ANTIOXIDANT, ANTIMICROBIAL ACTIVITY AND PHYTOCHEMICAL
ANALYSIS OF *PISTACIA VERA L.*SKIN

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

BY

MUHAMMED ÖMER ŞERMET

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

JUNE 2015

Approval of the thesis:

ANTIOXIDANT, ANTIMICROBIAL ACTIVITY AND PHYTOCHEMICAL ANALYSIS OF *PISTACIA VERA L.*SKIN

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ABSTRACT

ANTIOXIDANT, ANTIMICROBIAL ACTIVITY AND PHYTOCHEMICAL ANALYSIS OF *PISTACIA VERA L* SKIN

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June 2015, 62 pages

Pistacia vera L. commonly grows in Mediterranean region, mainly in the Southeast of Turkey. Traditionally it is used for the treatment of several diseases such as asthma, rheumatism and hypertension. Pistachios are very rich sources of phenolic compounds however the skins are excluded from the nut as waste. This study was designed to investigate the antioxidant and antimicrobial effects of *P. vera* skin and to evaluate its phenolic compounds.

P. vera skin extracts were prepared in different solvents and methanol was found to be the most suitable solvent in terms of the phenolic yield. Antioxidant capacities were examined by 1,1-diphenyl-2-picryl-hydrazyl (DPPH)$^{\bullet}$ scavenging method. Total phenolic and flavonoid contents were also determined. Furthermore, antimicrobial activities of extracts were detected by using Kirby-Bauer disc diffusion method together with MIC and MBC methods.

EC_{50} of radical scavenging capacity was determined as 0.0375±0.006 mg/ml. Further, total phenolic content was determined as 145.32±7.14 GAE mg/g and

flavonoid content as 92.74±9.24 µg QE/mg extract.

The highest antimicrobial activity of *P. vera* skin extract was observed against *S. aureus* with 17.1 ± 0.7 mm zone of inhibition. The MIC values against examined bacteria were ranging from 0.5 to 2.0 mg/ml. MBC values have also been determined as minimum 1.0 mg/ml for *Bacillus subtilis* and maximum 2.5 mg/ml for *Serratia marcences*. The results of Chromatographic (LC-MS/MS) analyses indicated that *P.vera* skin is very rich in quercetin and gallic acid.

Methanol extract of *P. vera* skin can be regarded as a strong antioxidant agent relative to the standards considering total phenol and flavonoid contents in addition to DPPH radical scavenging activity and moderate to strong antimicrobial agent against different bacteria regarding to the zone of inhibition, MIC and MBC values.

Keywords: *P. vera*, Antioxidant, Antimicrobial, Free Radicals.

ÖZ

PISTACIA VERA L. KABUĞUNUN ANTİOKSİDAN, ANTİMİKROBİYAL AKTİVİTESİ VE FİTOKİMYASAL ANALİZİ

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Haziran 2015, 62 sayfa

Pistacia vera L. Akdeniz bölgesinde ve daha yoğun olarak Türkiye'nin Güneydoğu bölgesinde yetişmektedir. Geleneksel olarak astım, romatizma ve yüksek tansiyon gibi hastalıkların tedavisi için kullanılagelmiştir. Antep fıstığı biyoaktif fenolik maddelerce çok zengin bir bitkidir. Bununla beraber kabuk kısmı atık olarak görülüp üretim aşamasında uzaklaştırılmaktadır. Bu çalışmada *P. vera* bitkisinin kabuğunun antioksidan kapasitesinin belirlenmesi ve gram pozitif ve gram negatif bakteriler üzerindeki antimikrobiyal etkilerinin belirlenmesi ve fenolik bileşiklerin araştırılması planlanmıştır.

P. vera kabuğu özütleri değişik çözücülerde hazırlanmış ve metanol, fenolik verim sonuçlarına göre en uygun çözücü olarak belirlenmiştir. Toplam fenolik ve topla

Metanol özütlerinin antioksidan kapasiteleri radikal yakalama kapasitesini ölçme methodu olan 1,1-diphenyl-2-picryl-hydrazyl (DPPH)$^{\bullet}$ methodu ile belirlenmiştir. Toplam fenolik ve toplam flavonoid madde miktarları da hesaplanmıştır. Antimikrobiyal aktivite tayini için ise disk difüzyon yöntemiyle birlikte MIC ve MBC değerleri belirlenmiştir.

Radikal yakalama kapasitesi DPPH EC_{50} değeri 0.0375 ± 0.006 mg/ml olarak hesaplanmıştır. Aynı zamanda toplam fenolik madde miktarı 145.32 ± 7.14 mg gallik aside eşdeğer madde/g ve total flavonoid miktarı 92.74 ± 9.24 µg kuersetine eşdeğer madde/ mg olarak hesaplanmıştır. Kromatografik (LC-MS/MS) analizler, *P. vera*'nın quercetin ve gallik asit açısından oldukça zengin kaynkalar olduğunu göstermiştir.

P. vera kabuk özütünün en etkili antimikrobiyal aktivitesi 17.1 ± 0.7 mm inhibisyon çapıyla *S. aureus*'a karşı gözlenmiştir. İncelenen bakterilerin minimum inhibe edici konsantrasyon (MIC) değerleri 0.5 ile 2.0 mg/ml arasında değişmektedir. Minimum bakterisidal konsantrasyon (MBC) değerleri ise en az *Bacillus subtilis* için 1 mg/ml ve en fazla *Serratia marcences* için 2.5 mg/ml olarak belirlenmiştir.

Anahtar Kelimeler: *P.vera.*, Antioksidan, Antimikrobiyal, Serbest Radikal.

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LIST OF ABBREVIATIONS

mg   : Milligram

mL   : Milliliter

μL   : Microliter

mm   : Millimeter

nm   : Nanometer

DPPH   : 2,2-diphenyl-1-picrylhydrazyl

GAE   : Gallic acid equivalent

LC MS/MS   : Liquid chromatography-tandem mass spectrometry

QE   : Quercetin equivalent

MIC   : Minimum inhibitory concentration

MBC   : Minimum bactericidal concentration

a.u.   : Absorbance unit

RSA   : Radical scavenging activity

ROS   : Radical Oxygen Species

RNS   : Radical Nitrogen Species

SD   : Standard deviation

TUBIVES   : Turkish Plants Data Service

CHAPTER 1

INTRODUCTION

1.1 FREE RADICALS AND OXIDANTS

Oxygen is a component vital for maintenance of metabolism of living organisms. As a response of ATP (adenosine triphosphate) generation by the mitochondria due to oxygen consumption of cells to generate energy, free radicals are emerged. Mainly in the form of ROS (reactive oxygen species) and RNS (reactive nitrogen species) resulting from the cellular processes, free radicals show beneficial effects on immune system and cellular responses at moderate or low levels. However when produced at high concentrations, they can damage cell membranes and other structures which is a detrimental process known as oxidative stress (Valko et al, 2004). The term ''oxidant'' is also used in general to describe all the free radicals and other non-radical reactive elements known collectively as ROS and RNS. Compared to non-radical species, radicals are usually more reactive but less stable. In fact, free radicals are defined as a compound which has a one or more single electron in its outer orbit (Droge, 2002).

Free radicals are produced in several ways:

1) By the cleavage of a chemical bond resulting each molecule with one electron.

2) By redox reactions

3) Also by cleavage of a radical to give another one.

The most notable examples of free radicals are hydroxyl ($OH\bullet$), superoxide ($O_2\bullet^-$), peroxyl ($ROO\bullet$), nitric oxide ($NO\bullet$), nitrogen dioxide ($NO_2\bullet$), and lipid peroxyl ($LOO\bullet$) molecules. Other examples of not free radicals but oxidants which can lead

to free radical reactions in organisms are hydrogen peroxide (H_2O_2), ozone (O_3), hypochlorous acid (HOCl), nitrous acid (HNO_2), singlet oxygen ($^1O_2$), peroxynitrite ($ONOO^-$) and lipid peroxide (LOOH) (Genestra, 2007). Biologically active free radicals are highly unstable molecules which have the capacity to react with several substrates such as proteins, lipids, polysaccharides and DNA.

1.1.1 Production of Free Radicals in the Human Body

Normal metabolic reactions in the human body or from outside sources such as exposure to ozone, X-rays, smoking, air pollutants, and industrial chemicals are mainly responsible for free radical and other ROS production (Valko et al, 2004). Free radical formation is observed continuously in the body as a result of both enzymatic and non-enzymatic reactions. Serving as free radicals source, enzymatic reactions include the ones in phagocytosis, in the respiratory chain, in prostaglandin synthesis and in the cytochrome P-450 system (Liu et al, 1999). Free radicals can be produced in reactions of oxygen in a nonenzymatic way, as well.
Internal origins of free radicals include (Ebadi, 2001)

- Peroxisomes
- Mitochondria
- Inflammation
- Xanthine oxidase
- Phagocytosis
- Arachidonate pathways
- Ischemia/reperfusion injury

Externally generated sources of free radicals include:

- Environmental pollutants
- Certain drugs, pesticides
- Industrial solvents
- Radiation

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- Smoking
- Ozone

1.1.2 Deleterious Activities of Free Radicals and Oxidants

Oxidants and free radicals generate oxidative stress which is a deleterious process that can seriously disrupt the cell membranes and other biological molecules such as lipids, proteins, lipoproteins and DNA when produced in excess amounts (Halliwell, 2007). Oxidative stress is a problem mainly observed when cells cannot adequately inactivate the excess of oxidants formed. That is to say, imbalance between the production and destruction of ROS/RNS is the main reason of oxidative stress. For instance, excess amount of peroxynitrite and hydroxyl radical may damage lipoproteins and cell membranes by a process called lipid peroxidation. Proteins can be damaged by ROS/RNS as well, leading to loss of enzyme activity and structural changes (Halliwell, 2007). Oxidative damage to DNA molecule can lead to the formation of different lesions which may cause mutations. DNA repair enzymes and antioxidants are used to fix these attacks as a defence mechanism (Pacher et al, 2007). Oxidative stress can cause several chronic or degenerative disorders if it is not regulated properly.

1.1.3 Oxidative stress and Human Diseases

Oxidative stress has been shown to have certain roles on human health in different conditions, including inflammations, certain cancers, atherosclerosis and the aging. It is more recently thought to make a serious contribution to most of the disorders such as arthritis, lupus erythematous, ischemic heart diseases, stroke, gastric ulcers, hypertension, neurological disorders including Parkinson's disease and Alzheimer's disease and more (Stefanis, 1997). (Fig 1.1).
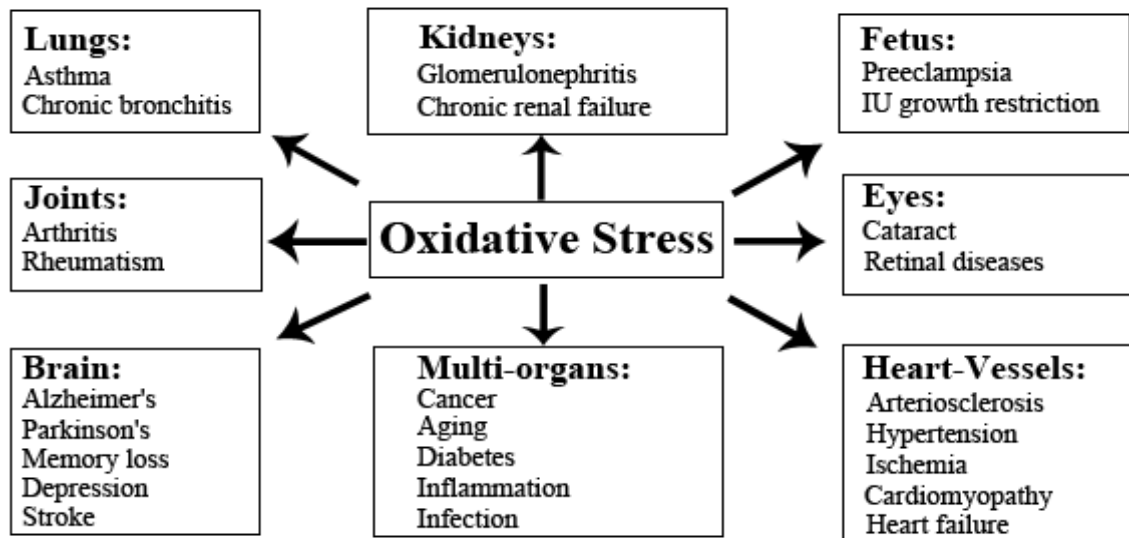
Figure 1.1 Oxidative stress-induced diseases in humans

1.1.4 Cardiovascular Diseases

Cardiovascular diseases are still the first reason for deaths in the world as well as in Turkey, constituting about half of deaths (TUIK, 2013). Oxidative stress is one of the most important inductors of cardiovascular diseases in general and more specifically atherosclerotic plaque formation. Oxidation of poly unsaturated fatty acids in low density lipoproteins (LDL) ,which is as a major part of the blood, play a vital role in atherosclerosis (Esterbauer, 1991). In addition, together with endothelial cells macrophages can release free radicals that is affecting lipid peroxidation (Neuzil, 1997). Continuous oxidation of lipids can lead to production of foam cells and plaque formation which are the main symptoms of atherosclerosis (Fig 1.2). Furthermore, oxidized LDL has toxic effects and can damage endothelial cells directly. Antioxidants such as vitamin E or β-carotene play significant roles in the prevention of various cardiovascular diseases by scavenging the free radicals and preventing lipid peroxidation in the arterial cell membranes.
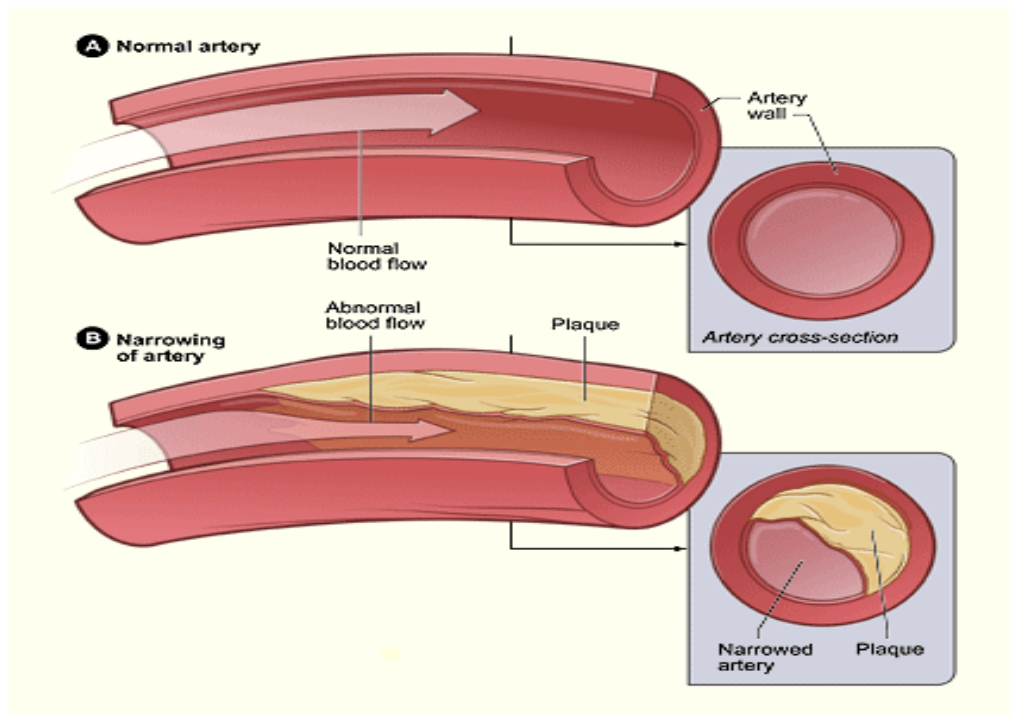
Figure 1.2 a) Blood flow in healthy artery b) Plaque formation inhibits regular blood flow

1.2 ANTIOXIDANTS

The body has different mechanisms to resist oxidative stress by generating antioxidants. Endogenous antioxidants are naturally generated in situ and exogenous antioxidants are externally supplied through diet. The main roles of antioxidants are to neutralize the excessive amount of free radicals. They are helpful for protecting the cells against toxic effects of oxidants and contribute to disease prevention.

1.2.1 Enzymatic

Endogenous compounds in cells can be further divided into enzymatic antioxidants and non-enzymatic antioxidants. The major enzymatic antioxidants which have direct roles in the neutralization of ROS and RNS are: catalase (CAT), superoxide dismutase (SOD), glutathione peroxidase (GPx) and glutathione reductase (GRx) (Willcox et al, 2004). SOD is the the first line of defense against attacks of free radicals. It catalyzes the dismutation of superoxide anion radical ($O_2•^-$) into

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hydrogen peroxide (H_2O_2) by reduction. The oxidant H_2O_2 is later transformed into water (H_2O) and oxygen (O_2) by the help of catalase CAT or GPx. The selenoprotein GPx removes hydrogen peroxide by using it to oxidize reduced glutathione (GSH) into oxidized glutathione (GSSG). By the help of NADPH, which act as a source of reducing power, glutathione reductase reproduces GSH from GSSG. Along with hydrogen peroxide, glutathione peroxidase also reduces lipid or nonlipid hydroperoxides while oxidizing GSH (Bahorun et al, 2006).

$$2O_2^- \cdot \ + \ 2H^+ \ \xrightarrow{\ SOD\ } \ H_2O_2 \ \xrightarrow{\ CAT\ } \ H_2O \ + \ O_2$$

RSH / RSSR / GPx / O_2 / H_2O
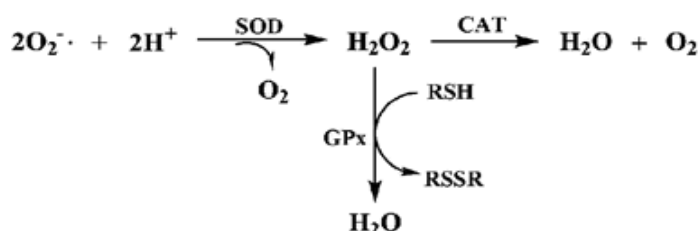
1.2.2 Non-Enzymatic

The non-enzymatic antioxidants are also categorized as metabolic antioxidants and nutrient antioxidants. Metabolic antioxidants, which belong to endogenous antioxidants, are generated by the body. Some examples of them are lipoid acid, glutathione, coenzyme Q10, L-arginine, melatonin, uric acid, bilirubin, transferrin, etc (Droge, 2002). Glutathione shows antioxidant activity due to the thiol group and act as a reducing agent. Melatonin is a powerful antioxidant that can easily cross cell membranes and the blood–brain barrier and different from other antioxidants, melatonin does not undergo redox reactions (Reiter et al, 1997).

However, nutrient antioxidants belong to exogenous antioxidants. They cannot be produced in the body so must be provided through diet or supplements. Vitamin E, vitamin C, carotenoids, omega-3 and omega-6 fatty acids, trace elements (selenium, manganese, zinc) and flavonoids can be given as examples to nutrient antioxidants. In particular, α-tocopherol form of vitamin E can be considered the most potent lipid-soluble antioxidant by preventing membranes from oxidation and by reacting

with lipid radicals produced in the lipid peroxidation chain reaction (Traber and Atkinson, 2007).

1.3 PHYTOCHEMICALS

Phytochemicals are protective and disease preventive chemicals found in plants which do not have nutrition value. Plants produce these chemicals as secondary metabolites for protecting themselves. They are not required to be consumed by the body for maintaining the metabolism properly. However, natural phytochemicals are shown to be effective in reducing free radical formation which is a significant contributor in the etiology of several diseases (Liu, 2003). There are more than five thousand phytochemicals. Some of the very popular examples of them are lycopene in tomatoes, beta carotene and other carotenoids, isoflavones in soy, ascorbic acid (vitamin C), folic acid, vitamin E and flavonoids in fruits.

In addition to antioxidant effects, some phytochemicals have also hormone-like actions. There is strong evidence that a diet rich in fruits and vegetables can prevent the formation of certain types of cancer by inhibiting the carcinogens and different chronic diseases such as diabetes, heart disease and high blood pressure (Craig WJ, 2009).

1.4 CONCEPTS OF FUNCTIONAL FOODS AND NUTRACEUTICALS

Recently it has been more clearly understood that nutrition has an important role for protection against chronic diseases. Functional foods can be considered as a food which can be used as a source of biological healing agent by contributing to the prevention against various diseases or regulating physiological functions in addition to supplying basic nutrients (López-Varela et al, 2002). A food can be considered as functional if it is satisfactorily showed to exert beneficial functions in the body beyond normal nutritional effects. The effects could be either maintenance/promotion of wellbeing or /and a reduction of risk of a pathologic condition or a disease (Roberfroid, 1999). The simplest examples of functional foods

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are constituted generally by whole foods. Carrots, broccoli and tomatoes are regarded functional since they contain significant amount of bioactive molecules like lycopene and B-carotene, and or fatty fish containing omega-3 fatty acids (DHA and EPA). "Nutraceutical" is defined "as a food or parts of food that provide medical or health benefits, including the prevention and treatment of disease" (Stephen DeFelice, 1979). Nutraceuticals may include dietary supplements, genetically modified "specific" foods and natural or synthetic herbal products. The interest in nutraceuticals has increased recently. This emphasize that people take more attention to scientific studies related with biological benefits of nutrition and act accordingly. Flavonoids can be regarded as one of the main nutraceutical ingredients found in plants. They can act as potent metal chelators and antioxidants. They have also been shown to exert antiviral, anticarcinogenic, hepatoprotective and anti-inflammatory effects (Tapas et al, 2008).

1.5 MICROORGANISMS AND DISEASES

1.5.1. Gram-positive Bacteria Used in This Study

1.5.1.1 *Staphylococcus aureus*

Characteristics: It is a type of bacteria commonly found on the skin and hair as well as in the noses and throats of people and animals. Most of the colonies have pigmented dull yellow color. It is seen more commonly among people with skin, eye, nose, or throat infections (Fig 1.3).

Infections: Skin infections like impetigo, scalded skin syndrome, cellulitis and pimples or more serious diseases such as pneumonia, endocarditis, meningitis and toxic shock syndrome. *Staphylococcus* is killed by cooking and pasteurization (Forbes et al, 2007).

Figure 1.3 *Staphylococcus aureus* (Rosenbach, 1884)

1.5.1.2 *Bacillus subtilis*

Characteristics: Rod-shaped, tolerable to extreme environmental conditions by the help of protective endospore, obligate aerobe (Fig 1.4). Used commonly as a model organism to study cell differentiation and bacterial chromosome replication.

Infections: In healthy people it can be used as a probiotic. It rarely causes food poisoning (Ryan et al, 2004). However, it may cause diseases in immunocompromised patients (Oggioni et al, 1998).
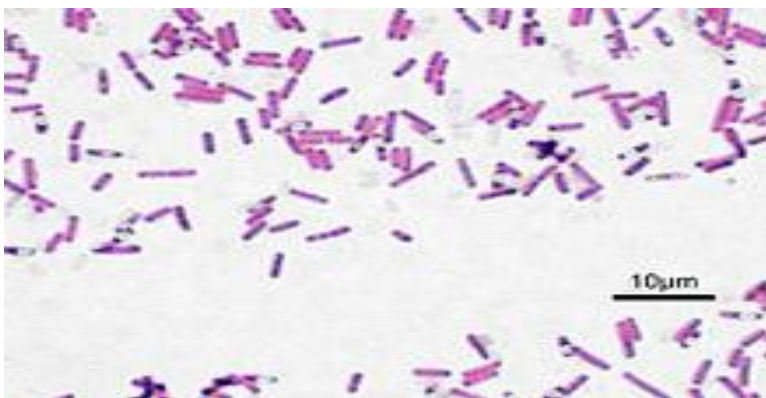
Figure 1.4 *Bacillus subtilis* (Tambe, 2005)

1.5.2 Gram-negative Bacteria Used in This Study

1.5.2.1 *Escherichia coli*

Characteristics: It is a gram-negative, facultatively anaerobic, rod-shaped bacterium which has flat, dry, pink colonies that is commonly seen in the lower intestine of warm-blooded organisms (Singleton, 1999) (Fig 1.5).

Infections: In fact, most strains of *E.coli* are not harmful and take part of the normal gut microflora. They produce vitamin K_2 which has an important role in maintaining healthy bone mineral density (BMD) (Bentley and Meganathan, 1982) and additionally prevent colonization of pathogenic bacteria in the intestine (Hudault et al, 2001). However, some strains can cause severe food poisoning (Vogt and Dippold, 2005), some virulent serotypes can cause gastroenteritis, urinary tract infections, and neonatal meningitis.

0157:H7 strain of *E.coli* produces a special toxin namely Shiga Toxin. It is responsible for hemolytic-uremic syndrome (HUS) (Evans et al, 2007).

Figure 1.5 *Escherichia coli*

1.5.2.2 *Pseudomonas aeruginosa*

Characteristics: Gram-negative, coccobacillus bacterium with unipolar motility. Aerobic but adapted to survive in partial or total oxygen depleted conditions (Ryan and Ray, 2004) (Fig 1.6).

Infections: *P. aeruginosa* usually infects the urinary tract, wounds, airway and also may be responsible for other blood infections (Cooper et al, 2003). It is the most common reason of burn injuries related infections. It is naturally resistant to a wide range of antibiotics and may show additional resistance after unsuccessful treatment.
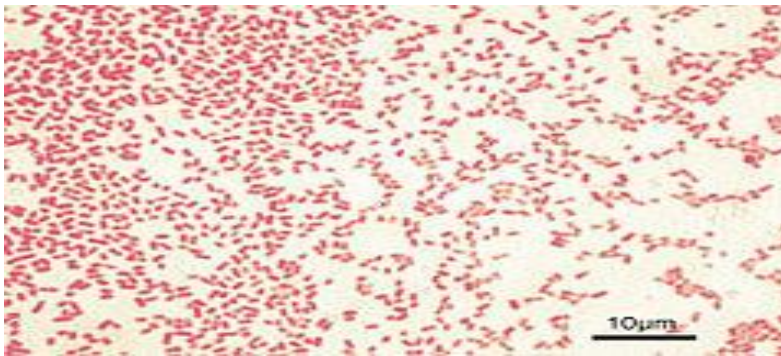
Figure 1.6 *Pseudomonas aeruginosa* (Tambe, 2005)

1.5.2.3 *Klebsiella pneumonia*

Characteristics: It is a gram-negative, rod-shaped, nonmotile, facultative anaerobic, encapsulated and lactose-fermenting bacterium (Fig 1.7). It has a distinctive yeasty odor in culture and has colonies of viscous appearance. Normally it is present in the microflora of the intestine, mouth and skin (Antoniadou et al, 2007) but if ingested, it can lead to destructive changes to human lungs.

Infections: It normally affects immunocompromised people such as hospitalized, diabetic patients or patients with chronic lung disease. *Klebsiella pneumoniae* may be responsible from most the community-acquired and hospital urinary tract infections (UTIs) together with E.coli. In fact, next to pathogenic E. coli strains, it is the second most virulent pathogen leading to UTI.

It is also a frequent cause of health care associated bloodstream infections. Pneumonia, specifically in the form of bronchitis, and septicemia (whole body inflammation) are main health conditions caused by *Klebsiella* outside the hospital. In spite of the antimicrobial therapy it has a death rate of about 50% and even can reach 100% for people with bacteremia (bacteria in the blood) and alcoholism (Greenwood et al, 2002).

Figure 1.7 *Klebsiella pneumoniae* (Murray et al, 1997)

1.5.2.4 *Serratia marcescens*

Characteristics: Gram-negative, motile, rod shaped, non-endospore forming, some strains easily identified with red pigmentation, relatively low virulence level. Phenotypically *Serratia* is one of the easiest genera to differentiate within the *Enterobacteriaceae* family. Unlike other enterobacteria, it is resistant to the antibiotics ampicillin, cephalothin and colistin (Auwaerter, 2007) (Fig 1.8).

Infections: *S. marcescens* has a role in several serious infections including ocular infection with high incidence in keratitis related with contact lenses (Das et al, 2007), urinary tract infection (Kawecki et al, 2013), lower respiratory tract infection (van der Vorm et al, 2002), bloodstream infection, wound infection and meningitis (Merkier et al, 2013). The organism has also been a rare cause of endocarditis and osteomyelitis (mostly in people employing intravenous drugs) and pneumonia (Jones, 2010).
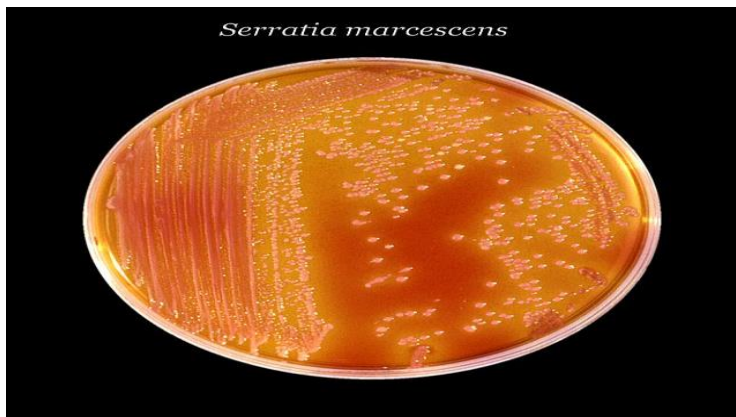
Figure 1.8 *Serratia marcescens* (Murray et al, 1997)

1.6 *PISTACIA VERA L.*

1.6.1 Taxonomic Hierarchy

Table 1.1 Taxonomy of *Pistacia vera*

Kingdom	Subkingdom	Superdivision	Division	Class
PLANTAE	VIRIDIPLANTAE	EMBRYOPHYTA	TRACHEOPHYTA	MAGNOLIOPSIDA

Superorder	Order	Family	Genus	Species
ROSANAE	SAPINDALES	ANACARDIACEAE	PISTACIA	*PISTACIA VERA L.*

Figure 1.9 *Pistacia vera* Tree (Ferguson, 2005)
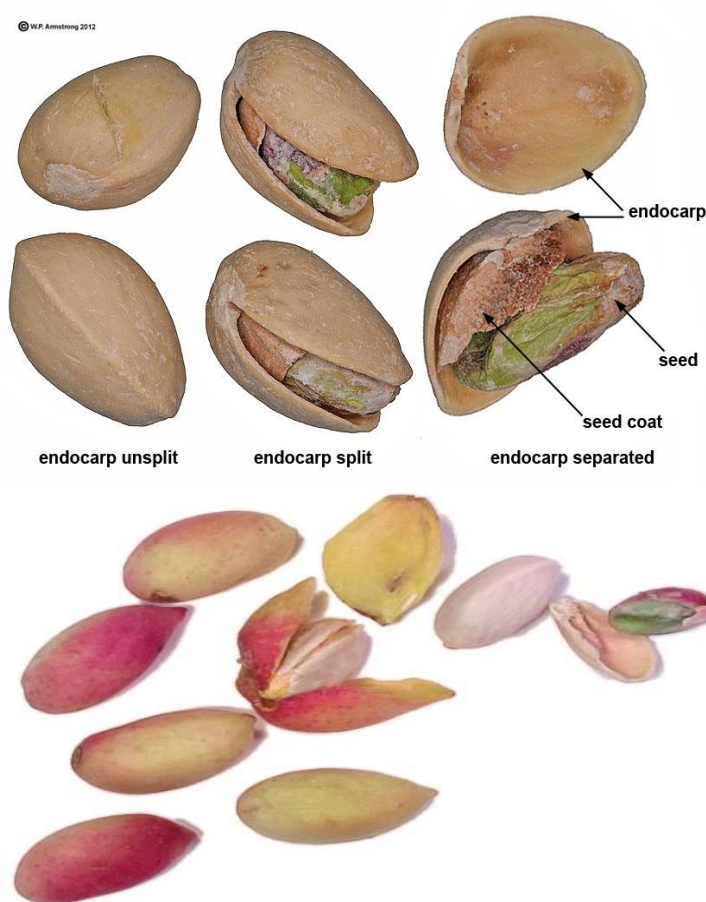
Figure 1.10 Parts of *Pistacia vera* fruit (Polito and K. Pinney, 1999)

The genus *Pistacia* is a member of the family Anacardiaceae that is composed of 70 genera and more than 500 species. Regular, healthy *Pistacias* have shrubs and trees that can grow up to 10-11 m tall. The tree has deciduous leaves. The plants are dioecious; that is, it has either male or female flowers. Among the species, *P. terebinthus L., P. atlantica desf., P. lentiscus* and *P.vera.* is native of Central Asia, Middle East and distributed throughout the Mediterranean region. *P. vera* is the only commercially cultivated species in the family (C. Kole et al, 2011).

Pistachios are Mediterranean plants, i.e. they thrive in dry, hot, desert-like condition and are very much tolerant of saline soil. In fact, they are more tolerant of alkaline (Na^+) and saline soil than most of the tree crops (Herrera, 1997). Pistachio trees can survive temperatures ranging between −10 °C in winter and 48 °C in summer. They

need intense and prolonged sunlight and well-drained soil. High humidity prevents growing of the tree and promotes severe diseases. Long and hot summers are crucial for proper maturation of the fruit.

So far, various parts of *Pistacia* plant have been studied for different effects pharmacologically. Along with therapeutic utilization, *Pistacia* species are utilized in commercial products such as usage of *P. vera* nut as a food additive (Marderosian and Beutler, 2010), fruit of *P. terebinthus* as snack food or in making caffeine-free coffee (menengic coffee) drink (G. Durmaz and V. Gokmen, 2011) and fruit of *P. lentiscus* as food colorants (Longo et al, 2007).

Nonetheless, *P. vera* are most of the time used after removal of skin parts. Skins are excluded from seeds in the industrial process. Compared to total weight of the nut skins make up 10% of the pistachio and considered as waste (Martorana et al, 2013).

Pistachios are very rich source of phenolic compounds, and so may be regarded as a functional food with unique properties. In a recent study, pistachio has been determined among the 50 product which have the highest antioxidant levels (B. L. Halvorsen et al, 2006). The various bioactive molecules observed in *P. vera* are flavanones, anthocyanins, phenolic acids, flavonols, flavan-3-ols, stilbenes, and isoflavons, (F.C. Lau et al, 2006). These phenolic compounds are known for their chemopreventive (D. Hou et al, 2004), cardioprotective and vasoprotective capacities (A. Agouni et al, 2009) as well as their high antioxidant activity.

In July 2003, the Food and Drug Administration (FDA) confirmed the first qualified health claim for seeds specifically lowering effects of heart disease risk as: "Scientific evidence suggests but does not prove that eating 1.5 ounces (42.5 g) per day of most nuts, such as pistachios, as part of a diet low in saturated fat and cholesterol may reduce the risk of heart disease".

1.6.2 Traditional Uses

Conventional usage and biological effects of *P. vera, P. atlantica*, *P. lentiscus* and *P. terebinthus* from Mediterranean Regions are showed in Table 1. Among them, *P. vera* fruit is consumed throughout the world that date back to 7000 BC (Marderosian and Beutler, 2010). In traditional Anatolian and Persian medicine, several fragments of *P. atlantica, P. lentiscus* and *P. vera* have been utilized for centuries as useful treatments for several disorders. While *P. vera* fruit is used against cardiac and stomach diseases and for its wound healing activity, the fruits of *P. atlantica* is mainly employed due to their sexual stimulatory effects and treatment of kidney and heart diseases (Aghili, 2009).

Table 2.2 Ethnomedicinal uses of selected Pistacia species in Mediterranean Region

Species	Regions	Plant part(s) used	Traditional uses and ethnobotanical reports	Reference(s)
	Turkey	Leaf	Eczema, diarrhea, throat infections, paralysis, kidney stones, Jaundice, asthma, stomach ache, astringent, anti-inflammatory, antipyretic, and stimulant	Giner-Larza et al,2001
Pistacia lentiscus	Italy	Leaf	Toothache, mycosis, herpes, abdominal and intestinal pain, rheumatism, antiseptic, cicatrizant, emollient, expectorant, and astringent	Scherrer et al,2005
	Greece	Resin	Stimulant, diuretic, hypertension, kidney stones, jaundice, cough, sore throat, eczema, and stomach ache	Hanlidou et al,2004
Pistacia atlantica	Turkey	Resin	Wound healing	Altundag et al, 2011
	Greece	Fruit	Mouth flavouring, tanning, and as fodder	Tzakou et al.2007
Pistacia terebinthus	Turkey	Leaf	Stomach ache, mycosis, and antidiabetic	Sezik et al,2001
		Resin	Urinary and respiratory antiseptic, asthma, antipyretic, and anti-inflammatory	Topcu at al,2007
	Spain	Leaf	Antiseptic,Odontalgia and Dislocated joint	Benítez et al,2010
Pistacia vera				
	Turkey	Resin	Asthma, stomach ache, and hemorrhoids	Orhan et al,2006

(Bozorgi et al, 2013)

1.6.3 *Pistacia vera* in Turkey

Wild trees of *Pistacia* species are spread in almost all parts of Anatolia. According to the TUBIVES records, South East Anatolia is the main region where *P. vera* can be grown (TUBIVES) and distribution with respect to districts where *P. vera* commonly grows is listed as: GAZIANTEP, MARDIN and SANLIURFA. The climate of this region is very suitable for growth of *Pistacia* trees or shrubs. The dispersion of the taxon over Turkey is demonstrated further in Figure 1.11.
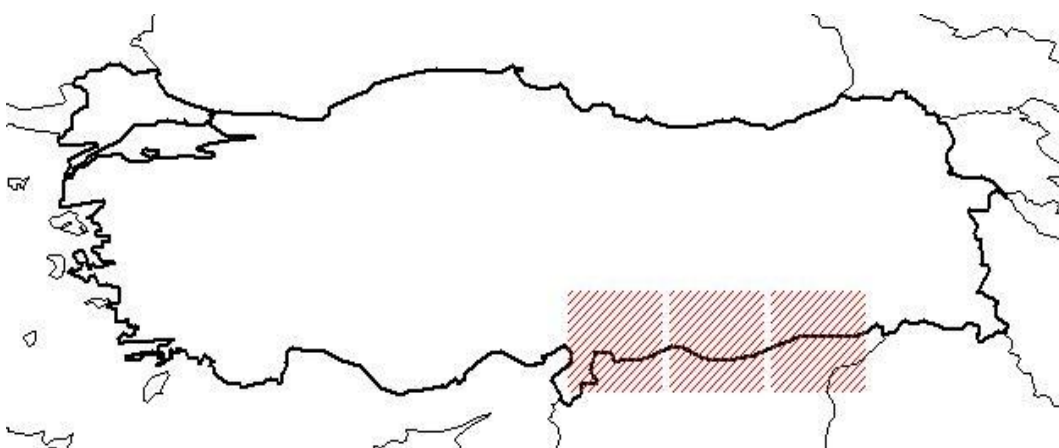
Figure 1.11 Dispersion of the taxon over Turkey based on grid (TUBIVES)

1.6.4 Phytochemical Studies

1.6.4.1 Phenolic Compounds

Various phenolic compounds have been isolated from *Pistacia vera*. Among them, Gallic acid, epicatechin and catechin were determined in skin as well as in seed of *P. vera*, leaves of *P. lentiscus* and *P. atlantica* (Romani et al, 2002). Isomers of resveratrol (Assimopoulou and Papageorgiou, 2005), and trans-resveratrol-3-O-β-glucoside were also characterized in *P. vera* (Grippi et al, 2008).

Several flavonoid compounds have been isolated and detected in various parts of these species. Naringenin, Quercetin, eriodyctyol, kaempferol, daidzein, apigenin, genistein and luteolin were isolated from fruits of *P. vera*. Main flavonoid

constituent of the seed was determined as Quercetin-3-O-rutinoside (Tomaino et al, 2010). During the ripening of the fruit, flavonoid content of *P. vera* has been shown to decrease (Ballistreri et al, 2009).

Among anthocyanins, cyanidin-3-O-glucoside and cyanidin-3-galactoside have been reported to be as main compounds of *P. vera* skin (Bellomo and Fallico, 2007).

1.6.5 Pharmacological Aspects

1.6.5.1 Antioxidant Activity

Between Pistacia species, *P. lentiscus* and *P. terebinthus* resins have been shown to have strong effects against oxidation of LDL in vitro (Andrikopoulos et al, 2003).

Fruit of *P. vera* has showed noticeable antioxidant effects comparable to the synthetic antioxidants (Goli et al, 2005). It is important to note that hydrophilic extract of *P. vera* nuts showed higher antioxidant potential than that of lipophilic extract (Gentile et al, 2007). Due to higher amount of phenolic compounds in skins, it has been shown that extract of *P. vera* skins had more antioxidant capacity with respect to seeds (Tomaino et al, 2010). Antioxidant activity of different parts from *P. vera* has also been reported (Hosseinzadeha et al, 2012).

1.6.5.2 Antimicrobial and Antiviral Activities

Pistacia have been shown considerable antimicrobial activity against several Gram negative and Gram positive bacteria. In a study with lipophylic extracts of *P. vera*, it is shown that several fragments obtained from *P.vera* had some antibacterial effect and more significant antifungal activity against C. albicans and C. parapsilosis. Seed and kernel extracts also exerted noticeable antiviral effects in vitro (Özçelik et al, 2005).

1.6.5.3 Effects on Atherosclerosis

In addition to antihyperlipidemic activity, Pistacia species show their anti-atherosclerotic effects by alleviating the atherosclerotic lesions directly. In an experimental atherosclerosis study in which rabbits are used as a model, methanolic extracts from *P. vera* fruits have shown favorable effects on HDL and LDL levels in addition to aortic intimal thickness. Morever, methanolic extracts have also revealed a remarkable mitigation in aortic surface lesions (Marinou et al, 2010).

1.7. PISTACHIO PRODUCTION

Turkey is one of the gene centers of pistachio and according to data obtained from Nestle Co. the third largest producer of pistachios in the world with 150,000 tonnes a year contributing around 15-20% of total world production following Iran and USA. However, there is a potential to increase production levels significantly with the employment of modern agricultural techniques.

1.8 SCOPE OF THE STUDY

Plants are fairly rich and bioavailable sources of polyphenols and traditionally used for several health effects. Among various species of Pistacia genus, *P. vera* is by far the most important one both biologically and economically throughout the world. Nonetheless, there were not significant and sufficient studies related with skin of *P. vera*. In this study we aimed to investigate antioxidant properties and antimicrobial effects of *P. vera* skin extracts against various gram positive and negative bacteria as an alternative solution to synthetic antibiotics. Microorganisms have gained resistance recently and synthetic antibiotics also causes destruction of the beneficial bacteria within the intestines by disrupting the balance of intestinal terrain. Natural antibiotics act in a unique mechanism through a variety of phytochemicals. They are more tolerable and less toxic for the body. In addition to its bactericidal action they stimulate the immune system, exhibit anti-inflammatory and anti-septic properties

In addition, we hope the results of this study can be used to produce a functional food product containing the skin of *P. vera* which can provide a significant economic value to Turkey.

CHAPTER 2

MATERIALS AND METHODS

2.1 MATERIALS

2.1.1 Chemicals

Nutrient agar and Nutrient broth were purchased from Merck (Darmstadt, Germany). 6 mm diameter antimicrobial susceptibility test discs being used in disc diffusion tests were purchased from Oxoid (Hants, UK).

Standard antimicrobial discs Amikacin (30 mcg) and Chloramphenicol (30 mcg) were bought from Bioanalyse.

Thermo Scientific Finnpipettes were used during the experimental research

Gallic acid, Quercetin, 2,2-diphenyl-1-picrylhydrazyl (DPPH), sodium carbonate (Na_2CO_3), sodium hydroxide (NaOH), aluminum chloride ($AlCl_3$) were purchased from Sigma Chemical Company (St.Louis, MO, USA). Folin Ciocalteu phenol reagent was purchased from Merck (Darmstadt, Germany)

Staphylococcus aureus (RSKK 95047, ATCC 43300), *Bacillus subtilis* (RSKK 0313, ATCC 6633), *Klebsiella pneumoniae* (RSKK 06017, ATCC 10031), *Pseudomonas aeruginosa* (RSKK 06021, ATCC 15442), *Escherichia coli* (RSKK 06015, ATCC 11229) and Serratia marcences (RSKK 579, Pasteur Institute) used for antibacterial experiments were supplied from Refik Saydam Hygiene Center.

Chloramphenicol powder which is used for MIC experiments was bought from Sigma-Aldrich (Germany).

Commercially available Amikacin solution is bought from Zentiva Co.

2.1.2 Apparatus

Thermo Scientific 96-Well Microtiter Microplates are used for micro-volume applications

For the spectroscopic experiments UV- 1700 PharmaSpec UV-VIS spectrophotometer (SHIMADZU) were used.

Rotary evaporator (Stuart RE300DB)

Stainless-steel blender (Waring, 32BL80),

M 420B incubator (Elektro.mag)

96-well plate reader ELISA (Elx808, Germany) was used in METU Biology Department,

2.2. METHODS

2.2.1 Preparation of Plant Extracts

Plant material was obtained from Yaylımlı Village, Adıyaman, Turkey. The skins were peeled off manually and air dried in shade. Dried skins were grinded with pestle and mortar. The samples were stored at 4^0C until they were used.

10 grams of dried ground plant material was added to 500 mL flasks, and solvents of 100 mL (water, acetone, ethanol and methanol) were poured to flasks. Each mixture was placed on a shaker and left for extraction for 24 hours for 125 rpm at 25 ^{0}C. Later, extracts were filtered and transferred to a round bottom flask of 250 mL. Later, solvents were separated by using a rotary evaporator (Stuart RE300DB). Arranged temperature of the water was set at 32 to 45 ^{0}C depending on the solvent type. Later, the weight of the dried sample was determined. Following, for each extract the yield is calculated. Since it gave the highest yield value, methanol was chosen as a type of solvent and we continued our experiments by using methanol.

Stock solution was prepared in methanol and stored in the freezer which is set at a temperature of -20 ^{0}C until the day of experiment.

2.2.2 Determination of Antioxidant Capacity

2.2.2.1 Determination of Total Phenolic Content

Colorimetric reactions are regarded as rapid, easy to operate and low-cost methods which are broadly used in the UV/VIS spectrophotometric analyses (Pelozo et al, 2008). A reference substance is needed for the colorimetric assay. In that sense, the total phenolic concentration in the plant extract can be determined properly. Polyphenols contained in plant extracts react with specific redox reagents (Folin-Ciocalteu reagent) and generate a blue-colored complex which can be detected and quantified by visible-light spectrophotometry. The reaction mostly provides accurate and specific data for different phenolic groups (Folin and Ciocalteu, 1927).

The concentration of phenolics in plant extracts was determined by using Folin-Ciocalteau method (Singleton and Rossi, 1965) with slight modification. The reaction mixture was prepared by mixing 50 % Folin phenol reagent (100 μL) and methanol extracts (100 μL) at different concentrations. Later, 2 % Na_2CO_3 (2 mL) was used into the tubes to cease the reaction.The same procedure was followed for blank solution. The only difference was that methanol was used instead of *Pistacia* extract. The absorbance values were measured at 750 nm after incubation period (30 min). As standard, Gallic acid solution was used at various concentrations and after several trials the calibration line was constructed. By using the Gallic acid standard curve, results were calculated as milligram of total phenolic compounds found in grams of extract as the Gallic acid equivalents (GAE). The results were recorded by taken the average of three independent experiments in duplicates.

2.2.2.2 DPPH Method

DPPH assay is broadly used and convenient method that presents true information about the scavenging activity of the sample against an effective free radical. In the radical form, DPPH (1,1-diphenyl-2-picryl-hydrazyl), is a quite stable molecule. Dissolved in ethanol, it has a violet color that can be characterized as an absorption band at 517nm. Whenever it is quenched and reduced by the extract or by any H donor, a decrease in the absorbance and change of colour to pale yellow is observed

due to the presence of picryl group in the compound. Hence, the antioxidant activity can be determined measuring this color change by UV spectrophotometer at 517 nm (Molyneux, 2003).
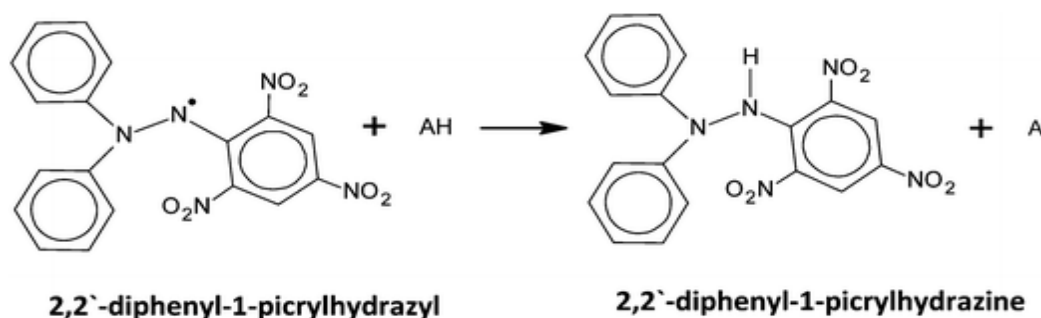
Figure 2.1 Schematic illustration of radical (oxidized) and non radical (reduced) form of DPPH Molecule

DPPH method was applied according to Hatano et al., 1988. 2,2-diphenyl-1-picrylhydrazyl (DPPH) is prepared in ethanol such that the concentration of the solution was 0.05 mg/mL. The concentration of DPPH is controlled at UV-Vis spectrophotometer which shows 1.3-1.4 absorbance value at 517 nm. Methanolic extracts concentrations (from 0.125 to 2.5 mg/ml) as 100 µL was added to 1.4 mL of DPPH solution. The solutions were incubated at dark (20 min) at 25 ^{0}C and the absorbance value was determined at 517 nm.

Radical scavenging activities of the extracts (RSA %) were found by using the formula RSA (%) = 100 * $(A_0 - A_1)/A_0$ where A_0 is the absorbance of the control which contain all reagents except *Pistacia* sample. A_1 shows the absorbance value of the sample tested. Accordingly, EC_{50} value that represents the concentration of extract causing 50% inhibition of the radical was calculated. For positive control, Quercetin standard was used. Various concentrations of Quercetin were tried to construct the calibration line properly.

2.2.2.3 Total Flavonoid Content

The principle is basically the generation of complexes with the keto group at 4th C and the hydroxyl groups at 3th and 5th C of flavonols and flavones in the presence of $AlCl_3$. Quercetin is chosen as standard material to be able to build the calibration curve efficiently. Quercetin standard solution was prepared at several concentrations and after several trials, the calibration curve was constructed (Duke, 2002).

Determination of total flavonoid content is of quite importance for several reasons. Apart from their antioxidative capacities, flavonoids have also strong anti-inflammatory potentials by interfering with different molecules such as lipoxygenase and cellular structures like epithelial cells, lymphocytes and macrophages (González et al, 2011).

The total flavonoid concentration was measured by the aluminum chloride colorimetric assay (Marinova et al, 2005) with some modification. The methanolic extracts (1 mL) was added to a 10 mL volumetric flask containing 4 mL of distilled water and mixed with 0.3 mL of 5% $NaNO_2$. After 5 min, 0.3 mL of 10% $AlCl_3$ was added. After another 6 min, 2 mL of 1 M NaOH was added and the total volume was made up to 10 mL with distilled water. The solution was mixed well and the absorbance was measured at 415 nm against a blank (1 mL of sample was replaced by 1 mL of methanol solvent). Orange to yellowish color was developed. A calibration curve was prepared with Quercetin as standard at various concentrations (100, 200, 300, 400, 500 μg/mL). The results were expressed as mg Quercetin equivalents (QE)/g of extract on dry weight (dw) basis. The results were recorded by taken the average of three independent experiments in duplicates.

2.2.3 Antibacterial Activity Tests

2.2.3.1 Preparation of Bacterial Cultures

The bacteria were subcultured on Nutrient agar (NA) to make sure to obtain pure isolated colonies and incubated for 24 h at 37 ^{0}C before being used and kept at 4 ^{0}C until used. Then, 5 isolated colonies of bacteria were transferred to previously prepared 5 mL Nutrient broth (NB) and incubated at 37 ^{0}C for approximately 2-4 hours until the amount of bacteria achieved the value of 1.0 x 10^8 cfu/mL. Contamination was checked by microscopic method. The pH of the broth was also assured to be between 7.2 and 7.4 at room temperature (25˚C) with a pH meter.

Concentration of bacteria was adjusted spectrophotometrically by matching the turbidity of inocula with the turbidity of McFarland standard of 0.5 (optical density: 0.08-0.10 at OD 600) to obtain a standardized number of colony forming unit (CFU) for all strains. Adequate light was adjusted to visually compare the inoculum tube and the 0.5 McFarland standard against a card with a white background and contrasting black lines (Fig 2.2). The optical densities of the bacterial suspensions were measured using UV-Vis spectrophotometer at a wavelength of 600 nm in addition to visual comparison. NB was used as the blank to calibrate the spectrophotometric measurements. When the bacterial density was under the desirable level, the duration of incubation period was increased; when the turbidity was too high, by using Nutrient Broth dilution process took place and the final concentration was set at 1.0 x 10^8 cfu/mL.
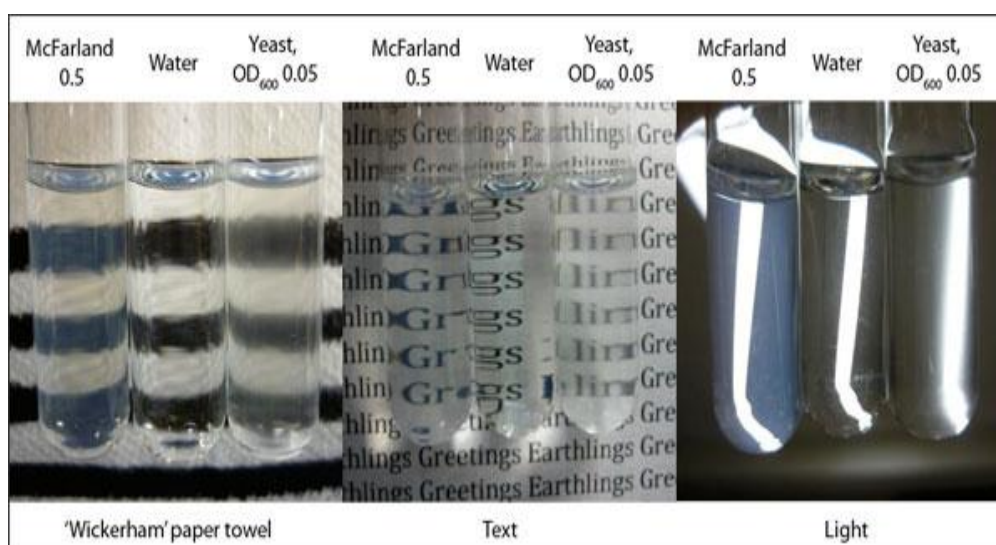
Figure 2.2 Representation of adjusted organism with 0.5 Mcfarland solution

2.2.3.2 Kirby Bauer Disc Diffusion Method

NB agars were prepared at aseptic conditions and transferred into 9 mm plates with 4 mm thickness. Later, plates were kept in cold room (4 ^{0}C) until the day of experiment. A 500 µL of bacteria (0.08 to 0.1 absorbance value at 600 nm which is equal to 1.0-2.0 x10^8 CFU/mL) were spread over the agar surface at aseptic conditions. The entire plate was covered by streaking the swab back and forth from edge to edge. The plate was rotated approximately 60° 2 times and repeated the swabbing procedure. This was done to ensure that the inoculum was evenly spreaded. After that 6 mm antibiotic discs were applied to the medium.

Discs impregnated with 20 µL solvent (methanol) and distilled water were used as negative control. They were also used to ensure that the discs were not contaminated. Together with negative controls, discs impregnated with commercial antibiotics which are used as positive control were placed on agar surface.

The choice of antibiotics was Amikacin (30 µg/disc) and Chloramphenicol (30 µg/disc). *Pistacia vera L.* skin extract in methanol which has a concentration of 115 mg/mL were impregnated to empty discs as 20 µL and then plates were kept in

incubator at 37 ^{0}C for 18 hours and the zones of inhibition were determined. Results included the diameter of discs (6 mm), as well.

2.2.3.3 Determination of Minimum Inhibitory Concentration (MIC)

Based on the preliminary screening, the extracts showing potent antibacterial activities were further subjected to minimum inhibitory concentration (MIC) assay.

Various final concentrations of the extracts ranging from 0.20 to 5.0 mg/ml (5, 2.5, 2, 1.5, 0.5, 0.25, 0.20) were prepared by adding appropriate quantities of each extract to pre sterilized nutrient broth and thoroughly mixing with the medium.
Determination of MIC was carried out using the broth dilution method with slight modification. The dilution factor needed was calculated and dilution was carried out to obtain a final concentration of 5.0 x 10^5 CFU/mL.

96-well plates were used for this microbial susceptibility testing. The designs of the 96-well plates were such that total volume of each well as 100 μL. Nutrient Broth with a volume of 100 μL was added to sterility control wells. A sterility control was used to ensure the sterility of the broth medium. From 1st to 7th well, broth medium with a volume 85 μL and methanol extracts of *Pistacia vera* skin with a volume of 10 μL starting from 5 mg/ml to 0.20 mg/ml was added followed by addition of 5 μL of bacteria (final concentration of 5.0 x 10^5 CFU/mL). To the 8th well, 90 μL of broth medium, 5 μL of bacteria and 5 μL of methanol were added for solvent control. The purpose of solvent control was to show if the solvent used had any inhibitory effect on each bacterial strain. After that, 95 μL of broth medium and 5 μL of tested microorganism inocula were added to growth control of the 96-well plate. The growth control was used to assess the viability of the microorganisms. Antibiotic sterility control contained 5 μL of antibiotic solution (100 μg/mL amikacin for *S. aureus, Klebsiella, P.aeruginosa, E.coli, S.marcences* and 100 μg/mL chloramphenicol for B. subtilis) and 95 μL of broth to understand whether there is a microbial contamination. The antibiotic growth control was made of 85 μL of NB, 10 μL of commercial antibiotic solution and 5 μL of bacterial were added for

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controlling the sensitivity of each bacterium to the antibiotic solutions. The 96-well plates was covered with a lid and incubated at 37ºC for 18 hours. Then, the results were confirmed by monitoring the absorbance at ELISA plate reader at 600 nm in addition to visual observance.

2.2.3.4 Determination of Minimum Bactericidal Concentration (MBC)

The MBC is the least concentration of a natural or synthetic antibacterial agent that is required to destroy all of the bacteria found in the sample. MIC test does not guarantee that all the microorganisms have been killed. It is possible that an antimicrobial agent with a specific concentration can inhibit the growth of microorganisms for a certain time but after a period the growth can be observed as well. That's why MBC test has quite importance to make sure to all bacteria has been eliminated. In order to measure the MBC, 96 well plate was prepared with 100 μL NB in each well. Then, from each previous MIC medium wells, 10 μL was moved into this plate and were incubated for 18 hours. Later, the absorption values of each well at 600 nm were monitored by ELISA reader and MBC was determined.

CHAPTER 3

RESULTS AND DISCUSSION

3.1 EXTRACTION OF *PISTACIA VERA L.*

10 g of powdered *Pistacia vera L.* skins were extracted separately in 100 mL methanol, ethanol, water and acetone and suspensions were placed in incubator at 125 rpm and 25 $^{\circ}$C for 24 h. Extraction mixtures were dried in rotary evaporator at various temperatures as stated in section 2.2.1. Final dry extract was found as 2.14 g and (%) yield was determined as 21.4% for methanol.

Since methanol showed the strongest yield value, it was chosen as type of solvent for further analyses. The different values of solvent yields were shown as in Table 3.1.

Table 3.1 Extraction yield of different solvents

	Solvent volume (mL)	Weight of dry *Pistacia vera* skin (g)	Total extract (g)	Yield (%)
Methanol	100	10	2.14	**21.4**
Ethanol	100	10	1.32	13.2
Water	100	10	1.86	18.6
Acetone	100	10	0.81	8.1

3.2 ANTIMICROBIAL ACTIVITY OF *PISTACIA VERA L.* SKIN

3.2.1 Kirby Bauer Disc Diffusion Test

Disc diffusion test was applied as previously stated in section 2.2.3.2. In brief, 500 µL of overnight cultures, (approximately 5.0 x10^8 CFU/mL) were spread over the agar plates. Discs containing 20 µL of 115 mg/mL of the *P. vera* extract, 20 µL of methanol and water along with commercially available antibiotic discs (30 µg/disc of

Amikacin and Chloramphenicol) were put onto sterile NB agar. After incubation period (18 hr) at 37 $^{\circ}$C, the zones of inhibition were determined. The highest antimicrobial activity was observed against *S. aureus* with 17.1 mm inhibition zone compared with antibiotic disc of Amikacin with 22.9 mm. Zone inhibition pictures of selected bacteria were shown in Figures 3.1, 3.2, 3.3 and 3.4. All zone of inhibition effectiveness results were summarized in Table 3.2 and Figure 3.5.

(a)

Figure 3.1 Inhibition zone diameters of *P. vera* skin extract against *P.aeruginosa* after 18 hours incubation period at 37 $^{\circ}$C

a) Inhibition zone of the extract above, antibiotic disc (30 mcg amikacin) below and negative controls of water and methanol with left and right, respectively.
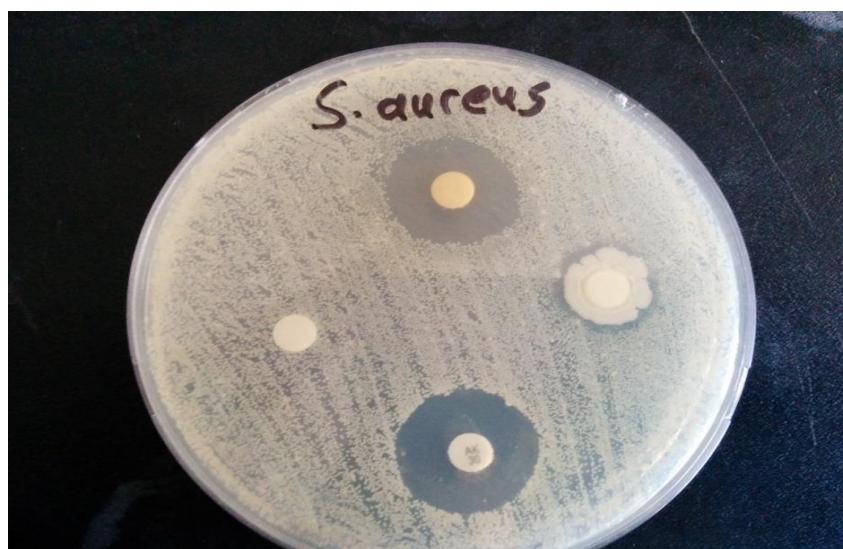
(b)

Figure 3.2 Inhibition zone diameters of *P. vera* skin extract against *S.aureus* after 18 hours incubation period at 37 °C

b) Inhibition zone of the extract above, antibiotic disc (30 mcg amikacin) below and negative controls of water and methanol with left and right, respectively.

(c)

Figure 3.3 Inhibition zone diameters of *P. vera* skin extract against *B.subtilis* after 18 hours incubation period at 37 °C

c) Inhibition zone of the extract above, antibiotic disc (30 mcg Chloramphenicol) below and negative controls of water and methanol with left and right, respectively.

(d)

Figure 3 4 Inhibition zone diameters of *P. vera* skin extract against *E.coli* after 18 hours incubation period at 37 $^{\circ}$C

d) Inhibition zone of the extract above, antibiotic disc (30 mcg Amikacin) below and negative controls of water and methanol with left and right, respectively.

Table 3.2 Antimicrobial activity (zone of inhibition) of *Pistacia vera* skin extract

Microorganisms	ZOI(mm)	ZOI (mm)[a]	ZOI (mm)
	Extract	**Amikacin**	**Chloramphenicol**
E. coli	10.2 ± 0.9	17.1 ± 0.8	NT
S. aureus	**17.1 ± 0.7**	22.9 ± 0.7	NT
P. aeruginosa	8.3 ± 0.4	20.8 ± 0	NT
B. subtilis	11.1 ± 1.1	NT	19.2 ± 1.4
S. marcences	7.4 ± 0.4	19.0 ± 0.5	NT
Klebsiella	9.2 ± 0.5	21.7 ± 0.8	NT

[a] The values are diameter of zone of inhibition at 115 mg/ml methanolic extract and 30μg/disc of Amikacin and Chloramphenicol. Diameter of inhibition zone (mm) includes disc diameter of 6 mm. Each data was collected by means of two independent experiments in duplicates
NT: Not Tested   ZOI: Zone of Inhibition

Inhibition zones of *P. vera* skin extracts against *Escherichia coli, Staphylococcus aureus, Pseudomonas aeruginosa, Bacillus subtilis, Serratia marcences* and

Klebsiella pneumoniae were measured and found as 10.2 mm, 17.1 mm, 8.3 mm, 11.1 mm, 7.4 mm and 9.2 mm, respectively as displayed in table 3.2.

The highest and the lowest antimicrobial activity were observed against *S. aureus* and *S. marcences* with 17.1 mm and 7.4 mm zone of inhibition, respectively. According to zone inhibition results, methanolic extracts of *P. vera* skin exert medium antimicrobial activity for selected bacteria except *S.aureus* at 115 mg/ml concentration. *S. aureus* showed high susceptibility compared to standard antibiotic, Amikacin.

In general, methanolic extracts of 115 mg/ml possessed moderate inhibitory effects against tested bacteria; the most reasonable explanation is that methanol is active in separating flavonoids and phenolics as well as to a certain extent essential oils which exist in *P. vera* and are strong antimicrobial agents. Still, the inhibitory effect has a direct relation with extract concentration. So, if the concentration were higher than 115 mg/ml, then bigger zones could likely be observed.

Inhibitory effects of *P.vera*ike *P. terebinthus* and *P. lentiscus* are known to be stronger against gram positive bacteria compared with gram negative bacteria (Magiatis et al, 1999) which were also be confirmed in our results, as well.

In 2007, Al-Bayati and Al-Mola studied the antimicrobial effect of the *P. vera* with the agar well diffusion assay. Aqueous extracts from *P. vera* nuts showed medium inhibitory effect on *P. aeruginosa*, *S. aureus*, *E. coli* and *K. pneumoniae* compared with the standard antibiotics using the concentration 200 mg/ml and high inhibitory effect against *B. cereus* compared with Cefalexin with an inhibition zone of 17.2 mm. The concentrations (12.5, 25 mg/ml) were inactive against tested bacteria. The results also showed the complete resistance of *S. marcescens* (Al-Bayati and Al-Mola, 2007).

Moreover, *E. coli* has demonstrated high susceptibility to ethanol extracts with respect to Gentamycin by an inhibition zone of 24.1 mm. Additionally, it was observed that ethanol extracts were significantly effective on *S. aureus, Ps. aeruginosa, Pr. vulgaris* and *B. cereus* compared with the standard antibiotics. However, they also noted that *S. marcescens* and *Sal. typhimurium* strains were totally resistant to all concentrations of ethanol extracts. It can be concluded from the study that ethanolic extracts were more effective compared to aqueous extracts in terms of antibacterial activity (Al-Bayati and Al-Mola, 2007).
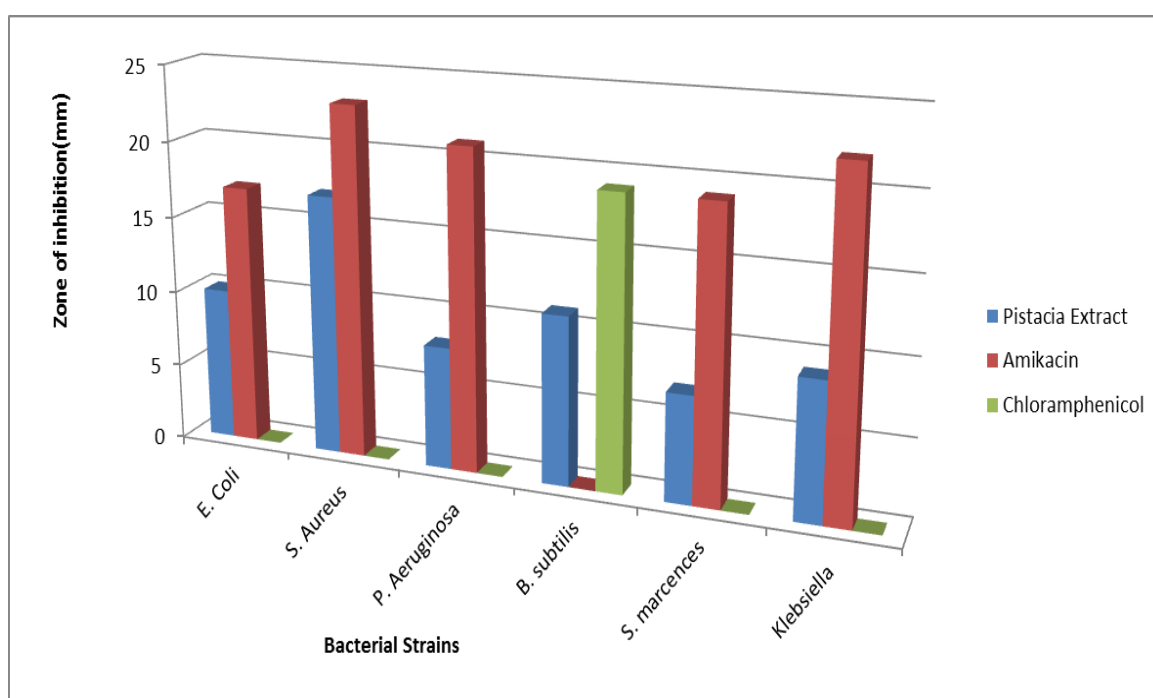
Figure 3.5 Antimicrobial activities of *P.vera.*skin extracts by disk diffusion method.

Diameter of inhibition zone (mm) includes disc diameter of 6 mm.

Each data was collected by means of two independent experiments in duplicates.

Regarding the harmful consequences of synthetic antibiotics to gut microbiota, *P. vera skin* can be used as an alternative natural antibiotic source against especially *S. aureus* causing infections such as scalded skin syndrome, impetigo and toxic shock syndrome (TSS).

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3.2.2 Minimum Inhibitory Concentration (MIC) of Extracts

Pistacia vera L. skin extracts were investigated for their growth inhibitory effect against *Escherichia coli, Staphylococcus aureus, Pseudomonas aeruginosa, Bacillus subtilis, Serratia marcences and Klebsiella pneumoniae* which are important pathogens.

As stated before, minimum inhibitory concentration shows the bacteriostatic effect of an antimicrobial agent. MIC values for *Pistacia vera L.* skin extracts were performed as stated in section 2.2.3.3 and the outcomes were documented in Table 3.3 together with Figures 3.6 and 3.7.

Table 3.3 Antimicrobial activity (MIC) of *Pistacia vera* skin extract

Microorganisms	MIC (mg/ml) of the extract
Escherichia coli	1.5
Staphylococcus aureus	2.0
Pseudomonas aeruginosa	2.0
Bacillus subtilis	1.0
Serratia marcences	1.0
Klebsiella pneumoniae	0.5

Pistacia vera skin extract has shown bacteriostatic effect on *Escherichia coli, Staphylococcus aureus, Pseudomonas aeruginosa, Bacillus subtilis, Serratia marcences and Klebsiella pneumoniae* with MIC values of 1.5, 2.0, 2.0, 1.0, 1.0 and 0.5 mg/mL, respectively.

The MIC values were to be considered close to each other. Still, among them, *Klebsiella pneumonia* was determined as the most susceptible to *P.vera* extract.

Figure 3.6 Determination of minimum inhibitory concentrations of *P. vera* skin extracts with 18 hours incubation against *E. coli, S. aureus and P. aeruginosa*.

Sterility control: Test the experiments if they were performed in aseptique conditions with broth only.

Growth control: Test of bacterial growth in the presence of nutrient broth and bacteria.

Antibiotic sterility control: Sterility of the antibiotics in the absence of bacteria.

Antibiotic control: 5 μL of Amikacin and Chloramphenicol were used as antibiotic with the concentration of 100 μg/mL in the presence of nutrient broth and bacteria

Solvent control: Test of bacterial growth in the presence of nutrient broth, bacteria and methanol to see whether the solvent is causing any inhibition.

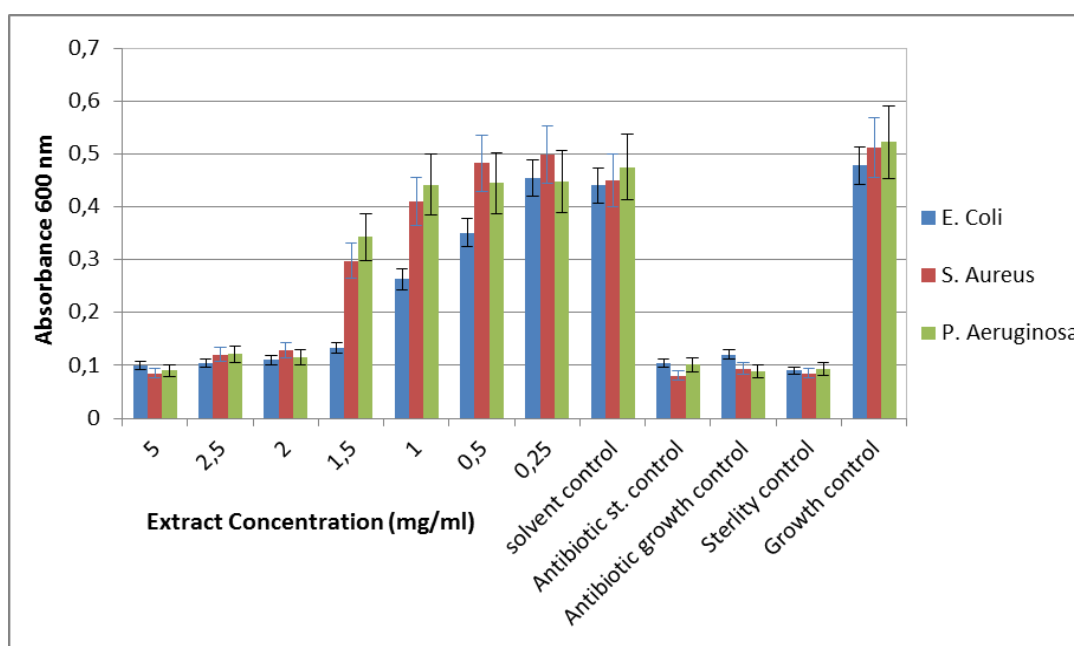Each data was obtained by taken the mean of two independent experiments in duplicates

Figure 3.7 Determination of minimum inhibitory concentrations of *P. vera* skin extracts with 18 hours incubation against *B. subtilis, S. marcences and K. pneumoniae*.

Sterility control: Test the experiments if they were performed in aseptique conditions with broth only.

Growth control: Test of bacterial growth in the presence of nutrient broth and bacteria.

Antibiotic sterility control: Sterility of the antibiotics in the absence of bacteria.

Antibiotic control: 5 μL of Amikacin and Chloramphenicol were used as antibiotic with the concentration of 100 μg/mL in the presence of nutrient broth and bacteria

Solvent control: Test of bacterial growth in the presence of nutrient broth, bacteria and methanol to see whether the solvent is causing any inhibition.

Each data was obtained by taken the mean of two independent experiments in duplicates

In a study by Alma et al, the antimicrobial activities of the essential oil of the gum from *P. vera* were compared with those of ampicillin, streptomycine, and nystatin used and it is found that the oil inhibits the growth of *E. coli,* one of the most common gram-negative poisoning bacteria, *S. aureus* and the yeast *C. Albicans*; however was not as effective on *B. cereus*, *P. aeruginosa*, and *K. pneumoniae* (Alma et al, 2004).

In an another study conducted by Ozcelik et al (2005), lipophylic (ethanol:hexane 1:1) extracts from non-processed fresh and dried skin of *P. vera* demonstrated an antibacterial activity with MIC values between 128–256 µg/ml against *E. coli* (ATCC 35218) and *P. aeruginosa*. (ATCC 10145). Moreover it had notable antifungal effect against *C. albicans* and *C. parapsilosis* with 16 µg/ml. Seed and kernel extracts showed potent antiviral activity.

In a study from Turkey, being potent antibiotic agents, sage infusion tea extract exerted 3 mg/mL of minimum inhibitory concentration (MIC) and 6 mg/mL of minimum bactericidal concentration (MBC). Rosehip was also found as an effective antimicrobial agent with a minimum inhibitory concentration of 3 mg/mL (Kümbet, 2010).

Considering the MIC results compared to previous studies, *P. vera skin* can be regarded as a moderate to effective antibiotic agent against gram positive and negative bacteria.

3.2.3 Minimum Bactericidal Concentration (MBC) of Extracts

MBC was determined as explained in section 2.2.3.4 and the outcomes were documented in Table 3.4

Table 3.4 Antimicrobial activity (MBC) of *Pistacia vera* skin extract

Microorganisms	MBC (mg/ml) of the extract
Escherichia coli	2.0
Staphylococcus aureus	2.0
Pseudomonas aeruginosa	2.0
Bacillus subtilis	1.0
Serratia marcences	2.5
Klebsiella pneumoniae	1.5

As it can be seen from Table 3.4, the bactericidal effects of *Pistacia vera L.* skin extracts against *Escherichia coli, Staphylococcus aureus, Pseudomonas aeruginosa, Bacillus subtilis, Serratia marcences and Klebsiella pneumoniae* are found as 2.0, 2.0, 2.0, 1.0, 2.5 and 1.5 mg/mL, respectively.

Bactericidal concentration was confirmed to be equal to bacteriostatic concentration for *S. aureus, P. aeruginosa and B. subtilis.* Since the MBC to MIC ratio does not exceed four, *Pistacia vera* skin can be considered as bactericidal agent against *S. marcences, K. pneumonia* and *E. coli,* as well. Among them, *S. marcences* was reported to be the most resistant one against *P. vera* skin in terms of MBC.

Bisignano et al (2013) investigated the antimicrobial activities of polyphenol-rich fractions from raw and roasted shelled pistachios (*Pistacia vera L.*) from California against several food and clinical isolates, ATCC strains of Gram-negative bacteria including *E. coli, P. aeruginosa, P. mirabilis* and Gram-positive bacteria like *L. monocytogenes, B. subtilis and S. aureus*. Extracts, which is dissolved and prepared in methanol/HCl 0.1% (v/v), from raw shelled pistachios were more active than those from roasted ones.

In the same study, *S. aureus* and *L. monocytogenes* were shown to be the most sensitive bacteria to raw pistachios with MIC values 125 and 31.25 μg mL^{-1}, respectively; followed by *B. subtilis* with a concentration of 500 μg mL$^{-1.}$. *P. aeruginosa* (ATCC 27853) and *E. coli* (ATCC 25922) had equal MIC and MBC values of 2.0 mg mL^{-1}.

The bactericidal concentrations of raw extracts against *L. monocytogenes* (ATCC 7644) and *S. aureus* (ATCC 6538P) were 1.0. and 2.0. mg mL^{-1}, respectively.

MIC and MBC of pistachio polyphenol-rich extracts against clinical isolates of methicillin-resistant *Staphylococcus aureus* (MRSA) were also determined and measured as 0.25 and 2.0 mg mL^{-1}, respectively.

In the study, it has shown that pistachio polyphenols have bactericidal effects on *S. aureus* and MRSA strains and the bactericidal activity of the extracts against *L. monocytogenes* could be employed to improve the quality and safety of food (Bisignano et al, 2013).

3.3 EXPLORATION OF ANTIOXIDANT ACTIVITY

3.3.1 Antioxidant Activity by DPPH Method

The *Pistacia vera skin* extracts were investigated as explained in section 2.2.2.2 and the outcomes were provided as EC_{50} values which shows the concentration of polyphenols needed to decrease the 50% of DPPH molecule in Table 3.5. EC_{50} values were computed by using the graph (Fig. 3.8.) showing % RSA versus final extract concentration.

A popular flavonoid Quercetin, which is broadly utilized as a reliable antioxidant in most of the studies, is used as standard in this experiment. Since Quercetin has very high antioxidant activity the graph could not be illustrated in the Figure 3.8 and graph of RSA (%) versus Quercetin concentration was displayed separately (Fig 3.9).

Table 3.5 Fifty percent effective concentration for DPPH radical scavenging capacities

Pistacia vera skin extract and Standard	*DPPH RSA EC50 (µg /mL)
Pistacia vera L. extract	37.5 ± 3.2
Quercetin	3.11 ± 0.1

DPPH RSA EC_{50}: Effective concentration of crude extract or fractions required for scavenging 50% of DPPH radical   *Mean of three independent experiments in duplicates

As it can be seen from Table 3.5, radical scavenging activity of *Pistacia vera L.* skin extract was determined with an EC_{50} value of 37.5 ± 3.2 µg /mL.
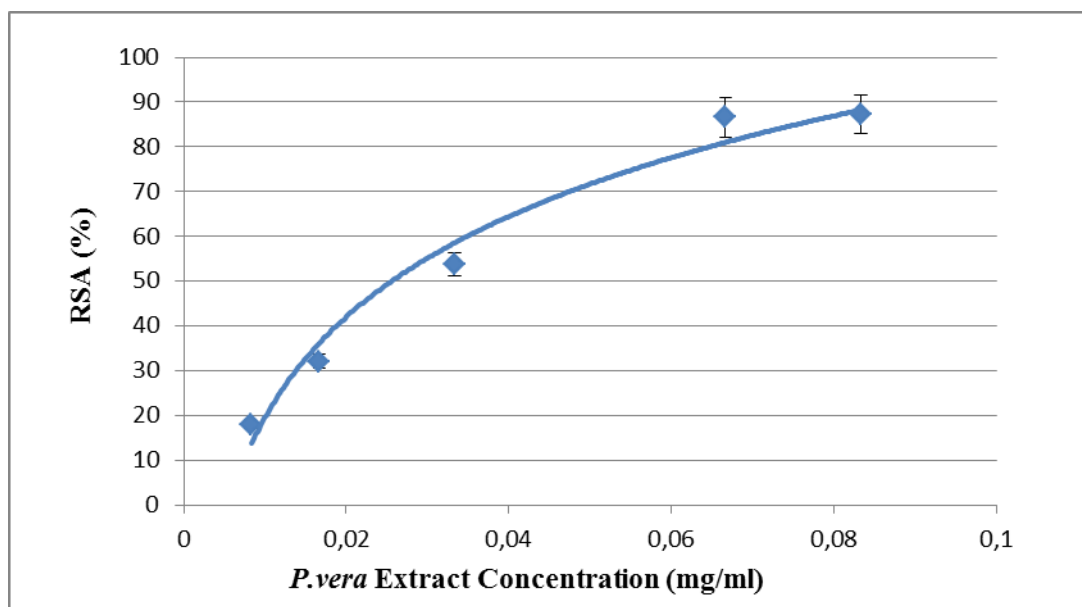
41

Figure 3.12 DPPH radical scavenging activity against concentration (mg/ml) of *Pistacia vera* skin extracts. DPPH radical scavenging activities were measured at 20th minute of incubation period at 517 nm
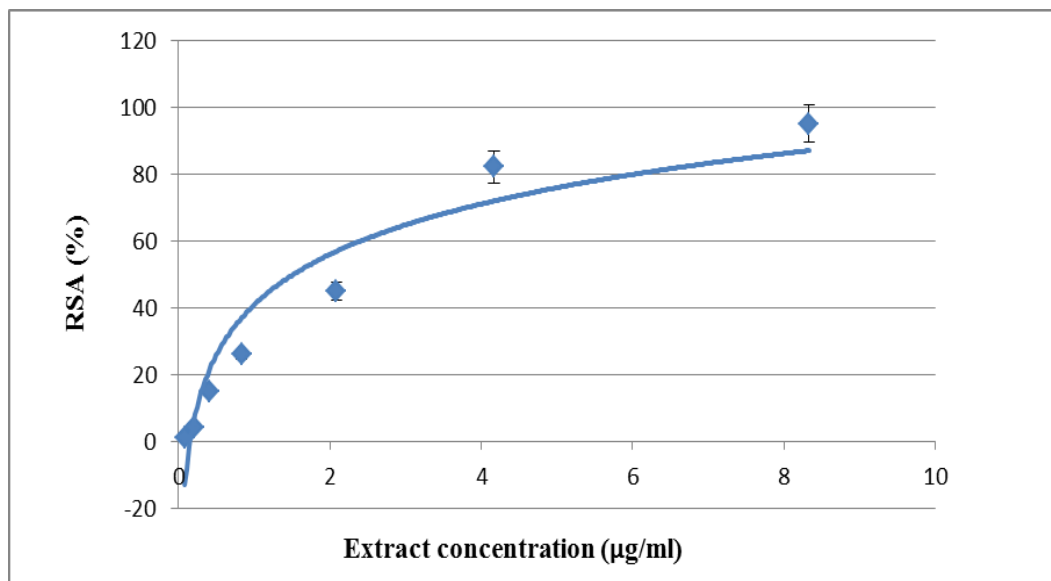

Figure 3.9 RSA (%) versus final concentration graph of Quercetin. Mean of three independent experiments in duplicates

Free radical scavenging activities of *P. vera* skin have been studied by Zoral and Turgay. DPPH (IC_{50}) value of the ethanol extract was found as 18.5 μg /mL (Zoral and Turgay, 2014).

In a recent study, Tomaino et al, 2010 calculated the concentration of pistachio required to scavenge 50 mmoles of initial DPPH found as 14.99 ± 1.43 mg and 0.019 ± 0.001 mg in the skin and seeds, respectively

When pistachio skin is compared to green tea which is one of the greatest antioxidant sources, it is seen that ethanolic extracts of Chinese green tea has IC_{50} values range between 28 and 130 μg leaf/ml (Tian, 2006). In addition, several extracts of hawthorn berries, which is another known potent antioxidant plant, have been shown to have IC_{50} values corresponding to 10 − 50 μg dry berries/ml (Bahourun et al, 1996).

Compared to Quercetin standard, there is about 10 fold difference in terms of radical scavenging activity. This result imply a strong antioxidative capacity of *P. vera* skin because usually between 10 to 100 fold difference is accepted as a potent antioxidant property relative to Quercetin which is one of the most powerful antioxidative bioactive compounds.

3.3.2 Total Phenol Content of Extract of *Pistacia vera L.* skin

In order to calculate total phenolic content responsible for antioxidant activity, we applied Folin-Ciocalteau method as explained in section 2.2.2.1. The obtained calibration curve was shown in Figure 3.10. Calculated values of *P. vera* extracts as GAE were documented in table 3.6. The equation used in calculation was found as y = 123.88x − 0.0596.
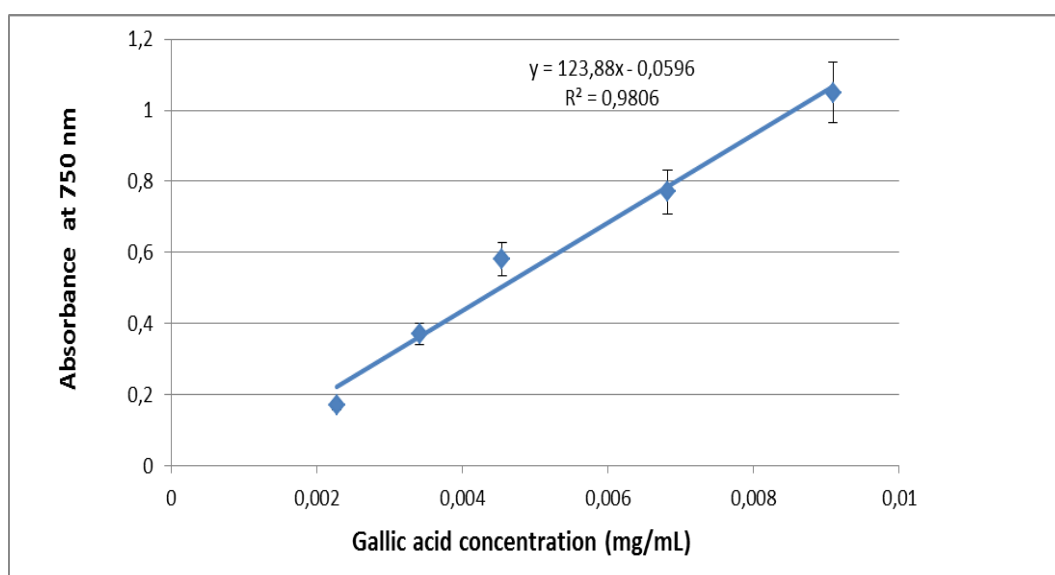
Figure 3.10 Gallic acid calibration curve to calculate total phenolic content of extracts in terms of Gallic acid equivalence.

Each data was collected by means of three independent experiments in duplicates

Table 3.6 Total phenolic content of *Pistacia vera* skin extract

Pistacia vera **Extract**	***TP GAE (mg/g)**
Pistacia vera L. skin	145.32 ± 7.14

TP GAE (mg/g): Total phenolic content mg equivalents of Gallic acid/ g of Pistacia extract
*Mean of three independent experiments in duplicates with standard deviation errors.

As it can be seen from Table 3.6, total phenol content of *P. vera* skin extract is calculated as 145.32 ± 7.14 mg Gallic acid equivalent per g of dry extract.

In a study with *Pistacia vera L.* from Turkey, Sagdıc et al (2008) showed methanol and aqueous extracts of Pistacia shell skins have a total phenolic content of 167.49 ± 0.48 mg GAE/g and 31.73±0.21 mg GAE/g dry extract, respectively.

In 2014, Zoral and Turgay (2014) studied total phenolic compounds in *P. vera* skin and determined the total phenolic content of aqueous extract as 24.79 mg GAE/g dry extract.

In a different study with Sicilian pistachio extracts from Bronte, the antioxidant activity in terms of total phenolic content was measured as 1.65 ± 0.08 and 116.32 ± 8.54 mg of GAE/g of fresh weight in the Folin-Ciocalteau assay, respectively in the seeds and skins (Tomaino et al, 2010).

Our results were very consistent in terms of total phenolic content with Sagdıc et al and Tomaino et al that used methanolic extracts of Antep and Sicillian Bronte *Pistacia vera*, respectively.

It is found that *P. vera* skin extract possessed quite strong antioxidant activities, associated with a very high total phenolic content which is greater than that of some well-known antioxidant-rich plant extracts.

For example, berries are known to exhibit high total phenolic contents and high antioxidant activity. Berries with a strong purple color, such as crowberry, blackberry, bilberry, and aronia, had phenolic contents range between 28.7 and 50.8 mg GAE /g. (Kahkönen et al, 1999).

In addition, in a recent study, green tea, one of the most potent antioxidant plants, was found to have a total phenolic content of 103.0 ± 0.3 mg GAE/g (Dutta et al, 2013).

The results simply imply that high amount of polyphenols found in skins of *P.vera* could be responsible for its strong antioxidant capacity.

Briefly, with respect to their more nutritionally bioactive profiles, it is better to use pistachios as a whole and unpeeled in the diet to be able to get benefit from its rich antioxidant bioactive components.

3.3.3 Total Flavonoid Content of the *Pistacia vera L.* Extract

Total flavonoid content was determined with Quercetin to be used as a choice of standard. Basically, by using this method it is possible to determine the flavonols and flavones. The curve of calibration of Quercetin was drawn against absorbance values at 415 nm. Obtained equation was employed to find the Quercetin equivalent amount of *Pistacia vera* skin (Fig 3.11). The computed outcomes as QE were listed in Table 3.7.
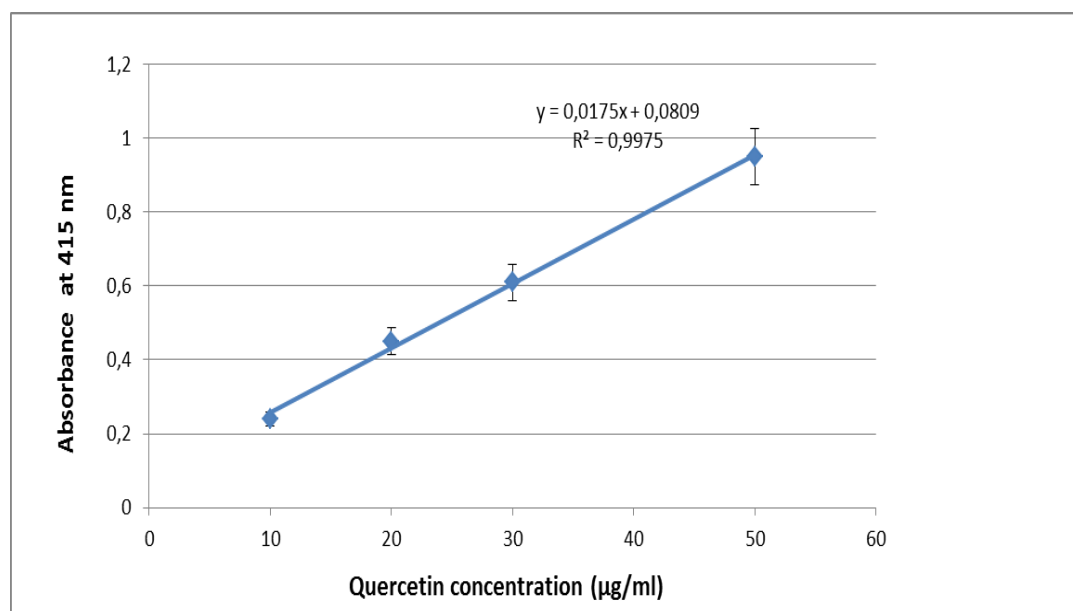
Figure 3.11 Quercetin calibration curve to calculate total flavonoid content of extracts in terms of Quercetin equivalence.

Each data was collected by means of three independent experiments in duplicates.

Table 3.7 Total flavonoid content of *Pistacia vera* skin extract

Pistacia vera **Extract**	***TF QE (µg/mg)**
Pistacia vera L. skin	92.74 ± 9.24

TF QE (µg /mg): Total flavonoid content µg equivalents of Quercetin / mg of Pistacia extract

*Mean of three independent experiments in duplicates with standard deviation errors

As it can be seen from Table 3.7, total flavonoid content of *Pistacia vera* skin extract is determined as 92.74 ± 9.24 µg equivalents of Quercetin / mg of dry extract.

In a recent research conducted, the total amount of flavonoids in *pistacia vera* skin is calculated as 70.27 ± 5.41 mg Catechin equivalent / g of dry extract. However, it is found only 0.46 ± 0.02 mg Catechin eq /g dry extract in seeds. (Tomaino et al, 2010).

Chen O and Blumberg J. (2008) listed several phytochemicals in nuts and determined total flavonoid content of pistachio nuts (seed) and hazelnuts as 0.12 mg/g.

Total flavonoid content method was less readily applied and therefore less researches were present in the literature. Still, total flavonoid content results also confirmed high antioxidant activity of pistacia vera skin compared to seed in addition to DPPH and total phenol content measurements.

3.4 CHROMATOGRAPHIC (LC-MS/MS) ANALYSES

The analysis was performed by METU Central Laboratory, Molecular Biology-Biotechnology Research and Development Center, Mass Spectroscopy Laboratory, Ankara, Turkey,with AGILENT 6460 Triple Quadrupole System (ESI+Agilent Jet Stream) coupled with AGILENT 1200 Series HPLC".

To further specify bioactive molecules that may be responsible for antioxidant and antimicrobial capacity, LC-MS/MS method was used. Components in methanolic extracts of pistachio skins were identified with that of known standards.

The reason that LC-MS/MS is chosen over HPLC is because it is more selective and sensitive technique in identifying the compounds. For these reasons, use of LC-MS technique is preferred commonly in academia and industry recently.

After a literature research, we preferred to identify five bioactive molecules. Among them, Gallic acid, Quercetin and Catechin were observed to be major phenolic molecules which were mainly responsible for the antioxidative and amtimicrobial activity of pistachio skin. Luteolin and Epicatechin values were under 5 ppm.

The results are demonstrated at table 3.8 below and chromatograms also provided in Figures 3.12, 3.13 and 3.14.

Table 3.8 Concentration (μg/g) of certain phytochemicals in *Pistacia vera* skin extract compared to Sicillian Pistachio

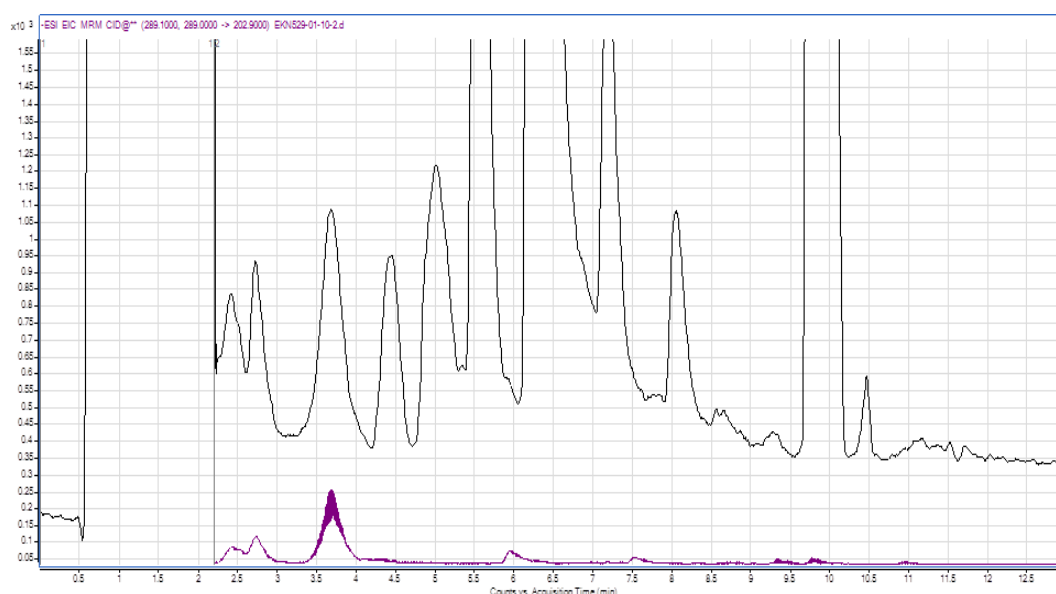| Type of *Pistacia vera l.* skin	Gallic acid	Catechin	Quercetin	Epicatechin	Luteolin
Tested Sample	7721.2 ± 227.2	12.617 ± 0.285	346.33 ± 4.344	1	5
Sicillan Pistachio (Tomaino et al, 2010)	1453.31± 97.6	377.45± 24.4	17.75± 0.65	104.8 ± 10.56	18.97 ± 0.87

Figure 3.12 Chromatogram of Catechin

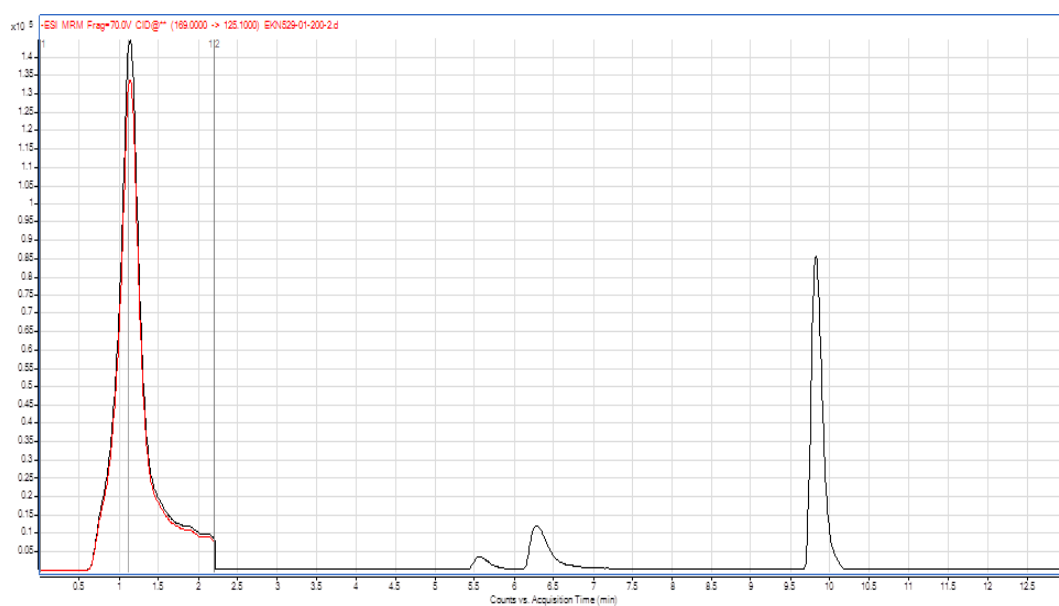*Pistacia vera* sample chromatogram and Catechin peak in the sample

48

Figure 3.13 Chromatogram of Gallic acid

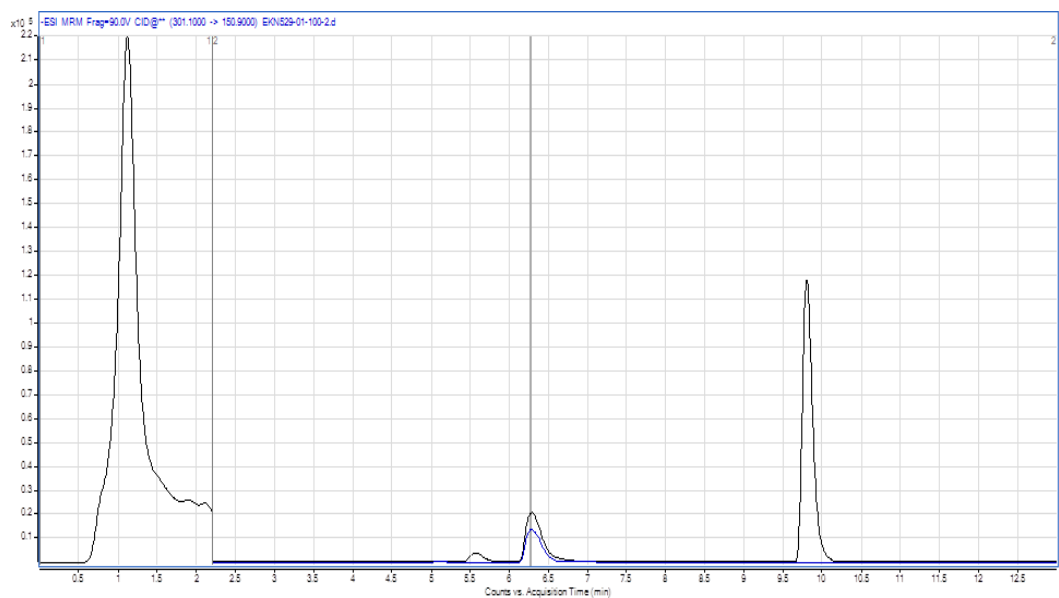*Pistacia vera* sample chromatogram and Gallic acid peak in the sample

Figure 3.14 Chromatogram of Quercetin

Pistacia vera sample chromatogram and Quercetin peak in the sample

As previously stated by Mandalari et al (2013), the main compounds identified in the pistachio nuts were Gallic acid, (+)-catechin and isoQuercetin.

Martorana et al (2013) studied and characterized the phenolic profile of pistachio seeds and skins by HPLC analysis. They identified 13 different phenolic compounds in the skin of *pistacia vera* which are gallic acid, catechin, epicatechin, quercetin, quercetin-3-O-rutinoside, eriodictyol, naringenin, luteolin, kaempferol, eriodictyol-7-Oglucoside, naringenin-7-O-neohesperidoside and anthocyanins cyanidin-3-O-galactoside and cyanidin-3-O-glucoside.

Both seed and skin extracts were shown to contain high levels of phenolic compounds, but the skin extract was about ten times richer in phenols than the seed extract. In fact, the anthocyanins cyanidin-3-O glucoside and cyanidin-3-O-galactoside were identified only in the skin extract as well as epicatechin and the aglycones kaempferol, Quercetin, naringenin, luteolin. In addition, being the only phenolic acid, the amount of Gallic acid and catechin is found much more higher in the skin than in the seed extract.

In pistachio skin, the quantity of cyanidin-3-O-galactoside was found as 5865.12 µg/g. Gallic acid as 1453.32 µg/g, catechin as 377.45 µg/g and eriodictyol rutinoside as 365.68 µg/g followed cyanidin-3-O-galactoside. It has also Quercetin value of 17.75 µg/g. (Tomaino et al, 2010).

With respect to study conducted by Tomaino et al, the concentration of catechin, epicatechin and luteolin found in the Bronte pistachio skin was shown higher but Gallic acid and Quercetin levels were lower than our measurement results.

Bisignano et al (2013) identified some phenolic compounds in raw and roasted salted pistachio from California which are Gallic acid, chlorogenic acid, catechin, epicatechin, Quercetin-3-*O*-rutinoside, isoQuercetin, eriodictyol glucoside, luteolin and daidzein. The quantity of total polyphenols were measured slightly higher in raw pistachios (6.7 mg / 100 g) with respect to roasted nuts (6.0 mg / 100 g).

As stated by Ballistreri et al (2009) roasting maybe responsible for the lower polyphenolic content compared to raw shelled nuts. In fact, being one of the most biologically active flavan-3-ols, catechin were found more than twice in terms of concentration in raw pistachios compared to roasted ones.

The results of antibacterial screening for Quercetin showed that Quercetin and its glycosides have strong antibacterial activity against the gram positive *S. aureus,* and the gram negative *E. coli* and *P. aeruginosa with* zone of inhibition values of 28 mm, 17 mm and 18 mm, respectively (A. M. Metwally et al, 2010). In the same study MIC values of Quercetin was found as 1.25 μg/mL for all strains.

In a study conducted by O. Oyedeji et al (2014) antibacterial activity of Gallic acid was determined as 30.3 mm for *S. aureus,* 31.0 mm for *B.subtilis*, 30.3 mm for *E.coli*, and *S.marcences*, 30.0 mm for K. pneumonia and 28.7 mm for *P. aeruginosa.* The MIC values ranged between 12.5 and 100 μg/mL.

In another study, Gallic acid had antimicrobial activity against the bacteria tested with MIC of 0.5 mg/mL for P. aeruginosa, 1.5 mg/mL for E. coli, 1.75 mg/mL for S. aureus, and 2.0 mg/mL for L. monocytogenes (Borges A et al, 2013).

CHAPTER 4

CONCLUSION

The results related with antioxidant and antimicrobial activities are mostly comparable to previous studies. However, there are some differences in the results which can stem from several reasons. These are related partly to differences in analysis procedures. To explain these different findings further, it is crucial to consider several factors that can affect biological properties and chemical profile of phytochemicals.

Regarding the genetic properties, Barreiras and colleagues (2008) showed quite noticeable diverseness of various plants in antioxidant capacity as well as biochemical compositions.

In addition, various different factors should also be taken into consideration. In fact, the antibacterial and antioxidant activities of plant extracts are strictly correlated to their chemical composition. This composition and their relative chemical concentrations may vary in plant extracts with respect to the geographical location of the plant, climatic and growth environments (temperature, soil, fertilizers, etc.), the part of the plant used, the season that plants were collected, the stage of plant development as well as processing and storage conditions (Kalemba and Kunicka, 2003; Nimri et al, 1999).

Additionally, culture conditions, e.g. the composition of the test medium, temperature and time of incubation, may also influence the result (Nasar-Abbas et al, 2004).

For *Pistacia vera*, it must be also noted that utilizing alcohol extraction result in obtaining a considerably higher polyphenol concentration and, consequently antioxidant activity, compared to the values achieved with water extraction.

In a general evaluation, methanol extract of *P. vera* skin can be regarded as a strong antioxidant relative to standards considering total phenol and flavonoid content in addition to DPPH radical scavenging activity and medium to strong antimicrobial agent against selected bacteria regarding zone of inhibition, MIC and MBC values.

The studies also showed that skin of pistachio has far more polyphenols than seed. One of the reasons that skin contains much more bioactive molecules with respect to seed may stem from the fact that phytochemicals naturally found in skin for protection of the plant against harmful living organisms and extreme climatic conditions.

In future studies, pistachio skin can be further analysed of its biochemical composition that is specifically responsible for its antioxidant and antimicrobial activity. In addition, according to results determined, being a natural antioxidant, skin of *P. vera* can be used to produce a functional food product or as a food additive to prevent biological or microbial spoilage against deterioration.

Moreover, after certain toxicity and clinical studies, *P.vera* skin extract may be used in skin care products to treat skin infections caused by *S.aureus* which can also result an economic improvement for the country since it is normally considered as waste product.

REFERENCES

Aghili M. H., Makhzan-al-Advia, Tehran University of Medical Sciences, Tehran, Iran, 2009, Edited by R. Rahimi, M.R. Shams Ardekani and F. Farjadmand.

Agouni, A.H. Lagrue-Lak-Hal, H.A. Mostefai, A. Tesse, P. Mulder, P. Rouet, F. Desmoulin, C. Heymes, M.C. Martinez, R. Andriantsitohain, Red wine polyphenols prevent metabolic and cardiovascular alterations associated with obesity in Zucker fatty rats (Fa/Fa). 2009, PLoS One 4, 1e8.

Al-Bayati Firas Abbas & Al-Mola Hassan Faesal, J.Edu. Sci. 2007, Vol. (19) No.(3) 59

Altundag K., Mohamed AS, Altundag O, Silay YS, Gunduz E, Demircan K. SRL172 (killed Mycobacterium vaccae) may augment the efficacy of trastuzumab in metastatic breast cancer patients, Med Hypotheses. 2005, 64(2):248-51

Andrikopoulos N. K.,. Kaliora A. C, Assimopoulou A. N., and Papapeorgiou V. P., "Biological activity of some naturally occurring resins, gums and pigments against in vitro LDL oxidation," Phytotherapy Research, 2003, vol. 17, no. 5, pp. 501–507.

Antoniadou A, Kontopidou F, Poulakou G et al. "Colistin-resistant isolates of Klebsiella pneumoniae emerging in intensive care unit patients: first report of a multiclonal cluster *J. Antimicrob. Chemother.* 2007, 59 (4): 786–90

Assimopoulou N. and Papageorgiou V. P., "GC-MS analysis of penta- and tetra-cyclic triterpenes from resins of Pistacia species. Part I. Pistacia lentiscus var. Chia," Biomedical Chromatography, 2005, vol. 19, no. 4, pp. 285–311.

Bahorun T, Soobrattee MA, Luximon-Ramma V, Aruoma OI. Free radicals and antioxidants in cardiovascular health and disease. Internet J Med Update 2006,1:1-17

Bahourun T, Gressier B, Trotin F, et al. Oxygen species scavenging activity of phenolic extracts from hawthorn fresh plant organs and pharmaceutical preparations. *Arzneim Forsch Drug Res.* 1996, 11:1086-1089.

Ballistreri G., Arena E., and Fallico B., "Influence of ripeness and drying process on the polyphenols and tocopherols of *Pistacia vera L.*," Molecules, 2009, vol. 14, no. 11, pp. 4358–4369.

Barreira JC, Ferreira IC, Oliveira MB, Pereira JA. Antioxidant activity and bioactive compounds of ten Portuguese regional and commercial almond cultivarsFood Chem Toxicol. 2008, 46(6):2230-5

Bellomo M. G. and Fallico B., "Anthocyanins, chlorophylls and xanthophylls in pistachio nuts (Pistacia vera) of different geographic origin," Journal of Food Composition and Analysis, 2007, vol. 20, no. 3, pp. 352–359.

Bentley R, Meganathan R "Biosynthesis of vitamin K (menaquinone) in bacteria". *Microbiological Reviews* 1982, 46 (3): 241–80.

Bisignano C. , Angela Filocamo , Richard M. Faulks , Giuseppina Mandalari, In vitro antimicrobial activity of *pistachio (Pistacia vera L.) polyphenols,* 2013, 341(1):62-7.

Chen O and Blumberg J. Phytochemical composition of nuts. Asia Pac J Clin Nutr. 2008, 7 (S1):329-332
Colburn, Anthocyanins inhibit activator protein 1 activity and cell trasformation: structure-activity relationsheep and molecular mechanisms. 2004, Carcinogenesis 25 29e36

Cooper M, Tavankar GR, Williams HD "Regulation of expression of the cyanide-insensitive terminal oxidase in *Pseudomonas aeruginosa*". *Microbiology* 149 (Pt 5) 2003, 1275–84.

Das S, Sheorey H, Taylor HR, Vajpayee RB. Association between cultures of contact lens and corneal scraping in contact lens related microbial keratitis. Arch Ophthalmol 2007, 125: 1182-1185.

DerMarderosian and J. A. Beutler, The Review of Natural Products, Wolters Kluwer Health, Missouri, Mo, USA, 6th edition, 2010

Dillard CJ, German JB. Phytochemicals: Nutraceuticals and human health. J Sci Food Agric. 2000, 80:1744–56.

Droge W. Free radicals in the physiological control of cell function. Review. Physiol Rev. 2002, 82:47-95.

Duke JA. Handbook of medicinal herbs. 2nd Ed. CRC Press, Washington DC. 2002 pp: 118

Durmaz G. and Gökmen V., "Changes in oxidative stability, antioxidant capacity and phytochemical composition of Pistacia terebinthus oil with roasting," Food Chemistry, 2011, vol. 128, no. 2, pp. 410–414.

Ebadi M. Antioxidants and free radicals in health and disease: An introduction to reactive oxygen species, oxidative injury, neuronal cell death and therapy in neurodegenerative diseases. 2001, Arizona: Prominent Press.

Esterbauer H, Pubi H, Dieber-Rothender M. Effect of antioxidants on oxidative modification of LDL.Ann Med. 1991, 23:573–81.

Evans Jr., Doyle J.; Dolores G. Evans. "Escherichia Coli". *Medical Microbiology, 4th edition*. The University of Texas Medical Branch at Galveston.

Folin, O.; Ciocalteu, V. On tyrosine and tryptophane determinations in proteins. J. Biol.Chem. 1927, 73, 627–650

Forbes, B.A., Sham, D.F. & Weissfeld, A. S. Bailey & Scott's diagnostic microbiology (12th ed.). 2007, St. Louis: Mosby.

Genestra M. Oxyl radicals, redox-sensitive signalling cascades and antioxidants. Review. Cell Signal. 2007, 19:1807-19

Gentile, L. Tesoriere, D. Butera et al., "Antioxidant activity of Sicilian pistachio (*Pistacia vera L.* var. Bronte) nut extract and its bioactive components," 2007, Journal of Agricultural and Food Chemistry, vol. 55, no. 3, pp. 643–648.

Goli H., Barzegar M., and. Sahari M. A, "Antioxidant activity and total phenolic compounds of pistachio (Pistachia vera) hull extracts," 2005, Food Chemistry, vol. 92, no. 3, pp. 521–525.

González R, Ballester I, López-Posadas R, Suárez MD, Zarzuelo A, Martínez-Augustin O, et al. Effects of flavonoids and other polyphenols on inflammation. Crit Rev Food Sci Nutr 2011, 51: 331–62.

Greenwood, D., Slack, R.C.B., & Peutherer, J.F. Medical microbiology: A guide to microbial infections: Pathogeneisis, immunity, laboratory diagnosis and control. 2002, 52: 322–48.

Grippi F., Crosta L., Aiello G. et al., "Determination of stilbenes in Sicilian pistachio by high-performance liquid chromatographic diode array (HPLC-DAD/FLD) and evaluation of eventually mycotoxin contamination," Food Chemistry, 2008, vol. 107, no. 1, pp. 483–488,

Halliwell B. Biochemistry of oxidative stress. Biochem Soc Trans. 2007, 35:1147-50

Halvorsen B.L., M.H. Carlsen, K.M. Phillips, S.K. Bøhn, K. Holte, D.R. Jacobs Jr., R. Blomhoff, Content of redox-active compounds (i.e., antioxidants) in food consumed in the United States. Am. J. Clin. Nutr. 84, 2006, 95e135.

Herrera E. *Growing pistachios in New Mexico,* New Mexico State University, Cooperative Extension Service, 1997, Circular 532

Hosseinzadeha H., Abolghasem S., Tabassib S., Moghadamc N. M., Rashediniac M., and Mehri S., "Antioxidant activity of *Pistacia vera* fruits, leaves and gum extracts," Iranian Journal of Pharmaceutical Research, 2012, vol. 11, no. 3, pp. 879–887.

Hudault S, Guignot J, Servin AL. "Escherichia coli strains colonising the gastrointestinal tract protect germfree mice against Salmonella typhimurium infection". 2001, *Gut* 49 (1): 47–55.

Jones RN. Microbial etiologies of hospital-acquired bacterial pneumonia and ventilator-associated bacterial pneumonia. Clin Infect Dis. 2010, 51 (Suppl1):S81-87

Kawecki D, Kwiatkowski A, Sawicka-Grzelak A, Durlik M, Paczek L, Chmura A, Mlynarczyk G, Rowinski W, Luczak M. Urinary tract infections in the early posttransplant period after kidney transplantation: etiologic agents and their susceptibility. Transplant Proc 2011, 43:2991-2993.

Kole, Wild Crop Relatives: Genomic and Breeding Resources Legume Crops and Forages, 2011, Springer, Heidelberg, Germany.

Lau F.C., B. Shukitt-Hale, J.A. Joseph, The beneficial effects of fruit polyphenols on juice on cognitive and motor deficits in aging. Nutrition 22, 2006, 295e302.

Liu T, Stern A, Roberts LJ. The isoprostanes: Novel prostanglandin-like products of the free radical catalyzed peroxidation of arachidonic acid. J Biomed Sci. 1999, 6:226–35.

Longo L., Scardino A., and Vasapollo G., "Identification and quantification of anthocyanins in the berries of Pistacia lentiscus L., Phillyrea latifolia L. and Rubia

peregrina L.," Innovative Food Science and Emerging Technologies, 2007, vol. 8, no. 3, pp. 360–364.

López-Varela S, González-Gross M, Marcos A. Functional foods and the immune system: A review. Eur J Clin Nutr. 2002, 56:S29–33.

Magiatis, P., Melliou, F., Skaltsounis, A., Chinou, I., Mitaku, S., Planta Med. Mahbubeh Bozorgi, Zahra Memariani, Masumeh Mobli, Mohammad Hossein Salehi Surmaghi, Mohammad Reza Shams-Ardekani, and Roja Rahimi Five Pistacia species (P. vera, P. atlantica, P. terebinthus, P. khinjuk, and P. lentiscus): A Review of Their Traditional Uses, Phytochemistry, and Pharmacology The Scientific World Journal Volume 2013, Article ID 219815

Maria Martorana , Teresita Arcoraci , Luisa Rizza , Mariateresa Cristani , Francesco Paolo Bonina , Antonina Saija , Domenico Trombetta , Antonio Tomaino In vitro antioxidant and in vivo photoprotective effect of pistachio (*Pistacia vera L.*, variety Bronte) seed and skin extracts 2013, *Fitoterapia 85 41–48*

Marinou K. A., Georgopoulou K., Agrogiannis G. et al., "Differential effect of *Pistacia vera* extracts on experimental atherosclerosis in the rabbit animal model: an experimental study," 2010, Lipids in Health and Disease, vol. 9, no. 73, pp. 1–9.

Marinova, F. Ribarova, M. Atanassova, Phenolics and total flavonoids in Bulgarian fruits and vegetables, Journal of the University of Chemical Technology and Metallurgy, 2005, 40, 255-260

Marja P. Kahkonen, Anu I. Hopia, Heikki J. Vuorela, Jussi-Pekka Rauha Kalevi Pihlaja, Tytti S. Kujala, and Marina Heinonen Antioxidant Activity of Plant Extracts Containing Phenolic Compounds J. Agric. Food Chem. 1999, 47, 3954 3962

Marone P, Bono L, Leone E, Bona S, Carretto E, Perversi L, Bactericidal activity of Pistacia lentiscus mastic gum against Helicobacter pylori, J Chemother. 2001, Dec; 13(6):611-4

Mehmet Hakkı Alma, Sıegfrıed Nıtz, Hubert Kollmannsberger, Metın Dıgrak, Fatıh Tuncay Efe, and Necmettın Yılmaz, Chemical Composition and Antimicrobial Activity of the essential Oils from the Gum of Turkish Pistachio (*Pistacia vera L.*), *J. Agric. Food Chem.* 2004, *52,* 3911-3914

Merkier AK, Rodríguez MC, Togneri A, Brengi S, Osuna C, Pichel M, Cassini MH; *Serratia marcescens* Argentinean Collaborative Group, Centrón D. Outbreak of a cluster with epidemic behavior due to *Serratia marcescens* after colistin administration in a hospital setting. J Clin Microbiol 2013, 51: 2295-2302

Metwally A. M., A. A. Omar, F. M. Harraz and S. M. El Sohafy, Phytochemical investigation and antimicrobial activity of *Psidium guajava* L. leaves, Pharmacogn Mag. 2010, Jul-Sep; 6(23): 212–218.

Neuzil J, Thomas SR, Stocker R. Requirement for promotion, or inhibition of α-tocopherol of radical induced initiation of plasma lipoprotein lipid peroxidation. Free Radic Biol Med. 1997, 22:57–71.

Oggioni MR, Pozzi G, Valensin PE, Galieni P, Bigazzi C "Recurrent septicemia in an immunocompromised patient due to probiotic strains of *Bacillus subtilis*". *J. Clin. Microbiol.* 1998, 36 (1): 325–6.

Olaoluwa Oyedeji, Festus O. Taiwo, Femi O. Ayinde, Olukayode Solomon Ajayi, Matthew Oziegbe, Monsuru T. Kelani, In vitro Antimicrobial and Antioxidant Analysis of Gallic acid from the Leaves of Ludwigia abyssinica A. Rich, European Journal of Medicinal Plants 2014, 4(9):1098-1112.

Ozcelik, M. Aslan, I. Orhan, and T. Karaoglu, "Antibacterial,antifungal, and antiviral activities of the lipophylic extracts of *Pistacia vera*," *Microbiological Research*, 2005, vol. 160, no. 2, pp. 159–164.

Pacher P, Beckman JS, Liaudet L. Nitric oxide and peroxynitrite in health and disease. Physiol Rev 2007, 87:315-424.

Pelozo, M.I.G.; Cardoso, M.L.C.; Mello, J.C.P. Spectrophotometric determination of tannins and caffeine in preparations from Paullinia. cupana var. sorbilis. Braz. Arch. Biol. Technol. 2008, 51, 447–451.

Rich A., In vitro Antimicrobial and Antioxidant Analysis of Gallic acid from the Leaves of Ludwigia abyssinica

Roberfroid MB. What is beneficial for health? The concept of functional food. Food Chem Toxicol.1999, 37:1034–41.

Romani, P. Pinelli, C. Galardi, N. Mulinacci, and M. Tattini, "Identification and quantification of gallyl derivatives, flavonoid glycosides and anthocyanins in leaves of Pistacia lentiscus L.," Phytochemical Analysis, 2002, vol. 13, no. 2, pp. 79–86.

Ryan, KJ; Ray, CG, eds. (2004). *Sherris Medical Microbiology* (4th ed.). McGraw Hill

Singleton P. *Bacteria in Biology, Biotechnology and Medicine* (5th ed.).1999, Wiley. pp. 444–454

Stefanis L, Burke RE, Greene LA. Apoptosis in neurodegenerative disorders. Curr Opin Neurol.1997, 10:299–305.

Tapas AR, Sakarkar DM, Kakde RB. Review article flavonoids as nutraceuticals: A review. Trop J Pharm Res. 2008, 7:1089–99.

Tian WX, Inhibition of fatty acid synthase by polyphenols. Curr Med Chem. 2006, 13(8):967-77.

Tomaino, M. Martorana, T. Arcoraci, D. Monteleone, C. Giovinazzo, and A. Saija, "Antioxidant activity and phenolic profile of pistachio (*Pistacia vera L.*, variety Bronte) seeds and skins," Biochimie, 2010, vol. 92, no. 9, pp. 1115–1122.

Valko M, Izakovic M , Mazur M, Rhodes CJ, Telser J. Role of oxygen radicals in DNA damage and cancer incidence. Mol Cell Biochem 2004, 266: 37-56

Van der Vorm ER, Woldring-Zwaan C. Source, carriers, and management of *Serratia marcescens* outbreak on a pulmonary unit. J Hosp Infect 2002, 52:263-267.

Vogt RL, Dippold L. "Escherichia coli O157:H7 outbreak associated with consumption of ground beef, June-July 2002". *Public Health Reports (Washington, D.C. : 1974)* 2005, 120 (2): 174–8.

Willcox JK, Ash SL, Catignani GL. Antioxidants and prevention of chronic disease. Review. Crit Rev Food Sci Nutr 2004, 44:275-95.

Zoral Betül, Turgay Özlem, *KSU J. Nat. Sci.* 2014 *17(2),*

Retrived   15.04.2015,   from   Nestle: http://www.nestle.com/csv/case-studies/AllCaseStudies/Sustainable-pistachio-production-Turkey