CHARACTERIZATION OF ANTIOXIDANT AND ANTIMICROBIAL ISOLATES FROM *Quercus brantii* L. EXTRACT

A THESIS SUBMITTED TO
THE GRADUATE SCHOOL OF NATURAL AND APPLIED SCIENCES
OF
MIDDLE EAST TECHNICAL UNIVERSITY

BY

CAN NEBİGİL

IN PARTIAL FULLFILLMENT OF THE REQUIREMENTS
FOR
THE DEGREE OF MASTER OF SCIENCE
IN
CHEMISTRY

FEBRUARY 2011

Approval of the thesis:

CHARACTERIZATION OF ANTIOXIDANT AND ANTIMICROBIAL ISOLATES FROM Quercus brantii L. EXTRACT

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ABSTRACT

CHARACTERIZATION OF ANTIOXIDANT AND ANTIMICROBIAL ISOLATES FROM Quercus brantii L. EXTRACT

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M.Sc., Department of Chemistry

Supervisor : Assoc. Prof. Dr. Nursen ÇORUH

February 2011, 95 pages

This study was designed for the investigation of antioxidative, and antimicrobial properties of *Quercus brantii L. (Q.brantii.)* seed extract. Phenolic profile of the total extract was determined by using High Performance Liquid Chromatography (HPLC) and confirmed by High Resolution Mass Spectroscopy (HRMS).

Solvent fractionation was performed to seperate bioactive compounds in total extract by using solubility differences. In comparison of fractions, ethyl acetate and diethyl ether phases have revealed highest antioxidant effect. Due to the low yield and high number of the molecules in ethyl acetate fraction, HRMS was used to characterize the compounds. On the other hand, in diethyl ether fraction, there was a single major

compound which showed high antioxidant activity. The major compound, was purified by column chromatography and characterized by NMR, IR and HRMS as methyl gallate.

E.coli, P.mirabilis, S.aureus, S.pyogenes bacterial strains were used to determine the antimicrobial activity of *Q. brantii* seed crude extract, fractions and isolated compound by disc diffusion, MIC and MBC methods. Isolated methyl gallate and ethyl acetate fraction displayed a significant effect on all bacterial strains as high as reference antibiotics.

Consequently, crude *Q. branti* extract, isolated methyl gallate and ethyl acetate fraction could be considered as powerful antimicrobial agents, and at the same time efficacious antioxidants.

Keywords: *Quercus brantii L.*, Acorn, Antioxidant, Free radical, RP-HPLC, Isolatated compounds, Characterization, Antimicrobial.

ÖZ

QUERCUS BRANTİİ L. ÖZÜTÜNDEN ANTİOKSİDAN VE ANTİMİKROBİYAL İZOLATLARIN KARAKTERİZASYONU

NEBİGİL, Can

Yüksek Lisans, Kimya Bölümü

Tez Danışmanı: Doç.Dr. Nursen ÇORUH

Şubat 2011, 95 sayfa

Bu çalışma, *Quercus brantii L.* meşe palamudu tohum özütünün antioksidan ve antimikrobiyal özelliklerini araştırmak için tasarlanmıştır. Toplam özütün fenolik profilini elde etmek için yüksek basınçlı sıvı kromatografisi kullanılmıştır ve yüksek çözünürlüklü kütle spectroskopisi ile doğrulanmıştır.

Toplam özüt içinde bulunan biyoaktif maddelerin çözünürlük farklarından yararlanılarak fraksiyonlama tekniği ile ayrım yapılmıştır. Diğer fraksiyonlarla karşılaştırıldığında etil asetat ve dietil eter fazları en yüksek antioksidan aktiviteyi göstermiştir. Düşük verim ve içinde bulunan maddelerin çokluğu sebebi ile etil asetat fraksiyonunun içinde bulunan maddeler yüksek çözünürlüklü kütle spektroskopisi yardımı ile tanımlanmıştır. Diğer taraftan dietil eter fraksiyonunda antioksidan

aktivite gösteren bir adet majör bileşik saptanmıştır. Majör madde, kolon kromatografisi ile saflaştırıldıktan sonra, NMR, IR ve yüksek çözünürlüklü kütle spektroskopileri kullanılarak, metil galat olarak karakterize edilmiştir.

E.coli, P.mirabilis, S.aureus, S.pyogenes bakterileri hatları kullanılarak, *Q. brantii* tohum özüt, fraksiyonlar ve izole edilen saf maddenin antimikrobiyal aktivitesi, disk difüzyon, minimum inhibe edici konsantrasyon ve minimum bakterisidal konsantrasyon yöntemleri ile saptanmıştır. İzole edilen metil galatlın ve etil asetat fraksiyonunun tüm bakteriler üzerinde referans antibiyotikler kadar etkili olduğu görülmüştür.

Sonuç olarak, *Q. brantii* özütü, izole edilen metil gallat ve etil asetat fraksiyonu etkili birer antimikrobiyal unsur ve aynı zamanda tesirli antioksidan olduklarını önerilebilir.

Anahtar sözcükler: *Quercus brantii L.*, Meşe palamudu, Antioksidan, Serbest radikal, Ters fazlı- yüksek basıçlı sıvı kromotografisi, Bileşiklerin izolasyonu, Karekterizasyon, , Antimikrobiyal.

ACKNOWLEDGEMENTS

I wish to express my sincere appreciation and thanks to my supervisor Assoc. Prof. Dr. Nursen Çoruh for her guidance, valuable advices, moral support and for enlightening my professional and academic vision throughout my study.

I would like to express my sincere thanks to Yeşim Kümbet for her endless support, patience and motivation throughout this work.

I would like to thank to NMR specialists Seda Karayılan and Zehra Uzunoglu for the NMR, Binnur Özkan and Çağtay Dengiz for mass experiments.

I would like to express my great thanks to all the members of Nursen Çoruh Research Group especially to Nizamettin Özdoğan and Burak Barut for their friendship and helps.

I wish to express my special thanks to Merve Gökçen Bekarlar for her support, friendship and encourage when I need.

I would like to thank to my friends Berk Müjde, Bahadır Doğan, Cansel Işıklı, Alper Kılıklı, Melek Sermin Özer, Tamer Tezel for their precious friendship.

I wish to express my appreciation to the academic staff of METU Department of Chemistry for their professional support and guidance to the students of Department of Chemistry.

Finally, I would like to give the biggest thanks to my mother and my sister who have made everything possible for me with their love, affection, support and guidance throughout my whole life. The completion of this study would not have been possible without them.

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LIST OF ABBREVIATIONS

mg:	Milligram
mL:	Milliliter
µL:	Microliter
mm:	Millimeter
nm:	Nanometer
ABTS:	2,2'-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid)
DPPH:	2,2-diphenyl-1-picrylhydrazyl
TEAC:	Trolox equivalent antioxidant capacity
GAE:	Gallic acid equivalent
CFU:	Colony forming unit
MIC:	Minimum inhibitory concentration
MBC:	Minimum bactericidal concentration
a.u.:	Absorbance unit
HPLC:	High pressure liquid chromatography
HPLC-DAD:	Photo diode array dedector
NMR:	Nuclear magnetic resonance spectroscopy
RSA:	Radical scavenging activity
SD:	Standard deviation

To you..

CHAPTER 1

INTRODUCTION

1.1 Quercus brantii Lindley

Turkey is one of the richest countries in the world regarding plant diversity. To date approximately 10,500 plant species have been found and 30% of the species are endemic. Since having multiple climate zones, Southern Anatolia has many ecological areas. This variety results a rich source of medicinal plants so that a great number of ancient Anatolian civilizations were assessed and still using in rural areas (Cakilcioglu, 2010).

Quercus species consist of 531 tree and shrub species distributed in northern hemisphere and widely distributed in Turkey. *Quercus brantii Lindley* is a common plant type for Turkey's dry forests which are placed in Southern and Southeastern Anatolia. They are found at high elevation between 350 to 1700 m in South Anatolia, mainly Southeastern Taurus including Şerafettin and Bitlis complex since their growth is best at volcanic sands and tuff (Kaya, 2001). It can be also found in Elazığ, Hakkari, Kahramanmaraş, Malatya, Mardin, Siirt and Şanlıurfa. It is known as "İran Palamut Meşesi" in Turkey.

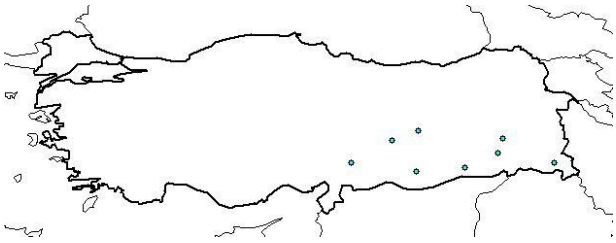


Figure 1 Distribution of the taxon over Turkey (TÜBİVES)

1.1.1 Botany

Acording to United States Department of Agriculture Quercus brantii Lindley is classified as (USDA):

Kingdom:	Plantae
Subkingdom:	Tracheobionta
Division:	Magnoliophyta
Class:	Magnoliopsida
Subclass:	Hamamelidae
Order:	Fagales
Family:	Fagaceae
Genus:	Quercus L.
Species:	Quercus brantii Lindley (Brant's oak)

It is a small tree which can grow up to 6 to 10 meters high (Aksekili, 2007). It blooms, with yellow or green flowers in males and yellow-pink flowers in female plants, from August through September, and its green seed pots split open to release the shiny brown seeds in September. The fruit of this plant is a rich glossy brown to blackish – brown nut 2-3 cm diameter. That brown fruits are called acorns (Agaclar).

1.1.2 Employment of Quercus brantii Lindley in field of medicine

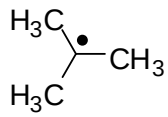
The use of acorn as a dietary intake has been known since end of 19^{th} century (Rakic, 2006). Besides, considering acorn as fruit many civilizations use it to cure diseases. According to José A. González et al., in western Spain, acorns are used in the treatment of diarrheal diseases by just eating (Gonzalez, 2010). In Bulgaria balm made by acorn is used against dermatitis and in Italy the tea of acorn is known for anti-inflammatory, mild antiseptic, anti-haemorrhagic affects (Leporatti, 2003). Not only its fruit but also other parts of the plant have medicinal values. For example, its

bark is used in the treatment of acute diarrhea, weeping eczema, febrile and infectious diseases and skin damage (Blumenthal, 1998).

1.2 Free radicals

Electrons of atoms or molecules occupy spatial volume elements are called orbitals. Each orbital contains maximum two electrons in opposite directions. Most chemical species in nature, have two electrons in each orbital, on the contrary, free radicals are the molecules or the molecular fragments which have an unpaired electron in their outer orbital. They are represented by the superscript dot, $R^{\bullet}$ (Halliwell, 1995).

There are two types of radicals upon their charge; neutral radicals **1** and charged radicals **2** and **3**.



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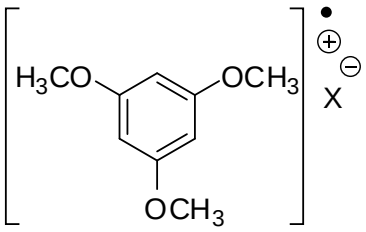

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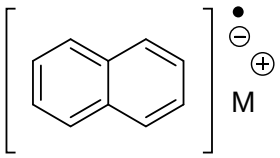


(3)

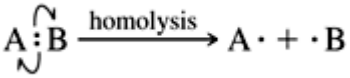
Moreover, radicals are classified in to two according to the position of the unpaired electron. If the unpaired electron is in the σ orbital like in 4, radical is called σ radical or if in it is in the π orbital radical is called π radical (Togo, 2004).

(4)

(5)

1.2.1 Radical formation

Radicals are generally formed by homolytic dissociation of covalent bond mainly by heat or light;



Without being radical, some molecules play role in the formation of free radicals like singlet oxygen or triplet oxygen. By the help of light, sensitizers or even other radicals present in the medium get dioxygen molecule to the excited state. The energy difference between ground state and singlet oxygen is 94.3 kJ/mol. Figure 2 show the molecular formation of singlet oxygen.

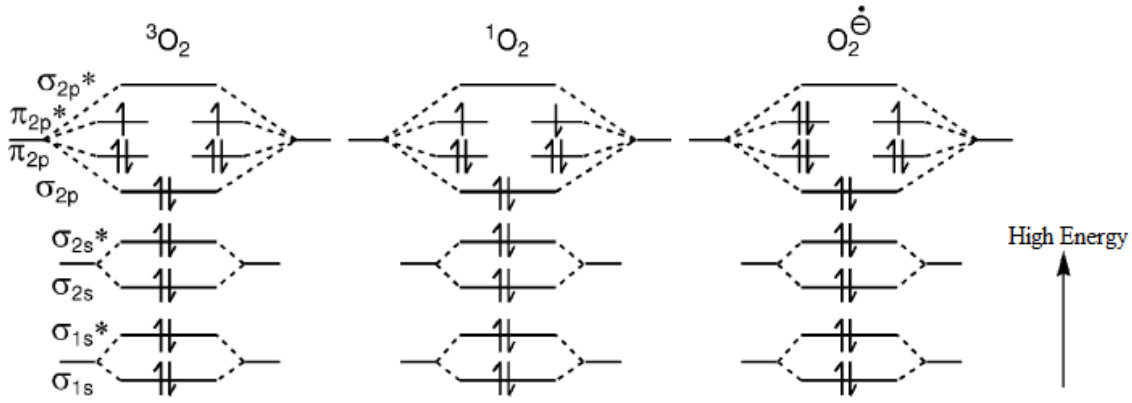


Figure 2 Electron configurations of molecular oxygen and related radical

Electrophilic singlet oxygen can directly react with electron rich compounds containing double bonds or with hydrocarbons and produce peroxides. These peroxides can easily dissociate and form oxygen radicals (Togo, 2004).

1.2.2 Radical reactions

Almost all small radicals are short-lived, highly reactive molecules. If these radicals collide with other molecules, they tend to react by pairing their unpaired electron in many ways (Solomon, 2007) as:

Addition reactions

$A^{\bullet} + B \rightarrow AB'$

Substitution reactions;

$A^{\bullet} + BC \rightarrow AB + C^{\bullet}$.

Elimination reactions;

$ABC^{\bullet} \rightarrow CB + A^{\bullet}$.

Oxidation reactions;

$A^{\bullet} + B \rightarrow A^{-} + B^{+\bullet}$

Reduction reactions;

$A^{\bullet} + B \rightarrow B^{-\bullet} + A^{+}$

1.2.3 Mechanism of radical reactions

Mechanisms of free radical reactions are composed of a cycle of repeating steps which form new reactive intermediates and these intermediates cause the next reaction to occur. This process is called chain reaction.

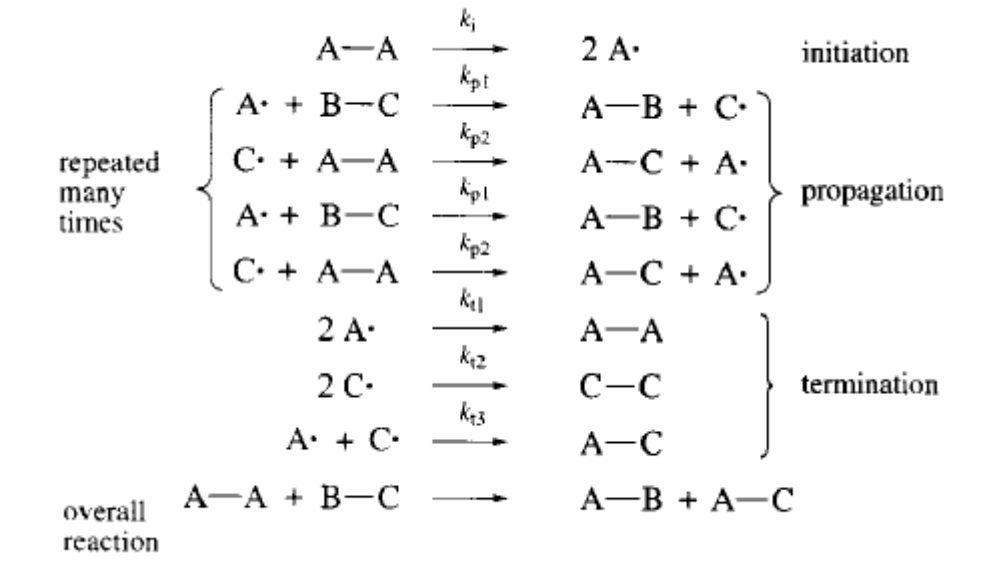
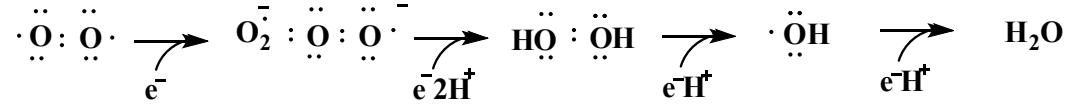


Figure 3 Radical reaction steps

The first step in this example where the $A^{\bullet}$ is formed, called initiation step. In this step radicals are created, generally by using light or heat. In the next four steps a sequence of two reactions are repeated. These sequences are called propagation step. Since chain reactions are characterized by chain length which is the number of propagation steps, this step identifies the reaction. Lastly, the reaction intermediates in propagation phase are destroyed in the termination step (Carey, 2008).

1.2.4 Free radical reactions in biological systems

In biological systems free radical system is mainly depend on oxygen, nitrogen and heavy metal containing molecules. Reactive oxygen species (ROS) are first discovered free radicals in biological materials. ROS such as superoxide, hydroxyl, alkoxy, peroxy and nitric oxide radicals are derived from molecular oxygen during the normal oxygen metabolism (Pala, 2007). Although molecular oxygen is not a radical, it has a critical role on producing oxygen radicals.



Molecular Oxygen **Superoxide Radical** **H_2O_2(Hydrogen Peroxide)** **(Water)**

$H_2O + OH^{\bullet}$

Production of highly reactive oxygen species (ROS) is an integral feature of normal cellular function like mitochondrial respiratory chain, phagocytosis, arachidonic acid metabolism, ovulation, and fertilization (Singh, 2004).

For example in respiratory process oxygen is used as an electron acceptor and it is reduced to water;

$$O_2 + 4e^- + 4H^+ \longrightarrow 2H_2O$$

Radical formation can be observed if the subsequent process is observed;

$$O_2 + e^- + H^+ \rightleftharpoons HO_2^{\bullet} \rightleftharpoons O_2^{\bullet} + H^+$$
$$HO_2^{\bullet} + e^- + H^+ \rightleftharpoons H_2O_2$$
$$H_2O_2 + e^- + H^+ \rightleftharpoons [H_3O_2] \rightleftharpoons H_2O + {}^{\bullet}OH$$
$$OH^{\bullet} + e^- + H^+ \rightleftharpoons H_2O$$

These generated or externally taken oxygen radicals cause imbalance between the production of reactive oxygen species and biological system's ability to readily detoxify this reactive intermediates. This situation is called oxidative stress. Oxidative stress can cause damage in all components of the cell. For example; ROS react with lipids on the cell membrane and cause lipid peroxidation. More dangerously, these species can react every part of DNA. It can damage both purine and pyrimidine bases and also deoxyribose backbone (Buonocore, 2010).

The second main radicals in biological systems are reactive nitrogen species (RNS). Nitrogen oxide, nitrogen dioxide and peroxynitrites are the common examples of them. Nitric oxide is a common reactive radical which has as an important role in oxidative biological signaling and in a large variety of diverse physiological processes, including neurotransmission, blood pressure regulation, defence mechanism, smooth muscle relaxation and immune regulation (Valko, 2007).

Although nitrogen oxide show protective effect in tissues, it can also form perozynitrate and once it threated with superoxide it forms peroxynitrate ion or peroxynitrous acid. Peroxynitrous acid can easily decay in to most reactive radical hydroxy radical and nitrogen dioxide radical and peroxynitrite ion which is an oxidizing free radical that can cause DNA fragmentation and lipid oxidation (Jung, 2009).

Third source of radicals in biological systems are transition metals, like iron and chromium. Since these two heavy metals can both gain or lose electrons easily, that makes them two common catalysts for redox reactions. Fenton reaction is a common example for iron catalyst radical reactions (Pala, 2007).

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

$Fe^{3+} + H_2O_2 \rightarrow Fe^{2+} + OOH^{\bullet} + H^{+}$

Iron is also the major component of red blood cells. In deoxyhemoglobin, iron is reduced in a ferrous state (Fe II), but, when deoxyhemoglobin is attached with oxygen and turn into hemoglobin, in this process, an intermediate structure occurs in which an electron is delocalized between ferrous ion and oxygen.

This intermediate result to, Fe (II) bound to oxygen and Fe (III) bound to superoxide. Sometimes the oxyhemoglobin molecule goes into decomposition and release superoxide anion (Buonocore, 2010):

Heme $Fe^{+2}-O^2 \rightarrow$ Heme $Fe^{+3}-O_2^{-}$

Heme $Fe^{+2}-O_2^{\bullet} \rightarrow O_2^{\bullet}$ + Heme Fe^{+3}

Chromium (III) is an essential trace element which plays an important role in regulating blood levels of glucose. On the other hand Chromium (IV) is toxic and carcinogenic at high doses. Figure 4 illustrates an example of reduction of Cr (III) to Cr (IV) in our body (Valko, 2006).

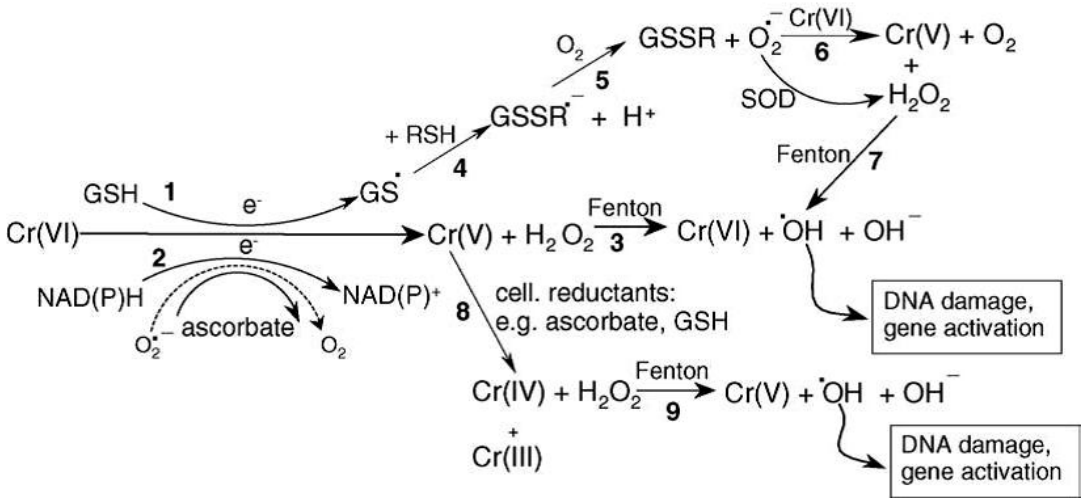


Figure 4 Role of chromium reactions in our body (Valko, 2006)

1.2.5 Free radical related disorders

Reactive oxygen species and reactive nitrogen species are formed through a variety of events and pathways have shown before. These highly reactive species interact various components of body and causes serious damages.

It has been estimated that a human cell is exposed to approximately 1.5×10^5 oxidative hits a day from free radicals and other reactive species. Radicals, such as hydroxyl radical can react with all vital components and elements in the cell like DNA molecule. Purines, pyrimidine bases and also the deoxyribose backbone can be target. Single or double stranded DNA breaks, base or deoxyribose modifications and DNA modifications are occurred via these reactions. Permanent modification of genetic material caused by oxidative damage incidents results the first step of mutagenesis, carcinogenesis, necrosis and ageing (Dizdaroglu, 2007).

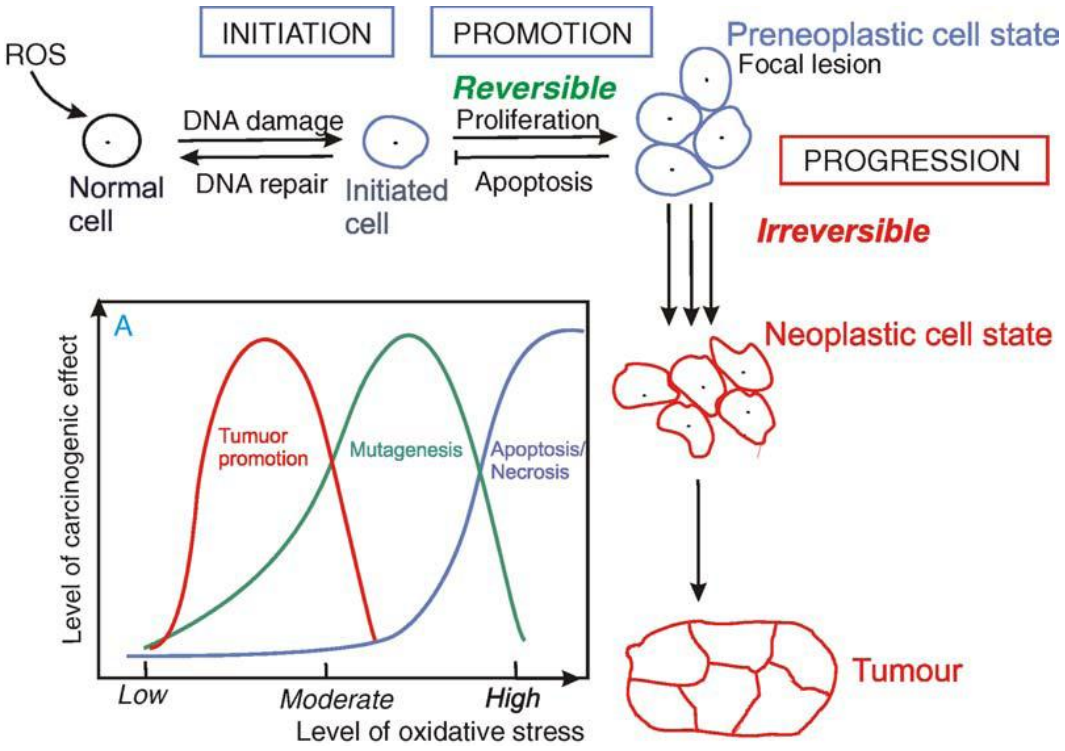


Figure 5 Role of free radicals at stages of carcinogenesis process (Valko, 2006)

Generated oxygen radicals are not only attack to DNA in the cell nucleus, but also attack to other cellular components including polyunsaturated fatty acid residues of phospholipids, which are extremely sensitive to oxidation. The experiments in the literature suggest that mitochondrial PUFA are a preferential target for iron-driven peroxidation. The deleterious process of the peroxidation of lipids is very important in arteriosclerosis, stroke, heart diseases, tumor supression and inflammation (Brown, 2001).

Reactive oxygen radicals are generated as by-products of many metabolic processes, including monoamine metabolism. The hydroxyl radicals have been implicated in dopaminergic neurotoxicity caused by MPTP (1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine). MPTP, which is not toxic, is a lipophilic compound. It can cross the blood-brain barrier. Once inside the brain, MPTP is metabolized into the toxic cation MPP^+ (1-methyl-4-phenylpyridinium) by the enzyme MAO-B of glial cells.

MPP^+ primarily kills dopamine-producing neurons in a part of the brain called substantia nigra and leads to Parkinson's disease (Obata, 2002).

1.3 Antioxidants

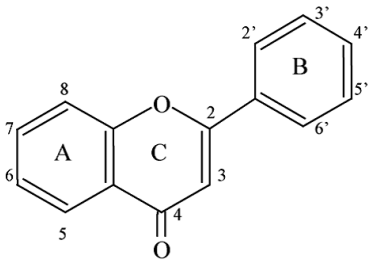
Halliwell & Gutteridge claims that, any substance that, when present at low concentrations compared with that of an oxidizable substrate, significantly delays or inhibits oxidation of that substrate is defined as antioxidants (Sies, 1997). The antioxidants effects have various functions besides reducing oxidant species like hydroperoxides and hydrogen peroxide, they can also scavenge active free radicals, and repairing and/or clearing the damage caused by them (Noguchi, 2000).

1.3.1 How antioxidants scavenge free radicals

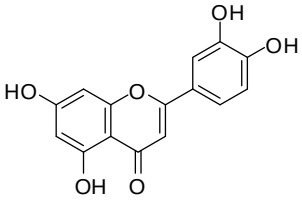
Antioxidants are mainly derivatives of secondary aromatic amines, phenols, organic phosphites and sulfides. In modern biomedical literature, both synthetic and natural antioxidants are represented by a broad class of phenolic compounds, whose valence saturated molecules containing an active hydrogen atom (InH). When these compounds react with active free radicals they turn into radicals which do not participate in chain propagation step.

$$R^{\bullet} + InH \rightarrow RH + In^{\bullet}$$

Since more substituted radical is most stable radical, steric effect of these radicals especially OH shielded ones, prevent the propagating chain reactions of these oxygen radicals (Kolotver, 2010). It is not the only fact that making phenolic compounds stable radicals. The ability of producing tautomeric dislocation of the radical position, substituents attached to compound, presence and location of double bonds and carbonyl groups are also important for the scavenging activity (Marin, 2002 & Cos, 2000).



If B-ring-localized semiquinones are investigated in order to demonstrate the how important of these options for antioxidant activity, it can be clearly observed that; (I) The o-dihydroxy (catechol) structure in the B ring which confers great stability to the aroxyl radicals and which participates in electron dislocation; (II) The double bond between carbons 2 and 3 in conjugation is responsible for electron dislocation from the B ring; and in the presence of both 3 and 5 hydroxyl groups for maximal radical scavenging capacity and strongest radical absorption; (III) When antioxidant activity of flavonoids is evaluated in liposomal systems, the 3-hydroxy group becomes less importance and an o-hydroxylation at A-ring is become a requirement for antioxidant activity (Heijnen, 2001).



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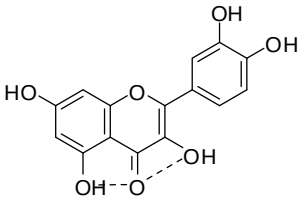

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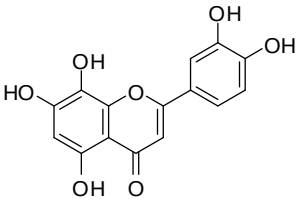


(III)

Moreover, these type of antioxidants can scavenge free radicals but they can also protect radical formation resulted by Fenton reaction. As discussed before, heavy metals like iron and cupper can produce free radicals. However, with the help of antioxidants like flavonoids, with their ability to chelate transition metals, these compounds inactivate iron ions and other biavalent compounds through complexation (Afanav'es, 1989).

1.3.2 Types of antioxidants

Antioxidants can be classified as enzymatic or non-enzymatic physiological antioxidants. Enzymatic antioxidants such as; superoxide dismutase, glutathione peroxidase, catalase, thioredoxine, glutaredoxin (Powers, 2000) are type of antioxidants which are enzymes that produced endogenously by various components of different cells. Non-enzymatic antioxidants such as; vitamins A,C,E, flavonoids and polyphenolic compounds are contributed from diet and help antioxidants system of body (Singh, 2004 & Powers, 2000).

1.3.2.1 Enzymatic antioxidants

The enzymatic antioxidant system includes enzymes, such as superoxide dismutase (SOD), glutathione peroxidase (GSHPx), catalase, etc. In example of this system; SOD catalyzes the dismutation of $O_2^{\bullet -}$;

$$2\ O_2^{\bullet -} + 2H^+ \xrightarrow{SOD} H_2O_2 + O_2$$

(Gutteridge, 1994)

Human cells have a manganese-containing SOD (Mn-SOD) in the mitochondria, whereas the copper- and zinc-containing SOD (Cu,Zn- SOD) is primarily present in the cytosol. Two enzyme systems exist to catalyze the breakdown of H_2O_2. Firstly, the enzyme catalase, which is located in the peroxisomes, converts H_2O_2 into H_2O and O_2.

$$2H_2O_2 \xrightarrow{Catalase} 2H_2O + O_2$$

Secondly, the group of selenium-containing glutathione peroxidases uses the remaining H_2O_2 as an oxidant to convert reduced glutathione (GSH) to oxidized glutathione (GSSG).

$$2GSH + H_2O_2 \xrightarrow{GSHPx} GSSG + 2H_2O$$

(Cos, 2000)

1.3.2.2 Non-enzymatic antioxidants

Although body produce non-enymatic antioxidants such as uric acid and bilirubin, remaining antioxidant need is supplied by endogenously mainly from plants. Phenolic compounds in fruits, vegetables and grains are natural sources of this need (Shaidi, 2004). They are considered as secondary metabolites of plants and are produced during growth derived by tyrosine and phenylalanine (Beckman, 2000).

1.3.3 Antioxidant compounds in plants

Resveratrol, in grape, peanut and pine, (Wang, 2010), α-tocopherol (vitamin E), in wheat, sunflower seed and oil, kiwi, mango (NIH), L-ascorbic acid (vitamin C), in green peppers, citrus fruits, strawberries, tomatoes (NIH), are common and vital examples of phenolic compounds. Plants also include great range of variety and diversity as a group of phytochemicals such as simple phenols, coumarins, stilbenes, phenolic acids, flavonoids, hydrolyzable and condensed tannins, lignans, and lingins. Figure 6 illustrates phenolic compounds in plants (Chi, 1991).

These isolated phenolic compounds can be either used as antioxidant or/and antibacterial compounds.

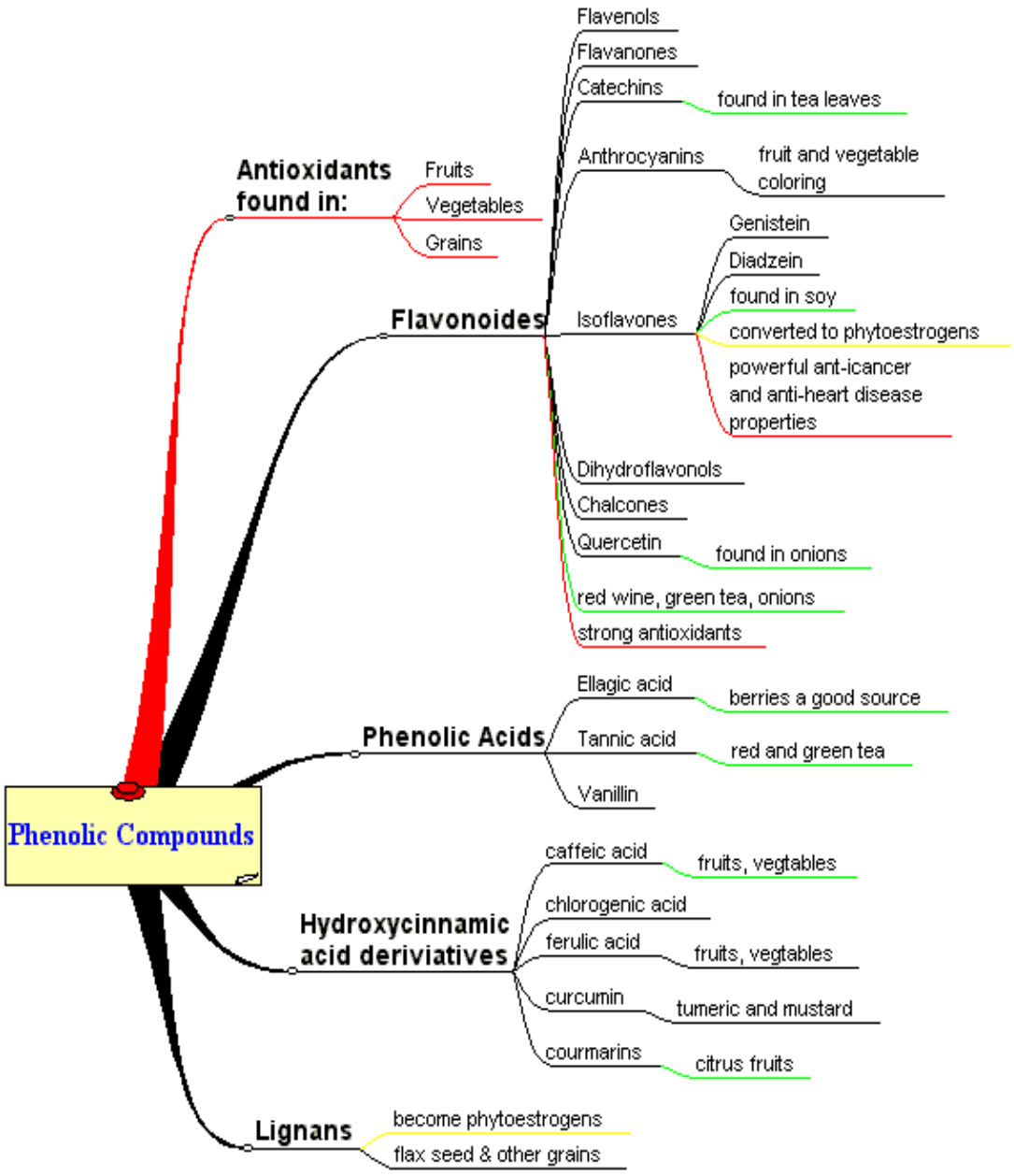


Figure 6 Phenolic compounds in food (Chi, 1991)

1.4 Antibacterial activity

In developing countries bacterial infections are commonly seen especially in rural areas due to sanitation and unhygienic conditions. With mutagenesis, in bacterias get resisted to the antibiotics and pathogens are becoming more dangerous not only in rural areas but also in big cities. This leads scientists to find effective compounds for pathogens. In order to test the new compounds whether they are effective or not firstly in vitro studies such as disc diffusion test, minimum inhibitory concentration test and minimum bactericidal concentration test are done against various bacterial strains.

1.4.1 Bacteria used in this study

4 bacterias were used in this study. *Escherichia coli*, *Staphylococcus aureus*, *Proteus mirabilis* and *Streptococcus pyogenes*.

1.4.1.1 *Escherichia coli*

Scientific classification of *Escherichia coli* is;

Kingdom: Bacteria

Phylum: Proteobacteria

Class: Gamma Proteobacteria

Order: Enterobacteriales

Family: Enterobacteriaceae

Genus: Escherichia coli

Species: *E. coli*

Escherichia coli is a gram negative rod-shaped bacterium that is commonly found in the lower intestine of warm-blooded organisms. From a genetic and clinical perspective, this bacterias biological importance to humans can be broadly categorized as commensal E. coli and intestinal pathogenic E. coli. Commensal E. coli is harmless intestinal colonizers. On the other hand, intestinal pathogenic E. coli having enteric or diarrheagenic strains, can play a resident role for other bacterias and associate with diseases such as urinary tract infections, neonatal meningitis and intestinal diseases (Johnson, 2005).

1.4.1.2 *Proteus mirabilis*

Scientific classification of *Proteus mirabilis* is,

Kingdom: Bacteria

Phylum: Proteobacteria

Class: Gamma Proteobacteria

Order: Enterobacteriales

Family: Enterobacteriaceae

Genus: *Proteus*

Species: *P. mirabilis*

Proteus species are part of the Enterobacteriaceae family of gram-negative bacilli. It is found in the human intestinal tract as part of normal human intestinal flora, along with *Escherichia coli* and *Klebsiella* species, of which *E. coli* is the predominant resident. *Proteus mirabilis* causes 90% of *Proteus* infections and can be considered a community-acquired infection. It results mainly urinary tract and kidney infection in body (Struble, 2009).

1.4.1.3 *Staphylococcus aureus*

Scientific classification of *Staphylococcus aureus* is,

Kingdom: Bacteria

Phylum: Proteobacteria

Class: Gamma Proteobacteria

Order: Enterobacteriales

Family: Enterobacteriaceae

Genus: *Staphylococcus aureus*

Species: S. *aureus*

Staphylococcus aureus is gram positive bacteria which is the one of the most common causes of nosocomial or community-based infections, leading to serious illnesses such as pneumonia, meningitis, osteomyelitis, endocarditis, toxic shock syndromewith high rates of morbidity and mortality. During recent years, the increase in the number of bacterial strains showing resistance to methicillin or oxacillin has become a serious clinical and epidemiological problem for several reasons (Bou, 2007).

1.4.1.4 *Streptococcus pyogenes*

Scientific classification of *Streptococcus pyogenes* is,

Kingdom: Bacteria

Phylum: Firmicutes

Class: Gamma Proteobacteria

Order: Lactobacilliales

Family: Streptococcaceae

Genus: *Streptococcus*

Species: *S.pyogenes*

Streptococcus pyogenes is gram-positive bacteria which grows in long chains and cause Group A streptococcal infections. The bacteria commonly produce large zones on blood agar plates by disrupting erythrocytes and release hemoglobin. For that reason the bacteria is also named as Group A (beta-hemolytic) Streptococcus. It is known by folk as beta microbe, causes tonsillar inflammation especially in children. In addition to tonsillitis, pharyngitis, cellulite, sinusitis, mastoiditis, pneumonia, ampiem, osteomyelitis, meningitis, streptococcal pyoderma, ecthyma, and streptococcal toxic shock syndrome, as nonsporulating complication acute rheumatic fever, and many more can be accounted among the diseases that the bacterium causes (Uzel, 2004 & Hanna, 2006).

1.4.2 Antibacterial properties of phenolic compounds

It has been reported that phenolic compounds have useful properties, including anti inflammatory activity, oestrogenic activity, enzyme inhibition, antimicrobial activity (Havsteen, 1983) antiallergic activity, antioxidant activity, vascular activity and cytotoxic antitumour activity (Grange, 1990). For a group of compounds phenolic compounds having relatively homogeneous structure, inhibit a perplexing number and variety of eukaryotic enzymes, inhibit or kill many bacterial strains such as reverse transcriptase and protease, and destroy some pathogenic protozoans. In the case of enzyme inhibition, this has been postulated because of the interaction of enzymes with different parts of the molecule, in example carbohydrate, phenyl ring, phenol and benzopyrone ring (Havsteen, 2002).

There are many examples of phenolic compounds in literature which shows high antioxidant and also high anti bacterial activities. In example; Silymarin is a flavonoid isolated from milk thistle had shown free radical scavenging activity in both vitro and vivo studies. It also showed beneficial effects on radiation injury of liver cells (Soto, 1998) and it is currently used as a drug. Its antibacterial effects are tested upon various bacterial strains like *Escherichia coli, Bacillus subtilis*, *Staphylococcus epidermidis*, *Proteus vulgaris*, *Candida albicans*, *Saccharomyces cerevisiae*, and *Trichosporon beigelii*. In both bacteria silymarin showed high antibacterial activity (Lee, 2003).

1.5 Scope of the work

This study was designed to investigate the antioxidative, and antimicrobial properties of seeds of *Q.brantii L.* extract for the first time. Isolation of bioactive compounds from the seeds were carried out by utilizing the HPLC-DAD analysis. Isolates then were identified and characterized. Furthermore, the isolated compounds were examined for their antioxidative activity and for their antimicrobial activity on four bacterial strains.

CHAPTER 2

MATERIALS AND METHODS

2.1 MATERIALS

2.1.1 Chemicals

Preparative chromatography grade methanol used in the preparation of extracts was purchased from Merck (Darmstadt, Germany).

For the solvent system performed with HPLC, HPLC grade methanol, acetonitrile, purchased from Merck (Darmstadt, Germany) and for obtaining ultra pure water (18.2 M ohm.cm) Milli-Q system (Milli-pore, Bedford, MA, USA) was used.

Disposable syringe filters (pore size: 0.22 μm and 0.45 μm Diameter: 33mm) were purchased from Millipore Corporation (Bedford, MA USA) before injection of extracts or standards.

For the fractionation process chromatography grade hexane, diethyl ether, ethyl acetate, methanol was purchased from Merck (Darmstadt, Germany), and magnesium sulfate was purchased from Sigma Chemical Company (St.Louis, MO, USA).

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

Gallic acid, ellagic acid, caffeic acid, citric acid, vanilic acid, rutin, quercetin, esculetin, esculin, scopoletin, epi-catechin, p-coumaric acid were, 2,2'-azino-bis (3-ethylbenzthiazoline-6-sulphonic acid) (ABTS), 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (trolox), 2,2-diphenyl-1-picrylhydrazyl (DPPH), sodium carbonate (Na_2CO_3), sodium hydroxide, aluminum chloride were purchased from Sigma Chemical Company (St.Louis, MO, USA). Folin Ciocalteu's phenol reagent was purchased from Merck (Darmstadt, Germany).

D_2O, CD_3OD, and $CDCl_3$ used in NMR experiments was purchased from Merck (Darmstadt, Germany).

Proteus mirabilis (code: RSKK 737, Pasteur Institute), *Escherichia coli* (code: RSKK 234, Pasteur Institute), *Staphylococcus aureus* (code: RSKK 95084, Pasteur Institute), *Streptococcus pyogenes* code: (RSKK 03019, ATCC 19615) used for antimicrobial assays was obtained from Refik Saydam Hygiene Center.

Luria Bertani agar and Luria Bertani broth were purchased from Merck (Darmstadt, Germany).

Ready to use agar plates, which contain sheep blood agar medium, for stock cultures of bacteria, were purchased from OR-BAK (Istanbul, Türkiye).

6 mm diameter discs for antimicrobial susceptibility test used in disc diffusion method were purchased from Oxoid (Hants, UK). Standard antimicrobial discs Gentamycin (10 mcg) and Kanamycin (30 mcg) were bought from Bioanalyse (50 susceptibility tests for in vitro diagnostic use).

Gentamycin powder (cell culture tested grade) was purchased from Sigma-Aldrich (Germany).

2.1.2 Apparatus

All HPLC analysis were carried out using a analytic and semi preperative apparatus equipped with an online degasser unit and detection systems both photodiode array (2996 Waters) detectors and a multi wavelength fluorescence detector (Waters, 2475). A multi solvent delivery system (Waters, 600E), 20mL and 100μl sample loops were used for semi-preparative and analytical HPLC sample injections, respectively. The manual sample injectors for semi-preparative (Hamilton 1005 LTN) and analytical (Hamilton model 700 Nr) were used. Stationary phases used for analytical methods was a Symmetry C18 reverse phase column, 5 μm, 4.6 x 150mm (Part No: WAT045905) was utilized. Data acquisition and quantification were carried out with Waters Empower Software.

All the spectroscopic studies were carried out with a Cary 50 Bio UV-VIS spectrophotometer (Varian). Other apparatus used for the experiments were; ultrasonic bath (Bandelin Sonorex); Oval-rotating incubator (Optic Ivymen System); rotary evaporator (Heidolph Laborota 4000); stainless-steel blender (Waring, 32BL80); NMR, C-NMR 100 MHz and H-NMR 400 MHz (Bruker-Spectrospin 400 ultrashield); FT-IR (Vertex 70 Bruker).

Class II Safety Cabinet (ESCO, Thailand) was used for antimicrobial assays. ELISA, 96-well microplate reader (Bio-tek, Elx808, Germany) was used from Prof. Dr. Mesude İşcan Laboratory in Biological Sciences Department, METU.

Scientific, 12 multichannel 5-50 µL, 12 multichannel 100-300 µL, and 8 multichannel 5-50 µL pipettes (Thermo Electron Corporation, Finnpipette) were used through out the experiments.

2.2 Methods

2.2.1 Crude extraction of *Q. branti* Lindley acorns

Seeds of *Q. branti* obtained from Van Yüzüncü Yıl University and dried under shade. They were ground with Waring blender. Samples, 50 g were extracted in methanol with 1:12 ratio for 24 hours in oval-rotating incubator rotating at 175 rpm at 30 °C. Extraction mixtures were filtered through a rough watman filter paper and filtrates were evaporated using rotary evaporator at 35 °C to dryness. Dried extract was weighed out and yield of extraction was calculated in percent. Extracts were stored at 4 °C in refrigerator.

2.2.2 Isolation of plant constituents

2.2.2.1 Fractionation of extract

Solubility of phenolic compounds is related to the polarity of solvent used in fractionation method. Plant constituents are differentiated according to that polarity difference between water and solvent of choice for partitioning the total extract. This process is called fractionation (Naczk, 2004). In this assay hexane, diethyl ether, ethyl acetate and methanol/water were used in the order of polarity as described in figure 10. Fractionation yields of each fraction were calculated and their radical scavenging and antibacterial activity were determined. Each fraction was analyzed through HPLC and chemical constituents were identified.

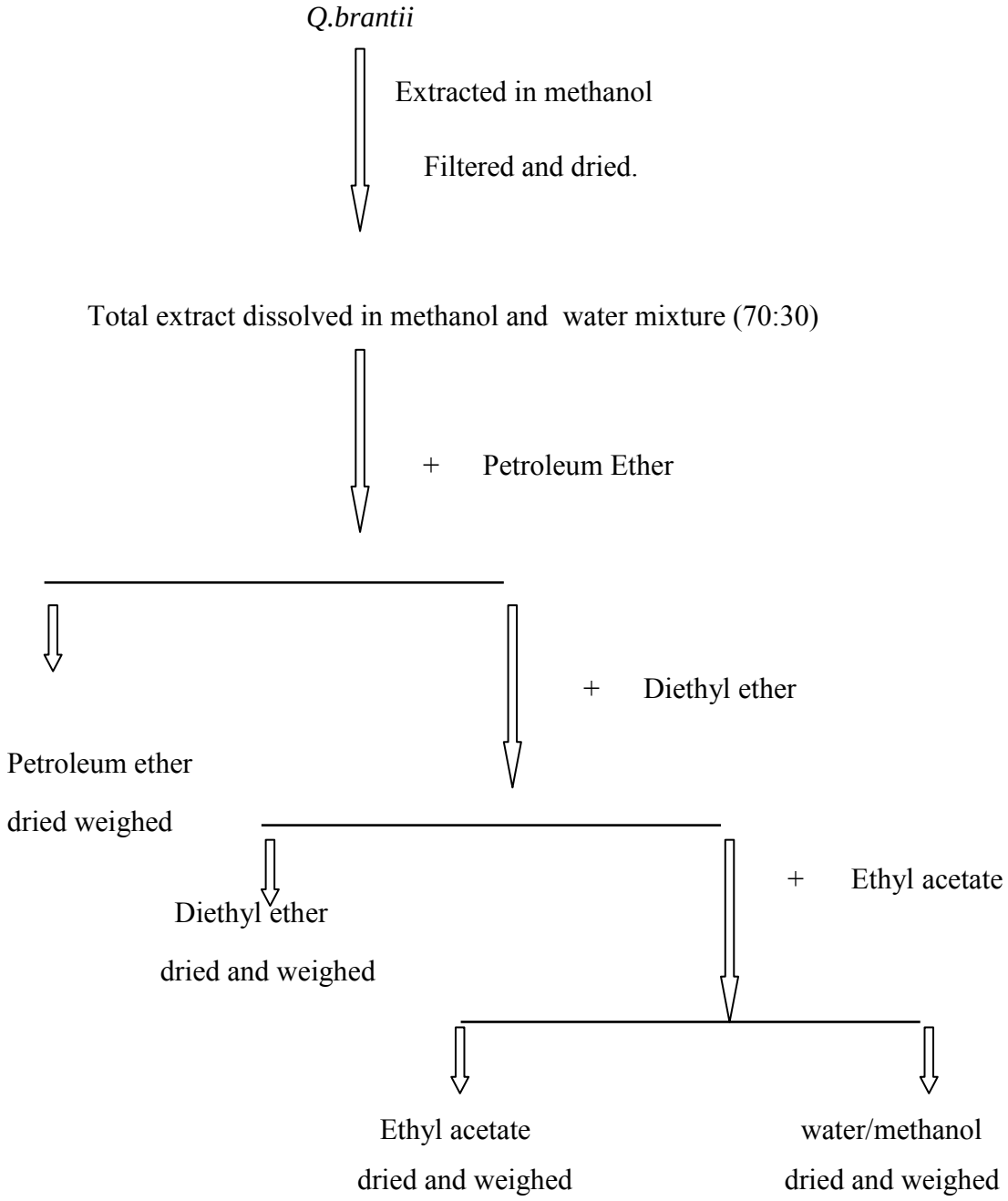


Figure 7 Fractionation process

2.2.2.2 Analytical High Performance Liquid Chromatography (HPLC) Analysis

Analytic HPLC–DAD was utilized to develop a method for the separation, and identification of bioactive compounds from crude extracts of *Q.brantii*. An optimal elution program giving a good resolution was composed of the following conditions for crude extract:

Mobile phase was consisted of two mixtures. Solution A was water, solution B was consist of acetonitrile, methanol and 2% acetic acid in a ratio of 2:2:1. The gradient was linear with 99% from 0 to 10^{th} min, 85% at 11^{th} min., 65% at 50^{th} min and 0% at 74^{th} 85^{th} minutes of solution A. Then column was washed with 100% solution B and re-equilibriated for 10 min. All runs were carried out at a flow rate of 1.3 mL/min and a constant column temperature of 30 °C.

Stationary phase was Reverse Phase Symmetry Column – C18. 4.6 x 150mm, 5µm (4.6mm: column internal diameter; 150mm: column length; 5 µm: column dimension)

Absorption spectra were observed in the range of 210-700 nm by diode array detector. Chromatograms of extracts and standards were obtained using following maximal absorption wavelengths in nm: 254, 280, 300 and 366.

2.2.2.3 Thin layer chromatography

TLC is a simple, quick, and inexpensive procedure that determines the number of the molecules in a mixture. Due to the attraction of the components of the mixture with stationary phase, Retention factor (R_f) differences occur. TLC is also used to support the identity of a compound in a mixture when the R_f of a compound is compared with the R_f of a known compound. TLC used to determine R_f of target compound in diethyl ether fraction

2.2.2.4 Column chromatography

In order to purify the phenolic compounds in target fractions, silica gel column chromatography was used. This is a common method depends on polarities of stationary phase, eluents and compounds. Polar compounds interact with silica and depending on the degree of eluent's polarity, cause separation of the molecules in fraction.

After determination of retention factors by thin layer chromatography, same eluent system was used in the column chromatography. Column size and type of silica were chosen according to amount and number the compounds in the mixture (Skoog, 1997).

2.2.3 Determination of antioxidant activities

2.2.3.1 Determination free radical scavenging capacity by DPPH method

DPPH (2,2-Diphenyl-1-picrylhydrazyl) radical was first discovered by Goldschmidt and Renn in 1922 and later it was introduced for chemical assays of antioxidant properties by Blois in 1958. Currently, this radical has become a popular tool for determining natural or synthetic compounds antioxidant activity (Viirlaid, 2009).

DPPH, a highly stable radical, is protonated and reduced when treated with antioxidants, as in the figure 7.

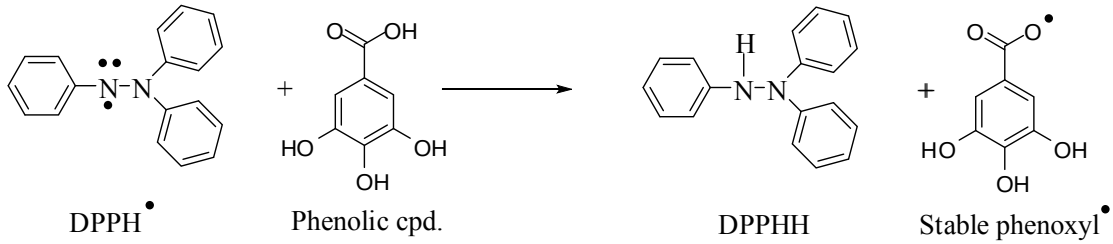


Figure 8 Reaction of DPPH radical with a flavonoid

Absorbance of DPPH radical (0.05 mg/mL DPPH in ethanol, 1.3 unit of absorbance) at 517 nm is monitored through its reaction with antioxidants (phenolic compounds) for a specified time.

We slidely modified the experiment as described by Çoruh et al. (Çoruh, 2007). 0.05 mg/mL of 2,2-diphenyl-1-picrylhydrazyl (DPPH) solution was prepared by dissolving 10 mg of DPPH in 200 mL ethanol. Its absorbance at 517 nm was measured and it gave about 1.4 absorbance unit. Various concentrations of extracts (0.3 mg/mL- 1.25 mg/mL) were also prepared by using ethanol.

After time optimization, 5 minute was determined for the complete reaction of radical and antioxidants in total extract. 100 µL of each extract solution added to 1.4 mL DPPH solution, waited for 5 minutes and measured absorbance at 517 nm results different absorbance units. Blank solution was consist of 100 µL ethanol and 1.4 mL DPPH solution, also incubated for 5 minutes and measured. This procedure was done triplicates and repeated 3 times.

Radical scavenging activity in % is calculated by the following equation;

RSA% (Radical Scavenging Activity) = $[(A_0-A_1)/A_0]*100\%$

A_0 is the absorbance of the blank including DPPH solution in ethanol without extract sample and A_1 is the absorbance of the DPPH solution reacted with extract concentrations after a specified time.

From the graph plotted by the calculated %RSA versus concentration of the analytes EC_{50} values which is the concentration of a drug, antibody or toxicant that induces a

response halfway between the baseline and maximum after some specified exposure time were calculated. It is commonly used to measure antioxidant's potency by following equation;

$$Y = Bottom + (Top - Bottom)/(1 + (X/EC_{50})^{(Hill\ coefficient)})$$

where Y is the RSA % value, x is the concentration, bottom is the lowest observed value, top is the highest observed value, and the Hill coefficient gives the largest absolute value of the slope of the curve.

2.2.3.2 Determination Free radical scavenging activity by ABTS method

ABTS method depends on scavenging of a radical cation, for the determination of antioxidant activity in an aqueous phase. Method inspects the ability of antioxidant compounds to scavenge free radicals as equivalent of trolox a vitamin E analogue (Rice-Evans, 1997).

The generation of 2,2-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) radical, includes the direct production of a green ABTS•1 chromophore through the reaction between ABTS and potassium persulfate ($K_2S_2O_8$) as illustrated in figure 8. According to literature this cationic radical gives absorption maxima at 415, 645, 734 and 815 nm. Addition of antioxidants reduces the created radical and the measurement decrease of the absorbance at 734 nm (0.70 ±0.02 a.u.) is observed. Reaction stops after 6 minutes and the absorbance value is stabilized.

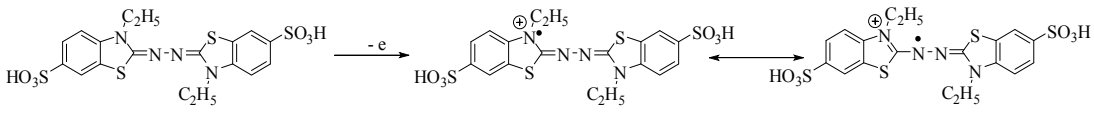


Figure 9 Preparation of ABTS radical

ABTS radical cation ($ABTS^{\bullet+}$) produced by preparing a solution containing 7 mM ABTS and 2.45 mM potassium persulfate. This mixture then was allowed to stand in dark at room temperature for 16 h.

Before using the solution, it was diluted by adding 100 mL 96% ethanol and made the solutions absorbance (0.70 ± 0.02 a.u).

With the difference between absorbance of ABTS solution which includes methanol (A_0) and absorbance of ABTS solution in presence of Trolox as standard or extract at various concentrations (A_1). ABTS radical scavenging in percent was calculated according to following equation;

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

After calculation of radical scavenging activity using trolox standard curve is plotted. Then plant extracts radical scavenging activities were determined as Trolox equivalents from the standard curve equation y = mx + n where y is radical scavenging activity (%), is concentration. X is solved as extract concentration in trolox equivalent.

2.2.3.3 Determination of metal reducing capacity by CUPRAC method

Method is based on spectroscopic observation of the redox reaction between synthesized chromogenic oxidizing agent and the corresponding antioxidant compounds in plant extracts at a pH of 7 which is represented in figure 9 (Apak, 2007).

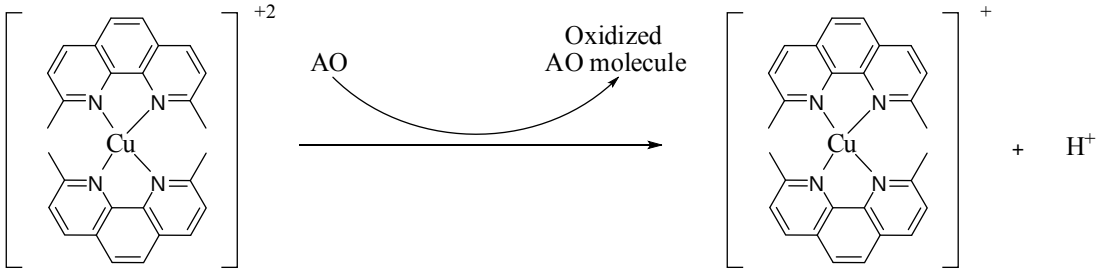


Figure 10 Reaction of CUPRAC chromophore with antioxidants

Metal reducing activity of plant extract was determined with the method described by Apak et al. with some modifications. Cupper reducing activity of acorn extract was done by preparing CUPRAC solution. 1.0 x 10^{-2} M $CuCl_2$, 1.0 amonium acetate buffer at pH 7.0 and 7.5 x 10^{-3} M neocuprine solution in 96% ethanol were prepared and addition of 0.25 mL each of them results the formation of chromogenic oxidizing agent at a neautral pH. Addition of 0.1 mL of various concentrations of analyte to 0.75 mL CUPRAC solution and diluting it with 1 mL water results absorbance change after 30 minutes at 450 nm by oxidation of phenolic compounds in analyte and reduction of the chromophore.

Same procedure was applied to trolox and quercetin. Absorbance 450 nm vs. concentration of trolox graph plotted. Then plant extracts radical scavenging activities were determined as trolox equivalents from the stand curve equation y = mx + n where y is radical scavenging activity (%), is concentration. X is solved as extract concentration as trolox equivalent.

2.2.4 Determination of total phenolic quantity

Total phenolic content of the plant was determined according to the method described by Singleton and Rossi (1965) with modifications. Gallic acid was used as a standard to calculate quantity of phenols as a gallic acid equivalent. Addition of Folin-Ciocaltaeu's reagent which contains sodium tungstate, sodium molybdate,

lithium sulfate, bromine to various extract concentration started the reaction between phenols and the reagent (Vinson, 2005). The method is based on the reduction of this unque reagent by phenolic compounds in plant extracts in 3 minutes. In order to stop the reaction 2% Na_2CO_3 was used and waited for 30 minutes at room temperature. All solutions vigorously votexed after each step. Absorbance values of mixtures were determined at 750 nm. Concentration vs absorbance graphs were plotted for both plant extract and standard gallic acid. By these graphs the amount of phenolic compounds in *Q.brantii* extracts was determined in gallic acid equivalents (GAE) as described for trolox equation above.

2.2.5 Determination of total flavonoid quantity

Bakar claims that the total flavonoid quantity is determined in the sample extracts by the reaction of sodium nitrite with aluminum chloride in the presence of flavonoids results colored flavonoid-aluminum complex. This complex can be monitored spectrophotometrically at 510 nm. The formation of that complex with flavon, flavonol and flavanol type compounds in the mixture. However, because of chelation deficiency of Al (III) with flavonoid glycosides, it gives negative error (Bakar, 2009).

Determination of total flavonoid content was done as slightly modified version which described by Zhishen et al. (1997). 0.2 mL various concentrations of catechin as starndard were diluted with water and 0.075 mL 5% $NaNO_2$ was added. Then waited for 5 minutes and 0.15 mL 10% $AlCl_3$ was added. 6 minutes later 0.5 mL 1 M NaOH was added and solution was made up to 3 mL with water. The solution was mixed well and the absorbance measured against blank solution at 510 nm immediately.

Same procedure was applied for extract solutions. Calibration curves of the standard and extract were plotted as absorbance at 510nm against concentration. Then total flavonoid content was calculated as catechin equivalent from catechin standard curve equation $y = mx + n$.

2.2.6 Antimicrobial tests

2.2.6.1 Disc diffusion test

Disc diffusion test, is one of the most common method, used for determining bacterial sensitivity and resistance to antimicrobial agents. Bacteria, stored at –80 ^{o}C are was suspended in 1.0 mL of related broth and streaked onto ready to use agar plate containing sheep blood medium, then incubated for 24 hours at 37 ^{o}C for short term bacterial stocks. Then stock plates were stored at 4 ^{o}C room and stocks were renewed each week to maintain the freshness. In all antimicrobial tests bacteria supplied from these stock agars.

Bacteria taken from stock agar are first standardized by monitoring their optical densities. Then they are spread on the agar in early stages of growth. Addition of analytes on empty filter discs results inhibition zones as indicated in figure 11. Measuring the inhibition zones of antimicrobial agents gives the number of bacteria affected by a known concentration of analyte. This method provides equal susceptibility of all cells to antimicrobial agents (Pollack, 2005).

The measurement is based on the diameter of the clearance zone of inhibition surrounding each disc. These clearance zones are measured in millimeters (mm) and reveals susceptibility or sensitivity to antimicrobial agent, or no clear zone indicates ineffectiveness or resistance, as sketched in Figure 11. The inhibition zone that is between susceptible and resistant is called as intermediate (Pollack, 2005).

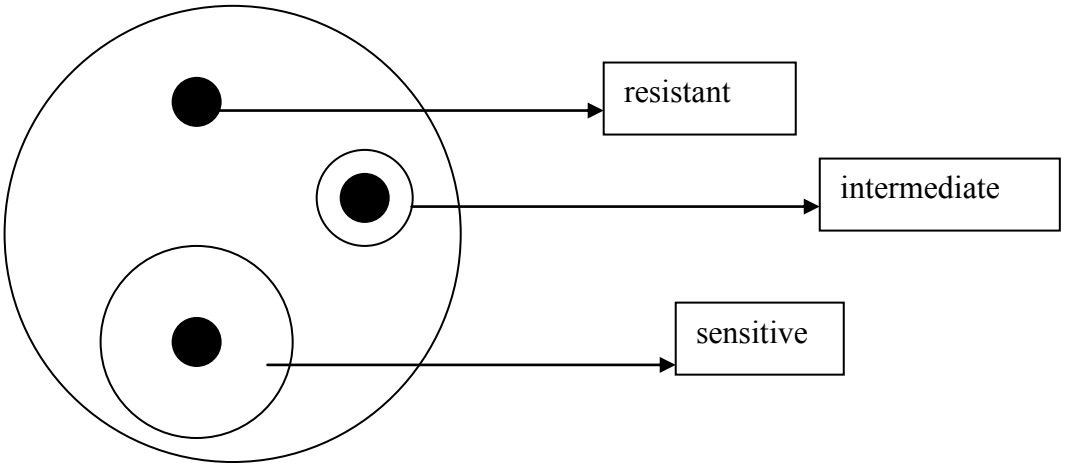


Figure 11 Representation of disc diffusion test; agar plate and discs

2.2.6.2 Determination of minimum inhibitory concentration

Observing the effect of an antimicrobial agent against a bacterial strain described by minimum inhibitory concentration (MIC) is the lowest concentration of antimicrobial compound, which is capable of inhibiting the growth of the bacteria of interest (Mann, 1997).

2.2.6.2.1 Solvent effect

This test was performed in order to determine the minimum inhibitory concentration (MIC) of crude extract, fractions and isolated compound in the presence of a suitable solvent without any inhibitory effect. This solvent should also dissolve all the analytes even at high concentrations.

In search of proper solvent 96-well plates were used and in each row contained various concentrations of solvents such as ethanol (EtOH), methanol (MeOH), dimethylsulfoxide (DMSO), ethyl acetate (EtOAc), acetonitrile, and double distilled water (ddH_2O) as in the Table 1.

The sterility control and growth control was also determined by using two columns. A 5 μL of the inoculated bacterial suspensions at 0.05 OD_{570} was added to each well except the column for sterility control. After 24 hours incubation at 37 ^{o}C, minimum bacterial inhibitory concentration of the solvents was determined and the solvent which would use in future experiment was determined.

Table 1: 5 µl of bacteria with 0.05 absorbance unit was added to each well and total volume was adjusted to 100 µl. All of the wells were prepared with respected solvents with same broth.

	9.75%	**4.88%**	**2.44%**	**1.22%**	**0.61%**	**0.31%**	**0.16%**	**0.08%**	**0.04%**	**0.02%**	**sterility control (broth only)**	**growth control (broth and bacteria)**
EtOH												
MeOH	20 µL MeOH, 175 µL broth	95 µL broth 50 % of well-1	95 µL broth 50% of well-2	95 µL broth 50% of well-3	95 µL broth 50% of well-4	95 µL broth 50% of well-5	95 µL broth 50% of well-6	95 µL broth 50% of well-7	95 µL broth 50% of well-8	50% of well-9	100 µL broth	95 µL broth
DMSO												
EtOAc												
Water												

2.2.6.2.2 Micro broth dilution method for the determination minimum inhibitory concentration

The most commonly used techniques for determining the minimum inhibitory concentration (MIC) of antimicrobial agents, that inhibit the growth (bacteriostatic activity) of bacteria, are agar dilution and broth dilution methods. Micro broth dilution method was performed according to "Nature Protocols" (Wiegand, 2008). Luria-Bertani (LB) broth was chosen for *E. coli, P. mirbailis S. pyogenes* and brain heart infusion (BHI) broth for *S. aureus* considering their growing capabilities in related broth. First column of 96-well plates were arranged with the stock concentration of extract, fractions and isolate. Two fold dilutions were applied to the wells starting from the first column of wells by using multichannel pipettes.

In order to observe the inhibitory effect bacteria, which were at the stationary phase of the growth, were diluted with selected broth. Then, diluted bacteria were inoculated to all of the columns equally, except sterility control columns.

Lastly, 96-well plates were incubated at 37 ^{o}C for 16 hours and absorbance was monitored with ELISA plate reader at 570 nm.

Table 2: 96-well plate design in MIC and MBC experiments. 5 µl of bacteria with 0.05 absorbance unit was added to each well and total volume was adjusted to 100 µl

	1	2	3	4	5	6	7	8	sterility control (broth only)	growth control (broth and bacteria)	Antibiotic sterility control (antibiotic and broth)	Antibiotic control
A												
B	20 µL Analyte, 175 µL broth	95 µL broth 50 % of well-1	95 µL broth 50% of well-2	95 µL broth 50% of well-3	95 µL broth 50% of well-4	95 µL broth 50% of well-5	95 µL broth 50% of well-6	50% of well-7	100 µL broth	95 µL broth	80 µL broth, 20 µL Antibiotic	75µL broth, 20 µL Antibiotic
C												
D												
E												

2.2.6.3 Minimum bactericidal concentration

Minimum bactericidal concentration (MBC) was defined as the lowest concentration of an antimicrobial agent that kills the bacteria (Jacobs, 1991). By performing this experiment the amount the antimicrobial agent that kills the bacteria was determined.

After performing the MIC experiment, as described in section 2.2.6.2, the incubated bacteria were transferred to 96 well plates which contains selected agar for specified bacteria by 12 multi-channel pipette (5-50 μL). The agars were prepared before the experiment and kept at 0 ^{0}C. Well plates were incubated for 16 hours again and minimum bactericidal concentration was determined as the lowest concentration in which did not contain visible living bacteria. ELISA plate reader was used at 570 nm to read the plate absorbance against blank agars to determine whether there were any bacteria in the plates.

2.3 Statistical analysis

All results are expressed as mean ± standard deviation (SD). Rejections were carried out according to Q test. Confidence interval around mean value was calculated at 95%.

CHAPTER 3

RESULTS AND DISCUSSION

3.1 Extraction of *Quercus brantii L.*

Powdered 50 g of *Q.brantii* seed sample was extracted in 600 mL methanol (1:12) by using oval-rotating incubator at 30 °C and 175 rpm overnight. Extracts then filtered through filter paper and solvent is evaporated by using rotary evaporator. Remaining crude extract is weighted and the percent yield is calculated. This procedure was performed 3 times. 7 grams of extract resulted about 17.5% yield.

3.1.1 Determination free radical scavenging activity of *Q.brantii* extract

Many types of Quercus species had reported having antioxidant activity in the literature. Quercus robur (English oak), Quercus cernel (Turkish oak) are the recent examples. On the other hand, for the first time Quercus brantii Lindley is investigated for its antioxidant activity, phenolic quantity or flavonoid quantity.

3.1.1.1 Free radical scavenging activity by DPPH method

The method performed as stated in 2.2.3.1. Radical scavenging activity (RSA) of each concentration was calculated by subtracting the solvent effect. RSA% vs. crude extract concentrations of the extracts (mg/ml) was plotted as shown in the figure 12.

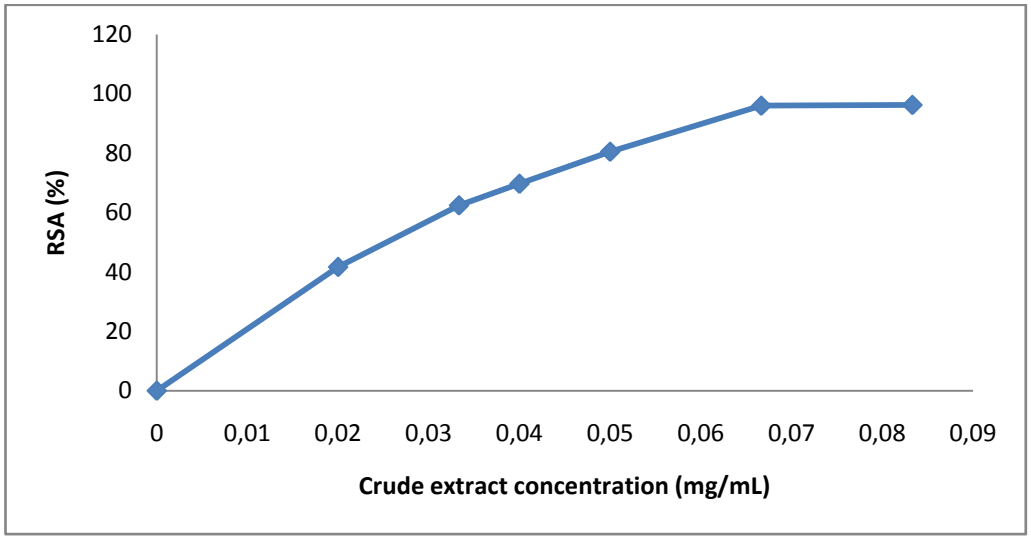


Figure 12 DPPH radical scavenging activity in percent versus acorn extract concentrations (mg/mL)

EC_{50} value of the *Q.brantii* extract was determined as 31.1 ± 0.19 µg /mL. DPPH scavenging activity of quercetin used as a standard was determined as in the literature, 8.09 ± 0.17 µg /mL. Rakic et al. have reported DPPH radical scavenging activities of Quercus robur (English Oak) and Quercus ceris (Turkish Oak) by slightly modified version of our experiment and stated EC_{50} values as 8.04 µg/mL and 8.88 µg/mL when quercetin having EC_{50} value of 2.75 µg/mL (Rakic, 2006)

3.1.1.2 Free radical scavenging activity by ABTS method

RSA% was calculated as stated in section 2.2.3.2. Trolox standard curve and total extracts graphs were plotted against RSA (%) versus concentration and shown in the figure 13 and 14

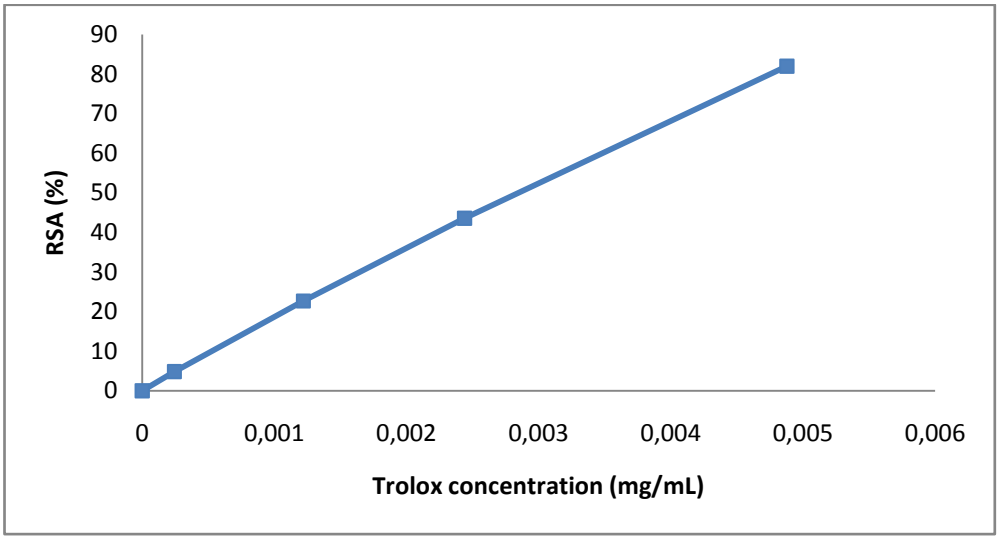


Figure 13 Trolox standard curve to calculate $TEAC_{ABTS}$ of the extracts and fractions.

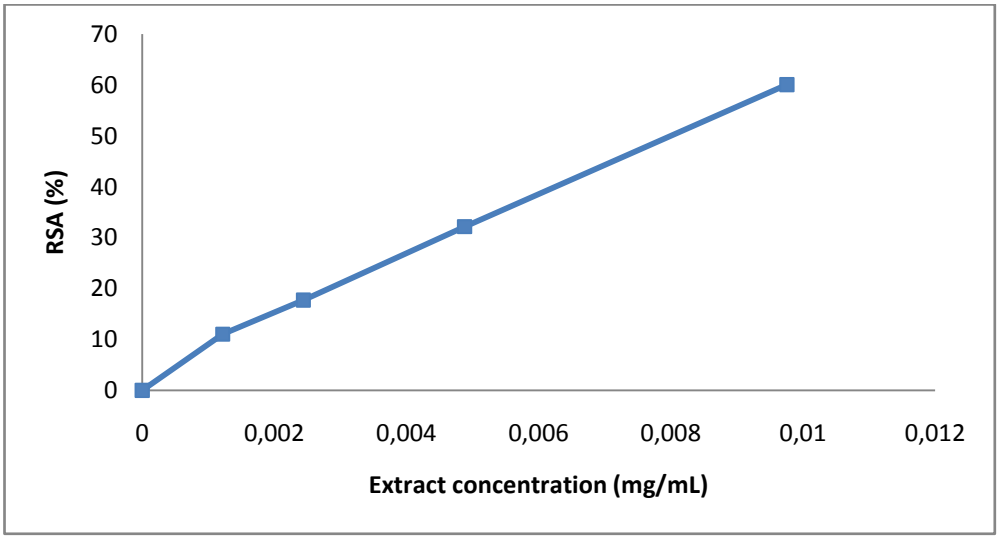


Figure 14 Radical scavenging activity in percent versus concentration of acorn extract (mg/ml)

This experiment repeated 3 times in triplicates. Equation of trolox standard curve was calculated as y = 16759x + 1.172, R^2 = 0.998 and total extracts equation was y = 5999x + 2.251, R^2 = 0.995. From these graphs $TEAC_{ABTS}$ value of acorn extract was derived as described in 2.2.2.2 and found to be 0.36 ± 0.01 mg trolox equivalent per mg of crude extract. Reference compound quercetin's $TEAC_{ABTS}$ value was determined as 2.90 ± 0.01 mg. Antioxidant activities of different acorns extracts were

stated as $TEAC_{ABTS}$ equivalent by Cantos et al. Results were trolox equivalent; Quercus suber 0.81, Quercus rotindifolia 0.81 and Quercus ilex 1.23 (Cantos, 2003).

3.1.1.3 Metal reducing antioxidant capacity by CUPRAC method

Different concentrations of plant extracts were prepared by using 96% ethanol. The standard Trolox and total extracts graph was plotted against absorbance vs concentration as shown in the figure 15.

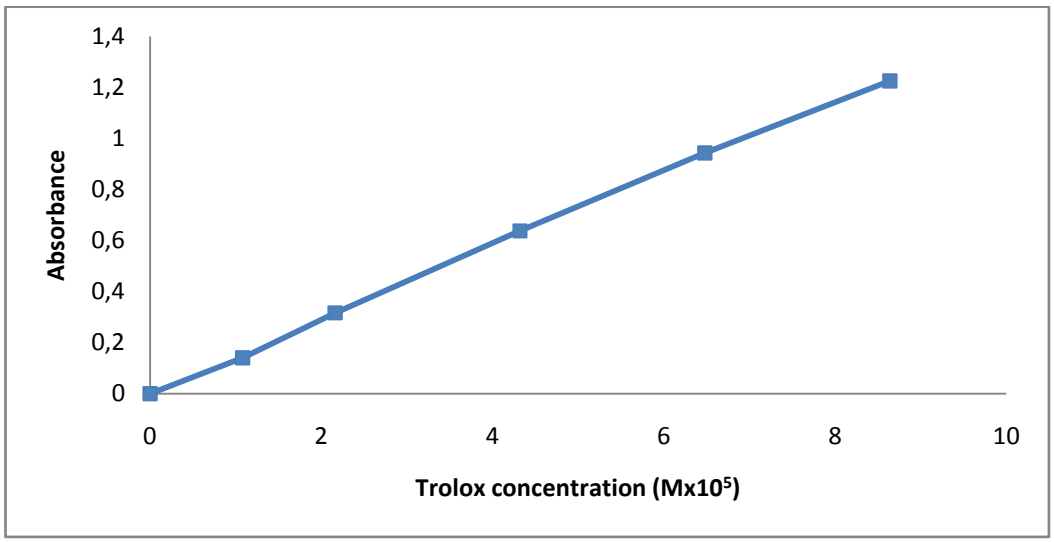


Figure 15 Trolox standard curve to calculate $TEAC_{ABTS}$ of the extracts and fractions

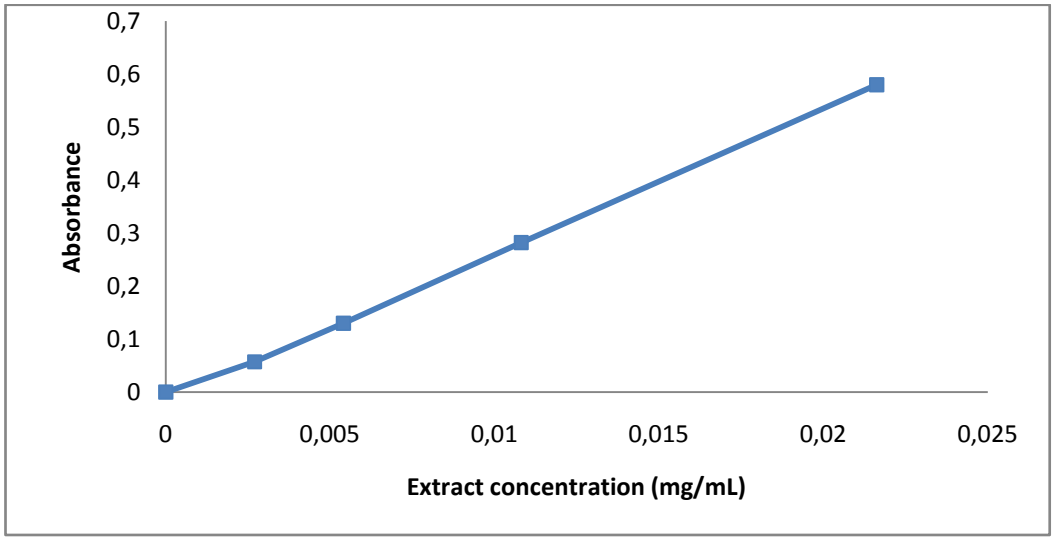


Figure 16 Absorbance versus concentration of acorn extract (mg/ml) to calculate $TEAC_{CUPRAC}$

This experiment was performed three times in triplicates as stated in 2.2.3.3. Equation of the Trolox was found as y = 0.143x + 0,001 with R^2 = 0.999 and equation of total was y = 27.15x – 0,010 with R^2 = 0.999. With the help of this equation $TEAC_{CUPRAC}$ value of total extract was founded as 0.43 ± 0.01 mg and standard Quercetin was founded as 4.52 ± 0.01 mg. Since this method is a recent one there no studies in the literature related to application of CUPRAC for this species.

3.1.2 Determination of total phenolic quantity

Concentration vs absorbance graphs were plotted for both plant extract and standard gallic acid. By these graph the amount of phenolic compounds in *Q.brantii* extracts was determined in gallic acid equivalents (GAE).

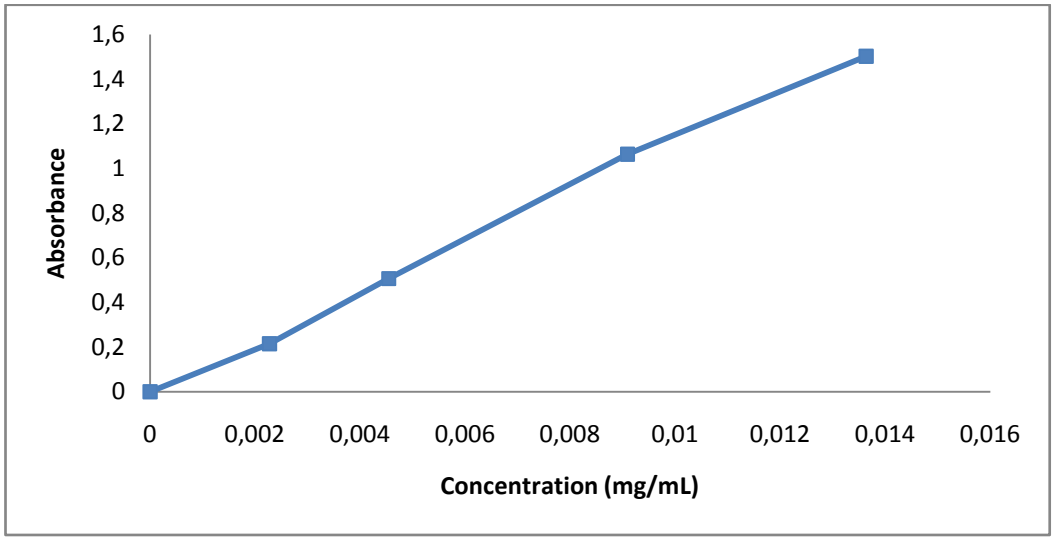


Figure 17 Gallic acid standard curve to calculate total phenolic content of acorn extracts

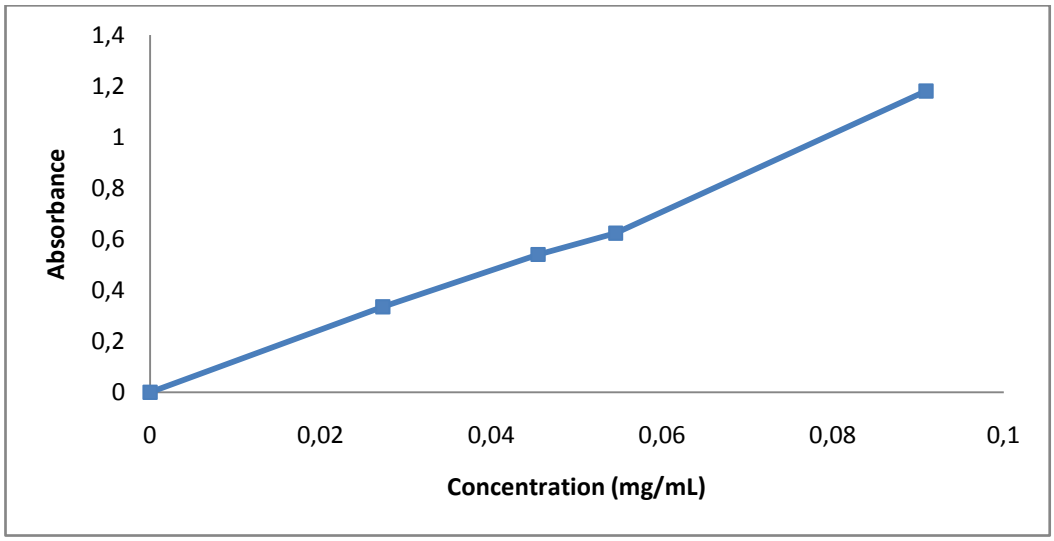


Figure 18 Acorn extract standard curve to calculate total phenolic content

This experiment was performed three times in triplicates as stated in 2.2.4. Equation of the gallic acid was found as $y = 112.07x - 0.009$ with $R^2 = 0.997$ and equation of total extract was $y = 12.82x - 0.024$ with $R^2 = 0.993$. With the help of these equations phenolic compounds in total extract was founded as 0.109 ± 0.006 mg gallic acid equivalent in per mg plant extract. Rakic et al. reported total phenolic

contents of Quercus robur (English Oak) and Quercus ceris (Turkish Oak) as gallic acid equivalents and results were 0.223 ± 0.004 and 0.247 ± 0.001.

3.1.3 Determination of total flavonoid quantity

The standard curve of catechin and crude methanol extract was shown in the figure 19 and 20.

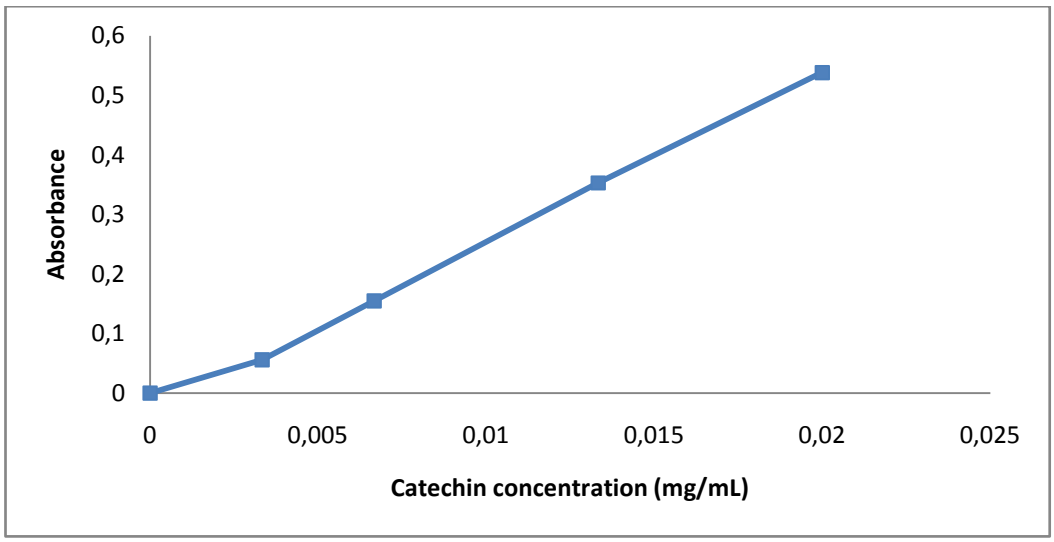


Figure 19 Catechin standard curve to calculate total flavonoid content of acorn extracts

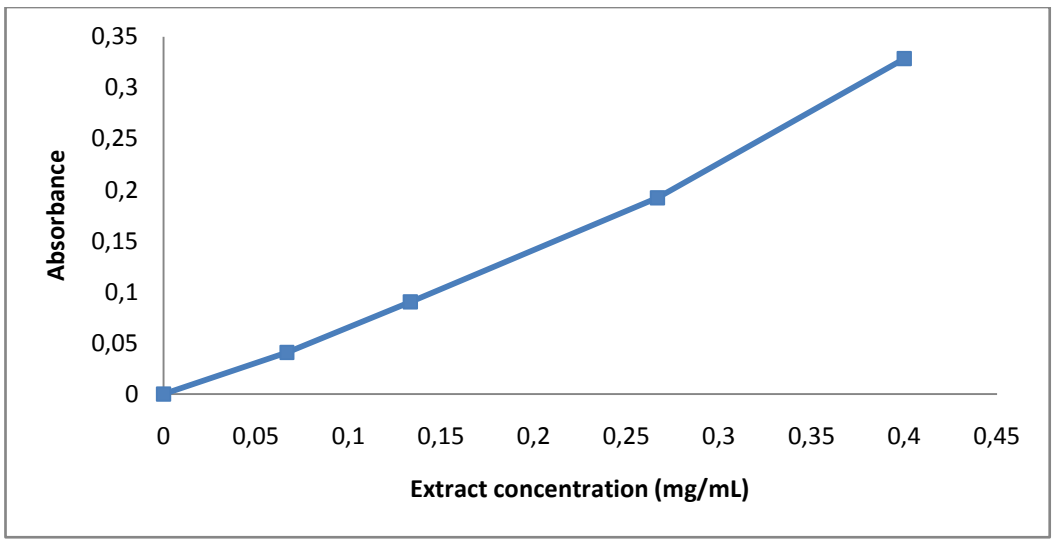


Figure 20 Acorn extract standard curve to calculate total flavonoid content

This experiment was performed as stated in 2.2.5. Equation of the catechin curve results y = 27.69x – 0.019 with R^2 = 0.996. Equation of the total extract was calculated as y = 0.819x – 0.011 with R^2 = 0.991. From these results (n=36) the amount of flavonoids in acorn extract calculated as 0.031 ± 0.001 mg catechin equivalent in per mg total extract.

3.1.4 Analytical High Performance Liquid Chromatography (HPLC) analysis

In order to isolate and characterize the compounds in *Q.brantii* extracts high performance liquid chromatography was performed. Many solvent systems has tried including different eluent mixtures such as methanol, formic acid, acetic acid, water, acetonitrile on both isocratic and gradient systems. Finally, separation of the compounds were optimized by a gradient system, consist of two different mobile phases as given in 2.2.2.2

Retention times of the peak can be clearly observed in the figure 21. Peak numbers and retention times are; 1 (8.5), 2 (20.1), 3 (22.1), 4 (24.8), 5 (25.2), 6 (29.6), 7 (30.9), 8 (37.5), 9 (41.3), 10 (42.6), 11 (45.3), 12 (47.3), 13 (51.8), 14 (60.3).

3.1.4.1 Analytical HPLC analysis of standards

Once, the conditions for acorn extract was optimized, standard compounds were applied to the same conditions to find out the structures of matching isolates by comparing the matching retention times. Table 3 illustrates the structures, retention times of used standards.

From the table 6 in Appendix A, by considering an RP column it could be driven that the polarity of the similar sized molecules increases with the number of polar substituents such as hydroxyl groups and hence the retention time of such a molecule

would be decreased. The difference between retention times of syringic acid and gallic acid or esculetin and scopoletin were also based on that fact.

Moreover, molecules having the same skeleton but larger substitiuents were compared, pore size of the column does not affect the retention time. As an example, esculin and esculetin are coumarine molecules whose skeleton is a benzene ring fused with pyrone. Instead of a hydroxy substituent esculin has a glucose ring. Although esculin molecule is larger, glucose group increases the molecules polarity and decreases the retention time, about 3 minutes. In the case of rutin and myricetin, difference in retention times increases dramatically up to 18 minutes.

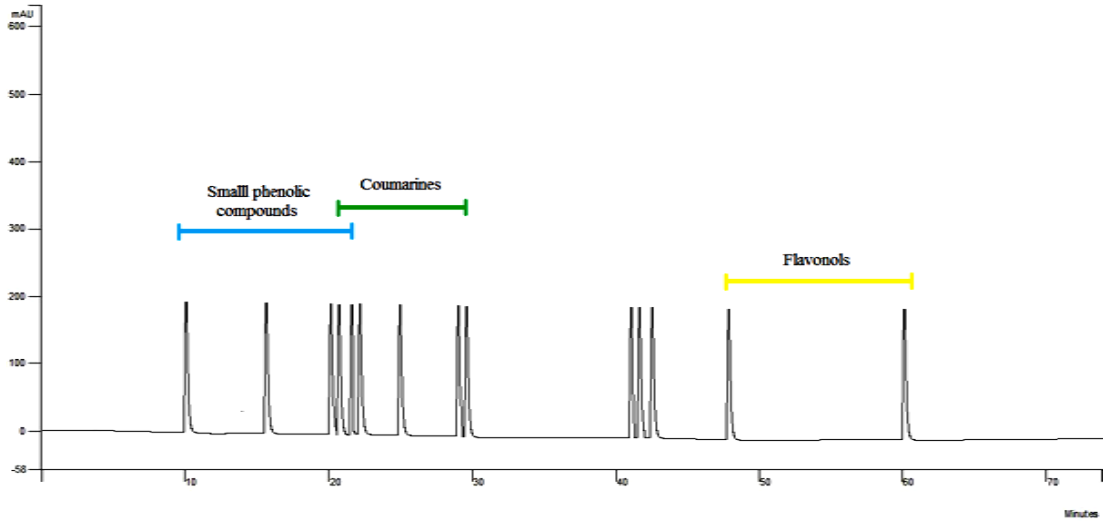


Figure 21 Representation of all standard molecules in same chromatogram

If we consider all standard molecules injected in the same solution, it results in borderlines between groups of different compounds as it is represented in figure 21. Between 10th and 22nd minutes small phenolic compounds having single aromatic ring with small substituents, from 21th to 31th minute coumarine type molecules without glycosides, and between 48th and 60th minutes flavonol derivatives were observed.

When retention times of standard molecules which stated in Appendix A compared with the total extract as illustrated in figure 22, peaks at 24.8 and 25.2 minutes

matches with epi-catectechin, 29.6 and 30.9 minutes matches with p-coumaric acid, 41.3 minutes matches with ellagic acid, 42.6 minutes matches with rutin and 60.3 minutes matches standard quercetin. The peak at 20.1 minute could be considered as a derivative of a small phenolic compound. Since 47.3 and 51.8 minutes are in the flavonol zone they could also be a flavonol compound.

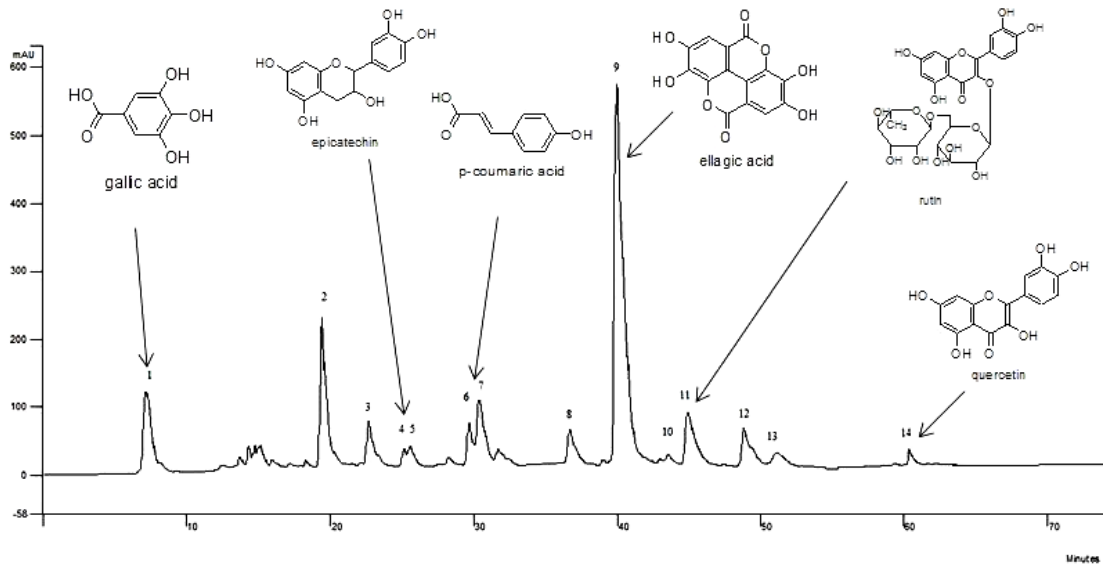


Figure 22 HPLC chromatogram of crude extract of *Q.brantii* and standards matched with peaks

To confirm the HPLC results of total extract High Resolution Mass Spectroscopy (HRMS) experiment was performed. As a result, gallic acid, epi-catechin, coumaric acid, ellagic acid, rutin and quercetin were determined in the extract solution.

In literature different quercus species were studied for their phenolic constituents by using HPLC. According to Fernandez et al. *Quercus suber* was found to contain, vanillin, gallic acid, ellagic acid and its derivatives, valoneic acid (Fernandez, 2011). Another quercus specie; *Quercus ilex* was reported by Skaltsa et al. having coumaric acid derivatives and kaempherol glucosides isolated (Skaltsa, 2010).

3.1.5 Fractionation of extract

After determining the phenolic compounds in *Q.brantii* extract using analytical HPLC and HRMS, fractionation method was employed in order to obtain high amount of characterized compounds for the determination of antioxidant and antimicrobial activities. Petroleum ether, diethyl ether, ethyl acetate and water with increasing polarities were selected for this purpose.

9.12 g of crude extract was dissolved in 200 mL 70% methanol and fractionation started in separatory funnel using 200 mL petroleum ether and repeated 3 times. First, the aqueous and organic phase difference occurred then, organic phase separated and evaporated. Then the remaining water phase was re-fractionated with total of 600 mL diethyl ether. Same procedure, applied and its weight was measured as 0.1455 mg. After diethyl ether, a total of 600 mL ethyl acetate was added to the remaining water phase. Evaporation of the organic phase gave 0.9042 g product, where as the aqueous phase was also evaporated and weighed as 6.478 g.

3.1.5.1 Petroleum ether fraction

The least polar solvent petroleum ether has dissolved waxy compounds in the crude extract. It was diluted with methanol and in order to see the ingredients of fractions same HPLC was used as described in 2.2.2.2. The results are in the figure 23.

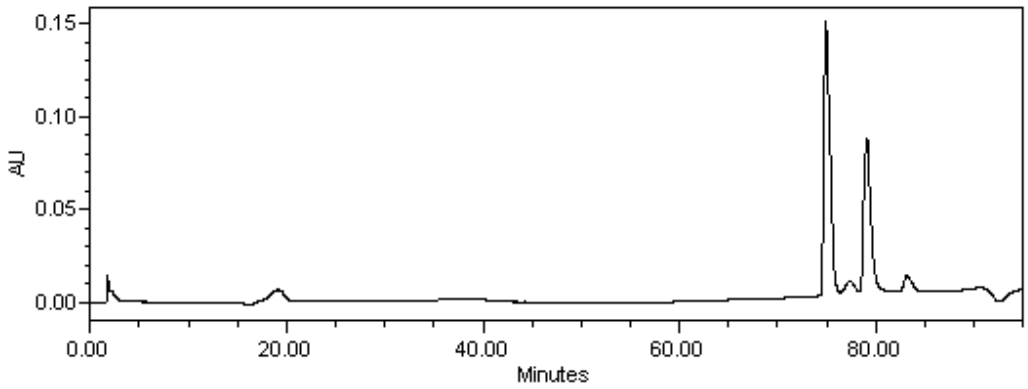


Figure 23 HPLC chromatogram of petroleum ether fraction

The peaks at 75, 77, 79 and 84 minutes were observed at 254 nm when the gradient program was washing the column with 100% eluent B. This result shows that the compounds in this fraction are non-polar and not phenolics.

However, this fractions antioxidant activity was determined.

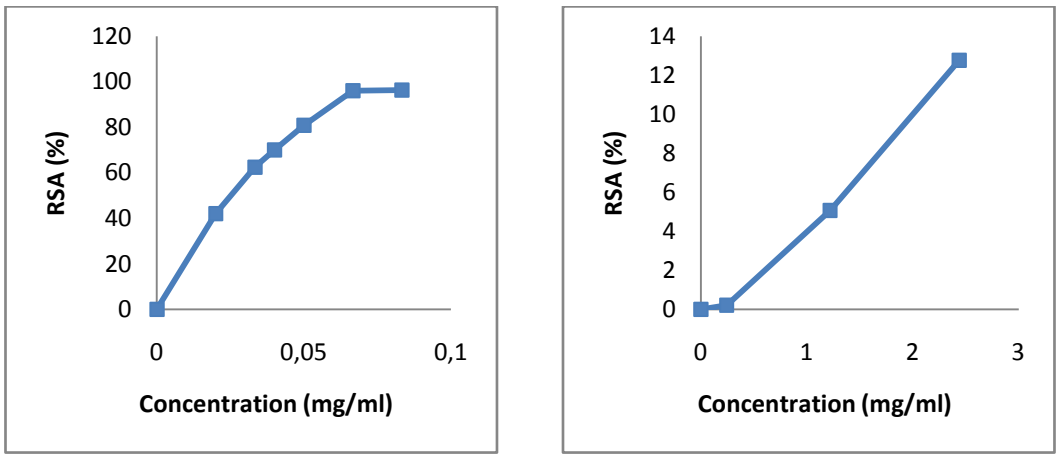


Figure 24 Radical scavenging activity in percent versus concentration (a) DPPH (b) ABTS

Radical scavenging activity of DPPH and ABTS radicals were determined as quiet low. EC_{50} value for 5 minute was 29.24 ± 0.01 µg/mL and $TEAC_{ABTS}$ was 0.001 ± 0.0001 mg trolox equivalent. The results demonstrated us that there was no antioxidant compounds in this fraction. CUPRAC test was also applied, however the

compounds in this fraction caused interference. Precipitation was observed which should not occurred and so the absorbance of the concentrations could not determine.

3.1.5.2 Diethyl ether fraction

Another solvent with low polarity was diethyl ether. Fortunately, in this fraction single major peak was observed at 20.3th minute with minor impurities. Although it was not matching with any of the selected standards, it was between the small phenolic compounds and coumarine region as shown in the figure 25.

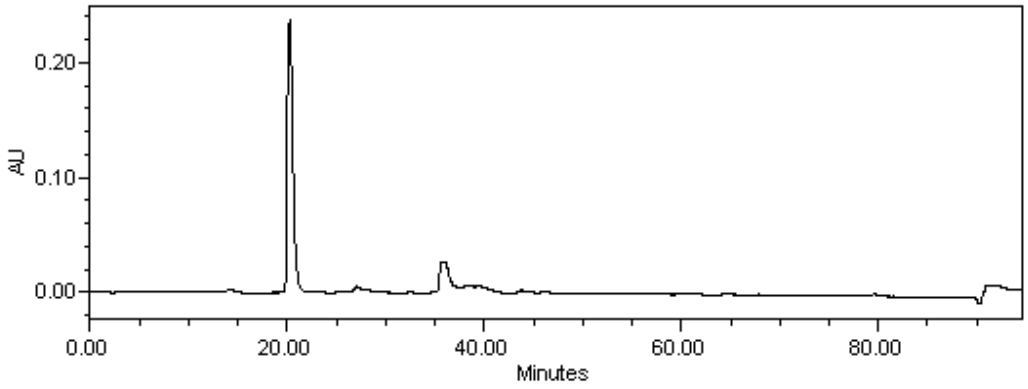
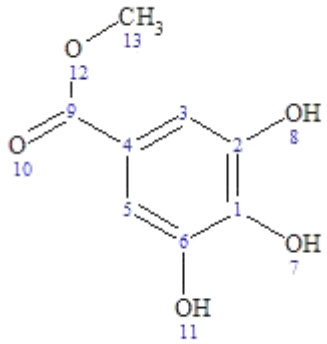


Figure 25 HPLC chromatogram of diethyl ether fraction

3.1.5.2.1 Column chromatography

The purification of the product was done by column chromatography. 50 cm glass column filled with 15 grams of Silica Gel 60 with particle size of 0.063–0.200 mm. Hexane/ethyl acetate (1:4) elution system was used to purify and separate compounds. 97 mg of target molecule was isolated. Thin layer chromatography was used to observe the purity. The fraction containing the target molecule was evaporated.

3.1.5.2.2 Characterization of isolated compound



Structure determination of isolated compound was done with 400 MHz H-NMR, 100 MHz C-NMR.

^{1}H NMR (400 MHz, MeOD) δ: 6.90 (s, 2H, H-3, 5), 3.70(s, 3H, H-13)

^{13}C NMR (100 MHz, MeOD) δ: 169.05, 146.38, 139.77, 121.54, 110.33, 52.32

Mass: 184.0208 m\z

IR: 3366, 3006, 2922, 1742, 1611, 1198, 767, 710

The structure elucidation was done by both 1D and 2D NMR spectra with the help of IR and mass spectra in comparison of literature. In proton NMR concerning ratio of integration values, two protons resonated in the aromatic region and three resonated at 3.7 ppm. The two protons in the aromatic region showed us the existence of substituted benzene ring and resonance at 3.7 ppm clearly indicates the presence of methoxy proton.

There were six different carbons in carbon NMR spectra. Resonance at 169.05 ppm resembles the carbonyl group. Four different carbons in the aromatic region, in addition to the two identical aromatic protons in proton NMR, pointed the existence of substituted and symmetrical benzene ring. Also, methoxy carbon resonated at 52.32 as expected.

In addition to 1D NMR, 2D NMR proves the structure. 2D NMR consists of COSY, DEPT, HSQC and HMBC. In COSY spectrum, which shows the proton-proton correlations over 2-4 bonds, there was no correlation between the 2 different protons of the molecule. HSQC shows proton-carbon correlations over one bond. The correlations observed in that spectra proves the result from the proton and carbon NMR spectra. In HMBC spectrum, which indicates the correlation of protons and

carbons over 2-4, carbons C-1, C-2, C-4, C-8 showed correlations with H-3, C-4 and C-8 with H-12.

The isolated compounds antioxidant activity is determined as described in 2.2.3 by DPPH, ABTS and CUPRAC methods and illustrated in figure 26.

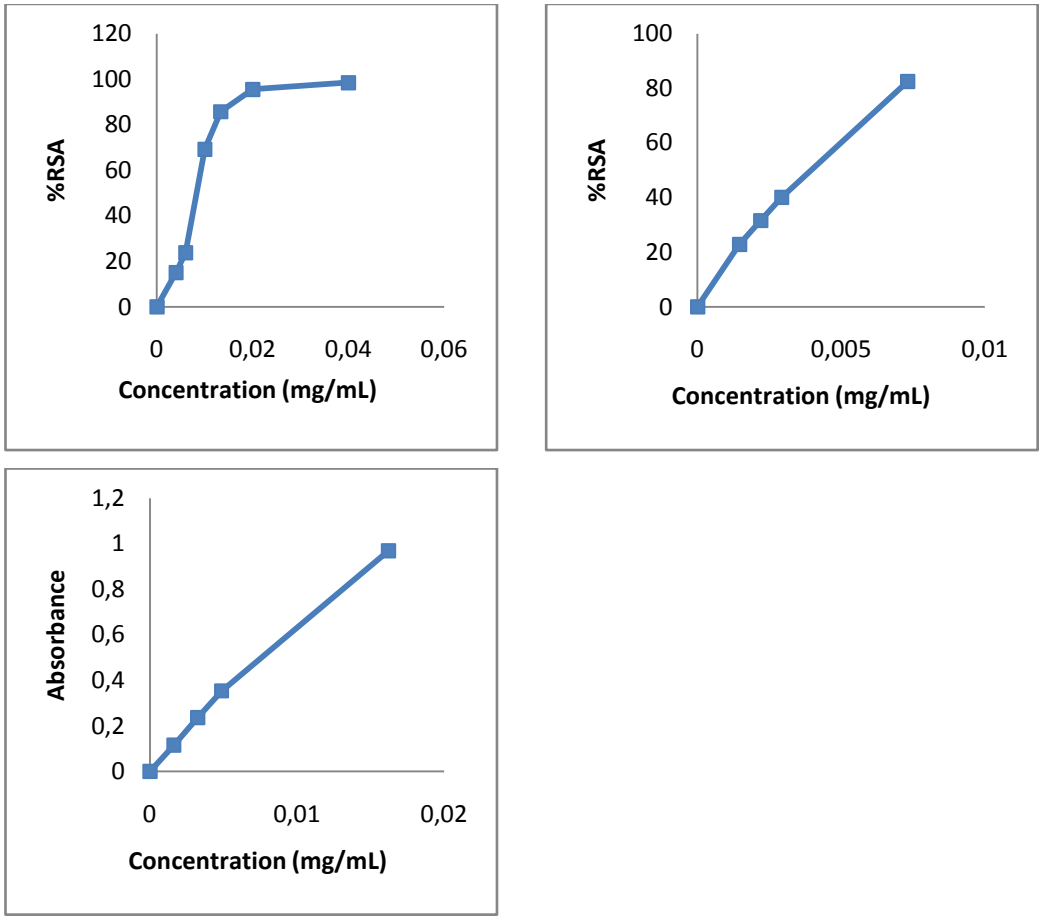


Figure 26 (a) radical scavenging activity in percent versus concentration (a) DPPH (b) ABTS and (c) metal reducing antioxidant activities of various concentrations of methyl gallate

EC_{50} value of the compound was calculated as 9.19 ± 0.04 µg/mL. TEAC values determined by ABTS and CUPRAC were 0.65 ± 0.01 mg/mg and 1.02 ± 0.01 mg/mg respectively.

In the literature, methyl gallate is known as strong antioxidant agent which was also isolated from another quercus specie Quercus salina (Kim, 2007) with $TEAC_{ABTS}$: 0.824 and DPPH EC_{50}: 1.90 when EC_{50} of quercetin was 2.51 (Cioffi, 2002).

3.1.5.3 Ethyl acetate fraction

Third polar solvent which used in fractionation process was ethyl acetate. Since it has high polarity, most of the phenolic compounds were expected to be concentrated in this fraction. According to HPLC analysis the separation was done successfully as shown in the figure 27.

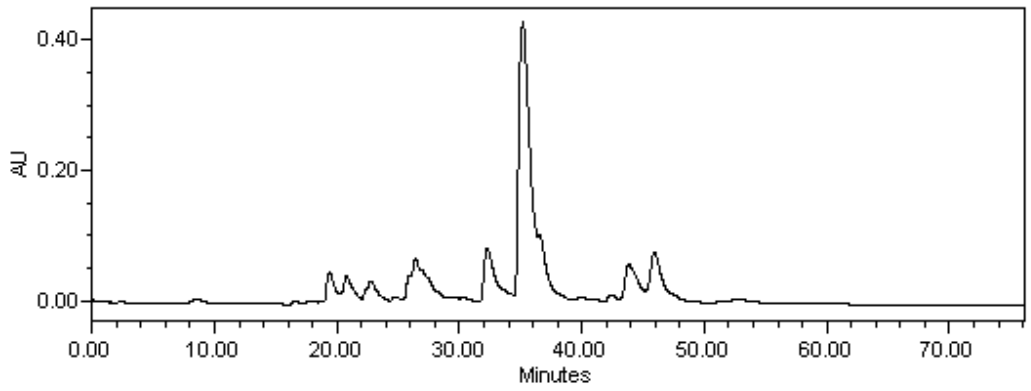


Figure 27 HPLC chromatogram of ethyl acetate fraction

HPLC analysis at 280 nm shows methyl gallate was remaining in that fraction because not enough solvent was used in the previous fractionation process. Another notable result is the peak which has the highest absorbance in acorn extract was not present in this fraction.

DPPH time optimization resulted in 5 minutes and R^2 of the ABTS and CUPRAC were 0.990 and 0.998 respectively. Figure 28 shows the plotted graphs as described in 2.2.3

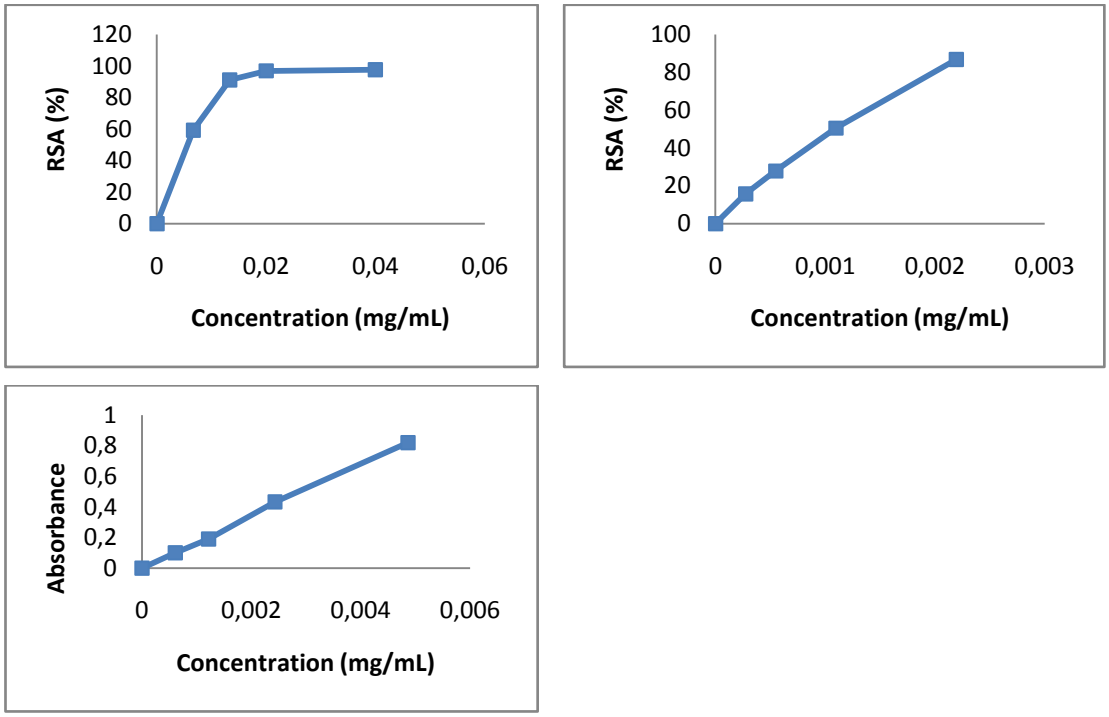


Figure 28 (a) radical scavenging activity in percent versus concentration (a) DPPH (b) ABTS and (c) metal reducing antioxidant activities of various concentrations of ethyl acetate fraction.

The antioxidants tests were resulted as; EC_{50} value of the fraction according to DPPH method was 7.91 ± 0.08 µg/mL. TEAC values determined by ABTS and CUPRAC were 2.32 ± 0.01 and 2.96 ± 0.01 respectively.

Due to the low yield of ethyl acetate fraction, isolation of the molecules by column chromatography could not be performed. However, these fractions exhibited high antioxidant activity determined by DPPH, ABTS and CUPRAC methods. Therefore, compounds in this fraction were characterized by High Resolution Mass Spectroscopy (HRMS).

All expected compounds derived from HPLC analysis (coumaric acid, methyl gallate, epi-catechin, ellagic acid and rutin) were confirmed by HRMS experiment.

3.1.5.4 Water/methanol fraction

The remaining part from the fractionation process was water/methanol fraction in other words aqueous phase. Rotary evaporator was used to remove the solvent at 50 C^o for 3 days and then remaining solvent was removed at room temperature in fume hood for 5 days. The solid part was taken and HPLC analysis was done.

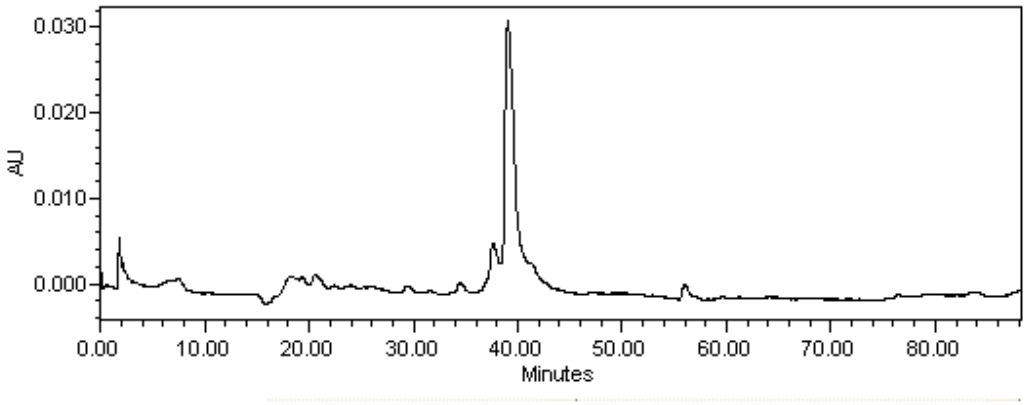


Figure 29 HPLC chromatogram of water/methanol fraction

It can be clearly observed that there is one major peak at 40.3 minute. According to selected standards this compound could be ellagic acid. NMR was employed to determine the structure. ^{13}C NMR spectrum indicates that there were no aromatic carbons instead most of the carbons in the mixture were in high field. Unfortunately, compound was not ellagic acid.

Its antioxidant potential was again determined by the methods as described in 2.2.3 DPPH methods with 10 minutes of optimized reaction time which was higher than both fractions and total extract. ABTS and CUPRAC graphs in figure 27 have revealed R^2 values of 0.993 and 0.999 respectively.

EC_{50} value of the fraction was determined as 128.5 ± 1.3 µg/mL and TEAC values were; $TEAC_{ABTS}$: 0.07 ± 0.01 and $TEAC_{CUPRAC}$: 0.15 ± 0.01.

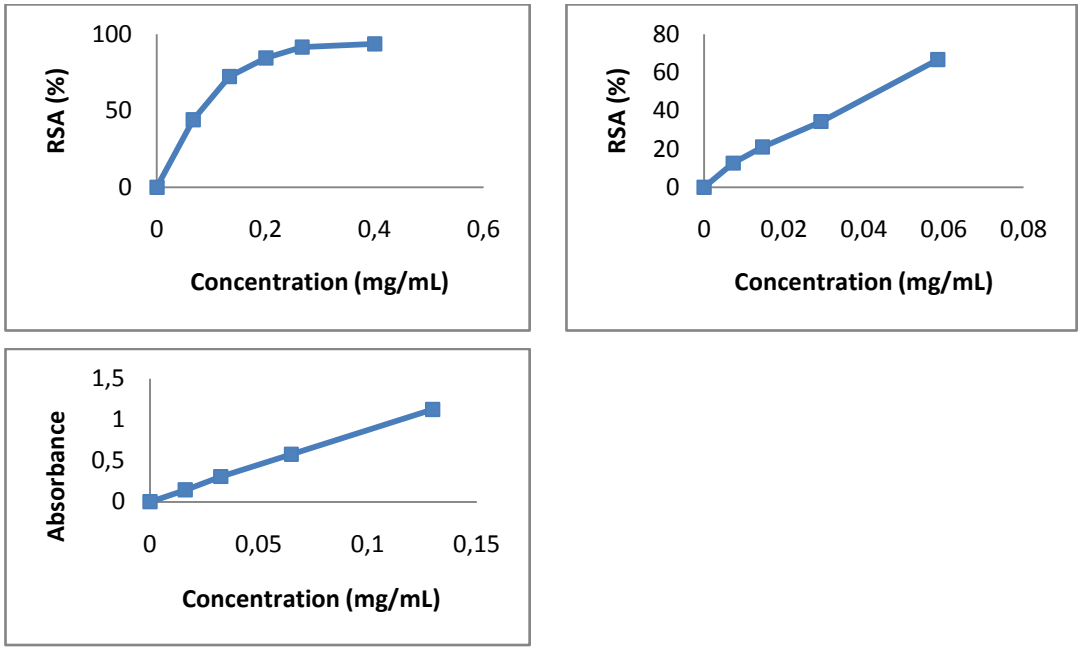


Figure 30 (a) radical scavenging activity in percent versus concentration (a) DPPH (b) ABTS and (c) metal reducing antioxidant activities of various concentrations of ethyl acetate fraction.

The low antioxidant activity and NMR analysis showed us this fraction was the mixture of sugars and trace amount of ellagic acid.

3.1.6 Antimicrobial tests

Antimicrobial activities of plant extract, fractions and isolate were studied against *E.coli*, *P.mirabilis*, *S.aureus*, and *S.pyogenes*. Bacteria, stored at -80 ^{o}C before, was suspended with related broth, streaked on to sheep blood agar plate and incubated at 37 ^{o}C for 24 hours. 3-4 colonies were taken from the grown bacteria and diluted with 10 mL broth and again incubated at 37 ^{o}C for 24 hours. Antibiotics that are used in antimicrobial tests were; Penicillin (P), Kanamycin (K), Chloramphenicol (C), Clindamycin (DA), Sulfamethoxazole (SXT), Gentamycin (CN).

Baceterial growth values were determined by the absorbance at 600 nm and by dilution absorbance of all bacterial solutions set up to 1 absorbance unit (au). 1 mL bacterial solutions of 1 au at 600 nm represents an approximately $5x10^8$ bacteria of *E.coli*, $2x10^9$ bacteria of *P.mirabilis* and $5x10^8$ for *S.pyogenes* in LB broth, also, 10^9 bacteria for *S.aureus* in BHI broth.

3.1.6.1 Disc diffusion test

Bacteria were grown as stated in 2.2.6.1. A 100 µL solution from the bacterial stock was spread on to corresponding agar plates and empty filter discs were placed on as described in section 2.2.6.1 figure 11. After the placement of discs on agar plates a 20 µL of various concentrations of plant extract, fractions and isolates were applied. Then plates were incubated at 37°C for 16 hours. After the incubation period, inhibition zone diameters were measured in millimeters. Inhibition zones of each bacteria for each analyte was displayed in figure 31-34. In all disc diffusion experiments, samples were prepared by dissolving 50 mg of total extract, 18 mg of isolate, 100 mg of petroleum ether fraction, 16 mg of ethyl acetate fraction and 60 mg of water/methanol fraction in 1 mL methanol. All experiments were duplicate and repeated 3 times.

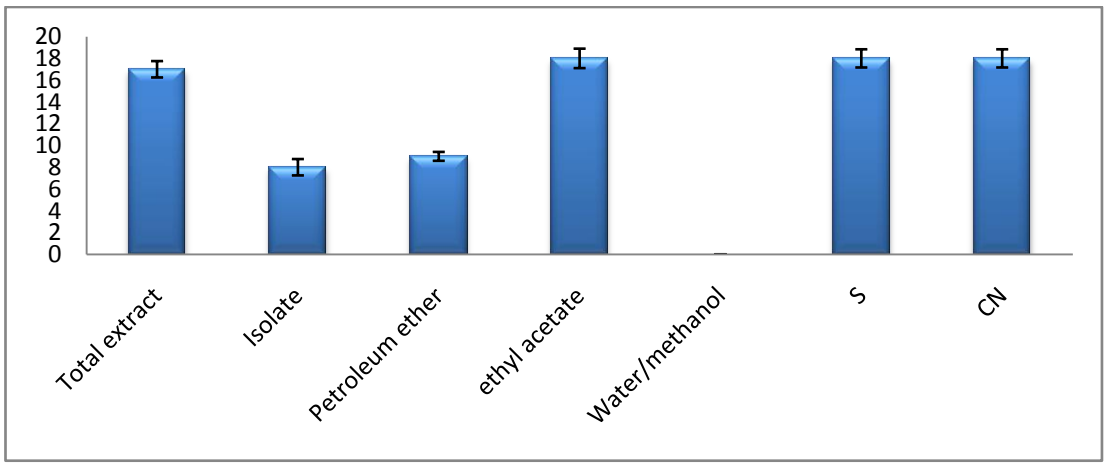


Figure 31 Antimicrobial activities of Total extract, fractions and Isolate by disk diffusion method *E.coli*. Diameter of inhibition zone (mm) includes disc diameter of 6 mm.

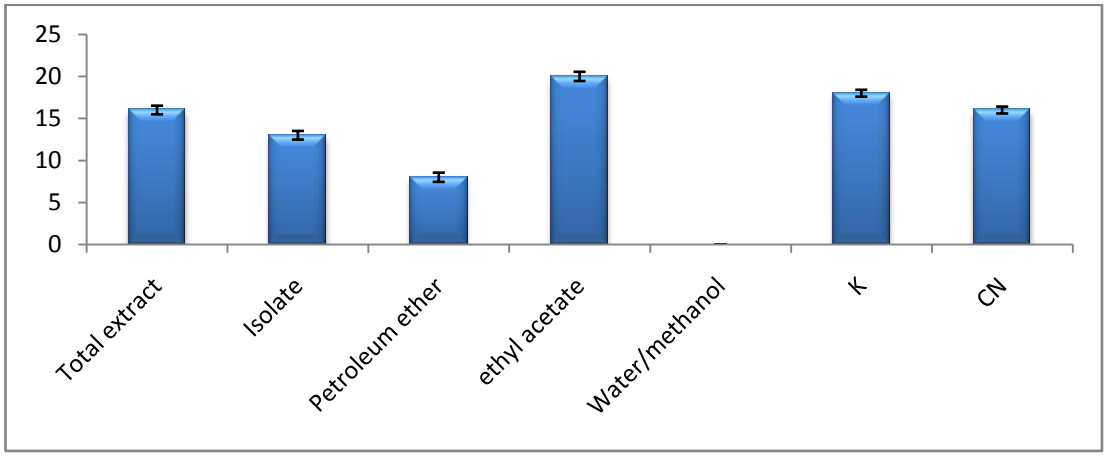


Figure 32 Antimicrobial activities of Total extract, fractions and Isolate by disk diffusion method P.mirabilis. Diameter of inhibition zone (mm) includes disc diameter of 6 mm.

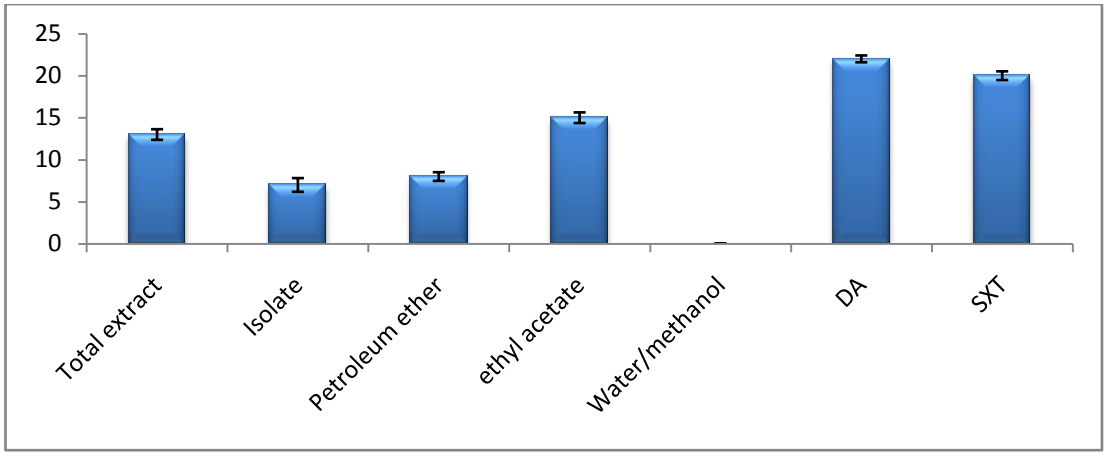


Figure 33 Antimicrobial activities of Total extract, fractions and Isolate by disk diffusion method S.aureus. Diameter of inhibition zone (mm) includes disc diameter of 6 mm.

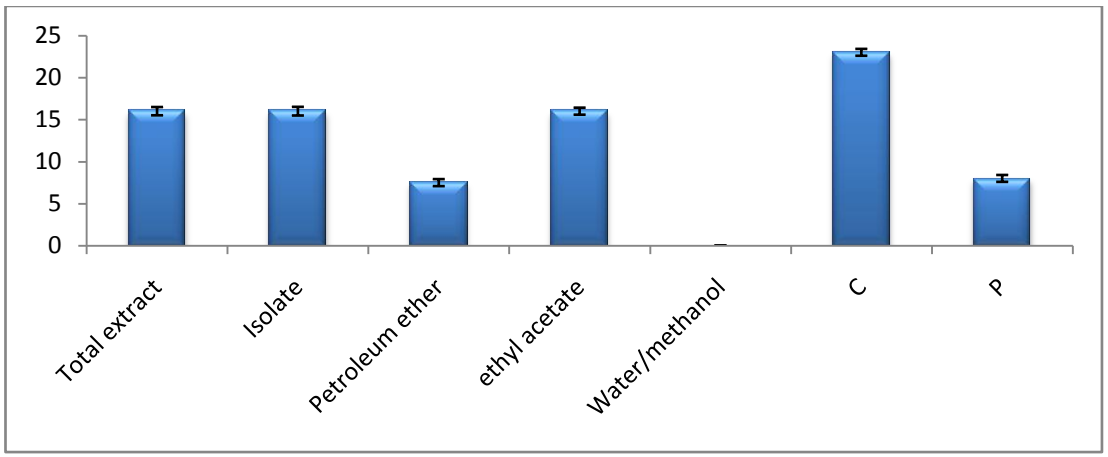


Figure 34 Antimicrobial activities of Total extract, fractions and Isolate by disk diffusion method S.pyogenes. Diameter of inhibition zone (mm) includes disc diameter of 6 mm.

In both bacterial strains ethyl acetate fraction and isolated compound, methyl gallate have dramatic inhibition zones comparing other fractions. Especially in *E.coli* and *P.mirailis* cases the effect of ethyl acetate fraction and isolate were as high as the antibiotics which are still in use to threat the diseases. Although, from the table 32 it could be understood that Kanamycin is a powerful antibiotic, however, in the inhibition zone against *P.mirabilis*, some bacterial growth was observed. So, these bacteria were gain resistance and the promising results of the analytes could be a solution in this case.

On the other hand, if we compare the ethyl acetate and isolate displayed significant effects in disc diffusion experiments, the effect of a single compound is more valuable than effect of a group of compounds in ethyl acetate fraction.

Sarafy et al. studied *Quercus branti Lindley*'s antimicrobial activity against eight bacterial strains including *Proteus mirabilis* and *Escherichia coli*. Although the strains of the bacteria was not mentioned, inhibition zones in disc diffusion tests was found to be 0.4 mg/mL methanolic extract was 8 mm for *E.coli* and 15 mm for *P.mirabilis* in 10^8 CFU (Safary, 2009).

Acording to Voravuthikunchai et al. Quercus infectiora acorn has inhibition zone of 23 mm against S.pyogenes and 12.5 mm against Escherichia coli (ATCC 25922). In this study 10^8 CFU was inoculated in to discs (Voravuthikunchai, 2008).

Serit et al. studied another Quercus species antimicrobial activity, Quercus acuta. The group also fractionated total extract with hexane, chloroform, ethyl acetate, water and observed antimicrobial activity on ethyl acetate and water fractions. 1 mg ethanolic extract of that quercus species had a zone of 20 mm in E.coli medium and did not show any activity against S.aureus (Serit, 1991).

On the other hand, Khan et al. performed fractionation with leaves of oak tree (Lithocarpus celebicus) and determined antimicrobial activity. A 20 mg of total extract, 27 mg of petroleum ether and 4 mg of ethyl acetate resulted 8, 14, 12 mm inhibition zones for S. aureus and 8, 14, 20 mm inhibition zones for E. coli (Khan, 2001).

3.1.6.2 Minimum inhibitory concentration

Minimum inhibitory concentrations were determined by using 96 well plates, as described in the methods part 2.2.6.2. All of the experiments were done in triplicates and repeated 2 times. Number of bacteria was determined at OD_{600} 0.06 au for 5 µL and calculated as; *E.coli*, approximately $1.5x10^5$ bacteria, *P.mirabilis*, approximately $6x10^5$ bacteria, and 0.05 au, for 5 µL *S.aureus* approximately $2.5x10^5$ bacteria, *S.pyogenes* approximately $1.25x10^5$ bacteria, was added to each well throughout these experiments.

3.1.6.2.1 Solvent effects

Inhibitory effects of various solvents must be determined to find the most suitable solvent with minimum inhibition. Ethanol, methanol, dimethyl sulfoxide, ethyl acetate, and ultra pure water were tested. Solvent concentrations were defined as solvent volume per total volume of medium solutions (Luria broth and Brain heart infusion broth) in 96-wells. Solvent effect studies were prepared in the concentration range of 0.02-10 %.

In order to find out the solvent with minimum inhibitory effect against bacteria, 20 µL of each respective solvent, 175 µL of broth, and 5 µL of bacteria with 0.05 OD_{570} were added to the wells, as shown in Table 1.

It was found that ethanol, methanol, dimethyl sulfoxide have only displayed the growth inhibitory effect (MIC) for concentrations of 10 % against all bacteria. All other solvents have displayed no growth inhibitory effect when used up to 10 %. Methanol was the best solvent choice, with minimum inhibitory effect and for the extract dissolution purposes in order to prepare as high extract concentrations as possible. Throughout the experiments methanol was 5% in concentrations and used as solvent.

3.1.6.2.2 Determination of minimum inhibitory concentration

To determine minimum inhibitory concentrations of crude extract, fractions and isolate, micro broth dilution method was performed as described in section 2.2.6.2.2. 175 µL broth and 20 µL analyte placed in to first column of 96 well plates. From 2nd to 7th column are filled with 95 µl broth and 8th column was empty. Two fold dilution made to each column. 10 mg/mL Penicillin used as antibiotic and placed in last two columns. Lastly 5 µL bacteria were added except 9th and 11th columns to observe the sterility. After 16 h incubation time, absorbance values were determined and graphs for each bacteria plotted as final concentration versus absorbance. Final extract concentrations were prepared based on the results in disc diffusion experiment and resuts were illustrated in Appendix D.

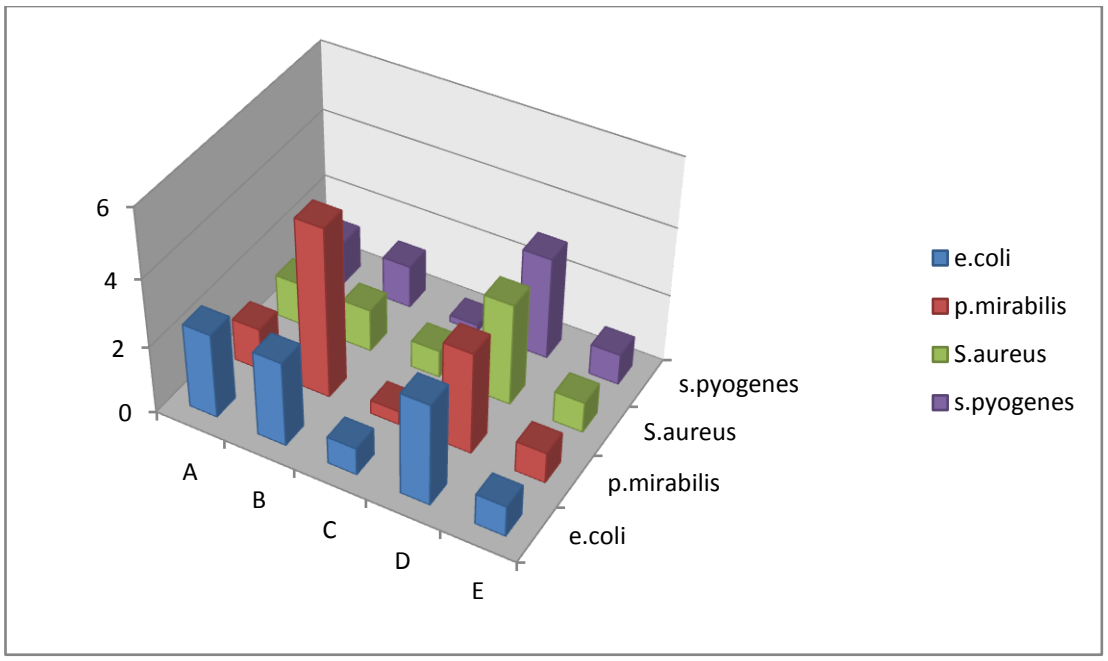


Figure 35 Determination of minimum inhibitory concentrations (mg/mL) A: Total extract, B: Petroleum ether fraction, C: Ethyl acetate fraction D: Water/methanol fraction E: Isolate against *E.coli*, *P.mirabilis, S.aureus* and *S.pyogenes*.

Total extract, petroleum ether, ethyl acetate, water/methanol fractions and methyl gallates minimum inhibitory concentrations were determined as 2.5, 2.5, 0.8, 3.0, 0.9 mg/mL for *E.coli*, 1.25, 5.0, 0.4, 3.0, 0.9 mg/mL for *P.mirabilis*, 1.25, 1.25, 0.8, 3.0, 0.9 mg/mL for *S.aureus* and 1.25, 1.25, 0.2, 3.0, 0.9 mg/mL for *S.pyogenes* respectively.

If the MIC concentrations of analytes compared with antibiotic which used in the experiment (Penicillin, 2 mg/mL), ethyl acetate and isolate shows significant effect as expected from the disc diffusion experiment. With the help of synergetic effect, ethyl acetate fraction always showed higher inhibition where as water/methanol fraction showed the lowest effect. Isolated methyl gallate showed also high effect on both bacterial strains.

In the literature, 0.8 mg/ml ethanolic extract of *Quercus infectoria* inhibited 10^4 CFU/mL *E.coli*. Comparing with *Quercus brantii* which inhibited 1.5 x 10^5 bacteria inhibited by 2.5 mg/mL it has less antimicrobial activity (Voravuthikunchai, 2008).

Sarafy et al. studied *Quercus branti Lindley*'s minimum inhibitory concentration against *Proteus mirabilis* and 4 other bacterias. They found could not found methanolic extracts MIC for P.mirabilis, however ethanolic extract MIC was 18 mg/ml for 10^6 CFU/ml *P.mirabilis* (Safary, 2009).

According to Binutu, minimum inhibitory concentration of isolated methyl gallate with *S.aureus* and *E.coli* resulted 0.250 mg/ml and 0.125 mg/ml respectively. In our experiments 10 folds more bacteria were used and found that 0.9 mg/ml inhibits 10^5 bacteria (Binutu, 2000). This compound have also inhibitory potential against herpes simplex virus in vitro, adhesion of human leukocytes, adhesion of cancer cells with vascular endothelial cells (Masibo, 2008).

3.1.6.3 Minimum bactericidal concentration

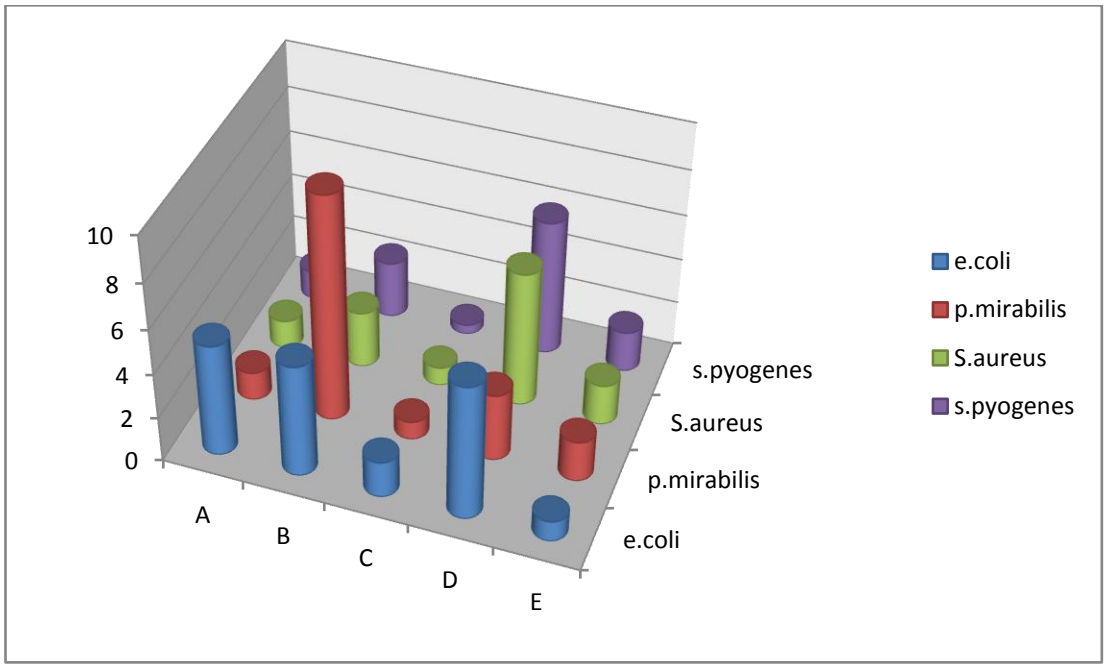


Figure 36 Determination of minimum bactericidal concentrations (mg/ml) A: Total extract, B: Petroleum ether fraction, C: Ethyl acetate fraction D: Water/methanol fraction E: Isolate against *E.coli, P.mirabilis, S.aureus* and *S.pyogenes*.

Total extract, petroleum ether, ethyl acetate, water/methanol fractions and methyl gallates minimum bactericidal concentrations were determined as 5.0, 5.0, 1.6, 6.0, 0.9 mg/mL for *E.coli*, 1.25, 10, 0.8, 3.0, 1.8 mg/mL for *P.mirabilis*, 1.25, 2.5, 0.8, 6.0, 1.8 mg/mL for *S.aureus* and 1.25, 2.5, 0.4, 6.0, 1.8 mg/mL for *S.pyogenes* respectively.

MBC experiments clearly show total extract is effective against all bacterial strains except *E.coli*. If the fractions were compared, as expected from MIC results, most effective analyte was ethyl acetate fraction.

In the literature, Sarafy et al. studied *Quercus branti Lindley*'s minimum bactericidal concentration against *Proteus mirabilis* and 4 other bacteria. They found could not found methanolic extracts MBC for *P.mirabilis*, however ethanolic extract MBC was 30 mg/ml for 10^6 CFU/ml P.mirabilis (Safary, 2009).

Quercus infectoria ethanol extract has MBC of 0.8 mg/ml for 10^4 CFU/mL *E.coli* and 1.6 mg/ml for 10^4 CFU/mL *S.aureus* (Voravuthikunchai, 2008).

Table 3 Comparison of DPPH EC_{50} (mg/mL), $TEAC_{ABTS}$ and $TEAC_{CUPRAC}$ results for all tested extracts, isolated compound methyl gallate and standard quercetin

	DPPH EC_{50} (μg/mL)	$TEAC_{ABTS}$ (mg/mg)	$TEAC_{CUPRAC}$ (mg/mg)
Total extract	31.04 ± 0.19	0.36 ± 0.01	0.43 ± 0.01
Petroleum ether fraction	29.24 ± 0.01	ND	NA
Ethyl acetate fraction	7.91 ± 0.08	2.32 ± 0.01	2.96 ± 0.01
Water/methanol fraction	128.5 ± 1.3	0.07 ± 0.01	0.15 ± 0.01
Methyl gallate	9.19 ± 0.04	0.65 ± 0.01	1.02 ± 0.01
Quercetin	8.09 ± 0.17	2.90 ± 0.01	4.52 ± 0.01

NA: not applicable, ND: not determined

Table 4: Comparison of disc diffusion (mm), MIC (mg/mL) and MBC (mg/mL) results for all tested extracts, isolated compound methyl gallate

	E.coli			*P.mirabilis*			*S.aureus*			*S.pyogenes*		
	Disc Diffusion (mm)	MIC (mg/mL)	MBC (mg/mL)	Disc Diffusion (mm)	MIC (mg/mL)	MBC (mg/mL)	Disc Diffusion (mm)	MIC (mg/mL)	MBC (mg/mL)	Disc Diffusion (mm)	MIC (mg/mL)	MBC (mg/mL)
Total extract	17	2.5	5.0	13	1.25	1.25	16	1.25	1.25	16	1.25	1.25
Petroleum ether fraction	9	2.5	5.0	8	5.0	10	8	1.25	2.5	7	1.25	2.5
Ethyl acetate fraction	18	0.8	1.6	15	0.4	0.8	20	0.8	0.8	16	0.2	0.4
Water/methanol fraction	ND	3.0	6.0	ND	3.0	6.0	ND	3.0	6.0	ND	3.0	6.0
Methyl gallate	8	0.9	0.9	7	0.9	1.8	13	0.9	1.8	16	0.9	1.8

Diameter of inhibition zone (mm) including disk diameter of 6 mm. ND: not determined

CHAPTER 4

CONCLUSIONS

All of the antioxidant and antimicrobial methods that were used in this study were summarized in Table 3 and 4. As a conclusion, methanolic extract of *Q. brantii* showed high antioxidant and antimicrobial effects.

Phenolic profile of the total extract was qualified by using HPLC. A new elution system was generated for this plant and with the help of standards and HRMS analysis phenolic content of the selected plant was characterized.

Fractionation was performed to separate the antioxidant compounds in total extract by utilizing their solubility differences. Ethyl acetate and diethyl ether fractions exhibited the highest antioxidant capacity. Due to the low yield and high number of compounds in ethyl acetate fraction, HRMS was used to characterize these molecules. On the other hand, in diethyl ether fraction there was a single major compound with a high antioxidant activity. The major compound, was characterized by NMR, IR and HRMS as methyl gallate.

Finally, in order to determine the bioactivity of the plant extract, fractions and isolated compound, disc diffusion, MIC and MBC experiments were performed against two gram-negative and two gram positive bacterial strains. The results were parallel to their antioxidant activities. Ethyl acetate fraction and methyl gallate showed a significant effect on all bacterial strains even as high as standard antibiotics in use.

Consequently, total extract, isolated product and ethyl acetate fraction could be considered as powerful antibacterial agents, and at the same time efficacious antioxidants.

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APPENDIX A

NAMES, STRUCTURES, HPLC CHROMATOGRAMS AND RETENTİON TIMES OF STANDARDS

Table 2 Names, structures, HPLC chromatograms and retention times of standards

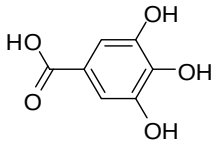
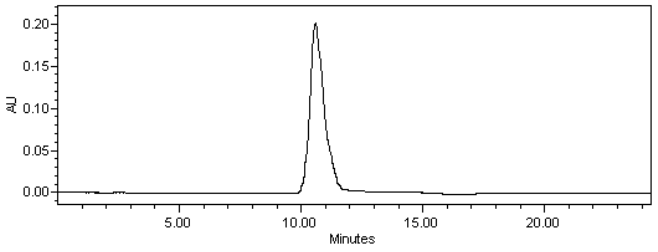
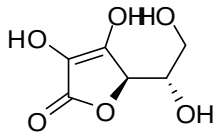
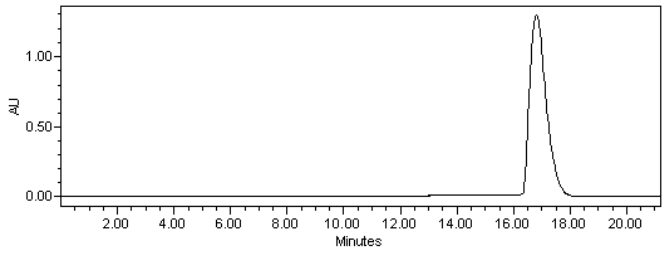
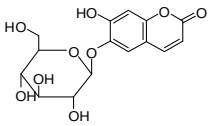
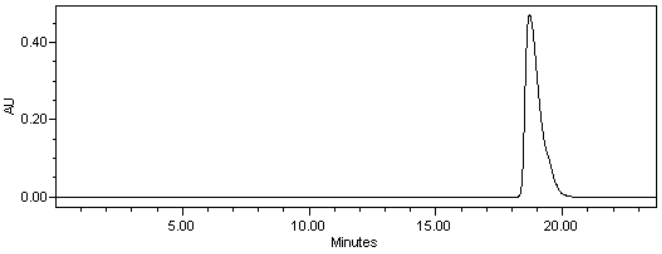
Structure & Name	Chromatogram	Retention time
 Gallic acid		10.7
 L- ascorbic acid		16.9
 Esculin		18.6

Table 2 (continued)

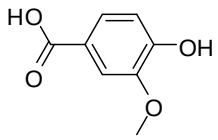
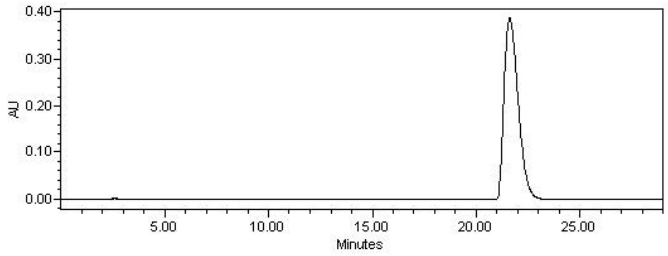
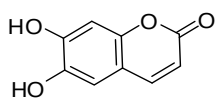
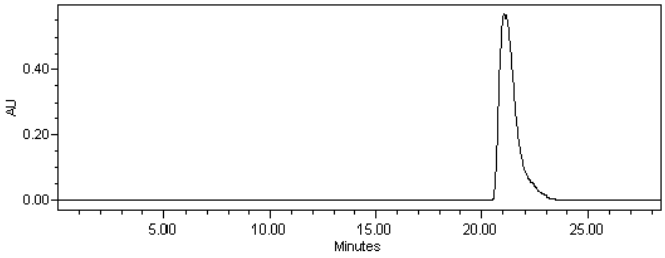
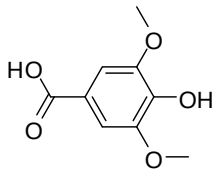
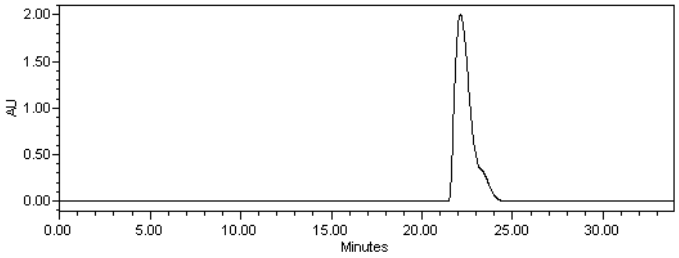
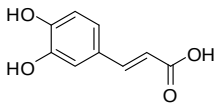
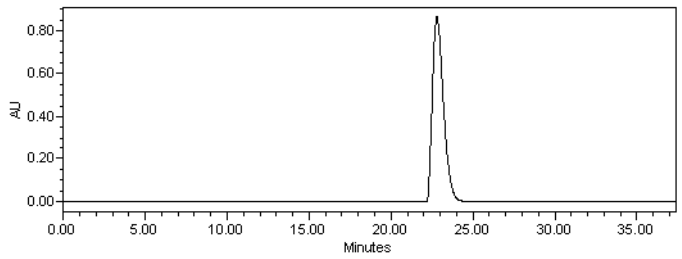
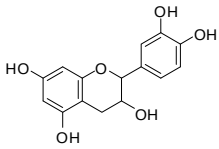
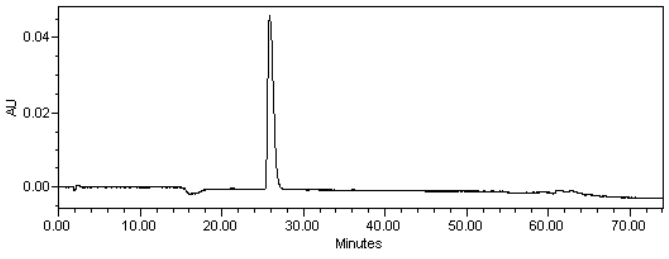
 Vanilic acid		21.5
 Esculetin		21.7
 Syringic acid		22.1
 Caffeic acid		23.0
 Epicatechin		25.9

Table 2 (continued)

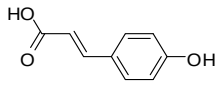
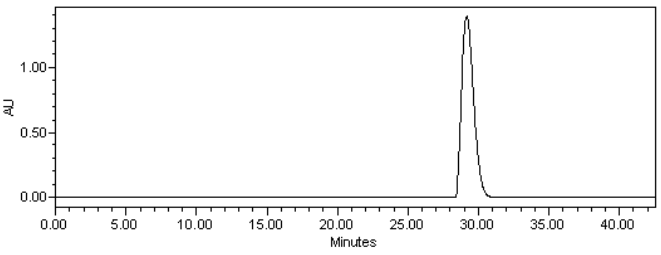
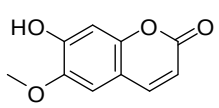
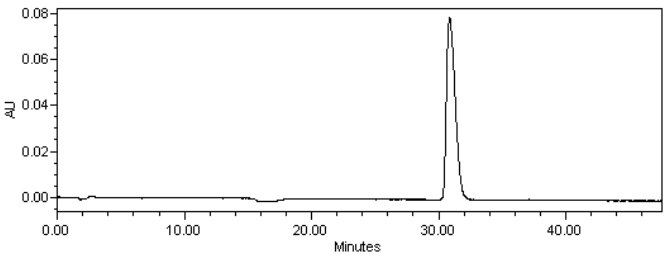
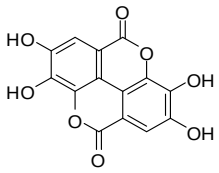
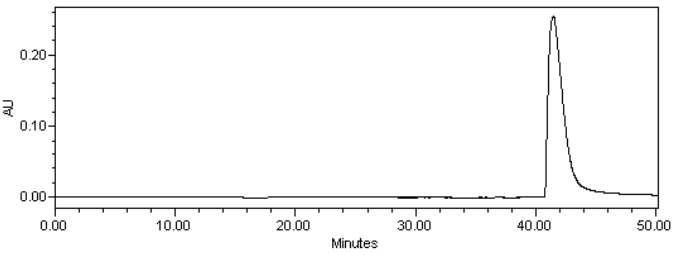
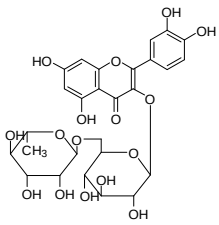
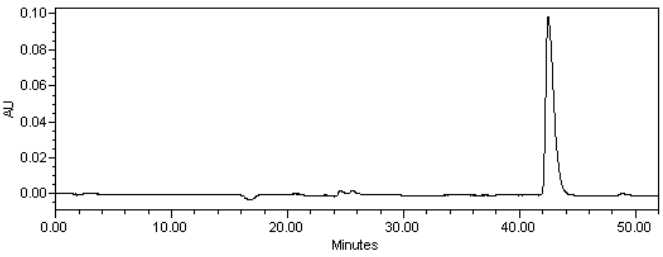
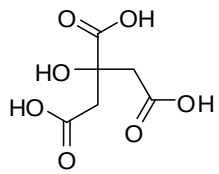
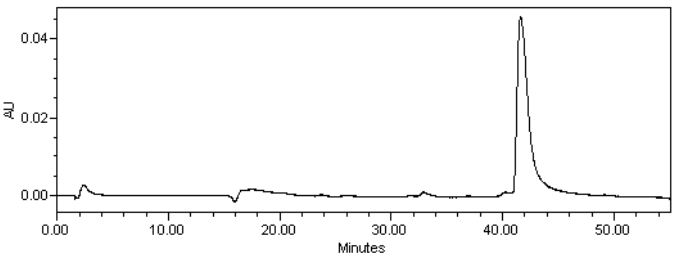
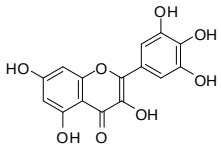
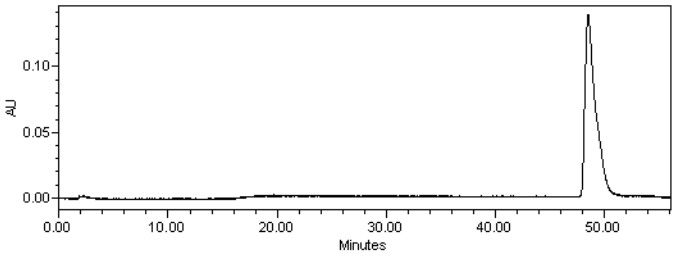
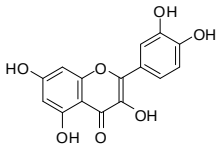
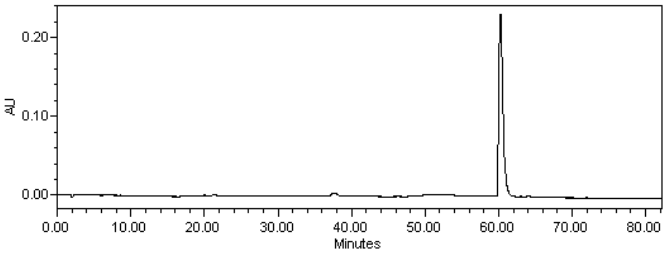
 p-coumaric acid		29.1
 Scopoletin		31.0
 Ellagic acid		41.6
 Rutin		42.7
 Citric acid		43.7

Table 2 (continued)

 Myricetin		48.6
 Quercetin		60.4

APPENDIX B

NMR, IR AND HRMS SPECTRA OF METHYL GALLATE

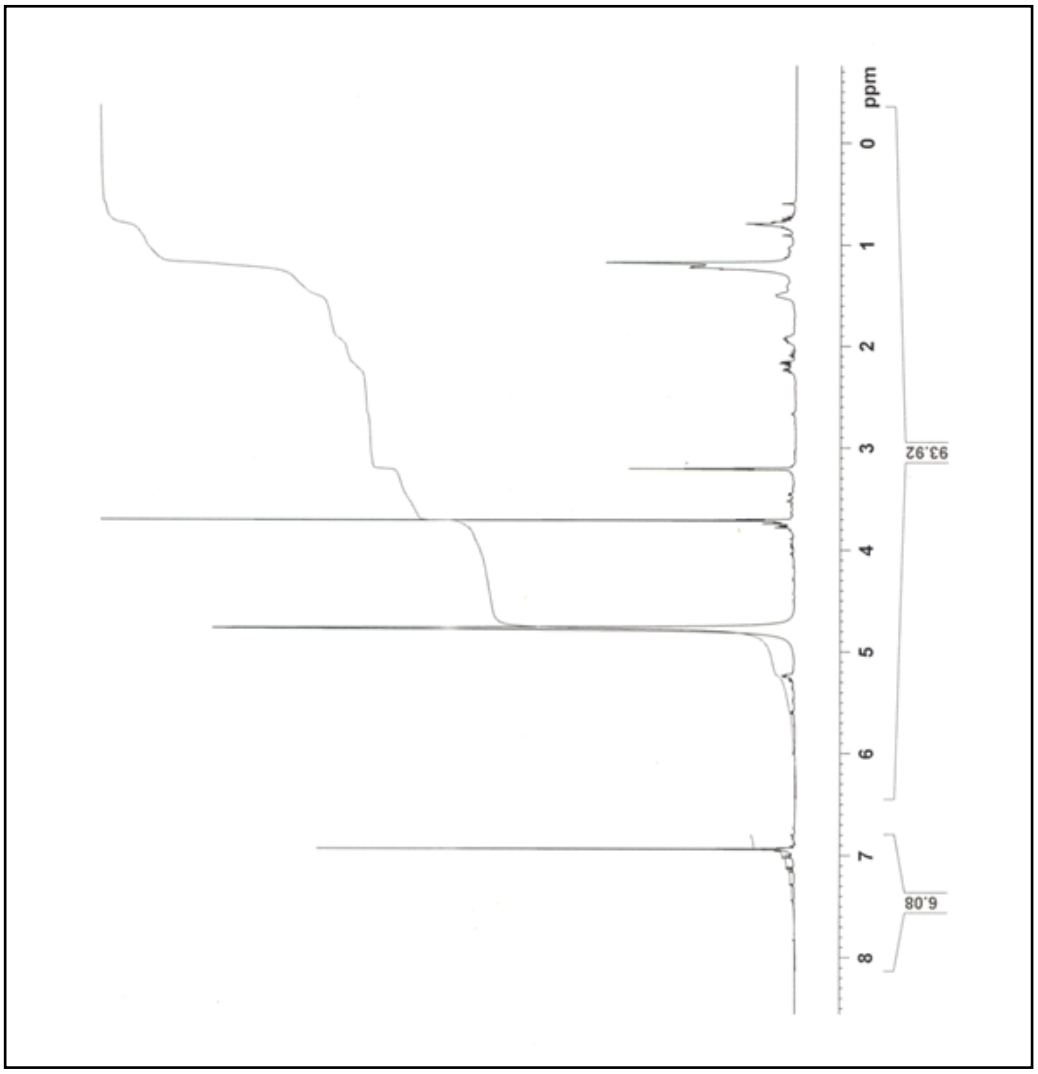


Figure 37 ^{1}H -NMR Spectrum of Methyl gallate

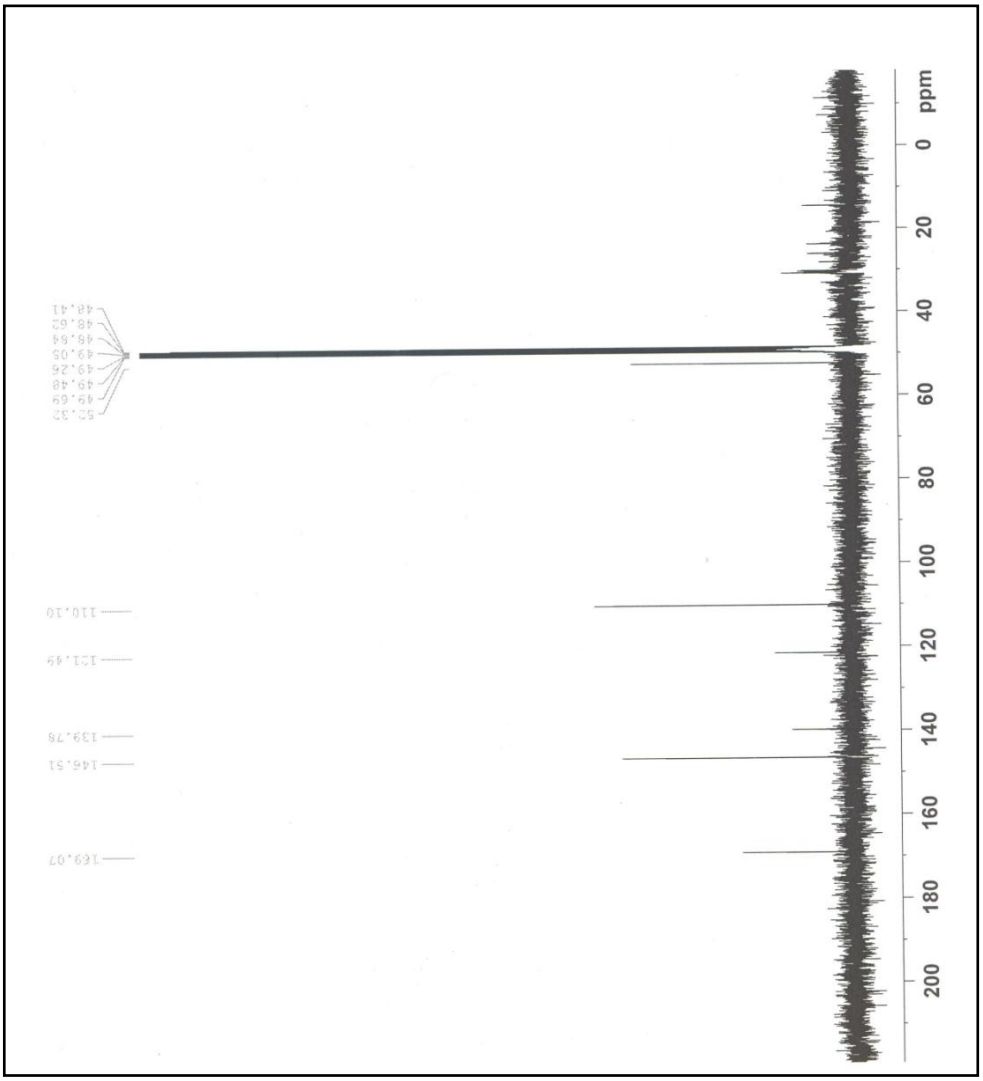


Figure 38 ^{13}C -NMR Spectrum of Methyl gallate

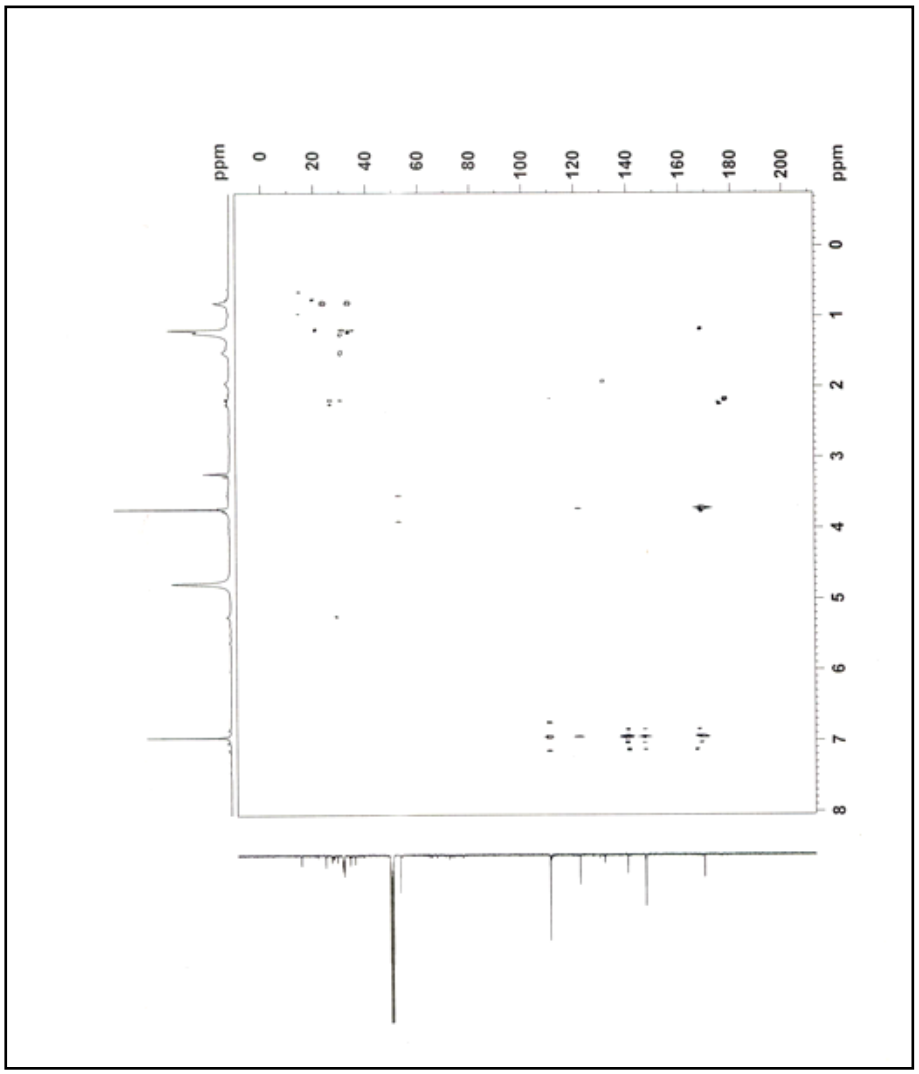


Figure 39 HMBC Spectrum of Methyl gallate

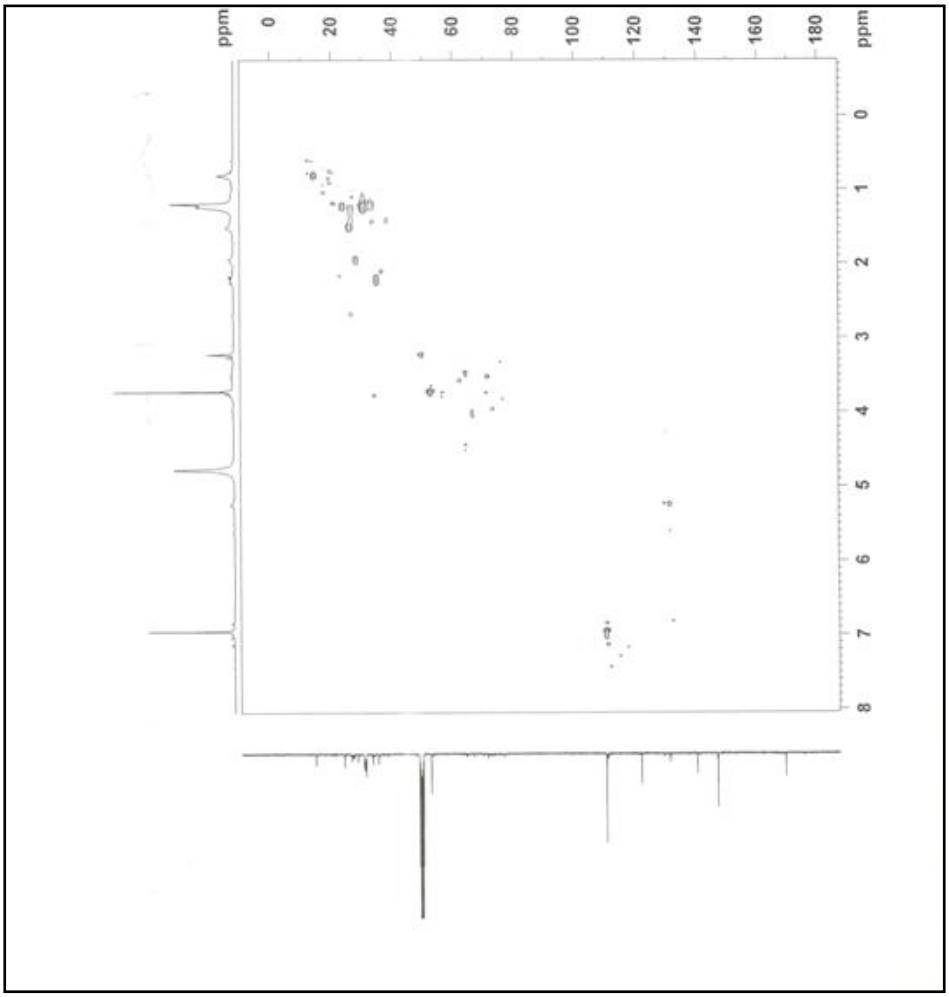


Figure 40 HSQC Spectrum of Methyl gallate

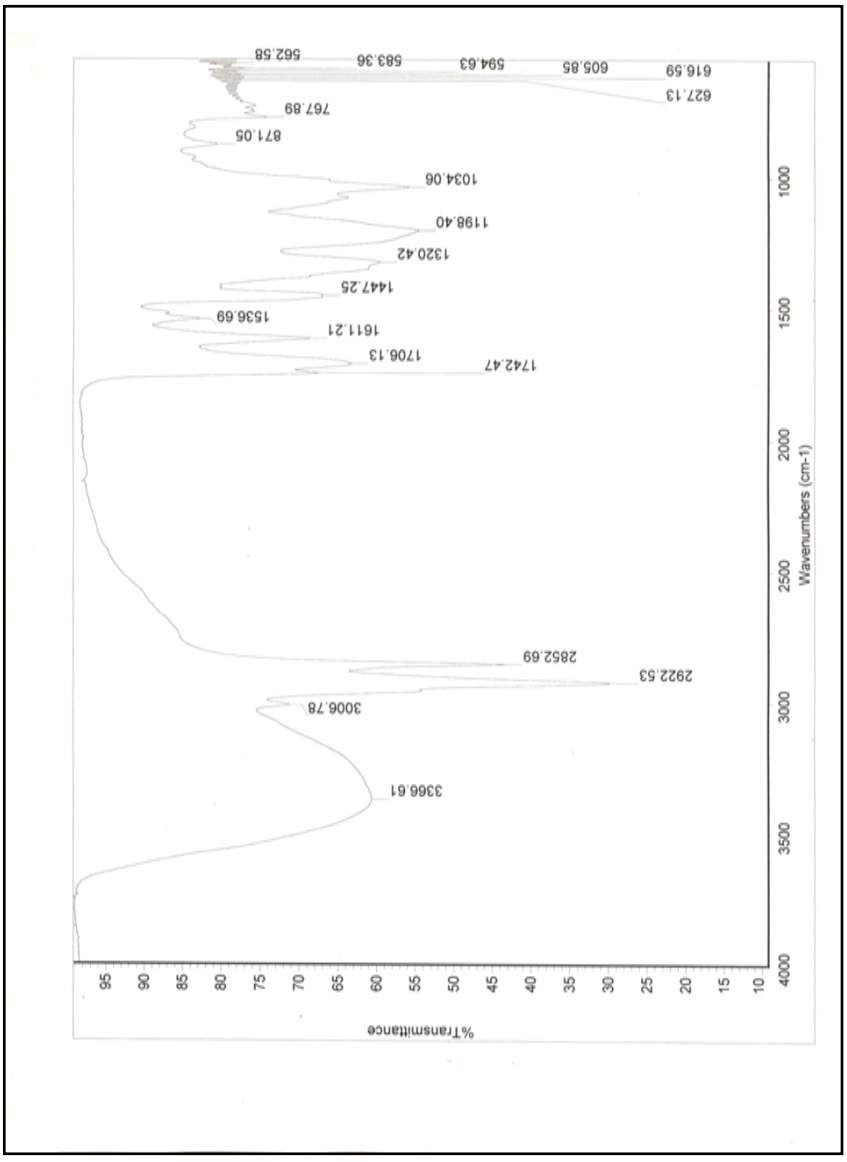


Figure 41 IR Spectrum of Methyl gallate

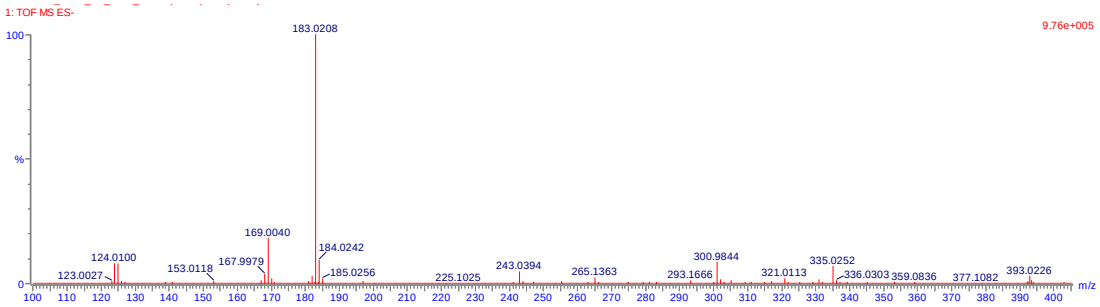


Figure 42 Mass spectrum of Methyl gallate

APPENDIX C

HRMS SPECTRA OF TOTAL EXTRACT AND ETHYL ACETATE FRACTION

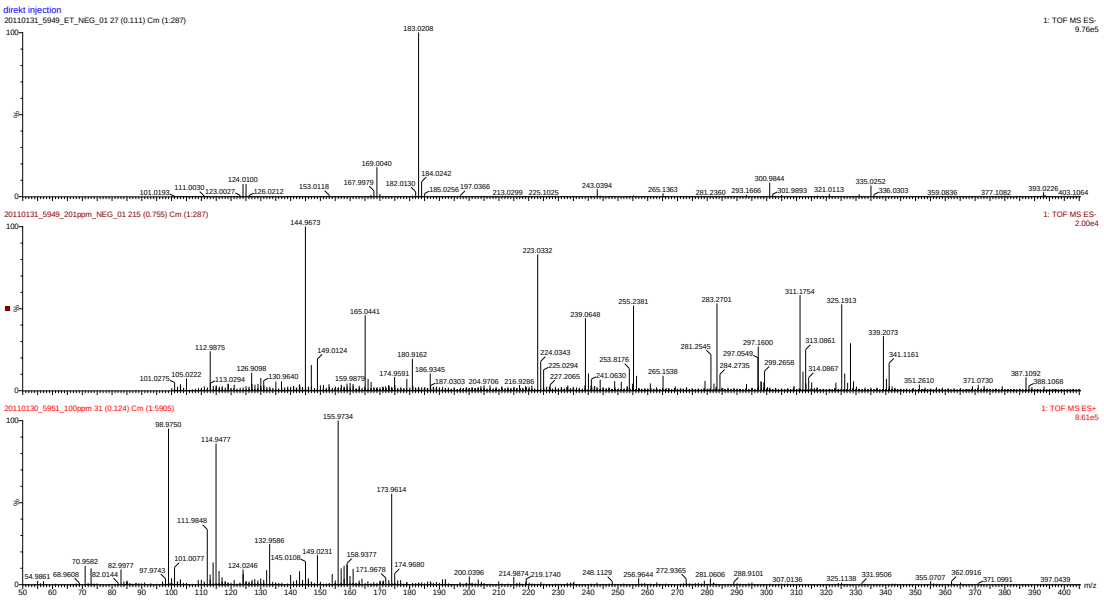


Figure 43 Mass spectrum of A: Ethyl acetate fraction, TOF^- B: Totat extract TOF^+ C: Totat extract TOF^-

APPENDIX D

MIC RESULTS OF TOTAL EXTRACT FRACTIONS AND METHYL GALLATE

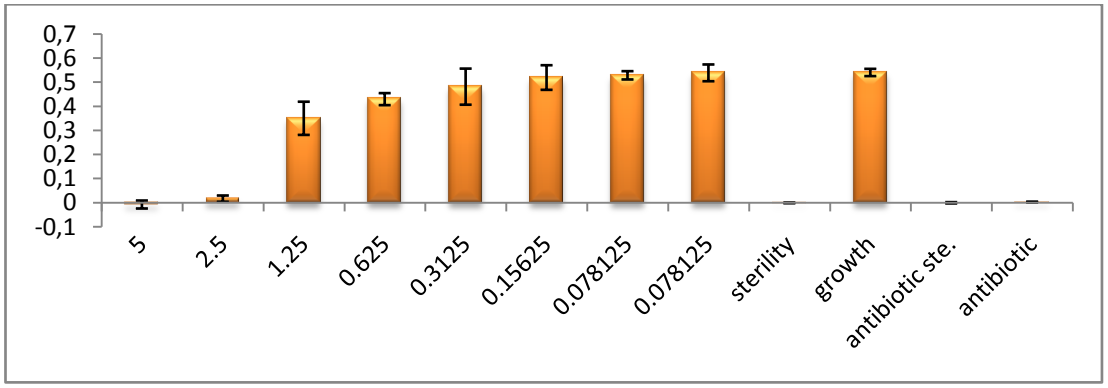


Figure 44 MIC of total extract on E.coli

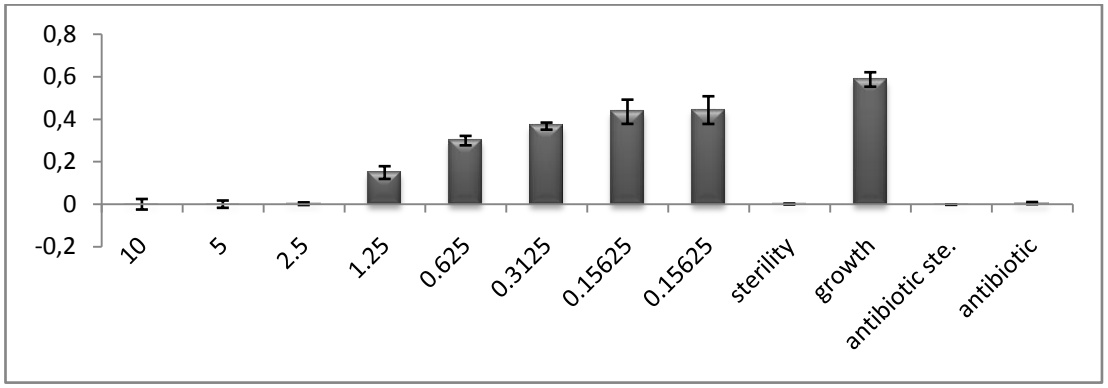


Figure 45 MIC of petroleum ether fraction on E.coli

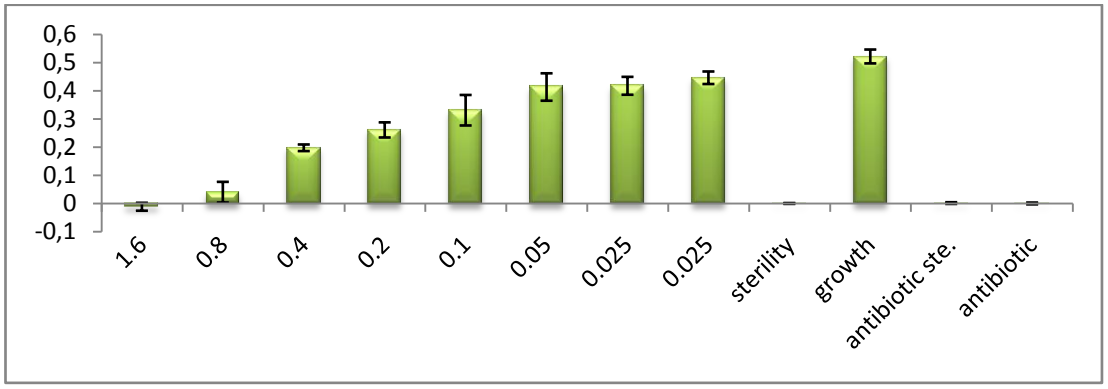


Figure 46 MIC of petroleum ether fraction on E.coli

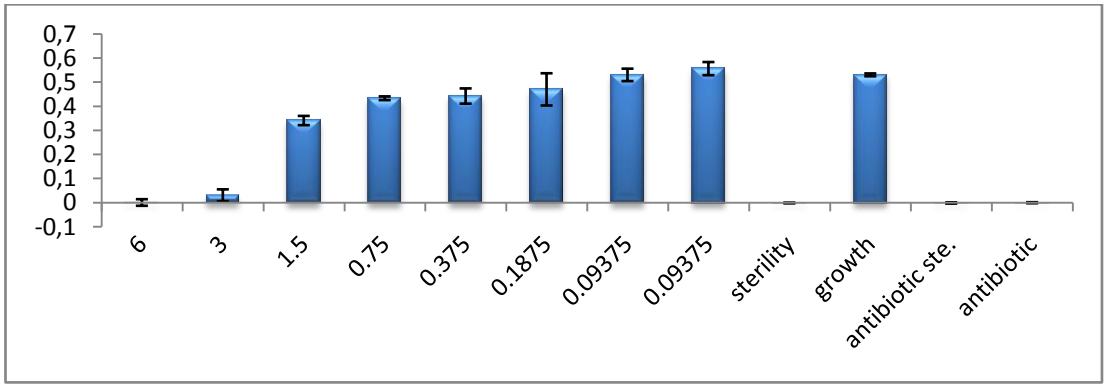


Figure 47 MIC of water/methanol fraction on E.coli

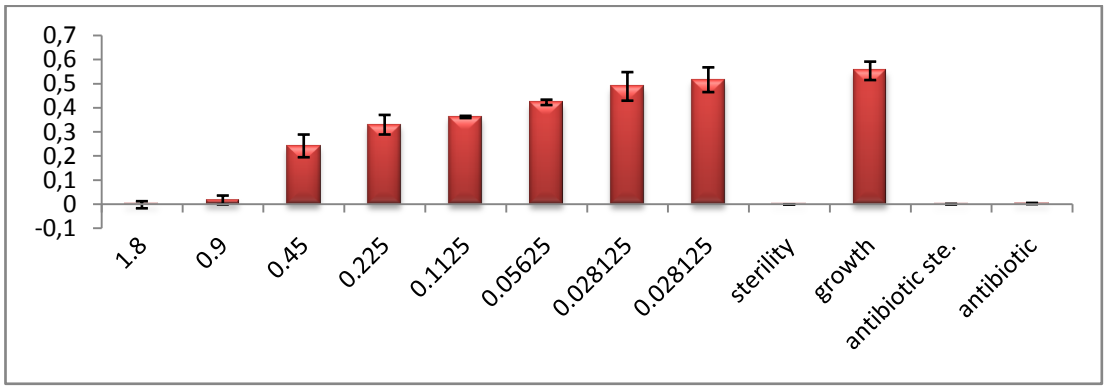


Figure 48 MIC of isolate on E.coli

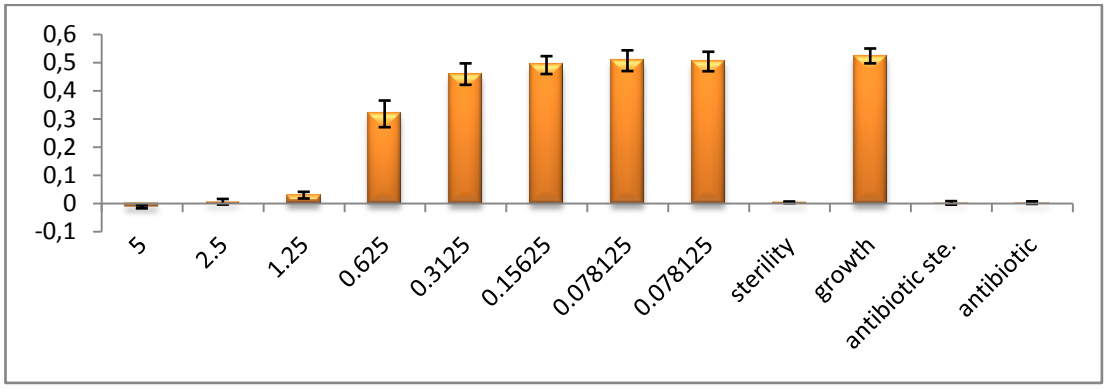


Figure 49 MIC of total extract on S.aureus

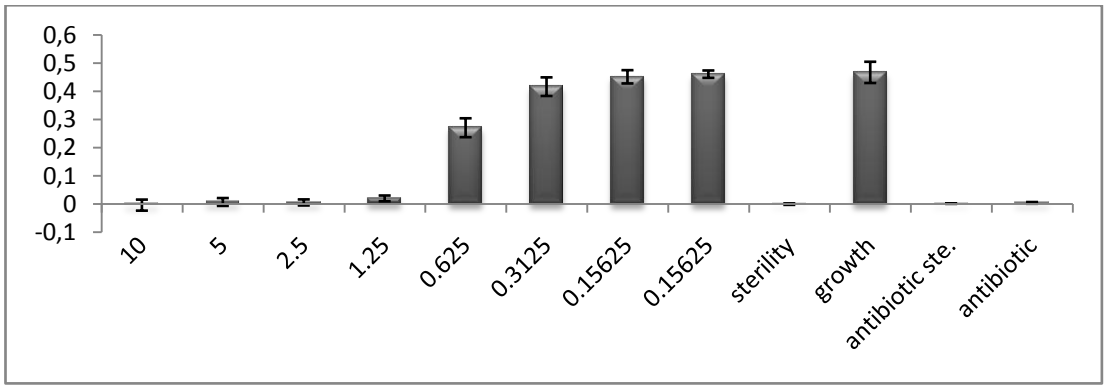


Figure 50 MIC of petroleum ether fraction on S.aureus

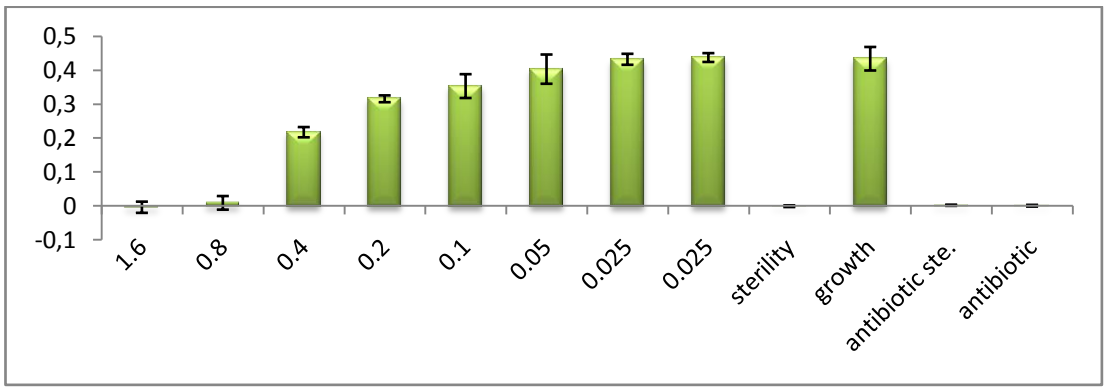


Figure 51 MIC of ethyl acetate fraction on S.aureus

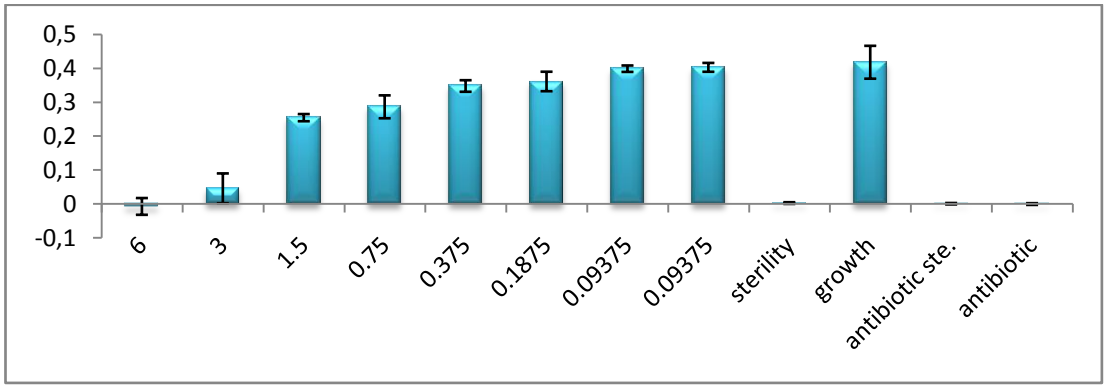


Figure 52 MIC of water/methanol fraction on S.aureus

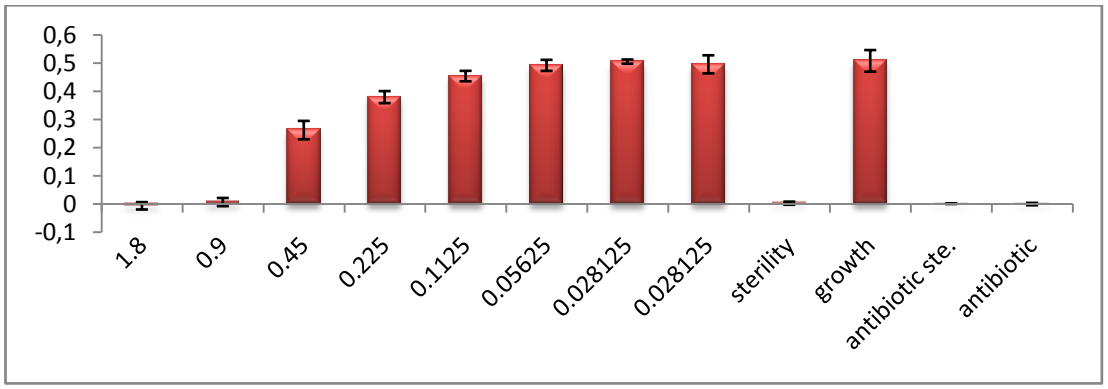


Figure 53 MIC of isolate on S.aureus

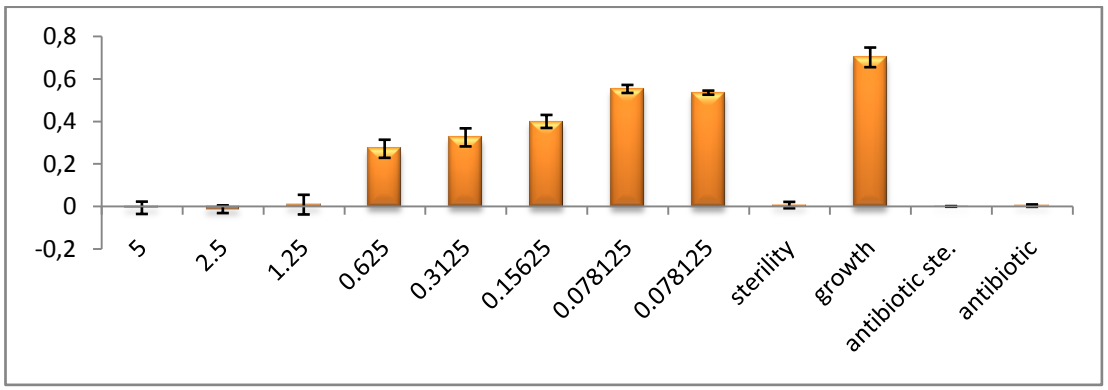


Figure 54 MIC of total extract on p.mirabilis

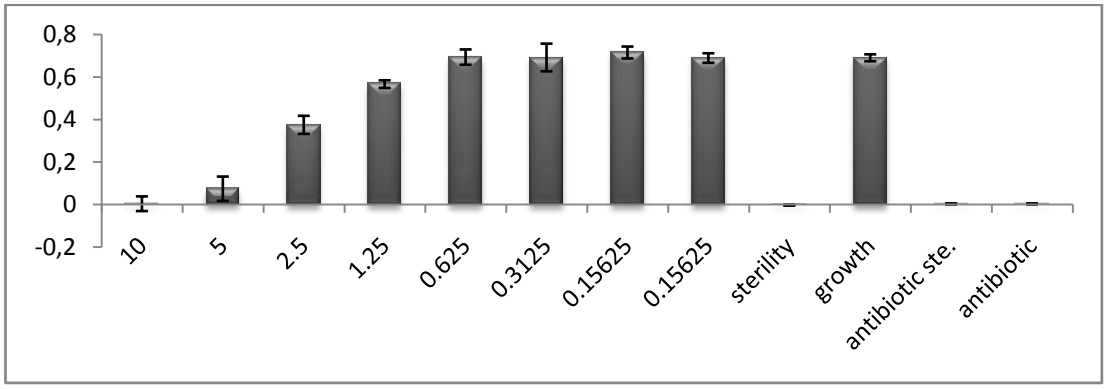


Figure 55 MIC of petroleum ether fraction on P.mirabilis

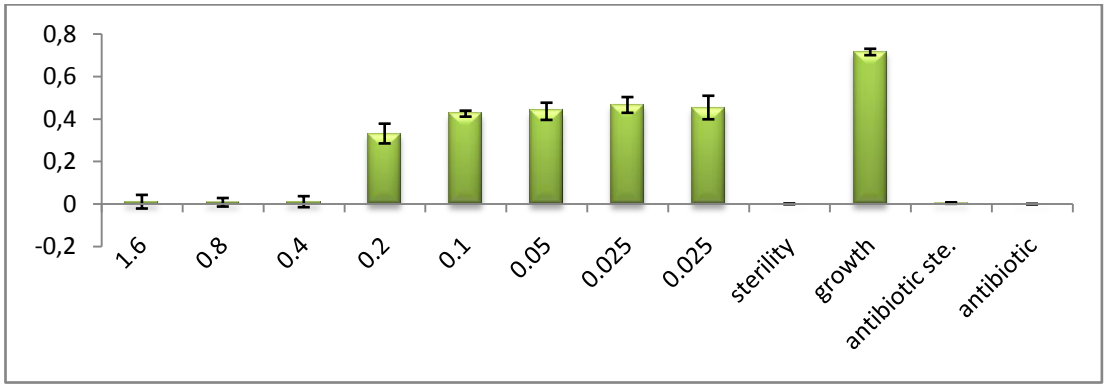


Figure 56 MIC of ethyl acetate fraction on P.mirabilis

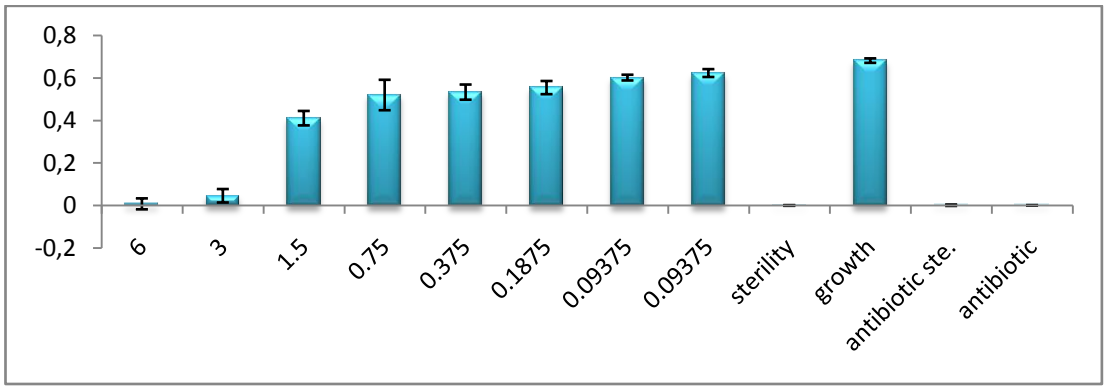


Figure 57 MIC of water/methanol fraction on P.mirabilis

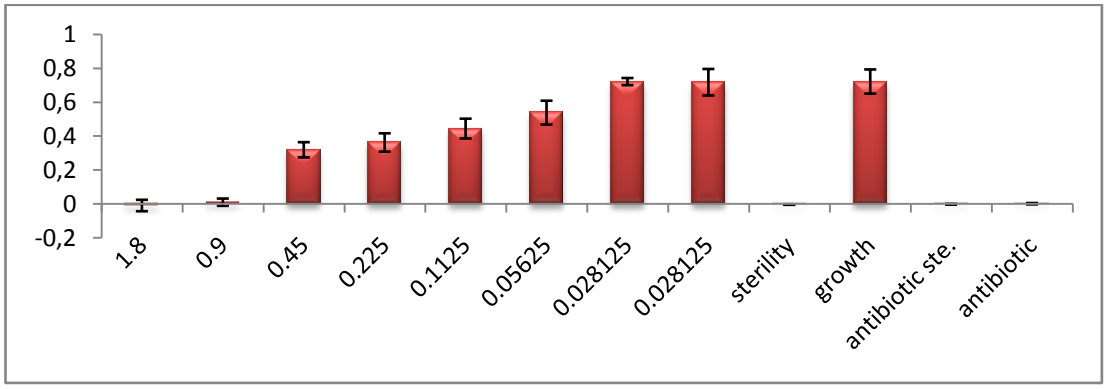


Figure 58 MIC of isolate on P.mirabilis

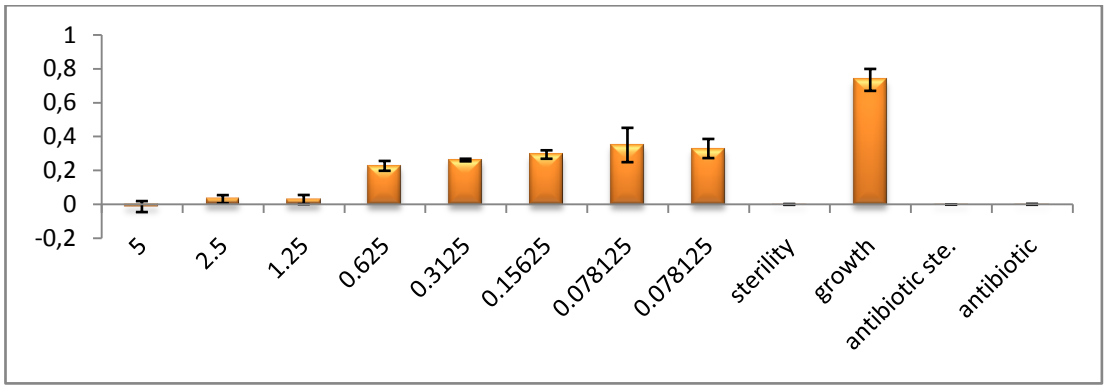


Figure 59 MIC of total extract on S.pyogenes

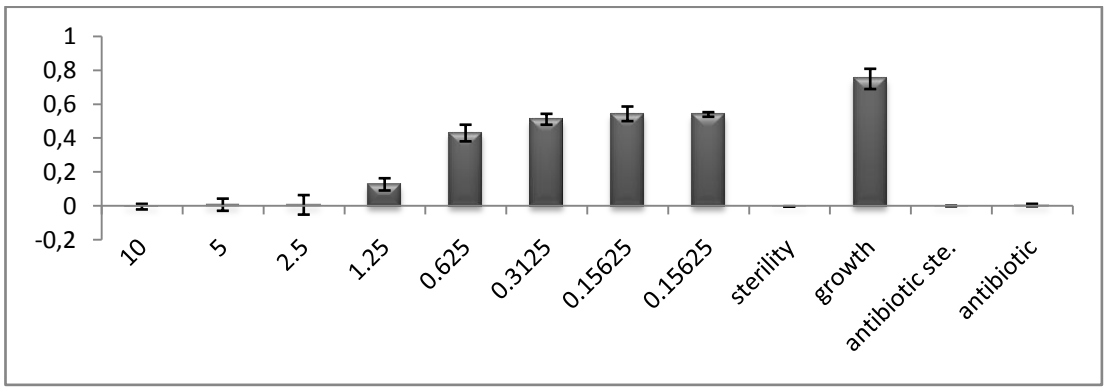


Figure 60 MIC of petroleum ether fraction on S.pyogenes

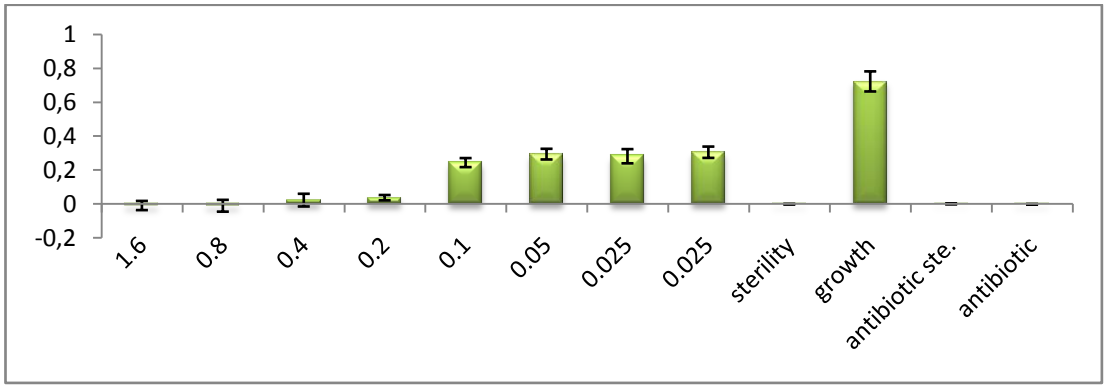


Figure 61 MIC of ethyl acetate fraction on S.pyogenes

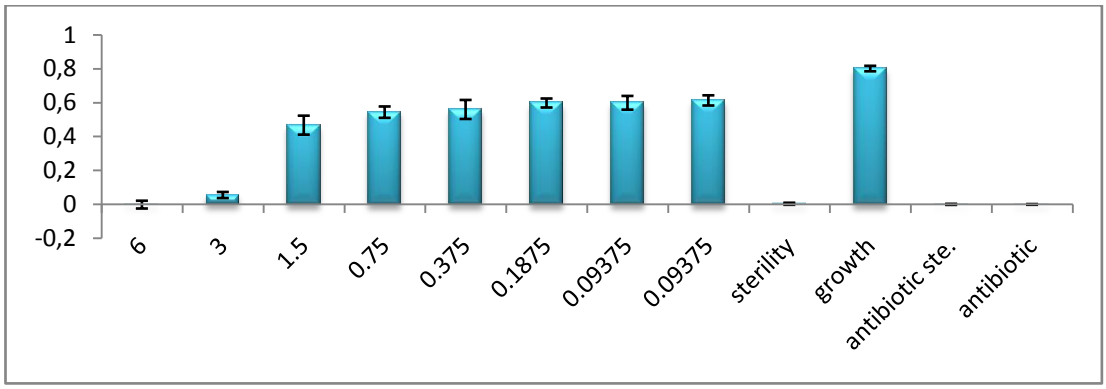


Figure 62 MIC of water/methanol fraction on S.pyogenes

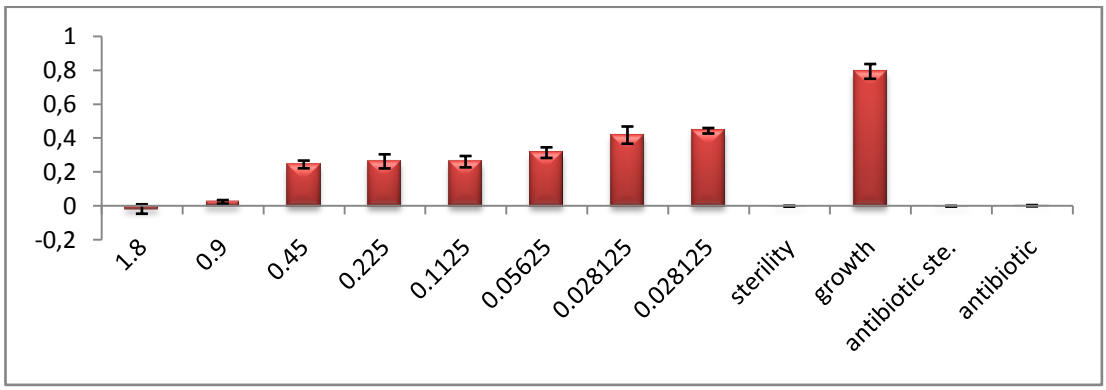


Figure 63 MIC of isolate on S.pyogenes