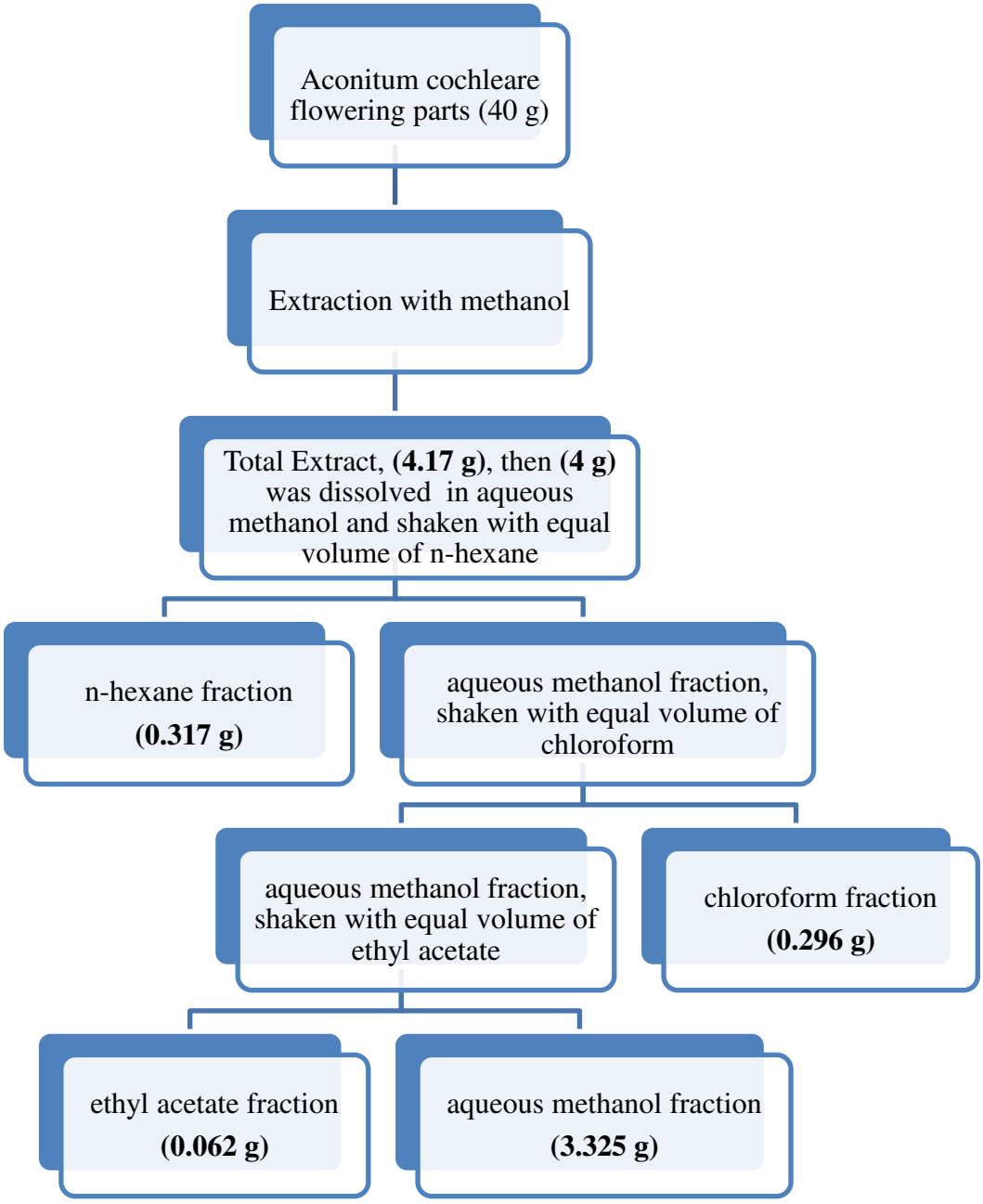


Figure 3.1: Flow chart for the extraction and fractionations of the methanol extract from the flowering part of *Aconitum cochleare*.

The purpose of methanol extraction is to ensure phenolic compounds concentrate, as they are present in very small quantities in the plants. While the fractionation procedure in order of increasing polarity ensures the phenolic compounds to be concentrated in the specific solvent extracts and enables us to easily study their nature. While n-hexane fraction dissolves in itself almost all of the lipid and chlorophyll matter (Özdoğan, 2013), ethyl acetate fraction tends to attract most of the phenolic compounds (Çoruh et al, 2014).

3.3. Antioxidant Capacity Determination

All the plants containing phenolic compounds have some antioxidants capacity. Therefore, its measurement is important in the first step. This estimation includes all the fractions separately and the whole extract too. Any fraction which does not show significant antioxidant effect will not be investigated further in highly sophisticated tests of RP-HPLC and HR-MS. The most used test for estimation of antioxidant capacity is DPPH test.

3.3.1. DPPH Method for Radical Scavenging

This test was performed as mentioned under the section 2.2.4 of method. The result obtained was plotted in the graph with concentrations versus the percentage of Radical Scavenging Activity (RSA). Also the effective concentration (EC_{50}) at 50 % of the activity was calculated. This EC_{50} is the concentration of the sample or the amount of sample required to produce half of its maximal effect. It is important to calculate the EC_{50} value as this gives an insight of the effectiveness of the sample as antioxidant, and that sample will show the highest antioxidant effect which will have the lowest EC_{50} value. The EC_{50} value is calculated from the plot RSA percentage versus concentration of samples as shown in figure 3.2.

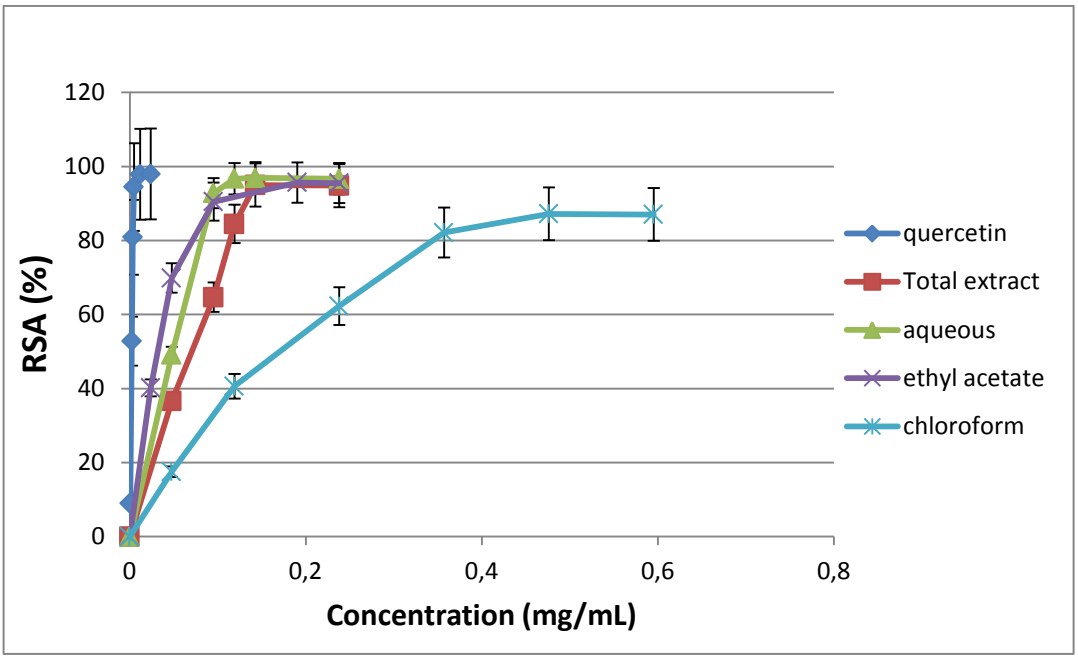


Figure 3.2: DPPH radical scavenging activity of total extract and fractions of *A. cochleare* and standard Quercetin, observed at 517 nm. Each point is the mean of two sets of triplicate.

This experiment was done to show the effectiveness of scavenging free radical. Quercetin was used as the standard and all the separated fractions, as well as the total crude extract were compared against it. Figure 3.2 shows that the graph of ethyl acetate is nearest to the standard; therefore, it has the highest amount of phenolic antioxidants and thus corresponds to the highest free radical scavenging activity in all the samples tested. Next to ethyl acetate is aqueous fraction graph, in Figure 3.2, which reaches its maximum level of activity with a little higher concentration than that of ethyl acetate. Therefore, from Figure 3.2 it can be concluded that, from the total extract, most of the phenolic antioxidants remain in the ethyl acetate and then in aqueous fraction, but as seen in table 3.1, the yield of ethyl acetate fraction is less than 2%, thus, indicating that the compounds fractionated with ethyl acetate solvent are mostly antioxidants, while less antioxidants remain in aqueous fraction.

Table 3.2: Comparison of EC_{50} values of different extracts and standard quercetin.

Sample	**DPPH** **EC_{50} (µg/mL)* ± SD**	**RSA (%)** **(concentration)**
total extract	69.186 ± 0.06158	94.86 (5 mg/mL)
n-hexane	ND	ND
chloroform	217.986 ± 0.08194	87.02 (12.5 mg/mL)
ethyl acetate	28.817 ± 0.05682	95.52 (3 mg/mL)
aqueous	45.749 ± 0.04377	96.66 (5 mg/mL)
quercetin	4.346 ± 0.12539	97.97 (0.5 mg/mL)

ND: Not determined.

* Mean of two independent experiments in triplicates.

From the table 3.2, our estimation about the concentration of antioxidants soluble in each fraction is proved right, with ethyl acetate fraction being the one, most concentrated with the antioxidant compounds, as it has the lowest EC_{50} value, and the chloroform fraction being vice versa. The % RSA and EC_{50} of n-hexane could not be determined because this fraction had the antioxidant compounds in very low concentration, and to reach its maximum effective concentration, the amount of the extract had to be increased to such high levels that other compounds present in it, created hindrance with the absorption measurement at given wavelength of 517 nm. From the table 3.2, the order of decreasing of the antioxidant activity in the given fractions is ethyl acetate > aqueous > total extract > chloroform with values 28.817 > 45.749 > 217.986 > 69.186 µg/mL respectively.

The standard used in this study, quercetin is a pure flavonoid compound and showed an EC_{50} value of 4.346 µg/mL

There is till now no such study in literature which showed the DPPH result of fractions of *Aconitum cochleare*. Also no study has shown the antioxidant property of methanol

extract of *A. cochleare* by this test. Therefore, it is an important step to name this species (*Aconitum cochleare*) in the row of other potential antioxidant plants.

3.4. Determination of Total Phenol Content

The Folin-Ciocalteu test was employed for the determination of total phenol in all the fractions. It is a sensitive test for phenol estimation, easy to perform and shows quite precise results. The Folin-reagent consists of a mixture of the acids, phosphomolybdic acid and phospohotungstic acid, which form a colored chelate or complex with the phenols when the latter is oxidized. The resulting solution shows a maximum absorbance at 750 nm and thus is observed at this wavelength.

The standard used in this experiment is gallic acid owing to its very easy solubility in the Folin-reagent. Therefore, the standard gallic acid curve of its absorbance versus the concentration of gallic acid is plotted first. Then the equation for the slope is obtained. Using this equation the phenolic contents can be calculated for all the samples.

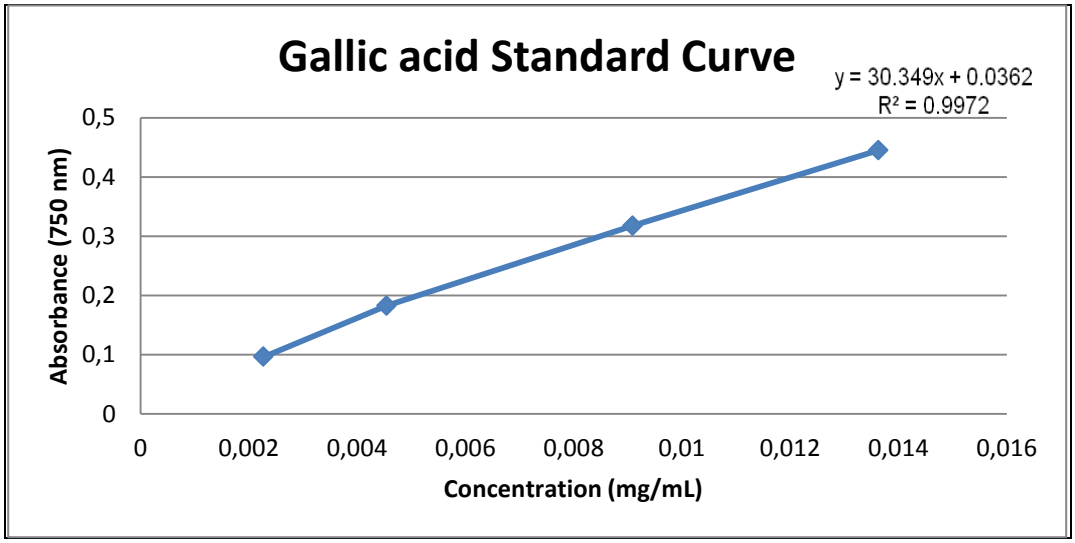


Figure 3.3: Gallic acid standard plot, concentration versus absorbance.

The standard curve of Gallic acid obtained by this experiment is shown in Figure 3.3 and the slope of this curve, also known as the linear regression of equation, was calculated to be $y = 30.349x + 0.0362$ with an $R^2 = 0.9972$.

Using this plot and the equation of the slope the phenol content of all the fractions were determined as Gallic Acid Equivalent (GAE) and expressed as µg/mg. This is shown in the Table 3.3.

Table 3.3: Total Phenolic Contents in *Aconitum cochleare* extract and fractions

Fraction/standard	Total Phenol GAE (µg/mg) ± SD
total extract	8.62 ± 0.001
n-hexane	2.53 ± 0.000
chloroform	6.80 ± 0.005
ethyl acetate	32.98 ± 0.01
aqueous	10.89 ± 0.001
quercetin	ND

ND: Not Determined
SD: Standard Deviation
GAE: Gallic Acid Equivalent

The results of this test correlates with the previous test i.e., the DPPH test. Table 3.3 shows that ethyl acetate fraction has the highest value of gallic acid equivalent; therefore 1 mg of dried ethyl acetate fraction is equivalent to almost 33 µg of gallic acid, followed by aqueous as in DPPH test, while n-hexane posses the least amount of antioxidants in it and its 1 mg is equivalent to only 2.53 µg of the standard used, that is, gallic acid. The GAE value of quercetin was not determined because our purpose was not to compare one standard with the other, rather to compare our fractions with the standard.

3.5. Determination of Total Flavonoid Content

The test for the determination of total flavonoid content of the plant extract involves the formation of colored complexes which are easily measured by their absorbance. Flavonoids present in a plant are responsible for the antioxidant activity of that plant; therefore, measuring their amount gives an insight about how strong a plant is, in terms of free radical scavenging activity.

In this test, the absorbance of the sample is compared to that of a standard. Rutin was used as the standard here, and its absorbance was measured and a graph was plotted, Figure 3.4, in which absorbance of the flavonoid complexes of rutin are shown against its different concentrations.

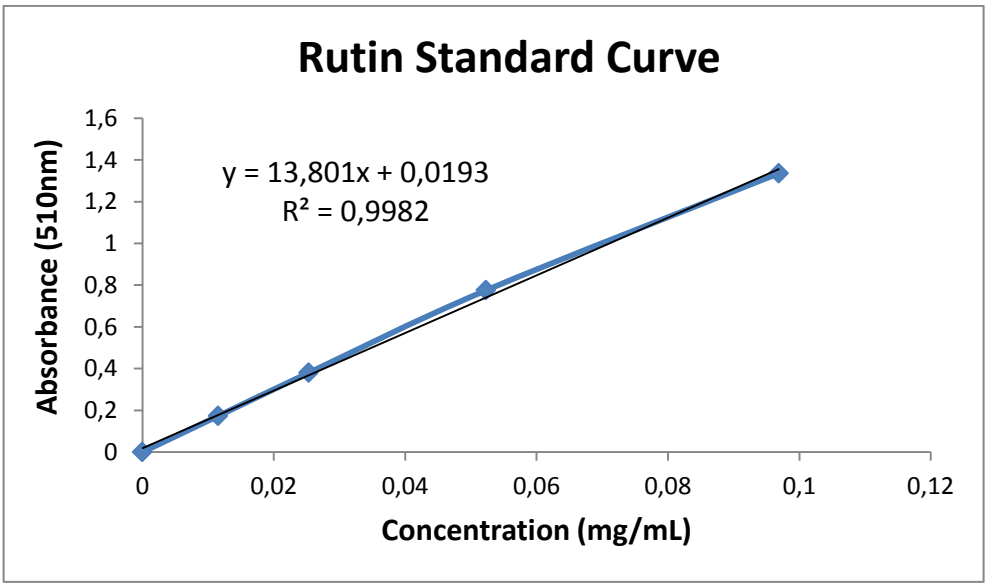


Figure 3.4: Rutin standard plot, concentration versus absorption. Three independent experiments in duplicates were performed.

The slope of the graph, linear regression equation, was calculated to be, y = 13.801x + 0.0193, while R^2 was measured as 0.9982. By using this linear equation of regression, rutin equivalent was calculated for all the sample fractions as well as the total extract. This is shown in Table 3.4.

Table 3.4: Total flavonoid content of samples, total extract and fractions.

Extracts	Total flavonoid content RE (μg/g) ± SD
Total extract	143.74 ± 22.68
n-hexane	ND
chloroform	107.60 ± 12.10
ethyl acetate	639.16 ± 28.52
aqueous	175.60 ± 20.67

SD: Standard Deviation

ND: Not Determined

RE: Rutin Equivalent

From the Table 3.4, it is concluded that ethyl acetate fraction has the highest amount of flavonoids in it. This corresponds that when taken in same concentration to start the experiment, ethyl acetate has the highest antioxidant activity, which is already proved in dpph test and total phenol content test and, therefore, the tests correlate with each other.

Ethyl acetate fraction has the highest rutin equivalent flavonoid content and the order of decreasing flavonoid content is ethyl acetate > total extract > aqueous > chloroform. The tests of total phenol content and dpph also correlate with this fact that the chloroform fraction has the least amount of antioxidant activity out of all the fractions.

As this plant, *A. cochleare*, has not been previously studied for the phenolic constituents, therefore, this test has also been performed for the first time on this plant.

3.6. Reverse Phase-High Pressure Liquid Chromatography

The total extract as well as the other fractions, except the n-hexane fraction, was analyzed by reverse phase-high pressure liquid chromatography (RP-HPLC). This was done in order to discover the number of phenolic compounds present in each fraction. The compounds present in any fraction, owing to their difference in nature of attraction towards the mobile and stationary phase, will show some retention and will elute at different times and this action is shown by peaks in the chromatogram.

First of all a number of standards were run separately to optimize their elution time on our mobile phase. A single standard was run three times and the mean of the elution time was calculated along with the standard deviation. The solution for each of the standard injection was prepared similarly with same concentrations i.e. 1 mg/mL of each standard separately in methanol.

Next two mixtures of these standards was prepared, one containing 11 standards and the other containing six standards, and run on the PR-HPLC to analyze the change in elution time of these standards when they are present in a mixture. This step was also repeated three times to optimize the retention time of each standard in the mixture. Figure 3.5 shows the injection of mixture of standards and their identification.

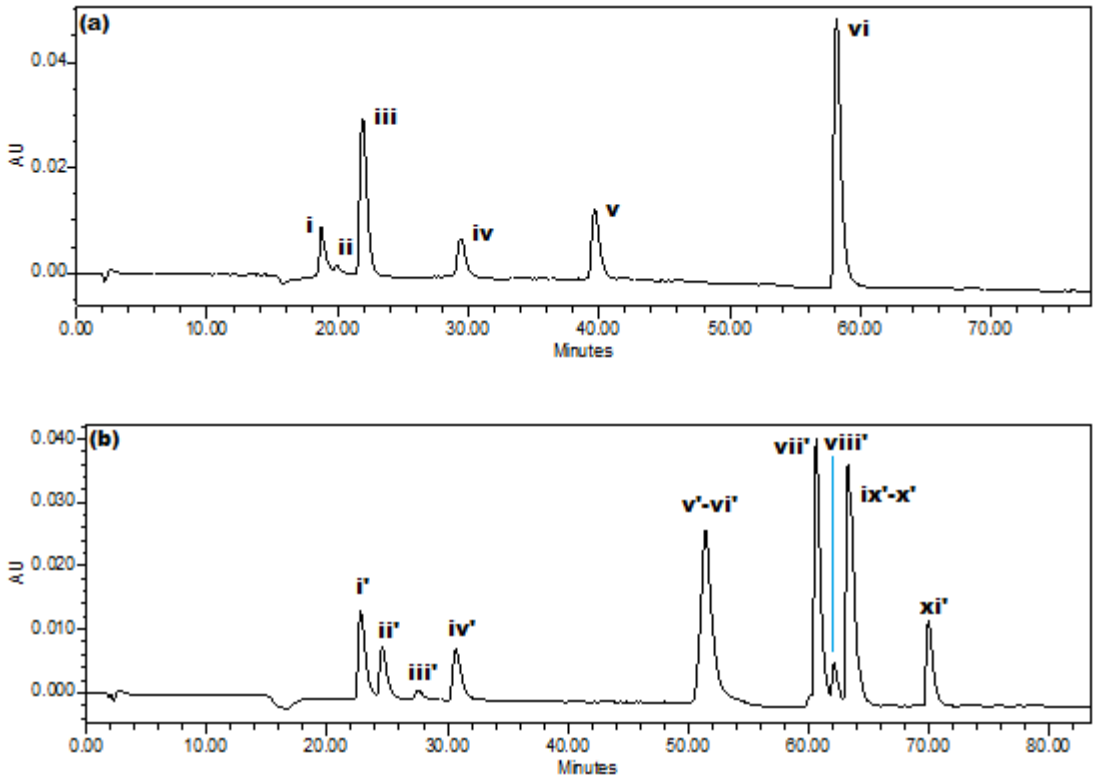


Figure 3.5: Chromatograms of RP-HPLC analysis, by using photo diode array detector, of 17 phenolic standard compounds in the form of two mixtures at 254 nm. Peaks are identified as follows, i) esculin, ii) catechin, iii) caffeic acid, iv) scopoletin, v) rutin, vi) quercetin; i') vanillic acid, ii') syringic acid, iii') epicatechin, iv') coumaric acid, v'-vi') resveratrol, myricetin, vii') luteolin, viii') naringenin, ix'-x') hesperitin, apigenin, xi') coumarin.

Table 3.5 gives a comprehensive detail of all the standards with their elution time. From the Figure 3.5 it is interpreted that in our mobile phase, a mixture of phenolic compounds elute in such a way that, the small phenols and the phenolic acids are eluted first, that is, in the first fifteen to thirty minutes, followed by the structurally bigger phenolic compounds like flavonoids. This is because, the mobile phase is more hydrophilic in nature in the beginning and slowly the trend shifts to hydrophobic in nature because of the gradient flow of mobile phase (mobile phase consists of 99 % of water in the first ten minutes, then the water content decreases to 85 % in the eleventh

minute and further decrease occurs gradually till the 50th minute where water consist of 65 % of the mobile phase and in this time all the phenolic acids and smaller phenolic compounds elute. As shown in Table 2.1, the hydrophilic nature of mobile phase then decreases more steeply to hydrophobic till the 74th minute, where the mobile phase has no water at all and is maximum hydrophobic in nature, bringing with it, the flavonoids, high molecular weight phenolic compounds). So the phenolic acids tend to get attracted to the hydrophilic mobile phase and elute first, while the flavonoids, which are more hydrophobic, interact with the stationary phase. These flavonoids and other high molecular weight phenolic compounds elute only when the mobile phase gets more hydrophobic than the stationary phase and thus brings all the other phenolic compounds out of the column.

Table 3.5: Phenolic compounds and their elution time

No.	Name	Molecular Formula	Elution time ± SD	Molecular weight
1	Esculin	$C_{15}H_{16}O_9$	18.535 ± 0.38	340.282
2	Catechin	$C_{15}H_{14}O_6$	20.122 ± 0.43	290.27
3	Caffeic Acid	$C_9H_8O_4$	22.239 ± 0.12	180.16
4	Vanillic Acid	$C_8H_8O_4$	22.690 ± 0.36	168.14
5	Syringic Acid	$C_9H_{10}O_5$	24.417 ± 0.56	198.17
6	Epicatechin	$C_{15}H_{14}O_6$	27.460 ± 0.32	290.27
7	Scopoletin	$C_{10}H_8O_4$	30.050 ± 0.23	192.16
8	Coumaric Acid	$C_9H_8O_3$	30.421 ± 0.11	164.16
9	Rutin	$C_{27}H_{30}O_{16}$	41.370 ± 0.43	610.52
10	Resveratrol	$C_{14}H_{12}O_3$	50.900 ± 0.22	228.24
11	Myrcetin	$C_{15}H_{10}O_8$	51.119 ± 0.06	318.34
12	Quercetin	$C_{15}H_{10}O_7$	59.351 ± 0.53	302.236
13	Luteolin	$C_{15}H_{10}O_6$	60.532 ± 0.39	286.24
14	Naringenin	$C_{15}H_{12}O_5$	61.975 ± 0.36	272.257
15	Hesperitin	$C_{16}H_{14}O_6$	63.172 ± 0.08	302.28
16	Apigenin	$C_{15}H_{10}O_5$	63.492 ± 0.45	270.24
17	Coumarin	$C_9H_6O_2$	69.888 ± 0.38	146.14

Next all the fractions in different concentrations were run on RP-HPLC and the obtained chromatogram was compared with the standards chromatogram Figure 3.5 to analyze which compound may be present in our fraction. The first to be analyzed chromatographically was ethyl acetate fraction. A solution of 5 mg/mL of ethyl acetate fraction in methanol was prepared and 15 µL of it was filtered and run on RP-HPLC. The resulting chromatogram was analyzed at different wavelengths of 254, 280, 320 and 360 nm, Figure 3.6.

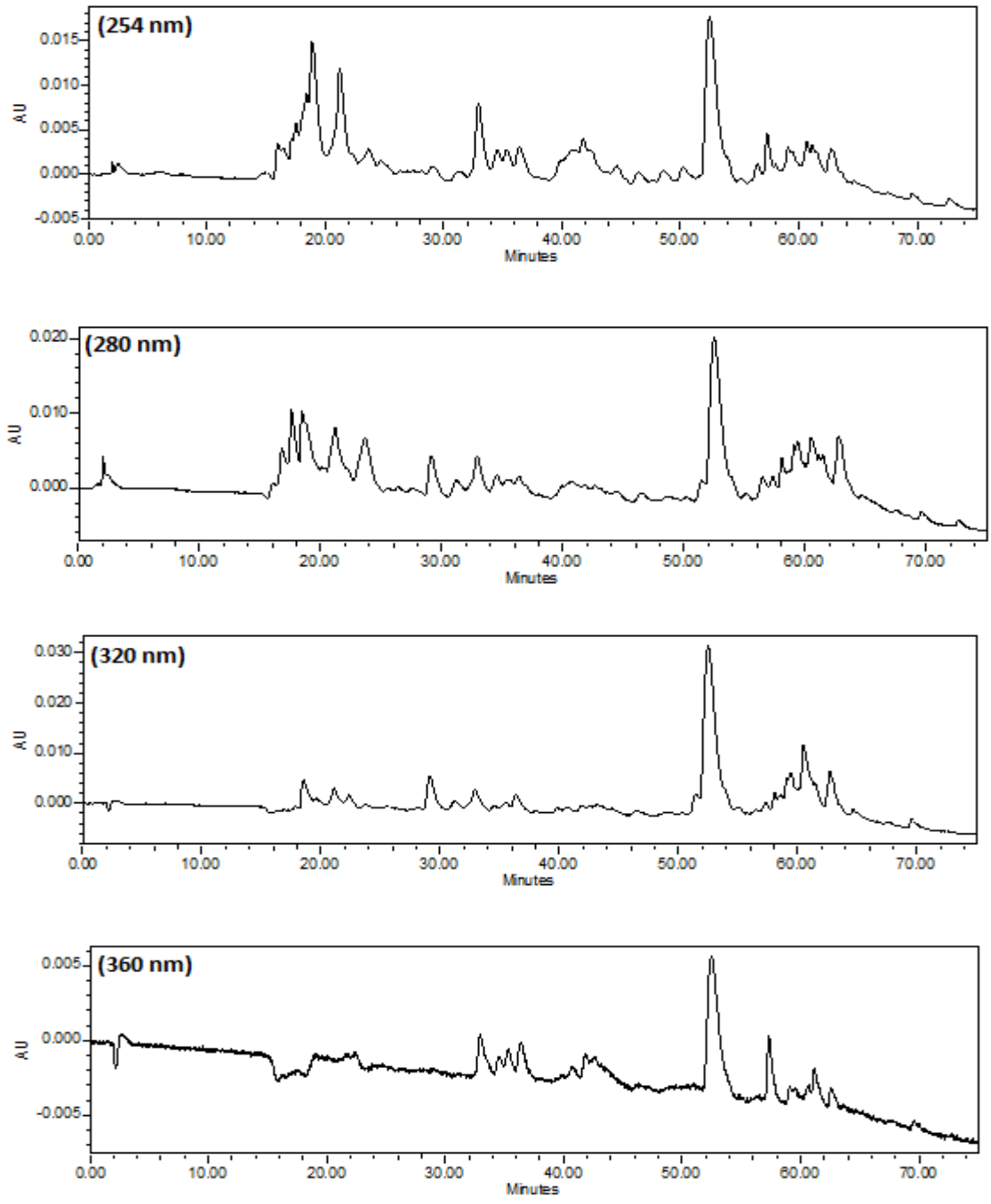


Figure 3.6: Ethyl acetate 5 mg/mL chromatograms, with RP-HPLC, at different wavelengths (254, 280, 320, 360nm).

Generally flavonoids exhibit two major absorption bands in the UV-Vis spectrum. One is the longer wavelength absorbance in the region of 320−385 nm and is called Band I, while the other is the shorter wavelength absorbance, in the region of 250−285 and is referred to as Band II. The Band I represent the absorption due to B-ring of the flavonoids, i.e., its attachment point and substituents attached to it, while the Band II represents the A-ring and substituents attached to it, Figure 3.7. The nature of C-ring also influences the absorption wavelength. Thus, a given chromatogram should be visualized at all wavelengths between both the band range and that wavelength is selected for further studies, at which the peaks of the phenolic constituents are meaningful with reference to the absorption spectrum of Band I and II (Özdoğan, 2013).

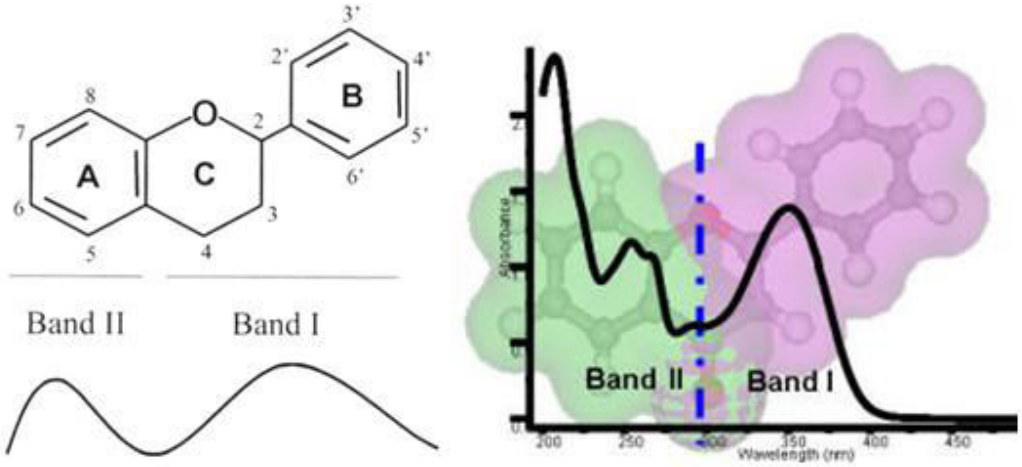


Figure 3.7: Basic structure of flavonoid with absorption band (Özdoğan, 2013)

The figure 3.8 shows the chromatogram of ethyl acetate fraction at 254 nm. This wavelength was selected because the peaks were better resolved at this wavelength. This chromatogram, Figure 3.8, shows some meaningful (signal to noise ratio higher than 2:1) number of peaks, 1, 2, 3, 4, and 5. The peak 4 represents the compound which is in highest concentration and forms the major part of the phenols of this fraction and is responsible for most of the action in DPPH scavenging, complex formation in total phenol test, with Molybdenum (VI) and reaction with aluminium chloride in total flavonoid test. The peaks 1, (elution time 18.442 ± 0.45 minutes) and peak 2, (elution

time 21.191 ± 0.39 minutes) represent compound/compounds in lesser concentration than compound eluting at peak 4, (elution time 52.473 ± 0.34 minutes). The peak 3, (elution time 32.922 ± 0.21 minutes) and 5, (elution time 57.560 ± 0.33 minutes) are of less importance as the compounds they represent account for only a very small fraction of the total phenols present in ethyl acetate fraction of the extract.

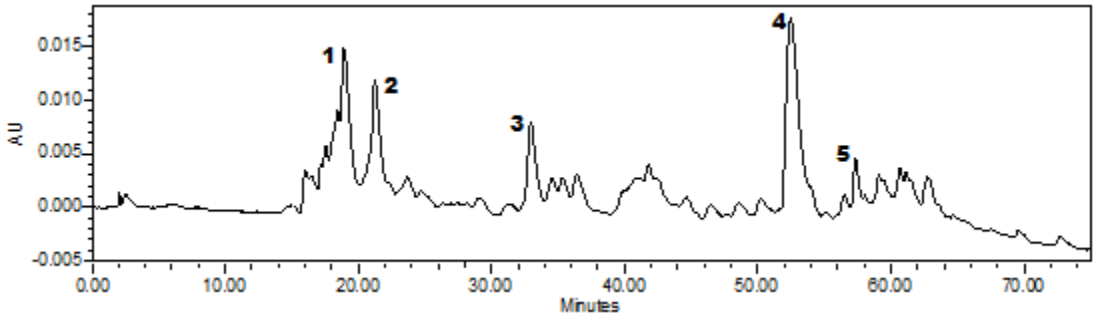


Figure 3.8: Chromatogram of ethyl acetate fraction when run on RP-HPLC and analyzed at 254nm.

This chromatogram, Figure 3.8 was compared with the chromatograms of mixture of phenolic standards, Figure 3.5 and it was assumed that due to elution at similar timings, the following phenolic compounds may be present in the ethyl acetate fraction, i.e. esculin, catechin, caffeic acid, myrcetin, quercetin. Therefore, to confirm their presence, co-injections were done with each standard separately.

A co-injection consists of ethyl acetate fraction in same concentration along with a specific amount of the standard added. This injection when run on RP-HPLC, the standard may increase any one of the peaks, indicating that it may be present in the given fraction or elute separately in the chromatogram, indicating that it is completely absent in the given fraction. The amount of standard added is dependent on its own absorption and varies from compound to compound.

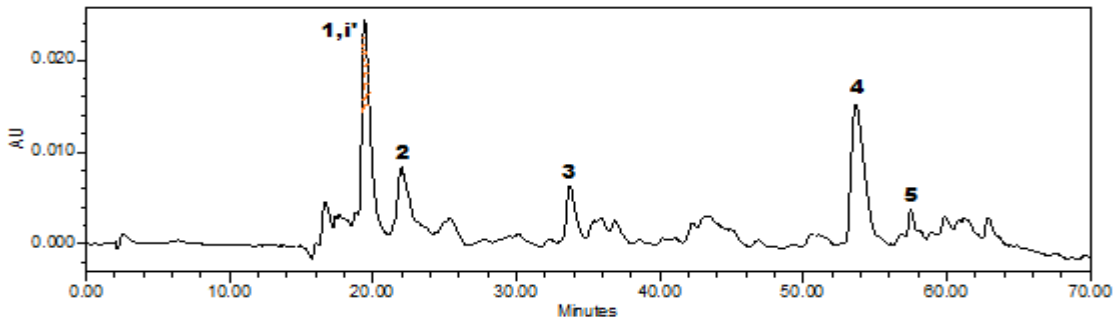


Figure 3.9: Co-injection of esculin standard with ethyl acetate fraction.

Figure 3.9 shows the chromatogram of co-injection of esculin with ethyl acetate fraction. As is evident from the shaded part in Figure 3.9, the first peak is increased in length while all the other peaks remain same, therefore, it is concluded that esculin may be present in this fraction. This has to be further confirmed by the High Resolution-Mass Spectroscopy, as there is a possibility that this can be some other compound present in the fraction which has the same physical characteristics and thus attraction towards mobile phase like esculin and thus elutes at the same time.

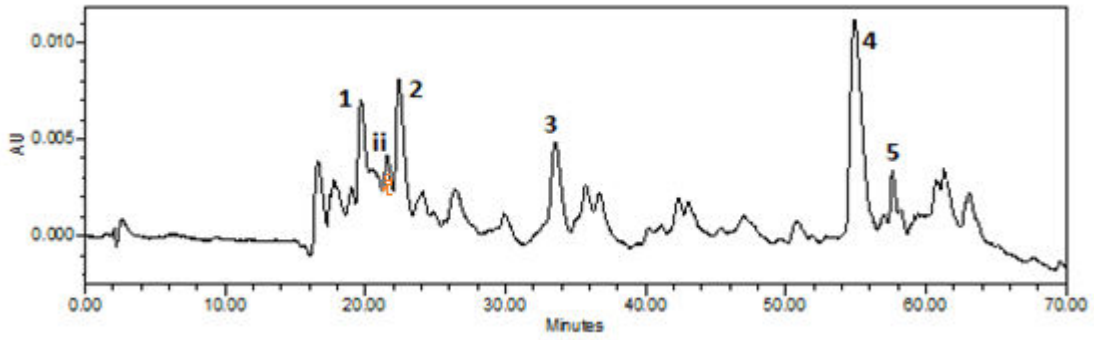


Figure 3.10: Co-injection of catechin standard with ethyl acetate fraction.

Next co-injection to be done was ethyl acetate with catechin standard. The co-injection was prepared by adding a specific calculated amount of catechin to the same 5 mg/mL of ethyl acetate solution in methanol and run on RP-HPLC. The resultant chromatogram

showed that it is not present in the ethyl acetate fraction and therefore elutes out as a separate peak in the chromatogram, Figure 3.10. All the rest of the peaks of the original ethyl acetate fraction are present as before in Figure 3.8.

The third standard which was verified by the co-injection was caffeic acid. A small calculated amount of caffeic acid was added to the ethyl acetate fraction separately and vortex mixed. This mixture was then run on RP-HPLC, resulting in a chromatogram showed in Figure 3.11.

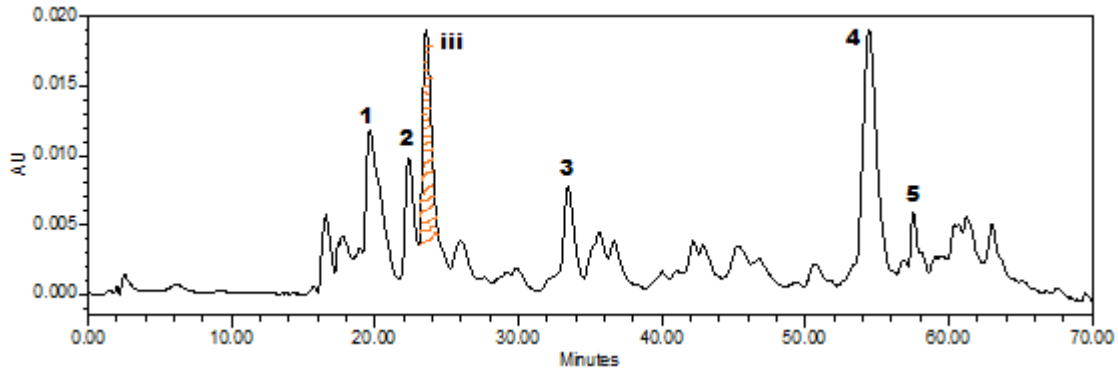


Figure 3.11: Co-injection chromatogram of caffeic acid with ethyl acetate.

This too is not present in the ethyl acetate fraction and therefore elutes as a separate peak, shaded peak, after the second meaningful peak in Figure 3.11. As this caffeic acid elutes after the second peak, therefore, all the standards tested above, Figure 3.5, which elute a little after caffeic acid in the standard mixture are sure to not present in this ethyl acetate fraction. Therefore, our next focus was on the peak no. 3 in the ethyl acetate chromatogram.

The third meaningful compound in ethyl acetate chromatogram elutes at 32.922 minutes Figure 3.8, and when comparing this with the standard chromatogram, it is observed that there is no phenolic compound in the standard graph which elutes around 32-33 minutes. Therefore, no co-injection could be done to check this peak.

The compound/compounds present in highest concentration in ethyl acetate fraction elute as peak 4 around 52.473 minutes, Figure 3.8. Myrcetin is the phenolic standard which elutes nearest to this peak Figure 3.5, at 51.12 minutes. Therefore a co-injection of myrcetin was prepared with the original ethyl acetate fraction 5 mg/mL and vortex mixed. A total of 15 µL of this was filtered and run on RP-HPLC, Figure 3.12 (a) and (b).

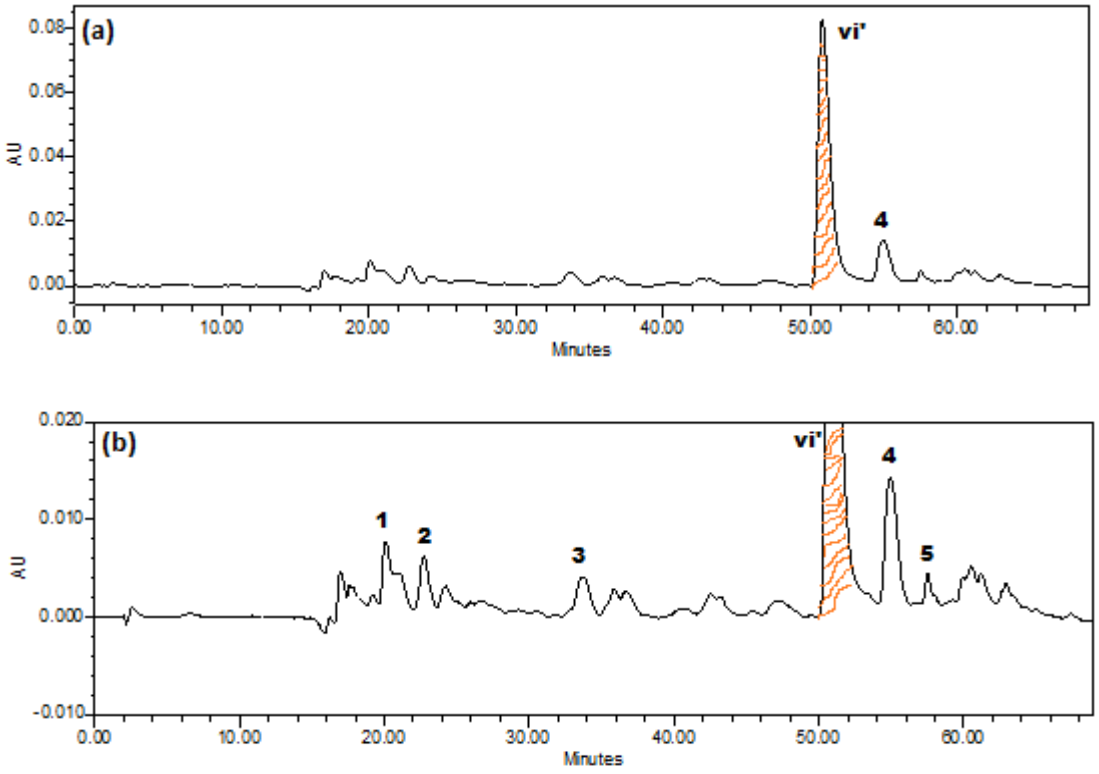


Figure 3.12 (a) and (b): Co-injection of myrcetin with ethyl acetate fraction.

Figure 3.12 (a) shows that myrcetin, shown as shaded peak, elutes as peak no vi', a little before our meaningful peak of ethyl acetate fraction, peak 4, and does not matches with the peak 4 of ethyl acetate fraction. This has two interferences, one it is not present in the sample fraction, secondly, all compounds eluting before myrcetin in the standard mixture may not assumed to be present in the sample fraction. In Figure 3.12 (a) the

other peaks of the ethyl acetate fraction seem very small. This is because myrcetin, even though it was added in very small quantities, showed high absorption and thus the other peaks became much smaller. But this picture when zoomed and viewed, Figure 3.12 (b), all the five important and meaningful peaks of the original ethyl acetate sample can be identified.

The next phenolic compound eluting in the standard mixture was analyzed. It is quercetin in Figure 3.5 (a), marked as vi. So quercetin was added in the ethyl acetate fraction, 5 mg/mL separately, mixed well, filtered and 15 µL of it was injected for RP-HPLC.

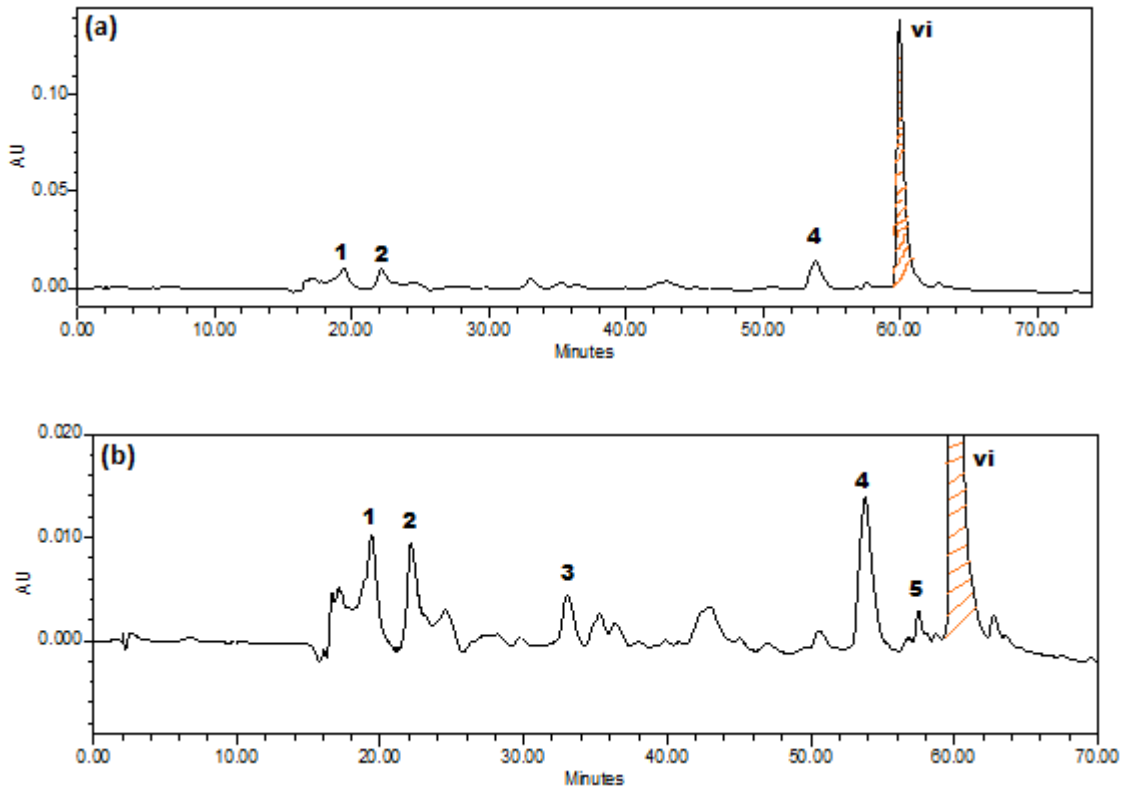


Figure 3.13 (a) and (b) co-injection of quercetin with ethyl acetate fraction.

The resultant chromatogram is shown in Figure 3.13 (a) and (b). In figure 3.13 (a), it is seen that the peak of quercetin, shaded peak, does not matches with the peak 4 or peak 5 of original ethyl acetate sample, Figure 3.8. The peaks of original ethyl acetate sample can be made out vividly in Figure 3.13 (a); while Figure 3.13 (b) is its zoomed version and all the five peaks can be identified clearly. Therefore, quercetin is also absent in the ethyl acetate fraction. Also this helps us to conclude that, all standards which elute after quercetin in the given standard chromatogram, Figure 3.5 (a) and (b), for example luteolin, hesperitin etc cannot be present in the ethyl acetate sample fraction. Therefore, from the given set of seventeen standards, only esculin is one which may be present in the ethyl acetate fraction of *A. cochleare* flowering parts.

The conclusion which can be made from the view of ethyl acetate original chromatogram, Figure 3.8, keeping in mind the nature of mobile phase is that, the peaks 1 and 2 are some low molecular weight phenolic compounds while the third peak may be of either a coumarin or flavonoid glycoside, The largest peak, i.e. peak 4 and also peak 5 is of some flavonoid(s), high molecular weight phenolic compounds.

Second sample to be analyzed on RP-HPLC was the aqueous fraction. It is an important fraction from the view of DPPH, determination of total phenol content and determination of total flavonoid content tests result in that it may contain high concentration of Phenolic compound/compounds.

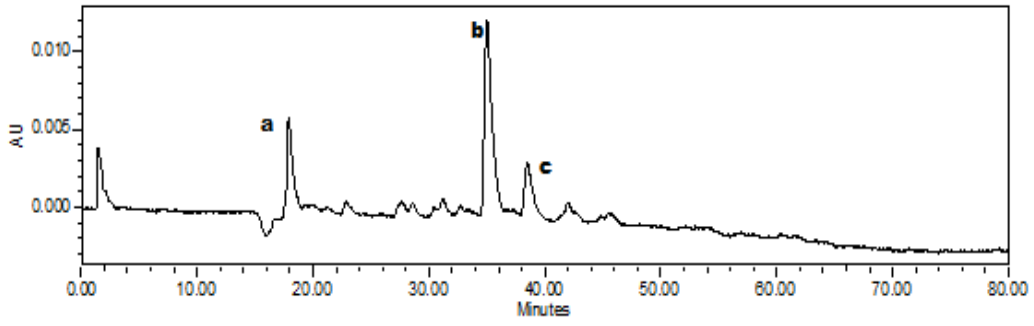


Figure 3.14: Aqueous fraction chromatogram resulting from RP-HPLC at 280nm.

The chromatogram of 5 mg/mL of aqueous fraction in methanol is shown in Figure 3.14. The three meaningful peaks obtained are marked as a, b and c. When this chromatogram was compared to the chromatogram of the mixture of phenolic standard compounds, there was no standard found to be eluting at the same time as these peaks in Figure 3.14. Therefore no co-injection could be done here. By looking at Figure 3.14 and keeping in mind the physical properties of the mobile phase during the whole injection time, this can be concluded that, all three peaks present are of compound or compounds which are may be phenolic acids or small phenols. This conclusion has been reached due to two reasons, one, the aqueous nature of the fraction will tend to dissolve mostly the acidic phenols or other small phenols. Secondly, the compounds have eluted in the first forty minutes of the injection, and from standard mixture, we know that, compound eluting in this time are phenolic acids or small phenols in nature. Next to be analyzed was the chloroform fraction.

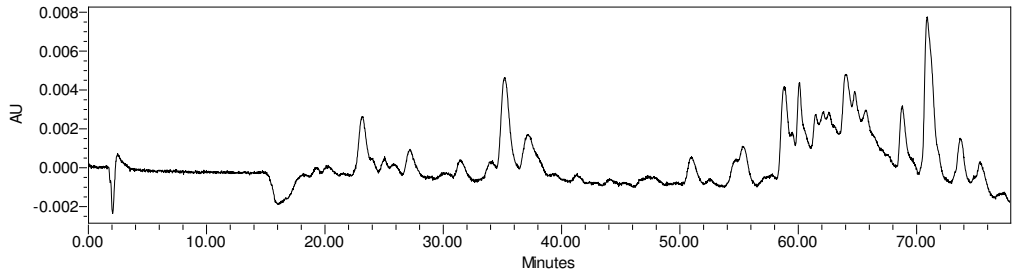


Figure 3.15: Chloroform fraction injection chromatogram observed at 280nm.

Figure 3.15 shows the chromatogram of chloroform fraction and it can be seen that there is no meaningful peak present in this fraction. Reason being that, the absorption is too low, suggesting that compounds are present in very small concentrations. Because this fraction has not shown impressive results in DPPH, determination of total phenol content and determination of total flavonoid content tests, therefore, it was not expected

that this fraction will show any meaningful peak. Thus, further analysis of this fraction was not performed.

The total extract was also run on RP-HPLC to verify our peaks of ethyl acetate and aqueous fractions.

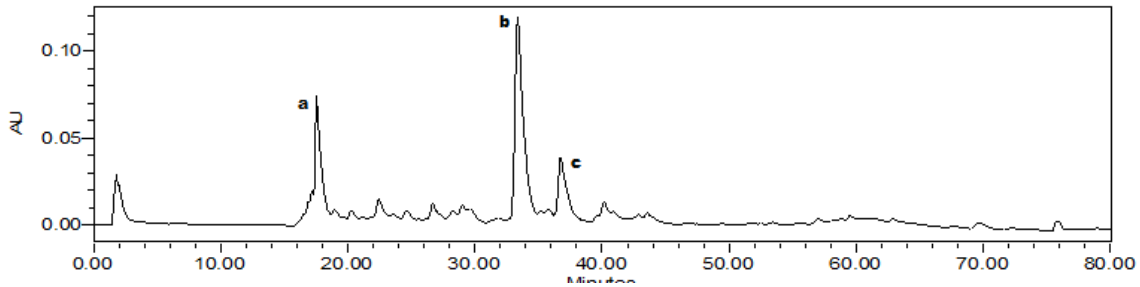


Figure 3.16: Chromatogram of total extract of *A. cochleare* flowering parts.

When the chromatogram of total extract Figure 3.16, was compared with that of ethyl acetate and aqueous chromatograms, it was observed that aqueous fraction chromatogram, Figure 3.14, is more or less the same picture of that of total extract. This is because, the yield, as seen in Table 3.1, shows that most of the total extract went to the aqueous fraction while ethyl acetate fraction received less than 2% of the total extract. Therefore, it was assumed that the peaks of ethyl acetate are present in the total extract chromatogram but the concentration being too low, corresponding to small peaks.

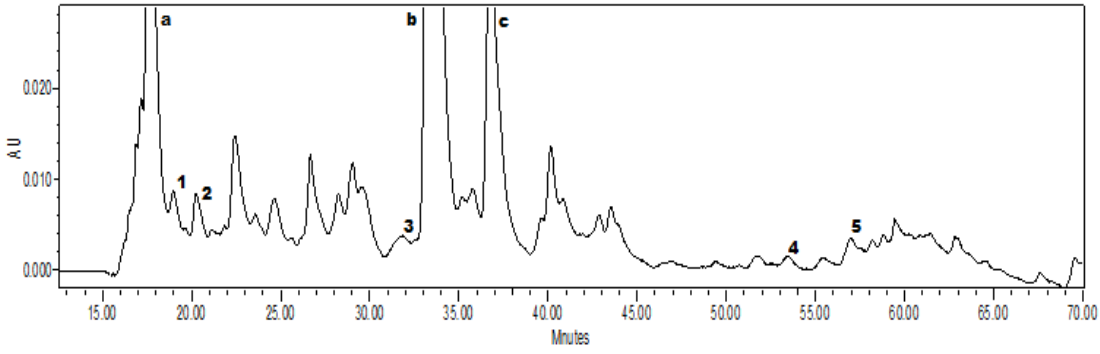


Figure 3.17: Chromatogram of total extract of *A. cochleare* flowering parts, enlarged.

Thus when this chromatogram, Figure 3.16, was enlarged and studied, the peaks of compounds present in ethyl acetate fraction could be easily identified, Figure 3.17, and marked.

Thus it can be concluded that the methanolic extract of *A. cochleare* flowering parts has many phenolic compounds, including the phenolic acids and flavonoids, one of them may be esculin.

To confirm the HPLC results of ethyl acetate fraction, HR-MS experiment was performed.

3.6. High Resolution-Mass Spectroscopy

Keeping in mind the good results of ethyl acetate fraction in the previous tests of DPPH, determination of total phenol, determination of total flavonoids and the number of peaks in chromatogram of RP-HPLC, this fraction was sent for (HR-MS), as HR-MS is a modern technique which identifies the molecular weights of compounds accurately and rapidly, along with its concentration. It reveals the retention time and virtual exact mass of the compound of interest. By this technique, as is shown in shown in Figure 3.18, almost 5 compound of interest were found. They are called compound of interest because they are present in such significant concentration, as to show some contribution in the aforementioned tests. The direct injection resulted in the following information (Figure 3.18) where the height of the line tells us the concentration of the compound and above it is the molecular weight of that compound.

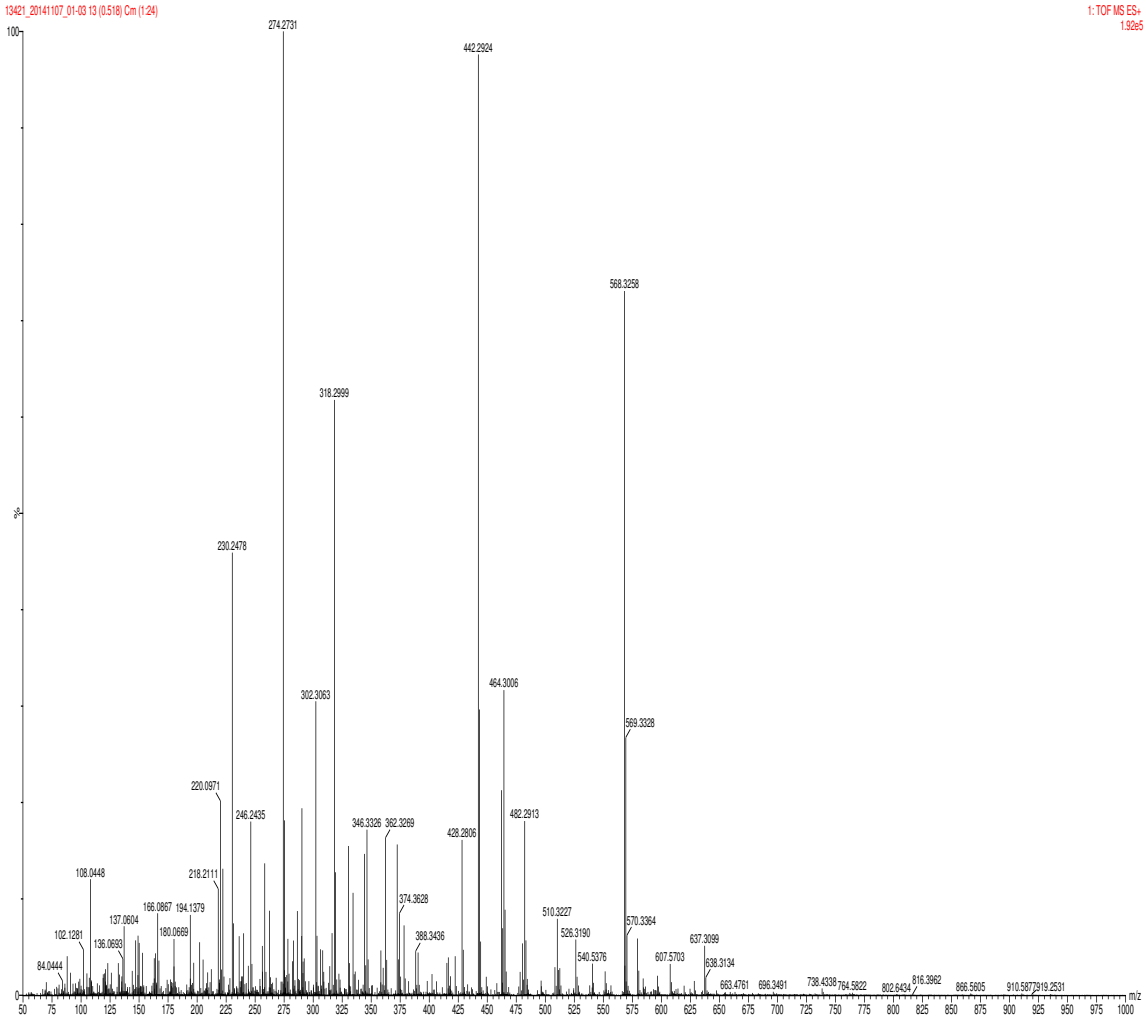


Figure 3.18: Direct injection of ethyl acetate fraction as viewed by High Resolution-Mass Spectroscopy.

The molecular weight of compounds which were present in highest concentrations was noted and by literature review some flavonoids were found which had the similar molecular weight. This is shown in Table 3.6.

Table 3.6: Interpretation of HR-MS results

Molecular Weight	**Possible compounds**
302.3063	Morin (302.2357) Flavonol
	Hesperetin (302.28) Flavanone
	Homoeriodictyol (302.27876) Flavanone
	Sterubin (302.28) Flavanone
274.2731	Afzelechin (274.26) Flavanol
	Fisetinidol (274.26) Flavanol
442.2924	Epicatechin gallate (442.37) Flavanol
318.2999	Gossypetin (318.23) Flavonol
	Myricetin (318.24) Flavonol
464.3006	Hyperoside (464.38) Flavonol glycoside
	Isoquercetin (464.38) Flavonol glycoside
	Myricitrin (464.37) Flavonol glycoside
	Spiraeoside (464.37) Flavonol glycoside

Although these standards were not present and, therefore, the co-injection with them could not be performed, still some conclusions can be made from the above data, keeping in mind the nature of our stationary and mobile phases. Firstly, the peak 3 in the Figure 3.8 is possible to be a flavonol glycoside (mol. weight = 464.3006), because from the standard injection, Figure 3.5 (a) and (b), glycosides of flavonoids elute between 30-45 minutes of the injection. Secondly, form Figure 3.18, compound with molecular weight of 274.2731 is present in highest concentration; therefore, it corresponds to peak 4 of Figure 3.8, as it is the largest peak. Also there is a possibility that peak 4 may be a complex peak, and if it is true, then the compound present in second highest concentration, that is, 442.2924, will also correspond to peak 4. Thus it is possible that peak 4 may have epicatechin gallate and either afzelechin or fisetinidol eluting at almost the same time, and because both are flavonol, they may show similar interaction with the mobile phase. If the above mentioned assumptions are true then the peak 1 and 2 in

Figure 3.8 corresponds to compounds with molecular weight 302.3063 and 318.2999 which may be flavonols as they are eluting in the beginning of the injection and thus have a good interaction with polar mobile phase.

CHAPTER 4

CONCLUSION

Most of the species of *Aconitum* genera have been used since long for different purposes, the most notable, for its poisonous effects. Also some of them were used for medicinal effects in curing. When under research, the main investigation was focused on alkaloids present in them. However, after the discovery of anti-oxidant effects in some species of *Aconitum,* investigation was done for the phenolic constituents present in them. Many phenols particularly the flavonoids were found in the flowers, roots and leaves of these plants.

A. cochleare has never previously been investigated for any phenolic constituent test or antioxidant properties due to phenols present in it. Therefore, in this study this was done.

The crude Methanolic extract of the flowering parts of the *A. cochleare* showed effective antioxidant properties, and therefore, it was fractionated. These fractions were then tested for their effectiveness in antioxidant activity. The DPPH and determination of total phenol test showed the ethyl acetate fraction to be the most effective, thus having the most antioxidant compounds in it, while the determination of total flavonoid test showed that most of the antioxidants in the ethyl acetate fraction are rutin like flavonoid. Rutin was used as standard in the total flavonoid test.

Further investigation by RP-HPLC showed that ethyl acetate fraction has a number of phenolic compounds, evident by peaks in the chromatogram. This chromatogram was compared with the chromatogram of phenolic standard compounds and co-injections of simultaneously eluting standards were done. Esculin compound showed presence

according to the co-injection in RP-HPLC. HR-MS determined the molecular weight of compounds present in highest concentrations in ethyl acetate fraction. Compounds with similar molecular weight were obtained from the literature and some conclusions were made by keeping in view the nature of mobile phase.

The presence of other standards assumed to be present in ethyl acetate fraction, by HR-MS, should also be confirmed by RP-HPLC. The antioxidants found in this plant open the door for further investigation of this plant for anticancer effects. Also this study has put an unused plant, *A. cochleare*, in the list of antioxidant compounds containing plants.

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