INVESTIGATION OF ANTIOXIDANT AND ANTIMICROBIAL EFFECTS OF PLANTAGO MAJOR LEAVES

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

BY

ÖZGE KAYA

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

SEPTEMBER 2011

Approval of the thesis:

INVESTIGATION OF ANTIOXIDANT AND ANTIMICROBIAL EFFECTS OF *PLANTAGO MAJOR* LEAVES

submitted by **ÖZGE KAYA** in partial fulfillment of the requirements for the degree of **Master of Science in Department of Biochemistry, Middle East Technical University** by,

Prof. Dr. Canan Özgen _____
Dean, Graduate School of **Natural and Applied Sciences**

Prof. Dr. G.Candan Gürakan _____
Head of Department, **Biochemistry,METU**

Assoc. Prof. Dr. Nursen Çoruh _____
Supervisor, **Chemistry Dept., METU**

Assist. Prof. Dr. A. Gülçin Sağdıçoğlu Celep _____
Co-Supervisor, **Family and Consumer Sci. Dept., Gazi Unv.**

Examining Committee Members:

Prof. Dr. Musa Doğan _____
Biology Dept., METU

Assoc. Prof. Dr. Nursen Çoruh _____
Chemistry Dept., METU

Prof. Dr. Orhan Adalı _____
Biology Dept., METU

Prof. Dr. Ceyhan Kayran _____
Chemistry Dept., METU

Assist. Prof. Dr. A. Gülçin Sağdıçoğlu Celep _____
Family and Consumer Sci. Dept., Gazi University

Date: 09.09.2011

I hereby declare that all information in this document has been obtained and presented in accordance with academic rules and ethical conduct. I also declare that, as required by these rules and conduct, I have fully cited and referenced all material and results that are not original to this work.

Name, Last name: Özge KAYA

Signature:

ABSTRACT

INVESTIGATION OF ANTIOXIDANT AND ANTIMICROBIAL EFFECTS OF *PLANTAGO MAJOR* LEAVES

KAYA, Özge
M.Sc., Department of Biochemistry
Supervisor: Assoc. Prof. Dr. Nursen ÇORUH
Co-Supervisor: Assist. Prof. Dr. A. Gülçin Sağdıçoğlu CELEP

September 2011, 96 pages

This study was designed to investigate *Plantago major* leaves, which is widely used in ethnobotanical uses in Turkey as is the case in all over the world, for their antioxidant capacities and antimicrobial effect on two gram-positive and two gram-negative bacteria.

Extracts of *P. major* leaves prepared in the methanol and fraction samples were collected in solvents in increasing polarity, from non-polar to polar, to separate bioactive compound in crude extract. Antioxidant capacities of the crude extracts and fractions were examined by radical scavenging methods, namely 2,2-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) ABTS and 1,1-diphenyl-2-picryl-hydrazyl (DPPH); as well, total phenolic and total flavonoid contents were determined. Furthermore, antimicrobial activities of crude extract and fractions were calculated in terms of minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) methods together with disc diffusion method.

Among the investigated samples, ethyl acetate phase had the highest radical scavenging capacity with the ABTS value of 2130.4±9.845 µmol equivalents of trolox/ g of ethyl acetate fraction DW, and with EC_{50} value of 0.011±0.001 mg/mL; further, the highest phenolic content was found in ethyl acetate phase with values of 0.452 GAE mg/mg of ethyl acetate fraction DW and 0.434± 0.0017 mg catechin equivalent in per mg of ethyl acetate fraction DW.

In general, it was found that the crude extract and aqueous phase of *Plantago major* had a weak antimicrobial effect on *S. aureus, P.mirabilis, S.pyogenes* and *E. coli,* along with the highest bacteriostatic and bactericidal upshot on selected bacterial populations in ethyl acetate fraction.

Keywords: *Plantago major*, Antioxidant, Antimicrobial, Free Radicals.

ÖZ

PLANTAGO MAJOR YAPRAKLARININ ANTİOKSİDAN VE ANTİMİKROBİYAL ETKİLERİNİN ARAŞTIRILMASI

KAYA,Özge,
Yüksek Lisans, Biyokimya Bölümü
Tez Yöneticisi: Doç.Dr. Nursen ÇORUH
Ortak Tez Yöneticisi: Yrd. Doç. Dr. A. Gülçin Sağdıçoğlu CELEP

Eylül 2011, 96 sayfa

Bu çalışma, Dünyada olduğu gibi Türkiye'de de geniş bir alanda etnobotanik olarak kullanılan *Plantago major* bitkisinin antioksidan kapasitesini belirlemek ve iki gram-pozitif iki gram negatif bakteri üzerindeki antimikrobial etkilerinin tesbiti için tasarlanmıştır.

Plantago major yapraklarının total özütü methanolde hazırlandıktan sonra total özüt içindeki biyoaktif maddelerin çözünürlük farklarından yararlanılarak total özüte artan polaritelerde apolardan polara doğru organik solventler uygulanarak fraksiyonlama işlemi yapılmıştır. Total özüt ve fraksiyonların antioksidan kapasiteleri radical yakalama kapasitesini ölçme methodları olan olan 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulphonic acid) ABTS ve 1,1-diphenyl-2-picryl-hydr`azyl DPPH methodları ile belirlenmiştir. Bunun yanısıra total fenolik ve total flavonoid madde miktarlarıda hesaplanmıştır. Antimikrobiyal aktivite tayininin belirlenemesi

için minimum inhibe edici konsantrasyon, minimum bakterisidal konsantrasyon ve disk difüzyon teknikleri kullanılmıştır.

Total özüt ve fraksiyonlar içinde en yüksek radikal sönümleme kapasitesini etil asetat fazı ABTS 2130.4±9.845 μmol trolox eşleniği/g, 0.011±0.001 mg/mL DPPH EC_{50} değerleri ile göstermiştir.Aynı zamanda toplam fenolik madde miktarı 0.452 GAEmg/mg ve total flavonoid miktarı 0.434 mg catechine eşdeğer madde/mg olarak hesaplanmıştır.

Genel olarak *Plantago major* bitkisinin *S. aureus, P.mirabilis, S.pyogenes* ve *E. coli* bakterileri üzerinde zayıf bir etkisi olduğu saptanmış. Etil asetat fraksiyonunun en etkili antimikrobiyal faz olmanın yanı sıra antioksidan olarak da en etkili olduğu gözlenmiştir.

Anahtar Kelimeler: *Plantago major*, Antioksidan, Serbest Radikal, Antimikrobiyal.

To my sister and my mother…

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 thank to my co-supervisor Assist. Prof. Dr. A. Gülçin Sağdıçoğlu Celep for her guidance and valuable advices.

I would like to also thanks to Prof. Dr. Mesude İşcan to give me opportunity to use her laboratory equipment.

I appreciate for patience, criticism and advice of my thesis examining committee members Prof. Dr. Musa Doğan, Prof. Dr. Orhan Adalı, Prof. Dr. Ceyhan Kayran, and Assist. Prof. Dr. A. Gülçin Sağdıçoğlu Celep, while reading and commenting on my thesis study.

I would like to express my great thanks to all the members of Nursen Çoruh Research Group Yeşim Kümbet, Nizamettin Özdoğan , Can Nebigil, and my friends Sule Sahin, Asligül Aksoy, Ayten Eroglu , Elif Asik, Emrah Sagır for their helps and friendship. I express my special thanks to research assistant Can Yılmaz helping me in the usage of ELISA

I would like to express my sincere thanks to Göktuğ Seymen, Neslihan Şalcı Akdoğan, Serap Altınay, Münire Ekmekçigil, Tevfik Akdoğan, Buket Çarbaş, Serdar Çarbaş, Yeşim Kümbet for their endless support, patience and motivation throughout this work.

Finally, I would like to express my deepest thanks to my family my mother, my sister, my cousin İrem Gözübüyük… for helping, encouraging and loving me all through my life. I cannot ask for more from them as they are simply perfect.

TABLE OF CONTENTS

LIST OF TABLES

TABLES

LIST OF FIGURES

FIGURES

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
MIC	: Minimum inhibitory concentration
MBC	: Minimum bactericidal concentration
CFU	: Colony forming units
a.u.	: Absorbance unit
HPLC	: High pressure liquid chromatography
HPLC-DAD	: Photo diode array detector
RSA	: Radical scavenging activity
ROS	: Radical Oxygen Species
RNS	: Radical Nitrogen Species
SD	: Standard deviation
HRMS	: High Resolution Mass Spectroscopy

CHAPTER 1

INTRODUCTION

In the last two decades, the public interest has been focused on medicinal and aromatic plants as they are a good candidate to be used as natural sources in pharmaceutical, food and cosmetic industries all around the world. The center of attention arises as the bioactive compounds present in those medicinal plants used as botanical drugs, functional foods and additives, dietary supplements and antimicrobial food packing materials, etc. Today, many of the bioactive plant metabolites, which has been used as a cure for many diseases like hypertension, cancer, cold flue, eczema and cholesterol for centuries, has been identified and isolated to be used in ethnopharmacy (Aziz et al., 2003; Littleton et al., 2005). Those bioactive chemicals are defined as phytochemicals that occur naturally in plants as secondary metabolites. Phytochemicals are known to posses many bioactivities such as antioxidant, antimicrobial, antivirus, anticancer, etc., (Xia et al., 2010; Duffy and Power, 2001). Among many available biological activities, usage of medicinal plants due to their antioxidant action on free radicals to reduce or delay the hazardous health effects is an emerging subject to many researchers. Free radical damage, which is associated with the oxidative process in living systems, drugs or in food can be pressurized by antioxidant chemicals (Pourmorad et al., 2006). The other significant biological action of medicinal plants is their antimicrobial activity against infectious diseases, which is the foremost deadly treat worldwide (Balandrin et al, 1993). The major problem is the developing multi drug resistance in both humans and newly emerging pathogens, as a result of indiscriminate usage of commercial antimicrobial drugs that have widely been applied in the treatment of infectious diseases.

This treat of getting immune to many synthetic antibiotics captured attention into search for new antimicrobial substances from other sources such as plants. Hence, plant based antimicrobial compounds became popular, and it is known that, now, almost half of the clinically used drugs are originated from natural products with one quarter coming from higher plants (Bandow et al, 2003).

Among many popular medicinal plants, *Plantago major* has accessed a scientific value, as it has been taken place in many historical uses as a wound healing remedy for centuries (Samuelson, 2000) As it has a wide dispersion all over the world, *P. major* has also been seen extensively in Turkey and there are many studies showing the ethnobotanical uses of this plant in Turkey (see Table 1.1). Thus, in this study, *P. major* leaf extract and fractionized products were examined for their antioxidant and antimicrobial properties against reduction of free radical standards and gram-positive and gram-negative bacteria, respectively.

1.1 *PLANTAGO MAJOR L.*

Plantago major is the member of the family of *Plantaginaceae,* which is a vastly diverse genus, including around 256 species. This plant was born in Eurasia but now naturalized elsewhere in the world. Even they can be found in many different habitats, they grow best in wet areas such as river beds, coastal areas, drains or seepage areas around buildings, hillsides, and can frequently be found at the side of the roads. As being a temperature-zone plant, they can be seen at extreme ranges almost from pole to pole, even they are very rare in lowlands. They grow on lands at sea level to 3500 m altitude, in their wild form (Zubair 2010, TUBIVES)

1.1.1 Botany of the *Plantago major* L.

Taxonomic Hierarchy

Kingdom ***Plantae***

Subkingdom ***Tracheobionta***

Division ***Magnoliophyta***

Class ***Magnoliopsida***

Subclass ***Asteridae***

Order ***Plantaginales***

Family ***Plantaginaceae***

Genus ***Plantago***

Species ***Plantago major*** L.

Plantago major has ovate to elliptic leaves that forms basal rosette and can grow up to 30 cm in length. Leaves are usually green with purple shades, glabrous or hairy. The leaf blade is either entire or toothed unevenly, and it contracts through the petiole. Leaf petiole has almost the same length as leaf blade. They have short, stout and erect herbaceous stems (Samuelsen, 2000). *Plantago major* may produce whitish color roots, which can grow up to 1m (Sagar and Harper, 1964). The total number of the leaves, the biomass buildup or the flowering time may vary greatly depending on the habitat of the plant, but, usually the flowering time is in between May and September for temperature zones (Sagar and Harper, 1964). Inflorescence of *P. major* is a spike, which is usually simple but sometimes branched and can grow up to 30 cm in length. They can bloom yellowish white flowers of 2–4 mm diameter. Grazing animals do not prefer to consume spikes since they are hard compared to succulent and soft leaves. (Zubair, 2010)

1.1.2 Ethnobotanical Studies on *P. major* in Turkey

Plantago major species have widely been used as traditional medicine because of having high degree of phenotypic content. The major morphological plasticity of *Plantago*

major has been biomass variations and water content as this was correlated in many studied traits. Also, the soil variables such as moisture content, soil salinity, sulphates, calcium carbonates, Ca^{++} ions and phosphorus in the soil has been found as the effective contributors to the phenotypic plasticity of *Plantago major* (Serag et al., 2010). Thus, the habitat of the plant has a great effect on the biological activity and *P. major* cultured in Turkey needs to be determined for its antioxidant capacity and antimicrobial effect on many human pathogenic bacteria.

Plantago major shows a wide diversity in Turkey. According to the TUBIVES records, Northern part of Turkey, Mid and South Anatolia are the regions where *P. major* are frequently seen(TUBIVES) and dispersion with respect to cities where *P. major* grows commonly is listed as: Adana, Düzce, Istanbul, Kars, Kastamonu, Ağri, Antalya, Artvin, Bursa, Diyarbakir, Giresun, Kirklareli, Ordu and Sivas. The distribution of the taxon over Turkey is illustrated further in Figure 1.

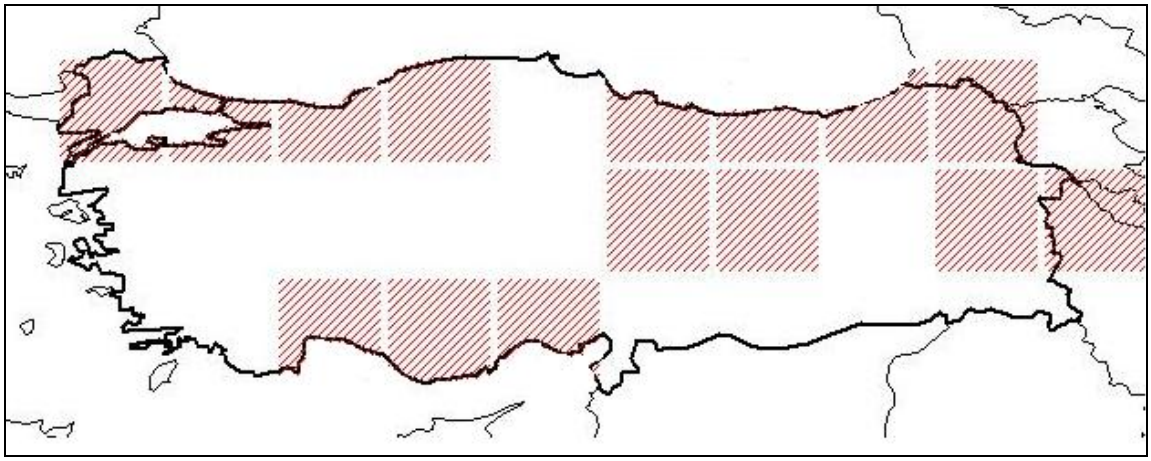


Figure1.1: Distribution of The Taxon over Turkey (TUBIVES) based on grid

Studies summarizing the ethnobotanical uses of *P. major* in Turkey has been listed in Table 1 As it can be seen in the Table 1, majority of public people have preferred to use the leaves of *P. major* as a folk medicine. It is usually applied externally for wound

healing or consumed as a drink for ulcer, cancer, kidney stone, and urethral inflammation treatments (Table 1).

1.1.3 Medicinal Investigation of *P. major*

Current studies have been focused on the medicinal effects of *P. major* leaves, especially on ulcer (Than et al, 1996; Yesilada et al., 1993), anti-inflammatory (Nathan and Hibbs, 1991; Flores, 2000) and cancer (Yokozawa et al, 1997) treatments. *P. major* leaves were investigated for healing effect against alcohol- and aspirin-induced gastric ulcer (Atta and El-Sooud, 2004; Than et al, 1996) on mice. In another study, the methanol and water extract of leaves were examined for ulcer inhibition capacity, and it was shown that water extract (1 g/kg) inhibited ulcer formation by 37% relative to 29% inhibition with methanol. The combined methanol- and water extract (1.2 g/kg) resulted in best inhibition with 40% reduced ulcer formation rate (Yesilada et al, 1993).

Similar to ulcer studies, *P. major* has also been showed to be successful against tumor formation and infection development. *P. major* leaf extract has an inducing effect on production of nitric oxide and tumor necrosis factor-alpha (TNF-∝) (Nathan and Hibbs, 1991). It is known that nitric oxide is to inhibit the synthesis of DNA and ATP; and tumor necrosis factor-alpha (TNF-∝) is one of the essential mediators of host inflammatory responses in natural immunity. Thus, *P. major* leaf extracts induces these immunity parameters; and along with this, it is fair to consider *P. major* as clinically relevant in numerous disease processes including tuberculosis, AIDS and cancer (Flores et al., 2000).

Also, great antioxidant capacity of the extract of *P. major* was assigned to the mechanisms that contribute to its wound healing properties (Yokozawa et al, 1997).

Table 1.1: The ethnobotanical uses of *P. major* in Turkey

REGION	LOCAL NAME	USED PART	TRADITIONAL USAGE	PREPARATION AND ADMINISTRATION	REFERENCES
KIRKLARELI, Kofcaz, Demirkoy, Merkez, Vize	Sinirliot, sinirotu,	Leaves	Boils	Heating ,External use	(Kultur 2006)
Vize	damarotu, damarliot, kesikotu, keskinotu,	Leaves	Diabetes	Decoction, Oral administration, drink one teacup two times a day for 1 week	
Demirkoy, Vize	bobvitsa		Wounds, cuts , hemostatic, corn	Fresh, External use	
Demirkoy			Goiter	Fresh, Ext., wrapping half hour in neck, once a day for 3 months	
Demirkoy			Asthma, cold, flu	Infusion , O.Ad., drink one teacup two times a day for 7–10 days	
Demirkoy, Merkez, Vize		Seeds	Cancer	Fresh, O.Ad., +honey, eaten 1–2 spoonful once a day for 30 days	
Demirkoy		Aerial parts	Blood depurative	Decoction, O.Ad., drink one teacup two times a day for 6 days	
ELAZIG	Bag Yapragi	Leaves	Constipation, hemmoroids	Decoction, External Use	(Cakicoglu et al., 2011)
AMASYA, Merkez	Sinir otu, Bag yapragi,Cigarca	Leaves	For prostatitis	Decoction is taken on an empty stomach	(Ezer et al., 2006)
Aktarla			For urethral inflammation	Decoction is taken on an empty stomach	
Oratbul, Yukaribuk, Bahcekent,Pekmezci			For abscess	Leaves are wilted over a fire and then applied to abscess	

Table 1.1 continued

ISTANBUL,CATALCA, Kabakca Belgradkoy, Kabakca Kabakca	Kara kabarcık, sinirli ot, sinir otu	Flowers Leaves	Diarrhea Boil Sedative	Decoction, internal use Heated, external use, wrapped in a cloth (with fresh leaves) Decoction, internal use	(Genc et al., 2006)
AFYONKARAHISAR, Cayhisar,Yorukmezari, cobanozu, Yagci, Ulukoy Y.Yenice	Siglek otu, Sigil otu	Leaves	Ripe and brust the boils Warts	The leaves are crushed and mixed with pine resin. The leaves are wrapped around warts to get rid of them.	(Kargioglu et al., 2008)
ERZURUM		Leaves Seed	Against stomachache, gastric ulcer, dyspnea For maturation of abscess, against rheumatism Against urticaria Against bronchitis, as analgesic	Powder or decoction Poultice Eaten fresh or powder (eaten aft er mixed with honey) Decoction	(Kaya et al., 2011
YALOVA	Sinirotu, Köpekdili	Leaves	For wound healing	Externally	(Kocyigit et al., 2006)
IGDiR , Kulluk ARDAHAN, Olcek KARS, Arpacay KARS,Cildir,Kaslikaya KARS , Yenidemirkapi, Gole EZURUM, Narman, Uzundere ARTVIN, Yusufeli, Morkaya	Boga yapragi,Belhaviz, Supazisi, Sinirliot, Sigil otu, Amin	Leaves	For maturation of abcess	Fresh, Externally use	(Sezik et al., 1997)
ANKARA, Haymana	Sigil otu	Leaves	Abcess	Fresh plant is applied on abscess, replace with a resh leaf when necessary until	(Sarper et al., 2009)

Table 1.1 continued

				complete recovery Fresh leaf is applied on abscess to drain the edema.	
RIZE	Damar otu	Leaves	Hypertension, Common cold, Kideny stone, Blood purfication	Infusion	(Toksoy et al., 2010)
ISTANBUL_SILE; **Imrenli, Karakiraz, Komurluk, Osmankoy** **Yenikoy** **Bickidere** **Imrenli**	Yedidamar otu	Leaves Flowers and leaves	Boil Eczema Cancer Stomachache	Crushed, External use, Wrapped in a cloth Eaten Decoction, internal use	(Tuzlaci et al., 2000)
Hakkari Semdinli; **Centrum, Tekeli Village, Yukarı Yiğitler Village**	Damar otu	Leaves	Wound, Abcess	Fresh, Raw	(Ozturk et al., 2011)

1.2 FREE RADICALS

Free radicals are atoms or molecules with unpaired electrons in the outermost shell, and may have positive, negative or neutral charge (Uppu et al., 2010). Because of the unpaired electrons, free radicals are prone to be highly chemically reactive and transient, and usually present a low steady state concentration (1nM-100μM) (Grune, 2005). Up to now, several studies have been revealed proving the advantageous use of free radicals in body such as their role in physiological functions in metabolic pathways, cell signaling, immune response; but harmful effects of free radicals causing potential biological damage in living systems has also been a known fact such as their role in variety of pathophysiological conditions (Valko et al, 2006). Free radical systems generally comprise the oxygen, nitrogen and other heavy metal containing molecules. The first discovered free radicals are reactive oxygen species (ROS) which are derived from univalent reduction of molecular oxygen through oxygen metabolism. Examples of these oxygen centered free radicals are superoxide, hydroxyl, alkoxy, peroxy and nitric oxide radicals (Pala and Tabakçıoğlu, 2007).

1.2.1 Free Radical Chain Reaction

Free radical reactions are a series of repeating chemical reactions in which free radicals present are used to generate new radicals to begin a new cycle. There are three steps of this chain reaction:

Initiation step: The first step is the formation of radicals (R· is repeat radical (Eqn. 1), RO· is alkoxy radical (Eqn.2), ROO· lipid peroxy radical (Eqn.3)). In biological systems, free radicals are produced when unsaturated lipid reacts with oxygen.

$$RH \rightarrow R\cdot + H\cdot \quad \ldots\ldots 1$$

$$ROOH \rightarrow RO\cdot + HO - \cdots \quad \ldots\ldots 2$$

$$2ROOH \rightarrow RO\cdot + ROO\cdot + H_2O \quad \ldots\ldots 3$$

Propagation step: In propagation step, new types of radicals are generated using the free radicals formed in initiation step. Lipids react with oxygen to form peroxi radicals (ROO·-Eqn.4), which reacts with unsaturated lipid (RH) to form peroxides (ROOH) and lipid radical (R·) back (Eqn.5). These two reactions are repeated until termination.

$$R\cdot + O_2 \rightarrow ROO\cdot \quad \ldots 4$$

$$ROO\cdot + RH \rightarrow ROOH + R\cdot \quad \ldots 5$$

Termination step: This step is the destruction of radicals. When unsaturated lipid (or fatty acids) amount decreases, radicals start to react with each other resulting in stable and non radical compounds (Eqns. 6, 7, 8).

$$R\cdot + R\cdot \rightarrow R - R \quad \ldots 6$$

$$R\cdot + ROO\cdot \rightarrow ROOR \quad \ldots 7$$

$$ROO\cdot + ROO\cdot \rightarrow ROOR + O_2 \quad \ldots 8$$

(Sen et al., 2010; Favier et al., 1995)

1.2.2-Sources of Free Radicals

There are many sources for free radical formation. UV radiations, X-rays, gamma rays and microwave radiation, metal-catalyzed reactions and oxygen free radicals present in the atmosphere considered as pollutants are the major exogenous contributors. Among many environmental factors like interaction with chemicals, automobile exhausts fumes, industrial effluents, burning of organic matter, or some pesticides and herbicides, there are many endogenous substances serving as a source for formation of free radicals such as ROS generated by mitochondrial cytochrome oxidase, xanthine oxidases, neutrophils and by lipid peroxidation or by arachidonic acid, platelets, macrophages metabolism or mitochondria-catalyzed electron transport reactions (Sen et al., 2010)

1.3 ANTIOXIDANTS

Antioxidant is defined as “any substance, when present in low concentration compared to that of an oxidizable substrate, significantly delays or inhibit the oxidation of that substrate” (Richer and Milbury, 2008)

As described previously, oxygen and nitrogen free radicals, ROS and RNS, are the products of cellular metabolism in body. Living systems align themselves with these species, since they can play a dual role as both harmful and advantageous species (Valko et al., 2006). At low/moderate concentrations, they have many beneficial physiological roles such as functioning in cellular responses against protection from infectious agents; or they induce mitogenic response when present at low/moderate concentrations. In reverse, high concentrations of free radicals may result in some harmful effects. For example, they can attack to membrane lipids by generating carbon radicals. Carbon radicals can react with oxygen to form peroxyl radicals, which in turn can conjugate with fatty acids of membrane lipids to produce new carbon radicals. This chain reaction mechanism is known as lipid peroxidation, and in biological systems, this occurs when ROS/RNS overproduction happens. This indicates that even one free radical may affect many molecules by initiating the lipid oxidation chain reaction and when there is a deficiency of enzymes or antioxidants of interest, auto-oxidation reactions increase exponentially. Thus, body has some defense mechanisms against the potential damaging nature of free radicals, such as enzymes of superoxide dismutase and catalase; or water-soluble and lipid-soluble molecular antioxidants (Croft, 2006)

1.3.1 Enzymatic Antioxidants:

Free radical reaction chain has three stages: initiation, propagation and termination. Body defense mechanism against damaging effect of free radicals includes antioxidant enzymes, which can be effective in each step of chain reaction mechanism to lower the burden of free radicals. They act on precursor molecules by inactivating them to prevent the ROS formation. (Mates et al., 1999)

1.3.1.1 Superoxide Dismutase (SOD)

One of two antioxidant enzymes that act at initiation step of auto-oxidation chain reaction is superoxide dismutase (SOD). This enzyme catalyses the reduction reaction of highly reactive O_2^- to less reactive H_2O_2 molecules (Eqn.9). There are three forms of SOD which are Cu/Zn containing-SOD found in cytoplasm, Mn containing-SOD found in mitochondria, and extra- cellular SOD (EC-SOD) in humans(Mates et al., 1999).

1.3.1.2 Catalase (CAT)

After dismutasing activity of SOD to convert O_2^- to H_2O_2, Fe- containing metallo enzyme of catalase (CAT) enzyme can decompose the produced H_2O_2 molecules to form water and molecular oxygen (Eqn.10). CAT, also, can react very efficiently with hydrogen donors such as methanol, ethanol, formic acid, or phenols with peroxidase activity (Eqn.11) (Mates et al., 1999; Garewal, 1997)

$$2H_2O_2 \xrightarrow{CAT} 2H_2O + O_2 \quad \ldots\ldots 10$$

$$ROOH + AH_2 \xrightarrow{CAT} H_2O + ROH + A \quad \ldots\ldots 11$$

1.3.1.3 Glutathione Peroxidase (GPX)

In addition to two metalloenzymes of SOD and CAT described above, the glutathione peroxidase (GPX) has an antioxidant capacity. Selenium-containing GPX acts during propagation stage of auto-oxidation chain reaction and catalyses the decomposition reaction of H_2O_2 by using the reduced glutathione (GSH) to form water and oxidized glutathione (GSSH). Thus, GPX has a very important role in antioxidative defense mechanisms (Eqn.12) (Mates et al., 1999, Garewal, 1997)

$$ROOH + 2GSH \xrightarrow{GPX} ROH + GSSG + H_2O \quad \ldots\ldots 12$$

1.3.2 Non- Enzymatic Antioxidants

There are two types of non-enzymatic antioxidants: metabolic antioxidants and nutritional antioxidants. Metabolic antioxidants are the endogenous. They are produced by normal metabolism in living systems. Examples are: lipoid acid, glutathione, L-ariginine, melatonin, uric acid, coenzyme Q10, bilirubin, metal-chelating proteins, transferrin or glutathione. Reduced glutathione (GSH) can be produced in normal cells, as well; they can be provided through diet. On the other hand, nutritional antioxidants are exogenous, which means they cannot be found in body but can be taken from outside. Examples are: omega-3 and omega-6 fatty acids, vitamin E and C, ad some trace amount of metals such as selenium, manganese, zinc. Those metals can only serve as antioxidant when incorporated into their respective enzymes. And the phytoconstituents, which are employing a vital role in preventing the free radical induced injuries in body (Sen et al., 2010).

1.4 PHYTOCHEMICALS

Up to now, more than 5000 phytochemicals have been identified but majority still remains undefined and is waiting to be discovered and characterized to fully understand their health benefits. Besides, as growing evidences accumulate, it becomes obvious that this is just a tip of iceberg, and even greater health advantages than what is currently understood. The interest on natural phytochemicals increases because they are effective on reduction of free radical formation which is the foremost contributor in the etiology of many diseases. (Liu, 2003). In order to suppress or prevent the oxidative stress in cells induced by free radicals, abundant amounts of antioxidants need to be consumed. The antioxidants (phytochemicals) can be taken by consuming various nutrient rich vegetables, fruits or grains. This may strengthen the defense mechanism against free radicals in cellular systems, and decrease the oxidative damage, and in parallel the risk of disease which can be related with oxidative damage In Figure 1.2 the types of the phytochemicals are summarized. In general, they are classified in 5 sub-classes which are: carotenoids, phenolics, alkaloids, nitrogen-containing compounds, and organosulfur

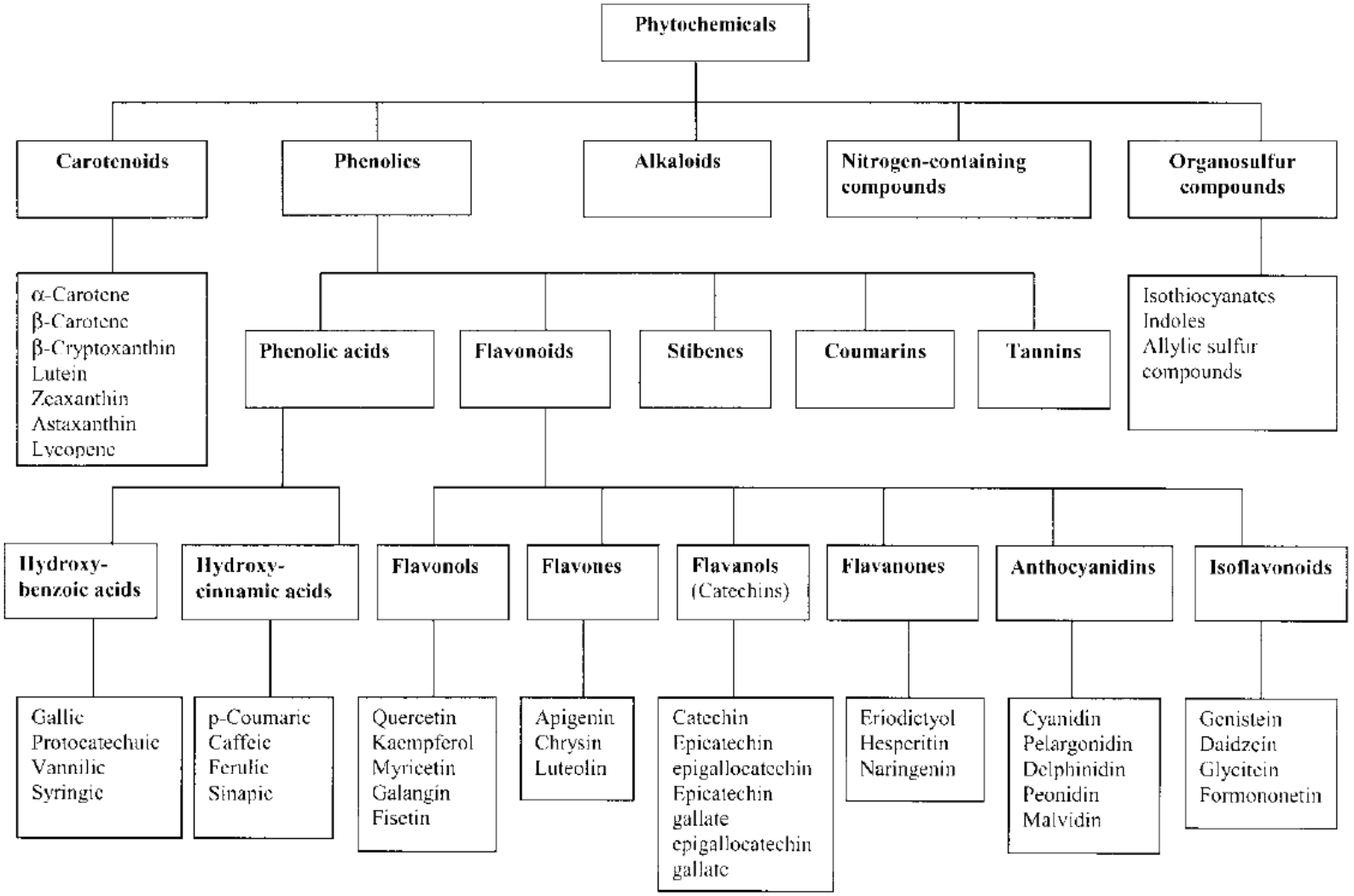


Figure1.2: Schematic diagram for types of phytochemicals (Liu, 2004)

compounds. Among those, phenolics and carotenoids are the ones that studied the most. (Liu, 2004)

1.5 ANTIOXIDANT CAPACITY EVALUATION METHODS USED

There is an increasing demand for antioxidants as they are capable of suppress the deleterious effects of free radicals in body, and natural sources of antioxidants are more preferable compared to synthetic ones (Abdalla and Roozen, 1999). Hence, the methods to estimate the efficiency of antioxidants become important as the interest in antioxidants from natural sources get popular (Schwarz et al., 2000)

1.5.1 DPPH Method:

The molecule of 1,1-diphenyl-2-picryl-hydrazyl (DPPH) radical has a spare electron over the molecule which makes the molecule stable as it does not dimerise like the most of other free radicals. This delocalization of the spare electron, also, gives the molecule a deep violet color which can be characterized as an absorption band at 517nm when dissolved in ethanol. When diphenylpicrylhydrazyl molecule is reduced by some H donor (diphenylpicrylhydrazine, reduced form of DPPH), the violet color disappears and turns into pale yellow color because of the picryl group present in the molecule (see Figure 1.3). Antioxidant capacity can be determined by measuring this color change as absorbance at 517nm (Molyneux, 2003)

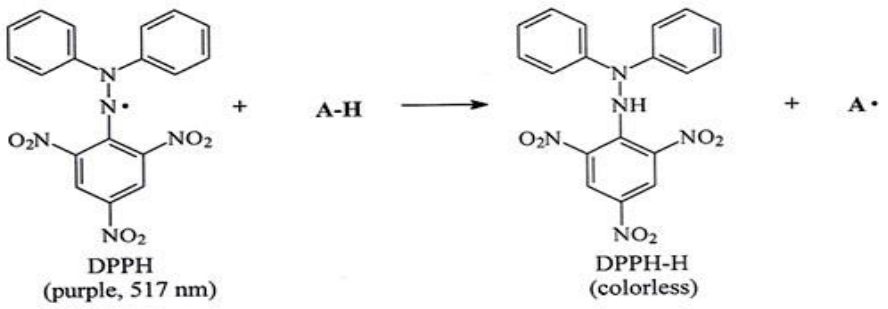


Figure 1.3: Schematic illustration of radical (oxidized) and non radical (reduced) form of DPPH molecule.

1.5.2 ABTS Method

ABST method starts with the oxidation of 2,2-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) radical cation ($ABTS^{\bullet+}$) by oxidizing agents and cation radical is later reacted with antioxidants to get reduced forms. As $ABTS^{\bullet+}$ accepts H ion from antioxidants, the color of solution changes from blue to clear which can be detected as an absorbance band at 734nm (Figure 1.4). The radical cation of $ABTS^{\bullet+}$ has characteristic absorption peaks at 414, 645, 734 and 815 nm but most of the investigators preferred to follow the color change at 734nm to avoid absorption contributions coming from other compounds that may present in plant extracts (Villano et al., 2004; Prior et al., 2005)

ABTS method has lot of advantageous as it is easy to apply, has a high reproducibility and diversity, and foremost it can be use for both hydrophilic and lipophilic antioxidant capacity determination in various media such as food extract or physiological fluids, because this reagent is soluble in both aqueous and organic solvent media (Apak et al, 2007).

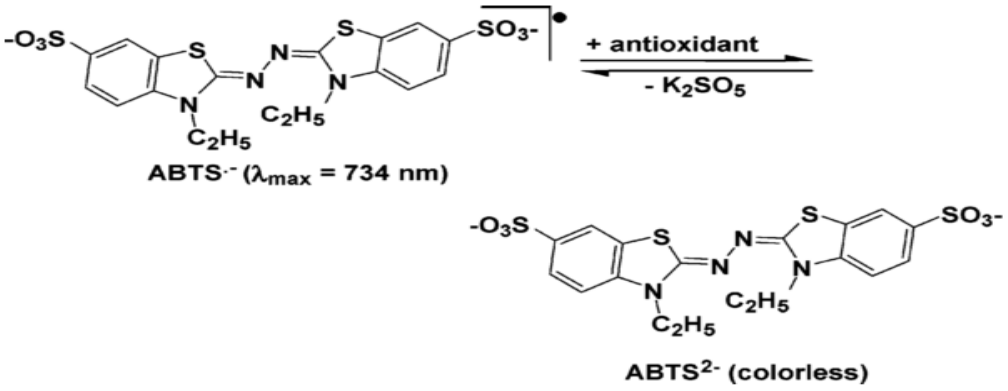


Figure 1.4: Schematic illustration of the reaction between the $ABTS^{\cdot+}$ radical and the antioxidant. (Huang and Prior, 2005)

1.5.3 Total Phenol

Total phenol determination assay depends on the oxidation of phenols by molybdotungstophosphoric heteropolyanion reagent (namely, Folin-Ciocalteu (FC) Reagent). As phenol oxidation happens, the yellow color of the molybdotungstophosphoric heteropolyanion reagent turns into molybdotungstophosphate blue, and this color change is recorded as absorbance at 750nm. In this method, the operating conditions have a great influence on results because of the low stability of the oxidizing reagent, and so as the blue color, in alkaline solutions, which is a must to react phenols with the reagent rapidly. Thus, a well known phenol, usually the gallic acid, is used as a reference standard in order to obtain satisfactory analytical results by providing identical conditions for gallic acid, the known phenol used as the reference standard.

Phenolic compounds can only react with FC reagent when medium is basic, because FC reagent is not only specific to phenolic compounds but also to nonphenolic compounds like vitamin C, Cu(I), etc. The mechanism involves the dissociation of phenolic proton to phenolate anion, which later reduces the FC reagent and blue color appears. And this blue compounds formed are independent of the specific structure of phenolic compounds, but valid for all phenols. Even FC reagent has a nonspecific affinity for phenol groups; this method is still easy to handle, convenient, and reproducible. That's why FC reagent became a routine assay to study phenolic antioxidants (Huang and prior, 2005)

1.5.4 Total Flavonoid

In order to determine the total flavonoids in samples, the classical aluminum chloride colorimetric test was applied. The basic principle of this method depends on the reaction between the aluminum chloride and C-4 keto group or either the C-3 or C-5 hydroxyl group of flavones and flavonols. The resultant has a characteristic absorption band at 510 nm as visually observable pink color appears. As well, $AlCl_3$ can react with the -

dihydroxyl groups in the A- or B-ring of flavonoids to form acid labile complexes (Chang et al., 2002) In fact, the AlCl3 colorimetric test is not a liable test to determine the flavonoids that do not have characteristic chelating functional groups for Al binding. Thus, this method is used to evaluate the flavones like chrysin, apigenin, luteolin, and flavonols like quercetin, myricetin, morin, rutin but it is not suitable for flavanones and flavanonols (Apak et al, 2007).

1.6 ANTIBACTERIAL ACTIVITIES

1.6.1 Gram-positive Bacteria Used in This study

1.6.1.1 *Staphylococcus aureus:*

Appearance: Medium to large; most of the colonies pigmented pale yellow smooth, whole, translucent yellow; most colonies beta-hemolytic.

Habitat: Normal flora of human anterior nares, nasopharynx, perineal area, and skin; can colonize various epithelial or mucosal surfaces.

Mode of transmission: Spread of patient's endogenous strain to normally sterile site by traumatic introduction (e.g., surgical wound or microabrasions).Also may be transmitted person to person by fomites, air or unwashed hands of health care workers, especially in the nosocominal setting. May be transmitted from infected skin lesion of heal care worker to person.

Virulance Factor: Produces and secretes toxins and enzymes that have a role in virulence acts on the cell membranes and mediate the cell destruction. Leucocidin (PVL) mediates destruction of phagocytes. Clumping factor, coagulase, and hyaluronidase enhance invasion and survival in tissues.

Spectrum of disease and infections: Infections generally involve intense suppuration and destruction (necrosis) of tissue. Infections can be generally grouped as localized skin infections such as folliculitis, various wound infections; deep infections that spread from skin to cause bacterimia.(Forbes et al., 2007)

1.6.1.2 *Streptococcus pyogenes:*

Appearance: Large colonies; translucent to opaque; flat; glossy; zone of beta hemolysis.

Habitat: Inhabits skin and upper respiratory tract of humans. Not considered part of normal flora but may be carried on nasal, pharyngeal, and some times, anal mucosa. Presence in specimens is almost always considered clinically significant.

Mode of transmission: Person to person by direct contact with mucosa or secretions, or by contaminated droplet produced by coughs, sneezes. Once exposed, recipient may become colonized, with subsequent development of infection.

Virulence Factor: Protein F mediates epithelial cell attachment, and M protein is anti phagocytic; produces several enzymes and hemolysins that contribute to tissue invasion and destruction.

Spectrum of disease and infections: Acute pharyngitis, impetigo, erysipelas, necrotizing faciitis, and myocitis; bacteremia with potential for infection any of several organs, pneumonia. (Forbes et al., 2007)

1.6.2 Gram-negative Bacteria Used in This study

1.6.2.1 *Escherichia coli:*

Appearance: Flat, dry, pink colonies enclosed with darker pink area

Habitat: Normal bowel flora of humans and other animals may also inhabit female genital tract.

Mode of transmission: Varies with the type of *E.coli* organism may be endogenous or spread person to person, fecal-oral spread between humans via contaminated food or water or consumption of undercooked beef or milk from colonized cattle.

Virulence Factor: Several, including endotoxin, capsule production and pilithat mediate attachment to host cell.

Spectrum of disease and infections: Urinary tract infections, bacteremia, nosocomial infections of various body sites. Most common cause of gram-negative nosocomial infections. (Forbes et al., 2007)

1.6.2.2 *Proteus mirabilis:*

Appearance: May swarm depending on the amount of agar in the medium. Characteristic foul smell

Habitat: Normal human gastrointestinal flora

Mode of transmission: Endogenous, or person to person spread, especially in hospitalized patients.

Virulence Factor: Several factors, including endotoxins, capsules, adhesion proteins, and resistance to multiple antimicrobial agent.

Spectrum of disease and infections: Wide variety of nosocomial infections of respiratory tract, urinary tract, blood and several other normally sterile sites; most frequently hospitalized and seriously debilitated patient(Forbes et al., 2007)

1.7 PHYTOCHEMICALS AS ANTIMICROBIAL AGENTS

The synthetic drugs have modest antimicrobial activity even they have been successfully designed and used as enzyme inhibitors, probably because of the complex structure of cell uptake of those drugs (Cushine, 2005). Thus, scientist tended to look for more effective plant based antimicrobial sources as an alternative to synthetic ones and now it is estimated that more 50% of the Western drugs are plant derived, which have been once used in crude form in traditional or folk healing practices. Plant based drugs has proven themselves as they serve relatively safer and more affordable treatments. Since this is the way it is, there has been an improved interest in natural products resulted from factors such as consumer's displeasure from conventional medicines and boosted confidence in natural products as being superior. Changing law enforcements to supply structure-function claims, national concerns about health care cost or even changes in advertising have contributed to enhanced interest in natural products. Thus, it is fair to consider including antimicrobials into medicines is rewarding for both standpoints of drug development and phytomedicines (Ciocan and Bara, 2007)

1.7.1 Major Groups of Antimicrobial Compounds from Phytochemicals

1.7.1.1 Simple Phenols and Phenolic Acids:

Simple phenols are the simplest bioactive phytochemicals comprise of a phenolic ring. The toxicity of phenols to microorganisms were found to depend on the number of hydroxyl groups bound to phenol group since increased hydroxylation results in increased toxicity. Some other researchers have proposed that more highly oxidized phenols are inhibitorier. The toxicity to microorganisms are bond to enzyme inhibition as a result of the reaction between oxidized compounds and sulfhydryl groups, or may it is just because of nonspecific interactions of phenols with the proteins. Essential oils are phenolic compounds having a C3 side chain at a lower level of oxidation and they are often referred as antimicrobial as well (Cowan, 1999)

1.7.1.2 Quinones:

Quinones have highly reactive characteristic which is unique in nature. They are composed of aromatic rings with two ketone substitutions. They are responsible from the process known as browning reaction seen in cut or injured fruits and vegetables. The antimicrobial property of quinines depends on their irreversible binding with nucleophilic amino acids in proteins, which usually results in function loss of protein bound. That's why quinines are very popular as antimicrobial agents. Cell-wall proteins, surface-exposed adhesins, and many membrane bound enzymes are targeted structures for quinones.

Potential targets in the microbial cell are surface-exposed adhesins, cell wall polypeptides, and membrane-bound enzymes. Quinones may also deliver substrates unavailable to the microorganism. As with all plant-derived antimicrobials, the possible toxic effects of quinones must be thoroughly examined (Ciocan and Bara, 2007)

1.7.1.3 Tanins:

Tanin is a general name of a class of polyphenolic compounds that binds to various organic compounds such as amino acids and proteins via hydrophobic effects or by hydrogen bonding or covalent bonding. Thus it is considered that the antimicrobial properties of tannin come from their ability to inactivate microbial adhesins, transport proteins or enzymes. Plant based tannin can be classified as hydrolyzable tannins, e.g gallic acid, non- hydrolyzable tannins, e.g., flavones. Tanins may be formed from flavan derivatives located in woody tissues of plants or from quinine units by polymerization. Besides their capability to form complexes with proteins, they were assigned to many other molecular activity in human such as host-mediated tumor activity, phagocytic cells stimulation or anti-infective actions (Cowan, 1999).

1.7.1.4 Alkaloids:

Alkaloids are one of the most effective therapeutically active plant derived compounds. They are generally composed of rings of carbon atoms with nitrogen atom in ring. Therapeutical efficacy comes from their very high toxicity capacity on microorganisms. That's why they usually applied externally instead of using as folk medicine. The antispasmodic, analgesic, and bactericidal properties of plant derived alkaloids and their synthetic derivates make them popular all over the world (Ciocan and Bara, 2007)

1.7.1.5 Flavones, Flavonoids, and Flavonols:

One of the undoubted role of favonoids and related polyphenols in plants is protecting against microbial invasion (Harborne and Williams, 2000). Flavonoids are polyphenolic compounds containing 15 C atoms with 2 ring structure bound by a 3-C bridge. They are sub-classed into 6 groups as flavonols, flavan-3-ols, flavones, isoflavones, flavanones, and anthocyanidins according to different forms of central C-ring (Fraga, 2010) Antibacterial property of flavonoids comes from the inhibitory effect of flavonoids on the DNA and RNA synthesis, which was proposed as intercalation or hydrogen bonding of the B ring of the flavonoids with the nucleic acid bases has an inhibitory action on nucleic acid formation. Another research claims that, flavonoids have an inhibitory effect on function of cytoplasmic membrane which was supported with evidence of reduced the fluidity of cell membranes when flavonoids (specifically sophoraflavanone G) are present. Another mechanism of antimicrobial activity of flavonoids is related with the energy metabolism inhibition. The theory is that the licochalcones may be interfering with energy metabolism in a way that it affects the energy required for metabolite uptake or macromolecule biosynthesis (Cushine and Lamb, 2005)

1.8 METHODS USED FOR EVALUATION ANTIBACTERIAL ACTIVITY

1.8.1 Minimum Inhibitory Concentration

Minimum inhibitory concentration (MIC) is described as the lowest concentration of an antibacterial substance that will provide a complete inhibition on the observable growth of a microorganism after 24h incubation. (MIC) is thought as ‘gold standard’ for establishing the susceptibility of organisms to antibacterial. For that reason it is used to evaluate the efficiency of all other methods of susceptibility testing. (Andrews, 2001) With the purpose of test natural substances, MIC is recommended as general standard method.

1.8.2 Minimum Bactericidal Concentration

The minimal bactericidal concentration (MBC) is described as the lowest concentration of an antibacterial substance killing the 99.9% of bacterial inoculums. As MIC is the inhibition effect of the antibacterial substance on the growth of the bacteria, it is possible that if antibacterial substance is removed, the bacteria can start to grow again. To find out the ability of the antibacterial substance to kill the bacteria, a growth test can be performed and this test is called as the MBC (Schwalbe et al., 2007).

1.8.3 Agar Disc Diffusion Assay

Disc diffusion test is a general antibacterial susceptibility testing procedure. The suspension pathogen that is including definite number of uncontaminated pathogen spread onto the whole surface of the proper agar medium to inoculate it. Paper discs impregnated with different antibiotic or substance thought has antimicrobial effect, are placed on to the inoculated agar medium and incubated. Meanwhile they penetrate to agar media, if the pathogen sensitive to the antibacterial substance growth inhibition is seen as apparent region around the disk. Some conditions like type of medium, density

of inoculums on the plates etc. for the procedure must be standardized in order to interpret the presence or diameter of the apparent region correctly (Singelton, 2004).

1.9 SCOPE OF THE STUDY

This study was designed to investigate the antioxidative and antimicrobial properties of *P. major* leaves extracts. Purification of bioactive compounds from the leaves was carried out by applying the fractionation method. Isolates in the most active fraction then were tried to identify. Furthermore, the crude extracts and fractions were examined for their antioxidative activity and for their growth inhibitory or bacterisiodal effects on *S. aureus, E. coli, P. mirabilis,* and *S. pyogenes*.

CHAPTER 2

MATERIALS AND METHODS

2.1 MATERIALS

2.1.1 Chemicals

Chromatography grade methanol using in the extraction assay and HPLC assay, chromatography grade hexane, chloroform, diethyl ether, ethyl acetate, which were used for fractionation, and acetonitrile for HPLC were taken from Merck (Darmstadt, Germany), and magnesium sulfate was taken from Sigma Chemical Company (St.Louis, MO, USA).

To obtain ultra pure water (18.2 M ohm.cm) Milli-Q system (Milli-pore, Bedford, MA, USA) was used.

Not reusable syringe filters (pore size: 0.22 μm and 0.45 μm) were taken from Millipore Corporation (Bedford, MA USA) before injection of extracts and sterilize solvent used for antimicrobial studies.

Gallic acid, trolox, caffeic acid, catechin, tannic acid, rutin, quercetin, 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 (Na2CO3), sodium hydroxide, aluminum chloride were taken from Sigma Chemical

Company (St.Louis, MO, USA). Folin Ciocalteu's phenol reagent was taken from Merck (Darmstadt, Germany).

Streptococcus pyogenes code: (RSKK 03019, ATCC 19615), *Proteus mirabilis* (code: RSKK 737, Pasteur Institute), *Staphylococcus aureus* (code: RSKK 95084, Pasteur Institute), *Escherichia coli* (code: RSKK 234, Pasteur Institute) used for antibacterial experiments were taken from Refik Saydam Hygiene Center.

Luria Bertani agar, Luria Bertani broth, Brain-Heart Infusion agar, Brain Heart Infusion broth, and Muller-Hinton agar and Muller-Hinton broth were taken from Merck. (Darmstadt, Germany)

Ready to use sheep-blood agar plates, to ensure long cultivation of bacteria, were bought from OR-BAK (Istanbul, Türkiye).

6 mm diameter empty sterile discs for antibacterial activity test used in agar diffusion assay were taken from Oxoid (Hants, UK).

Standard antibiotic discs Gentamycin (10 mcg), Trimethoprim, Clindamycin, Penicilin, Tetracyclin and Kanamycin (30 mcg) were bought from Bioanalyse (50 susceptibility tests for in vitro diagnostic use).

Gentamycin powder which is used for MIC experiments (cell culture tested grade) was taken from Sigma-Aldrich (Germany).

Penicillin G Potassium powder which is used for MIC experiments (cell culture tested grade) was taken from (St.Louis, MO, USA)

2.1.2 Apparatus

For the spectroscopic experiments Cary 50 Bio UV-VIS spectrophotometer (Varian) were used.

Apparatus which are used throughout this study were; rotary evaporator (Heidolph Laborota 4000) ultrasonic bath (Bandelin Sonorex); stainless-steel blender (Waring, 32BL80); Oval-rotating incubator (Optic Ivymen System).

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.

Nuve EN-500 incubator, Rotary evaporator (Heidolph Laborata 4000

12 multichannel 5-50 μL, 8 multichannel 5-50 μL, and 12 multichannel 100-300 μL pipettes (Thermo Electron Corporation, Finnpipette) were used during the antibacterial activity tests.

HPLC analysis were performed using an analytic apparatus including online degasser unit, detection systems with both photodiode array (2996 Waters) detectors and a multi wavelength fluorescence detector (Waters, 2475). A multi solvent delivery system (Waters, 600E), 100μl sample loops were used for analytical HPLC sample injections. The manual sample injectors for 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 performed with Waters Empower Software. HRMS analysis were performed with Waters SYNAPT MS System, in Central Laborary, METU.

2.2 METHODS

2.2.1 Extraction

Plantogo major leaves were gifted from Van Yüzüncüyil University identified by Fevzi Özgökçe and a sample of the plant kept at the Herbarium of Biology Department of the Van Yuzuncuyil University. A classical methanol extraction method was applied. Briefly, 160 grams of shade dried plant leaves were ground to get similar size of powdered plant material with a Waring blender. A 40 g sample of ground leaves portion was mixed with 400 mL methanol in dark colored bottles for the extraction process. Each sample bottle was first subject to ultrasonification for 30 minutes in an ultrasonic bath. Ultrasonicated sample was then incubated in oval-rotating incubator at a fixed 180 rpm for 24 h at 25^{o}C. Resulted suspension was filtered through white watman filter paper. Pellet was re-extracted using methanol repeatedly for 2 more times. But each time the amount of the methanol was 100mL decreased. Three filtrates were collected to remove the methanol by keeping in rotary evaporator at 30^{o}C. The extraction yield was calculated as the weight percentage. Crude methanol extract was kept at -20^{o}C until further experiments.

2.2.2 Fractionation of Crude Extract

The fractionation of crude extracts was performed by using solvents in increasing polarity from non-polar to polar. The fractionation method described by Naczk and Shahidi (2004) was adapted. First, a 3.7 g of crude extract was dissolved in a total 300 mL of water /methanol mixture (1:1 v/v) and immediately a 300 mL hexane was added in a separatory funnel, and mixture was vigorously shaken and kept steady until organic and aqueous phase were separated. Aqueous phase, at the bottom of the separatory funnel, was drained into a beaker for the further fractionation steps; on the other hand the organic phase (hexane) was transferred into rotary evaporator for evaporation. This separation procedure on the aqueous phase was repeated until organic phase was completely transparent.

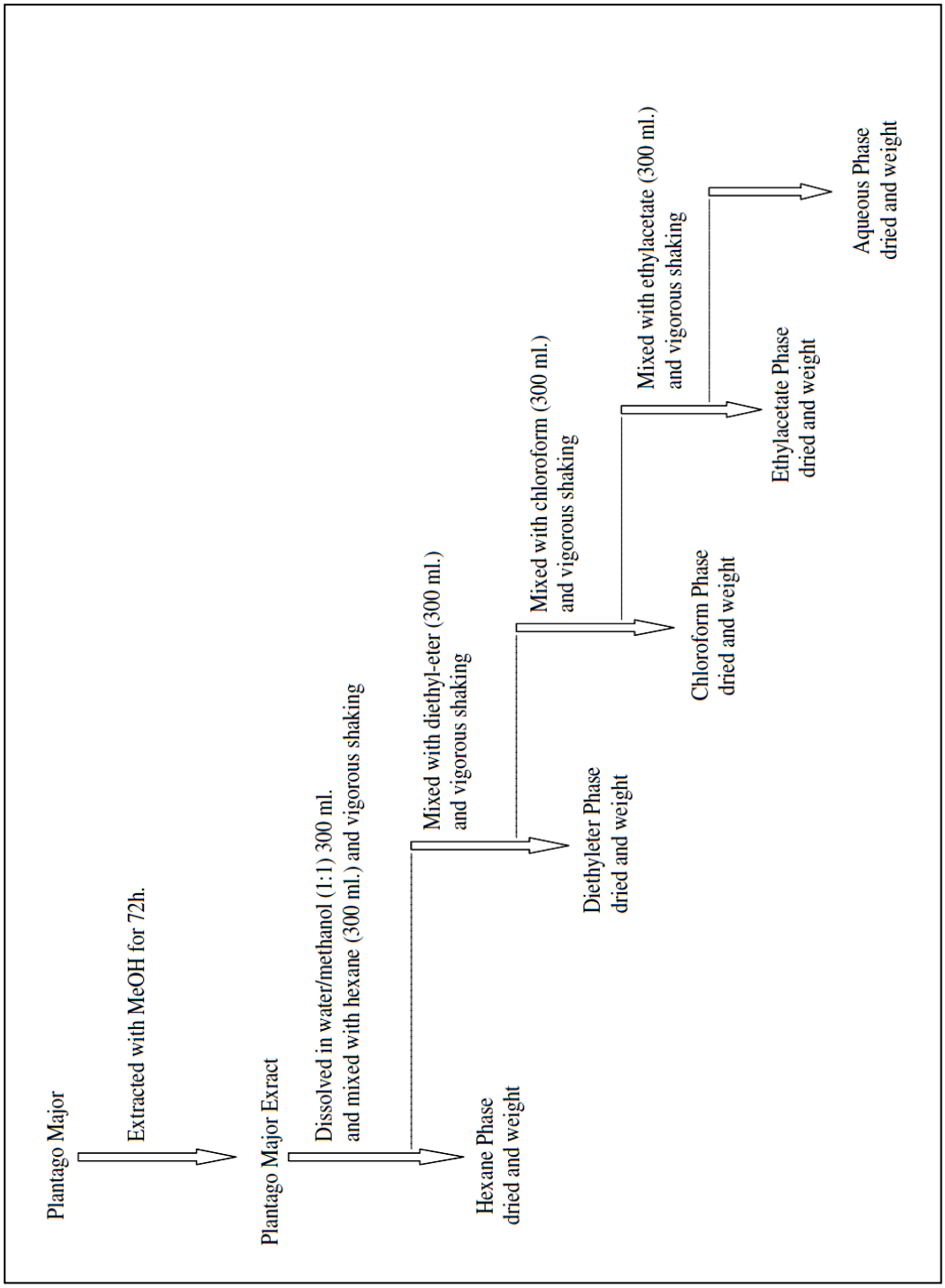


Figure 2.1 Schematic illustration of the extraction and fractionation procedure.

The trace amount of water left in the organic phase was absorbed by using anhydrous magnesium sulfate at the end of each step. The organic phase (hexane) collected from separation method was evaporated to dryness evaporating and was stored at +4 ^{o}C until analysis. Fractionation steps were further continued with aqueous phase mixed with organic solvents in increasing polarity. The second fractionation steps were carried out with, mixing diethyl ether and aqueous phase. The other applied organic solvents were chloroform and ethyl acetate in given order. Each fractionated phase was evaporated to dryness and was kept at +4^{o}C. The yield of each fractionation step was determined in terms of (w/w) % per 3.7 g crude extract. The residual aqueous phase was liyophilized and kept for further analysis. Figure 2.1 summarizes the extraction and fractionation procedures.

2.2.3 Analytical High Performance Liquid Chromatography (HPLC) Analysis

Ethyl acetate fraction which has the heights antioxidant capacity worthed to analyze for its bioactive compounds was analyzed by using HPLC-DAD. The operating conditions were arranged as described previously described in our laboratory: "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 10th min, 85% at 11th min., 65% at 50th min and 0% at 74th 85th minutes of solution A. Then column was washed with 100% solution B and re-equilibriated for 15 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" (Nebigil, 2011).

2.2.4 Determination of Antioxidant Capacity

Antioxidant capacities of the crude extract and fractions were evaluated using DPPH radical scavenging, ABTS methods and calculating the total phenolic content and total flavonoid content.

2.2.4.1 DPPH Method

DPPH procedure was taken from (Blois, 1958) with a modified protocol adapted from Coruh et. al was utilized to decide DPPH radical scavenging capacity (Coruh et al., 2007). A 0.05 mg/mL DPPH solution was prepared by dissolving 15 mg of DPPH radical in 300 mL absolute ethanol to get approximately 1.4 absorbance unit at 517nm. Varying concentrations of crude extract and fraction mixtures in methanol were prepared and EC_{50} values were determined in order to compare the EC_{50} values of known standard of quercetin prepared at varying concentrations in methanol. The time needed to the complete the reaction was optimized for each standard, crude extract and fractions, separately. The absorbance values were recorded against ethanol after adding 100µL of sample (standards, crude extract and fractions) into 1400µL DPPH radical solution at the end of previously optimized time period. All the experiments were carried out in duplicates and repeated for three different assays.

The percentage of radical scavenging capacity (RSA %) was calculated according to the following equation:

$$RSA\% = [(A_0 - A_1)/A_0] \times 100 \ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots . (1)$$

Where, A_0 is the absorbance unit of the blank, which is 1400µL DPPH radical solution with 100 µL pure methanol, A_1 is the absorbance unit of mixture of 100µL of quercetin standards/crude extracts/fractions at varying concentration in 1400 µL DPPH solution at optimized incubation time.

EC_{50} values were calculated in order to evaluate the antioxidant capacity. EC_{50} values were thought as the concentration of substance needed to reduce the % 50 DPPH activity by means of color change. EC_{50} values were calculated from Equation 2 by using the graph of RSA (%) versus final concentration of substance (mg/ml).

$$y = BOT + \left[(TOP - BOT) / \left(1 + \left({}^{x}/_{EC_{50}} \right) \right)^{slope} \right] \ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots (2)$$

Where, y is the percent activity (%), x is the corresponding concentration (mg/ml), TOP corresponds to the highest observed RSA (%) and BOT is the lowest observed RSA (%) values.

2.2.4.2 ABTS Method

2,2'-azino-bis(3-ethylbenzthiazoline-6-sulphonic acid) ABTS method was performed as previously described by Re et al (Re et al., 1998) ABTS radical cation ($ABTS^{\cdot+}$) was formed by utilizing the reaction between ABTS and potassium persulfate. The antioxidant capacity values were given in terms of trolox equivalent antioxidant capacity (TEAC) value. TEAC values for crude extract and fragments were calculated by taking the ratio of slopes of RSA (%) versus concentration (mg/ml) graphs for samples to standard (trolox). A stock mixture with final concentration of 7mM ABTS and 2.45mM potassium persulfate in ultra pure water was prepared and kept in dark room overnight before use (Figure 2.2) The working solution was prepared by diluting 1 mL of ABTS stock solution in 100mL pure ethanol in order to get approximately 0.70 (±0.02) absorbance unit at 734 nm. A 2.5 mM stock trolox solution was prepared in methanol and serial dilutions were prepared by taking 2.0, 4.0, 6.0, 8.0 mL from stock trolox solution and completing to 10 mL with methanol. A 10 μL from each diluted trolox solution having varying concentrations (final concentrations of 5.0, 10.0, 15.0 and 20.0 μM trolox in the cuvette) was added in 1ml ABTS solution as described above after the dilution in 100 mL ethanol and resultant absorbance (trolox + ABTS) were recorded at

sixth min. The percentage of the radical scavenging capacity of standard trolox and samples were determined using equation (3),

$$RSA\% = [(A_2 - A_3)/A_2] \times 100 \ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots . (3)$$

in this case, A_2 was defined as the absorbance unit of the blank which is 10µL pure methanol (in the absence of plant extract) in 1ml radical solution, A_3 was the absorbance unit of 10µL of trolox standards/crude extract/fractions having varying concentrations in 1ml radical solution at the optimized incubation time (t=6min).

All the experiments were carried out in duplicates and repeated for three different experimental preparations.

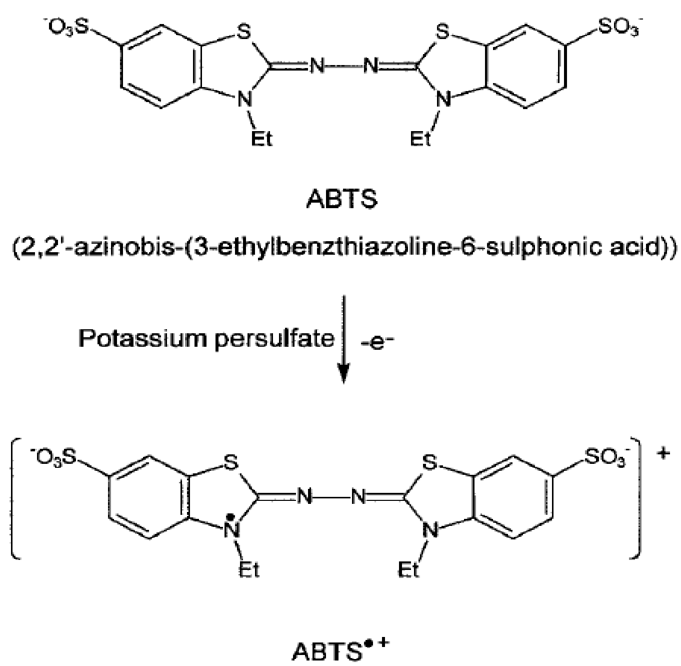


Figure 2.2: Formation of ABTS radical cation (Pannala et al., 2001)

2.2.5 Determination of Total Phenolic Content

Total phenol content of the crude extract and fractions were evaluated according to the folin-ciocaltaeu method proposed previously by Singleton and Rosi (1965). For this, varying concentrations of gallic acid a, well known phenol, as a standard, crude extracts and fractions were prepared in methanol. 100µL of samples were added to the 100µL of %50 folin-ciocaltaeu reagent in a test tube, vortex mixed for a few seconds and mixed with 2000µL of 2% Na_2CO_3. The mixture was set aside at in dark at room temperature for 30 minutes. At the end of the incubation time, absorbance values at 750nm were recorded against blank, which was prepared 100µL pure methanol instead of sample. The curve of gallic acid concentration (prepared as 0.05, 0.1, 0.2, 0.3 mg/ml) versus absorbance was used as calibration curve to calculate the amount of phenolic content of dry samples in terms of gallic acid equivalent (GAE) value. All the experiments were carried out in duplicates and repeated for three different experiments.

2.2.6 Determination of Total Flavonoid Content

Modified aluminum chloride method proposed previously by Zhishen et al (1999) and Dewanto et al. (2002) was used to determine the total flavonoid content of crude extract and fraction samples. Briefly, catechin standards/crude extract/fractions in various concentrations were prepared in methanol. 125µL of samples were put into to a test tube, mixed with 625µl of water and 37.5µl 5% $NaNO_2$ and vortexed. Next at 5^{th} min, 75µl %10 $AlCl_3$ was added and vortexed then at 11^{th} min, 250µl 1.0M NaOH together with 387µL water were added to get final volume of 1.5 mL. The mixture was left in dark room at room temperature for another 10 min. At the end of the incubation time, the absorbance values at 510nm were read against blank, which was prepared by using 125µL pure methanol instead of sample. The catechin standard curve drawn for recorded absorbance value versus known concentrations of standard (mg/ml) was used to evaluate total flavonoid content of crude extract and fraction samples in terms of catechin equivalents.

2.2.7 Antimicrobial tests

2.2.7.1 Preparation of Microbial Stock:

Selected bacterial populations, which were *Proteus mirabilis (*RSKK 737, Pasteur Institute), *Escherichia coli (*RSKK 234, Pasteur Institute), *Staphylococcus aureus* (RSKK 95084, Pasteur Institute), *Streptococcus pyogenes* (RSKK 03019, ATCC 19615) were bought from Refik Saydam Hygiene Center. A pellet of *E. coli* and *P. mirabilis* were suspended in 1 ml of Luria-Bertani (LB) broth, for *S. aureus* Brain Heart Infusion (BHI) broth and for *S. pyogenes* Mueller-Hinton (MHB) broth was used to prepare 1 ml of bacterial suspension. Suspensions were used to streak onto the sheep blood agar plates prepared previously in our lab from these new agar plates were prepared and were incubated at 37^{o}C for 24 h and individual colonies selected by needle head were spread onto new sheep blood agar plates in every two weeks, to keep the freshness of selected bacteria and use morphologically similar colonies. A pellet of bacteria of interest was further suspended in 1 mL specified broth mediums and 100 µL suspensions were transferred into 10 mL specified broth mediums for optimal growth at 37^{o}C and fixed 180 rpm for 24 h. in sterile falcon tubes. Cultures were used in minimum inhibitory concentration (MIC) and agar disk diffusion assay analyses. For long term storage, 24 h-grown cultures were diluted to obtain 0.6 absorbance unit at 570nm, and 100µL of diluted suspension were completed to 1 mL with sterile % 20 (v/v) glycerol solutions and stored at -80^{o}C.

2.2.7.2 Determination of the Minimum Inhibitory Concentration:

2.2.7.2.1 Solvent effect

The solvent to dissolve the crude extract, fractions, and phenolic compounds samples was selected according to the following criteria: First, the inhibitory effect of the selected solvent was tested using minimum inhibitory concentration (MIC) method and later the solubility in selected solvent was determined. Tested solvents were: ethanol,

methanol, ethyl acetate, dimethylsulfoxide, and ultra pure water. For MIC analysis, 5 x 12 (row x column) wells of standard 96-well plates were used. First column of each plate was loaded with 175µL of broth of interest and rest was filled with 95 µL broth of interest until the 9^{th} column, 10^{th} column was left empty for sterility control of pipettes and 100µL broth was pipetted into the 11^{th} column for the sterility control of broth and finally 95µL of broth was added to the 12^{th} column for the growth control. 20 µL of 5 different solvents were added in 1^{st} column of broth loaded well plates. Pipette mixed solvent plus broth blends were serially diluted 2-fold by transferring 95 µL blend into 95 µL broth. This procedure was applied for each of 5 different solvents (5 rows) listed above for 4 different selected bacteria (4 plates). Each column except the one for pipette sterility and sterility control inoculated with 5µL bacterial suspension (overnight cultures having approximately 0.05 absorbance at 570nm corresponding to 10^5 CFU/mL) and incubated 37^oC for 24 h. Growths of bacteria of interest were visually determined. MIC analyses for solvent selection were repeated three times for each solvent. This procedure was explained schematically in Table 2.1.

Table 2.1: 96-well plate design in minimum inhibitory experiments.
5µL bacteria were inoculated to each well except pipette sterility and sterility control

	9.75%	4.88%	2.44%	1.22%	0.61%	0.31%	0.16%	0.08%	0.04%	Pipet sterility control	sterility Control (broth only)	growth control (broth and bacteria)
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
EtOH												
DMSO												
EtOAc												
Water												

2.2.7.2.2 Minimum Inhibitory Concentration (MIC) Value Evaluation

In this study, the NCCLS protocol (National Committee for Clinical Laboratory Standard) was slightly tailored to establish MIC values for samples (Wiegand et al., 2008) Standard 96-well plates were loaded with broth mediums chosen previously for each bacterial colonies as described in section 2.2.7.1 to determine the solvent effect. This time, crude extract and fraction samples together with standard phenolic were dissolved in methanol and 20µL of this mixture pippetted into the first column of well plates. Two fold dilutions were applied as explained in previous section except the serial dilution was ended at 7^{th} column. 8^{th} column left empty for pipette sterility control; 9^{th}, 10^{th}, 11^{th}, and 12^{th} columns were used for sterility control of the broth, the growth control, the antibiotic sterility control and the antibiotic growth control, respectively. Plates were incubated at 37^{o}C for 16 h and bacterial growth was evaluated at 570nm using ELISA plate reader against blank including all but bacteria. This procedure was explained schematically in Table 2.2

Table 2.2: 96-well plate design in minimum inhibitory experiments.

	1	2	3	4	5	6	7	Pipet sterility control	sterility control	growth control	Antibiotic sterility control	Antibiotic control
A	20 µL Analyte, 175µL broth 5µL bacteria	95 µL broth 50% of well-1 5µL bacteria	95 µL broth 50% of well-2 5µL bakteria	95 µL broth 50% of well-3 5µL bacteria	95 µL broth 50% of well-4 5µL bakteria	95 µL broth 50% of well-5 5µL bacteria	95 µL broth 50% of well-6 5µL bacteria	50% of well-7	100 µL broth	95 µL broth 5µL bacteria	95 µL broth 5µL Antibiotic	95 µL broth 5µL Antibiotic 5µL bacteria suspension
B												
C												
D												
E												
F												
G												
H												

2.2.7.2.3 -Minimum Bactericidal Concentration (MBC) Value Evaluation

Before the MIC values were monitored, 10µL of mixtures from each well were transferred into another well-plate which were pre-loaded with agar medium specific to studied bacterial colonies. Plates were incubated at 37^{o}C for 24 h and growth was determined by using ELISA plate reader operated at 570 nm against blank mixture without bacteria. The minimum bactericidal concentration was determined as the lowest concentration of crude extract/fraction/standard phenol needed to kill bacteria of interest.

2.2.7.3 Agar Disk Diffusion Assay

The NCCLS protocol (National Committee for Clinical Laboratory Standard) with little modifications was adapted to perform the agar disk diffusion assay (Wiegand et al., 2008). 100 µL of overnight cultures of bacteria (10^8 CFU/mL) was spread onto agar plates. 20µL of crude extract and fraction samples together with standard phenolic were dissolved in methanol and pippetted onto discs with 6mm diameter. Discs were placed into sterilized 96-well plates for impregnation, methanol was evaporated and dried discs were placed onto the inoculated agar plates. For positive control, antibiotic impregnated discs were also placed onto agar plates and plates were incubated at 37^{o}C for 16 h. The antimicrobial agent activity was determined by measuring the area of inhibition zone which was observed as a transparent area enclosing the discs.

2.3 Statistical analysis

All results are expressed as mean ± standard deviation (SD). Comparison of means was analyzed by Student's t test and differences were considered significant when $p<0.05$.

CHAPTER 3

RESULTS AND DISCUSSION

3.1 EXTRACTION OF *PLANTAGO MAJOR* LEAVES

160 g of powdered *P. major* leaves were extracted in total of 3600mL methanol and shook in ultrasonic bath for 30 minutes. Suspension was later incubated in oval-rotating incubator at a fixed 180 rpm for 24 h and 25 ^{o}C. Extraction mixture was dried in rotary evaporator at 30^{o}C as described in section 2.2.1. Resultant dry extract was weighted as 27.3 g and (%) yield was calculated as 17.06.

3.1.1 Prufication of Crude Extract with Fractionation

After the extraction of crude extract from the *P. major*, 3.70 g of crude extract was mixed with 300 mL water-methanol (1:1) and mixture was poured in separatory funnel and same volume of hexane was added to the mixture. When aqueous and organic phase separation obtained, organic phase and aqueous phase were separated from each other, then, organic phase was evaporated. Same procedure was carried on with other organic solvents (diethyl ether, chloroform, and ethyl acetate) and at each time, the remaining aqueous phase was re-fractionated with the other organic solvent. At the end, the remaining aqueous phase was lyophilized. After the fractionation with hexane, diethyl ether, chloroform ethyl acetate, and aqueous solvents, dried matter was weighed out as 0.1980, 0.133, 0.068, 1.080, and 2.03 g respectively.

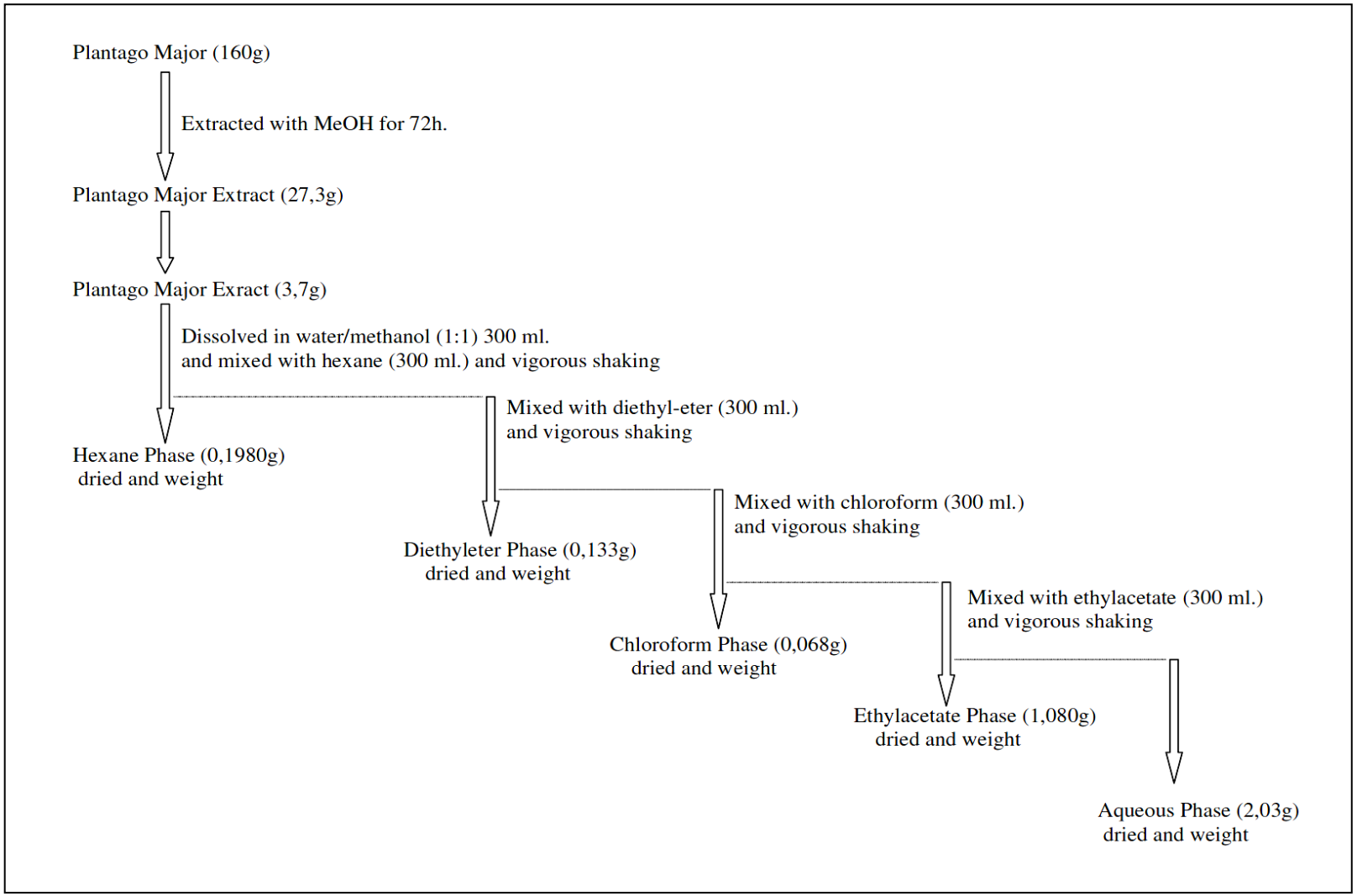


Figure 3.1: Schematically illustration of extraction and fractionation steps and yield as gram

3.2 EXPLORATION OF ANTIOXIDANT CAPACITIES

Antioxidants were explored by using DPPH radical scavenging, ABTS methods and determined in terms of the total amount of phenolics and flavonoids, which are well known compounds with strong antioxidant properties**.**

3.2.1 Antioxidant capacity by ABTS Method

Antioxidant capacities of the crude extracts and the fractions of the *P. major* were determined according to their radical scavenging potential with ABTS method as described previously in section 2.2.4.2. The concentration of trolox standard versus RSA (%) graph was shown in Figure 3.2, below. The linear regression equation driven from this curve was further used to calculate the trolox equivalent antioxidant concentration (TEAC) values for the samples of crude extract and fractions. For this, the slopes of regression models of samples (determined using the same procedure as done for trolox standard calibration curve for each sample-see Appendix A) were divided to the slope of trolox standard in order to calculate the TEAC values of each sample in terms of μmol Trolox equivalent/g extract. Results were listed in Table 3.1.

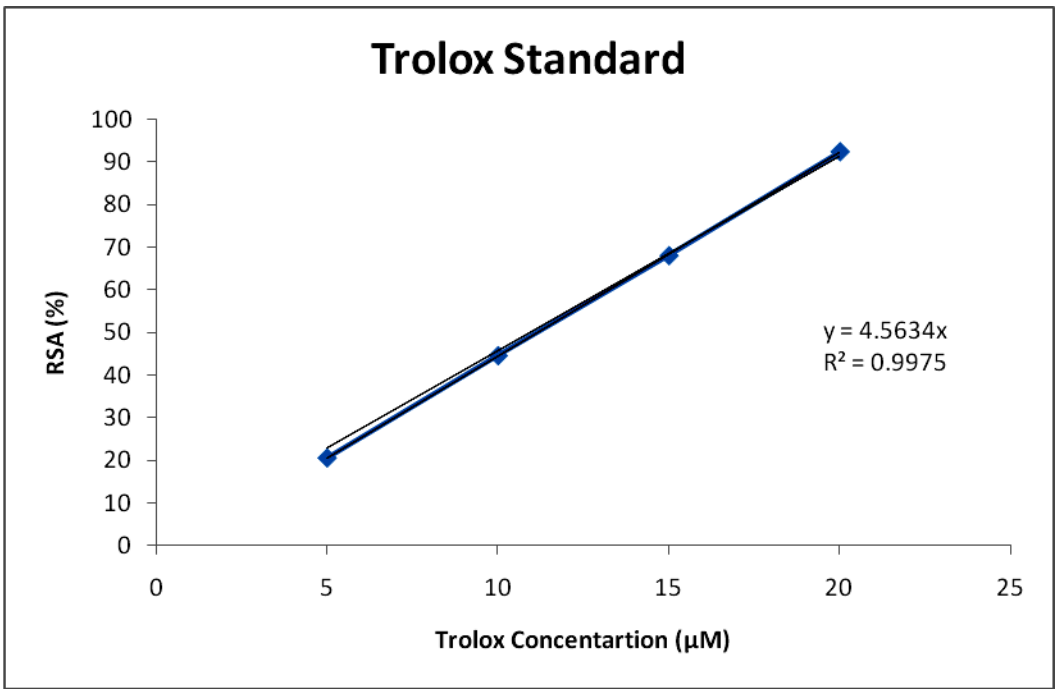


Figure 3.2 Trolox Standard Curve (at 734 nm). Experiments were performed in triplicates in three times.

The higher TEAC value means the higher antioxidant capacity of material under study. TEAC values calculated for crude extract and for each fraction of hexane, diethyl ether, chloroform, ethyl acetate in increasing polarity, and at last aqueous phase were found as 392.75, 49.316, 94.219, 49.37, 2130.4, 270.57 µmol/g, respectively. Among the investigated fractions, ethyl acetate has given the highest TEAC value indicating that ethyl acetate fraction has the highest radical scavenging capacity.

Table 3.1 Trolox equivalent antioxidant capacities (TEAC) of crude extract and fractions

P. major Crude Extract and Fractions	*TEAC value (µmol/g)
Crude Methanol extract	392.75±3.215
Hexane fraction	49.316±0.430
Diethyl ether fraction	94.219±0.987
Chloroform fraction	49.37±0.215
Ethyl acetate fraction	2130.4±9.845
Aqueous phase fraction	270.57±1.732

TEAC value: Radical scavenging activity µmol equivalents of trolox/g of crude extract and fractions (DW)
*Means of three independent experiment in duplicates

There is no study for comparing the antioxidant capacity of different fractions obtained from *P. major* fractionation using different solvents with varying polarities. However, one research group has reported the TEAC value as 37.77 µmol/g for *P. major* leaf crude extract in 80% methanol (Gan et al). They looked for 50 different Chinese medicinal plants which are known as being effective on rheumatoid disease treatment. Among them, *P. major* displayed a moderate antioxidant capacity when leaves were treated with 80% methanol for 24 h (Gan et al). They reported the TEAC value for dry weight of plant material, not the weight of the extracted material; in the view of these circumstances, we re-calculated the TEAC value of crude extract in terms of dry weight of initial plant material (160 g powdered plant leaf in 3600 mL methanol) and we obtained 1.77 fold higher TEAC value compared to findings of (Gan et. al., 2010) with a value of 67.01 µmol/g dry weight of *P. major*.

In another study, *P. major* tea was prepared by decoction of 2 g tea in 150 mL hot water and the TEAC value was given as 838 µmol/150 mL (5.59 µmol/mL) of hot water (Speisky et al., 2006). Again, when the TEAC values for current study were back calculated for a total plant material (160 g powder *P. major* leaves in total 3600 mL methanol), it was found that radical scavenging capacity was 3.12 fold higher (5.59

µmol/mL hot water versus 160g/ 3600 mL methanol x 392.75 µmol/g = 17.46 µmol/mL methanol in this study than that of Speisky groups, which was an expected outcome because it is a known fact that hot water is not a suitable solvent for many bioactive compounds in plants.

3.2.2 Antioxidant capacity by DPPH Method

DPPH is another radical scavenging evaluation method for medicinal plant material and the results were given in terms of EC_{50} values which represent the amount of bioactive compound needed to reduce to 50% of original DPPH reagent (Table 3.2). EC_{50} values were calculated with the help of RSA (%) versus final concentration (mg/mL) of the crude extract and fraction curves, which were illustrated in Figure 3.3. Quercetin, which is a well known antioxidant in flavanoid structure and widely used in the DPPH experiments as a positive control, was chosen in this study, because of its very high antioxidant capacity the data could not be displayed in the figure 3.3 and RSA (%) versus final concentration graph of quercetin, was illustrated in Appendices B.

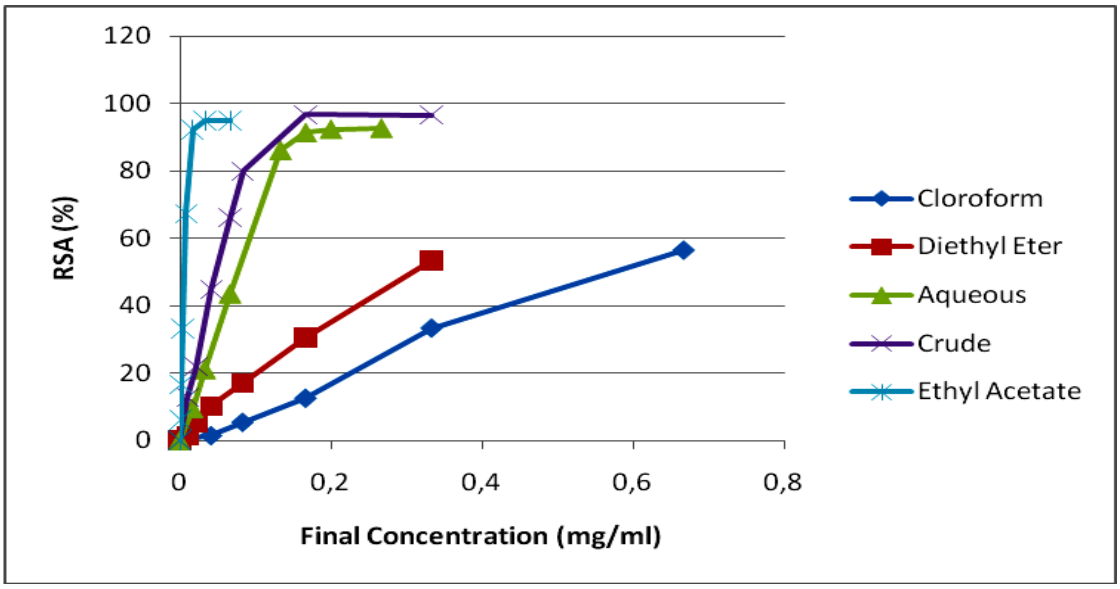


Figure 3.3 DPPH radical scavenging activity in percent versus the final concentration of crude extract and fractions (mg/mL) at 517 nm.

As it can be seen from Table 3.2, among the samples of crude extract and fractions, the lowest EC_{50} value, which means the highest radical scavenging activity, was observed for ethyl acetate fraction sample with an EC_{50} value of 0.0107±0.0002 mg/mL. Ethyl acetate fraction samples have also resulted in the highest TEAC values as evaluated in ABTS method. The rest of the EC_{50} values, from lowest to highest, were 0.062 ±0.003 and 0.089 ± 0.0021 mg/mL, for crude extract and aqueous phase, respectively. EC_{50} values could not be determined for the lower polarity solvent fractions of hexane, diethyl ether, and chloroform, even when the samples have been dissolved at their maximum solubility in methanol. The reactions of DPPH radical were not completed for these solvents even at maximum concentration, which indicates a poor separation capacity of bioactive compounds in plant materials for the solvents with lower polarity.

Table 3.2 Fifty percent effective concentration for DPPH radical scavenging capacities.

P. major Crude Extract and Fractions	*DPPH RSA EC_{50} (mg/mL)
Crude Methanol Extract	0.062 ±0.003
Hexane Fraction	NA
Diethyl Ether Fraction	NA
Chloroform Fraction	NA
Ethyl acetate Fraction	0.0107 ± 0.0003
Aqueous phase Fraction	0.089 ± 0.002
Quercetin	0.0077 ± 0.0002

DPPH RSA EC50: Effective concentration of crude extract or fractions required for scavenging 50% of DPPH radical
*Mean of three independent experiments in duplicates
NA: Not applicable

In the study of Pourmorad et. al.,(2006) DPPH scavenging for some of the medicinal plants collected in Iran was compared for their EC_{50} values. They reported the EC_{50} value of *P. major* leaf extract as 0.32 mg/mL for crude extract by incubating 48h in methanol (1:10(v/v)). Researchers used two different standards which were quercetin and butylated hydroxyanisole (BHT); and stated the EC_{50} values as 0.01mg/mL for

quercetin and 0.054 mg/mL for BHT. In the present study, EC_{50} was found 11.6 times more than the quercetin standard, while Pourmorad et. al.'s work resulted in 32 times more EC_{50}, which points to approximately 3 fold higher antioxidant capacity in crude extract in the present study (was much higher antioxidant capacity when plant extract in ethyl acetate results were considered).

Another research group has investigated the *P. major* leaves collected from Serbia (Beara et al., 2009). They extracted plant material in 80% aqueous methanol for 72 h and calculated the EC_{50} value as 0.00535 mg/mL while it was 0.00828 mg/mL for BHT standard. The crude extract was further washed with petroleum ether which is non polar solvent. The EC_{50} values for ethyl acetate fraction samples in the currents study are comparable with their outcomes, and in this case, they found 2.1 fold higher radical scavenging capacity. However, it is known that BHT has lower antioxidant capacity compared to quercetin standard (almost 5 times higher EC_{50} values were reported in previous reference- see Pourmorad et. al). The difference can be explained by this fact. Additionally, in another study from Serbia (Stanisavljevic´ et al., 2008) the EC_{50} value was found almost 6 times higher (EC_{50} = 0.360±0.001 mg/mL) with respect to our findings. In that study, they applied ultrasonic bath for *P. major* leaf extraction in ethanol. This difference may be due to the extraction method selected but most probably because of the plant material collected. Even, the last two studies have collected their plant material from the same region (Serbia), they reported very different antioxidant capacities for *P. major* leaves (almost 12 times lower scavenging capacity was found in the second study), indicating the importance of starting plant material and also the extraction method applied (solvents with higher polarity are more suitable to gather the bioactive compounds). For example, a research group from Scotland has given EC_{50}value of 0.034 mg/mL while quercetin has EC_{50} value of 0.0028 mg/mL for *P. major* leaves. They followed the strategy of applying non-polar solvents first and going up to higher polarities for fractionation. The expected antioxidant capacity was much lower than our findings when compared with ethyl acetate phase seeing that it was 1.3 fold higher with respect to quercetin standard, and it was 12.4 fold higher than standard's EC_{50} value in the study of Kumarasamy et. al (Kumarasamy et al., 2007).

Depending on these findings, it is fair to consider that starting with highly polar methanol, then applying solvents with increasing polarity is a better fractionation procedure to collect the phenolics in *P. major* leaves.

3.3 TOTAL PHENOL CONTENT OF CRUDE EXTRACT AND FRACTIONS OF *P. major*

In order to find out the correlation between the radical scavenging activity and phenolic content of the crude extract and the fraction samples, Folin-Ciocalteau method was applied as previously described in section 2.2.5. The calibration curve of gallic acid against absorbance value at 750nm was drawn and the equation driven from the regression analysis was used to calculate the phenolic content of the crude extract and fraction samples in terms of gallic acid equivalent value (GAE). The resulted calibration curve was demonstrated in figure 3.4 and computed GAE values of samples were listed in table 3.3. The linear regression curve equation is y= 98.017x- 0.0142.

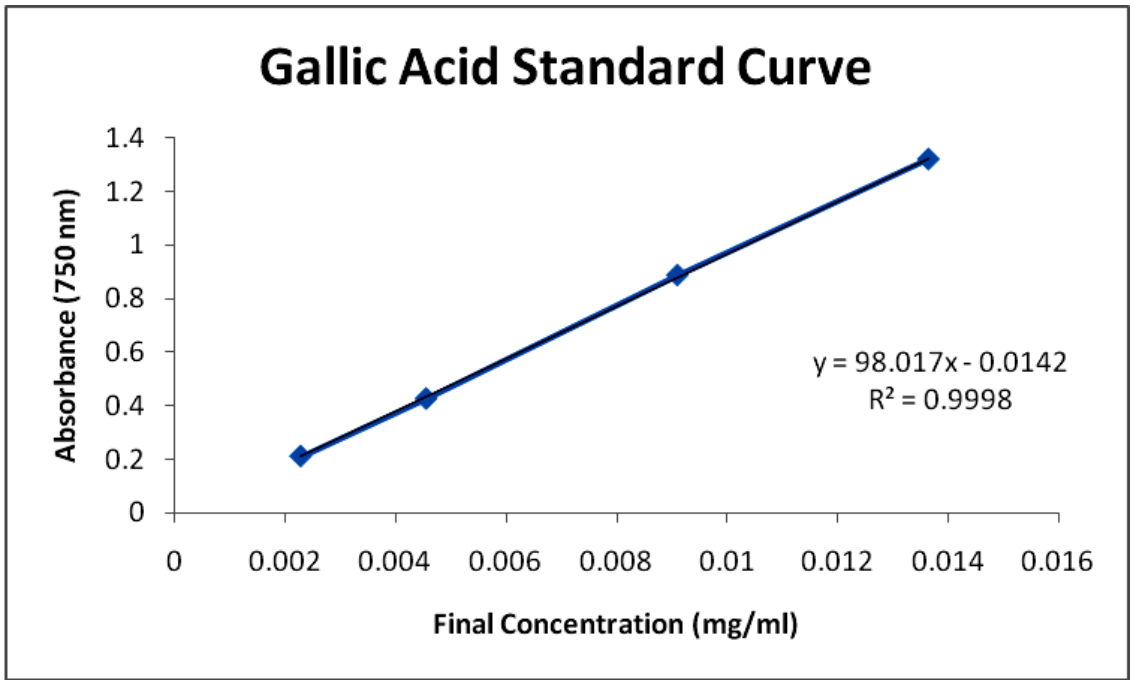


Figure 3.4: Gallic acid standard curve. Experiments were performed three independent experiments in triplicates.

As is the case in previous analysis, the highest total phenolic content was observed in ethyl acetate fraction samples with a value of 0.452±0.011 GAE mg/mg extract. The GAE values were 0.116 ±0.001 and 0.052 ± 0.0012 mg/mL, for crude extract and aqueous phase, respectively (Table 3.3). The maximum solvable dry extract in methanol was used to determine the GAE values for hexane, diethyl ether and chloroform fractions, though, no meaningful results were observed for those solvent fractions

Table 3.3: Total phenolic content of crude extract and fractions.

P. major Crude Extract and Fractions	*TP GAE (mg/mg)
Crude Methanol Extract	0.116±0.001
Hexane Fraction	NA
Diethyl Ether Fraction	NA
Chloroform Fraction	NA
Ethyl Acetate Fraction	0.452 ± 0.011
Aqueous phase Fraction	0.052 ± 0.001

TP GAE (mg/mg): Total phenolic content mg equivalents of gallic acid/ mg of crude extract and fractions
*Mean of three independent experiments in duplicates with standard deviation errors.
NA: Not applicable

Just like with radical scavenging evaluation in terms of EC_{50} values for *P. major* leave extract collected in Iran (Pourmorad et al., 2006), the GAE values were reported as 0.031±0.004 mg/mg GAE, which is 3.7 times lower than that of our results (Pourmorad et al., 2006). Similarly, in the studies of Gan et. al. (2010), Beara et. al. (2009), and Stanisavljevic et. al. (2008), a lot lower GAE values were reported for *P. major* leaf extract, which were 0.0062 ± 0.18 GAE mg/mg, 0.042 mg ± 0.001 GAE mg/mg, and 0.069±0.1 GAE mg/mg, respectively. It is interesting that, Beara et. al. and his co-workers obtained almost 2 fold higher TEAC value compared to our results, but claimed 0.36 fold lower total phenolic content in their samples, indicating the possible advanced antioxidant capacity of present phenolics in their extracts. On the contrary, Gan et. al. (2010) has shown a lot less GAE value, indicating less scavenging capacity of existing

phenolic compounds in their extracts. Likewise, Stanisavljevic; et al. found 1.66 fold less GAE value, pointing out less activity of phenolics existing in the extract samples.

3.4 TOTAL FLAVONOID CONTENT OF CRUDE EXTRACT AND FRACTIONS

The compounds in *P. major* extract samples of current study give absorbance at 415 nm which interfere with the readings of reaction used to determine the total flavonoid content. Thus, total flavonoid content was determined at 510 nm and catechin standard (giving absorbance at 510) was selected instead of quercetin (giving absorbance at 415 nm). Generally, this method is used to evaluate the flavones such as chrysin, apigenin, luteolin, and flavonols such as quercetin, myricetin, morin, rutin, but it is not suitable for flavanones and flavanonols (Apak, 2007). The calibration of catechin concentration values versus absorbance values at 510 nm was plotted and the equation driven from regression analysis was used to estimate the catechin equivalent values of crude extract and fraction samples (see figure 3.5). The calculated catechin equivalent values were given in table 3.4, below. Flavonoid content was evaluated to verify and clarify the correlation between the antioxidant capacities, total phenol and total flavonoid results.

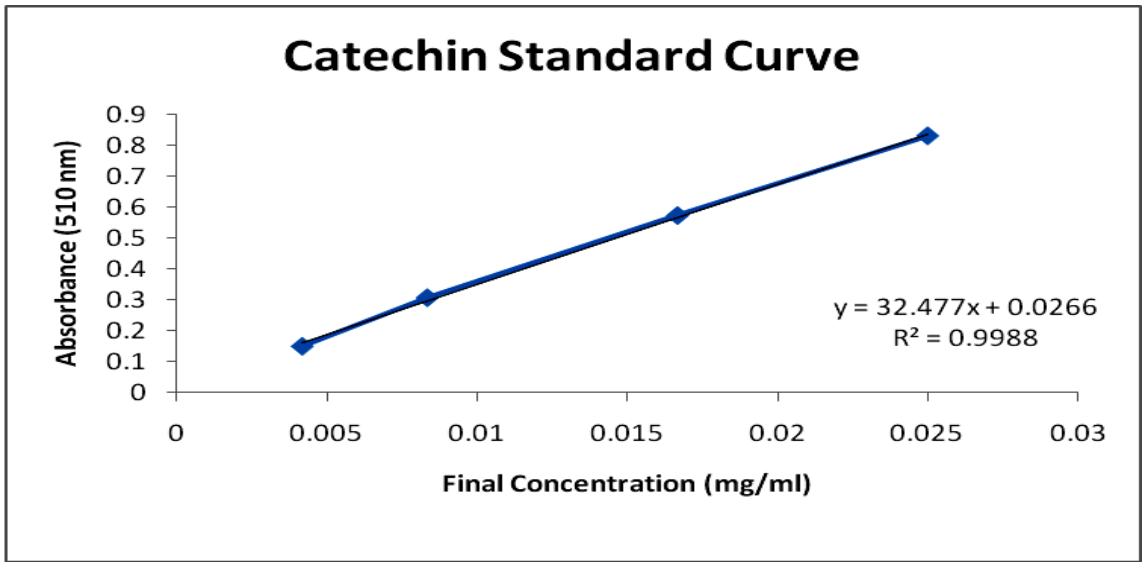


Figure 3.5: Catechin standard curve. Experiments were performed three independent experiments in triplicates.

As in the case of earlier results, the ethyl acetate fraction ended in the highest catechin equivalent value of 0.4340 ± 0.0017 mg / mg dry matter collected from ethyl acetate fraction, while it was 0.0867 ± 0.0018 and 0.0549 ± 0.0014 mg catechin equivalent per mg dry weigh of matter in crude extract and aqueous phase, respectively. Again, no consequential catechin equivalent values were computed for the hexane, diethyl ether and chloroform solvent fractions; even the samples were dissolved at an amount measured as weight of matter that gives maximum solubility in methanol.

Table 3.4: Total phenolic content of crude extract and fraction

P. major Crude Extract and Fractions	*TF Catechin Equivalent (mg/mg)
Crude Methanol Extract	0.0867 ± 0.0018
Hexane Fraction	NA
Diethyl ether Fraction	NA
Chloroform Fraction	NA
Ethyl acetate Fraction	0.4340± 0.0017
Aqueous phase Fraction	0.0549 ± 0.0014

TP Catechin Equivalent (mg/mg): Total flavonoid content mg equivalents of catechin/ mg of crude extract and fractions
*Mean of three independent experiments in duplicates,
NA: Not applicable

Pourmorad et al. (2009) found the total flavonoid content of *P. major* as 0.02515±0.18 mg quercetin equivalent in per mg dry extract. Similarly, Stanisavljevic et al. (2008), reported the total flavonoid content of *P. major* as 0.040±0.009 rutin equivalent in per mg dry extract. In both of these studies, results are in agreement with our findings and also they calculated the total flavonoid content very close to the total phenolic compound. Beara et al. (2009), on the other hand, got the total flavonoid content of *P. major* leaf extract as 0.00531±0.0008 mg of quercetin equivalent /mg of dry weight of crude extract. They explained this perceptible difference by proposing a weak relationship between total phenolic and flavonoid content; thus they claimed that other natural products present in *Plantago* extracts, besides phenolic compounds, can be involved in the scavenging of these radical species.

Table 3.5 Comparison of the TEAC (µmol/g), DPPH EC_{50} (mg/mL), TP GAE (mg/mg), TF Catechin Equivalent (mg/mg)

P. major Crude Extract and Fractions	*DPPH RSA EC_{50} (mg/mL)	*TEAC value (µmol/g)	*TP GAE (mg/mg)	*TF Catechin Equivalent (mg/mg)
Crude Methanol extract	0.062 ±0.003	392.75±3.215	0.116±0.001	0.0867 ± 0.0018
Hexane fraction	NA	49.316±0.430	NA	NA
Diethyl ether fraction	NA	94.219±0.987	NA	NA
Chloroform fraction	NA	49.37±0.215	NA	NA
Ethyl acetate fraction	0.011 ± 0.001	2130.4±9.845	0.452 ± 0.011	0.4340± 0.0017
Aqueous phase fraction	0.089 ± 0.002	270.57±1.732	0.052 ± 0.001	0.0549 ± 0.0014
Quercetin	0.0077 ± 0.0002	ND	ND	ND

TEAC value: Trolox equivalent antioxidant capacity of plant extract in µmol trolox equivalent / g of crude extract or fraction DW
DPPH RSA EC_{50}: Effective concentration of crude extract or fractions required for scavenging 50% of DPPH radical
TP GAE (mg/mg): Total phenolic content mg equivalents of gallic acid / mg of crude extract and fractions
TF Catechin (mg/mg): Total flavonoid content mg equivalents of catechin / mg of crude extract and fractions
*Mean of three independent experiments in duplicates
NA: Not applicable
ND: Not determined

3.5 ANTIMICROBIAL ACTIVITY

Antimicrobial activity of the crude extract, fractions and the antioxidant standard chemicals, which are tannic acid (a polyphenol), catechin (a flavan 3-ols), quercetin (a flavonoid), rutin (a flavonoid glycoside), caffeic acid (a hydroxynnamic acid), and trolox (a water soluble vitamin E derivative) were tested for their bacteriostatic and bactericidal effect on two gram (+), which are *S. aureus* and *S. pyogenes*; and two gram (–) bacteria, which are *P. mirabilis* and *E. coli*.

3.5.1 Solvent Effect

Five of the most widely used solvents in inoculation applications for antimicrobial tests, namely methanol, ethanol, dimethylsulfoxide DMSO, ethyl acetate and ultra pure water, were tested in order to determine the best one in terms of inhibitory effect on four selected bacteria (*S. aureus*, *S. pyogenes*, *P. mirabilis* and *E. coli*) and solubility of extracted matter in those solvents (see section 2.2.7.2.1). As expected, none of the selected solvents showed an inhibitory effect on selected bacteria which was observed visually. Thus, according to the second criteria, which is the resolving power on the used materials, methanol was selected (results were determined as visual observation according to the precipitate amount in solvents). Absorbance versus concentration (%) in the final mixture of methanol (MIC values of methanol) was further shown in figure 3.6.

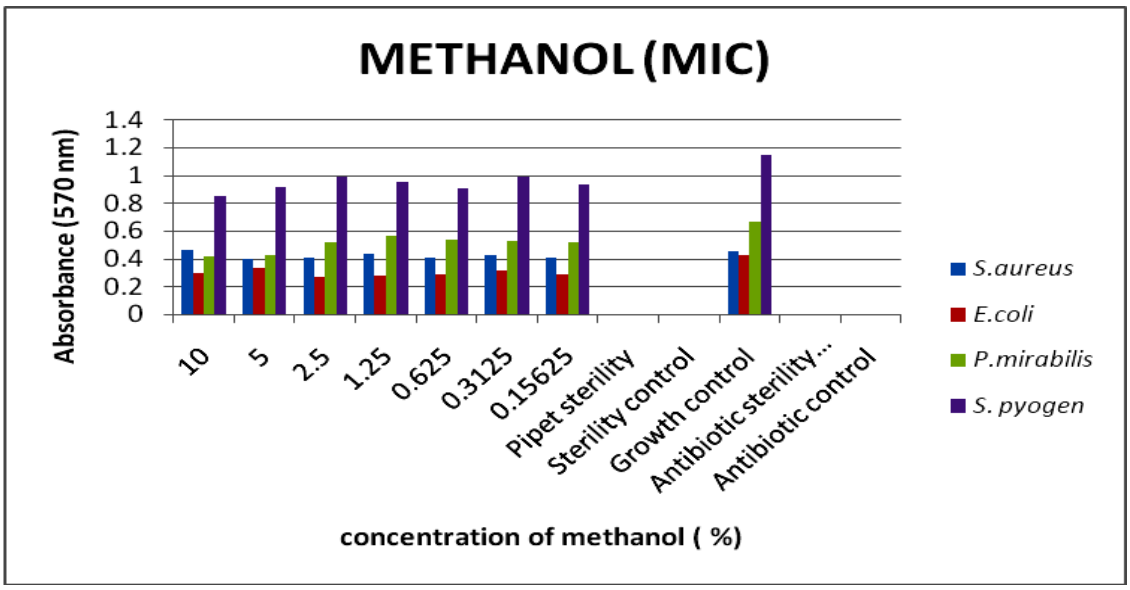


Figure 3.6: Evaluation of methanol effect on growth of *S. aureus, E. coli, P. mirabilis, S. pyogenes* after 16 h incubation.

3.5.2 Antimicrobial Effect of Crude Extract and Fractions

MIC values, as mentioned earlier, indicate the bacteriostatic effect of the antimicrobial agent on the selected bacteria. MIC values for crude extract and fractions were determined as described in section 2.2.7.2.2 and the results were shown in figure 3.7-9 for crude extract, ethyl acetate, and aqueous phase, respectively. Other fractions (hexane, diethyl ether and chloroform) did not show any bacteriostatic effect on selected bacteria.

The final concentrations of samples in methanol were 0.3125-20.0 mg/mL for the crude extract; 0.016-1.0 mg/mL for hexane, diethyl ether, and chloroform fractions, 0.078-5.0 mg/mL for ethyl acetate, 0.046-3.0 mg/mL for the aqueous fractions. The maximum limit of concentration values were determined according to saturated amounts dissolved in methanol.

Growth curves of bacteria used in this study and calculated CFU numbers which were determined for earlier thesis works were taken as reference (Gerdan, 2009; Kumbet,

2010; Barut 2010). Depending on the information in referred plots, 5 µL of bacteria collected from overnight cultures having approximately 0.05 au at 570nm (corresponding to 10^5 CFU/mL) were inoculated into the wells which were assigned to antimicrobial activity of plant extracts, growth control and antibiotic growth control. MIC values were found as the minimum concentration of matter of interest which resulted in no bacterial growth after 16 h incubation at 37^{o}C. MIC value also demonstrates the zero absorbance units at 570 nm; this is the ideal minimum inhibitory concentration for that material.

The crude extract was shown bacteriostatic effect on *S. aureus*, *P. mirabilis*, and *S. pyogenes* with MIC values of 5.0, 5.0, 10.0 mg/mL, respectively. Hexane, diethyl ether, chloroform didn't show any bacteriostatic effect on *S.aureus*, *P. mirabilis*, *E. coli* and *S. pyogenes*, except chloroform fraction with 1.0 mg/mL MIC value had an inhibitory effect on *S. pyogenes*. Ethyl acetate fraction was the most powerful fraction among the examined samples for bacteriostatic effect on all four of the selected microorganisms; and the MIC values were 1.250 mg/mL for *S. aureus*, 5.0 mg/mL for *E. coli*, 1.25 mg/mL for *P. mirabilis*, and finally 2.5mg/mL for *S. pyogenes*. Also, the aqueous fraction showed a good but not best bacteriostatic effect on two bacteria *S. aureus*, and *P. mirabilis* with the same MIC value of 3.0 mg/mL.

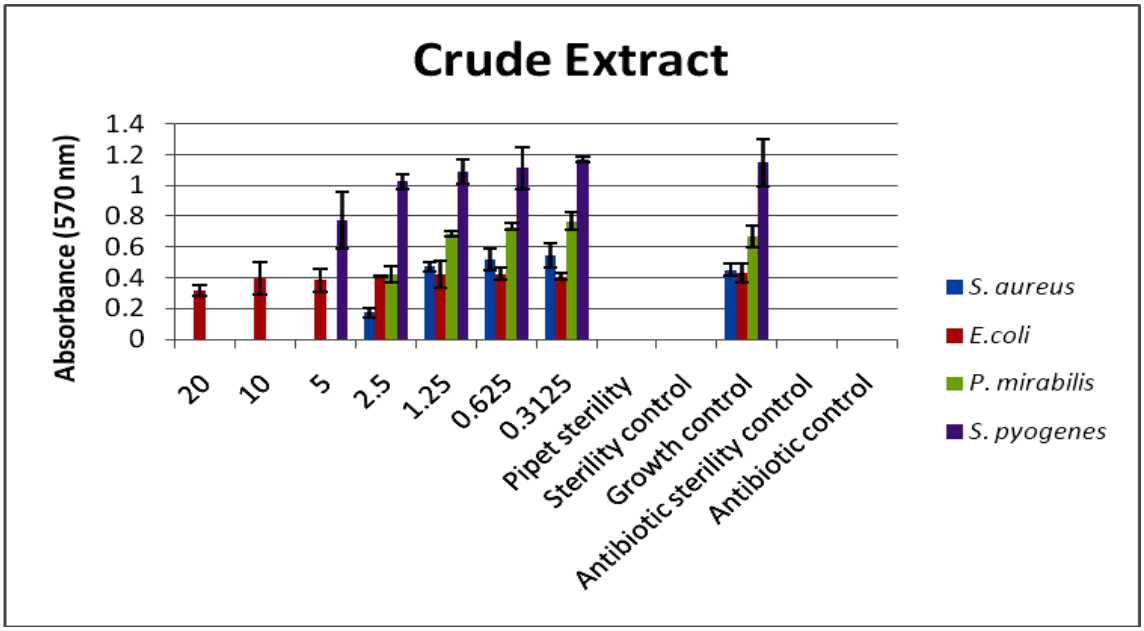


Figure 3.7: Evaluation of minimum inhibitory concentration of crude extract on *S.aureus, E.coli, P. mirabilis, S. pyogenes* after 16 h incubation

Pipette sterility: 8th column include only 95%of the 7th column for sterility control of pipette
Sterility control: test experiments if they were performed in aceptique conditions in the presence of broth only.
Growth control: Test of bacteria growth in the presence of only broth and bacteria
Antibiotic sterility control: Sterility of Gentamicin or Penicillin G in the absence of the bacteria
Antibiotic control 5µL Gentamicin or Penicilin G was used as antibiotic in the concentration of 10 mg/mL
Each data obtained three independent experiments in duplicates.

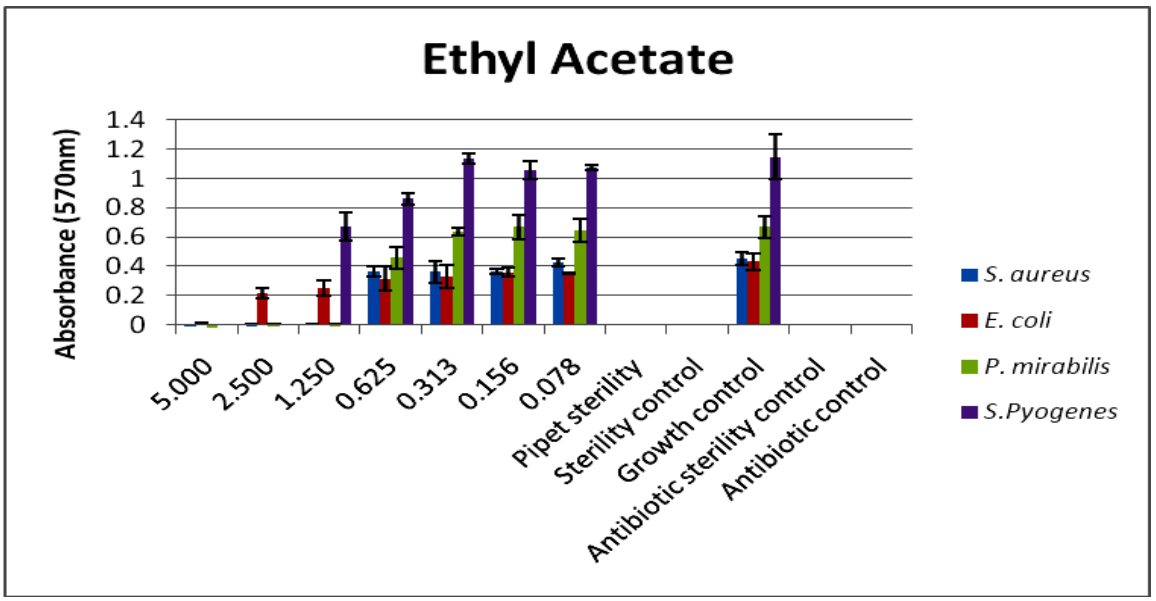


Figure 3.8: Evaluation of minimum inhibitory concentration of ethyl acetate fraction on *S.aureus, E.coli, P. mirabilis, S. pyogenes* after 16 h incubation

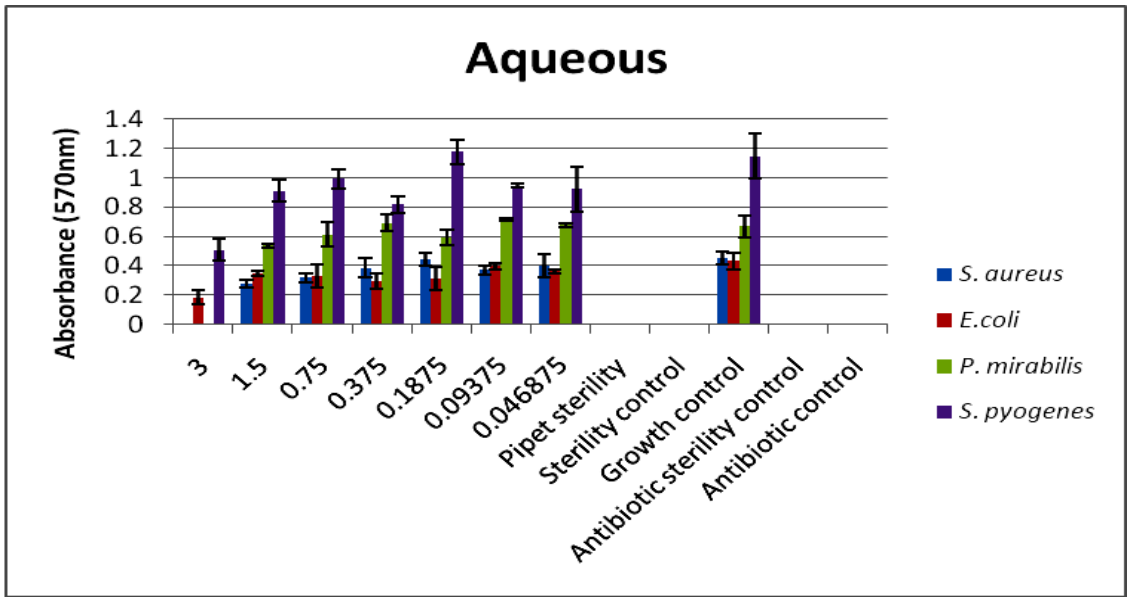


Figure 3.9: Evaluation of minimum inhibitory concentration of aqueous fraction on *S.aureus, E.coli, P. mirabilis, S. pyogenes* after 16 h incubation

Holetz et al. worked with the 13 Brazilian medicinal plants (including *P. major*) to evaluate their antimicrobial activity on some bacteria including *S. aureus* ATCC 25923 and *E. coli* ATCC 25922. They extracted the leaves of the plant with ethanol/water (9:1) and applied the crude extract directly on *S. aureus* and *E. coli*. The highest material concentration used in MIC analysis was 1.0 mg/mL, which did not show any effect on *E. coli* but inhibited the growth of *S. aureus.* Thus, they concluded that *P. major* has a weak activity against *S. aureus* (Holetz et al. 2002). In this current study, MIC value of crude extract was 5.0 mg/mL, while it was much lower for ethyl acetate fraction with a value of 1.250 mg/mL for *S. aureus*. A research group from University of Kebangsaan Malaysia has investigated the morphological changes of the cell wall of *S. aureus*, and *E. coli* under electron microscope after exposing the cells to methanol and ethanol extract of *P. major* having a varying concentration between 100 and 200 mg/mL of dry matter. They observed that *S. aureus* cells got sticky and shrunken, while in E. coli cells blebs formation and indentations (as compare to control without plant extract) were observed (Sharifa et al. 2008). Depending on former observations and our results, it can be suggested that *P. major* is effective on both gram (-) and (+) bacteria, even this effect differs morphologically.

Ravn et al. using agar dilution assay method, studied the minimum inhibitory effect of three isolated compounds from *P. major*, which are plantamajoside (a caffeic acid ester), forsythiaside, and chlorogenic acid, found MIC values as 2.0, 1.5, and 2.5 mg/mL, for *S. aureus* (SO2A), and >2.5, >2.5, and 2.5 mg/mL for *E .coli* (MI30), respectively (Ravn et al. 1988). These findings indicate that, even the isolates were used directly in the study of Ravn et. al (1988); the inhibitory capacities were almost the same for crude extract and fraction samples compared to the present study given in this thesis.

A more recent study from Bilecik in Turkiye, reported about several medicinal plant antimicrobial effects on the following strains of *Staphylococcus aureus* ATCC 6538, *Staphylococcus epidermidis* ATCC 12228, *Escherichia coli* ATCC 8739, *Klebsiella pneumoniae* ATCC 4352, *Proteus mirabilis* ATCC 14153, *Pseudomonas aeruginosa* ATCC 1539, *Shigella flexneri* and *Candida albicans* ATCC 10231. They found that

minimum inhibitory effect for *P. major* leaf extracts prepared in Petroleum ether and ethanol as 0.156 mg/mL and 0.019 mg/mL for *S. aureus* (ATCC 6538), and the MIC values as 1.25mg/mL, and 1.25mg/mL for E. coli (ATCC 8739) also 1.25 mg/mL for both the extracts against *P. mirabilis* (ATCC 14153) (Unsal et al., 2010).

As far as to our knowledge, apart from these three studies reporting the MIC values of *P. major* leaf extracts against *E. coli* and *S. aureus, P. mirabilis* there is no work investigating the inhibitory effect of extract of on *S. pyogenes.* Although all three of the mentioned studies reported their results against different strains of the bacteria of interest. Whereas, *Streptococcus pyogenes* are known to cause 700 million infections each year together with severe hospitalization cases which results in 25% mortality (Aziz et. al., 2010). Similarly, *P. mirabilis* are known to cause significant infections in humans accompanying another gram (-) bacteria of *Escherichia coli (*(Forbes et al., 2007). Both of them are treatable with most of the antibiotics but with some resistance. The MIC values in our results are promising and with further refinement to increase the inhibitory activity of plant based extracts. Therefore it would be fair to consider the *P. major* leaf extracts as an alternative to present synthetic antibiotics in the treatments of *P. mirabilis* and *S. pyogenes* related infections.

3.5.3 Antimicrobial Effect of Antioxidant Standards

Minimum inhibitory concentrations (MIC) of the selected phenolic compounds were determined by using the technique of micro broth dilution as previously described in section 2.2.7.2.2. Figure 3.10-16 shows the effect of the tannic acid, catechin, quercetin, rutin, caffeic acid and trolox (a water soluble vitamin E derivative) these compounds on *S. aureus*, *E. coli*, *P. mirabilis*, *S. pyogen* in terms of absorbance of microbial growth at 570 versus the concentration of the phenolic compounds in the broth as mg/mL.

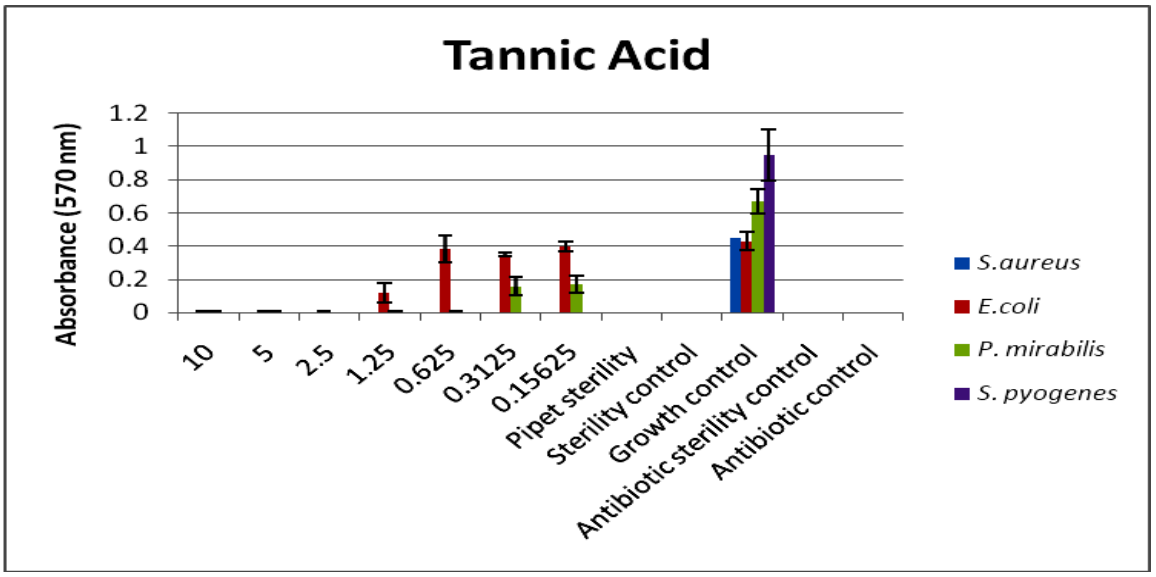


Figure 3.10: Evaluation of minimum inhibitory concentration of tannic acid on *S.aureus, E.coli, P. mirabilis, S. pyogenes* after 16 h incubation

Pipette sterility: 8th column include only 95%of the 7th column for sterility control of pipette
Sterility control: test experiments if they were performed in aceptique conditions in the presence of broth only.
Growth control: Test of bacteria growth in the presence of only broth and bacteria
Antibiotic sterility control: Sterility of Gentamicin or Penicillin G in the absence of the bacteria
Antibiotic control 5µL Gentamicin or Penicilin G was used as antibiotic in the concentration of 10 mg/mL
Each data obtained three independent experiments in duplicates.

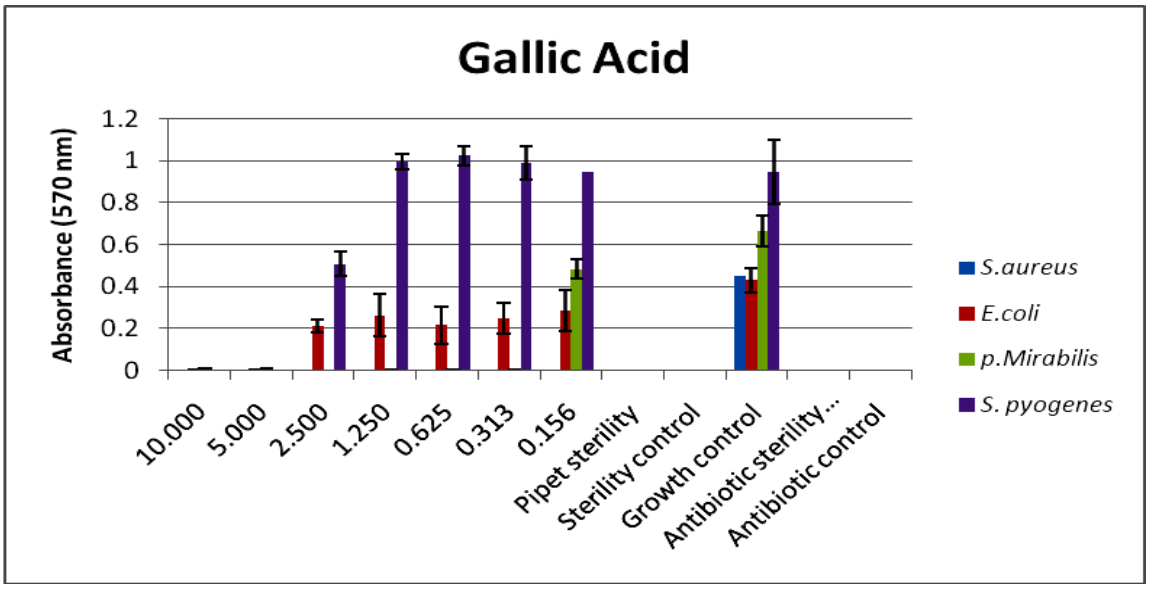


Figure 3.11: Evaluation of minimum inhibitory concentration of gallic acid on *S.aureus, E.coli, P. mirabilis, S. pyogenes* after 16 h incubation

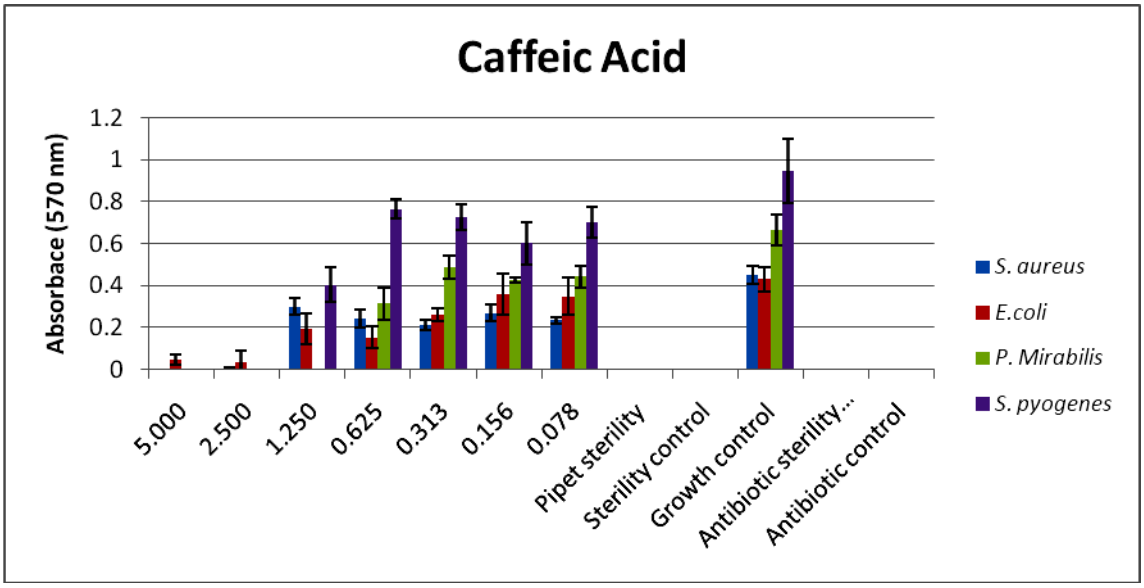


Figure 3.12: Evaluation of minimum inhibitory concentration of caffeic acid on *S.aureus, E.coli, P. mirabilis, S. pyogenes* after 16 h incubation

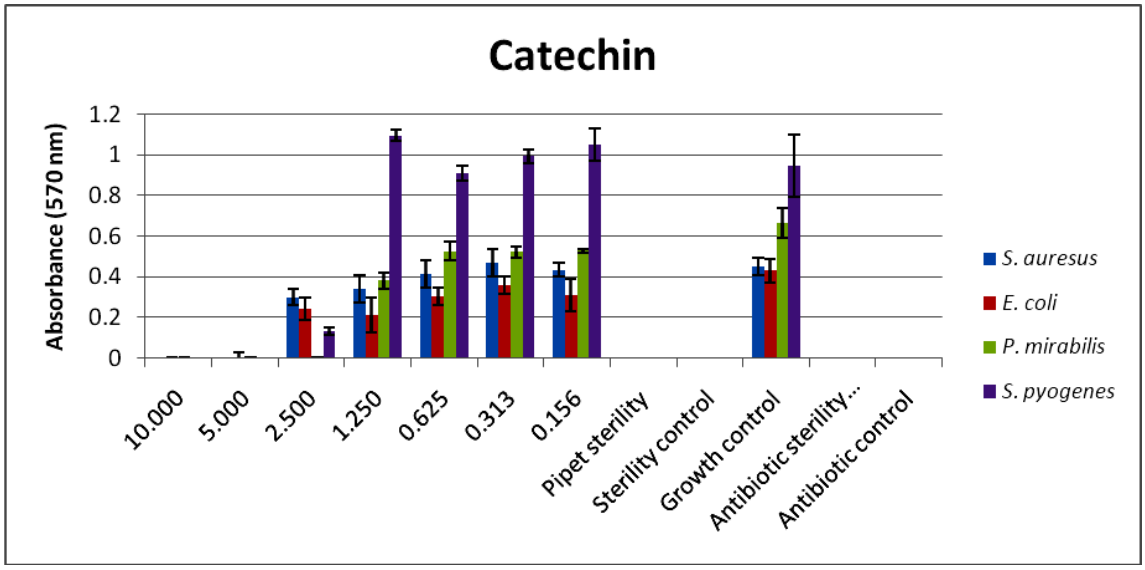


Figure 3.13: Evaluation of minimum inhibitory concentration of catechin on *S.aureus, E.coli, P. mirabilis, S. pyogenes* after 16 h incubation

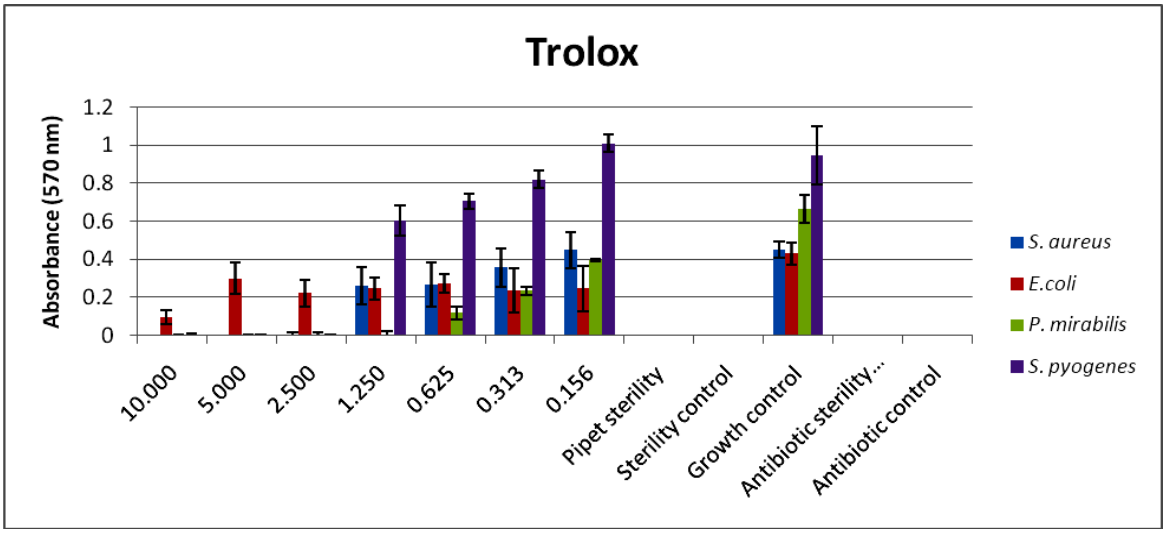


Figure 3.14: Evaluation of minimum inhibitory concentration of trolox on *S.aureus*, *E.coli*, *P. mirabilis*, *S. pyogenes* after 16 h incubation

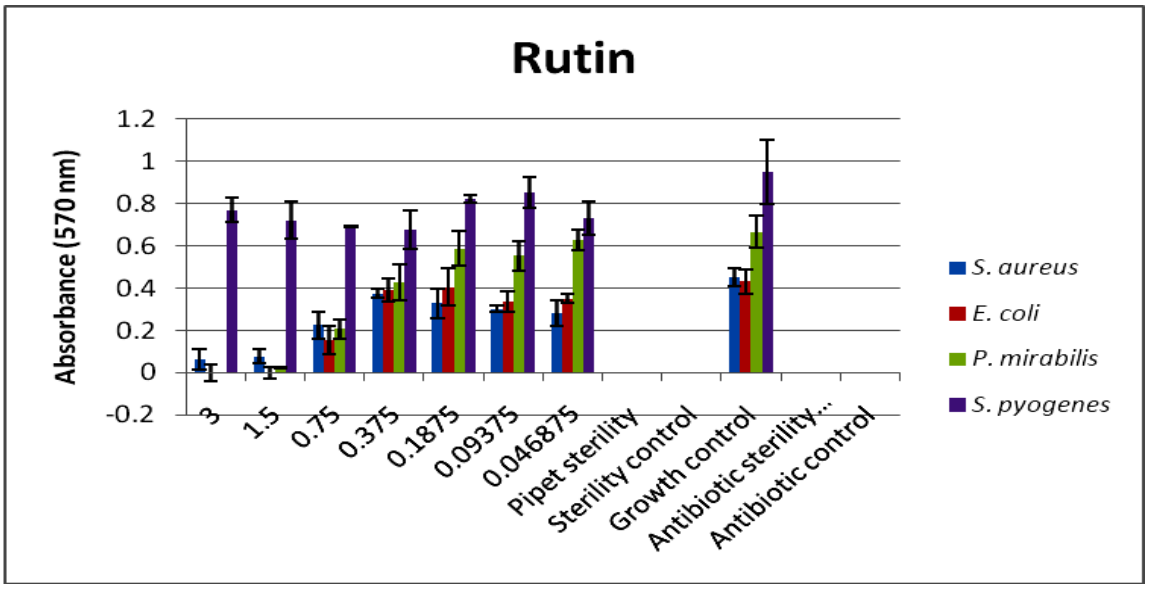


Figure 3.15: Evaluation of minimum inhibitory concentration of rutin on *S.aureus*, *E.coli*, *P. mirabilis*, *S. pyogenes* after 16 h incubation

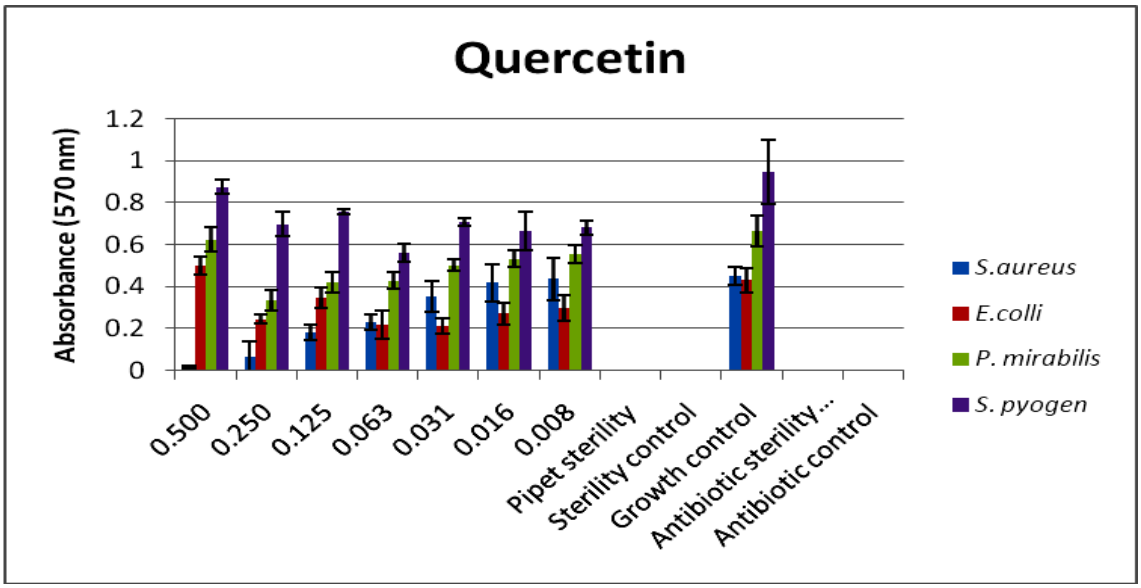


Figure 3.16: Evaluation of minimum inhibitory concentration of quercetin on *S.aureus, E.coli, P. mirabilis, S. pyogenes* after 16 h incubation

The concentration range was arranged in between 0.15625-10.0 mg/mL for the tannic acid, gallic acid, catechin, and trolox; 0.078-5.0 mg/ mL for caffeic acid, 0.046-3.0 mg/mL for rutin, and 0.008-0.5mg/mL for quercetin. Inoclulation of bacteri were performed the same as described previously in section 3.5.2.

Among the investigated standard antioxidant compounds, tannic acid had the most powerful growth inhibitory action on the selected bacteria with MIC values of 0.15625 mg/mL for *S. aureus* and *S. pyogenes*; 0.625 mg/mL for *P. mirabilis*, and 2.5 mg/mL for *E. coli*. The minimum inhibitory concentration for the gallic acid, caffeic acid, and catechin, were determined as 0.15625, 2.5, 5.0 mg/mL for *S. aureus*; 5.0, 2.5, 5.0 mg/mL for *E. coli*; 0.3125, 1.1250, 2.5 mg/mL for *P. mirabilis*; and 5.0, 2.5, 5.0, 2.5 mg/mL for *S. pyogenes*, respectively. Trolox shows bacteriostatic effect for *S. aureus*, *P. mirabilis*, and *S. pyogenes* with 2.5, 1.25, 2.5 mg/mL MIC values respectively but there was no effect on *E. coli*. Rutin was also found effective on *S. aureus*, *E. coli* and *P. mirabilis* with 1.5 mg/mL MIC value; however, it didn't show any inhibitory result on *S. pyogenes* in investigated concentration range. Lastly, quercetin only illustrated inhibitory effect on *S. aureus* with 0.5 mg/mL inhibitory concentration.

Aguri and his co-workers worked with the 47 different phenolic compounds, some were isolated from plants and some were purchased standard phenolics containing tannic acid, gallic acid, catechin, rutin, caffeic acid; and determined the antimicrobial effects of listed phenolics on *S. aureus* (ATCC29213), *E .coli* (ATCC25922) and *P. mirabilis* (ATCC7001) (Aguri et. al., 2006). Their findings are in agreement with our results for tannic acid, catechin, rutin, caffeic acid, except gallic acid, which was 0.6 mg/mL (MIC) for *E. coli*, but we found it as 5.0 mg/mL (MIC) in the current study. The same research group has also reported the MIC values for 10 different phenolic compound including tannic acid against 10-27 different strains of *S. aureus, Salmonella, E.coli,* and *Vibrio*, in another study. What they found is surprising yet expected, as they showed that MIC values of tannic acid were different but in range of 0.8 mg/mL to 3.2 mg/mL (MIC) for the 23 different strains of the *E.coli* investigated. These results put the case clearly that phenolics (even they are purchased standards) may have different inhibitory action on even different strains of same bacteria. More surprisingly, they reported two different MIC values of 1.34 mg/mL and 0.8 mg/mL for the same strain of *E. coli*, cultivated at different times, which was also the case for the same strain of *S. aureus*, where MIC values were 0.4 mg/mL and 0.1 mg/mL for tannic acid, when bacterial cultures were cultivated in 2006 or 2004, respectively (Aguri et. al., 2006; Aguri et. al., 2004). Hence, despite the quality of the extracted/ isolated phenolics, the bacterial strains, and even the cultivation medium are important factors on MIC value determination experiments.

Binnuty et al. studied antibacterial effect of isolated gallic acid compound from the leaves of *Mezoneuron benthamianum* on the gram (+) *Staphylococcus aureus* (ATCC 29213), and gram (-) *Escherichia coli* (ATCC 25922) and found the MIC values as 0.25 mg/ mL for *S. aureus* and 1.0 mg /mL for the *E. coli* (Binnuty, 2000), which were found as 0.156 mg/mL for *S. aureus* and 5.0 mg/mL for *E. coli* in our work. However, as stated above, the MIC values are ranging widely for different strains or even for different operators as reported in the literature. For examples Henis et. al, has observed no inhibitory effect up to tannic acid or gallic acid concentrations of 1.0 mg/mL, while, in our case, 2.5 mg/mL tannic acid and 5.0 mg/mL gallic acid were effective for *E. coli* (Henis et al., 1964). Similarly, for the other standards like caffeic acid much higher MIC

value of 27.6 mM for E. coli was evaluated (when MIC values were re-calculated in units of mM) compared to 8mM reported value of Barbet et. al. (2000) for *E. coli*.
(Barbet, 2000)

Kajiya et al. evaluated the antibacterial activities of the catechin and catechin derivatives with different alkyl chain lengths against gram (+) bacteria including *S. aureus* (IAM1011) and found out minimum inhibitory concentrations as 12.8mg/mL for catechin. On the other hand, catechin derivatives which have different alkyl chain lengths showed lower MIC values up to 0.025 mg/mL. They presumed that the antibacterial activity of the (+)-catechin derivatives and their interaction with the bacterial membrane are closely correlated (Kajiya, 2004).

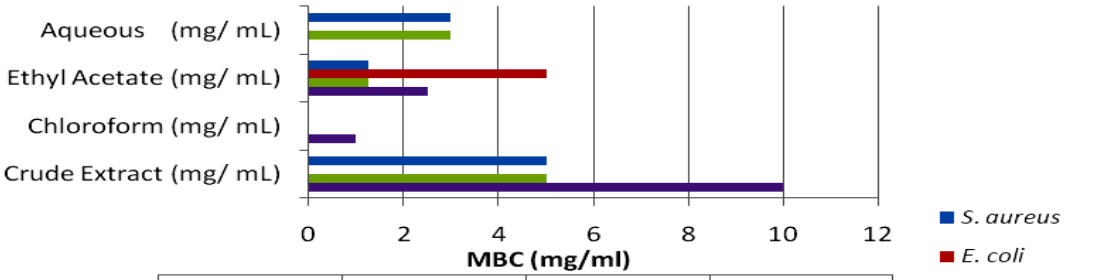
Gatto et al examined the antimicrobial activity of quercetin and its derivatives using different clinically isolated microorganisms, including five gram (+) strains (*S. aureus*, *B. subtilis*, *Listeria ivanovi, Listeria monocytogenes, Listeria serligeri*) and five different gram (-) strains (*Escherichia coli*, *Shigella flexneri*, *Shigella sonnei*, *Salmonella enteritidis*, *Salmonella tiphymurium*); and quercetin concentration up to 0.1 mg/mL did not show antimicrobial activity against any of these bacteria, because of the low solubility feature of quercetin in methanol (Gatto et al., 2001), in the current study it was found only effective on *S. aureus* with the MIC value of 0.5mg/ mL.

3.5.4 Minimum Bactericidal Concentration

In order to evaluate whether the observed crude extract/fraction samples or standard phenolic compounds' MIC values were bacteriostatic or bactericidal, MBC/MIC ratios should be calculated. The MBC is the minimal concentration of antimicrobial compound that kills the inoculums and can be determined from broth dilution MIC tests by sub culturing some to agar media as described in section 2.2.7.2.3. An antimicrobial substance regarded as the bactericidal if the ratio of MBC/MIC is not more than four (French, 2006)

3.5.4.1 Bactericidal evaluation of the crude extract and fractions

The crude extract was shown bactericidal effect on *S. aureus*, *P. mirabilis*, and *S pyogenes* with the 5.0, 5.0, 10.0 mg/mL, respectively, indicating what inhibited the growth was also bactericidal. Additionally, chloroform fraction resulted in bactericidal effect with 1.0 mg/mL MBC value on *S. pyogenes*. As well, the aqueous fraction showed the bacteriostatic effect on just two bacteria *S. aureus*, and *P. mirabilis* with the same value of 3.0 mg/mL (MBC). Again, the ethyl acetate fraction was the most powerful fraction among the bactericidal examination of the crude extract and fraction samples and resulted in 1.250 mg/mL (MBC) for *S. aureus*, 5.0 mg/mL (MBC) for *E. coli*, 1.25 mg/mL (MBC) for *P. mirabilis*, and finally 2.5 mg/mL (MBC) for *S. pyogenes*. For all samples, the MBC/MIC ratio was found as 1:1, indicating that all growth inhibition concentration was also bactericidal. Results are shown in figure 3.17.



	Crude Extract (mg/ mL)	Chloroform (mg/ mL)	Ethyl Acetate (mg/ mL)	Aqueous (mg/ mL)
S. aureus	5		1.25	3
E. coli			5	
P. mirabils	5		1.25	3
S. pyogenes	10	1	2.5	

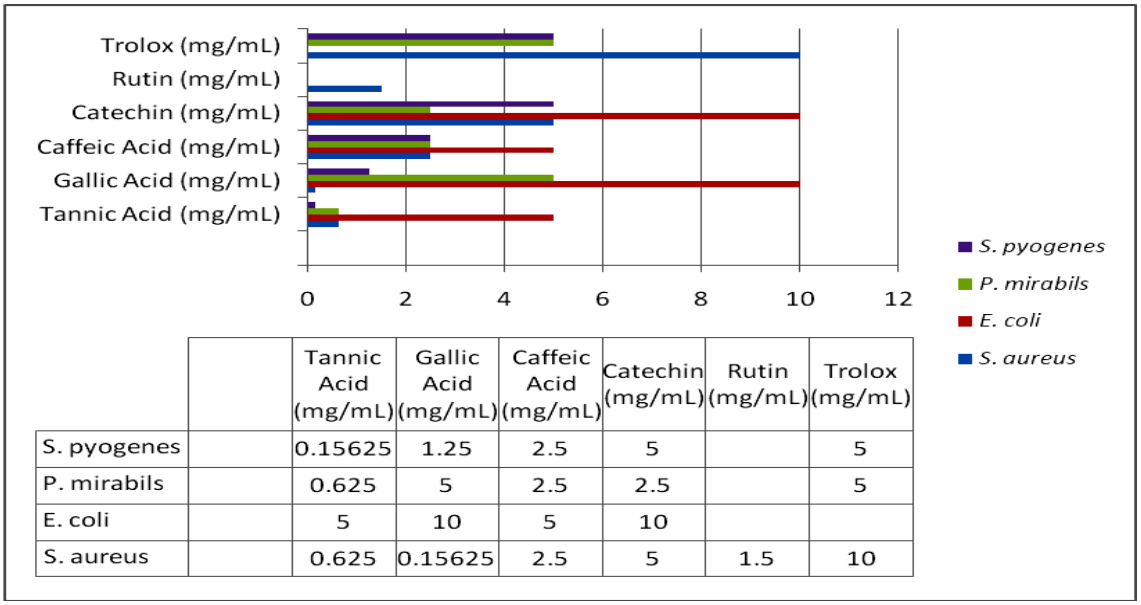
Figure 3.17: Minimum bactericidal concentration of crude extract,and chloroform, ethyl acetate, aqueous fractions

3.5.4.2 Bactericidal Evaluation of Standard Antioxidants

Minimum Bactericidal concentration (MBC) determinations for the phenolic compounds were examined according to the micro broth dilution method described in section 2.2.7.2.3.

Tannic acid and gallic acid was found the more efficient then the other phenolic. The minimum bactericidal concentration of the tannic acid, gallic acid, caffeic acid, and catechin were evaluated as 0.15625, 0.15625, 2.5, 5.0 mg/mL for *S. aureus*; 2.5, 5.0, 2.5,2.5 mg/mL for *E. coli*; 0.625, 0.3125, 1.1250, 2.5 mg/mL for *P. mirabilis*; 0.15625, 5.0, 2.5, 5.0mg/mL for *S. pyogenes* respectively. Trolox show bactericidal effect on *S. aureus*, *P. mirabilis*, *S. pyogenes* with the 10.0, 5.0, 5.0 mg/mL (MBC) respectively but it did not show any bacteriostatic or bactericidal effect on *E. coli*. Rutin shows bactericidal effect only on *S. aureus* with the 1.5 mg/mL (MBC). And lastly Quercetin did not show bactericidal effect on *S. aureus* which is only bacteria quercetin shows bacteriostatic effect on with 0.5 mg/mL (MIC).

According to these result shown in figure 3.18. Phenolic compounds used in this study are most effective on the *S. aureus* and least effective on the *E. coli*.



		Tannic Acid (mg/mL)	Gallic Acid (mg/mL)	Caffeic Acid (mg/mL)	Catechin (mg/mL)	Rutin (mg/mL)	Trolox (mg/mL)
S. pyogenes		0.15625	1.25	2.5	5		5
P. mirabils		0.625	5	2.5	2.5		5
E. coli		5	10	5	10		
S. aureus		0.625	0.15625	2.5	5	1.5	10

Figure 3.18: Minimum bactericidal concentration of trolox, catechin, cafeic acid, gallic acid, tannic acid, rutin

3.5.5 The Agar Disc Diffusion Assay (Kirby-Bauer Test) for total extract and fractions.

Kirby-Bauer test was applied as previously described in section 2.2.7.3. 100µL of overnight cultures (corresponds to approximately $5x10^8$ Colony Forming Units (CFU)/mL bacteria) was spread onto the corresponding agar plates. Discs which were previously impregnated with the total extract (4.0 mg), hexane fraction (0.2 mg), diethyl ether fraction (0.2 mg), chloroform fraction (0.2 mg), ethyl acetate fraction (1.0 mg), and aqueous fraction (0.6 mg) were placed onto inoculated agars. After the 16 hours of incubation, inhibition zones were measured. As a positive control, the antibiotic discs were placed onto agar plates.

The inhibition zone was only observable for *S. aureus* and *S. pyogenes*. For *S. aureus* 7.7±0.5 mm inhibition zone was measured with the total extract, and 8.5±0.8 mm with ethyl acetate fraction. For *S. pyogenes* inhibition zone was only observed with ethyl acetate fraction as 8±0.05 mm. These results were illustrated in figure 3.19

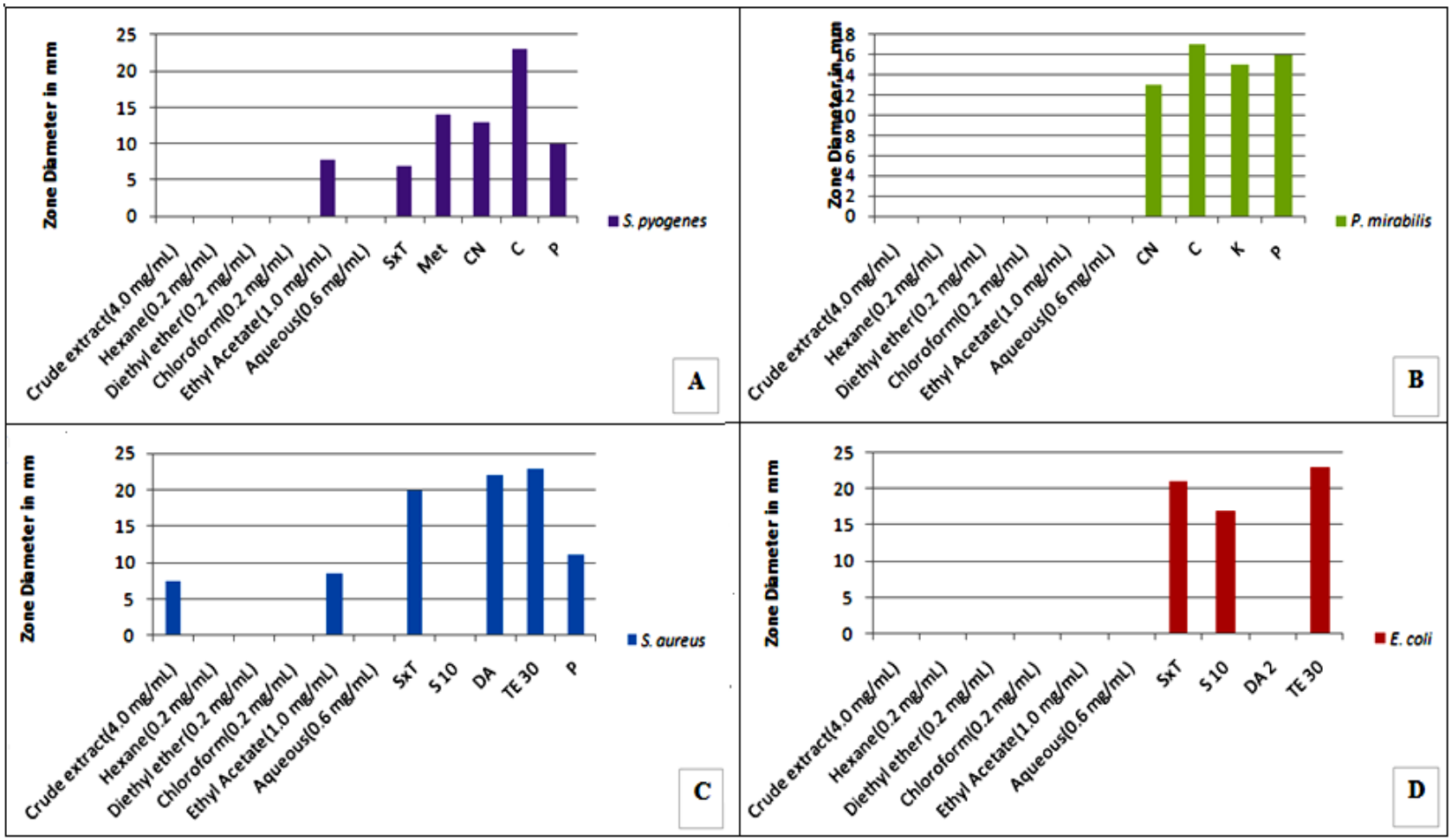


Figure 3.19: Evaluation of antimicrobial susceptibilities of crude extract , fraction and antibiotics with agar disc diffusion assay.inhibition zones(mm) include the 6mm disc diameter.A: *S. pyogenes*, B: *P. mirabilis*, C: *S. aureus* and D: *E.coli*
SXT: Trimethoprim, DA: Clindamycin, P: Penicilin, TE: Tetracyclin, K: Kanamycin, CN:Gentamicin

Stanisavljevic et al. studied antimicrobial effect of the *P. major* ethanol extract on the *S. aureus* ATCC 6538, and *E. coli* ATCC 25922 with the agar well diffusion assay by cutting a hole with 10mm diameter in inoculated agar plates (loaded cell= 10^6 CFU/mL) and filling it with 30µL total extract in methanol (20mg/mL). They calculated the inhibition zone as 10.7±0.3 and 11.7±0.4 mm for *S. aureus* and *E. coli*, respectively. (Stanisavljevic et al., 2008)

Lezama et al. performed disk diffusion assay for *P. major* leaves extracted in four different solvents, which were methanol, water, hexane, and chloroform extractions, against *E. coli* and *B. subtilis*. They used cell suspensions with 10^8 CFU/mL concentration as initial load and placed discs impregnated with 0.40, 0.20, 0.10, 0.05 and 0.025 mg/mL of the plant extracts collected by four different solvents. According to their results, water and methanol extracts of the *P. major* did not show any inhibitory effect on *E. coli*, on the other hand, hexane extract inhibited the growth of *E. coli* in a dose-dependent fashion as 34.0, 30.0, 24.0, 18.0, 13.0 mm zone diameter for 0.40, 0.20, 0.10, 0.05 and 0.025 mg/mL, respectively; then again, it was 12.0, and 9.0 mm inhibition zones for for 0.40 and 0.20 g/mL plant extract, respectively. Contrary to *E. coli*; water and methanol extract of the *P. major* showed an inhibitory effect on *B. subtilis*. Consequently, they interpreted these results as 'finding may be related with the polarity of the compounds in the extract and their capability to penetrate cell walls with hydrophilic environments (Gram-positive bacteria) and hydrophobic or lipophilic environments (Gram-negative bacteria), respectively' (Lezama et al., 2005).

3.5.6 The Agar Disc Diffusion Assay (Kirby-Bauer Test) for standard phenolics

Kirby-Bauer test was applied as previously described in section 2.2.7.3. Briefly, 100µL of overnight cultures, (approximately $5x10^8$ CFU/mL bacteria) were spread onto the corresponding agar plates. Discs, which were previously impregnated with the tannic acid (2.0 mg), gallic acid (2.0 mg), catechin (2.0 mg), trolox (2.0 mg), caffeic acid (1.0 mg), rutin (0.6 mg), and quercetin (0.1 mg), were placed onto inoculated agar. After 16 hours of incubation at 37^{o}C, the diameter of inhibition zones were measured.

Among the investigated phenolic compounds, tannic acid resulted in the highest inhibition zone with a diameter of 13.5±0.35 mm for *S. aureus*, 12.9±0.22 mm for *P. mirabilis*, and a diameter of 12.0±0.5 mm for *S. pyogenes*. Second highest vales were obtained from gallic acid with diameter values of 10.7±0.8, 8.5±0.4, 10.25±0.6 mm for *S.aureus, P. mirabilis, S. pyogen*, respectively. Trolox and catechin standards were only effective on *S. pyogenes* with zone diameter values of 9.3±0.4 and 8.8±0.25 mm respectively. Results were illustrated in figure 3.20.

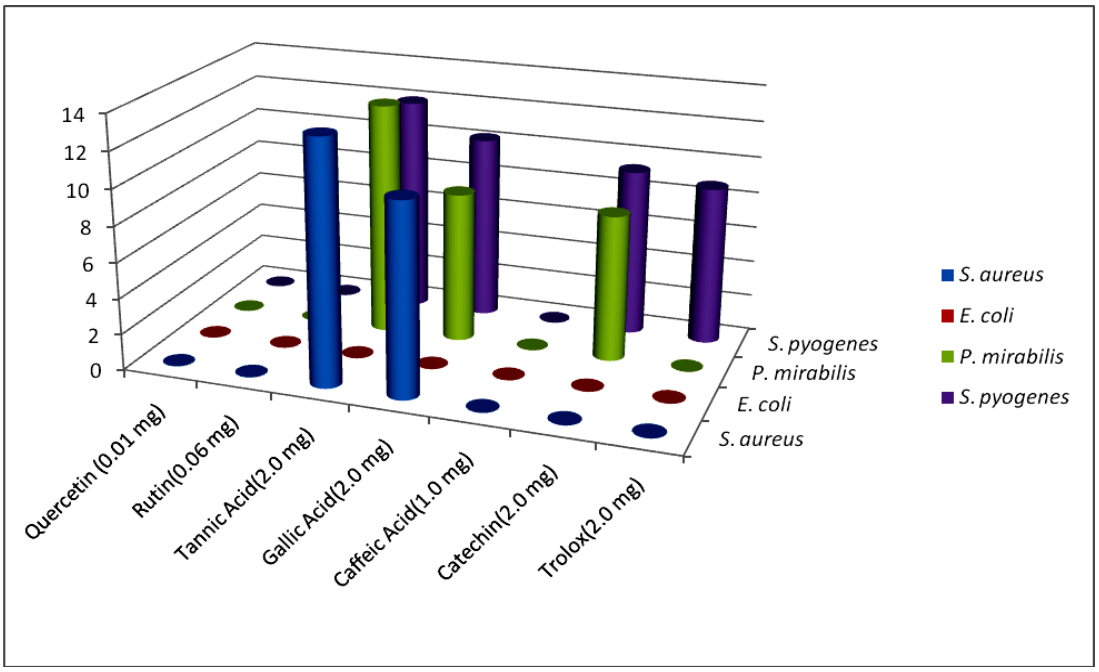


Figure 3.20: Evaluation of antimicrobial susceptibilities of phenolic compound with agar disc diffusion assay. Inhibition zones(mm) include the 6mm disc diameter.

In the study of Rauha et al. some phenol compounds including quercetin, catechin, caffeic acid, gallic acid, and rutin were tested for their antimicrobial effect on some bacterial strains inculding *S. aureus*DSM 20231, *E. coli* ATCC 8739, and E. *coli* ATCC 11775 with the hole-plate diffusion method, 500 mg of each sample added to the holes. Among them only quercetin shows moderate antimicrobial effect on *S.aureus*. Others did not show any inhibitory effect (Rauha et al., 2000).

All antimicrobial effectiveness tests are summarized in table 3.6

Table 3.6 Comparison of MIC, MBC, Disc Diffusion method for crude extract, fractions and phenolic compounds.

	S. aureus			E. coli			P. mirabilis			S. pyogenes		
	MIC (mg/mL)	MBC (mg/mL)	DD (mm)	MIC (mg/mL)	MBC (mg/mL)	DD (mm)	MIC (mg/mL)	MBC (mg/mL)	DD (mm)	MIC (mg/mL)	MBC (mg/mL)	DD (mm)
Crude extract	5.0	5.0	7.5±0.5	—	—	—	5.0	5.0	—	10.0	10.0	—
Hexane	—	—	—	—	—	—	—	—	—	—	—	—
Diethyl ether	—	—	—	—	—	—	—	—	—	—	—	—
Chloroform	—	—	—	—	—	—	—	—	—	1.0	—	—
Ethyl Acetate	1.250	1.25	8.5±0.86	5.0	5.0	—	1.25	1.25	—	2.5	2.5	7.75±0.5
Aqueous	3	3	—	—	—	—	3	3	—	—	—	—
Tannic acid	0.15625	0.625	13.5±0.35	2.5	5.0	—	0.625	0.625	12.9±0.22	0.15625	0.15625	12.0±0.5
Gallic acid	0.15625	0.15625	10.7±0.83	5.0	10.0	—	0.3125	5.0	8.37±0.4	5.0	5.0	10±0.5
Caffeic acid	2.5	2.5	—	2.5	5.0	—	1.250	2.5	—	2.5	2.5	—
Catechin	5.0	5.0	—	5.0	10	—	2.5	2.5	8.15±0.15	5.0	5.0	7.5±0.4
Trolox	2.5	10	—	—	—	—	1.250	5.0	—	2.5	5.0	8.8±0.2
Rutin	1.5	1.5	—	—	—	—	1.5	1.5	—	—	—	—
Quercetin	0.5	—	—	—	—	—	—	—	—	—	—	—

3.6 ANALYTICAL HIGH PERFORMANCE LIQUID CHROMATOGRAPHY (HPLC) ANALYSES

Ethyl acetate fraction, which has the highest antioxidant capacity and antimicrobial activity, was further analyzed with HPLC-DAD in order to identify the bioactive compounds present. Separation of the compounds was optimized by a gradient system, consisting of two different mobile phases, as previously described in section 2.2.3. Three major peaks were obtained from HPLC analysis of this fraction with the maximum absorbance at 330nm. Their retention times were 48.08, 50.1, and 51.9 min (figure 3.21). However, interpretation of these peaks was not clear because of poor fitting with existing standards which are previously giving in the master thesis study of Nebigil (2006) from our laboratory.

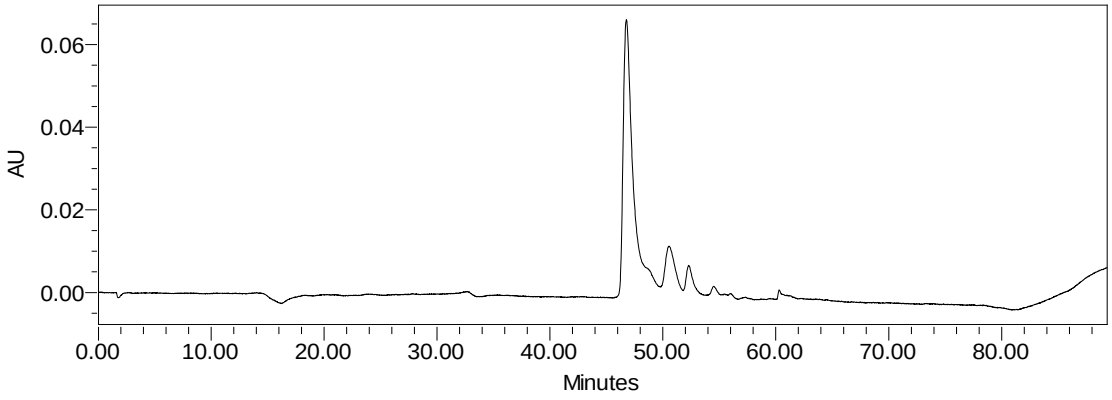


Figure 3.21: HPLC chromatogram of ethyl acetate fraction

Thus, in order to characterize the phenolic compounds, ethyl acetate phase was further analyzed with high resolution mass spectroscopy and when we compare the MS results with the literature, results are confirmed that the flavones of luteolin 7-glucoside, and hispidulin 7-glucuronide were present in the ethyl acetate fraction.

In the literature Kawashty et al., found that hispidulin 7-glucuronide, and luteolin 7-glucoside in the *P. major* as a major component (Kawashty, 1994).

Shimoi and co workers investigated the intestinal absorption of luteolin and luteolin 7-O-glucoside in rats by HPLC. The absorption analysis using rat everted small intestine demonstrated that luteolin was converted to glucuronides during passing through the intestinal mucosa and that luteolin 7-O-glucoside was absorbed after hydrolysis to luteolin (Shimoi et al., 1998).

CHAPTER 4

CONCLUSION

There is abundance of literal evidences saying that *Plantago major* leaves are effective on treatment of many diseases such as wound healing, ulcer or cancer. Hereby, it was aimed to determine the phytochemicals responsible for that pronounced antioxidant and antimicrobial effect when the plant was used as folk medicine. Based on the knowledge of having diverse solubility of phytochemicals in different solvents with different polarities, in this thesis study, we have started out to investigate the extraction quality and fractionation capacity of *P. major* leaves by using the classical methanol extraction method which was followed by fractioning with solvents in increasing polarity, in order of hexane, diethyl ether, chloroform, ethyl acetate. Final aqueous phase was also run to conclude on possible phytochemicals present.

Among the entire investigated samples, ethyl acetate fraction was found as the most effective in both DPPH and ABTS radical scavenging capacity measurements, indicating that the concentrated form of existing phenolics were generally dissolved in this mid-polarity solvent, after collected with highly polar methanol. As supporting evidence, total phenolic content and the total flavonoid content were the highest in ethyl acetate fraction; conversely, other solvents of hexane, diethyl ether, and chloroform fractions did not show neither radical scavenging activity nor total phenolic and flavonoid contents.

According to the minimum inhibitory concentration (MIC) results, only crude extract, ethyl acetate fraction and aqueous phase showed a weak antimicrobial effect on the bacteria used. Nonetheless, ethyl acetate fraction most effectively inhibited the

growth of the selected bacterial populations same as in the case of antioxidant capacity determination. Similarly, hexane, diethyl ether and chloroform fractions did not show any growth inhibition.

Minimum bactericidal concentration (MBC) findings showed a hundred percent compatibility with the MIC results which means that what is bacteriostatic in crude extract, aqueous phase and ethyl acetate fraction samples were also bactericidal (MBC/MIC =1). Another probative evidence came from disc diffusion test, which resulted in zone of inhibition on pre-inoculated agar plates with selected bacteria when crude extract and ethyl acetate fraction samples, but not aqueous phase samples, were impregnated onto discs. This test is something related with the maximum solvable amount of bio-active compounds in solvents, and results can be explained with poor solubility of agar in aqueous phase. Other than that, zone of inhibition was only observable when agar plates were inoculated with the two of gram positive bacteria which were *S. aureus* and *S. pyogenes*, not with that of gram negative ones. This may also be an outcome of insufficient dispersion of bio-active material onto solid media to kill gram negative bacteria.

As a final step, it was tried to identify and quantify the existing phenolic compounds in ethyl acetate phase, thus HPLC analysis was conducted, however, poor separation of observed peaks lead us to perform high resolution mass spectroscopy to characterize these peaks. MS results confirmed that the flavones of Luteolin 7-glucoside and hispidulin 7-glucuronide were present in the ethyl acetate fraction.

Depending on these findings, we concluded that the crude extract and ethyl acetate fraction of *P. major* leaves owe the antioxidant capacity to high concentration of listed flavonoid compounds, even they have a weak antimicrobial activity on bacterial populations studied.

REFERENCES

Abdalla A. E., Roozen J. P. (1999). Effect of plant extracts on the oxidative stability of sunflower oil and emulsion. *Food Chemistry,* 64, 323-329

Aguri T. T., Anaka T. T., Kouno I. (2006). Antibacterial spectrum of plant polyphenols and extracts depending upon hydroxyphenyl structure, *Forestry,* 29, 2226-2235

Andrews J. M. (2001). Determination of minimum inhibitory concentrations. *Journal of Antimicrobial Chemotherapy,* 48, 5-16

Apak R. , Güçlü K., Demirata B., Özyürek M., Çelik S. E., Bektaşoğlu B., Berker K. I., Özyurt D. (2007). Comparative evaluation of various total antioxidant capacity assays applied to phenolic compounds with the CUPRAC assay. *Molecules,* 12, 1496-1547

Atta A.H., El-Sooud K.A. (2004) The antinociceptive effect of some Egyptian medicinal plant extracts. *Journal of Ethnopharmacology,* 95: 235–238.

Aziz R. A., Sarmidi M. R., Kumaresan S., Taher Z. M., Yee F. C. (2003). Phytochemical processing: the next emerging field in chemical engineering aspects and opportunities. *Jurnal Kkejuruteraan Kimia Malaysia,* 3, 45–60

Balandrin M. F., Kinghorn A. D., Farnsworth N. R. (1993). Plant-derived natural products in drug discovery and development. *American Chemical Society*, 2-10

Bandow J. E., Brötz H., Leichert L. I. O., Labischinski H., Hecker M. (2003). Proteomic approach to understanding antibiotic action. *Antimicrobial Agents and Chemotherapy,* 47, 948-955

Barber M. S., McConnell V. S., DeCaux B. S. (2000). Antimicrobial intermediates of the general phenylpropanoid lignin specific pathways. *Phytochemistry,* 54, 53-56

Barut B. (2011) Antioxidant capacities of selected fruits and herbal teas consumed in regular diet and their antimicrobial activities against Staphylococcus aureus, M.Sc Thesis. Middle East Technical University., Turkey.

Beara I. N., Lesjak M. M., Jovin D., Balog K. J., Anackov G. T., Orcic D. Z., Mimica-Dukic N. M. (2009). Plantain (plantago L.) species as novel sources of flavonoid antioxidants. *Journal of Agricultural and Food Chemistry,* 57, 9268-9273

Binutu O. A., Cordell G. A. (2000). Gallic acid derivatives from mezoneuron benthamianum. *Pharmaceutical Biology*, 38, 284-286

Blois, M.S., (1958). Antioxidant determinations by use of a stable free radical. *Nature*, 181, 1199-1200

Cakilcioglu U., Khatun S., Turkoglu I., Hayta S. (2011). Ethnopharmacological survey of medicinal plants in Maden (Elazig-Turkey). *Journal of Ethnopharmacology*, 1-18

Chang CC., Yang MH., Wen HM., Chern JC. (2002). Estimation of total flavonoid content in propolis by two complementary colorimetric methods. *Journal of Food and Drug Analysis,* 10, 178-182

Ciocan I. D., Bara I. I. (2007). Plant products as antimicrobial agents. *Secţiunea Genetică şi Biologie Moleculară,* 151-156

Çoruh N., Celep A. G. S., Özgökçe F. (2007). Antioxidant properties of prangos ferulacea (L.) Lindl., chaerophyllum macropodum boiss. and heracleum persicum desf. from apiaceae family used as food in Eastern Anatolia and their inhibitory effects on glutathione-S-transferase. *Food Chemistryt*, 100, 1237-1242

Cowan M. M. (1999). Plant products as antimicrobial agents. *Clinical Microbiology reviews,* 12, 564-582

Croft K. D. (1998).The Chemistry and biological effects of flavonoids and phenolic acidsAnn. *N. Y. Acad. Sci,* 854, 435-442

Cushnie T. P. T., Lamb A. J. (2005). Antimicrobial activity of flavonoids. *International Journal of Antimicrobial Agents,* 26, 343-356

Dewanto V., Wu X., Adom K. K., Liu R. H. (2002). Thermal processing enhances the nutritional value of tomatoes by increasing total antioxidant activity. *Journal of Agricultural and Food Chemistry,* 50, 3010-3014

Duffy CF, Power RF. (2001). Antioxidant and antimicrobial properties of some Chinese plants extracts*; Int J of Antimicrob Agents*, 17, 527-529

Ezer N., Arisan Ö. M. (2006). Folk medicines in Merzifon (Amasya, Turkey). *Tübitak,* 30, 223-230

Favier A.E., Cadet J., Kalyanaraman B., Fontecave M., Pierre J. L. (1995) Analysis of free radicals in biological systems , 1st Edition.; Birkhäuser Verlag, Basel-Boston Berlin,42

Flores R.G., Calderon C.L., Scheibel L.W., Guerra P.T., Padilla C.R., Guerra R.T., Weber R.J. (2000) Immunoenhancing properties of Plantago major leaf extract. *Phytotheropy Research,* 14: 617–622.

Forbes A. B., Sahm F. D., Weissfeld A. S. (2007). Diagnostic Microbiology, 12th Edition,*Bailey&Scott's*, Missouri,265-350)

Fraga C. G. (2010). Plant phenolics and human healt: Biochemistry, Nutrition, and Pharmacology, *John Wiley & Sons*, Inc., Canada, 2-10

French G. H. (2006). Bactericidal agents in the treatment of MRSA infections—the potential role of daptomycin, *Journal of Antimicrobial Chemotherapy*, 58, 1107–111

Gan RY., Kuang L., Xu XR., Zhang Y., Xia EQ., Song FL. (2010). Screening of natural antioxidants from traditional chinese medicinal plants associated with treatment of rheumatic disease. *Molecules,* 15, 5988-5997

Garewal S. H. (1997). Antioxidant and Disease Prevention,1st Edition;CRC Press, New York, 38

Gatto M. T., Falcocchio S., Grippa E., Mazzanti G., Battinelli L., Nicolosi G., Lambusta D., Saso L. (2001). Antimicrobial and anti-lipase activity of quercetin and its C2-C16 3-O-acyl-esters. *Bioorganic & Medicinal Chemistry*, 10, 269-272

Gerdan O. F., (2010) Antioxidant and antimicrobial activities of dietary plants against group A B-Haemolytic streptococci, M.Sc Thesis. Middle East Technical University., Turkey.

Genç G. E., Özhayat N. (2006). An ethnobotanical study in Çatalca (European part of Istanbul) II. *Turkish Journal Pharmaceutical Sciences,* 3 (2), 73-89

Grune T. (2005). Oxidant and Antioxidant Defense System; Springer, Berlin, 2nd Edition, 35

Harborne J. B., Williams C. A. (2000). Advances in flavonoid research since 1992. *Phytochemistry*, 55, 481-504

Henis Y., Tagari H., Volcani R. (1963). Effect of water extracts of carob pods, tannic acid, and their derivatives on the morphology and growth of microorganisms. *American Society for Microbiology,* 12 (3), 204-209

Holetz F. B., Pessini G. L., Sanches N. R., Cortez A. G., Nakamura C. V., Filho B. P. D. (2002). Screening of some plants used in the brazilian folk medicine for the treatment of infectious diseases. *Mem Inst Oswaldo Cruz,* 97, 1-5

Huang D., Ou B., Prior R. L. (2005). The Chemistry behind Antioxidant Capacity Assays. *Journal of Agricultural and Food Chemistry,* 53, 1841-1856

Kajiya K., Hojo H., Suzuki M., Nanjo F., Kumazawa S., Nakayama T. (2004). Relationship between antibacterial activity of (+)-catechin derivatives and their interaction with a model membrane. *Journal of Agricultural and Food Chemistry,* 52, 1514-1519

Kargıoğlu M., Cenkci S., Serteser A., Konuk M., Vural G. (2010).Traditional uses of wild plants in the middle aegean region of turkey. *Springer Science+Business Media,* 38, 429-450

Kawashty S.A., Gamal-El-Din E., Abdalla M.F., Saleh N.A.M. (1994) Flavonoids of Plantago species in Egypt. *Biochemical Systematic and Ecology* , 22, 729– 733.

Koçyiğit M., Özhayat N. (2006). Wild plants used as medicinal purpose in Yalova (Northwest Turkey). *Turkish Journal Pharmaceutical Sciences,* 3 (2), 91-103

Kumarasamy Y., Byres M., Cox P. J., Jaspars M., Nahar L., Sarker S. D. (2007). Screening seeds of some scottish plants for free radical scavenging activity. *Wiley InterScience*, 21, 615-621

Kültür Ş. (2007). Medicinal plants used in Kırklareli Province (Turkey). *Journal of Ethnopharmacology,* 111, 341-364

Kümbet Y. (2010). Investigation of antioxidant activities of fruit juices and herbal teas and their antimicrobial effects on proteus mirabilis**,** M.Sc Thesis. Middle East Technical University., Turkey.

Lezama R. V., Aguilar R. T., Ramos R. R., Avila E. V., Guti´errez Ma. S. P. (2006). Effect of Plantago major on cell proliferation in vitro. *Journal of Ethnopharmacology*, 103, 36-42

Littleton J., Rogers T., Falcone D. (2005). Novel approaches to plant drug discovery based on high throughput pharmacological screening and genetic manipulation. *Life Sciences*, 78, 467–475

Liu R. H. (2003). Health benefits of fruit and vegetables are from additive and synergistic combinations of phytochemicals. *American Journal Clinical Nutrition*, 78, 517s-520s

Liu R. H. (2004). Potential synergy of phytochemicals in cancer prevention: mechanism of action. *American Society for Nutritional Sciences*, 3479-3484

Mates J. M., Gomez C. P., Castro I. N. (1999). Antioxidant enzymes and human diseases. *Clinical Biochemistry*, 32 (8), 595–603

Molyneux P. (2004). The use of the stable free radical diphenylpicrylhydrazyl (DPPH) for estimating antioxidant activity, *J. Sci. Technol*, 26, 211-219.

Naczk M., Shahidi F. (2004). Extraction and analysis of phenolics in food. *Journal of Chromatography A*, 1054, 95–111

Nathan C.F., Hibbs J.B.Jr. (1991) Role of nitric oxide synthesis in macrophage antimicrobial activity. *Curr op Immunol* 3: 665–670.

Nebigil C. (2011) Characterization of antioxidant and antimicrobial isolates from *Quercus brantii L.* extract, M.Sc Thesis. Middle East Technical University., Turkey.

Özgen U., Kaya Y., Coşkun M. (2004). Ethnobotancal studies in the villages of the district of Ilıca (province Erzurum), Turkey. *Economic Botany*, 58 (4), 691-696

Öztürk F., Ölçücü C. (2011). Ethnobotanical features of some plants in the district of Şemdinli (Hakkari-Turkey). *International Journal of Academic Reserch*, 3 (1), 117-121

Pala F. S., Tabakçıoğlu K. (2007). Free radicals: Our enemies or friends?. *Advances in Molecular Biology*, 1, 63-69

Pannala A. S., Chan T. S., O'Brien P. J., Rice-Evans C. A. (2001). Flavonoid B-Ring Chemistry and Antioxidant Activity: Fast Reaction Kinetics. *Biochemical and Biophysical Research Communications,* 282, 1161-1168

Pourmorad F., Hosseinimehr S. J., Shahabimajd N. (2006).Antioxidant activity, phenol and flavonoid contents of some selected Iranian medicinal plants. *African Journal of Biotechnology*, 5 (11), 1142-1145

Rauha J., Remes S., Heinonen M., Hopia A., Kahkonen M., Kujala T., Pihlaja K., Vuorela H., Vuorela P. (2000). Antimicrobial effects of Finnish plant extracts containing flavonoids and other phenolic compounds. *International Journal of Food Microbiology*, 56, 3–12

Ravn H., Brimer L. (1988). Structure and antibacterial activity of plantamajoside, a caffeic acid sugar ester from plantago major subsp. major. *Phytochemtstry*, 27 (11), 3433-3437

Re R., Pellegrini N., proteggente A., Yang M, Rice- Evans C. (1998). Antioxidant activity appliying an improved ABTS radical cation decolorization assay. *Free Radical Biology &Medicine*, 26:1231-1237

Richer A. C., Milbury P. E. (2008) Understanding the Antioxidant Controversery, 1st Edition; Praeger,Westport Conneticut London,38

Prior, L. P., Wu X.., Schaich K.. (2005). Standardized methods for the determination of antioxidant capacity and phenolic in foods and dietary supplements, *Agricultural Food Chemistry*, 76,245-256

Sagar G. R., Harper J. L. (1964). Biological flora of the british isles: plantago major L., P. media L. and P. lanceolata L.. Journal of Ecology,52(1),189-221

Samuelson A. B. (2000). The traditional uses, chemical constituents and biological activities of Plantago major L. A review, *Journal of Ethnopharmacology,* 71, 1-21

Sarper F., Akaydin G., Şimşek I., Yeşilada E. (2009). An ethnobotanical field survey in the haymana district of Ankara province in Turkey. *Tübitak,* 33, 79-88

Schwalbe R., Moore L., Goodwin A. C. (2007) Antimicrobial Susceptibility Testing Protocols, Taylor&Francis Group., New York, 79

Schwarz K., Bertelsen G., Nissen L. R., Gardner P. T., Heinonen M. I., Hopia A., Huynh-Ba T., Lambelet P., McPhail D., Skibsted L. H., Tijburg L. (2001). Investigation of plant extracts for the protection of processed foods against lipid oxidation. Comparison of antioxidant assays based on radical scavenging, lipid oxidation and analysis of the principal antioxidant compounds. *European Food Research and Technology,* 212, 319-328

Serag, M.M.S., Amina Z. A., Mahitab M.A. (2010) Ecological study on *Plantago major* l. ın the different habitats of Damietta, *J. Plant Production, Mansoura University,* 1 (7): 1007-1031

Sen S., Chakraborty R., Sridhar C., Reddy Y. S. R., De B. (2010). Free radicals, antioxidants, diseases and phytomedicines: current status and future prospect. *International Journal of Pharmaceutical Sciences Review and Research,* 3, 91-97

Sezik E., Yeşilada E., Tabata M., Honda G., Takaishi Y., Fujita T., Tanaka T., Takeda Y. (1997). Traditional medicine in Turkey VIII. folk medicine in east Anatolia; Erzurum, Erzincan, Ağrı, Kars, Iğdır provinces. *Economic Botany,* 51 (3), 195-211

Shimoi K., Okada H., Furugori M., Goda T., Takase S., Suzuki M., Hara Y., Yamamoto H., Kinae N. (1998) Intestinal absorption of luteolin and luteolin 7-O-b-glucoside in rats and humans. *FEBS Lett*. 438, 220–224.

Sharifa A.A., Neoh Y. L., Iswadi M. I., Khairul O., Abdul Halim M., Jamaludin M., Mohamed Azman A.B., Hing H. L. (2008). Effects of methanol, ethanol and aqueous extract of plantago major on gram positive bacteria, gram negative bacteria and yeast. *Annals of Microscopy,* 8, 42-4

Singelton P. (2004) Bacteria in Biology Biotechnology and Medicine, 6th Edition.; John Wiley &Songs, Ltd., USA, 470

Singleton, V. L., Rossi, J. A. (1965). Colorimetry of total phenolics with phosphomolybdic phosphotungstic acid reagents. *American Journal of Enology and Viticulture*, 16, 144-158

Speisky H., Rocco C., Carrasco C., Lissi E. A., Alarcon C. L. (2006). Antioxidant screening of medicinal herbal teas. *Phytotherapy Research,* 20, 462-467

Stanisavljevic I. T., Stojicevic S. S., Velickovic D. T., Lazic M. L., Veljkovic V. B. (2008). Screening the antioxidant and antimicrobial properties of the extracts from plantain (Plantago Major L.) leaves. *Separation Science and Technology,* 43, 3652–3662

Than A., Myint M.M.S., Myint W., Myint T., Hlaing S.S. (1996) The anti-ulcerogenic acticity of Plantago major L. *Myanmar Health sciences Research Journal*, 8: 74–77

Toksoy D., Bayramoglu M., Hacisalihoglu S. (2010). Usage and the economic potential of the medicinal plants in Eastern Black Sea Region of Turkey. *Journal of Environmental Biology*, 31 (5), 623-628

Tuzlacı E., Tolon E. (2000). Turkish folk medicinal plants, part III: Şile (Istanbul). *Fitoterapia*, 71, 673-685

Uppu R.M., Murthy S.N., Pryor W.A., Parinandi N.L. (2010). Free Radicals and Antioxidant protocols, 2nd Edition.; Humana Press, UK, 3-5

Valko M., Leibfritz D., Moncol J., Cronin M. T. D., Mazur M., Telser J. (2006). Free radicals and antioxidants in normal physiological functions and human disease. *International Journal of Biochemistry and Cell Biology*,45-78

Valko M., Rhodes C. J., Moncol J., Izakovic M., Mazur M. (2006). Free radicals, metals and antioxidants in oxidative stress-induced cancer. *Chemico-Biological Interactions*, 160, 1–40

Villano D., Pachon F. M. S., Toroncoso A. M, Parrilla M. C. G. (2004). The antioxidant activity of wines determined by the ABTS method: influence of sample dilution and time. *Talanta*, 64, 501-509

Yesilada E., Sezik E., Fujita T., Tanaka S., Tabata M. (1993) Screening of some Turkish medicinal plants for their antiulcerogenic activities. *Phytotherapy Research*, 7: 263–265.

Yokozawa T., Dong E., Liu Z.W., Shimizu M. (1997) Antioxidant activity of flavonols In vitro. *Phytotherapy Research*, 11: 446–449.

Wiegand I., Hilpert K., Hancock R.E.W. (2008). Agar and broth dilution methods to determine the minimal inhibitory concentration (MIC) of antimicrobial substances; *Nature Protocols*, 3, 163 - 175.

Xia EQ., Deng GF., Guo YJ., Li HB. (2010). Biological activities of polyphenols from grapes. *International Journal of Molecular Sciences*, 11, 622-646

ZhishenJ., Mengcheng T., Jianming W. (1999). The determination of flavonoid content in mulbery and their scavenging effects on superoxide radicals. *Food Chemistry*, 64, 555-559

Zubair M. (2010). Genetic and environmental effects on polyphenols in *Plantago major*, 1-30.

(n.d). TUBIVES Retrived 15.08.2011, from TUBIVES: http://turkherb.ibu.edu.tr/index.php?sayfa=1&tax_id=8193

APPENDIX A

Radical scavenging activities of crude extract and fractions with ABTS

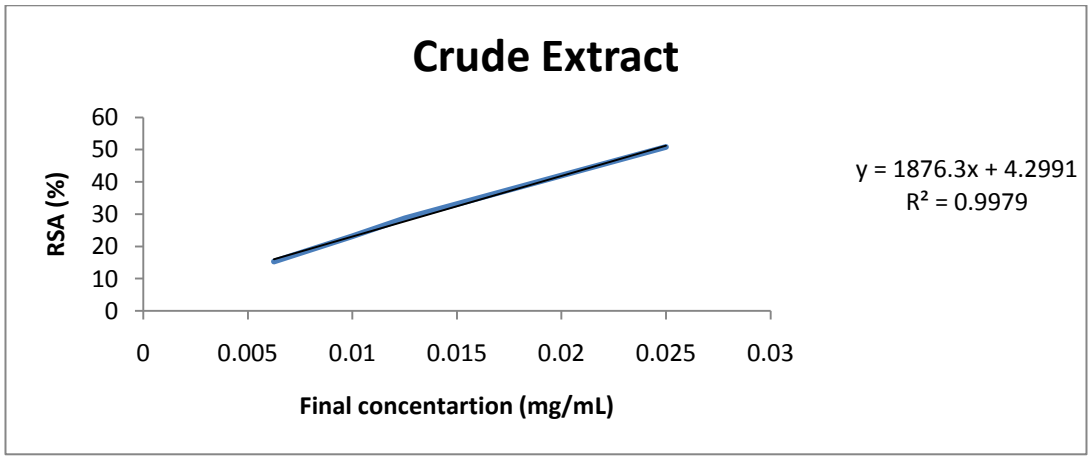


Figure A.1 Radical scavenging activities of crude extract with ABTS

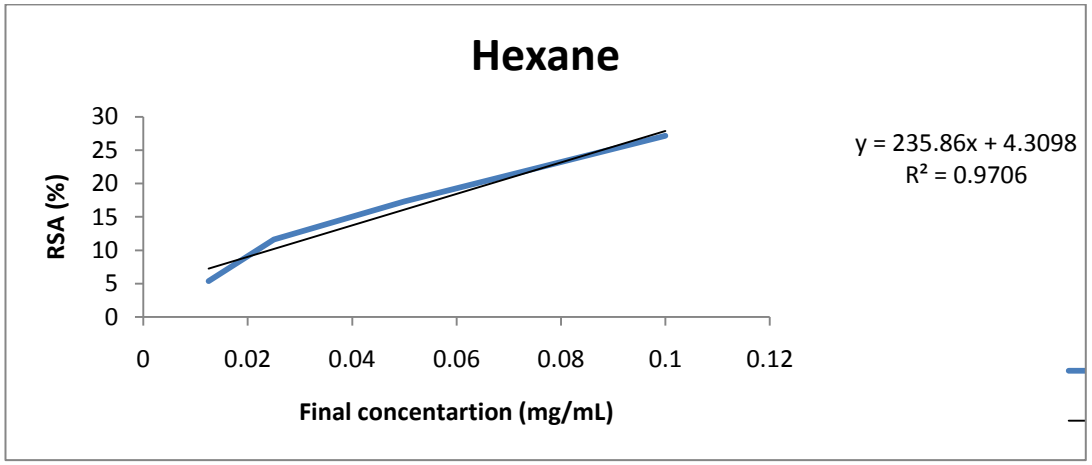


Figure A.2 Radical scavenging activities of hexane fraction with ABTS

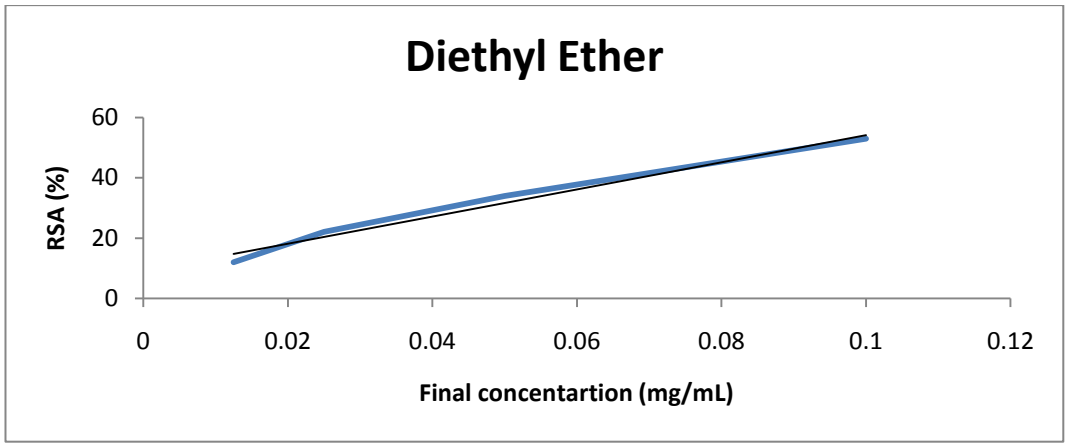


Figure A.3 Radical scavenging activities of diethyl ether fraction with ABTS

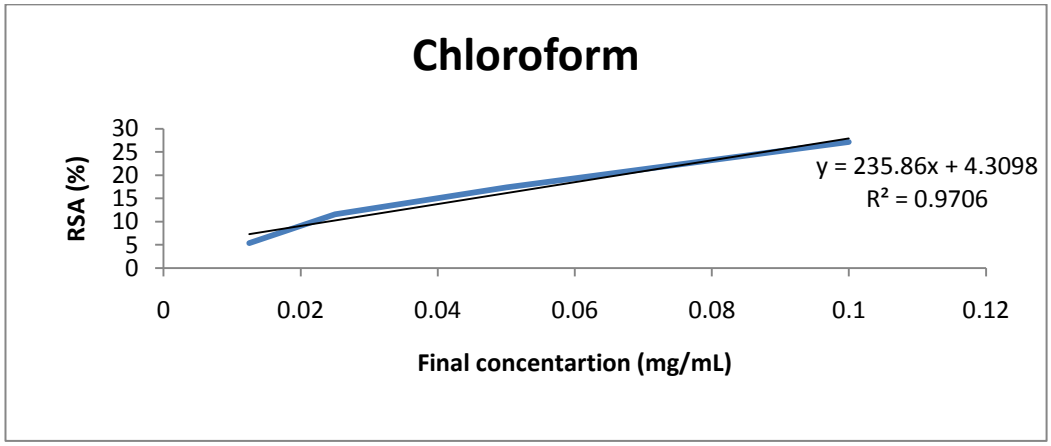


Figure A.4 Radical scavenging activities of chloroform fraction with ABTS

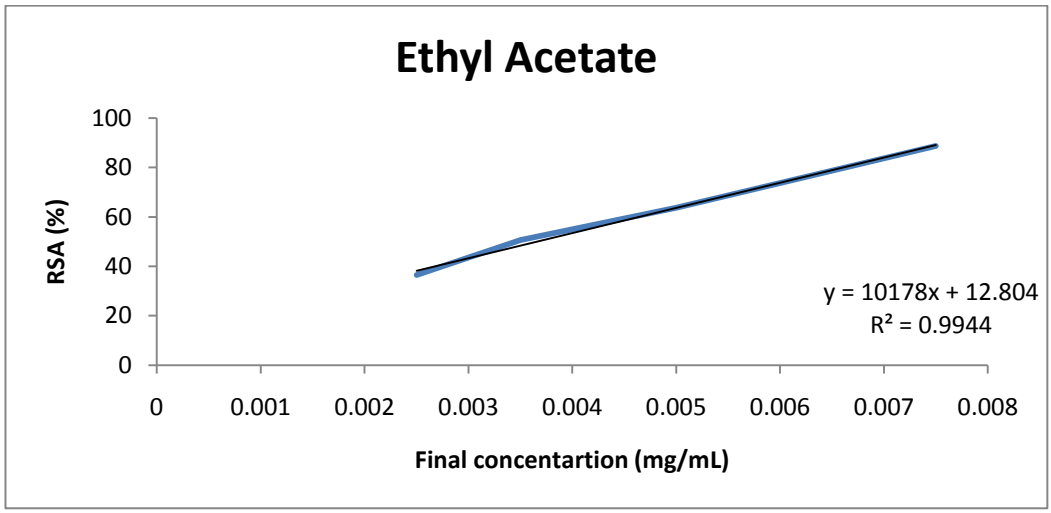


Figure A.5 Radical scavenging activities of ethyl acetate fraction with ABTS

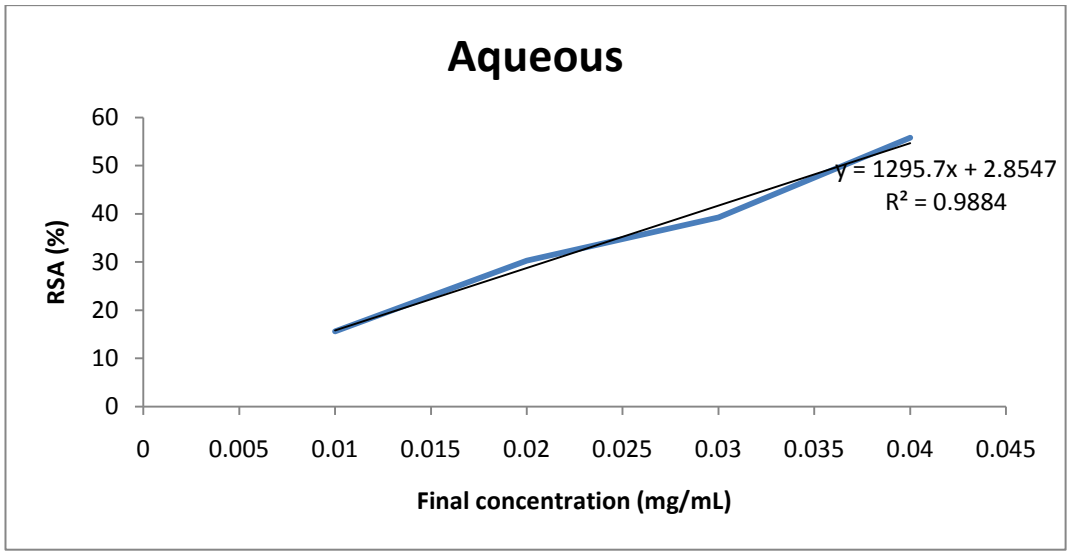


Figure A.6 Radical scavenging activities of aqueous phase with ABTS

APPENDIX B

RSA (%) versus final concentration graph of quercetin

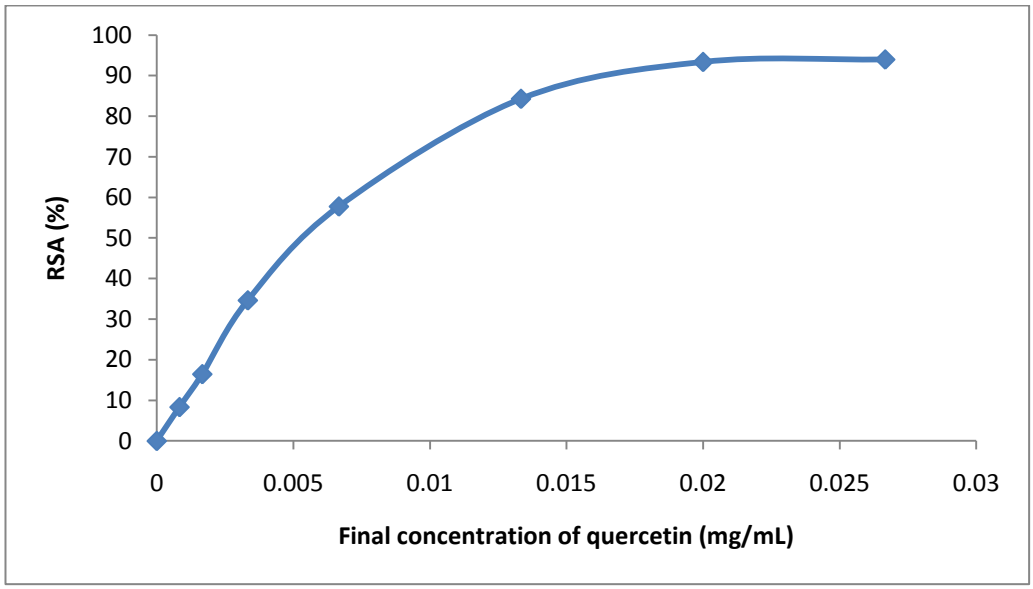


Figure B.1 RSA (%) versus final concentration graph of quercetin

APPENDIX C

UV- Scan for crude extract and fractions

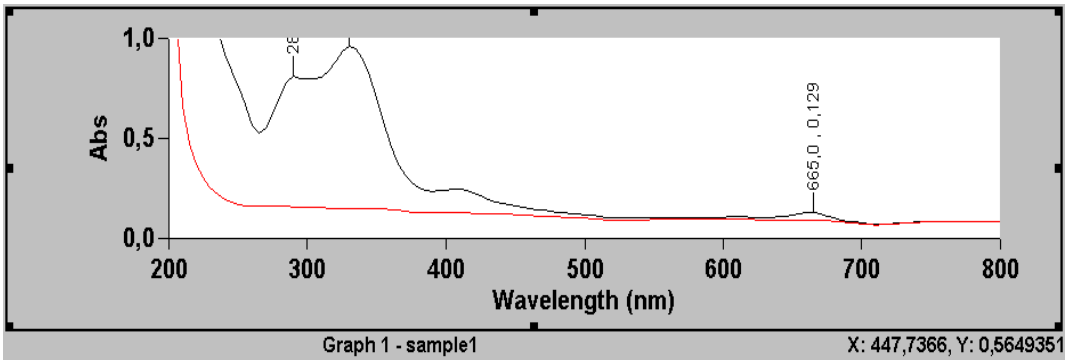


Figure C.1 UV-Scan of crude extract

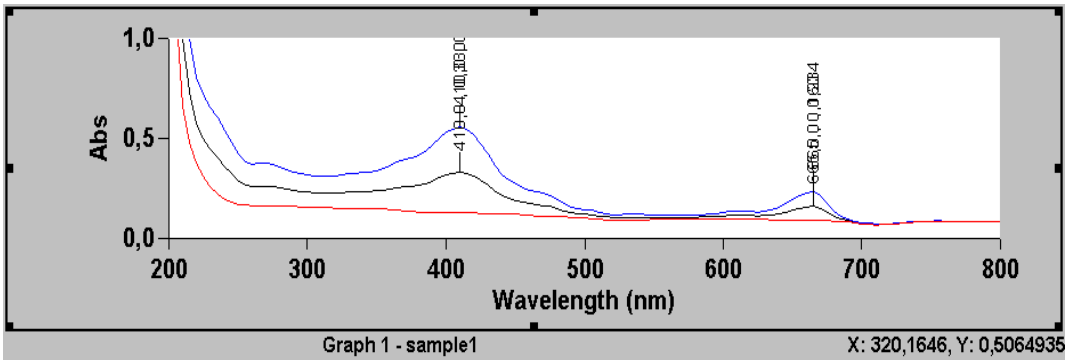


Figure C.2 UV-Scan of hexane fraction

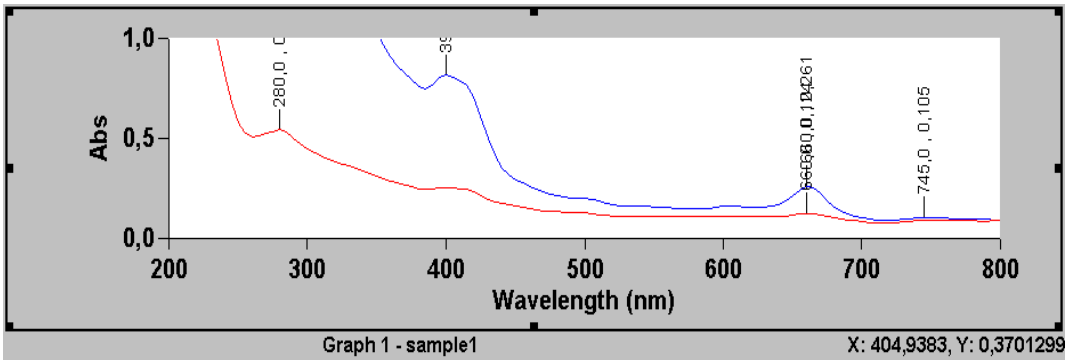


Figure C.3 UV-Scan of diethyl ether fraction

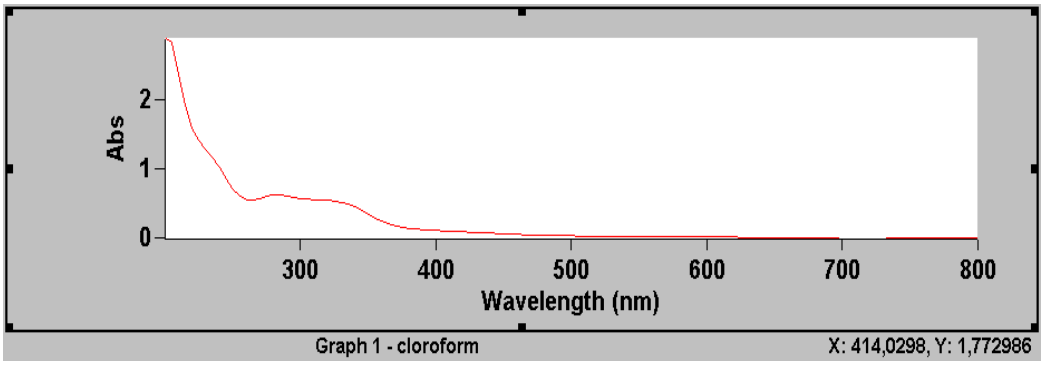


Figure C.4 UV-Scan of chloroform fraction

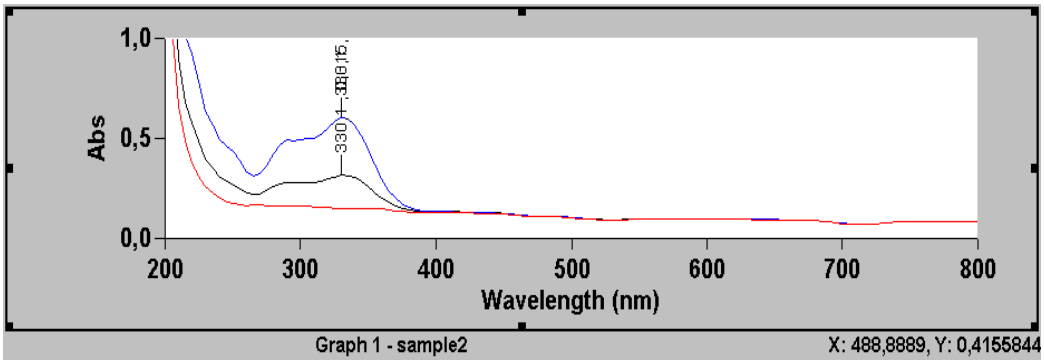


Figure C.5 UV-Scan of ethyl acetate fraction

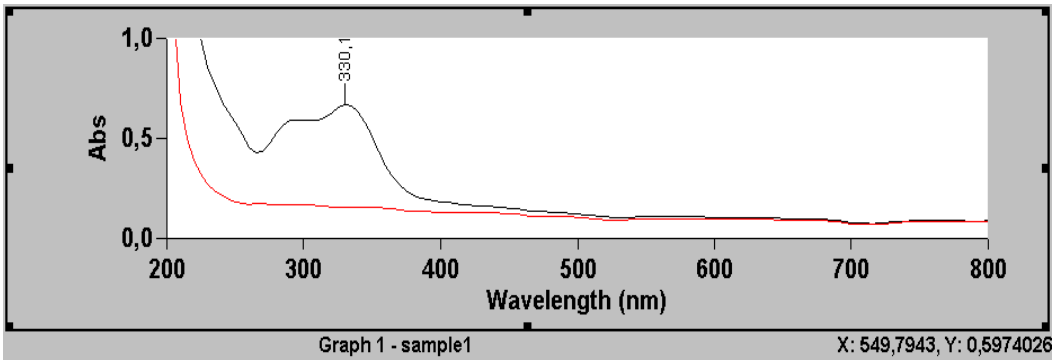


Figure C.6 UV-Scan of aqueous fraction

APPENDIX D

HRMS Spectra for hispidulin 7-glucuronide and luteolin 7-glucoside

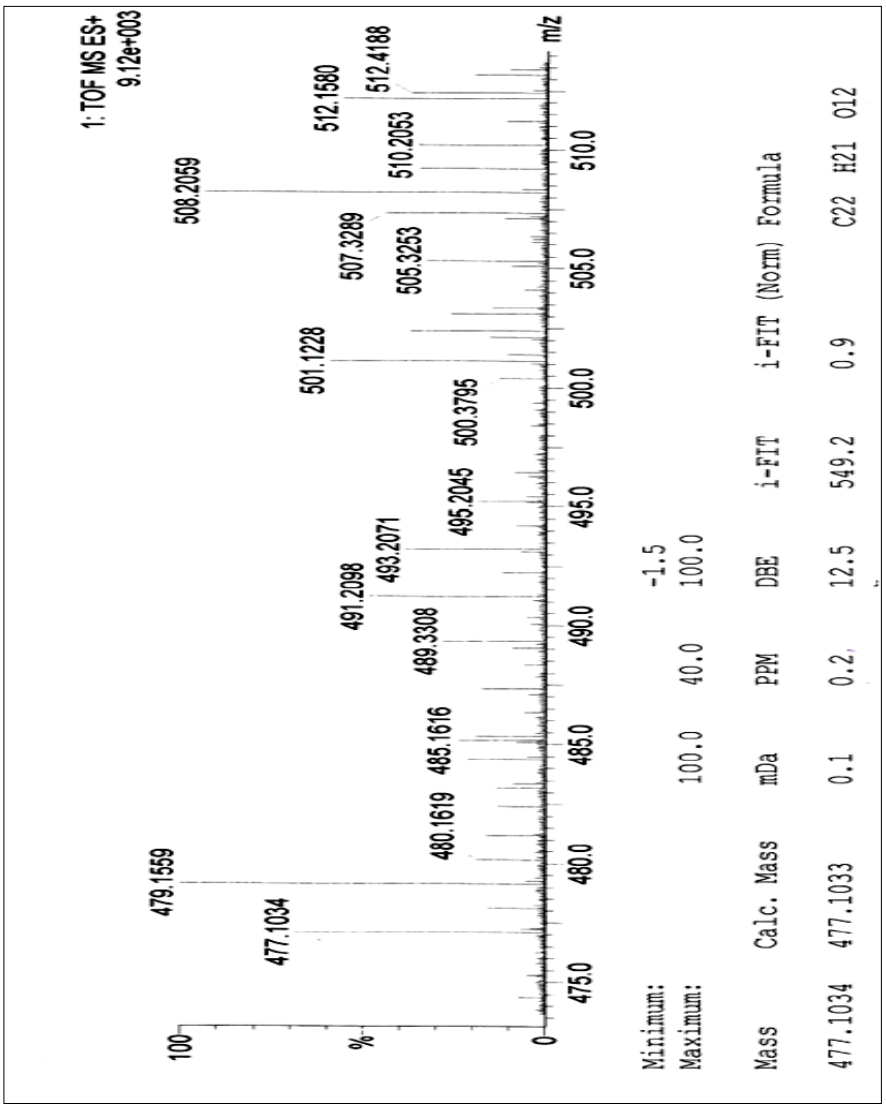
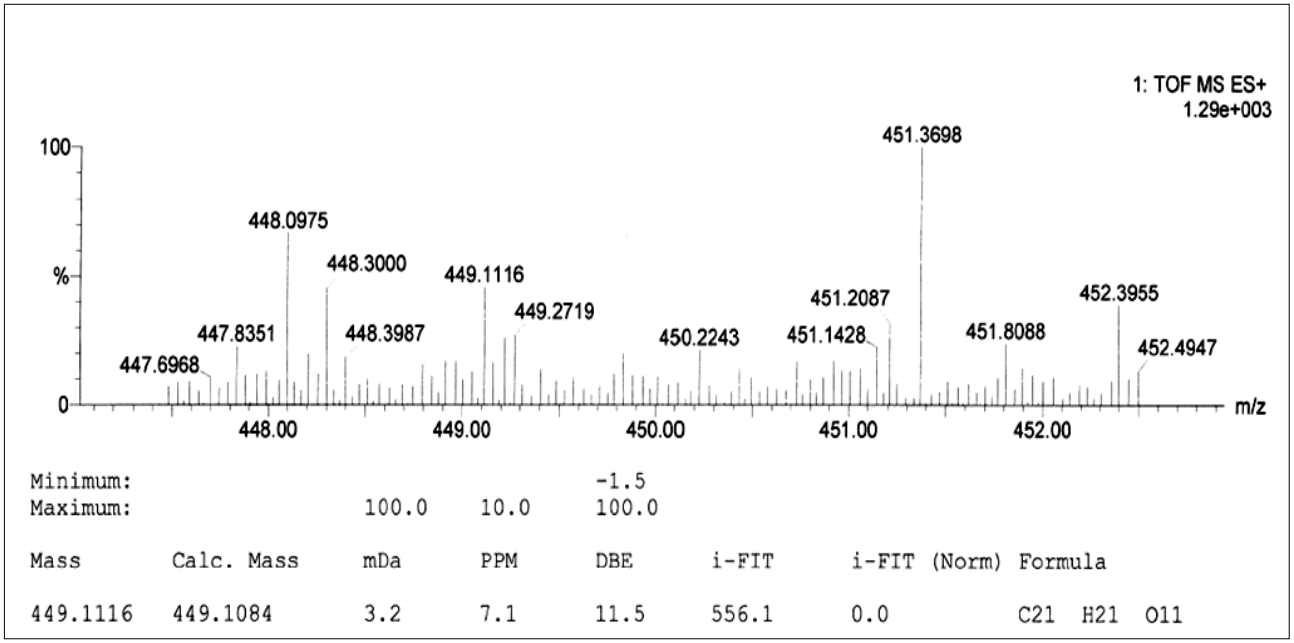


Figure D.1 Mass Spectrum for hispidulin 7-glucuronide



Minimum:				-1.5			
Maximum:		100.0	10.0	100.0			
Mass	Calc. Mass	mDa	PPM	DBE	i-FIT	i-FIT (Norm)	Formula
449.1116	449.1084	3.2	7.1	11.5	556.1	0.0	C21 H21 O11

Figure D.2 Mass Spectrum for Luteolin 7-glucoside