ISOLATION AND CHARACTERISATION OF ANTIOXIDANT COMPOUNDS IN YELLOW ROSE ROOT EXTRACTS

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ABSTRACT

ISOLATION AND CHARACTERISATION OF ANTIOXIDANT COMPOUNDS IN YELLOW ROSE ROOT EXTRACTS

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A phytochemical investigation on methanolic extract of roots of **yellow rose** led to the isolation of a catechin gallate. The crude extract first underwent fractionation using petroleum ether, chloroform, ethylacetate, butanol with water as solvents in their respective order. The emerging solvent fractions were subjected to further separation using lipophilic sephadex (LH-20), and silica gel column chromatography to isolate pure compounds. Analytical thin layer chromatography (TLC) was used to confirm the presence of a catechin in butanol fraction. Purified catechin compound was subjected to 2,2-diphenyl-1-picrylhydrazyl (DPPH) experiment to determine its Radical Scavenging Capacity, which was found quite promising. Chemical structure of purified compound was established by using Nuclear Magnetic Resonance (NMR) and Mass Spectrometry (MS) experiments.

Key words: Yellow rose, catechin gallate, DPPH, fractionation.

ÖZ

SARI GÜL KÖK ÖZÜTLERİNDE ANTİOKSİDAN MADDELERİN İZOLASYON VE KARAKTERİZASYONU

Yusuf Kyejjusa
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Sarı gül köklerinin methanol özütünde yapılan fitokimyasal araştırma sonucunda, katekin gallet izole edilmiştir. Ham özüt önce petrol eteri kullanılarak, daha sonra sırasıyla, kloroform, etilasetat ve butanol ile su fraksiyonlanmıştır. Elde edilen çözücü fraksiyonları lipofilik sefadeks (LH-20) kullanarak daha ileri ayrıştırmaya tabii tutuldu ve silika jel kolon kromatografisi saf maddeleri izole etmek için kullanıldı. Butanol fraksiyonunda katekin varlığı ince tabaka kromatografisi (İTK) kullanılarak gösterilmiştir. Saflaştırılan Katekin maddesi radikal Süpürme Kapasitesinin tanımlanması için 2,2 difenil-1-pikrilhidrazil (DPPH) deneyine tabii tutulmuş ve oldukça umut verici bulunmuştur. Saflaştırılmış maddenin kimyasal yapısı Nüklear Magnetic Rezonans (NMR) ve Kütle spectrometresi (MS) deneyleri ile tanımlanmıştır.

Anahtar kelimeler: Sarı gül, DPPH, fraksinasyon.

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I dedicate this piece of work to my parents; Yusuf Kitaka , Noor Najjuma & Mastulah Nakku. May their soul rest in eternal peace.

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INTRODUCTION

1.1 Plants and medicine

Plants form the main ingredients of medicines in traditional systems of healing and have been the source of inspiration for several major pharmaceutical drugs. Roughly 50,000 species of higher plants (about 1 in 6 of all species) have been used medicinally. This represents by far the biggest use of the natural world in terms of number of species[1].

Most species are used only in folk medicine, traditional systems of formal medicine using relatively few (e.g. 500-600 commonly in Traditionally Chinese Medicine) . Around 100 plant species have contributed significantly to modern drugs. The use of medicinal plants is increasing worldwide, related to the persistence and sometimes expansion of traditional medicine and a growing interest in herbal treatments[1].

The medicinal uses of plants grade into their uses for other purposes, as for food, cleaning, personal care and perfumery. Plants are used in medicine to maintain and augment health - physically, mentally and spiritually - as well as to treat specific conditions and ailments[1].

1.2 Yellow Rose

The Rosaceae family includes about 100 genera that grow almost widespread globally[2]. One of these genera is *Rosa* that includes about 150 species[3]. Among them is the genus *Rosa hemisphaerica* J. Herrm (R. hemisphaerica), known commonly a*s yellow rose*. It is a native to Asia, Minor and Southwest Asia [4-8]. *R. hemisphaerica was* introduced from Persia into Europe by a French scientist in 1807[9]. *R. hemisphaerica* is of VRS238 genotype. The fruit weight is 2.26 ± 0.10 g with dimensions of 15.45 ± 0.61 mm in length and 19.26 ± 0.26 mm in width. The percentage fresh ratio is 95.35 ± 0.35. The percentage of soluble content of the fruits is 23.85 ± 1.7, with ascorbic content of

604 ± 18 mg/100mL[10]

1.3 Classification

In taxonomy, *R. hemisphaerica* is classified as follows[11]

Kingdom	:	Plantae
Subkingdom	:	Tracheobionta
Division	:	Magnoliophyta Cronquist, Takht. & Zimmerm. ex Reveal
Class	:	Magnoliopsida Brongn.
Subclass	:	Rosidae Takht.
Order	:	Rosales Perleb
Family	:	Rosaceae Juss.
Genus	:	Rosa Linnaeus
Species	:	*Rosa hemisphaerica* J. Herrm.

1.4 Medicinal uses of *Rosa hemisphaerica*

Many of the *rosa species* have been used in traditional medicine to treat anxiety[4-7]. The essential oil of *Rosa moschata* has antimicrobial properties[12, 13]. The essential oil of *Rosa damascena* Mill. is one of the most valuable and important base material in the flavour and fragrance industry, and several therapeutic effects, including antispasmodic, relaxant, and stomachic, have been described for its flowers[9].

In Iran, *R. hemisphaerica* grows in Azarbayejan, Kordestan, Mazandaran, Semnan, Lorestan, and Isfahan provinces[14]. The flowers of this plant have an unpleasant smell.

A literature survey revealed that the essential oil of the aerial part of this plant in central Iran has not been chemically studied to date[2].

1.5 Phenolic compounds in plants

P**henols**, sometimes called **phenolics**, are defined as a class of chemical compounds consisting of a hydroxyl (¯**OH)** group bonded directly to an **aromatic hydrocarbon group**. The simplest of the class is phenol (C_6H_5OH).

Phenolic compounds are considered as secondary metabolites that are synthesized by plants during normal development and in response to stress conditions such as infection, wounding, and UV radiation, among others[15-18]. These compounds occur ubiquitously in plants and are a very diversified group of phytochemicals derived from phenylalanine and tyrosine[19, 20]. Plant phenolics include simple phenols, phenolic acids, which include benzoic and cinnamic acid derivatives, coumarins, flavonoids, stilbenes, hydrolyzable and condensed tannins, lignans, and lignins. In plants, phenolics may act as phytoalexins, antifeedants and attractants for pollinators.

Phenolics may also act as contributors to the plant pigmentation, antioxidants, and protective agents against UV light, among others.

In food, phenolics may be responsible for its bitterness, astringency, colour, flavour, smell, and oxidative stability of the respective products. In addition, health-protecting capacity of some and anti-nutritional properties of other plant phenolics

are of great importance to producers, processors and consumers[21]. **Figure 1.1** demonstrates the types of phenolic compounds found in food.

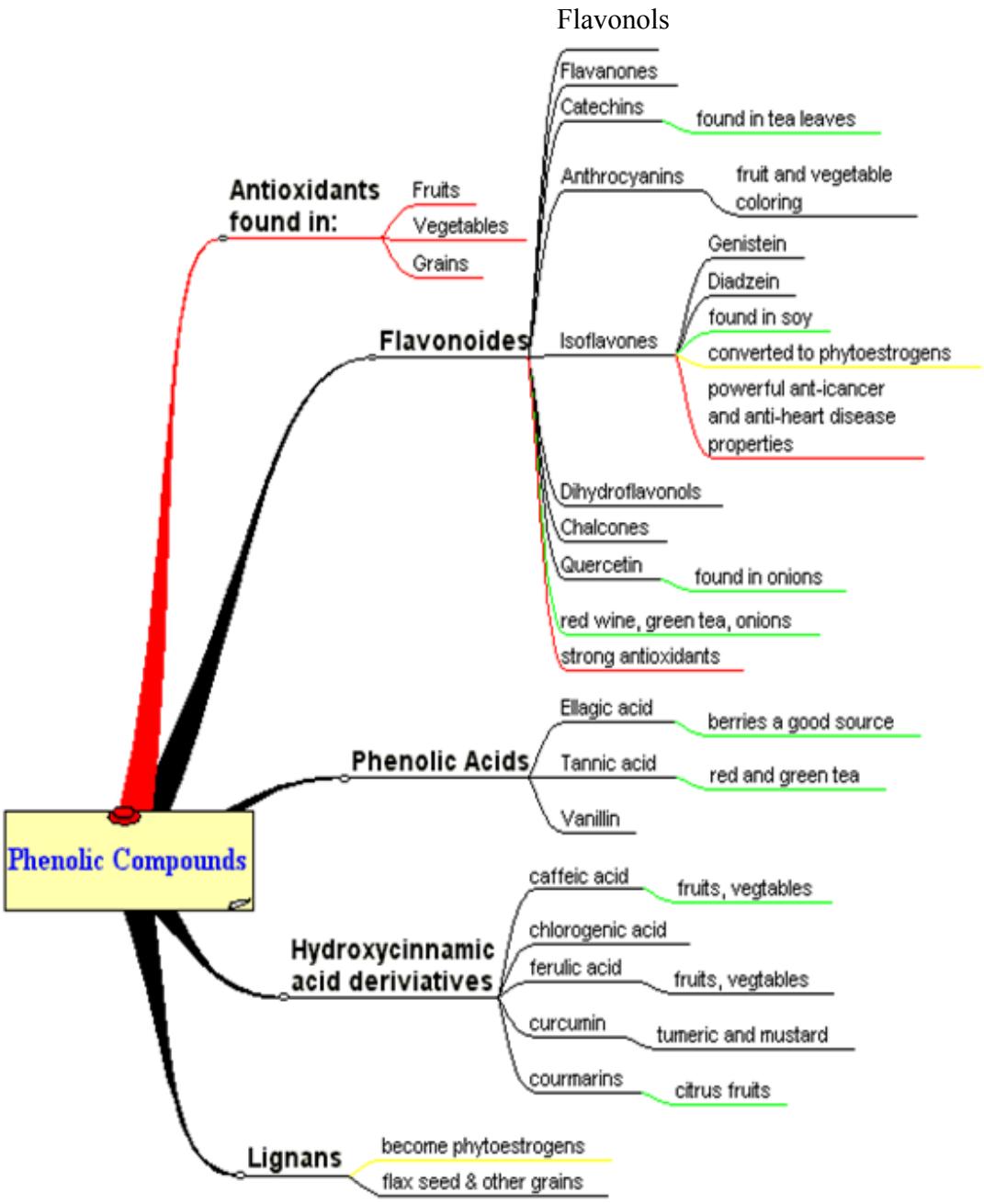


Figure 1.1 Phenolic compounds in food[22].

1.5.1 Flavonoids

Flavanoids are 3-ring phenolic compounds consisting of a double ring attached by a single bond to a third ring[23]. In leaves they block far-ultraviolet (UV) light (which is highly destructive to nucleic acids and proteins), while selectively admitting light of blue and red wavelengths which is crucial for photosynthesis[23]. Among flavonoids are water soluble pigments, such as anthocynanins (**Figure 1.2**), that are found in cell vacuoles. Water soluble flavonoids, especially anthocyanins, are responsible for the colours of a number of flowers ranging from blue to red, depending on the pH of the watery sap in the vacuoles.

Duck-weeds, of family Lemnaceae family, contain many kinds of flavonoids, including red anthocyanins in some species. By using 2D paper chromatography, duck-weed species' components have been separated and identified by their unique flavonoid content[23].

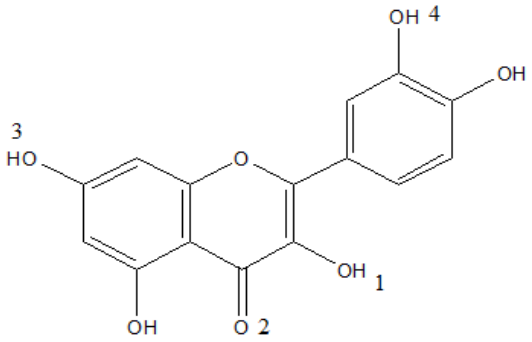
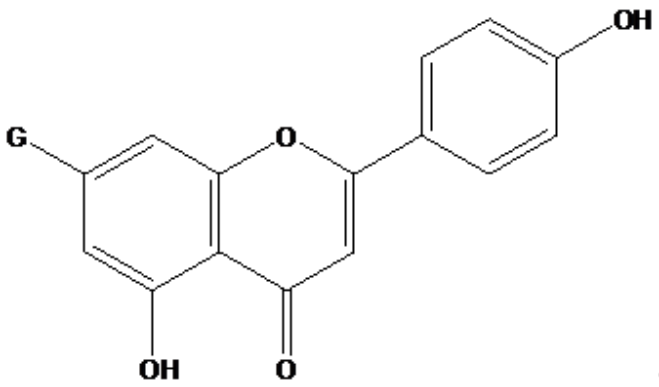


Figure 1.2 Structure of a flavonoid[23]

1. Remove OH at 1: Flavone
2. Replace OH at 1 with 3rd ring: Isoflavone
3. Replace O at 2 with an H: Anthocyanin

4. Replace OH at 3 with glucose, remove OH at 4, remove OH at 1: Glocoside, like the one in **Figure 1.3**. Glucoside applies only if the exact sugar is not specified. Without sugar, the molecule is called aglycone.



G = Glucose

Figure 1.3 Structure of apigenin-7-monoglucoside[23].

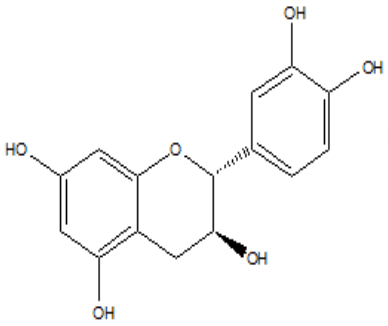
Flavonols are colorless or yellow flavonoids found in leaves and many flowers[23]. Quercetin is the yellow flavonol pigment of oak pollen. The fall colouring of deciduous trees may involve carotenoid pigments (terpenes) as well as flavonoids. In some trees, such as red maple (**Acer rubrum**) and scarlet oak (**Quercus coccinea**), colourless flavonols are converted into red anthocyanin as the chlorophyll breaks down[23].

Flavonoids with glucose side chains are called glucoflavonoids or glucosides (glycoside if the sugar is not specified), while the flavonoid component without sugar is called an aglycone[23]. Some nutritionists recommend flavonoids (bioflavonoids and isoflavones) in order to maintain healthy tissues and promote the proper balance of hormones and antioxidants in the body.

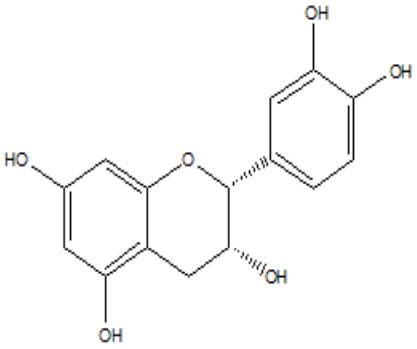
They may be obtained from a good diet of fruits, vegetables and soy protein or as dietary supplements.

1.5.2 Catechins

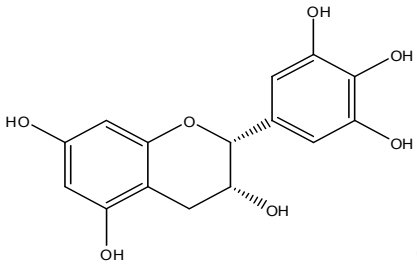
Some plants secrete bioflavonoids which inhibit growth and seed germination of nearby plants of a different species. This phenomenone, called allelopathy, has been well documented in chaparral shrubs that secrete terpenes which inhibit the germination of wild-flower seeds[24]. One potent allelopathic flavonoid is called Catechin whose different structures are given in **Figure 1.4**.



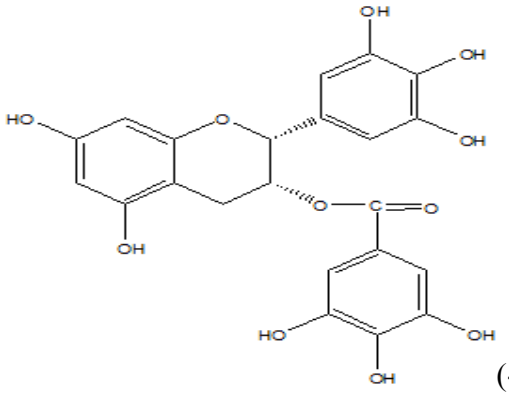
(+) - Catechin (C)



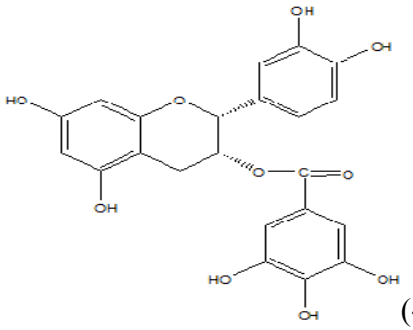
(-) - Epicatechin



(-) – Epigallocatechin (EGC)



(-) – Epicatechin gallate (ECG)



(-) – Epigallocatechin gallate (EGCG)

Figure 1.4 Chemical structures of catechins[23]

The chemical structure of catechin is synonymous to the floral pigment anthocyanin. The middle ring of catechin lacks a double bond, subsequently the molecule has two additional atoms of hydrogen. Catechin has got two mirror images, in a positive (+) form and a negative (-) form. The (+) catechin is an antioxidant found mainly in green tea.

1.5.2.1 Sources of catechins

Table 1.1 shows catechin content (in mg/100g food) of some common foodstuffs[25].

FOOD	CATECHINS
Chocolate	46-61
Beans	35-55
Apricot	10-25
Cherry	5-22
Grape	3-17.5
Peach	5-14
Apple	10-43
Red-raspberry	2-48
Strawberry	2-50
Blackberry	9-11
Green tea	10-80
Black tea	6-50
Red wine	8-30
Cider	4

From **Table 1.1** above, it can be inferred that good sources of catechins are; tea, chocolate, red wine, apples, and berries.

Catechins constitute about 25% of the dry weight of fresh tea leaf[26], although total catechin content varies widely depending on growing location, seasonal/ light variation, and the altitude of the area.

Catechins are also present in the human diets such as in chocolate[27], fruits, vegetables and wine and are found in many other plant species[28, 29]. The major dietary catechins are catechin, gallocatechin, epicatechin, epigallocatechin, epicatechin 3-gallate and epigallocatechin 3-gallate.

Epigallo-catechin gallate is by far the most abundant catechin in all tea types. One stereoisomer, (-)-catechin, is released from the roots of the invasive weed, spotted knap-weed. It acts as an herbicide to inhibit competition by a wide range of other plant species[28]. This phytotoxic compound prevents seed germination and growth. The (-) catechin induces oxidation and cellular death in root cells of plants in the neighbourhood.

1.5.2.2 Health benefits of catechins

The health benefits of catechins have been studied extensively in humans and in animal models. Reduction in atherosclerotic plaques was seen in animal models[30].

Reduction in carcinogenesis was seen *in vitro*[31]. Epigallocatechin-3-gallate is an antioxidant that reportedly helps to protect skin from UV radiation-induced damage and tumour formation[32].

Green tea catechins have also been shown to possess antibiotic properties due to their role in disrupting a specific stage of the bacterial Deoxyribonucleic acid (DNA) replication process[33]. Catechins, when combined with habitual exercise, have been shown to delay some forms of aging.

Mice fed catechins showed decreased levels of aging. Oxidative stress was lowered in cell mitochondria, as well as increase in Messenger ribonucleic acid (mRNA) transcription of mitochondria related proteins[34].

1.6 Free radicals

The antioxidants are the compounds that prevent some toxic materials, especially celled free radicals, in the body[35]. A free radical is easily formed when a covalent bond between entities is broken and one electron remains with each newly formed atom[36]. Free radicals are considered highly reactive due to presence of unpaired electron(s). In biological systems, free radicals are normally formed when oxygen interacts with certain biological molecules.

1.6.1 Mechanism of free radical reactions as illustrated in autoxidation

Interest in the process responsible for the deterioration of manufactured goods such as rubber, waxes in the past fifty years has stimulated the study of autoxidation[37, 38]. Autoxidation refers to the spontaneous free radical reaction of organic compounds with oxgygen[39].

In living systems, autoxidation of lipids, known as lipid peroxidation[40, 41] has been implicated in DNA and protein modification, radiation damage, aging and age pigment formation, modification of membrane structure, tumour initiation, and in the deposition of arterial plague associated with low-density lipoprotein modification[40-46].

Autoxidation is a free radical chain process consisting of chain initiation, propagation and termination steps, shown in **Figure 1.5**.

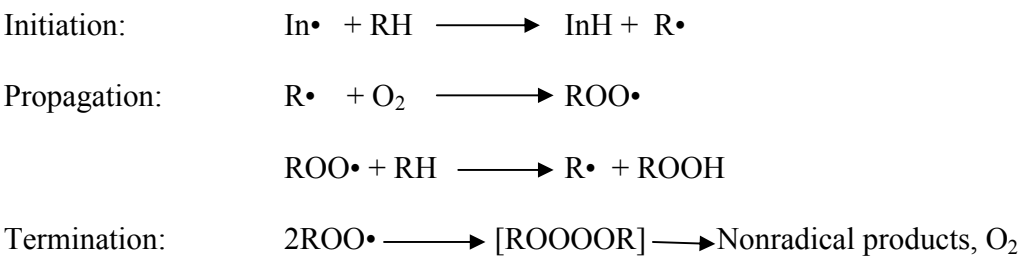


Figure 1.5 Chain sequence of free radical autoxidation[47].

The key event in the initiation step is the formation of the lipid radical, R•. This occurs by thermal or photochemical homolytic cleavage of an RH bond or by hydrogen atom abstraction from R-H by an initiator free radical.

The propagation step normally begins with the addition of molecular oxgen to R•, and the second step of propagation, the rate determining step, is abstraction of a hydrogen atom from RH by peroxyl radical, ROO• to generate ROOH and another radical, R•.

Propagation steps observed in autoxidation can be more complicated than they appear

in **Figure 1.5**. In general the propagation steps observed in free radical chain processes include radical coupling with oxygen, atom or group transfer, fragmentation, rearrangement and cyclisation[48].

Termination step involves side reactions that can stop the chain reaction are called termination steps. These termination steps involve the destruction of the free-radical intermediates, typically by two of them coming together. It is at this stage that antioxidants come into play, to stop the highly reactive species from destroying life supporting organs.

Autoxidation is an oxidation type of reaction defined in terms of hydrogen atom transfer as shown in the last row of **Table 1.2** below.

Table 1.2 Types of free radical reactions[49]

Type of Reaction	General Equation	Example
Addition	$X\cdot + Y \rightarrow [XY]\cdot$	Addition of OH· to guanine in DNA
Reduction	$X\cdot + Y \rightarrow Y^{-}\cdot + X^{+}$	Reduction of O_2 to $O_2^{-\cdot}$ by paraquat radical
Oxidation (electron acceptance)	$X\cdot + Y \rightarrow X^{-} + Y^{+}\cdot$	Oxidation of ascorbic acid
Oxidation(Hydrogen atom transfer)	$X\cdot + YH \rightarrow XH + Y\cdot$	Reaction of alfa - tocopherol with lipid peroxy radical

1.6.2 Types of free radicals

There are numerous types of free radicals that can be formed within the body. The most common reactive oxygen species (ROS) include: the superoxide anion (O^{2-}), the hydroxyl radical (OH^{-}), hydrogen peroxide (H_2O_2), lipid peroxide, singlet oxygen and hypochlorous acid which are also strong oxidising agents[50]. Superoxide anionsare formed when oxygen (O_2) acquires an additional electron, leaving the molecule with only one unpaired electron.

Within the mitochondria O^{2-} is continuously being formed. The rate of formation depends on the amount of oxygen flowing through the mitochondria at any given time. Hydroxyl radicals are short-lived, but the most damaging radicals within the body[49]. **Table 1.3** summarises the most common free radicals in biological systems.

Table 1.3 Most common free radicals in biological systems[49].

Name	Formula	Comments
Hydrogen atom	$H^{\cdot}$	The simplest free radical known
Superoxide	$O_2^{-\cdot}$	An oxygen-centered radical.
Hydroxyl	$OH^{\cdot}$	An oxygen-centered radical; the most highly reactive oxygen radical known.
Thiyl electron	$RS^{\cdot}$	General name for a group of radicals with an unpaired residing on sulfur
Peroxy, alkoxyl	$RO_2^{\cdot}$, $RO^{\cdot}$	Formed during the breakdown of organic peroxides

Once formed these highly reactive radicals can start a chain reaction. Their chief danger comes from the damage they can do when they react with important cellular components such as DNA, or the cell membrane. Cells may function poorly or die if this occurs[49].

Oxygen centered free radicals contain two unpaired electrons in the outer shell. When free radicals steal an electron from a surrounding compound or molecule, a new free radical is formed in its place. In turn the newly formed radical then looks to return to its ground state by stealing electrons with antiparallel spins from cellular structures or molecules. Thus the chain reaction continues and can be ″thousands of events long″[51].

The electron transport chain (ETC), a system found in the inner mitochondrial membrane, utilises oxygen to generate energy in the form of adenosine triphosphate (ATP).

Here oxygen acts as the terminal electron acceptor within the ETC. Literature suggests that anywhere ranging from 2 to 5% of the total oxygen intake during both rest and exercise free radicals are capable of forming the highly damaging superoxide radical via electron escape[52]. During exercise oxygen consumption increases 10 to 20 fold to 35-70 mL/kg/min[49]. In turn, electron escape from the ETC is further enhanced. Thus, when calculated, 6 to 3.5 mL/kg/min of the total oxygen intake during exercise can form free radicals[53]. Electrons appear to escape from the ETC at the ubiqunone-cytochrome-C level[52].

1.6.3 Importance of free radicals

Most studies focus only on the negatives associated with free radical production. However, free radicals are naturally produced by some systems within the body and have beneficial effects that cannot be overlooked.

The immune system is the benefactor of this since it is the main body system that utilises free radicals. A foreign invader, that can be a pathogen or a damaged tissue, is marked with free radicals by the immune system. This determines which tissue needs to be eliminated from the body.

1.7 Antioxidants in body defence system

Antioxidant means "against oxidation." Antioxidants aim at protecting lipids from peroxidation by radicals. Antioxidants are of significant importance here because they are willing to give up their own electrons to free radicals. Once a free radical gains the electron from an antioxidant it no longer needs to attack the cell and the chain reaction of oxidation is broken[53]. After donating an electron, an antioxidant becomes a free radical by definition.

Antioxidants in this state are not harmful because they have the ability to accommodate the change in electrons without causing any havoc.

Naturally the human body has an elaborate antioxidant defence system. These antioxidants are manufactured within the body and can also be extracted from the food humans eat such as vegetables, seeds, meats, and oil, fruits, nuts, among others. There are two lines of antioxidant defence within the cell. The first line, found in the fat-soluble cellular membrane consists of vitamin E, beta-carotene, and coenzyme Q[54].

Of these, vitamin E is considered the most potent chain breaking antioxidant within the membrane of the cell. Inside the cell water soluble antioxidant scavengers are present. These include vitamin C, glutathione peroxidase, superoxide dismutase (SD), and catalase[53].

1.8 Aim of the study

This study was aimed at investigating the antioxidant properties of *Rosa hemisphaerica* J. Herrm (yellow rose) root extracts, isolating the bioactive polyphenolic compounds from the root extract using fractionation, column chromatography and analytical TLC. Isolated phenolic compounds were examined for their antioxidant activity using 2,2-diphenyl-1-picrylhydrazyl (DPPH) method. The bioactive compound was futher characterised by using Nuclear Magnetic Resonance (NMR) and Mass Spectrometry (MS) techniques.

CHAPTER 2

MATERIALS AND METHODS

2.1 Materials

Silica gel 60 F_{254} (Merck, 0.2mm) Thin Layer Chromatography (TLC) plates were used. Lipophilic sephadex (LH-20, 25-100 μm) manufactured by Pharmacia, purchased from Sigma was used for gel filteration. Silica gel (Merck, 60-120 mesh) was used for column chromatography. The columns used were 3cm x 35cm for silica gel, and 5.0cm x 100cm for lipophilic sephadex (LH-20) gel filteration. They were made from the glassware workshop in chemistry department of Middle East Technical University (METU), Ankara-Turkey.

2.1.1 Chemicals

Preperative chromatography grade ethanol and methanol used in Thin Layer Chromatography (TLC), and in the preparation of extracts were purchased from Merck (Darmstadt, Germany). Water which was distilled twice, was purified using a Milli – Q system (Millipore, Bedford, MA, USA). Solvents; petroleum ether, chloroform, ethyl acetate, butanol used in the fractionation were all purchased from Merck (Darmstadt,
Germany) and were of analytical grade. 2,2-diphenyl-1-picrylhydrazyl (DPPH), gallic acid, sodium carbonate, were purchased from Sigma Chemical Company, (St. Louis, MO, USA). Folin - Ciocalteu's phenol reagent was purchased from Merck (Darmstadt, Germany). Dimethyl sulfoxide (DMSO) solvent and the internal standard,

Tetramethylsilane (TMS) used in Nuclear Magnetic Reasonance (NMR) experiments, were purchased from Merck (Darmstadt, Germany). Ninhydrin was purchased from Aldrich (Taufkirchen,Germany).

2.1.2 Instruments

The following instruments were used in this study: Rotary evaporator: Laborata 4000-Efficient; Heidolph instrument (GmbH & Co. KG, Germany) was used to remove residual solvents from the extract and its fractions during fractionation. Cary 50 Bio UV visible spectrophotometer (Varian) used in taking UV-VIS absorbance measurements, Bandelin Sonorex was used for ultrasonic bath.

Blender: Waring model 32BL80 (New Hartford, CT, USA) was used to grind the dry roots prior to extraction. Nuclear Magnetic Resonance (NMR) spectrometry experiments were performed from the chemistry department of METU.

The sample was dissolved in Dimethyl sulfoxide (DMSO) as the solvent. ^{1}H-NMR, ^{13}C-NMR, Correlation Spectrometry (COSY), Heteronuclear Multiple Quantum Correlation (HMQC) and Heteronuclear Multiple Bond Correlation (HMBC) experiments were recorded from the NMR laboratory (C-35) with a Bruker Biospin Ultrashield 400mHz NMR instrument. Tetramethylsilane (TMS) was the internal standard.

The mass spectrum was recorded on Thermo Scientific Trace GC Ultra made by Thermo Electron S.p.A, Strada Rivoltana, 20090 Rodano (Milan), Italy. It is equipped with Xcalibur Software (made by Thermo-Finnigen). It's a GC/MS with a quadruple detector. It also has a TR5NS column which is 30m long. It is found in laboratory DZ-36, Chemistry Department of Middle East Technical University (METU) in Prof. Cihangir Tanyeli's laboratory.

2.2 METHODS

2.2.1 Collection and preparation of *R. hemisphaerica* roots sample

R. hemisphaerica roots were collected during the summer of 2007 from Erek Mountain near Kevenli lying 38° 28' 54.53" N 43° 27' 2.10" E in Van region, eastern part of Turkey. In the laboratory, the roots were washed, and placed on clean filter papers to dry under the shade at room temperature. They were then kept in a dark place for some time till they were completely dry.

2.2.2 Extraction and fractionation of constituents

With some modification, procedures put forward by Kandil et al. were followed during fractionation and isolation stages[55]. 220 g of clean, dry roots of *R. hemisphaerica* roots were ground to obtain a 3-4 mm particle size with a blender, and extracted thrice with 200 mL of methanol (each time) using a sonicator (40 mins) at room temperature. The combined methanolic extracts were evaporated under reduced pressure at 37°C temperature to dryness with the help of rotary evaporator. It was then left in fume chamber overnight to completely dry before taking its wieght. It was then redissolved in methanl and some little water added (about 5 mL to ensure complete solubility).

The total volume was 100 mL. It was then partitioned into; petroleum ether, chloroform, ethyl acetate, n-butanol and water-soluble fractions. TLC experiment showed no flavonoids in petroleum ether, chloroform and water fractions. The remaining two fractions, starting with one from butanol were eluted (using lipophilic Sephadex LH-20) with chloroform (solvent A); increasing amounts of chloroform + methanol (9:1, solvent B) were added up to 100% B. At this point all colored materials were removed from the column.

Seven different fractions were collected. These fractions were concentrated under vacuum at 37 ºC to remove volatile solvents.

They then underwent additional chromatography on silica gel. Each step of this fractionation was monitored by TLC on silica gel plates with methanol: chloroform (1:9), using sulphuric acid: methanol (5:95) spray solution followed by heating at 100ºC in each instance.

Another plate was sprayed with Ninhydrin (2, 2-Dihydroxyindane-1, 3-dione) reagent to test for presence of amines. Further fractionation and purification of subfractions was accomplished by repeating the procedure, but varying solvent composition to achieve optimal separations. The same procedures were applied to ethyl acetate fraction.

2.3 Evaluation of antioxidant activity

2.3.1 DPPH Assay

The DPPH test, which utilises a redox reaction with the 2, 2-Diphenyl-1-Picrylhydrazyl (DPPH) radical can be used to determine the anti-oxidative capacity of extracts. The radical has a violet colour due to the unpaired nitrogen atom electron and, after the reaction with the oxygen atom of a radical scavenger, the reduced 2, 2-Diphenyl-1-picrylhydrazin (DPPH-H) is formed which is yellow as seen in **Figure 2.1** below.

The colour change can be followed spectrophotometrically at **517nm** and in this way the antioxidative potential of a substance or a plant extract can be determined[56].

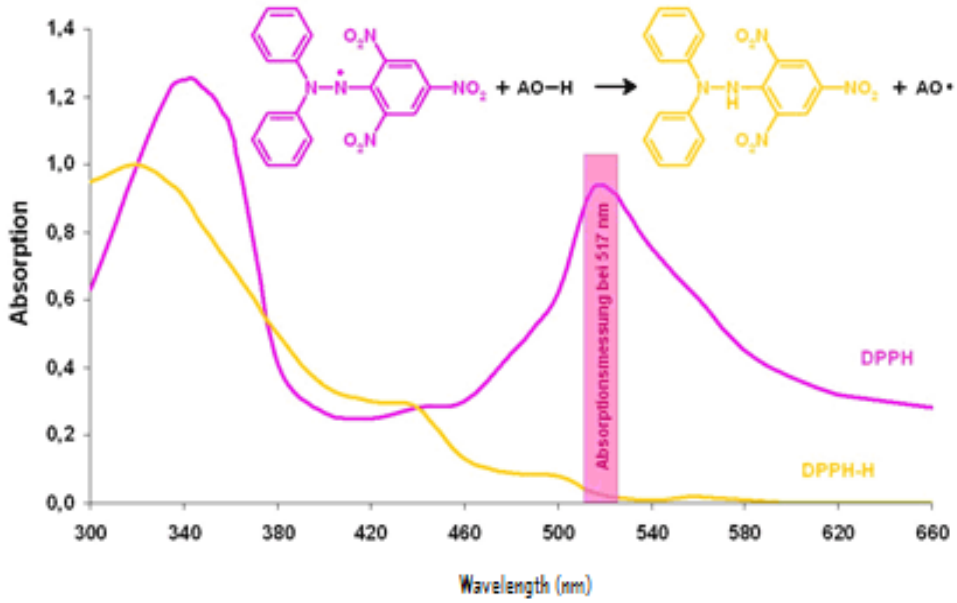


Figure 2.1 The DPPH experimeriment[56].

2.4 Determination of total phenolic content

The method put forward by Singleton and Rossi was followed in determining the total phenolic content of the crude extract of *R. Hemisphaerica* roots[57]. Ethanolic extracts were prepared in varying concentrations, ranging from 0.1 to 0.5 mg/mL. 0.1 mL of each extract concentration was thoroughly mixed with 2 mL of 2% aqueous solution of Na_2CO_3 and vigorously vortexed. Immediately after, 0.1 mL of 50% Folin-Ciocalteu's phenol reagent was added, and vigorously vortexed again. The incubation clocked 30 mins from the time Folin's reagent was added.
The same procedure was also applied to the standard solutions of gallic acid, prepared in concentrations varying between 0.05 – 0.3 mg/mL. In both the extract and gallic acid standards, each concentration was repeated twice.
Absorbance of each of the two sets was measured at the end of the incubation time.

The results were recorded in terms of milligrams of total phenolic content per milligrams of extract as gallic acid equivalents (GAE).
Absorbance measurements were preceded by a blank callibration reading that consisted of 2 mL of 2% Na_2CO_3, 0.1 mL of ethanol and 0.1 mL of 50% Folin's reagent.

2.5 NMR spectrometry

For ^{1}HNMR, 5 mg of sample/5 mL of the solvent was required and it lasted for 5 mins. For ^{13}CNMR 15 mg of sample/5 mL of the solvent was required, lasting for 15 hrs (overnight). Correlation spectrometry (COSY) experiment that gives information about pairs of protons that are J-coupled. It was done by correlating the ^{1}H-^{1}H profiles. Heteronuclear Multiple Quantum Correlation (HMQC) and Heteronuclear Multiple Bond Correlation (HMBC) were consequently done following the profiles of ^{1}HNMR and ^{13}CNMR experiments.

2.6 Mass spectrometry

The instrument follows an automated flow injection system. A sample volume of 2μL was injected. The onset of the analysis was at 80°C, lasting for 3 minutes.
This was folllowed by a 20°C/min gradient temperature increase, to the maximum temperature of 270°C, attained after 12.5 minutes from the onset. It then took 5 minutes for the instrument at maximum temperature to clean the column. The flow rate was set at 1.5 mL/min, split flow was 30 mL/min and the Split Ratio was 20.

CHAPTER 3

RESULTS AND DISCUSSION

3.1 Yield of plant extracts

From the 220 g of ground roots of *R. hemisphaerica* dry weight, a yield of 16.07 g (7.13%) of crude extract was obtained. Out of this, 10.63 g was air dried and dissolved in minimum amount of methanol. A little amount of water was added to ensure total solubility of all sample components. The total volume was then about 100 mL. Butanol fraction had a dry weight of 7.494 g. From this, 4 g underwent gel filteration with both lipophilic sephadex (LH-20) and silica gel, and only 0.085 g of **compound 1** was obtained which corresponds to 2.13% yield. The yield is so small and obtaining the **compound 1** from this plant species is uneconomical. The subsquent fractionations and their yields are summarised in **Figure 3.1**

3.2 Column chromatography and thin layer chromatography

With some modifications, method followed by Kandil[55] was followed in the sequential fractionation and isolation shown in **Figure 3.1.**

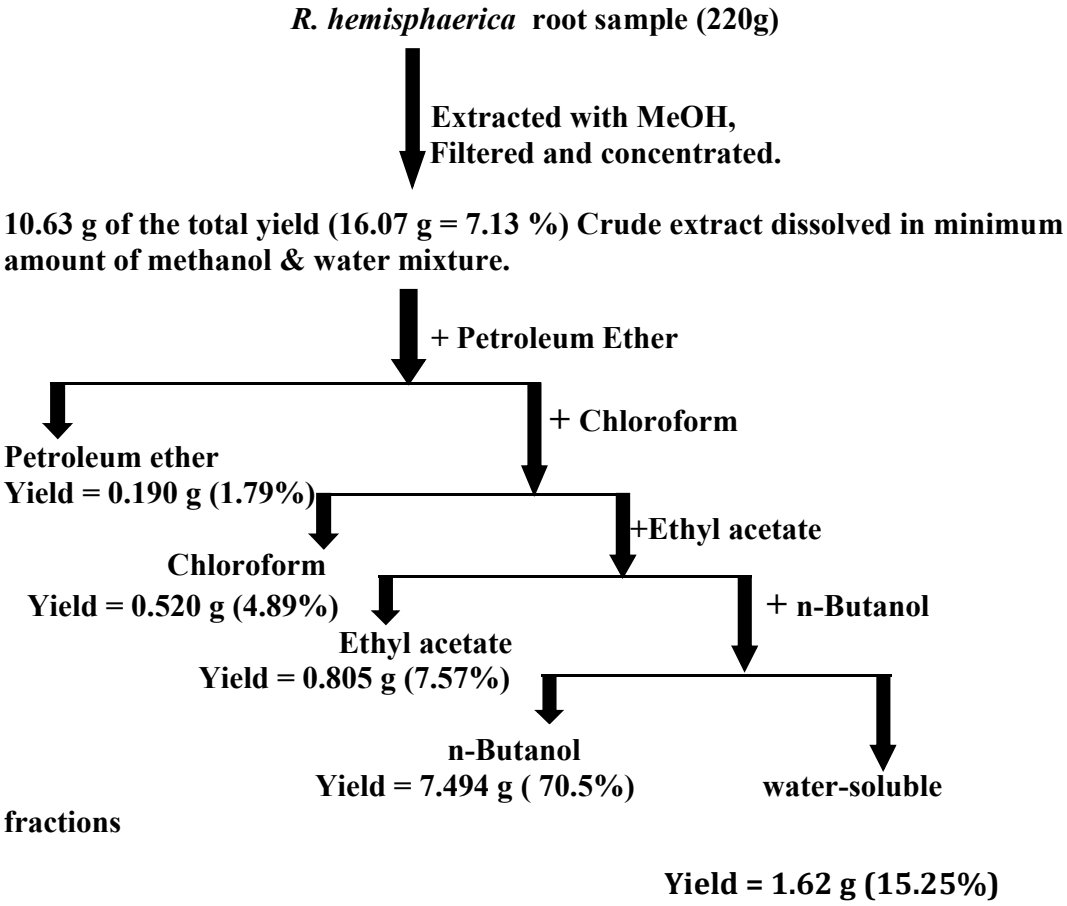


Figure 3.1 Sequential organic extractions and fractionation of *R. hemisphaerica* root sample.

Contents of each of the fractions above where closely monitored with Thin Layer Chromaphy (TLC) results of which are shown in **Figure 3.2** below.

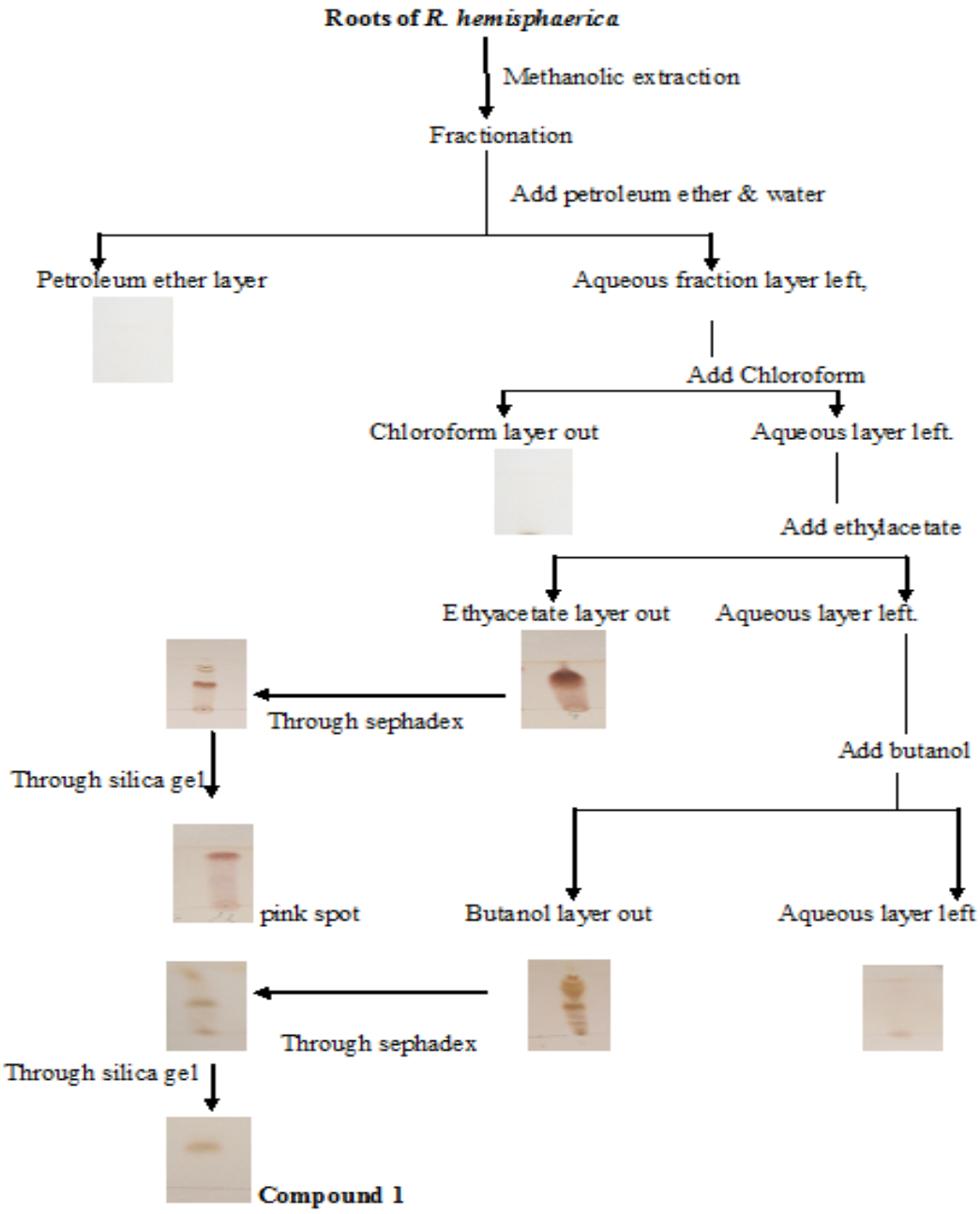


Figure 3.2. Scheme showing sample analysis by Thin Layer Chromatography (TLC) technique

In all stages of this experiment, precoated Kieselgel 60 F_{254} TLC plates (Merck, German) were used for analytical TLC. Solvent system for all through was methanol: chloroform (10:90). For visibility of the spots, TLC spots were sprayed with 5% methanolic sulphuric acid followed by heating at 100 ^{o}C. As it can be seen from **Figure 3.2** above, TLC results of both petroleum ether and chloroform did not yield any positive results. Yellow TLC spots, characteristic of saponin like compounds were found in ethylacetate fraction. Traces of the same saponin-like compounds were also found in butanol fraction.

Butanol fraction underwent gel filteration with lipophilic sephadex (LH-20) using Methanol: Chloroform (1:1). Test tubes giving yellow TLC spots were combined and subjected to further isolation with silica gel column chromatography using Methanol: Chloroform (15:85) to obtain pure **compound 1** (34mg, R_f – 0.52)**.** Upon spraying followed by heating at 100ºC, compound 1 appeared on the TLC plate as a pale yellow spot. All these procedures are summarised by the figure shown above. The pale yellow TLC spot signals presence of flavonoids hence the need to characterise **compound 1**. The saponin-like compounds obtained from both ethylacetate and butanol fractions was, however, not characterised because it had a an EC_{50} value of 0.292 ± 0.007 (μg/mL) which is a considerably very low radical scavenging capacity.

From the appearance of TLC and intensity of the spots, there were other compounds that could be isolated, but where ignored because they where present in minute quantities that were not enough to perform any characterisation on them.

3.3 Free radical scavenging activity by DPPH method

With some modifications, the method proposed by Blois was followed in this experiment[58]. A 0.05 mg/mL DPPH solution was used. At that concentration DPPH solution produces approximately 1.3 absorbance units at 517 nm.

Dry samples of crude extract, compound 1, and samples from the various solvent fractions were each separately dissolved in ethanol. 0.1 mL solutions of each ethanolic extracts above was added to 1.4 mL of 0.05 mg/mL DPPH. The initial concentrations were 0.10, 0.15, 0.30, 0.45, 0.75 and 1.00 mg/mL. After 20 mins of incubation time at room temperature, absorbance was recorded at 517 nm. Percentage of Radical Scavenging Activity (%RSA) was used to calculate the fifty percent Effective Concentrations (EC_{50}) for each extract at 50% scavenging activity[58].

Radical scavenging activity was calculated by the following formula:

$$\%RSA = [(A_0-A_1)/A_0] \times 100$$

Where : A_0 : absorbtion of blank sample (t=0 min);

A_1 : absorption of tested extract solution (t=20 min)

3.3.1 Optimization of time and temperature for DPPH radical scavenging activity of *R. hemisphaerica* root extract

Optimisation studies were carried out for the parameters of extract preparation, to determine the optimum incubation temperature and duration for DPPH radical scavenging activity of the sample. The results were tabulated in **Table 3.1**. According to the these results, optimum temperature was 25 ^{o}C. Secondly, incubation time was examined by testing at 0, 5, 10, 15, 20, 30, 45 minutes durations. The observed EC_{50} results, recorded in **Table 3.2**, were almost constant from 20 to 45 minutes, and hence 20 minutes was chosen as the optimum incubation duration.

Table 3.1 Optimization of temperature for the DPPH radical scavenging activity of root extract of *R. Hemisphaerica.*

Temperature	**Mean value of DPPH RSA as EC_{50} (mg/mL) ± SD**
25 °C	0.024 ± 0.008
50 °C	0.083 ± 0.009
70 °C	0.232 ± 0.065

Table 3.2 Optimization of time for the DPPH radical scavenging activity of root extract of *R. hemisphaerica.*

Time/ mins	**Absorbance at 517nm**	**%RSA ± SD**
0.0	1.2364	17.76 ± 1.31
5.0	0.9875	34.32 ± 2.03
10.0	0.3218	78.60 ± 1.47
15.0	0.2161	85.63 ± 4.10
20.0	0.1426	90.51 ± 4.99
30.0	0.1427	90.51 ± 5.10
45.0	0.1427	90.51 ± 5.12

Thus 20 minutes was the optimised reaction time for the DPPH free radical scavenging experiment.

3.4 Antioxidant capacities of the plant extracts

Antioxidant capacities of *R. hemisphaerica root* extract was investigated by measuring both DPPH scavenging activity and total phenolic contents. Measurements of DPPH percent scavenging activities were carried out on crude extract, compound 1and quercetin (standard) as it was done to ethyl acetate, butanol and water fractions at different concentrations ranging between the values 0.075 – 1.000 mg/mL . Results were used to to plot graphs of DPPH percentage radical scavenging activity (% RSA) versus extract concentrations (**Figure 3.3** and **Figure 3.4**), from which values of EC_{50} in **Table 3.3** were obtained.

Table 3.3 Results of Radical Scavenging Activity measured by the DPPH experiment expressed in terms of EC_{50} values:

Fraction	**Mean EC_{50} (µg/mL) ± SD**
Crude extract	0.0234 ± 0.002
Quercetin	0.0088 ± 0.0002
Ethylacetate	0.0217 ± 0.0002
Butanol	0.0209 ± 0.0012
Aqueous	0.0275 ± 0.0018
Compound 1	0.0239 ± 0.0001

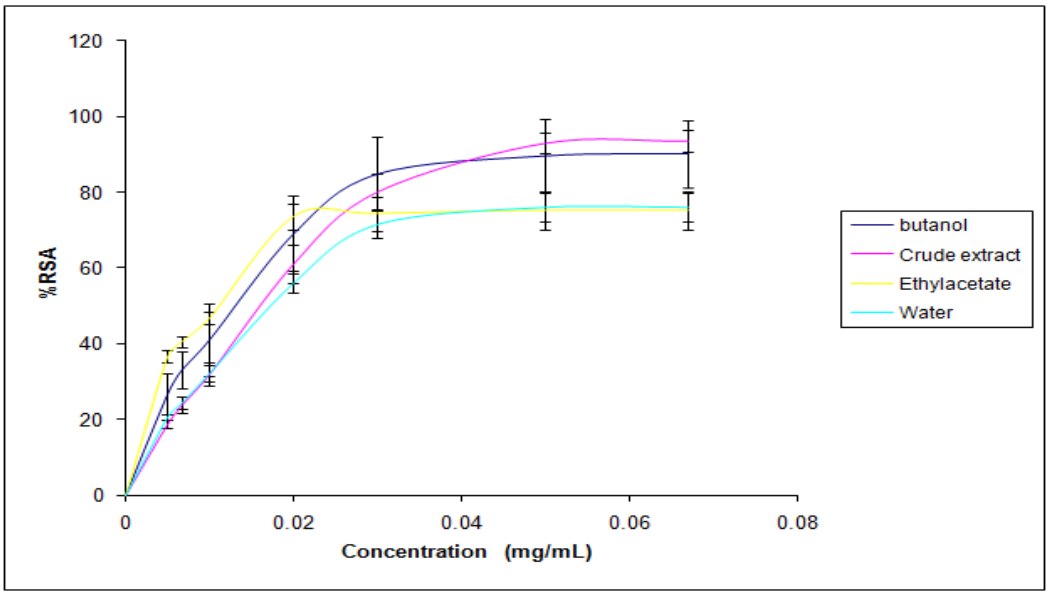


Figure 3.3 Percentage DPPH scavenging activities of crude extract, water, butanol and ethylacetate fractions, of roots of R. *hemisphaerica.*

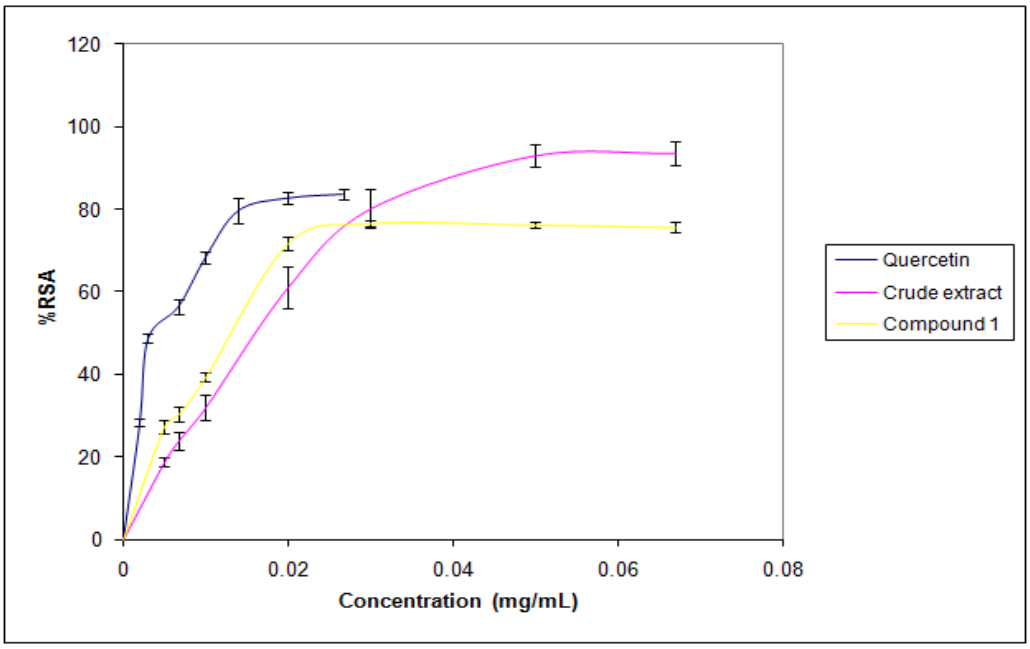


Figure 3.4 Percentage DPPH scavenging activities of quercetin, compound 1 and crude extract of roots of ***R. hemisphaerica***.

Each concentration was prepared in duplicate and every experiment was repeated three times (n=6) and Standard Deviation (SD) was found to be in the range of 0.00009 to 0.00199.

Amongst all fractions, the highest DPPH radical scavenging capacity was displayed by butanol fraction with an EC_{50} of 0.0209 ± 0.0012µg/mL. The lowest one was shown by water fraction with an EC_{50} value of 0.0275 ± 0.0018µg/mL.

This explains why **compound 1** shows a commanding radical scavenging activity. Quercetin was selected as a standard since it was quite often used in the literature as a reference.

3.5 Total phenolic content

Gallic acid was the standard in the experiment to determine total phenolic content of plant extracts, as proposed by Singleton and Rossi[57]. The results showed 0.224 ± 0.007 µg phenolic equivalents of gallic acid (GAE) per mg of extract.

3.6 Results of spectrometric measurements

A UV visible spectrum of a methanolic extract solution of **compound 1** shows a λmax peak at 280 nm (**Figure 3.5**), giving an indication that **compound 1** is a catechin.

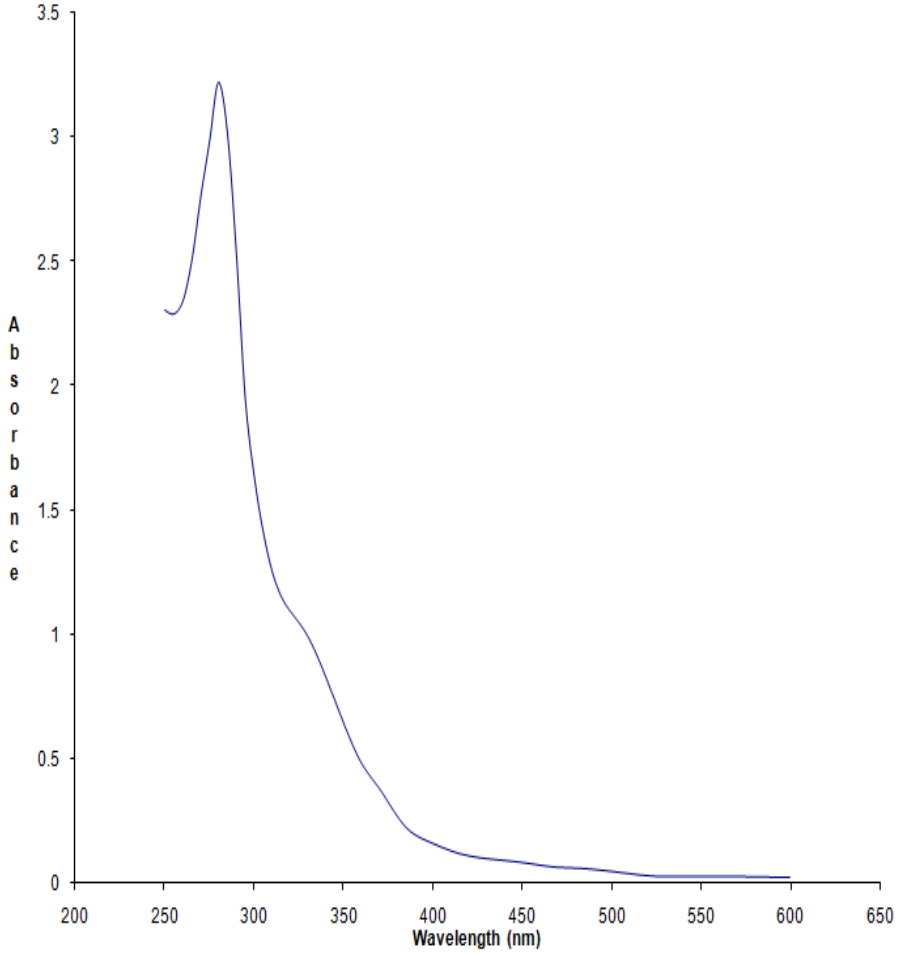
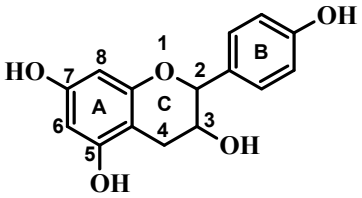


Figure 3.5 UV-VIS spectrum of compound 1

The numbering system in catechin shown below will be adapted during the explanation of results.



Numbering system in catechin.

For ^{1}H-NMR experiment, 5 mg of **compound 1** was dissolved in 5 mL of DMSO solvent. The experiment lasted for 5 minutes using a Brucker Biospin Ultrashield 400 MHz. Singlet signals at δ **5.45** ppm and δ **5.67** ppm in the ^{1}H NMR spectrum (**Figure 3.6**) revealed the peaks of 5, 7-dihydroxycatechin as **compound 1**. A singlet at δ **6.44** ppm and the two doublets at **6.49** ppm and **6.36** ppm (**J** = 2.0 Hz) showed 3, 4-dihydroxy functional structure corresponding to an aromatic ring B of catechin. The two proton singlets at δ **6.46** and **δ 6.48** ppm were assigned to the galloyl moiety.

The ^{1}H NMR spectrum is as given in **Figure 3.6** below.

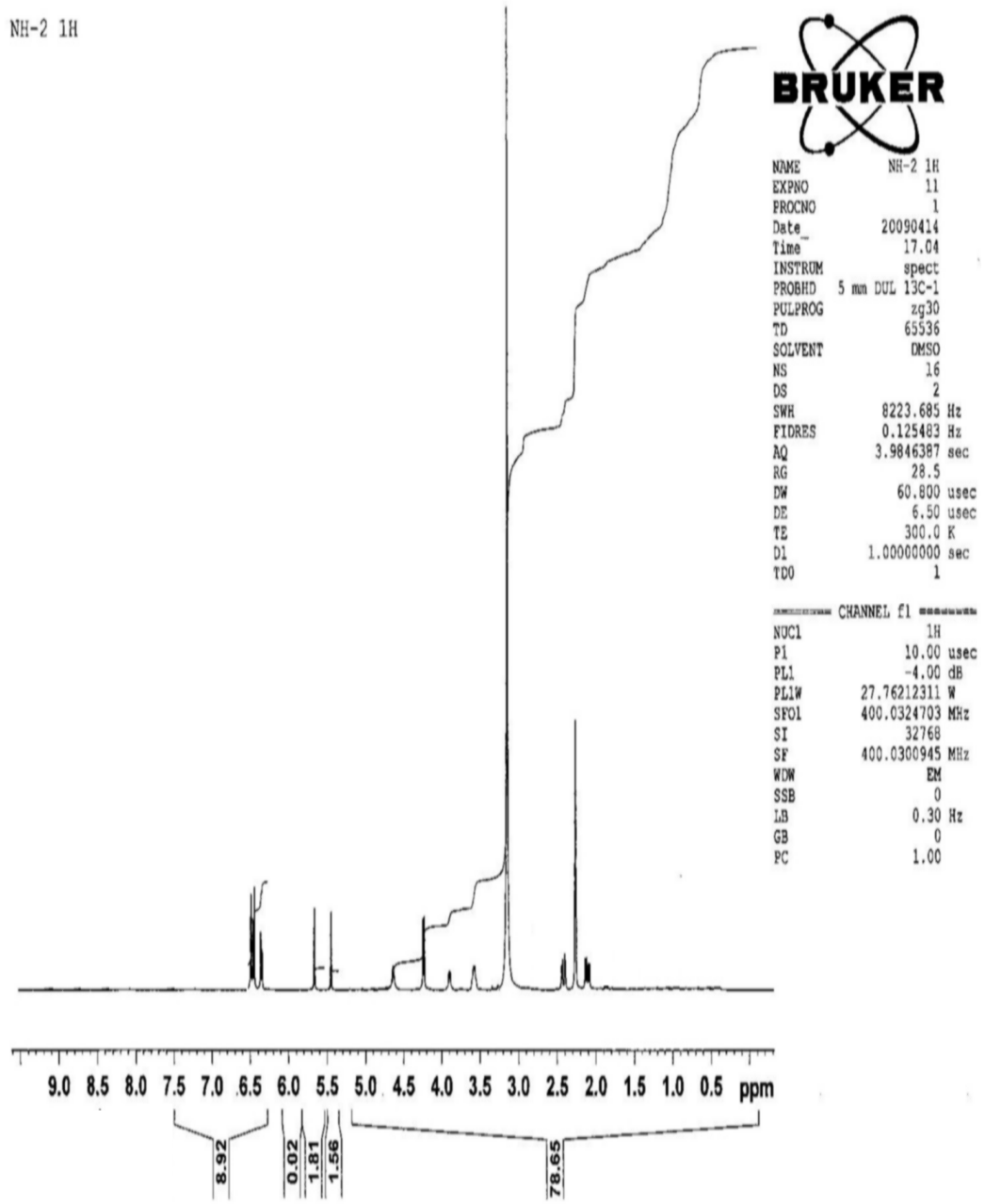


Figure 3.6 [1]HNMR spectrum of compound 1.

For ^{13}CNMR experiment, 15 mg of **compound 1** was dissolved in 5 mL of DMSO solvent. The experiment lasted for 17 hrs (overnight) using a Brucker Biospin Ultrashield 400 MHz. Only 100 MHz was used. The signal at δ **166.9** in the ^{13}CNMR (**Figure 3.7**) was assigned for the carbonyl of the galloyl moiety.

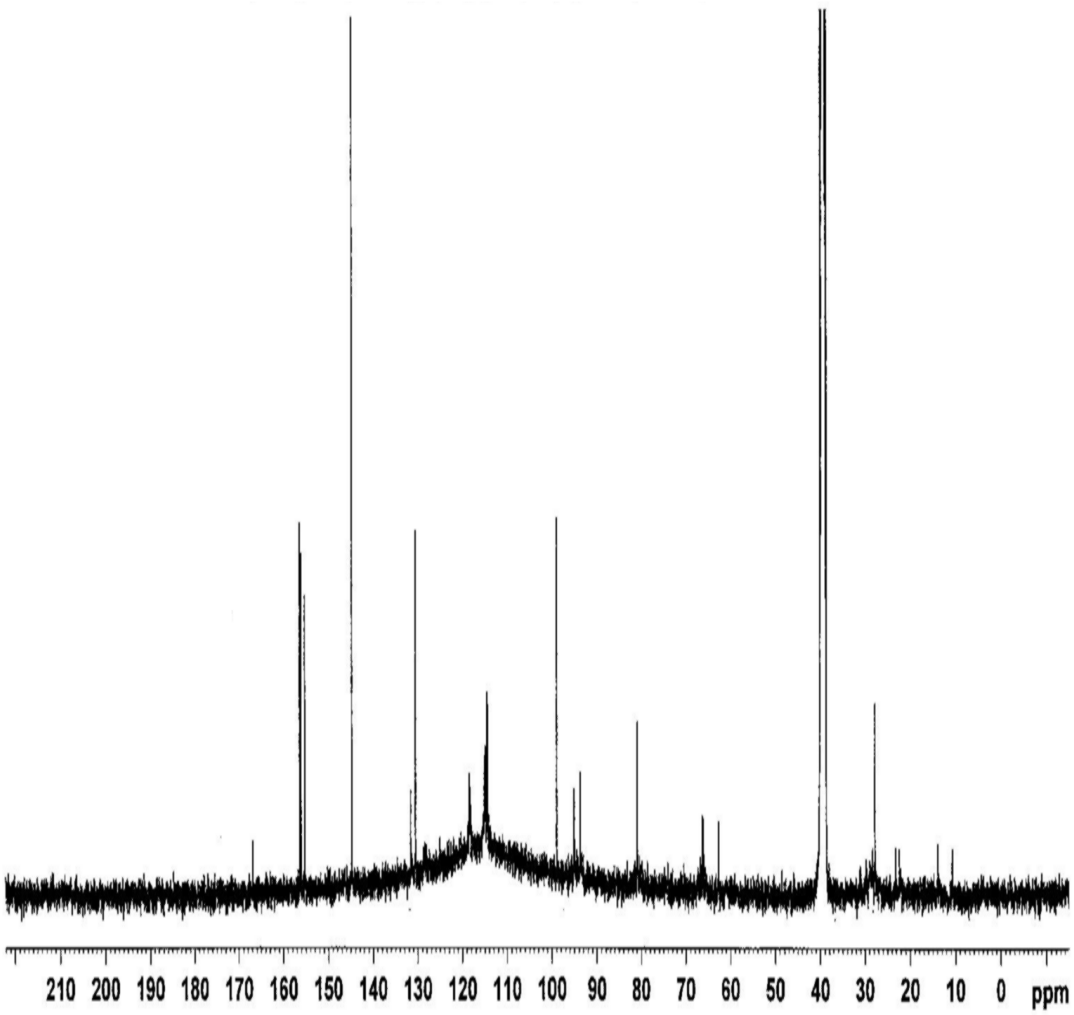


Figure 3.7 ^{13}C-NMR spectrum of compound 1.

Thus from ^{1}HNMR and ^{13}CNMR spectra, the structure of **compound 1** is as in **Figure 3.8** below. Chemical shifts of ^{1}HNMR are marked in red while the blue numbers correspond to the chemical shifts of ^{13}CNMR.

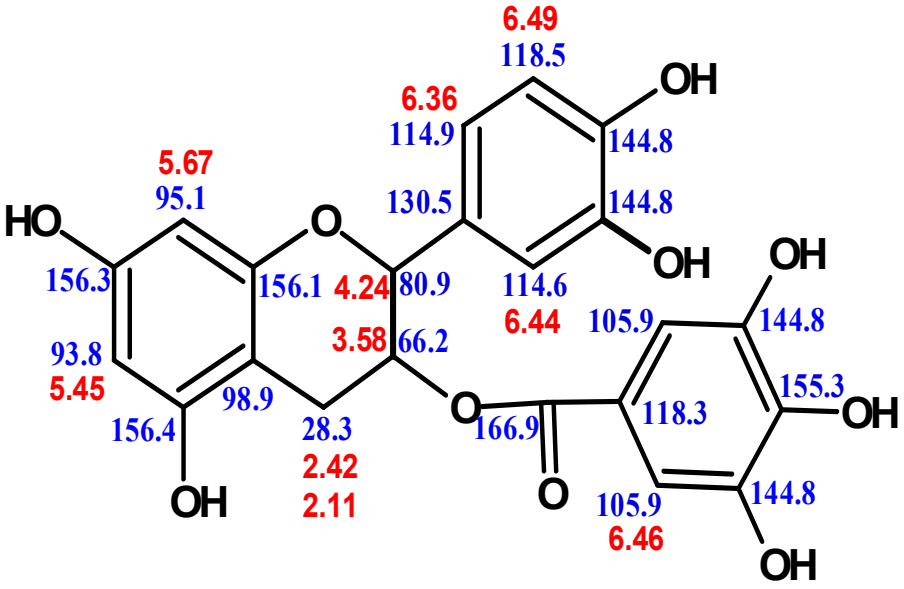


Figure 3.8 Structure of **compound 1** from ^{1}HNMR and ^{13}CNMR spectra**.**

The fragment at m/z 138 is reported in literature[59] as specific for all the catechins and catechin derivatives.

The fragmentation pathway observed in the mass spectrum of the molecule is as shown in **Figure 3.9**.

The MS of **compound 1** showed $[M-H]^{+}$ ion peak at m/z 441 (**Figure 3.10**). The spectra presented the characteristic fragment originated by the Retro Diels-Alder reaction described for catechins (**Figure 3.11)**.

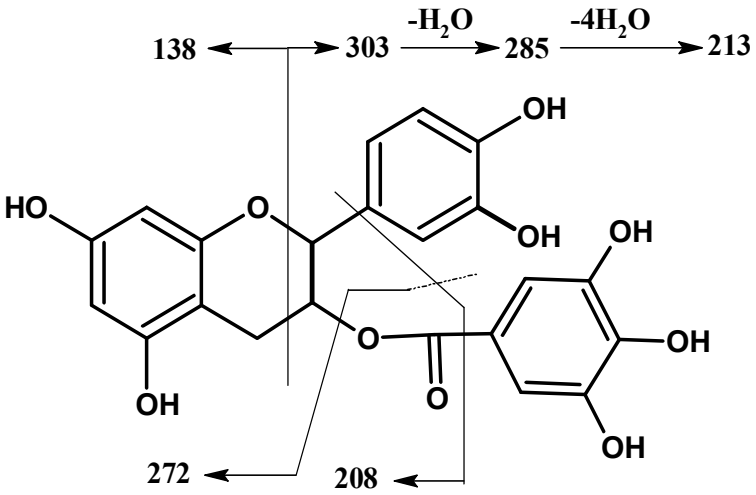


Figure 3.9 Fragmentation observed in the mass spectrum of **compound 1.**

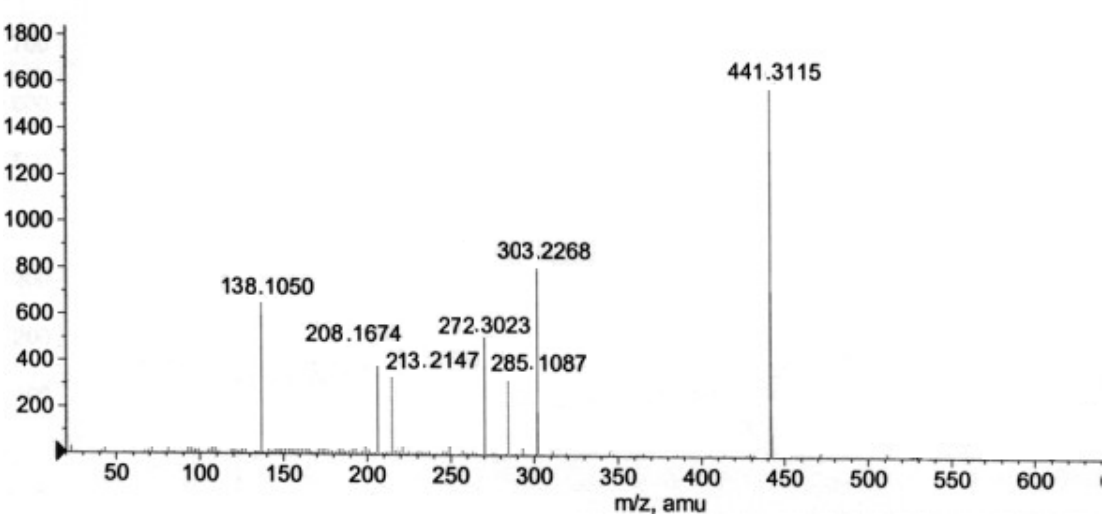


Figure 3.10 MS spectrum of **compound 1.**

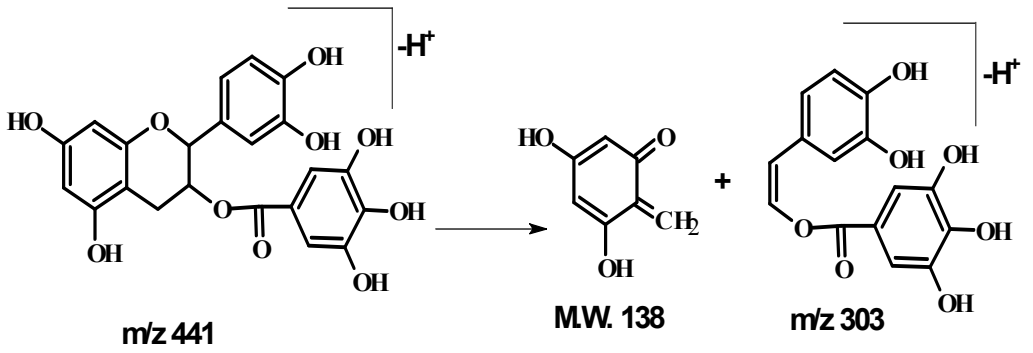


Figure 3.11 Retro Diels-Alder reaction described for catechins.

A major fragment peak at m/z 272 in the spectrum of **compound 1** confirmed the presence of the flavon-3-ol moiety with galloyl residue in the molecule. The fragment ion at m/z 285 corresponds to the loss of water moiety (18 amu) from the ion peak at m/z 303. And the loss of four water moieties from this ion peak gives the ion peak at m/z 213. Fission with the removal of both the tri-substituted benzene ring from the galloyl moiety and also ring B in flavon-3-ol moiety gives the ion peak at m/z 208.

In NMR spectrometry, the assignment of protons in this compound was facilitated by determination of correlation spectrometry spectrum ^{1}H-^{1}H COSY (**Figure 3.12**), HMQC (**Figure 3.13**) and HMBC (**Figure 3.14**).

COSY: Proton 2 of **compound 1** (δ **4.24, J =7.6** Hz **)** is coupled to proton 3 at (**3.58, J =7.4** Hz**)**. Proton 3 is, in turn coupled to both protons at 4 **(2.42, J= 15.8, 5.6** Hz**)** and **(2.11, J= 16.0, 8.0** Hz**).**

The two protons at 4 are thus *diastereotopic protons*. Proton 2 (δ **4.24)** is coupled to proton 3 at (**3.58),** but in this instance is a doublet (**J =7.6** Hz **).**

Proton 3 is again seen to be coupled to both protons at position 4 **(2.42** and **2.11).** Each of the protons at 4 is coupled to the other. **Figure 3.12** shows the **COSY** spectrum

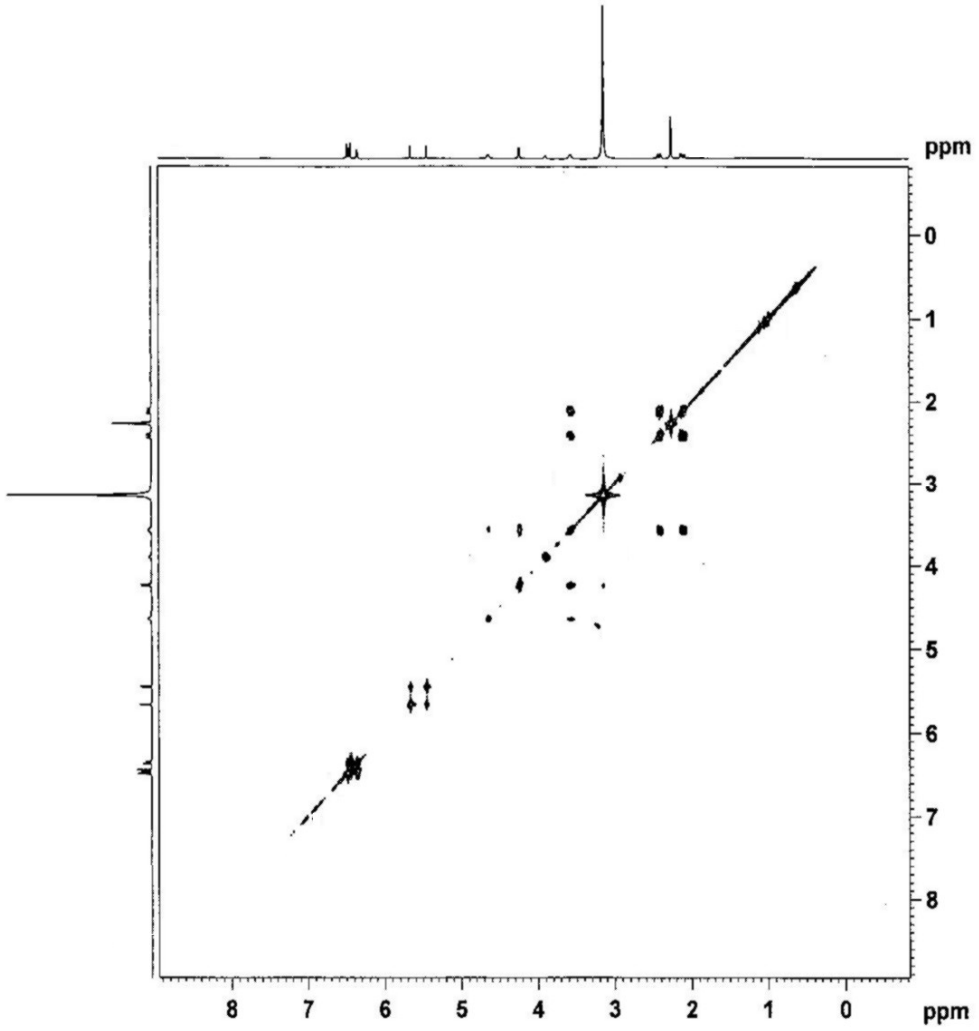


Figure 3.12 ^{1}H-^{1}H COSY spectrum of compound 1.

Heteronuclear Multiple Quantum Correlation (**HMQC**) experiment gives information about strong proton-carbon J-coupling. A strong proton-carbon J-coupling indicates that the proton is directly bonded to the carbon. It can beclearly seen here that two *diastereotopic protons* at 4 are attached to the same carbon atom. They resonate as an AB system because of the stereogenic centre at neighbouring position 3.

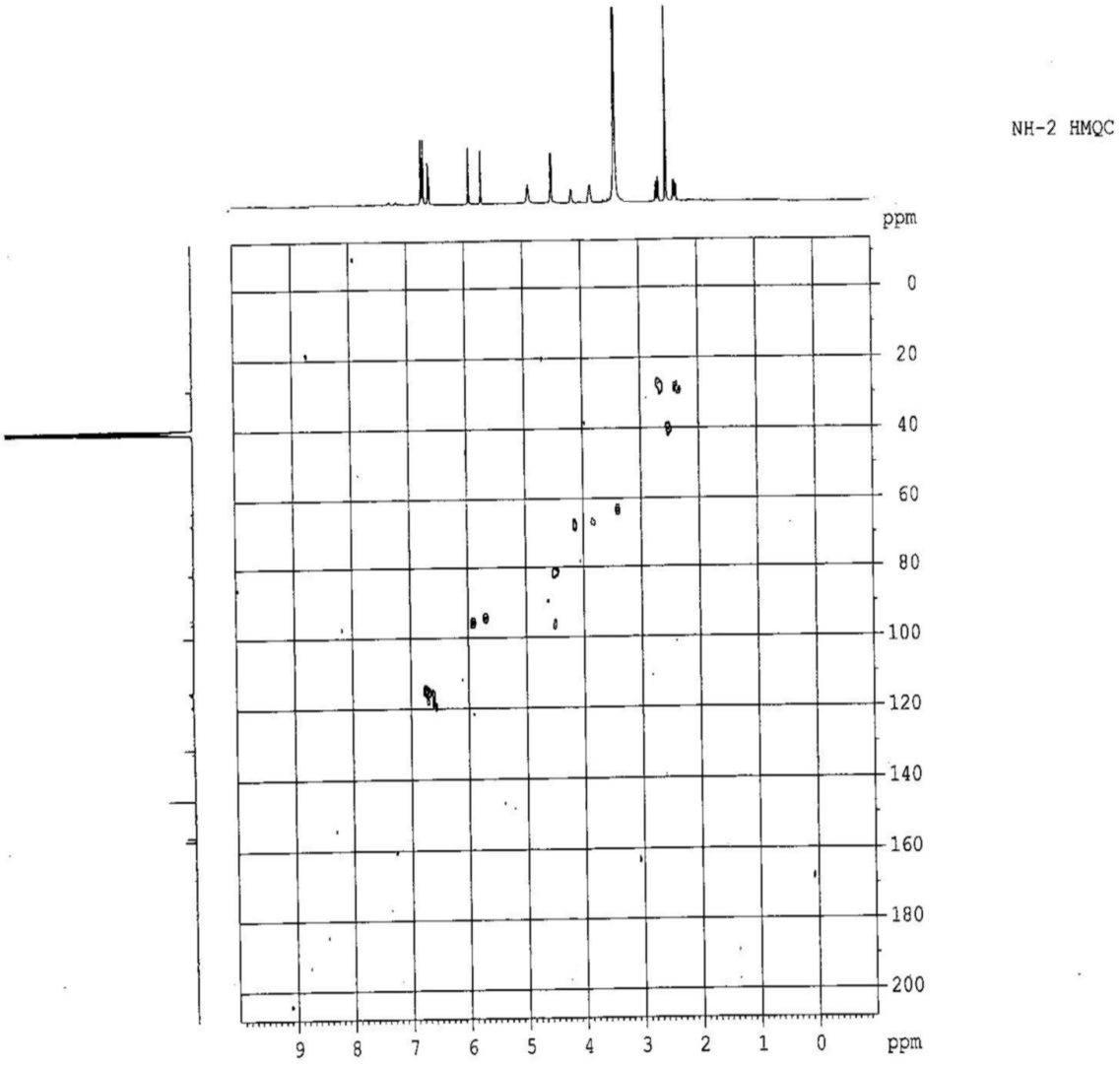


Figure 3.13 HMQC spectrum of compound 1.

Heteronuclear Multiple Bond Correlation (**HMBC**) gives information about weak proton-carbon J-couplings. It gives information about long range coupling between protons and carbons. The HMBC interaction of H-3 with δ **166.9** indicated the position of the attachment between the flavon-3-ol moiety with the galloyl. The chemical shift and coupling constants of this compound was similar to those previously reported[60]. **Figure 3.14** shows its spectrum.

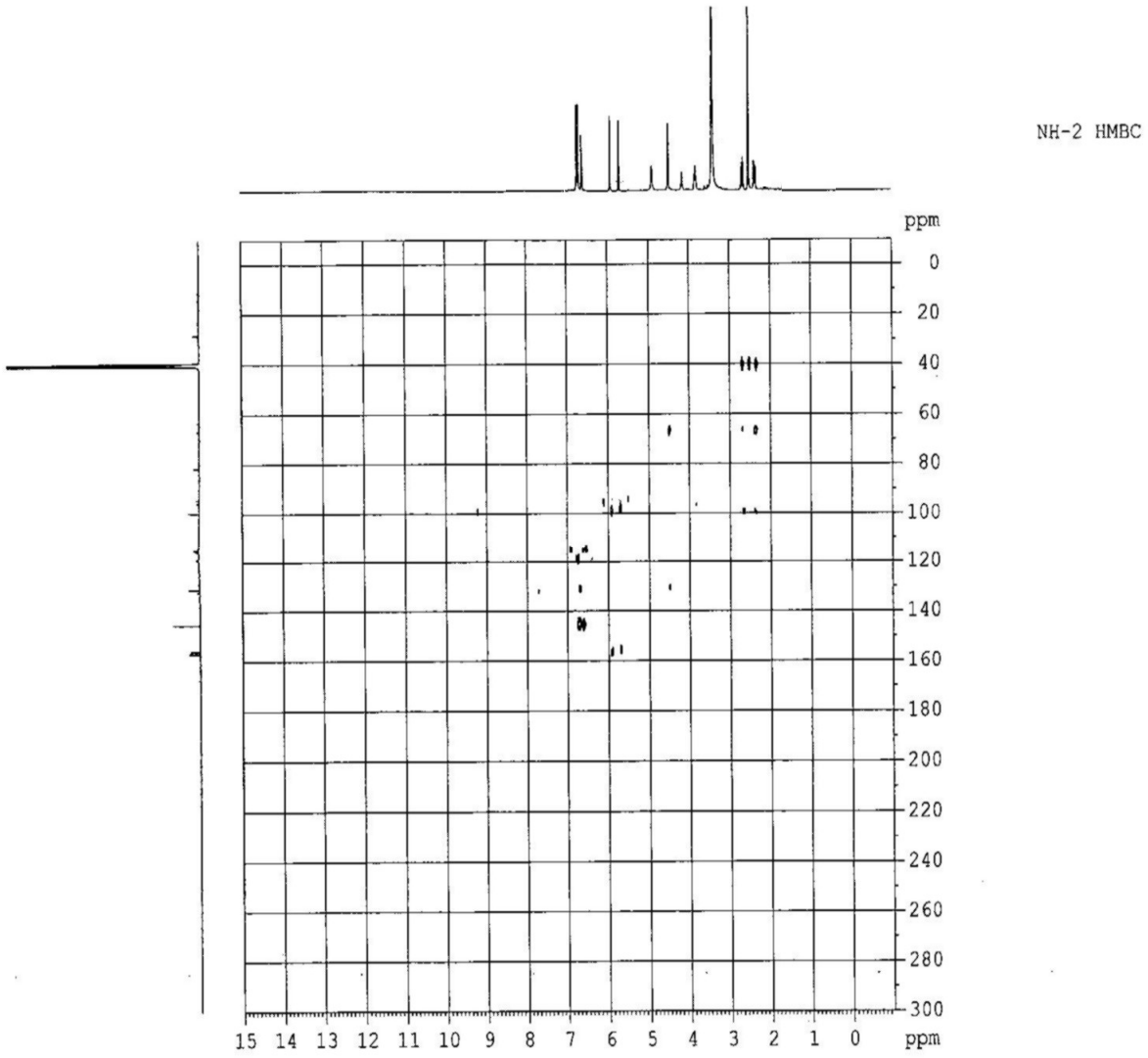


Figure 3.14 HMBC spectrum of compound 1.

All the long-range correlation observed are as shown in the **Table 3.4**

Table 3.4 Summary of NMR data of compound 1.

Carbon No.	^{13}CNMR δc, ppm	^{1}HNMR δ_H, ppm	COSY Correlations	HMBC Correlations
Flavon				
2	80.9	4.24 (7.6)	H-3	*C-1', C-3*
3	66.2	3.58 (7.4)	H-2 , H-4	-
4	28.3	2.42 (15.8, 5.6) 2.11 (16.0, 8.0)	H-3 , H-4b H-3 , H-4a	C-3 ,C-10 C-3 ,C-10
5	156.4	-	-	-
6	93.8	5.45 (2.0)	-	C-5, C-7, C-8
7	156.3	-	-	-
8	95.1	5.67(2.0)	-	C-7,C-9,C-10
9	156.1	-	-	-
10	98.9	-	-	-
1'	130.5	-	-	-
2'	114.6	6.44	-	C-3', C-4'
3'	144.8	-	H-2', H-6'	C-1', C-4'
4'	144.8	-	-	-
5'	118.5	6.49 (2.0)	H-6'	C-1', C-4'
6'	114.9	6.36 (2.0)	H-5'	*C-2' , C-1'*
Galloyl				
1''	118.3	-	-	-
2'' - 6''	105.9	6.46, 6.48	-	C-1'' , C-3'' ,C-5''
3'' - 5''	144.8	-	-	-
4''	155.3	-	-	-
C=O	166.9	-	-	-

The fragmentation pattern observed in the mass spectrum of compound 1 is well in agreement with the structure assigned (**Figure 3.7**).

CHAPTER 4

CONCLUSION

R. hemisphaerica root methanolic crude extract was fractionated in solvents of different polarities which included petroleum ether, chloroform, ethylacetate, butanol and water. TLC experiments on petroleum ether and chloroform fractions gave negative results. Butanol fraction was chromatographed on lipophilic sephadex (LH-20) using methanol and chloroform in 1:1 ratio as eluent and later fractions with yellow TLC spots were combined and further chromatographed on silical gel using Methanol: Chloroform (15:85) to obtain **compound 1** which was identified as catechin was indicated by intensification of the pale yellow colour on spraying the silica gel TLC spot with 5% methanolic sulphuric acid. This was further confirmed by its UV absorption (**Figure 3.5**), which showed λmax (MeOH) at 280 nm.**.** Antioxidant capacities of extracts were measured by utilizing DPPH radical scavenging and total phenolic content. In Figure 3.3 and Table 3.3 it can be seen that the 50% effective concentration (EC_{50}) of crude extract was found as 0.0234 ± 0.002µg/mL while butanol fraction revealed an EC_{50} value of 0.0209 ± 0.0012µg/mL. Compared with the EC_{50} value of **compound 1**, **0.02385 ± 0.00009 µg/mL**, it can be concluded that much of antioxidant activity of the crude extract comes from butanol fraction.

And the antioxidant activity of butanol fraction was mostly due to compound 1. The antoxidant capacity of compound 1, was not quite as effective as quercetin with an EC_{50} value of 0.0088 ± 0.0002µg/mL. Even though, it had significantly high EC_{50} value. From this, coupled with its considerably high amount in the butanol fraction, it was found worth characterising. NMR and MS techniques where employed in characterisation.

The **compound 1** was found with an elemental composition of $C_{22}H_{18}O_{10}$ as judged from its mass spectrum, with a $[M-H]^+$ ion at m/z 441 (**Figure 3.10**) and ^{1}HNMR (**Figure 3.6**) data. From all the above we have all reasons to conclude that **compound 1 is a catechin 3-O-gallate.**

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