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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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) 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 marcescens*. 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 ANTIOKSİDAN, ANTİMİKROBİYAL AKTİVİTESİ VE FİTOKİMYASAL ANALİZİ**

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*P. vera* kabuğunu çeşitli çözücülerde hazırlanmış ve metanol, fenolik verim sonuçlarına göre en uygun çözücü olarak 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'*ın quercetin ve gallik asit açısından oldukça zengin kaynaklara sahip olduğunu göstermiştir.

*P. vera* kabuk özütünün 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 *Bacillus subtilis* için 1 mg/ml ve en fazla *Serratia marcescens* için 2.5 mg/ml olarak belirlenmiştir.

**Anahtar Kelimeler:** *P. vera*, Antioksidan, Antimikrobiyal, Serbest Radikal.
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<tr>
<td>mg</td>
<td>Milligram</td>
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<tr>
<td>mL</td>
<td>Milliliter</td>
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<tr>
<td>μL</td>
<td>Microliter</td>
</tr>
<tr>
<td>mm</td>
<td>Millimeter</td>
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<tr>
<td>nm</td>
<td>Nanometer</td>
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<tr>
<td>DPPH</td>
<td>2,2-diphenyl-1-picrylhydrazyl</td>
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<tr>
<td>GAE</td>
<td>Gallic acid equivalent</td>
</tr>
<tr>
<td>LC MS/MS</td>
<td>Liquid chromatography-tandem mass spectrometry</td>
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<tr>
<td>QE</td>
<td>Quercetin equivalent</td>
</tr>
<tr>
<td>MIC</td>
<td>Minimum inhibitory concentration</td>
</tr>
<tr>
<td>MBC</td>
<td>Minimum bactericidal concentration</td>
</tr>
<tr>
<td>a.u.</td>
<td>Absorbance unit</td>
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<td>RSA</td>
<td>Radical scavenging activity</td>
</tr>
<tr>
<td>ROS</td>
<td>Radical Oxygen Species</td>
</tr>
<tr>
<td>RNS</td>
<td>Radical Nitrogen Species</td>
</tr>
<tr>
<td>SD</td>
<td>Standard deviation</td>
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<tr>
<td>TUBIVES</td>
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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•), superoxide (O₂•⁻), peroxyl (ROO•), nitric oxide (NO•), nitrogen dioxide (NO₂•), and lipid peroxyl (LOO•) molecules. Other examples of not free radicals but oxidants which can lead
to free radical reactions in organisms are hydrogen peroxide ($\text{H}_2\text{O}_2$), ozone ($\text{O}_3$), hypochlorous acid (HOCl), nitrous acid (HNO$_2$), singlet oxygen ($^1\text{O}_2$), 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
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 erythematosus, ischemic heart diseases, stroke, gastric ulcers, hypertension, neurological disorders including Parkinson's disease and Alzheimer's disease and more (Stefanis, 1997). (Fig 1.1).
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 first line of defense against attacks of free radicals. It catalyzes the dismutation of superoxide anion radical (O$_2^{•−}$) into
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).

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, $\alpha$-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
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).
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).
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](image)
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)](image)

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)](image.png)

### 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).
1.6 *PISTACIA VERA L.*

1.6.1 Taxonomic Hierarchy

**Table 1.1** Taxonomy of *Pistacia vera*

<table>
<thead>
<tr>
<th>Kingdom</th>
<th>Subkingdom</th>
<th>Superdivision</th>
<th>Division</th>
<th>Class</th>
</tr>
</thead>
<tbody>
<tr>
<td>PLANTAE</td>
<td>VIRIDIPLANTAE</td>
<td>EMBRYOPHYTA</td>
<td>TRACHEOPHYTA</td>
<td>MAGNOLIOPSIDA</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Superorder</th>
<th>Order</th>
<th>Family</th>
<th>Genus</th>
<th>Species</th>
</tr>
</thead>
<tbody>
<tr>
<td>ROSANAE</td>
<td>SAPINDALES</td>
<td>ANACARDIACEAE</td>
<td>PISTACIA</td>
<td><em>PISTACIA VERA L.</em></td>
</tr>
</tbody>
</table>

Figure 1.8 *Serratia marcescens* (Murray et al, 1997)

Figure 1.9 *Pistacia vera* Tree (Ferguson, 2005)
The genus *Pistacia* is a member of the family Anacardiaceae that is composed of 70 genera and more than 500 species. Regular, healthy *Pistacia* 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

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

(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.

![Image of Turkey map with marked regions]

**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, eriodictyol, 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. Moreover, 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$_2$CO$_3$), 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 marcescens (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 Yaylüm 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°C 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°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°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°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 % Na₂CO₃ (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).

![Image of DPPH Molecule]

**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 °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 * (A₀ – A₁)/A₀ where A₀ is the absorbance of the control which contain all reagents except *Pistacia* sample. A₁ shows the absorbance value of the sample tested. Accordingly, EC₅₀ 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 4\textsuperscript{th} C and the hydroxyl groups at 3\textsuperscript{th} and 5\textsuperscript{th} C of flavonols and flavones in the presence of AlCl\textsubscript{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\textsubscript{2}. After 5 min, 0.3 mL of 10\% AlCl\textsubscript{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 \textmu g/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°C before being used and kept at 4°C until used. Then, 5 isolated colonies of bacteria were transferred to previously prepared 5 mL Nutrient broth (NB) and incubated at 37°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.
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 °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 °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
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 °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

<table>
<thead>
<tr>
<th>Solvent</th>
<th>Weight of dry Pistacia vera skin (g)</th>
<th>Total extract (g)</th>
<th>Yield (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Methanol</td>
<td>10</td>
<td>2.14</td>
<td>21.4</td>
</tr>
<tr>
<td>Ethanol</td>
<td>10</td>
<td>1.32</td>
<td>13.2</td>
</tr>
<tr>
<td>Water</td>
<td>10</td>
<td>1.86</td>
<td>18.6</td>
</tr>
<tr>
<td>Acetone</td>
<td>10</td>
<td>0.81</td>
<td>8.1</td>
</tr>
</tbody>
</table>

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 x10⁸ 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 °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.

![Image](a)

**Figure 3.1** Inhibition zone diameters of *P. vera* skin extract against *P. aeruginosa* after 18 hours incubation period at 37 °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.
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.

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.
Figure 3.4 Inhibition zone diameters of *P. vera* skin extract against *E. coli* after 18 hours incubation period at 37 °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

<table>
<thead>
<tr>
<th>Microorganisms</th>
<th>ZOI(mm)</th>
<th>ZOI (mm)*</th>
<th>ZOI (mm)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Extract</td>
<td>Amikacin</td>
<td>Chloramphenicol</td>
</tr>
<tr>
<td><em>E. coli</em></td>
<td>10.2 ± 0.9</td>
<td>17.1 ± 0.8</td>
<td>NT</td>
</tr>
<tr>
<td><em>S. aureus</em></td>
<td>17.1 ± 0.7</td>
<td>22.9 ± 0.7</td>
<td>NT</td>
</tr>
<tr>
<td><em>P. aeruginosa</em></td>
<td>8.3 ± 0.4</td>
<td>20.8 ± 0.0</td>
<td>NT</td>
</tr>
<tr>
<td><em>B. subtilis</em></td>
<td>11.1 ± 1.1</td>
<td>NT</td>
<td>19.2 ± 1.4</td>
</tr>
<tr>
<td><em>S. marcences</em></td>
<td>7.4 ± 0.4</td>
<td>19.0 ± 0.5</td>
<td>NT</td>
</tr>
<tr>
<td><em>Klebsiella</em></td>
<td>9.2 ± 0.5</td>
<td>21.7 ± 0.8</td>
<td>NT</td>
</tr>
</tbody>
</table>

* 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. marcescens* 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.](image)

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).
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>
<thead>
<tr>
<th>Microorganisms</th>
<th>MIC (mg/ml) of the extract</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Escherichia coli</em></td>
<td>1.5</td>
</tr>
<tr>
<td><em>Staphylococcus aureus</em></td>
<td>2.0</td>
</tr>
<tr>
<td><em>Pseudomonas aeruginosa</em></td>
<td>2.0</td>
</tr>
<tr>
<td><em>Bacillus subtilis</em></td>
<td>1.0</td>
</tr>
<tr>
<td><em>Serratia marcences</em></td>
<td>1.0</td>
</tr>
<tr>
<td><em>Klebsiella pneumoniae</em></td>
<td>0.5</td>
</tr>
</tbody>
</table>

*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 aseptic 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. marcescens* and *K. pneumoniae*.

Sterility control: Test the experiments if they were performed in aseptic 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, streptomycin, 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 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>
<thead>
<tr>
<th>Microorganisms</th>
<th>MBC (mg/ml) of the extract</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Escherichia coli</em></td>
<td>2.0</td>
</tr>
<tr>
<td><em>Staphylococcus aureus</em></td>
<td>2.0</td>
</tr>
<tr>
<td><em>Pseudomonas aeruginosa</em></td>
<td>2.0</td>
</tr>
<tr>
<td><em>Bacillus subtilis</em></td>
<td>1.0</td>
</tr>
<tr>
<td><em>Serratia marcescens</em></td>
<td>2.5</td>
</tr>
<tr>
<td><em>Klebsiella pneumoniae</em></td>
<td>1.5</td>
</tr>
</tbody>
</table>
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 marcesces* 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. marcesces*, *K. pneumonia* and *E. coli*, as well. Among them, *S. marcesces* 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>
<thead>
<tr>
<th><em>Pistacia vera skin extract and Standard</em></th>
<th><em>DPPH RSA EC50 (µg/mL)</em></th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Pistacia vera L. extract</em></td>
<td>37.5 ± 3.2</td>
</tr>
<tr>
<td>Quercetin</td>
<td>3.11 ± 0.1</td>
</tr>
</tbody>
</table>

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.
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 (IC₅₀) 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 IC₅₀ 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 IC₅₀ 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

<table>
<thead>
<tr>
<th><em>Pistacia vera</em> Extract</th>
<th><em>TP GAE (mg/g)</em></th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Pistacia vera</em> L. skin</td>
<td>145.32 ± 7.14</td>
</tr>
</tbody>
</table>

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, Sagdic 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 $1.65 \pm 0.08$ and $116.32 \pm 8.54$ 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 Sagdic et al and Tomaino et al that used methanolic extracts of Antep and Sicilian 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. (Kakkönen 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 $103.0 \pm 0.3$ 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](image)

*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

<table>
<thead>
<tr>
<th><em>Pistacia vera</em> Extract</th>
<th><em>TF QE (μg/mg)</em></th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Pistacia vera</em> L. skin</td>
<td>92.74 ± 9.24</td>
</tr>
</tbody>
</table>

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 $92.74 \pm 9.24 \mu g$ equivalents of Quercetin / mg of dry extract.

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

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 antimicrobial 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 Sicilian Pistachio

<table>
<thead>
<tr>
<th>Type of <em>Pistacia vera</em> l. skin</th>
<th>Gallic acid</th>
<th>Catechin</th>
<th>Quercetin</th>
<th>Epicatechin</th>
<th>Luteolin</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tested Sample</td>
<td>7721.2 ± 227.2</td>
<td>12.617 ± 0.285</td>
<td>346.33 ± 4.344</td>
<td>1</td>
<td>5</td>
</tr>
<tr>
<td>Sicilian Pistachio (Tomaino et al, 2010)</td>
<td>1453.31 ± 97.6</td>
<td>377.45 ± 24.4</td>
<td>17.75 ± 0.65</td>
<td>104.8 ± 10.56</td>
<td>18.97 ± 0.87</td>
</tr>
</tbody>
</table>

**Figure 3.12** Chromatogram of Catechin

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