ANTIOXIDANT CAPACITIES OF SELECTED FRUITS AND HERBAL TEAS CONSUMED IN REGULAR DIET AND THEIR ANTIMICROBIAL ACTIVITIES AGAINST STAPHYLOCOCCUS AUREUS

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Approval of the thesis:

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

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

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

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

Examining Committee Members:

Prof. Dr. Mesude İşcan _____
Biology Dept., METU

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

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

Prof. Dr. Candan Gürakan _____
Food Engineering Dept., METU

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

Date: 04.02.2011

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

Name, Last name: Burak BARUT

Signature :

ABSTRACT

ANTIOXIDANT CAPACITIES OF SELECTED FRUITS AND HERBAL TEAS CONSUMED IN REGULAR DIET AND THEIR ANTIMICROBIAL ACTIVITIES AGAINST STAPHYLOCOCCUS AUREUS

BARUT, Burak
M.Sc., Department of Biochemistry
Supervisor: Assoc. Prof. Dr. Nursen ÇORUH

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Staphylococcus aureus is one of the major causes of food-borne pathogenesis. Antibiotic consumption for these pathogens has been increasing year by year world-wide. In order to decrease the use of synthetic antibiotics, fresh fruits and dry herbs consumed as beverages in regular diets were examined as potential natural antibiotics for the treatment of food based infections against *Staphylococcus aureus*.

Herbs consumed as tea infusions including *Pimpinella anisum L.* (anise), *Anthemis arvensis L.* (camomile), *Rosa canina L.* (rosehip), *Salvia fruticosa Mill* (sage) and fresh fruit juices including *Vitis vinifera L.* (grape), *Citrus sinensis L*. (orange), *Prunus persica L.* (peach) and *Punica granatum L.* (pomegranate) were selected as samples of hot or cold consumed beverages in our daily diets. Extracts of fresh fruit juices were prepared in methanol, on the other hand, tea infusions of herbs were filtered and lyophilized. Antioxidant capacities of the plant samples were investigated by radical scavenging methods, namely 2'2-azinobis-(3-ethylbenzothiazoline-6-

sulfonic acid) (ABTS) and 2,2-diphenyl-1-picrylhydrazyl (DPPH) as well as determination of total phenolic and flavonoid contents. Furthermore, antimicrobial activities of plant samples were determined by minimum inhibitory concentration and minimum bactericidal concentration methods along with disc diffusion method.

Trolox equivalent antioxidant capacities (TEAC) of the herbal tea infusions obtained by ABTS radical scavenging method were ranged between 48.38 ± 1.242 and 715.73 ± 4.265 (µmol Trolox equivalent (TE)/g of extract) while, TEAC values of fresh fruits juices were between 26.86 ± 0.217 and 73.55 ± 0.973 (µmol Trolox equivalent (TE)/g of extract). Moreover, EC_{50} values of the tea infusions obtained by DPPH radical scavenging method were ranged between 0.05 ± 0.001 and 1.53 ± 0.004 (mg/mL) while, EC_{50} values of the fruit juices were 1.6 ± 0.014 and 2 ± 0.093 (mg/mL). Total phenolic content of the plant samples tested in this study were varied from 1.383 (µg gallic acid equivalent (GAE)/mg of extract) to 159.167 (µg gallic acid equivalent (GAE)/mg of extract) and total flavonoid content varied from 0.111 (µg quercetin equivalent (QE)/mg of extract) to 201.15 (µg quercetin equivalent (QE)/mg of extract).

Sage, orange and grape displayed higher antimicrobial activities with values of 1.5, 6 and 6 mg/mL minimum inhibitory concentrations and 1.5, 24 and 24 mg/mL minimum bactericidal concentrations, respectively. Inhibition zone diameters of sage, orange and grape were found to be 9, 9 and 11 mm.

Keywords: *Staphylococcus aureus,* Radical scavenging, Total phenol, Total flavonoid, Antimicrobial activity

ÖZ

GÜNLÜK DİYETTE TÜKETİLEN BAZI MEYVELERİN VE BİTKİ ÇAYLARININ ANTİOKSİDAN KAPASİTELERİ İLE STAPHYLOCOCCUS AUREUS ÜZERİNDE ANTİMİKROBİYAL ETKİLERİNİN ARAŞTIRILMASI

BARUT, Burak
Yüksek Lisans, Biyokimya Bölümü
Tez Yöneticisi: Doç. Dr. Nursen ÇORUH

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Staphylococcus aureus gıda kaynaklı hastalıklara yol açan en önemli patojenlerden biridir. Dünya çapında bu patojenlere karşı antibiyotik tüketimi her geçen yıl artmaktadır. Sentetik antibiyotik kullanımını azaltmak için, günlük diyette tüketilen meyve suları ve bitki çayları gibi bazı içeceklerin gıda kaynaklı hastalıklara yol açan *S. aureus* organizmasının tedavisinde doğal antibiyotik olarak kullanılabilme potansiyelleri incelenmiştir.

Günlük diyette sıcak veya soğuk tüketilen içeçekler olarak bitki çaylarından *Pimpinella anisum L.* (anason*)*, *Anthemis arvensis L.* (papatya), *Rosa canina L.* (kuşburnu), *Salvia fruticosa Mill* (adaçayı) ve meyve sularından *Vitis vinifera L.* (üzüm*)*, *Citrus sinensis L*. (portakal), *Prunus persica L.* (şeftali) and *Punica granatum L.* (nar) seçilmiştir. Meyve suyu özütleri metanolde hazırlanıp liyofilize edilirken, bitki çayları filtreden geçirilip liyofilize edilmiştir. Bitki örneklerinin antioksidan kapasitelerinin tayini için radikal sönümleme

metotları olan 2,2’-azinobis-(3-etilbenzotiyazolin-6-sülfonik asit) (ABTS) ve 2,2-difenil-1-pikrilhidrazil (DPPH) metotlarının yanı sıra total fenolik ve total flavonoid madde miktarları da saptanmıştır. Antimikrobiyal aktivite tayini ise disk difüzyon testi, minimum inhibe edici konsantrasyon ve minimum bakterisidal konsantrasyon metotlarıyla gerçekleştirilmiştir.

ABTS radikal sönümleme metoduyla elde edilen bitki çaylarının trolox eşleniği antioksidan kapasiteleri (TEAK) 48.38 ± 1.242ve 715.73 ± 4.265 (µmol trolox eşleniği (TE)/g özüt) değerleri arasında değişirken, taze meyve örneklerinin değerleri 26.86 ± 0.217 ve 73.55 ± 0.973 (µmol trolox eşleniği (TE)/g özüt) aralığındadır. Yine, DPPH radikal sönümleme metoduyla bitki çaylarının EC_{50} verilerinin 0.05 ± 0.001 ve 1.53 ± 0.004 (mg/mL) değerleri arasında değiştiği tespit edilmişken, taze meyve örneklerinin EC_{50} verileri 1.6 ± 0.014 ve 2 ± 0.093 (mg/mL) aralığında bulunmuştur. Ayrıca, çalışmada kullanılan bitkilerin total fenolik madde miktarları 1.383-159.167 (µg gallik asit eşleniği/mg özüt) aralığında yer alırken, total flavonoid miktarları ise 0.111-201.15 (µg quercetin eşleniği/mg özüt) aralığındadır.

Adaçayı, portakal ve üzüm en yüksek antimikrobiyal etki gösteren bitki özütleri olarak bulunmuş olup sırasıyla, minimum inhibe edici konsantrasyonları 1.5, 6 ve 6 mg/mL, minimum bakterisidal konsantrasyonları ise 1.5, 24 ve 24 mg/mL’dir. Ayrıca bu bitkilerin inhibe ettikleri alanların çapları 9, 9 ve 11mm’dir.

Anahtar Kelimeler: *Staphylococcus aureus,* Radikal sönümleme, Total fenol, Total flavonoid, Antimikrobiyal aktivite

To my family

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LIST OF ABBREVIATIONS

mg Milligram

mL Milliliter

µL Microliter

mm Millimeter

BHI Brain Heart Infusion

ABTS 2,2'-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid)

DPPH 2,2-diphenyl-1-picrylhydrazyl

RSA Radical Scavenging Activity

TP Total Phenolics

TF Total Flavonoids

TEAC Trolox equivalent antioxidant capacity

GAE Gallic acid equivalent

QE Quercetin equivalent

CFU Colony forming unit

MIC Minimum inhibitory concentration

MBC Minimum bactericidal concentration

DW Dry weight

CHAPTER 1

INTRODUCTION

1.1 Free Radicals

Free radicals are the electrically charged molecules known as natural by-products of human metabolism which are continuously produced by the use of oxygen, like respiration and some cell-mediated immune functions. Free radicals are able to pass through cellular membranes to react with the nucleic acids, proteins, and enzymes present in the cells of human body. In other words, these electrically charged molecules attack our cells which result in oxidative stress. Oxidative stress affects the cells negatively and might cause the cells to lose their structure and function, even it might destroy our cells (Govindarajan, 2005).

1.2 Antioxidants

Antioxidants are the substances that are capable of reducing or preventing the oxidative stress. Moreover, they are capable of counteracting the damaging effects of electrically charged molecules, free radicals, in our cells and tissues. Therefore, it is believed that, antioxidants protect against several diseases including cancer, arteriosclerosis, heart disease (Bandyopadhyay, 2006). Vegetables, fruits and medicinal herbs displayed important antioxidant capacities (Zheng, 2001; Shobana, 2000; Madsen, 1995; Kahkonen, 1999). Phenolic compounds are the major constituent responsible for antioxidant activities (Shan, 2005).

1.3 Plant Phenols

Phenols are the organic compounds that has hydroxyl (-OH) group attached to benzene ring. The plant phenols have various kinds of secondary metabolites possessing an aromatic ring with one or more hydroxy substituents and they are obtained from the shikimate pathway and phenylpropanoid metabolism. Plant phenols have been divided into major groups which are namely flavonoids, phenolic acids, hydroxycinnamic acid derivatives and lignans as presented in Figure 1.1. They can be characterized by the number of constitutive carbon atoms within the structure of the basic phenolic skeleton. Flavonoids are the most widespread and diverse of group of plant phenolics (Ryan, 1999).

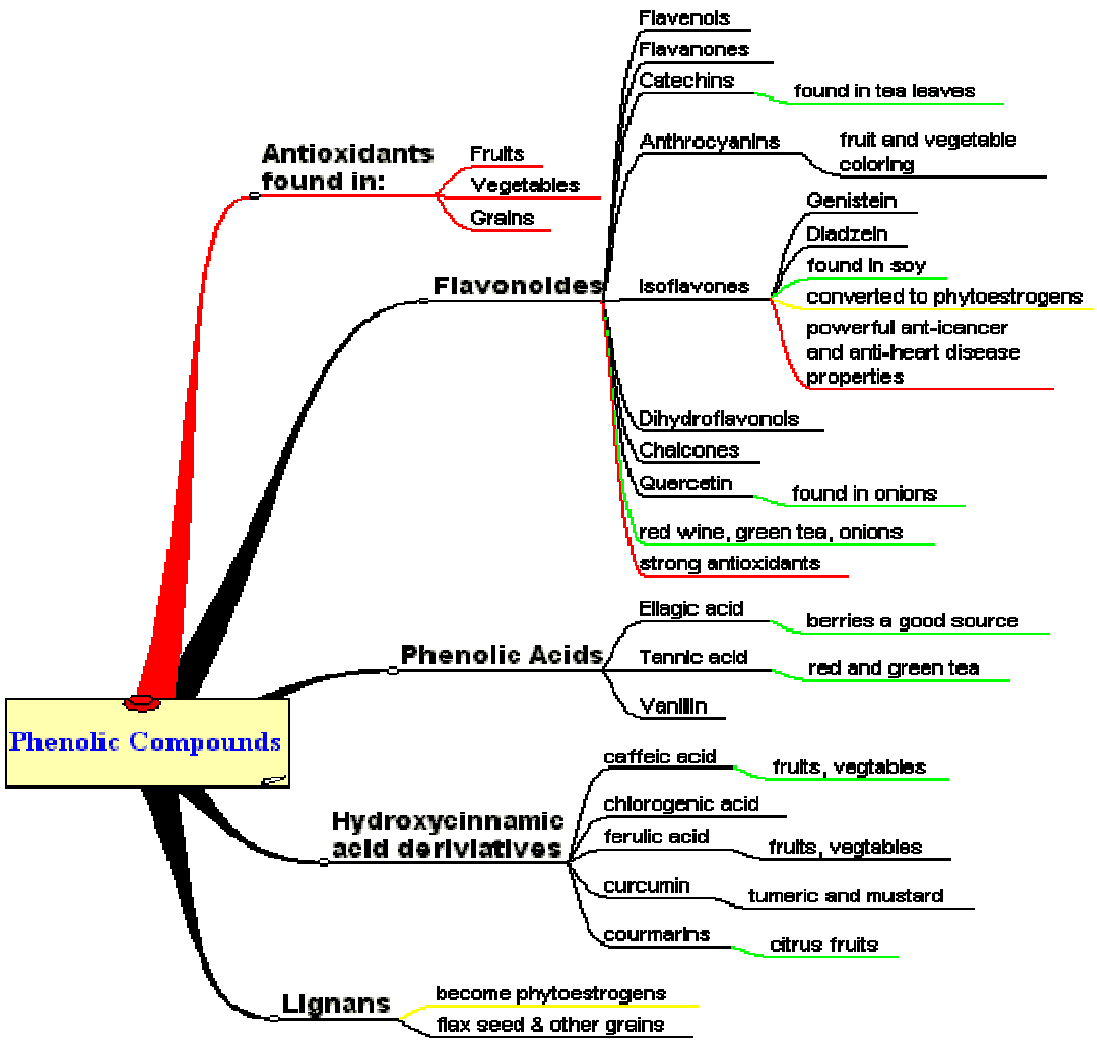

Figure 1.1 Plant phenolic compounds (Chi, 1997).

1.4 Medicinal Plants in Treatment of Microbial Infections

The use of plants for healing of certain diseases had appeared long before the mankind discovered the existence of microbes. Currently, widely accepted antimicrobial principles were adapted in the past, centuries ago. Antibacterial effects of the plants had been known for years. Mankind has used plants for treating common infectious diseases and some of these alternative natural medicines are still in part of the treatment of various illnesses (Rios, 2005). Modern medicine has arisen from traditional medicine after the chemical and pharmaceutical screening appeared. Since, the use of synthetic compounds has increased, use of plant material in modern medicine declined. Nevertheless, synthetic compounds can cause unwanted side effects in human body. Therefore, using synthetic drugs are less favorable than using natural compounds that are obtained from plants and plants appeared to be the major source of medicinal compounds which have therapeutic properties. (Dalal, 2010).

Plant materials are the major part of our diet. They are rich natural sources of antioxidants, antimicrobial compounds as well as other nutrients (Hänninen, 2000). The substances that have the ability to inhibit the growth of bacteria or the ability to kill them without harming the host cells are regarded to be candidates for producing new antimicrobial agents (Ahmad, 2001).

Therefore, in this study, antimicrobial effects against *S. aureus* and antioxidant properties of some of the fruit juices and herbal teas that are widely used in our daily diet will be studied.

1.5 Herbal Teas and Fruit Juices in Regular Diet

In this study, fresh fruits and herbal teas in our daily diet were selected to be tested since these fruits and herbs have great range of valuable chemical substances. The list of the plant materials are given below;

Sage: *Salvia fruticosa Mill*
Anise: *Pimpinella anisum L.*
Rosehip: *Rosa canina L.*
Camomile: *Anthemis arvensis L.*
Grape: *Vitis vinifera L.*
Orange: *Citrus sinensis L.*
Peach: *Prunus persica L.*
Pomegranate: *Punica granatum L.*

Grape: Grape and its seeds contain flavonoids including catechin, epicatechin, procyanidins and anthocyanins. They also contain phenolic acids (gallic acid and ellagic acid) and stilbenes (resveratrol and piceid). Grape seeds and grape itself can show health functional activities with these compounds (Yilmaz, 2006). Baydar *et. al* determined the antimicrobial effects of *V. vinifera* against *S. aureus* with disc diffusion method. *V. vinifera* seed extracts was found to be important and economical antibacterial agents (Baydar, 2006).

Orange: Orange juice is characterized by its rich phenolic profile and antioxidant properties. Mainly, orange juice was found to contain anthocyanins, cinnamates, and ascorbic acid (Lo Scalzo, 2004).

Peach: Peach is one of the most popular and widely consumed fruit all over the world because of the high nutrient level inside and pleasant flavor. Peach also contains high concentration of phenolic compounds (Wang, 2006).

Pomegranate: Pomegranate is one of the ancient fruit that takes part in our diet as fresh fruit and juice. Wide range of phytochemicals has been linked to the health benefits of pomegranate. The phytochemicals in pomegranate structure are mainly polyphenols including ellagitannins and anthocyanins (Patel, 2008). Duman and his co-workers tested the antimicrobial activities of six *P. granatum* varieties by disc diffusion and minimum inhibitory

concentration determination methods against *S. aureus*. MIC values were ranged between 40 µg/mL and >90 µg/mL (Duman, 2009).

Sage: *Salvia fruticosa* is a perennial herb which belongs to Lamiaceae family. *S. fruticosa* is thought to be one of the most important *Salvia* species used for medicinal purposes (Arikat, 2004). Askun *et. al* examined the MIC value of methanolic extract of *S. fruticosa* which was collected in Balıkesir. It was found to be 5120 µg/mL (Askun, 2009).

Anise: Chemical studies on *Pimpinella anisum* L. displayed that it contained anethole (Chandler, 1984; Fujita, 1960), estragole (Zargari, 1989), eugenol (Monod, 1950), pseudoisoeugenol (Reichling, 1995), methylchavicol and anisaldehyde (Wagner, 1984), coumarins, scopoletin, umbelliferon, estrols (Burkhardt, 1986), terpene hydrocarbons (Kartnig, 1975), polyenes and polyacetylenes (Schulte, 1970) as the major compounds (Gülçın, 2003).

Rosehip: Fresh rosehip has rich vitamin C content and it is widely used for food production. In addition to vitamin C content, rosehip contains carotene, tocopherol (Nicoara, 1974; Valadon, 1975; Stepanov, 1983; Biacs and Daood, 1994; Ivanov and Aizetmueller, 1998), flavonoids (Jennen, 1972; Jennen, 1973; De Vries, 1974; Marshall, 1975; Asen, 1982; De Vries, 1980), amino acids (Izhaki, 1998), fruit-acids and tanning substances as well (Muhitch and Fletcher, 1984). Shiota *et. al* examined the antimicrobial activity of *R. canina* extracts on *S. aureus* in the presence of various antimicrobial compounds. They concluded that *R. canina* extracts signifacantly reduced the MIC values of selected antimicrobial compounds (Shiota, 2006).

Camomile: There are three main classes of metabolites have been detected from *Anthemis arvensis L*. such as polyacetylenes (Christensen, 1992), flavonoids (Williams, 2001) and sesquiterpene lactones (Bulatović, 1998) so far (Vučković, 2006).

1.6 *Staphylococcus aureus*

Staphylococcus aureus is a non-motile, sphere-shaped, Gram positive bacterium (coccus) 0.5 to 1.0 μm in diameter. Microscopic examination of *S. aureus* shows that bacteria appear in pairs, short chains, or grapelike clusters (*staphylo* means grape in Greek) (Bremer, 2004).

S. aureus can be found closely associated with the human body. They may also be detected in many parts of our environment, such as dust, water, air and faeces and on clothing or utensils. Even though, *S. aureus* is a significant pathogen, many healthy people carry it in their bodies. *S. aureus* population might be located in nose, throat, perineum or skin as a part of the normal population of microorganisms in body. The *S. aureus* carrier rate can be different in different populations. The nasal passages are stated that 10-50% of the healthy population harbours *S. aureus.* (Bremer, 2004).

The Scientific Classification of *S. aureus* is given as;

Domain : Bacteria
Kingdom : Eubacteria
Phylum : Firmicutes
Class : Bacilli
Order : Bacillales
Family : Staphylococcaceae
Genus : *Staphylococcos*
Species : *S. aureus*

Binomial nomenclature: *Staphylococcus aureus* (Rosenbach, 1884)

Figure 1.2 Colonies of *Staphylococcus aureus*

Staphylococcus aureus causes several infections including food borne illnesses, various types of skin eruptions and inflammations (boils, acne, styes, etc.) and wounds, minor damage around fingernails, respiratory infections and enteritis (Bremer, 2004).

Staphylococcal food poisoning (staphyloenterotoxicosis; staphyloenterotoxemia) is the condition caused by the *S. aureus* enterotoxins. Syptoms of the early stages of staphylococcal food poisoning commonly occur fast and in various acute cases, based on the personal sensibility to toxins, amount of contaminated food eaten by the individual and the general health status of the individual. The most frequent symptoms of the staphylococcal food poisoning are nausea, vomiting, retching, abdominal cramping, and prostration. Some of the affected individuals might not always exhibit all the symptoms associated with the food poisoning and recovering from staphylococcal food poisoning generally takes two days. In more acute cases, symptoms can be more severe. Headache, muscle cramping, and

transient changes in blood pressure and pulse rate can be seen. Moreover, it takes three days or even more in severe cases for complete recovery. (FDA; U.S. Food and Drug Administration, 2009). In the U.S., there were 127 outbreaks involving 7,082 cases of food borne illnesses caused by bacterial pathogens in 1983 and 14 outbreaks involving 1,257 cases were caused by *S. aureus*. These outbreaks caused by *S. aureus* were followed by 11 outbreaks (1,153 cases) in 1984, 14 outbreaks (421 cases) in 1985, 7 outbreaks (250 cases) in 1986 and one reported outbreak (100 cases) in 1987. (FDA U.S. Food and Drug Administration, 2009)

Also, outbreaks and cases of food-borne poisoning caused by some Gram-positive bacteria in France between 1999 and 2000 are given in the table below (Haeghebaert, 2002).

Table 1.1 Causative agents of food-borne disease outbreaks recorded in France between 1999 and 2000. Frequencies of each type of agents are given in percent. (Haeghebaert, 2002)

Causative agents	**Outbreaks (N=530)**	**Cases (N=6451)**	**Hospitalizations (N=872)**	**Death (N=7)**
Salmonella sp. (Enteritidis, Typhimurium, Heidelberg and other serotypes)	63.8	47.7	16.8	100
Staphylococcus aureus	16	25.6	17.1	0
Clostridium pefringens	5.1	12.3	0.5	0
Bacillus cereus	2.8	3.7	10.0	0
Histamine	3.8	1.4	30.4	0
Other pathogens (*Campylobacter* sp., *Dinophysis*, *C. botulinum*, *Shigella* sp., Calicivirus, HAV, *Vibrio* sp., *E. coli*, etc.)	8.5	9.2	7.6	0

1.7 Methods for Determination of Antioxidant Capacities

1.7.1 ABTS Method

2,2′-azinobis (3-ethylbenzothizoline-6-sulfonic acid) radical cation ($ABTS^{+\bullet}$) has been widely used for investigating the antioxidant capacities. The reaction between antioxidant and ABTS radical cation is monitored at 734 nm (Henriquez, 2002). ABTS method can be applied to both lipophilic and hydrophilic antioxidants, including flavonoids, hydroxycinnamates, and carotenoids. ABTS radical cation is formed by oxidation of ABTS with potassium persulfate and $ABTS^{+\bullet}$ is reduced by antioxidants (Re, 1998). The best results are derived from generation of ABTS radical cation in the presence of peroxodisulfate with an ABTS/peroxodisulfate concentration ratio equal (or higher) to two (Henriquez, 2002).

1.7.2 DPPH Method

Scavenging of stable 2,2-diphenyl-1-picrylhydrazyl (DPPH) free radicals can be used to investigate the antioxidative capacities of compounds in a short time (Bandonien, 2002). The principle of DPPH test is donation of hydrogen atoms from antioxidants to the stable DPPH free radicals. The reduction reaction of DPPH radical results in corresponding hydrazine accompanied by color change. The color of the solution changes from violet to yellow which is screened spectrophotometrically at 517 nm (Stanojević, 2009).

1.7.3 Determination of Total Phenols

Total phenolic compounds are determined by using Folin-Ciocalteu reagent. Total phenol, Folin-Ciocalteu assay is based on electron transfer from phenolic compounds to the mixture of molybdenum and tungsten oxides to form blue molybdenum-tungsten complex which can be examined

spectrophotometrically at 750 nm (Ainsworth, 2007; Waterhouse, 2002; Peñarrieta, 2007). Oxidation of phenolic compounds can be achieved only at alkaline pH in a solution of sodium carbonate (Peñarrieta, 2007).

1.7.4 Determination of Total Flavonoids

The most widespread method used for investigation of total flavonoid content is a spectrophotometric assay which relies on the generation of complexes between aliminium ions and hydroxyl and carbonyl groups of flavonoids (Popova, 2004). Pink colored aliminium ion-flavonoid complex can be formed at alkaline pH (Peñarrieta, 2007), and the complex is monitored spectrophotometrically at 510 nm.

1.8 Methods for Determination of Antimicrobial Activities

1.8.1 Disc Diffusion Test: The Kirby-Bauer Method

Disc diffusion test in other words antimicrobial susceptibility testing (AST) is one of the most significant assays performed in many clinical microbiology laboratories. It is critical for selecting the best antimicrobial drug for the treatment and for epidemiological monitoring. Even though, suitable methods for detecting resistance to antimicrobials are available, disc diffusion technique is still most widely used (Felmingham and Brown, 2001). Moreover, the disc diffusion test is very effective and drug combinations can be changed easily (Berke and Tierno, 1996).

AST is performed according to a standard the Clinical and Laboratory Standards Institute (CLSI; formerly the NCCLS) as reference procedure (Lestari, 2008). In short, bacterial suspension of interest is spread on the surface of agar. Then, antibiotic discs are placed carefully on the agar surface. After the overnight incubation, inhibition zones of bacteria are

measured with the help of a ruler or a caliper. The inhibition zones are recorded in millimeter. The susceptibility category which can be classified as sensitive, intermediate susceptible or resistant, is investigated by comparing the zone of inhibition with the zone diameter breakpoint as recommended by the CLSI. ([National Committee for Clinical Laboratory Standards, 2000], [National Committee for Clinical Laboratory Standards, 2000] and [National Committee for Clinical Laboratory Standards, 2000]) (Lestari, 2008).

1.8.2 Minimum Inhibitory Concentration

Minimum inhibitory concentrations (MICs) can be described as the lowest concentration of an antimicrobial agent that inhibits the visible growth of a microorganism after overnight incubation. In diagnostic laboratories, MICs are used for approving the unusual resistance when disc diffusion methods do not work (Andrews, 2001).

Resistance of bacterial pathogens to antimicrobial drugs can cause significant problems while treating animals and humans infected by pathogens. Investigation of Minimum Inhibitory Concentrations of certain antibiotics for certain bacteria plays a crucial role in order to determine the antibiotic resistance of bacteria of interest (Islam, 2008).

1.8.3 Minimum Bactericidal Concentration

Minimum Bactericidal Concentrations (MBCs) can be defined as the lowest concentration of an antimicrobial agent that will prevent the growth of bacteria. In other words MBC is the minimum concentration of an antimicrobial compound that kills the organism (Andrews, 2001).

1.9 Purpose of the Study

The purpose of this study was to determine the antioxidant and antimicrobial activities of the tea infusion and fresh fruit juice extracts that are commonly consumed in regular diet. For antimicrobial effects of the daily consumed plant extracts, a Gram (+) Staphylocci, *Staphylococcus aureus* was tested. The microorganism used in our antimicrobial experiments is the major cause of food borne poisoning illnesses. Since the overuse of the antibiotics might result in resistance of the bacteria, alternative natural sources of antimicrobial compounds become more and more critical. In conclusion, investigation of antimicrobial activities of herbal teas and fresh fruits is significant and they might offer suitable natural antibiotics against treatment pathogens.

CHAPTER 2

MATERIALS AND METHODS

2.1 MATERIALS

2.1.1 Chemicals

The bacterium *Staphylococcus aureus* (collection from Pasteur Institute 1072), which was used for antimicrobial activity determination studies, was obtained from Refik Saydam Hygiene Center (RSHC No: RSKK 95084).

Brain-Heart Infusion agar and Brain-Heart Infusion broth were bought from Merck (Darmstadt, Germany).

Agar plate medium for the identification of *Staphylococcus aureus* including %5 sheep blood agar were bought from OR-BAK (Istanbul, Turkey).

Antimicrobial susceptibility testing discs (6 mm diameter) used in disc diffusion test were bought from Oxoid (Hants, UK).

For antimicrobial susceptibility testing, standard antimicrobial discs Trimethoprim (23.75 µg), Clindamycin (2 µg), Penicillin (10 µg) and Tetracycline (30 µg) were bought from Bioanalyse, Ankara, TURKEY (50 susceptibility discs for in vitro diagnostic use).

Cell culture tested grade in powdered form of Penicillin G potassium salt was bought from Sigma Chemical Company, (St.Louis, MO, USA).

12 multichannel 5-50 µL, 12 multichannel 100-300 µL, and 8 multichannel 5-50 µL Thermo Scientific Finnpipettes were used during the experiments.

HPLC grade ethanol, methanol, ethyl acetate, acetonitrile, hexane and acetone were bought from Merck (Darmstadt, Germany). Cell culture grade of dimethylsulfoxide was bought from AppliChem.

Milli-Q system (Milli-pore, Bedford, MA, USA) was used to obtain ultrapure water and Milli-pore walled system was used to obtain distilled water in order to prepare plant extracts.

Disposable syringe filters with pore sizes of 0.22 µm and 0.45 µm Diameter is bought from Millipore Corporation (Bedford, MA USA).

2,2'-azino-bis (3-ethylbenzthiazoline-6-sulphonic acid) (ABTS), 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (trolox), 2,2-diphenyl-1-picrylhydrazyl (DPPH), sodium carbonate (Na_2CO_3), were bought from Sigma Chemical Company (St.Louis, MO, USA). Folin Ciocalteu's phenol reagent was purchased from Merck (Darmstadt, Germany).

Tea bags and fresh fruits were bought from local markets in Ankara, Turkey. Rosehip infusion tea was prepared from fresh rosehips (*Rosa canina* fruits). It was dried and grounded to prepare rosehip infusion tea. Lipton (Unilever Ltd., Istanbul, Turkey) sage (*Salvia fruticosa*) herbal tea, Arifoglu brand (Istanbul, Turkey) anise (*Pimpinella anisum*) herbal tea, Dogadan brand (Ankara, Turkey) camomile (*Anthemis arvensis*) herbal tea in the form of 20 packages of tea bags had been bought from local markets in Ankara, Turkey. Fresh fruits of grape (*Vitis vinifera*), orange (*Citrus sinensis*), peach (*Prunus persica*), and pomegranate (*Punica granatum*) were bought from local markets in Ankara, Turkey. Herbal tea species were identified in Biological Sciences Department, METU.

2.1.2 Apparatus

Class II Safety Cabinet used for sterile antimicrobial experiments was bought from ESCO, Thailand.

Spectrophotometric analysis was done via Cary 50 Bio UV-VIS spectrophotometer (Varian, USA).

Bio-tek ELISA reader (Elx808-Bio-tek, Germany) (METU, Biology Department, Prof. Dr. Mesude İşcan Lab.) was used for determination of minimum inhibitory concentration and minimum bactericidal concentration experiments for 96-well plates.

Other experimental apparatus were used in this study are listed below;

- Nuve EN-500 incubator
- Bandelin Sonorex (ultrasonic bath)
- Optic Ivymen System (incubator and shaker)
- Rotary evaporator (Heidolph Laborota 4000)
- Blender: Waring model 32BL80 (New Hartford, CT, USA)
- Philips Cucina HR1840 Food Processor
- Lyophilizator (Heto-Holten Model Maxi-Dry Lyo)

2.2 METHODS

2.2.1 Sample Preparation

Fresh fruits that will be used in our experiments were bought from local stores. Grape, orange, peach and pomegranate were washed, dried and kept at 4 ℃ in dark until use. Anise, sage and camomile were bought in the form of teabag from stores. However, rosehip was bought in the dried form from a local market. Commercially avaible herbal teas including rosehip, anise, sage and camomile were stored at room temperature in dark until use.

40 grams of anise, camomile and sage were weighed. Fresh rosehip fruits were washed and dried at room temperature. Firstly, herbal teas were infused in 480 mL boiled distilled water for one hour at room temperature. Then, infused tea solutions were incubated at 37 ℃ for 24 hours with rocking-incubator at 180 rpm to increase the yield. After incubation of the tea infusions, solutions were filtered through filter paper and filtrates were lyophilized. The resultant lyophilized herbal tea extracts were stored at 4 ℃ in the dark.

For the fresh fruits, 474.9 grams of grapes, 1241.8 grams of orange, 655 grams of peach and 1665.5 grams of pomegranate arils were processed with the help of Philips Cucina HR1840 Food Processor in order to separate juice from the pulp. After obtaining the fruit juices, they were filtered through filter paper and filtrates were lyophilized. The resultant lyophilized fresh fruit extracts were stored at 4 ℃ in the dark.

2.2.2 Preparation of Microbial Strains and Stocks

The ß-haemolytic staphylococci, *Staphylococcus aureus* Pasteur 1072 strain which was used for in microbiological studies were bought from Refik Saydam Hygiene Center in pellet form.

The *S. aureus* pellet was suspended in 1 mL of Brain Heart Infusion (BHI) broth. Suspended pellet was spread onto blood agar medium aseptically and incubated at 37 ℃ for 24 hours. After incubation, the plates were stored at 4 ℃ for short term storage.

For long term storage at -80 ℃, morphologically similar colonies of *S. aureus* were picked and transferred aseptically from 4 ℃ blood agar plates. Those colonies were suspended into 1 mL BHI broth. After suspension of colonies, 100 µL were taken and inoculated into 100 mL BHI broth. The inoculum was incubated at 37 ℃ at 180 rpm until the microbial cells reach mid-log phase in which the OD_{570} of 0.6. 100 µL of bacterial suspension was taken and added onto filter sterilized pre-chilled 25 % 4.9 mL glycerol. Final mixture of glycerol and bacterial suspension was mixed gently. Then 250 µL of the bacterial solutions were delivered to pre-chilled centrifuge tubes for long term storage in -80 ℃ freezer.

2.2.3 Bacterial Growth Curve

Bacterial colonies were inoculated into 100 mL BHI broth and incubated at 37 ℃ at 185 rpm for 24 hours in order to reach a final OD_{570} of 0.03. The bacterial suspension with OD_{570} of 0.03 was used in bacterial growth curve determination experiments. At the time of 0, 2, 4, 6, 8, 10, 12, 14, 16, 18, 20, 22, 24 hours, the absorbance measurements were carried out. Simultaneously, bacterial colony counts were performed at time of 4, 8, 12, 16, 20, 24 hours. Bacterial samples were diluted with phosphate buffer saline (PBS). Four proper dilutions of the samples were performed and they were spread onto BHI agar plates. The plates were incubated at 37 ℃, until the colonies were observable; it took approximately 14-16 hours for the colonies to be visible on plates.

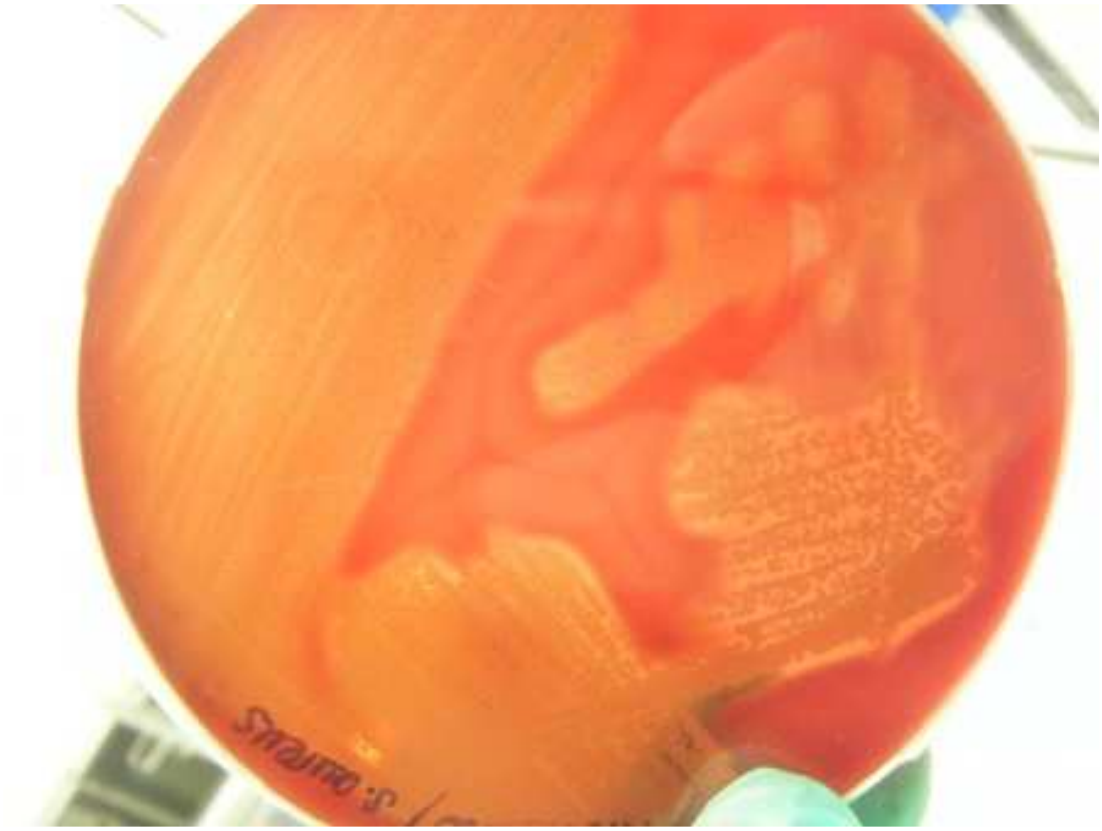

Figure 2.1 Bacterial strain of *Staphylococcus aureus* used in antimicrobial experiments was purchased from Refik Saydam Hygiene Center.

2.2.4 Antibacterial Activity Studies

In order to prevent contamination of the bacterium of interest, the antimicrobial activity studies were performed aseptically. By this way, the potential risks of the antimicrobial activity studies for human health were minimized.

2.2.4.1 Kirby-Bauer Disc Diffusion: Antibacterial Susceptibility Test

According to the given instructions by Merck, 100 mL autoclaved BHI agar was prepared and poured into sterile 100 mm plates. The plates were left to cool down and they were stored at 4 ^{o}C. Overnight grown bacteria in BHI broth were diluted to obtain 1 x 10^{8} colony forming units in 1 mL. Therefore,

final OD_{570} was arranged to 0.1 and 100 µL of inoculated bacteria were spread onto the agar aseptically. After spreading the bacteria, sterile paper discs and standard antibiotic discs (Trimethoprim, Clindamycin, Penicillin, and Tetracycline) were located onto the agar carefully. Then, 20 µL plant extracts with a concentration of 60 mg/mL were applied on filter discs. The plates were incubated at 37 ^{o}C for approximately 16 hours. Inhibition zone diameters were measured and recorded in millimeters. Filter discs with methanol and water were used as controls. All the experiments were performed three times in duplicates.

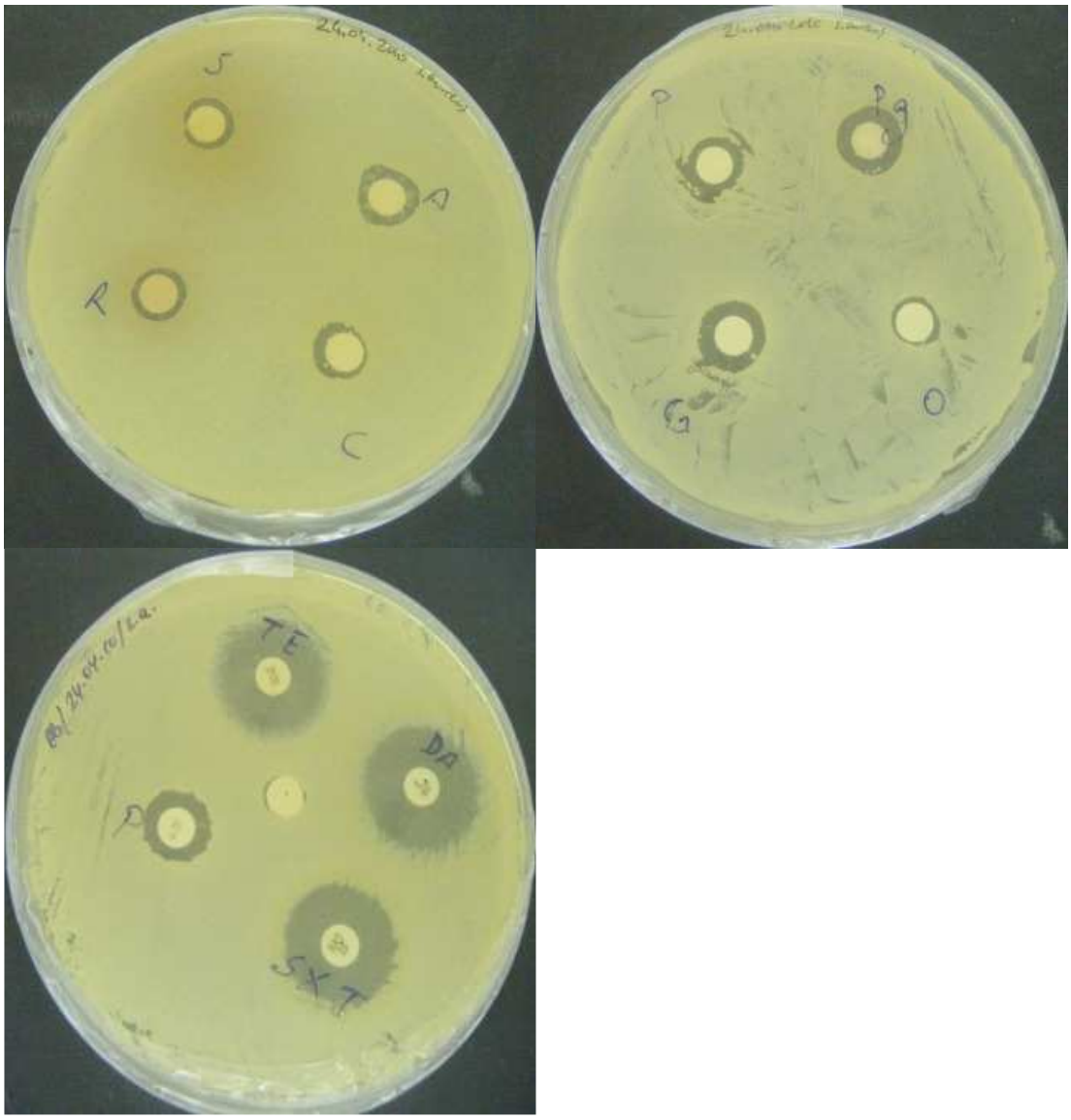

Figure 2.2 Inhibition zones of tea infusion extracts, fruit juice extracts and selected antibiotics after 16 hours incubation period

2.2.4.2 Determination of Minimum Inhibitory Concentrations

2.2.4.2.1 Solvent Effects

Minimum inhibitory concentration (MIC) of eight different solvents was determined by using 96-well plates. The first columns of each plate were selected as growth control columns. 95 µL BHI broth and 5 µL of bacteria were put in growth control columns. The second columns were selected as sterility control columns in which no bacteria was added. It contained only 100 µL broth. Column 3 contained 180 µL broth and the columns 4 to 11 contained 95 µL broth. However, column 12 was empty. 20 µL of different solvents (ethanol, methanol, dimethylsulfoxide-DMSO, ethyl acetate, acetonitrile, cyclohexane, acetone and double distilled water) were added each row at column 3, so that the final concentration was arranged to 10 % solvent (v/v). 95 µL solvent-broth solutions were transferred to next column, column 4. Two-fold dilutions were carried out until the 11^{th} column. Since 12^{th} column was empty, only 95 µL solvent-broth solution was transferred to 12^{th} column from column 11 as given in Table 2.1. The final concentrations of the solvents in the wells were 10%, 5%, 2.5%, 1.25%, 0.63%, 0.31%, 0.16%, 0.08% and 0.04%. After arrangement of the concentrations of the solvents, 5 µL of inoculum containing approximately 5×10^5 cells were added to wells except the wells of second column. The plates were then incubated for 16 hours at 37 ^oC and bacterial growth was investigated. Minimum inhibitory concentration of solvents can be defined as the lowest concentration of solvents at which no bacterial growth was observed. All the experiments were performed three times in triplicates.

Table 2.1 5 µL of *S. aureus* was inoculated to each wells and the final volume was arranged to 100 µL.

	1	2	3	4	5	6	7	8	9	10	11	12
	Growth Control	**Sterility Control**	**10 %**	**5 %**	**2.5 %**	**1.25 %**	**0.63 %**	**0.31 %**	**0.16 %**	**0.08 %**	**0.04 %**	**0.04 %**
	95 µL broth	100 µL broth	180 µL broth 20 µL plant extract	95 µL broth	95 µL broth	95 µL broth	95 µL broth	95 µL broth	95 µL broth	95 µL broth	95 µL broth	95 µL broth
Ethanol												
Methanol												
DMSO												
Ethyl Acetate												
Acetonitrile												
Cyclohexane												
Acetone												
ddH2O												

2.2.4.2.2 Preparation of Stock Solution Concentrations of Plant Extracts

Stock solutions of tea infusion and fruit juice extracts were limited with their solubilities prepared with methanol and the concentrations of stock solutions are given in the Table 2.2.

Table 2.2 Stock solutions (mg/mL) prepared in methanol to get subsequent dilutions for determination of antimicrobial activities of the extracts.

Name of the extract	Stock concentration (mg/mL)
P. anisum	**960**
A. arvensis	**960**
R. canina	**960**
S. fruticosa	**120**
V. vinifera	**960**
C. sinensis	**960**
P. persica	**960**
P. granatum	**960**

2.2.4.2.3 Determination of Minimum Inhibitory Concentration by Micro Broth Dilution Method

Minimum inhibitory concentration experiments were done according to the National Committee for Clinical Laboratory Standards protocol with some modifications (Wiegand, 2008). 96-well plates were used for this purpose. The first column again was assigned for growth control in which 95 µl BHI

broth and 5 µL inoculum were put. The second column was identified as sterility control column and it only contained 100 µL of BHI broth.

Third column, antibiotic sterility column, contained 95 µL of BHI broth and 5 µL of antibiotic (Penicillin G) solution. 90 µL of broth, 5 µL of antibiotic solution and 5 µL of *S. aureus* inoculum were put in the wells at 4^{th} column in order to screen the sensitivity of the bacterium to the selected antibiotic.

180 µL BHI broth and 20 µL plant material were added to the 5^{th} column. Moreover, 95 µL of BHI broth were placed to the wells starting from 6^{th} column to the 11^{th} column. 12^{th} column was left empty. 95 µL broth-plant extract solutions were transferred from 5^{th} column to the 6^{th} column which resulted in two-fold dilution of plant extract in the wells at 6^{th} column. Serial two-fold dilutions were achieved until the column 11. Lastly, 95 µL broth-plant extract solutions were transferred from 11^{th} column to the 12^{th} and last column.

Finally, 5 µL of *S. aureus* inoculum containing approximately 5 x 10^5 cells were added to all wells except sterility control and antibiotic sterility control wells.

After adding *S. aureus* inoculum to the wells, 96-well plates were incubated at 37 ^oC for 16 hours. Minimum inhibitory concentration of a plant extract can be defined as the lowest concentration of the plant extract that inhibits the growth of bacteria. Absorbance values were measured at 570 nm by using ELISA plate reader. All the experiments were performed three times in duplicates.

Penicillin G was chosen since it is widely used to treat Gram positive bacterial infections. 2 mg/mL Penicillin G stock solution was prepared with sterile ultrapure water. Final concentration of selected antibiotic in antibiotic sterility control wells was 0.05 mg/mL in each well.

2.2.4.2.4 Determination of Minimum Bactericidal Concentration by Micro Agar Dilution Method

Minimum bactericidal concentration (MBC) experiments were done according to the National Committee for Clinical Laboratory Standards protocol with some modifications (Wiegand, 2008). 10 µL of solution was taken from the 96-well plates previously prepared in minimum inhibitory concentration experiments. And then, with the help of Thermo Scientific Finnpipettes (12 multichannel 5-50 µL), 10 µL of solution was added onto the wells in which 100 µL of BHI agar had been prepared previously.

Minimum bactericidal concentration (MBC) of a plant extract can be defined as the lowest concentration of the plant extract that inhibits the formation of bacterial colonies on the agar surface. Absorbance values were measured at 570 nm by using ELISA plate reader. All the experiments were performed three times in duplicates.

2.2.5 Antioxidant Activity Tests

2.2.5.1 ABTS Method

2,2'-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid), ABTS method was carried out according to study described previously by Re *et. al* (1999).

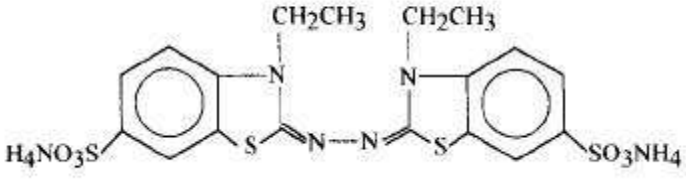

Figure 2.3 2,2'-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid) (Campos, 1997).

ABTS was dissolved in 20 mL of water in order to prepare 7 mM stock solution. Reaction of ABTS solution with potassium persulfate ($K_2S_2O_8$) with a final concentration of 2.45 mM in the stock solution was resulted in ABTS radical cation ($ABTS^{\bullet+}$). Then, the mixture kept in the dark for 12-16 hours at room temperature before use.

ABTS radical cation, $ABTS^{\bullet+}$ solution was prepared from stock solution by hundred-fold dilution. 100 mL methanol was added to 1 mL of stock solution and the resulting OD_{734} value was 0.70 ±0.02. 1.0 mg/mL of tea infusion and fruit juice extracts were set and 2 mL $ABTS^{\bullet+}$ solution were mixed with various volumes of plant extracts resulting in different concentrations of herbal tea infusions and fruit juices. Absorbance values of $ABTS^{\bullet+}$ solution mixed with different volumes of plant extracts were measured by Cary 50 Bio UV-VIS spectrophotometer. Absorbance measurements were carried out every minutes starting from 1st to 6th minute. After 6th minute, percent inhibition of ABTS by antioxidants didn't change since the rate of forward reaction and the rate of reverse reaction were equal as given in Figure 2.4.

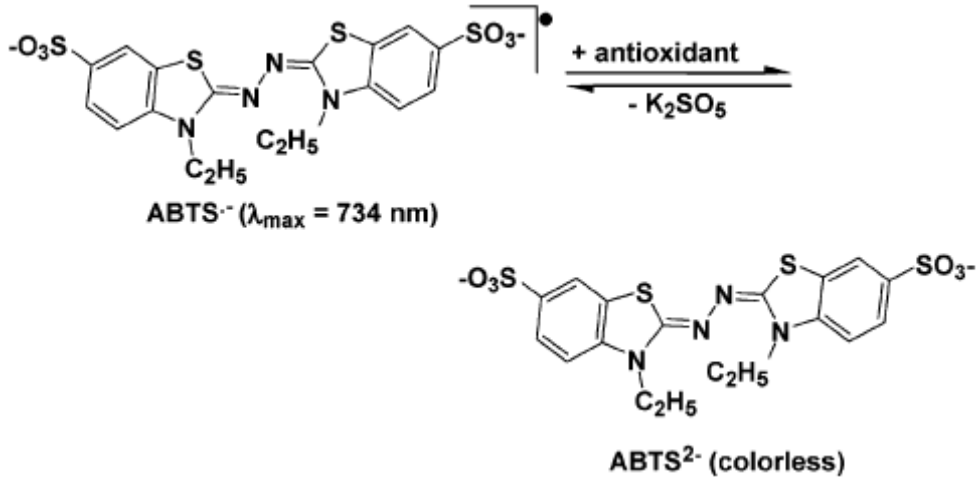

Figure 2.4 Reaction of ABTS radical and antioxidants (Apak, 2007).

In ABTS experiments, trolox standard was used as a reference. Different concentrations of trolox (5, 10, 15, 20 µM) were prepared to obtain the trolox equivalent antioxidant capacity (TEAC) values. Trolox concentration versus

percent inhibition graph was plotted in order to find out the TEAC values of selected plant extracts. For this purpose, the graphs of plant extract concentrations versus percent inhibitions were also plotted. If the ratio of the slope of the extract concentration versus percent inhibition graph to the slope of the trolox concentration versus percent inhibition graph was multiplied by dilution factor (x 100), TEAC value of the plant extract would be determined. All the experiments were performed three times in duplicates.

The percent inhibitions of trolox standard and plant extracts were calculated according to the equation;

$$\% \text{ Inhibition} = [(A_0 - A_1)/A_0] \times 100 \quad (1)$$

A_0; Absorbance value of $ABTS^{\bullet+}$ and methanol

A_1; Absorbance value of $ABTS^{\bullet+}$ and selected plant extract

2.2.5.2 DPPH Method

0.05 mg/mL 2,2-diphenyl-1-picrylhydrazyl (DPPH)/ethanol solution was prepared and its absorbance value was found to be approximately 1.4 at 517 nm.

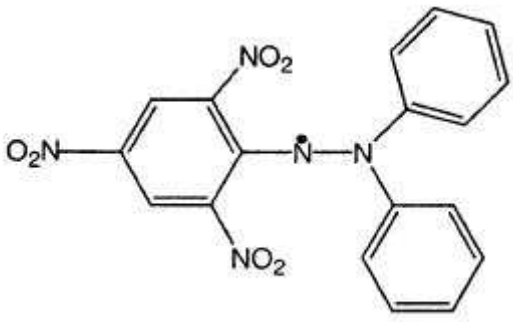

Figure 2.5 2,2-diphenyl-1-picrylhydrazyl (DPPH) (Litwinienko, 2002).

Different concentrations of the plant extracts were tested for their radical scavenging activities. 100 µL of plant extract was mixed with 1.4 mL of DPPH solution. Incubation time for *V. vinifera*, *C. sinensis*, *P. persica* and *P. granatum* was 15 minutes and incubation time for *P. anisum*, *A. arvensis*, *R. canina* and *S. frutioca* was 30 minutes. During incubation time all the samples were kept in dark at room temperature. Absorbance values were measured at 517 nm. Blank solution was also prepared by mixing 100 µL of methanol with 1.4 mL of DPPH solution. All the experiments were performed three times in duplicates.

Radical scavenging activity (RSA) of the plant extracts were calculated according to the equation;

$$\% \text{ RSA} = [(A_0 - A_1)/A_0] \times 100 \quad (2)$$

A_0; Absorbance value of the blank solution (DPPH + methanol)
A_1; Absorbance value of the plant extract (DPPH + plant extract)

After obtaining the data from radical scavenging activity of different concentration of plant extracts, % RSA versus final concentration of the plant extract graph was plotted. EC_{50} values for each plant extracts were calculated after plotting % RSA versus logarithm of plant extract concentration. Statistical analysis was done according to Minitab Release 14 software.

2.2.5.3 Determination of Total Phenols

Total phenolic content was determined according to the modified version of the method of Singleton and Rossi described previously (1963). Different concentrations of plant extracts were prepared. 100 µL of plant extract was added to 100 µL of 50 % Folin–Ciocalteu's phenol reagent. It was vortexed

vigorously. After vortexing, 2 mL aqueous solution of 2 % Na_2CO_3 was added in order to stop the reaction. The final solution was again vortexed vigorously.

100 µL of methanol was used rather than plant extracts for the preparation of blank solution. Again 100 µL Folin–Ciocalteu's phenol reagent was added and vortexed. Finally, 2 mL aqueous solution of 2 % Na_2CO_3 was mixed and the final solution vortexed vigorously.

Final mixtures of plant extracts and blank solution were incubated for 30 minutes and absorbance values were measured at 750 nm. The absorbance value of the blank solution was subtracted from the absorbance of the plant extracts. All the experiments were performed three times in duplicates.

For the determination of total phenolic content, gallic acid standard was used as a reference. Different concentrations of gallic acid (0.05, 0.1, 0.2 and 0.3 mg/mL) were needed to construct gallic acid standard curve. With the help of gallic acid curve, total phenol content of the plant extracts could be determined in gallic acid equivalents (GAE).

2.2.5.4 Determination of Total Flavonoids

Total flavonoid content was determined according to the method of Zhishen *et. al* (1999) with some modifications. Different concentrations of plant extracts were prepared. 250 µL of plant extract was mixed with 1250 µL of water. Then, 75 µL of 5% $NaNO_2$ was added to the mixture and vortexed vigorously. After 5 minutes, 150 µL of 10% $AlCl_3$ was also added and the mixture was vortexed vigorously. 500 µL of 1M NaOH was mixed, after 6 minutes of addition of 10% $AlCl_3$. Finally, total volume of the mixture was completed to 3 mL by adding 775 µL of water. The final 3 mL mixture was vortexed vigorously.

250 µL of methanol was used rather than plant extracts for the preparation of blank solution. Same procedure as described above was applied to the blank solution.

Final mixtures of plant extracts and blank solution were incubated. At the time of 21th minute, absorbance values were measured at 510 nm. The absorbance value of the blank solution was subtracted from the absorbance of the plant extracts. All the experiments were performed three times in duplicates.

For the determination of total flavonoid content, quercetin standard was used as a reference. Different concentrations of quercetin (0.05, 0.1, 0.2 and 0.3 mg/mL) were needed to construct quercetin standard curve. With the help of quercetin standard curve, total flavonoid content of the plant extracts could be determined in quercetin equivalents (QE).

CHAPTER 3

RESULT & DISCUSSION

3.1 Extraction of Plant Materials

Here, information obtained from extraction of 8 different samples of selected fruit juices and herbal teas is given.

3.1.1 Extraction of Tea Infusions

Total weight of selected tea infusions, total infusions volume, total weight of extracts and extraction yields were calculated and given in the table 3.1.

Table 3.1 Data obtained from the extraction of tea infusion extracts.

Tea infusions	***P. anisum***	***A. arvensis***	***R. canina***	***S. fruticosa***
Total weight (g)	**40**	**40**	**40**	**40**
Infusion volume (mL)	**480**	**480**	**480**	**480**
Total extract (g)	**8.00**	**23.70**	**22.20**	**6.20**
Yield (%)	**20.00**	**59.25**	**55.50**	**15.50**

3.1.2 Extraction of Fresh Fruits

Total weight of selected fresh fruits, total fresh fruits volume, total weight of extracts and extraction yields were calculated and given in the table 3.2.

Table 3.2 Data obtained from the extraction of fresh fruit extracts.

Fresh Fruits	***V. vinifera***	***C. sinensis***	***P. persica***	***P. granatum***
Total weight (g)	**474.9**	**1241.8**	**655**	**1665.5**
Juice (mL)	**371**	**524**	**452**	**407**
Total extract (g)	**42.4**	**51.6**	**52**	**59.2**
Yield (%)	**11.43**	**9.85**	**11.50**	**14.55**

3.2 Antimicrobial Activity of Plant Extracts on *Staphylococcus aureus*

Antimicrobial effects of the tea infusion and fruit juice extracts were studied on the bacterium *Staphylococcus aureus*.

3.2.1 Bacterial Growth Curves

In antimicrobial experiments, the equation obtained from colony count versus optical density graph for the bacteria of interest is critical. In Figure 3.1, a change in number of bacterial colonies in time is given. Then, logarithm of these colony numbers was calculated and the logarithm colony forming unit versus time graph was plotted as seen in Figure 3.2. Moreover, changes in optical density in time which indicates the bacterial growth was given in Figure 3.3. Finally, using Figure 3.1 and 3.3, the graph which relates the colony forming units (CFU) to the optical density was obtained and given in Figure 3.4. With the help of this graph, number of colonies of the bacteria of interest can be standardized. Therefore, one could use the same number of bacterial colonies in every single experiment.

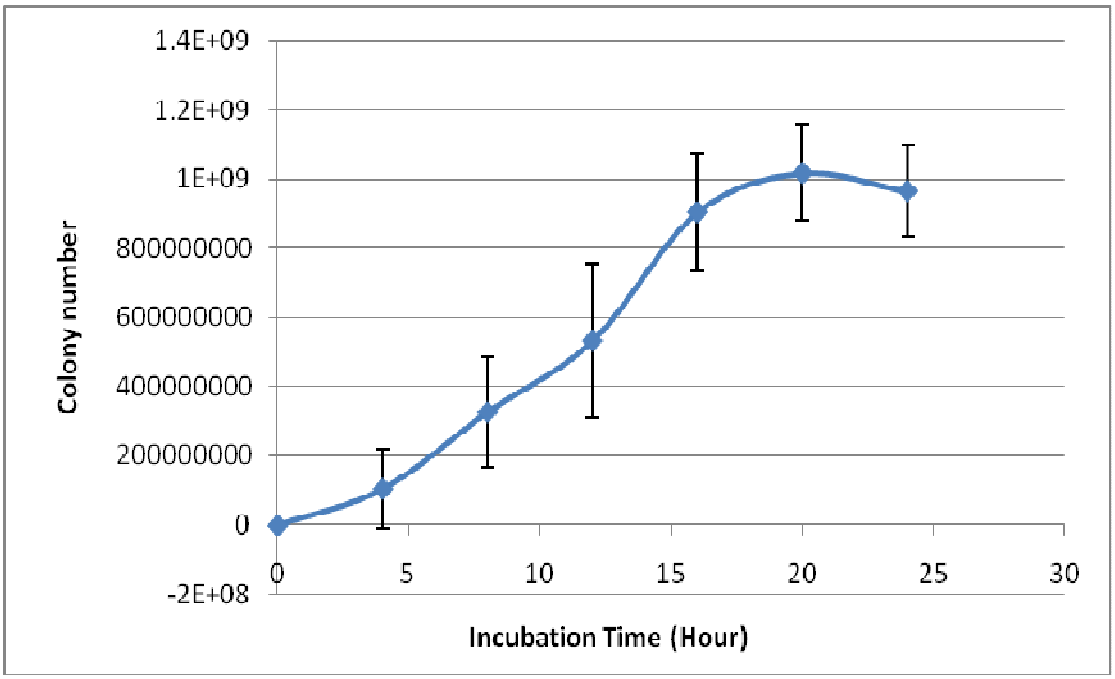

Figure 3.1 Graphical represation of colony numbers at various time intervals starting with a final OD_{570} of 0.03. (Each point represents the mean of three experiments and the vertical bars represent mean of standard errors).

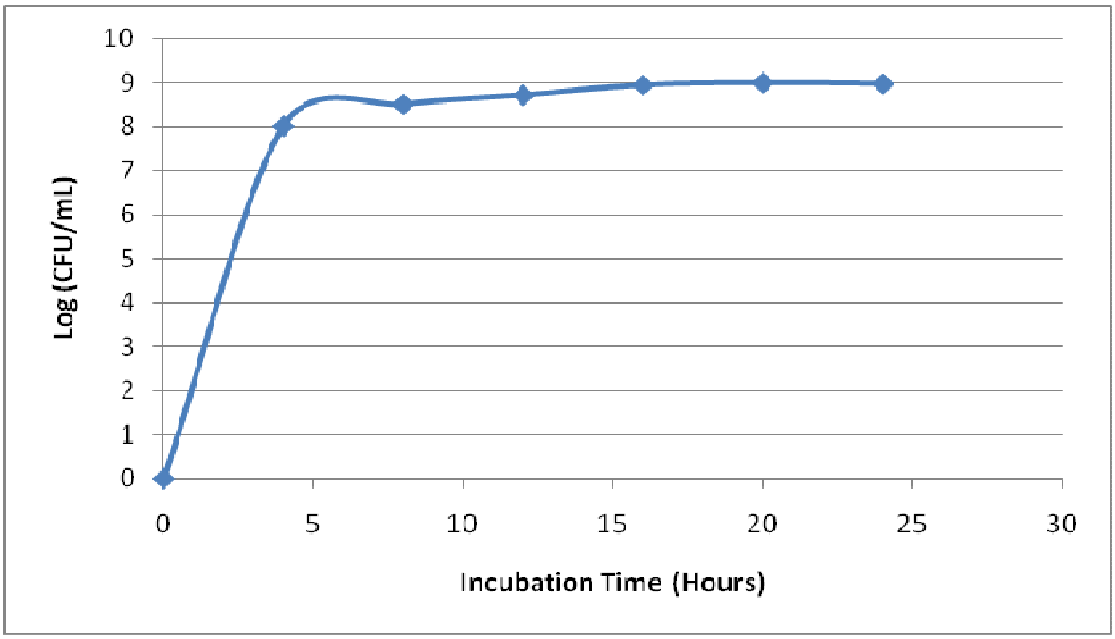

Figure 3.2 Graphical represation of logarithm of colony forming unit per mL at various time intervals starting with a final OD_{570} of 0.03. (Each point represents the mean of three experiments and the vertical bars represent mean of standard errors).

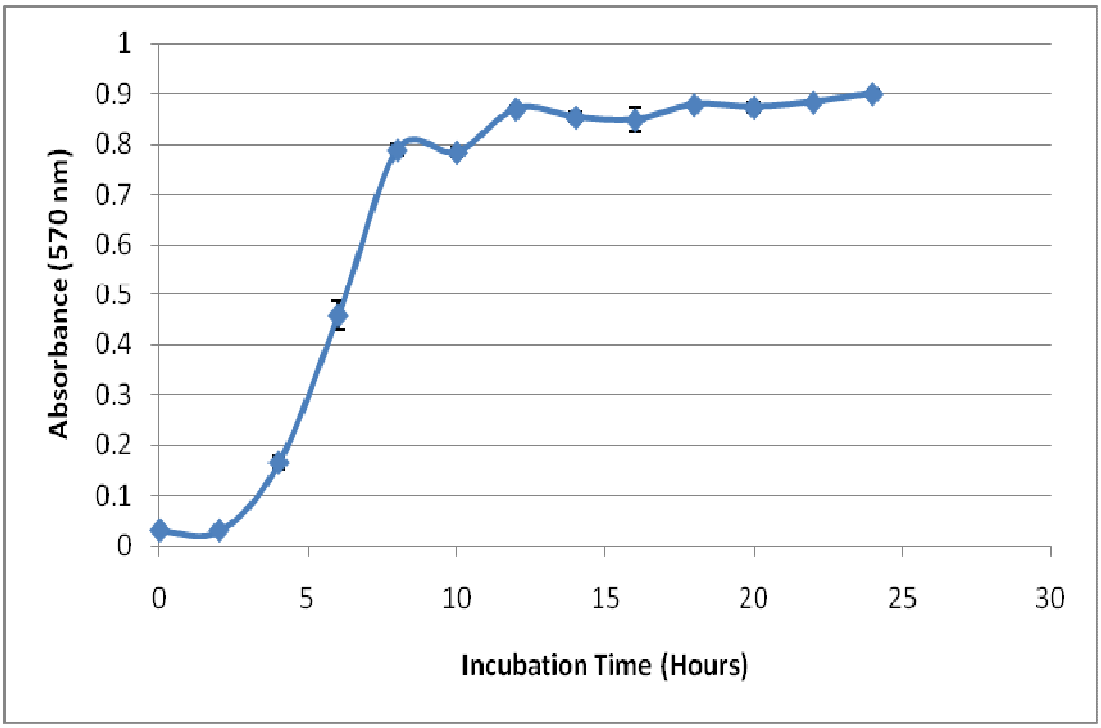

Figure 3.3 Graphical represation of absorbance at 570 nm at various time intervals starting with a final OD_{570} of 0.03. (Each point represents the mean of three experiments and the vertical bars represent mean of standard errors).

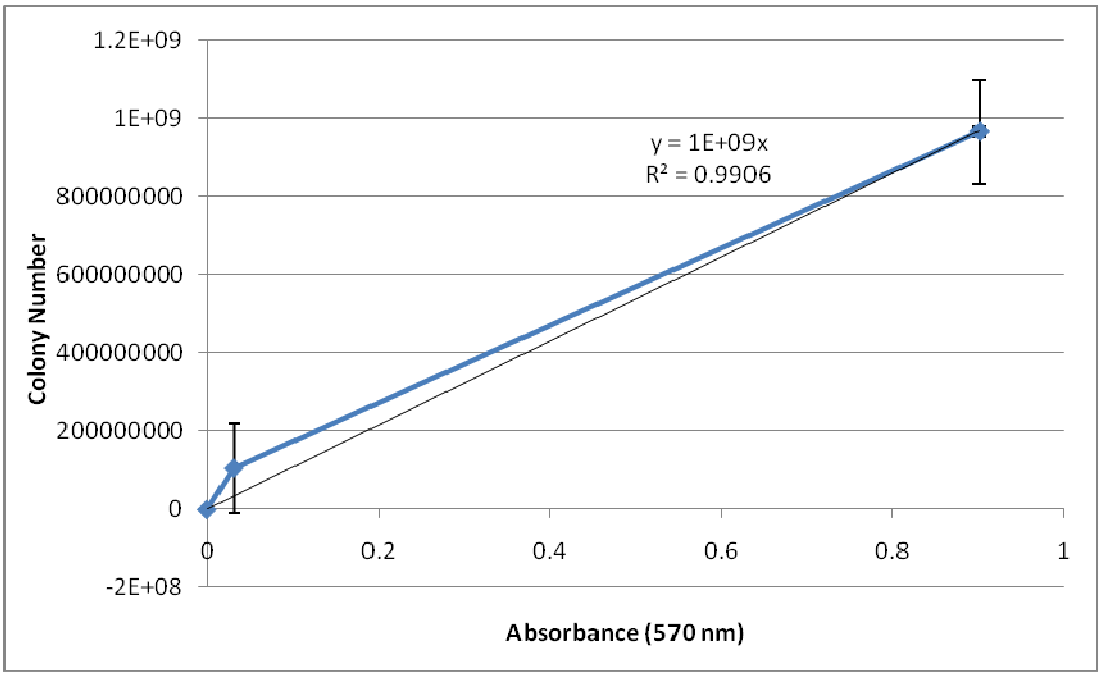

Figure 3.4 Graphical represation of colony numbers at OD_{570} which is derived from Figure 3.1 and 3.2. Using the equation of this graph, it can be estimated that 1 OD_{570} is approximately $1x10^9$ CFU/mL.

Figure 3.4 is the graphical illustration of bacterial colony number with respect to optical density at 570 nm. The trend line equation of the graph was calculated as $y = 1 X 10^{9}x$. Calculation of this equation is significant for estimating the colony forming unit per mL for the values of OD_{570}.

3.2.2 Minimum Inhibitory Concentrations

Minimum inhibitory concentration experiments were realized using 96-well plates as described previously in section 2.2.4.2. All experiments were performed three times in duplicates.

3.2.2.1 Effects of Solvents

The effects ethanol, methanol, dimethylsulfoxide, ethyl acetate, acetonitrile, cyclohexane and ultrapure water on the growth of *S. aureus* were investigated, in order to find out appropriate solvent with the least inhibitory effect. Concentrations of these solvents were ranged from 0.04 % (v/v) to 10 % (v/v) in BHI broth solution. Results were tabulated in Table 3.3, as observed from the table; none of the solvents had shown any inhibitory effects on bacterial growth therefore any of the solvents could have been selected for the rest of the studies. When considering the extraction procedure, methanol should have been the best option for micro broth dilution experiments.

Table 3.3 Minimum inhibitory concentrations of the solvents. None of the solvents had shown any inhibitory effect.

	Growth Control	Sterility Control	10%	5%	2.5%	1.25%	0.63%	0.31%	0.16%	0.08%	0.04%
EtOH	+	-	+	+	+	+	+	+	+	+	+
MeOH	+	-	+	+	+	+	+	+	+	+	+
DMSO	+	-	+	+	+	+	+	+	+	+	+
EtAc	+	-	+	+	+	+	+	+	+	+	+
Acetonitrile	+	-	+	+	+	+	+	+	+	+	+
Cyclohexane	+	-	+	+	+	+	+	+	+	+	+
Acetone	+	-	+	+	+	+	+	+	+	+	+
ddH_2O	+	-	+	+	+	+	+	+	+	+	+

3.2.2.2 Minimum Inhibitory Concentration of Plant Extracts

The inhibitory effects of herbal infusion tea and fresh fruit juice extracts on the growth of bacterium, *Staphylococcus aureus* were investigated.

3.2.2.2.1 Minimum Inhibitory Concentration of Tea Infusion Extracts

Growth inhibition capacities of the daily consumed herbal infusion teas were studied on *S. aureus* by the method described as in section 2.2.4.2.3.

The final concentrations of the selected tea infusion extracts in the 96-well plates were between 1.5 – 96.0 mg/mL.

S. aureus growth curve was taken as a reference and 0.01 OD_{570} which equals to 5 $x10^5$ colony forming units were used for the inoculation of each well.

Effects of the selected tea infusion extracts on *S. aureus* were screened at 570 nm. Plant extract concentration able to inhibit bacterial growth, could be identified as MIC value, at which the absorbance (OD_{570}) value dropped to zero. Bacterial growth might be inhibited by more than one plant extract concentrations. For this condition, lowest concentration would be accepted as the minimum inhibitory concentration. Inhibition of bacterial growth was investigated in the presence of tea infusion extracts as displayed in Figure 3.5.

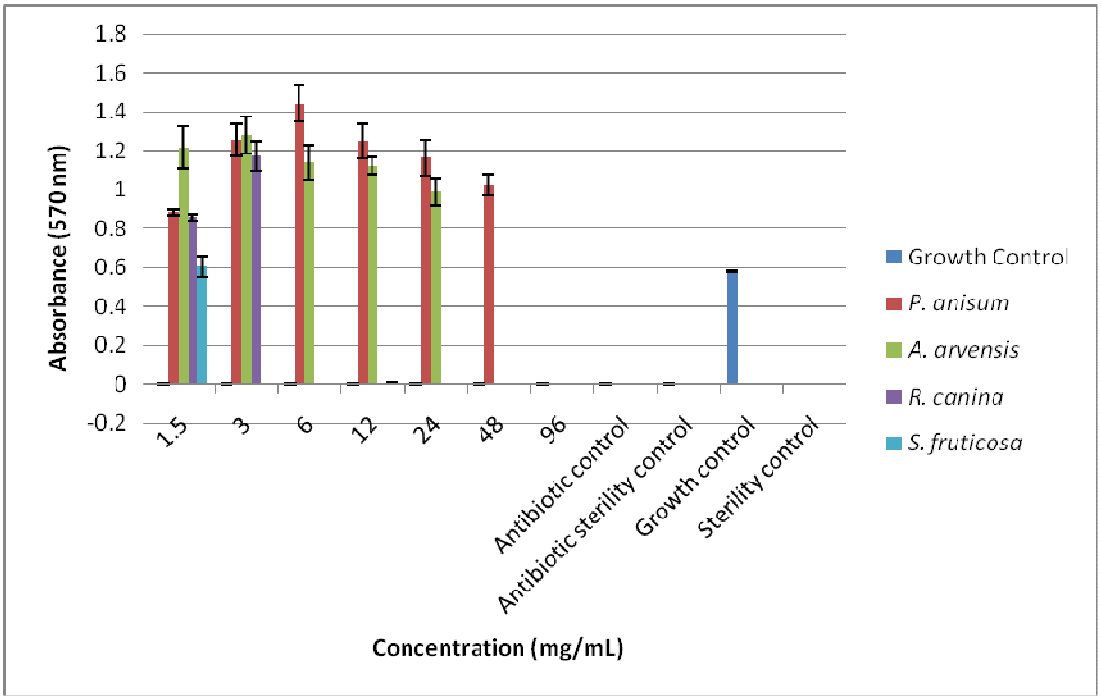

Figure 3.5 Investigation of minimum inhibitory concentration of tea infusion extracts on *Staphylococcus aureus* after 16 hours incubation.

Antibiotic control: 0.05 mg/mL Penicillin G was used as reference antimicrobial agent in the presence of *S. aureus* (OD_{570} = 0.01).
Antibiotic Sterility Control: Penicillin G sterility was examined without addition of inoculum
Growth Control: Growth of S. aureus was tested in Brain-Heart Infusion broth
Sterility Control: Experiments were conducted in aseptique conditions with only sterile Brain-Heart Infusion broth

S. fruticosa was found to be the most active antimicrobial extract among the tested herbal infusion tea extracts. Minimum inhibitory concentration of the *S. fruticosa* was 3 mg/mL at which optical density was zero. *R. canina* was the second active extract with a minimum inhibitory concentration value of 6 mg/mL. *A. arvensis* and *P. anisum* followed *R. canina* with increasing minimum inhibitory concentrations of 48 mg/mL and 96 mg/mL, respectively. Results are given in the Figure 3.6.

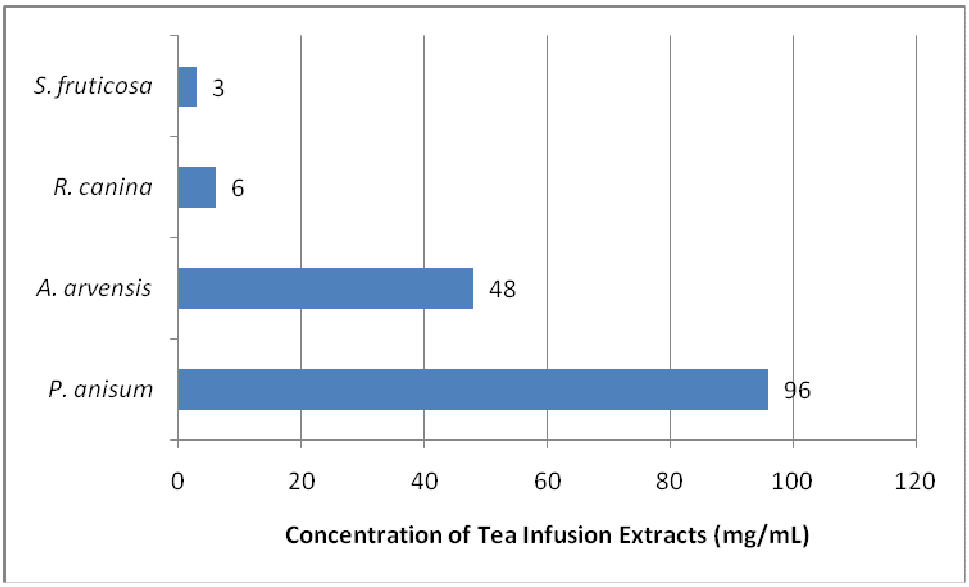

Figure 3.6 Minimum inhibitory concentrations of tea infusion extracts

Mahady and his co-workers investigated the minimum inhibitory concentration of plant extracts that are widely used in treatment of gastrointestinal disorders on *Helicobacter pylori*. Extracts of *Myristica fragrans* were the most effective againts *H. pylori* with a MIC value of 12.5 µg/mL. Moreover, minimum inhibitory concentration of *Pimpinella anisum* was found to be 100 µg/mL and they called *P. anisum* was weakly active against *H. pylori* (Mahady, 2005).

Askun *et. al* worked on the extracts of *Salvia fruticosa*, *Salvia tomentosa*, *Sideritis albiflora*, *Sideritis leptoclada*, and *Origanum onites. S. fruticosa* showed the best antimicrobial activity against Gram (-) bacteria. Minimum inhibitory concentrations of *S. fruticosa* were 640 µg/mL against *Salmonella typhimurium* and *Enterobacter aerogenes* (Askun, 2009). Because of its high activity towards Gram (-) bacteria, *S. fruticosa* tea is widely used to treat common cold and stomachache (Kirimer, 1999; Sezik, 2001).

3.2.2.2.2 Minimum Inhibitory Concentration of Fruit Juice Extracts

Growth inhibition capacities of the daily consumed fruit juices were studied on *S. aureus* according to the method as described previously in section 2.2.4.2.3.

The final concentrations of the selected fruit juice extracts in the wells were between 1.5 – 96 mg/mL.

S. aureus growth curve was taken as a reference and 0.01 OD_{570} which equals to 5 $x10^5$ colony forming units were inoculated to the wells.

Effects of the selected fruit juice extracts on *S. aureus* were screened at 570 nm and inhibition of bacterial growth was investigated in the presence of fruit juice extracts as displayed in Figure 3.7.

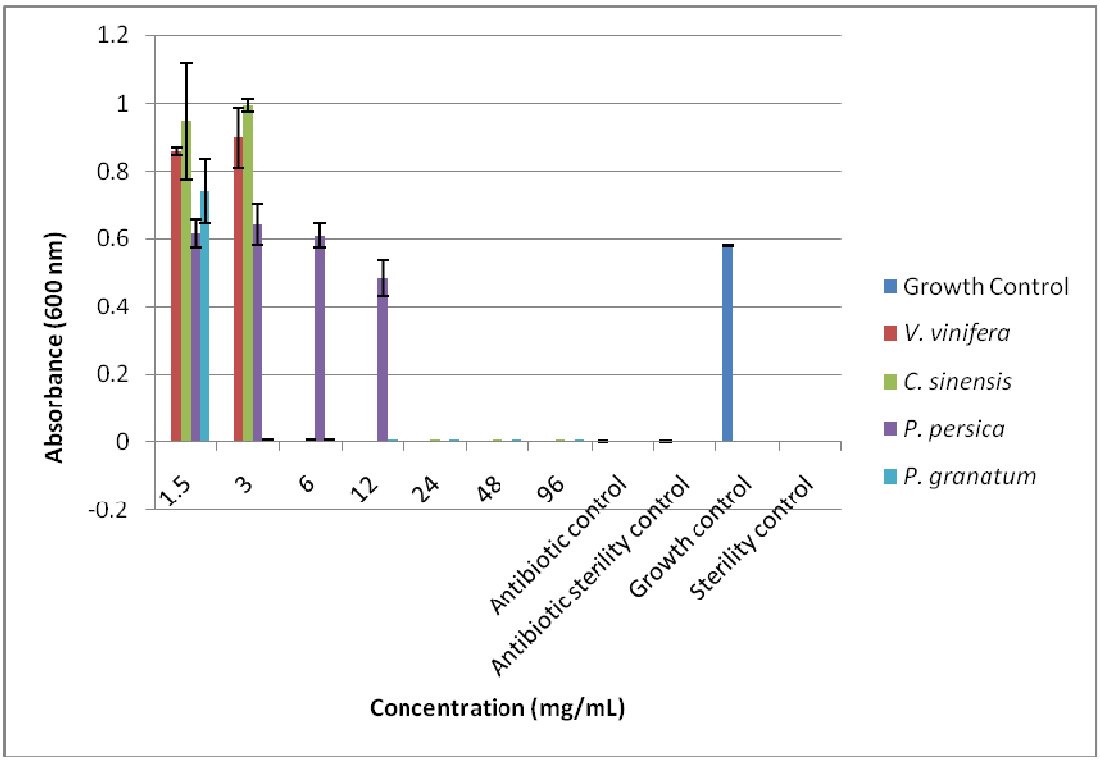

Figure 3.7 Investigation of minimum inhibitory concentration of fruit juice extracts on *Staphylococcus aureus* after 16 hours incubation.

Antibiotic control: 0.05 mg/mL Penicillin G was used as reference antimicrobial agent in the presence of *S. aureus* (OD_{570} = 0.01).
Antibiotic Sterility Control: Penicillin G sterility was examined without addition of inoculum
Growth Control: Growth of S. aureus was tested in Brain-Heart Infusion broth
Sterility Control: Experiments were conducted in aseptique conditions with the presence of sterile Brain-Heart Infusion broth

P. granatum was found to be the most active antimicrobial plant extract among the tested fruit juices. Minimum inhibitory concentration of the pomegranate was 3 mg/mL at which optical density was zero.

V. vinifera and *C. sinensis* were the second active antimicrobial plant extracts with minimum inhibitory concentrations values of 6 mg/mL. *P. persica* had the highest minimum inhibitory concentration value, 24 mg/mL, which means it was the least active antimicrobial plant extract. Results are given in the Figure 3.8.

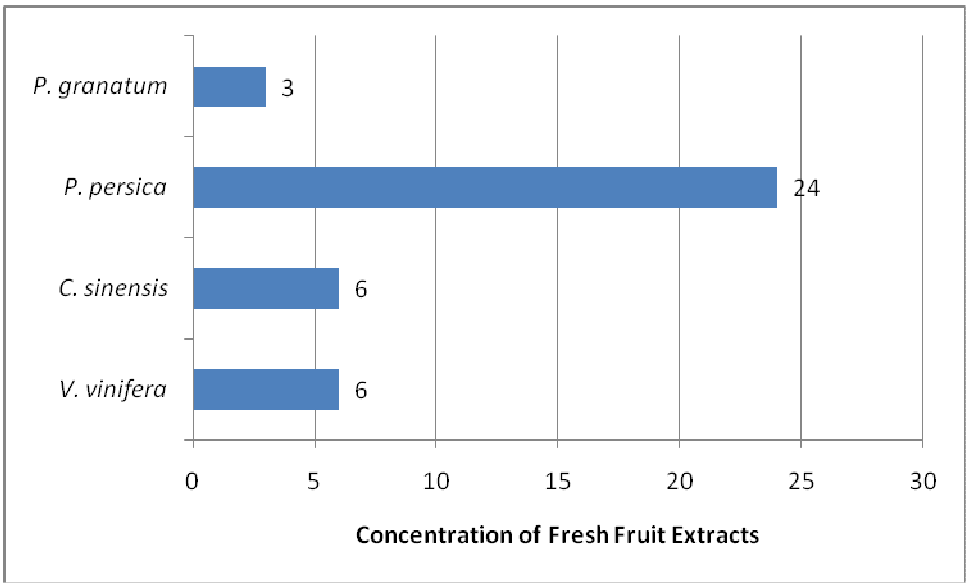

Figure 3.8 Minimum inhibitory concentrations of fruit juice extracts

Negi and Jayaprakasha studied the minimum inhibitory concentration of grapefruit (*Citrus paradisi*) peel extracts. They concluded that the Gram (-) bacteria were more resistant than Gram (+) bacteria and the MIC values for Gram (+) bacteria were between 250-3000 µg/mL, while the MIC values for Gram (-) bacteria ranged from 400 to 5000 µg/mL (Negi, 2001).

In 2009, Voravuthikunchai *et. al* reported the effects of some medicinal plants against 3 strains of *Escherichia coli* O157:H7, *Escherichia coli* O26:H11, *Escherichia coli* O111:NM, and *Escherichia coli* O22. Among the plant material used, extracts of *Quercus infectoria* and *Punica granatum* were found to have a high antimicrobial activity against all *Escherichia coli* O157:H7 strains. Minimum inhibitory concentrations of aqueous and ethanolic extracts of *Punica granatum* were 3.12 mg/mL (Voravuthikunchai, 2009).

3.2.2.3 Minimum Bactericidal Concentration of Plant Extracts

The lowest concentration of plant extracts that has the ability to kill bacteria was investigated. The plant extract solutions obtained from micro broth dilution assay used for determining the minimum bactericidal concentration of herbal tea infusion and fresh fruit juice extracts. Bactericidal concentration of an antimicrobial agent is critical, especially for the treatment of antimicrobial infections, in order to find out at which concentration bacteria of interest can be killed.

3.2.2.3.1 Minimum Bactericidal Concentration of Tea Infusion Extracts

Minimum bactericidal concentrations of tea infusion extracts were investigated by micro agar dilution method after applying micro broth dilution as described in section 2.2.4.2.4. Minimum bactericidal concentration of tea infusion extracts were determined by observing the lowest concentration at which no visible bacterial colonies could be seen.

Bactericidal effects of the selected herbal tea infusion extracts on *S. aureus* were monitored at 570 nm. *S. fruticosa* was found to be the most effective bactericidal against *S. aureus* among the other herbal teas. Minimum bactericidal concentration of the *S. fruticosa* was 3 mg/mL while *A. arvensis* was 48 mg/mL; *P. anisum* and *R. canina* were 96 mg/mL. Results are given in the Figure 3.9.

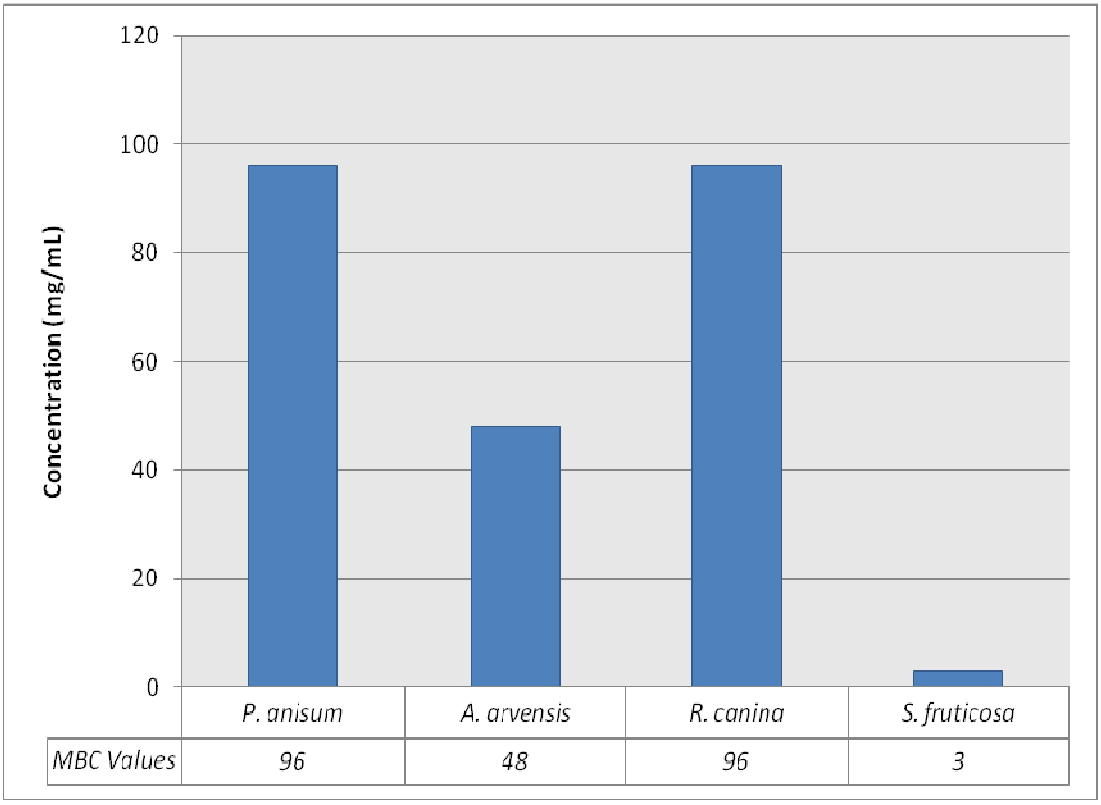

Figure 3.9 Minimum bactericidal concentrations of tea infusion extracts at 570 nm (Experiments were performed three times in duplicates).

Karamanoli *et. al* studied oregano, lavender, sage, and rosemary essential oils in order to determine minimum inhibitory concentration against *Pseudomonas syringae* and *Erwinia herbicola*. Also, minimum bactericidal concentrations of sage and oregano oils were investigated. MBC value of sage essential oil was found to be 2 mg/mL and MBC value of oregano oil was 0.4 mg/mL (Karamanoli, 2000).

Zu *et. al.* examined the antimicrobial activities of mint (*Mentha spicata* L., Lamiaceae), ginger (*Zingiber officinale* Rosc., Zingiberaceae), lemon (*Citrus limon* Burm.f., Rutaceae), grapefruit (*Citrus paradisi* Macf., Rutaceae), jasmine (*Jasminum grandiflora* L., Oleaceae), lavender (Mill., Lamiaceae), chamomile (*Matricaria chamomilla* L., Compositae), thyme (*Thymus vulgaris* L., Lamiaceae), rose (*Rosa damascena* Mill., Rosaceae) and cinnamon (*Cinnamomum zeylanicum* N. Lauraceae) against *Propionibacterium acnes*. Minimum inhibitory concentrations of selected plants were equal to minimum

bactericidal concentrations. They have found that the most active plant extracts were cinnamon and thyme with MIC & MBC values of 0.016 (% v/v). Minimum bactericidal concentration of rose, chamomile, lavender grapefruit, lemon, ginger, mint and jasmine were 0.031, 0.125, 0.125, 0.250, 0.250, 0.250, 0.250 and 0.500 (% v/v), respectively.

3.2.2.3.2 Minimum Bactericidal Concentration of Fruit Juice Extracts

Minimum bactericidal concentrations of fruit juice extracts were investigated by micro agar dilution method after applying micro broth dilution as described in section 2.2.4.2.4. Minimum bactericidal concentration of fruit juice extracts were determined by observing the lowest concentration at which no visible bacterial colonies could be seen.

Bactericidal effects of the selected fruit juice extracts on *S. aureus* were monitored at 570 nm. Minimum bactericidal concentration of *V. vinifera*, *C. sinensis* and *P. persica* was found to be 24 mg/mL while *P. granatum* was 96 mg/mL. Results can be seen in the Figure 3.10.

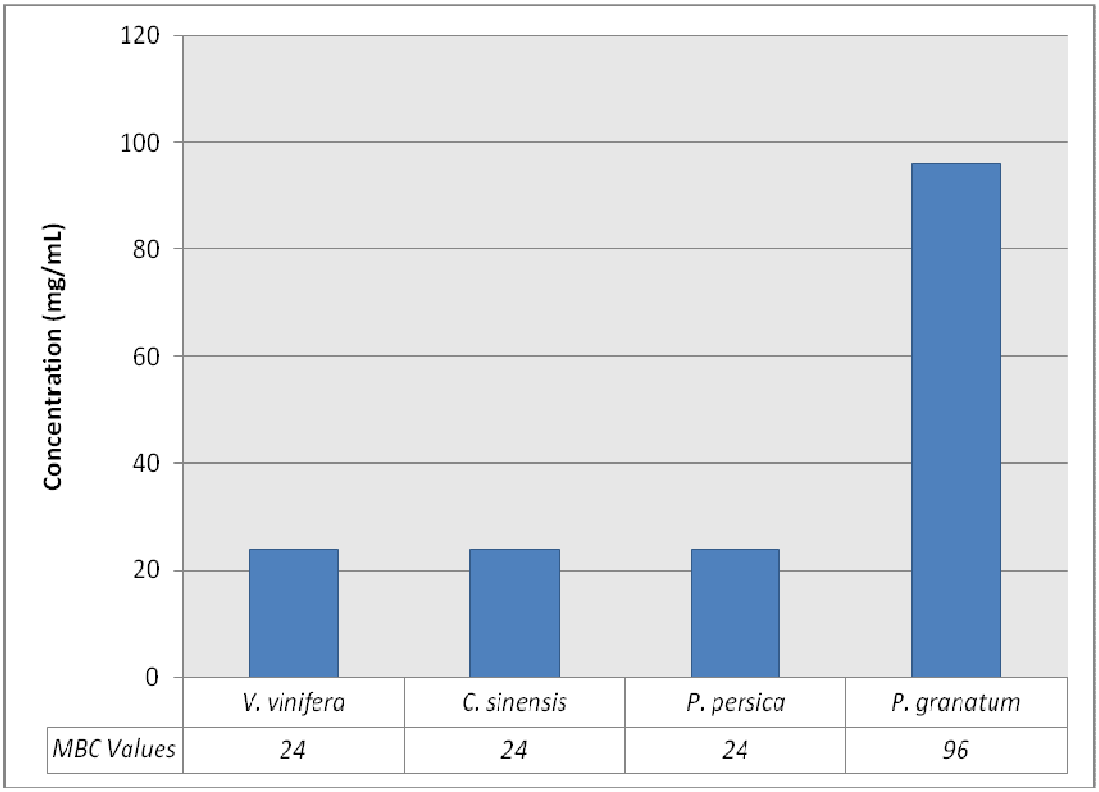

Figure 3.10 Minimum bactericidal concentrations of fruit juice extracts at 570 nm (Experiments were performed three times in duplicates).

Omoregie *et. al* searched for the antimicrobial activity of *Punica granatum* barks and leaves against various pathogen bacteria (including *S. aureus*) and fungi. Minimum bactericidal and fungicidal concentrations ranged from 1.25mg/mL to 10mg/mL (Omoregie, 2010).

Rodriguez *et. al* examined 28 edible plant extracts including peach, lemon, sour orange and seedless lime for their antimicrobial activities against *Campylobacter jejuni* and *Campylobacter coli*. The bacteria tested in this study were the major threats for food borne gastroenteritis. According to the minimum bactericidal concentration results, lime, plum, and sour orange peel were found to be most effective plant extracts with MBC values of 2 to 3 mg/mL (Rodriguez, 2010).

3.2.2.4 Antibacterial Susceptibility Test by Disc Diffusion

Kirby-Bauer method was applied to plant extracts in order to examine the antibacterial susceptibility as described in the section 2.2.4.1.

3.2.2.4.1 Antibacterial Susceptibility Test of Tea Infusion Extracts by Disc Diffusion

Staphylococcus aureus were spread on BHI agar petri plates and 6 mm discs were located on agar surface as described in the section 2.2.4.1. 20 µL of 60 mg/mL tea infusion extracts were applied on filter discs. After application of tea infusion extracts, the plates were incubated at 37 ℃ for 16 hours and inhibition zone diameters were measured in millimeters. Diameters of the inhibition zones of tea infusion extracts are given in Figure 3.11.

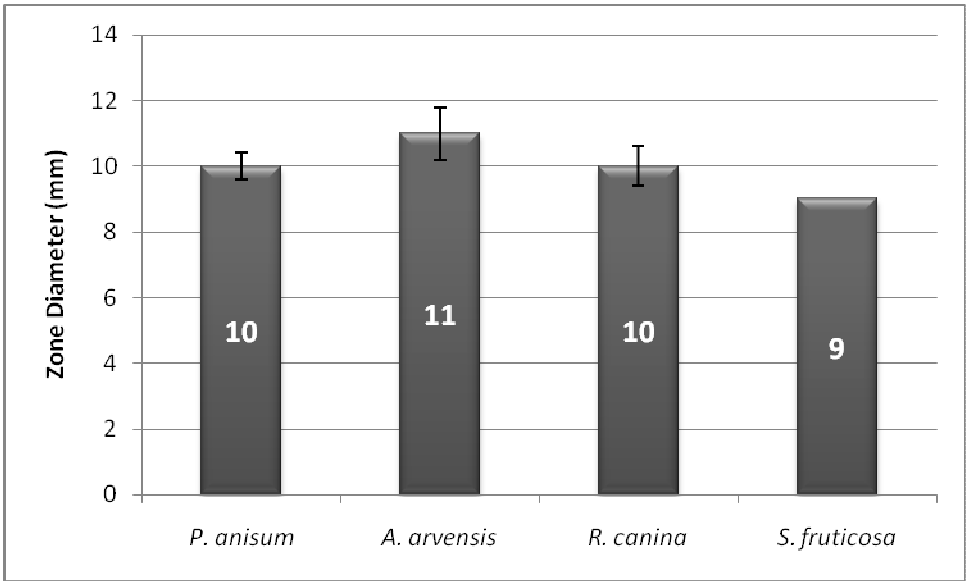

Figure 3.11 Determination of antibacterial susceptibilities of tea infusion extracts. Diameter of the empty discs (6 mm) was also included to the diameter of inhibition zones. Experiments were performed three times in duplicates.

Diameters of the inhibition zones of tea infusion extracts are given above in Figure 3.11. Inhibition zones for *P. anisum*, *A. arvensis*, *R. canina* and *S. fruticosa* were found to be 10 mm, 11 mm, 10 mm and 9 mm, respectively.

3.2.2.4.2 Antibacterial Susceptibility Test of Fruit Juice Extracts by Disc Diffusion

Staphylococcus aureus were spread on BHI agar petri plates and 6 mm discs were located on agar surface as described in the section 2.2.4.1. 20 µL of 60 mg/mL fruit juice extracts were applied on filter discs. After application of fruit juice extracts, the plates were incubated at 37 ℃ for 16 hours and inhibition zone diameters were measured in millimeters. Diameters of the inhibition zones of fruit juice extracts are given in Figure 3.12.

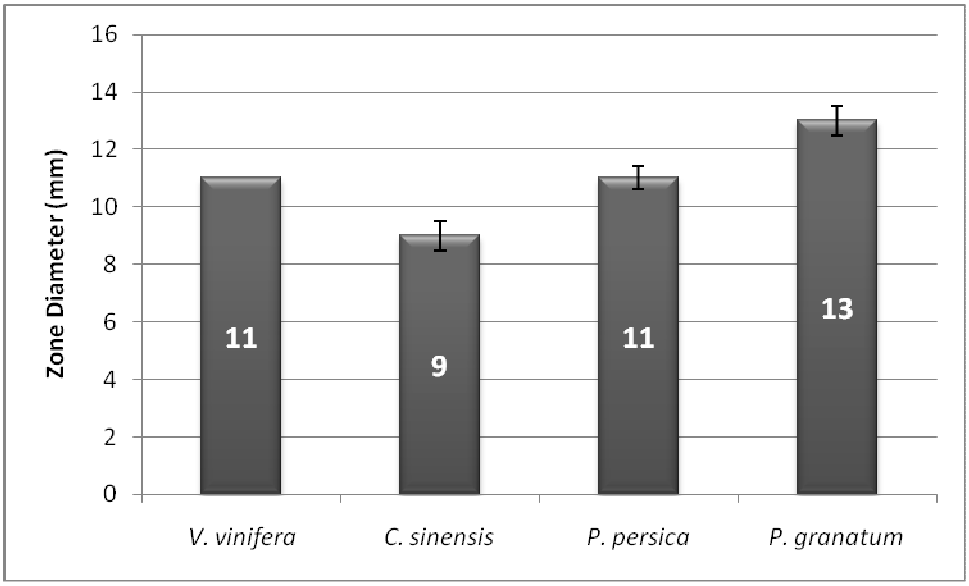

Figure 3.12 Determination of antibacterial susceptibilities of fruit juice extracts. Diameter of the empty discs (6 mm) was also included to the diameter of inhibition zones. Experiments were performed three times in duplicates.

Inhibition zones for *V. vinifera*, *C. sinensis*, *P. persica* and *P. granatum* were found to be 11 mm, 9 mm, 11 mm and 13 mm, respectively.

3.2.2.4.3 Antibacterial Susceptibility Test of Selected Antibiotics by Disc Diffusion

S. aureus were spread on BHI agar petri plates and four antibiotic discs were located on agar surface. Trimethoprim, clindamycin, penicillin and tetracycline discs were used. Agar petri plates containing the antibiotic discs were incubated as tea infusion and fruit juice extracts were done. Diameters of the inhibition zones of selected antibiotic discs are given in Figure 3.13.

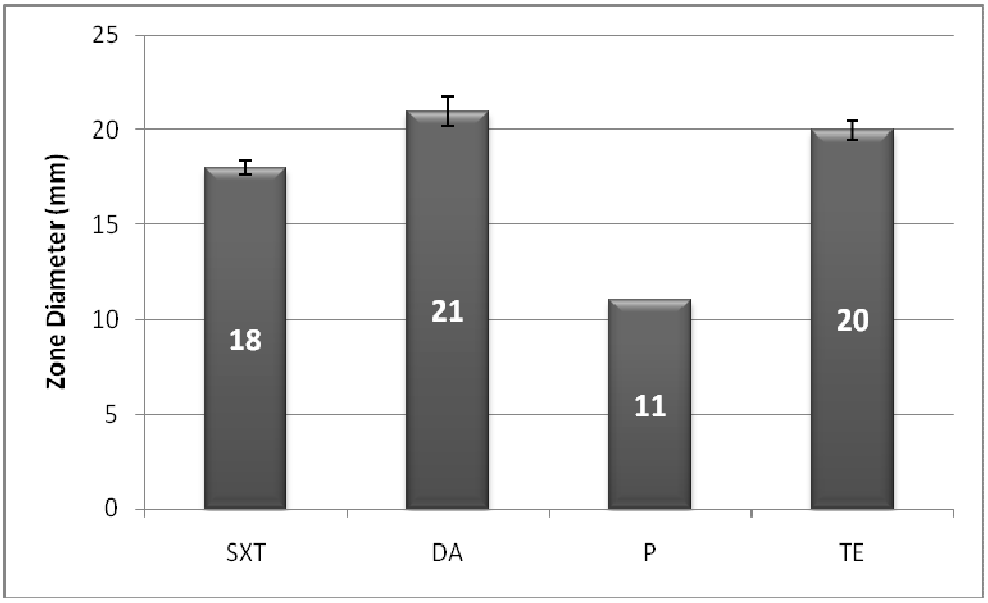

Figure 3.13 Determination of antibacterial susceptibilities of selected antibiotics. Diameter of the antibiotic discs (6 mm) was also included to the diameter of inhibition zones. Experiments were performed three times in duplicates.
SXT: Trimethoprim
DA: Clindamycin
P: Penicillin
TE: Tetracyclin

Inhibition zones for Trimethoprim (23.75 µg), Clindamycin (2 µg), Penicillin (10 µg) and Tetracycline (30 µg) were found to be 18 mm, 21 mm, 11 mm and 20 mm, respectively.

According to antibacterial susceptibility testing results, almost all of the plant extracts were effective as much as penicillin against *S. aureus*.

3.3. Investigation of Antioxidant Capacities

3.3.1 Investigation of Antioxidant Capacity by ABTS Method

Antioxidant capacities of the extracts that were obtained from tea infusions and fruit juices were studied by ABTS [2,2'-azino-bis(3-ethylbenzthiazoline-6-sulphonic acid)] radical scavenging method. ABTS method was revised according to Re *et. al* explained as described in section 2.2.5.1. Antioxidant capacities were calculated in the form of trolox equivalence antioxidant capacity (TEAC). Trolox concentration versus percent inhibition was plotted as shown in Figure 3.14.

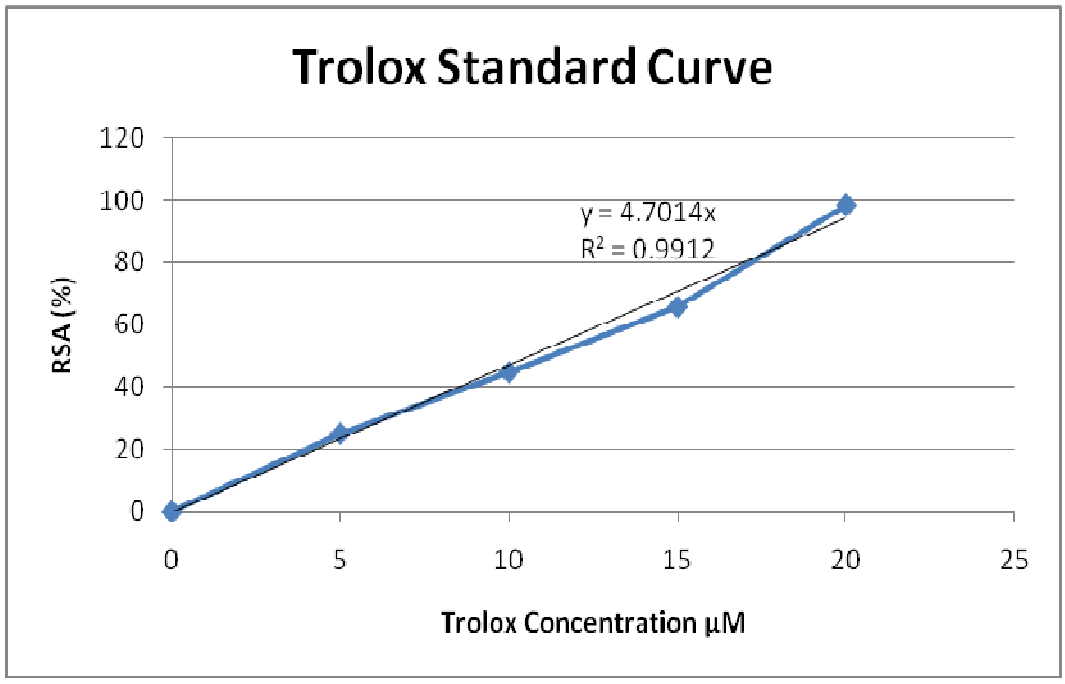

Figure 3.14 Trolox standard curve (at 734 nm). Experiments were performed three times in duplicates.

3.3.1.2 Investigation of Radical Scavenging Capacities of Tea Infusion Extracts by ABTS Method

Trolox equivalent antioxidant capacity (TEAC) values of selected tea infusion extracts were calculated according to the percent radical scavenging activity and concentration of tea infusion extracts as given in 3.15.

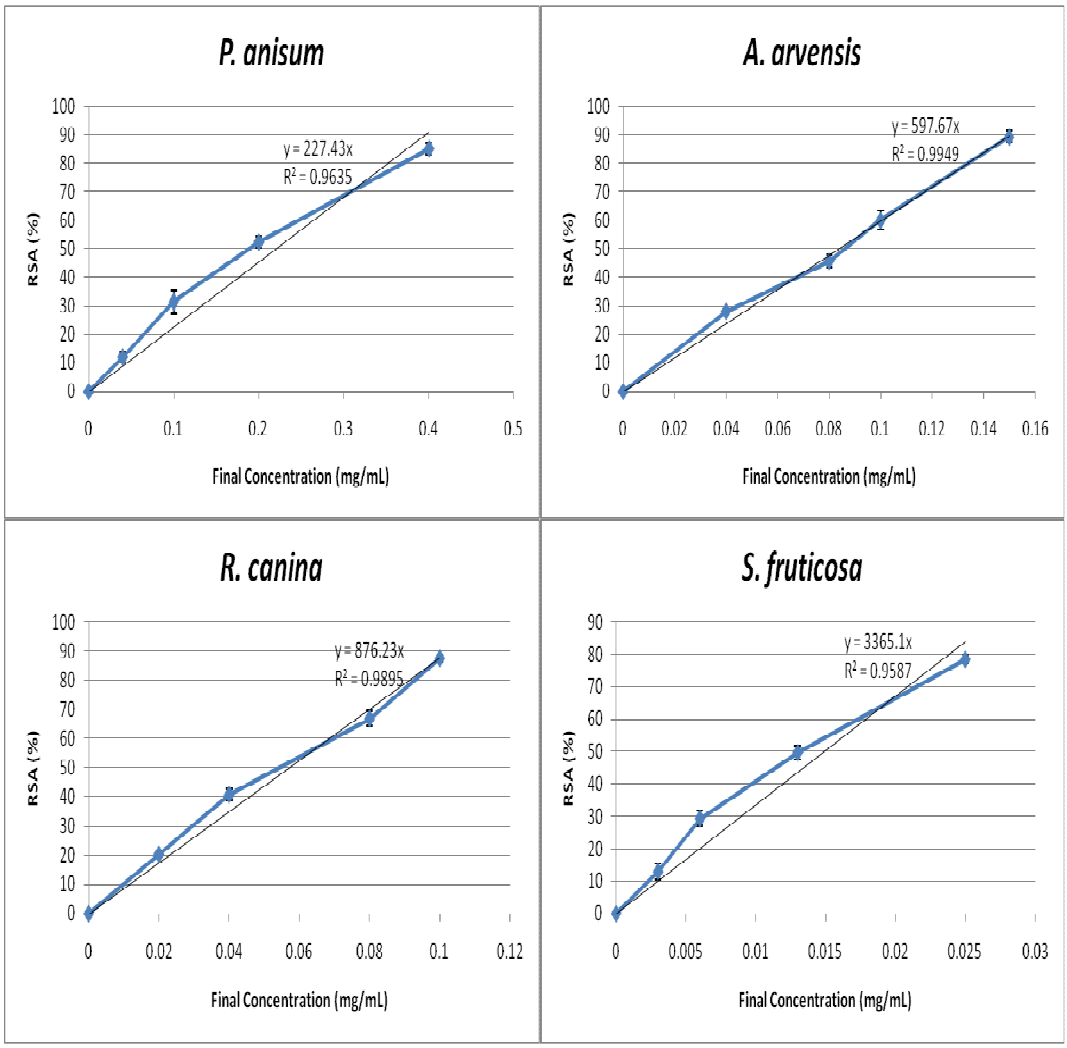

Figure 3.15 Radical scavenging activities of tea infusion extracts at 734 nm. Experiments were performed three times in duplicates.

In Table 3.4, the TEAC values and inhibition percentages of tea infusion extracts are given. Higher TEAC values indicate the higher antioxidant activities. Among the herbal teas, *S. fruticosa* was found to have the highest

antioxidant capacity with a TEAC value of 715.73. *R. canina*, *A. arvensis* and *P. anisum* had TEAC values of 186.38, 127.13 and 48.38 µmol Trolox Equivalent (TE) per gram of plant extract.

Table 3.4 Trolox equivalent antioxidant capacities (TEAC) of tea infusion extracts.

Extract	**TEAC Value[a] (µmol/g extract) ± SD**	**Inhibition[b] % ± SD**
P. anisum	48.38 ± 1.242	85.06 ± 2.053
A. arvensis	127.13 ± 1.69	89.35 ± 2.014
R. canina	186.38 ± 1.882	87.43 ± 1.294
S. fruticosa	715.73 ± 4.265	78.49 ± 1.17

TEAC value: Radical scavenging activity µmol trolox equivalents /g of extract (DW)
[a]Mean of three independent experiments in duplicates with standard deviation errors
[b]Scavenging capacities at the time of 6th minute

Miliauskas *et. al* observed the antioxidant activities of medicinal and aromatic plant extracts including some of the Salvia species. According to the results obtained from ABTS radical scavenging method, extracts of *Geranium macrorrhizum* and *Potentilla fruticosa* were the most efficient $ABTS^{+\bullet}$ scavengers (the absorbance value measured at 6th minute is close to 0). Moreover, antioxidative activity of *Salvia officinalis* extract was lower than *P. fruticosa* and *G. macrorrhizum*. *S. officinalis* absorbed 89% of ABTS radical. *Salvia glutinosa*, *Salvia sclarea* and *Salvia pratensis* extracts also showed strong antioxidant activities (Miliauskas, 2004).

Su *et. al* examined the ABTS radical scavenging capacities of black pepper-corn, nutmeg, rosehip, cinnamon and oregano extracts. According to this study, all of the plant extracts were found to have significant ABTS radical scavenging capacities. 80% methanol extracts of plants showed ABTS

radical scavenging ability ranged from 1064 µmol Trolox Equivalent (TE) per gram (g) of plant material to 23 µmol Trolox Equivalent per gram of plant material. Cinnamon was the best ABTS radical scavenger ability whereas, black peppercorn had the worst. The ABTS radical scavenging activity of rosehip was found to be 190 µmol TE/g (Su, 2007).

3.3.1.2 Investigation of Radical Scavenging Capacities of Fruit Juice Extracts by ABTS Method

Trolox equivalent antioxidant capacity (TEAC) values of selected fruit juice extracts were calculated according to the percent radical scavenging activity and concentration of fruit juice extracts as given in 3.16.

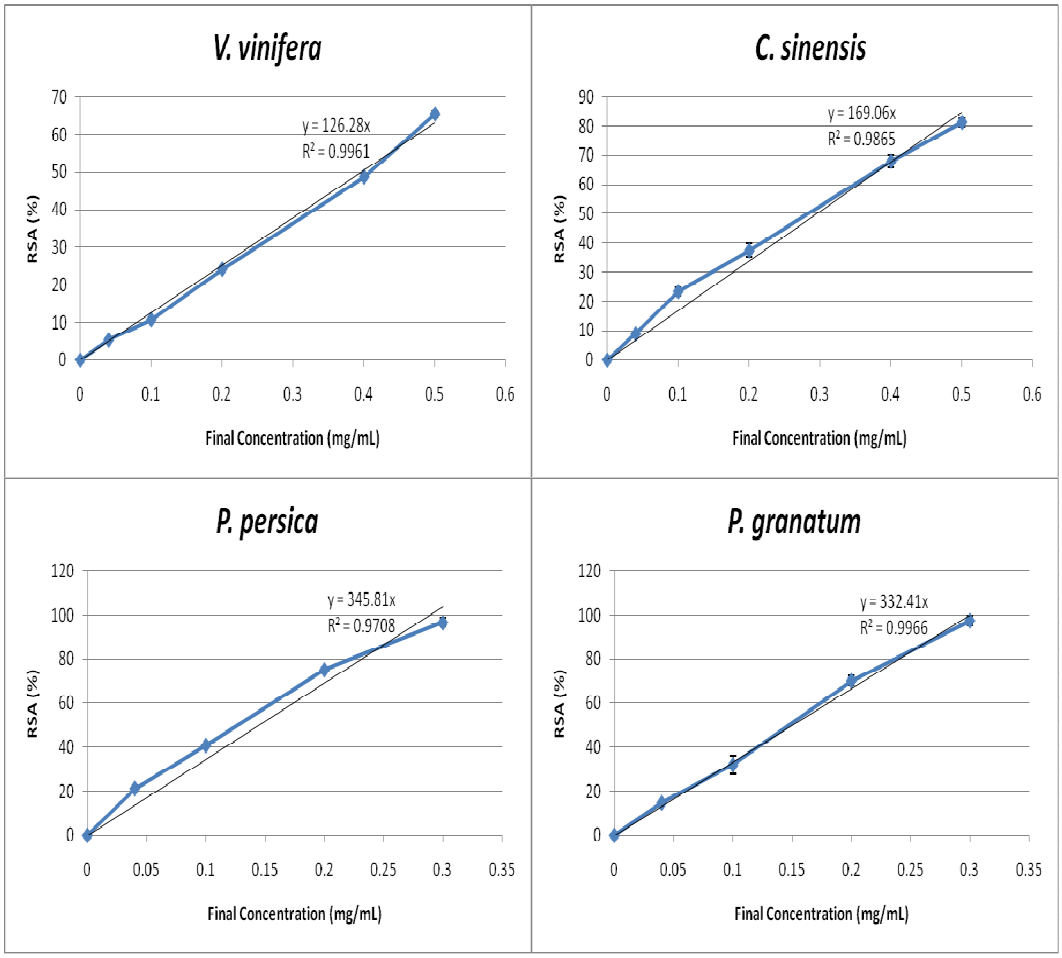

Figure 3.16 Radical scavenging activities of fruit juice extracts at 734 nm. Experiments were performed three times in duplicates.

In Table 3.5, the TEAC values and inhibition percentages of fruit juice extracts are given. Higher TEAC values indicate the higher antioxidant activities. Among the fruits, *P. persica* and *P. granatum* were found to have the highest antioxidant capacities with TEAC values of 73.55 and 70.71 µmole TE/g of extract. *V. vinifera* and *C. sinensis* had TEAC values of 26.86, and 35.96 µmole TE/g of extract.

Table 3.5 Trolox equivalent antioxidant capacities (TEAC) of fruit juice extracts.

Extract	**TEAC Value[a] (µmol/g extract) ± SD**	**Inhibition[b] % ± SD**
V. vinifera	26.86 ± 0.217	65.39 ± 0.769
C. sinensis	35.96 ± 0.887	81.33 ± 1.526
P. persica	73.55 ± 0.973	96.67 ± 1.928
P. granatum	70.71 ± 0.457	97.52 ± 2.268

TEAC value: Radical scavenging activity µmol trolox equivalents /g of extract (DW)
[a]Mean of three independent experiments in duplicates with standard deviation errors
[b]Scavenging capacities at the time of 6th minute

Okonogi *et. al* compared the antioxidant activities and cyctotoxities of eight different fruits grown in Thailand. The antioxidant capacities were investigated by DPPH and ABTS methods. By using ABTS method, they confirmed that all of the fruit extracts tested in this study showed free radical scavenging property in different ratios. The TEAC value of the pomegranate extract showed the highest antioxidant activity with a TEAC value of 4.07 mM TE / mg of plant material (Okonogi, 2007).

Yemis and his co-workers investigated the antioxidant activities and total phenolic contents of grape seed extracts. Various grape seeds were collected from different locations in Turkey. According to this study, antioxidant capacities of different grape seed extracts were ranged between 2.46–4.14 µmol TE per mg of extracts (Yemis, 2007).

3.3.2 Investigation of Antioxidant Capacity by DPPH Method

Antioxidant capacities of the extracts that were obtained from tea infusions and fruit juices were studied by DPPH radical scavenging method.

3.3.2.1 Investigation of Radical Scavenging Capacities of Tea Infusion Extracts by DPPH Method

DPPH radical scavenging activities of tea infusion extracts were measured according to the method as previously explained in section 2.2.5.2. Quercetin, whose antioxidant capacity is known widely, was used as standard for DPPH radical scavenging activity experiments. Percent DPPH radical scavenging activity versus extract concentration graph was plotted and the results are given in Figure 3.17.

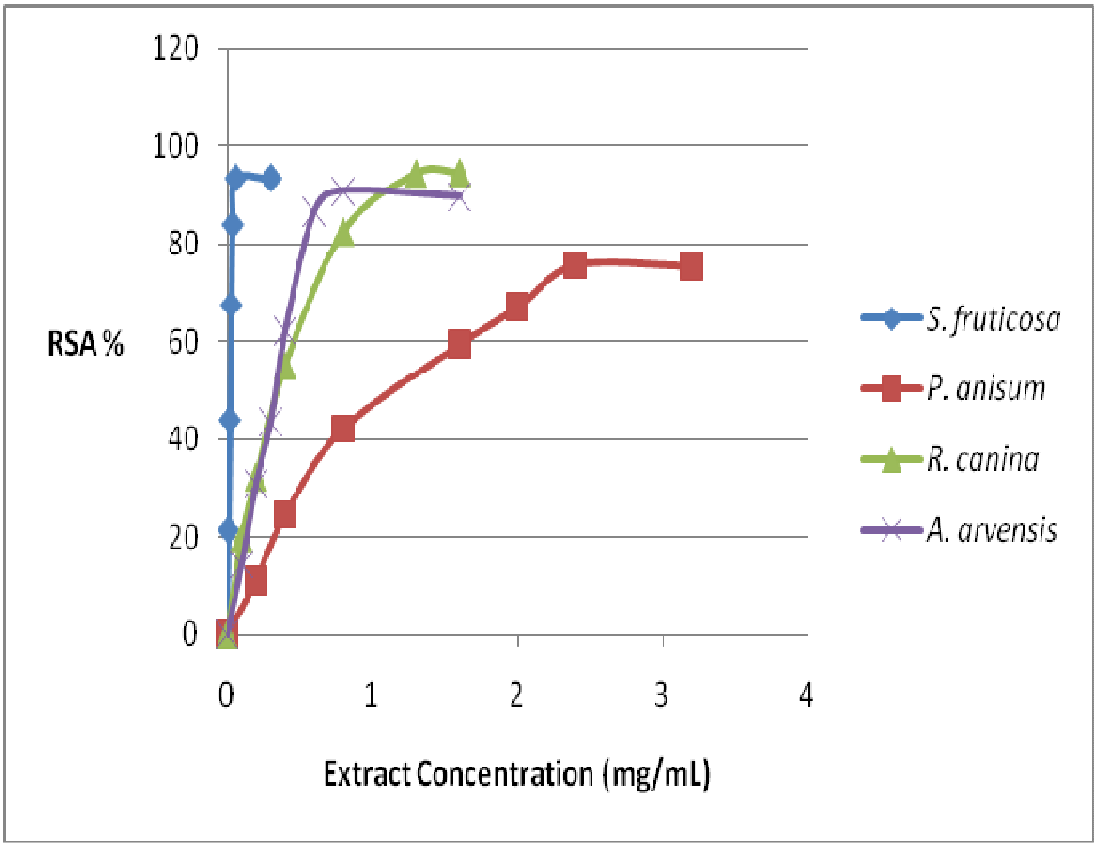

Figure 3.17 DPPH radical scavenging activity in percent versus extract concentrations (mg/mL) of tea infusion extracts. DPPH radical scavenging activity values were obtained after 30 minute of incubation time, at 517 nm.

EC_{50} value of 0.05 mg/mL of *S. frutioca* demonstrates that it has the most effective radical scavenging activity. *A. arvensis*, *R. canina* and *P. anisum* are following *S. frutioca* with an increasing order of EC_{50} values. Fifty percent effective concentrations for radical scavenging activities of tea infusion extracts are given in Table 3.6.

Table 3.6 Fifty percent effective concentrations for DPPH radical scavenging activities.

Tea infusions	**[a]DPPH RSA EC_{50} (mg/mL)**
P. anisum	1.53 ± 0.004
A. arvensis	0.47 ± 0.006
R. canina	0.57 ± 0.011
S. fruticosa	0.05 ± 0.001
Quercetin	0.008 ± 0.0002

DPPH RSA EC_{50}: Effective concentration of plant extracts required for scavenging 50 % of DPPH radical
[a]Mean of three independent experiments in duplicates with standard deviation errors

Senol *et. al* studied the dichloromethane extracts of *Salvia* taxa. The best radical scavenger against DPPH was *Salvia fruticosa* with 89.23% of inhibition and it is found to be the most promising *Salvia* species (Senol, 2010).

Nickavar *et al.* compared the EC_{50} values (with 95% confidence intervals) of the selected seven extracts of Umbelliferae fruits. *Pimpinella anisum* has the highest DPPH scavenging activity with an EC_{50} value of 109.80 µg/mL. Therefore, *P. anisum* can be used as a natural source to develop the antioxidants and free radical scavengers. Also it can be a useful source of materials for food preservatives and human health among the other selected Umbelliferae fruits (Nickavar, 2009).

In the reducing power assay, rosehip, *R. canina,* exhibited a high antioxidant capacity at all concentrations. This finding suggests that *R. canina* has a high antioxidant effect and, DPPH scavenging activity results of *R. canina* supports this finding. DPPH radical scavenging increased with the increasing concentration of *R. canina* (1, 2 and 3%). At 3%, 96% DPPH radical was inhibited by *R. canina*. However, DPPH radical scavenging activity did not increase at higher concentrations (4 and 8%). The scavenging activity of *R. canina* drastically decreased at these concentrations. This

information claims that *R. canina* may also behave like a prooxidant in different systems depending on the concentration of the extract (Kilicgun, 2010).

Djeridane *et. al* examined the antioxidant capacity of phenolic compounds in some Algerian medicinal plants. It was found that these medicinal plants showed high antioxidant activity than the common nutritional plants. *Artemisia arvensis* and *Artemisia campestris* were found to be the most efficient antioxidants among the tested plants (Djeridane, 2000).

3.3.2.2 Investigation of Radical Scavenging Capacities of Extracts of Fruit Juices by DPPH Method

DPPH radical scavenging activities of fruit juice extracts were measured according to the method as previously explained in 2.2.5.2. Quercetin, whose antioxidant capacity is known widely, was used as standard for DPPH radical scavenging activity experiments. Percent DPPH radical scavenging activity versus extract concentration graph was plotted and the results are given in Figure 3.18.

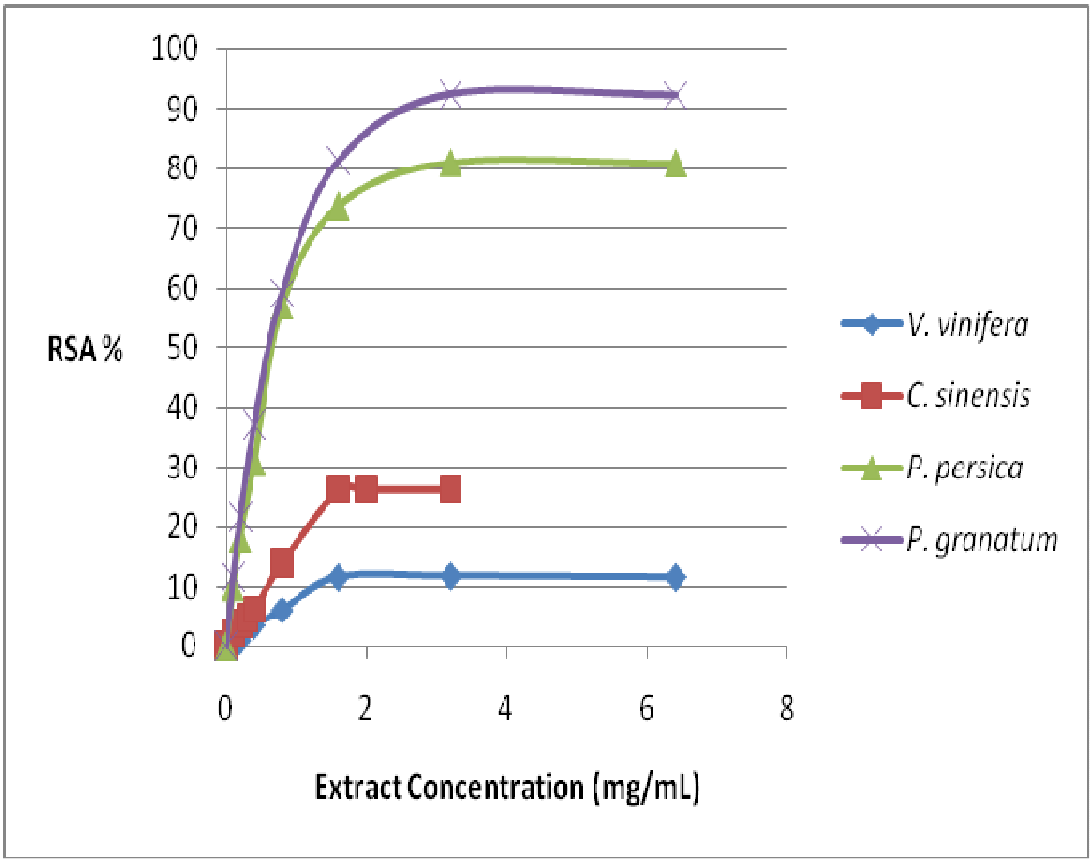

Figure 3.18 DPPH radical scavenging activity in percent versus extract concentrations (mg/mL) of fruit juice extracts. DPPH radical scavenging activity values were obtained after 15 minute of incubation time, at 517 nm.

P. granatum has the highest DPPH radical scavenging activity among other fruit juices. EC_{50} value of the *P. granatum* is 1.6 mg/mL. Moreover, *P. persica* has an EC_{50} value around 2 mg/mL. Fifty percent effective concentrations for radical scavenging activities of fruit juice extracts are given in Table 3.7.

Table 3.7 Fifty percent effective concentrations for DPPH radical scavenging activities

Fruit Juices	**[a]DPPH RSA EC_{50} (mg/mL)**
V. vinifera	Not Applicable
C. sinensis	Not Applicable
P. persica	2 ± 0.093
P. granatum	1.6 ± 0.014
Quercetin	0.008 ± 0.0002

DPPH RSA EC_{50}: Effective concentration of plant extracts required for scavenging 50 % of DPPH radical
[a]Mean of three independent experiments in duplicates with standard deviation errors

In 2010, Abd Ghafar reported that *Citrus hystrix* was the best DPPH radical scavenger with respect to other citrus species. *C. hystrix* fresh juice with a concentration of 35 mg/100 mL was required to scavenge 50% of initial concentration of DPPH radical which indicates its EC_{50} value (Abd Ghafar, 2010).

Martos and his co-workers studied the antioxidant properties of *Punica granatum*. According to them, the WFB (direct extraction from arils and peel) samples had a higher inhibition capacity against DPPH radical than the AB (direct extraction only from arils) samples. Increasing the concentration of both AB and WFB resulted in an increase in radical scavenging activity. 100 g/L of AB and WFB showed approximately 75% and 92% inhibition, respectively (Martos, 2010).

3.3.3 Investigation of Total Phenolic Content of Plant Extracts

Total phenolic content of the selected fruit juice and tea infusion extracts were determined by gallic acid standard curve which is previously described

in section 2.2.5.3 Gallic acid standard curve was plotted in order to calculate total phenolic content of selected extracts in terms of gallic acid equivalence. Absorbance versus Gallic Acid concentration graph is given in the Figure 3.19.

The Folin-Ciocalteau method has a major drawback. Interference of the sugars can be problematic while determining the total phenolic content for fruits since they have high levels of sugar.

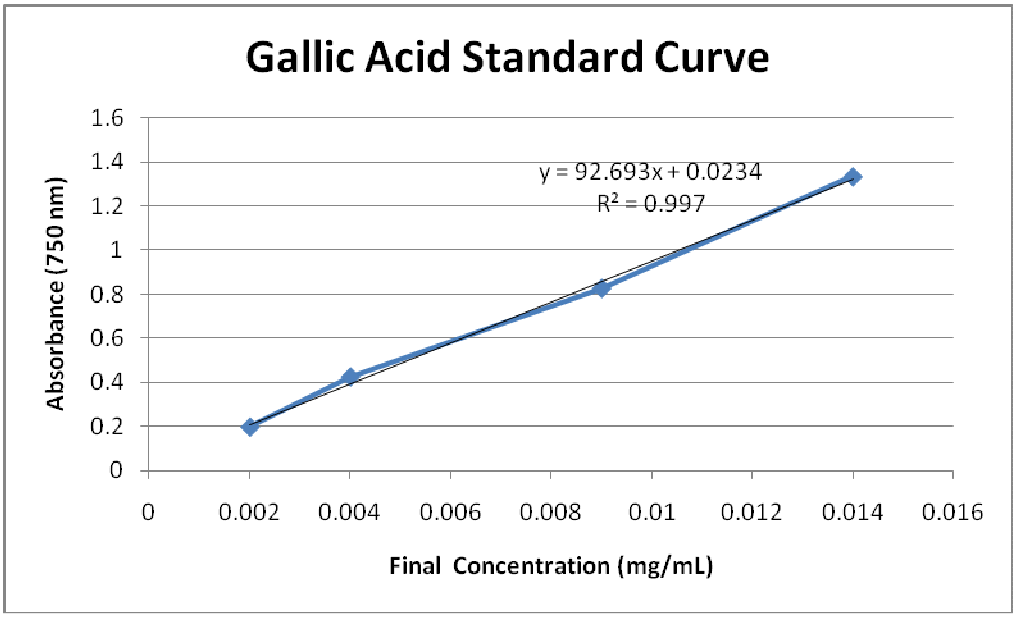

Figure 3.19 Gallic acid standard curve. Experiments were performed three times in duplicates.

3.3.3.1 Investigation of Total Phenolic Content of Tea Infusion Extracts

The total phenolic content of the tea infusion extracts were studied in order to find out the relationship between antioxidant capacity and total phenolic content of the selected herbs. It is thought that phenolic compounds are the major compounds that are responsible for antioxidant activity. *S. fruticosa* contained the highest amount of phenolic compounds, it had 159.17 µg gallic acid equivalent (GAE) in mg of extract. Also, *S. fruticosa* was found to be the most potent ABTS and DPPH radical scavenger with respect to other herbs

tested. In addition to this information, *P. anisum* had the least DPPH scavenging activity and it had the least phenolic content too. Total phenolic content of the tea infusion extracts are given in Table 3.8.

Table 3.8 Total phenolic content of tea infusion extracts.

Tea infusions	**[a]TP GAE (µg/mg)**
P. anisum	9.42 ± 0.515
A. arvensis	16.83 ± 2.608
R. canina	24.75 ± 1.603
S. fruticosa	159.17 ± 14.461

TP GAE (µg/mg): Total phenolic content µg equivalents of gallic acid/mg of plant extract
[a]Mean of three independent experiments in duplicates with standard deviation errors

Pizzale *et. al* reported that the average concentration of phenolic compounds (g of gallic acid/kg of extract) in sage samples was found to be 81.3 ±15.4 g/kg. *S. fructicosa* had the highest amount of total phenols (113.4g/kg), whereas *S. officinalis* had the lowest amount total phenols (46.4g/kg) (Pizzale, 2002).

In 2005, Proestos *et. al* studied the total phenol content of some of the plant extracts including anise (*Pimpinella anisum*) and the total phenolics in anise extract was 1.8 ± 0.1 mg of gallic acid/g of dry sample. Moreover, the content of phenolic acids was found to be 1.1 ± 0.01 (mg/100 g of dry sample) gallic acid and 0.8 ± 0.02 (mg/100 g of dry sample) caffeic acid (Proestos, 2005).

3.3.3.2 Investigation of Total Phenolic Content of Fruit Juice Extracts

The total phenolic content of the fruit juice extracts were studied and *P. persica* contained the highest amount of phenolic compounds, it had 18.1 µg Gallic Acid Equivalent (GAE) in mg of extract. *P. granatum* had

approximately half of total phenol content that peach contained. It had 8.6 µg gallic acid equivalent in mg of extract. *C. sinensis* and *V. vinifera* followed them with the 6.4 and 1.3 µg gallic acid equivalent in mg of extract, respectively which can be seen in the Table 3.9.

Table 3.9 Total phenolic content results of fruit juice extracts
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Fruit Juices	**[a]TP GAE (µg/mg)**
V. vinifera	1.38 ± 0.103
C. sinensis	6.36 ± 0.303
P. persica	18.05 ± 0.996
P. granatum	8.64 ± 0.564

TP GAE (µg/mg): Total phenolic content µg equivalents of gallic acid/mg of plant extract
[a]Mean of three independent experiments in duplicates with standard deviation errors

The phenolic content of the some Turkish pomegranate varieties was studied by the Folin-Ciacalteu method and the range of phenolic content of pomegranate varieties were between 1555.33 and 541.47 mg/L. Samples with the highest phenolic content had a sour taste and the one with the lowest phenolic content had a sweet taste (Hepaksoy, 2009).

Baydar *et. al* claimed that the amounts of total phenolic content of *Vitis vinifera* L. extracted with the different solvent mixtures ranged from 627.98 to 667.87 mg GAE/g in grape seed extracts. And it ranged from 29.55 to 45.44 mg GAE/g in grape bagasse extracts. Grape seed extracts contained the highest levels of phenolic compounds, nevertheless grape bagasse extracts contained the lowest (Baydar, 2004).

3.3.4 Investigation of Total Flavonoid Content of Plant Extracts

Total flavonoid content of the selected fruit juice and tea infusion extracts were determined by quercetin standard curve which is previously described in section 2.2.5.4 Quercetin standard curve was plotted in order to calculate total flavonoid content of selected extracts in terms of quercetin equivalence. Absorbance versus Quercetin concentration graph is given in the Figure 3.20.

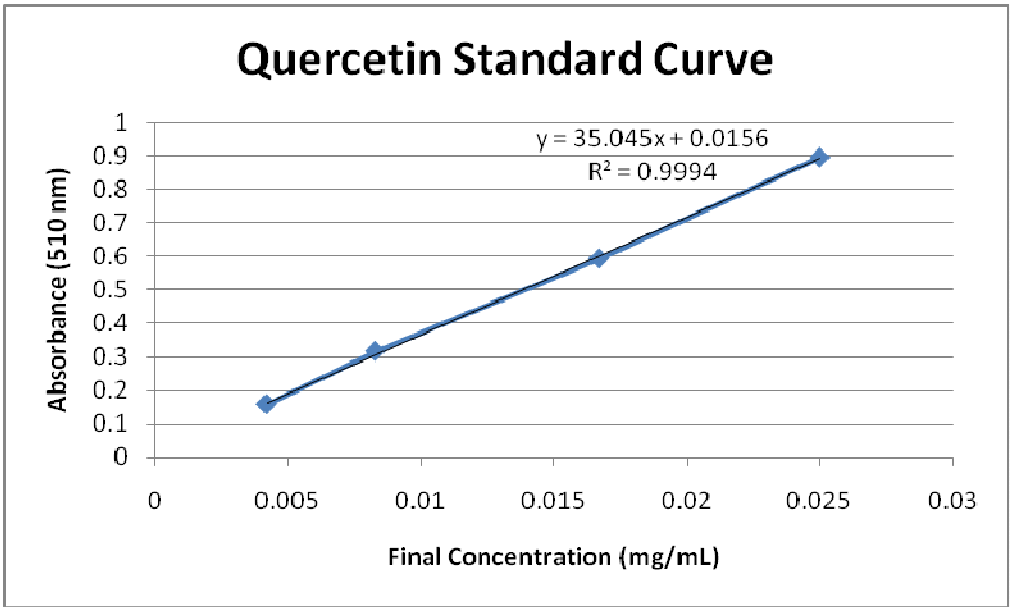

Figure 3.20 Quercetin standard curve. Experiments were performed three times in duplicates.

3.3.4.1 Investigation of Total Flavonoid Content of Tea Infusion Extracts

The total flavonoid content of the tea infusion extracts were studied in order to enlighten the relationship between total phenolic content and total flavonoid content of the selected herbs. Flavonoids are the major group of plant phenols. *S. fruticosa* contained the highest amount of phenolic compounds and total flavonoid content. It had 159.17 µg Gallic Acid Equivalent (GAE) in mg of extract and 201.15 µg Quercetin Equivalent (QE)

in mg of extract. Total flavonoid content of the selected herbs are given in Table 3.10.

Table 3.10 Total flavonoid content of tea infusion extracts.

Tea infusions	**[a]TF QE (µg/mg)**
P. anisum	1.57 ± 0.129
A. arvensis	9.33 ± 0.289
R. canina	9.03 ± 0.725
S. fruticosa	201.15 ± 15.045

TF QE (µg/mg): Total flavonoid contents µg equivalents of µg quercetin/mg of plant extract
[a]Mean of three independent experiments in duplicates with standard deviation errors

Papageorgiou *et. al* has suggested that the seasonal profiles of the flavonoid content of the *Salvia fruticosa* grown in Greece differed with respect to the sample collection time. (February, May, and August of 2005 and 2006). It was also suggested that quercitrin and luteolin contents of *S. fruticosa* increased with advancing development stages. The amount of quercitrin and luteolin in sage extracts reached the highest level in May during the flowering period (9.60 and 2.75 mg/g dry plant for quercitrin and luteolin, respectively, in 2005 and 3.10 and 3.80 mg/g dry plant for quercitrin and luteolin, respectively, in 2006). The amount of flavonoids was the lowest level during the late fruiting period (Papageorgiou, 2008).

Ghazghazi *et. al* studied the flavonoid content of the *Rosa canina* samples that were collected from different locations in Tunisia. They found that the lowest concentration of the total flavonoid content was (0.11 ± 0.01 mg/mL) and the highest concentration was (0.41 ± 0.01 mg/mL) (Ghazghazi, 2010).

Djeridane *et. al* reported that all the plants tested were rich in flavonoids. *Anthemis arvensis* had 13.20 mg/g rutin equivalent of the crude extract. It

was also stated that hydroxycinnamic and hydrobenzoic derivatives were the major flavonoids in all of the *Artemisia* species belonging to Asteraceae family including *Anthemis arvensis* (Djeridane, 2006).

3.3.4.2 Investigation of Total Flavonoid Content of Fruit Juice Extracts

The total flavonoid content of the fruit juice extracts were studied and *P. persica* contained the highest amount of flavonoid content as well as total phenolic content. It had 18.05 µg Gallic Acid Equivalent (GAE) in mg of extract and 1.26 µg Quercetin Equivalent (QE) in mg of extract. Total flavonoid content of the selected fruit juices are given in Table 3.11.

Table 3.11 Total flavonoid content of fruit juice extracts.

Fruit juices	**[a]TF QE (µg/mg)**
V. vinifera	0.11 ± 0.013
C. sinensis	0.18 ± 0.041
P. persica	1.26 ± 0.081
P. granatum	1.14 ± 0.109

TF QE (µg/mg): Total flavonoid contents µg equivalents of µg quercetin/mg of plant extract
[a]Mean of three independent experiments in duplicates with standard deviation errors

Abd Ghafar *et. al* studied the flavonoid content of four citrus species, *Citrus hystrix, C. aurantifolia, C. microcarpa* and *C. Sinensis*. *C. hystrix* had the highest amount of flavonoid content (22.25 ± 0.20 mg hesperidine equivalent/100 mL of juice) while *C. sinensis* had the lowest amount of flavonoid content (2.99 ± 0.09 mg hesperidine equivalent/100 mL of juice) (Abd Ghafar, 2010).

Marinova *et. al* studied the total phenol and flavonoid contents as well as total flavonoid ratio to phenolics. *Vitis vinifera* had 36.5 mg catechin

equivalent/100g fresh mass and the ratio was found to be 0.2 (Marinova, 2005).

ABTS, DPPH, total phenol, total flavonoid, MIC, MBC and disc diffusion tests results of tea infusion and fruit juice extracts were compared and given in Table 3.12. *S. fruticosa* was found to be the most active antioxidant with the highest amounts of phenolics and flavonoids. It was also found to be the most active antimicrobial compound with the lowest MIC and MBC values. Therefore, *S. fruticosa* could be regarded as the most promising plant sample tested in this study. Moreover, plant phenols and flavonoids might play a role in exhibiting high antioxidant and antimicrobial activity. Previously in our lab, a study carried out with a different Gram (+) bacteria, *Streptococcus pyogenes*. It was concluded that *Streptococcus pyogenes* was most effectively inhibited by *S. fruticosa* and *A. arvensis* with MIC values of 3 mg/mL and with the MBC values 16 mg/mL and 4 mg/mL, respectively.

Table 3.12 TEAC (µmol/g), DPPH EC_{50} (mg/mL), TP GAE (µg/mg), TF QE (µg/mg), MIC (mg/mL), MBC (mg/mL) and disc diffusion (mm) values for the tea infusion and the fresh fruit extracts.

	TEAC Value[a] (µmol/g)	DPPH EC_{50}[b] (mg/mL)	TP GAE[c] (µg/mg)	TF QE[d] (µg/mg)	MIC (mg/mL)	MBC (mg/mL)	Disc diffusion (mm)
P. anisum	48.38 ± 1.242	1.53 ± 0.004	9.41 ± 0.515	1.57 ± 0.129	6	96	10 ± 0.4
A. arvensis	127.13 ± 1.69	0.47 ± 0.006	16.83 ± 2.608	9.33 ± 0.289	48	48	11 ± 0.8
R. canina	186.38 ± 1.882	0.57 ± 0.011	24.75 ± 1.603	9.03 ± 0.725	96	96	10 ± 0.6
S. fruticosa	715.73 ± 4.265	0.05 ± 0.001	159.17 ± 14.46	201.15 ± 15.045	3	3	9
V. vinifera	26.86 ± 0.217	NA	1.38 ± 0.103	0.11 ± 0.013	6	24	11
C. sinensis	35.96 ± 0.887	NA	6.36 ± 0.303	0.18 ± 0.041	6	24	9 ± 0.5
P. persica	73.55 ± 0.973	2 ± 0.093	18.05 ± 0.996	1.26 ± 0.081	24	24	11 ± 0.4
P. granatum	70.71 ± 0.457	1.6 ± 0.014	8.64 ± 0.564	1.14 ± 0.109	3	96	13 ± 0.6
Quercetin	ND	0.008 ± 0.0002	ND	ND	ND	ND	ND
Trimethoprim	-	-	-	-	-	-	18 ± 0.4
Clindamycin	-	-	-	-	-	-	21 ± 0.8
Penicillin	-	-	-	-	-	-	11
Tetracycline	-	-	-	-	-	-	20 ± 0.5

[a]TEAC value: Trolox equivalent antioxidant capacity of plant extracts in µmol trolox equivalents/g of extract (DW).
[b]DPPH RSA EC_{50} : Effective concentration of plant extracts which scavenge 50 % of DPPH radical.
[c]TP GAE: Total phenolic contents of plant extracts in µg equivalents of gallic acid/mg of plant extract.
[d]TF QE: Total flavonoid contents of plant extracts in µg equivalents of quercetin/mg of plant extract.
NA: Not Applicable
ND: Not Determined

CHAPTER 4

CONCLUSION

As a conclusion, fruit juices and herbal teas that are widely consumed in regular diet were examined for their antioxidant and antimicrobial activities. *Staphylococcus aureus* was the choice of bacterium among the food-borne pathogens. Main purpose was to reduce the intake of antibiotics, and improve the immune system against Staphylococcal infections, by the consumption of beverages commonly used.

Among the tea infusion extracts, *S. fruticosa* was found to be the best ABTS radical scavenger. *P. persica* displayed the highest ABTS radical scavenging capacity among fruit juice extracts. Moreover, *S. fruticosa* was also found to be the best DPPH radical scavenger of all tea infusion extracts. However, *P. granatum* exhibited the highest DPPH radical scavenging capacity among fruit juice extracts.

Total phenolic and flavonoid content determination findings also supported the findings of ABTS and DPPH experiments. *S. fruticosa* had the highest amount of total phenolic compounds and total flavonoids among herbal teas. Furthermore, *P. persica* and *P. granatum* was found to have higher amount of total phenolic compounds and total flavonoids than other fruit juice extracts.

According to the MIC tests, the growth of *S. aureus* was inhibited most effectively by *S. fruticosa* of all tea infusion extracts. The concentration of *S. fruticosa* which inhibited the growth of *S. aureus* was measured as 3 mg/mL.

Minimum inhibitory concentration of *S. fruticosa* was also found to be equal to the minimum bactericidal concentration. 3 mg/mL of *S. fruticosa* killed the bacteria, so that no growth could be seen. Furthermore, the most effective growth inhibition was achieved by *P. granatum* with a value of 3 mg/mL among fruit juice extracts. Although, pomegranate succeeded the most effective growth inhibition, the ability of killing bacteria of interest was not as high as other fruit juice extracts. *V. vinifera*, *C. sinensis* and *P. persica* displayed the highest bactericidal activity with a MBC value of 24 mg/mL.

The extraction yield of *S. fruticosa* was 15.50 % (w/w) which accounts for 1.94 mg of sage tea needed to inhibit the growth of 5 $x10^5$ colony forming units of *S. aureus*. Similarly, the extraction yield of *P. granatum* was 14.55 % (w/w) and the amount of pomegranate juice that showed bacteriostatic effect on *S. aureus* was found to be 20.6 mL. Furthermore, 3.87 mg of sage tea killed 5 $x10^5$ colony forming units of *S. aureus*. Also, 208.7 mL peach juice and 210 mL grape juice were the effective amounts which killed the same number of bacterial colonies.

Disc diffusion test was also applied to screen the antimicrobial activity. *P. granatum* exhibited the highest antimicrobial activity. It achieved 12.5 mm diameter of inhibition zone. *P. persica*, *V. vinifera* and *A. arvensis* followed *P. granatum* with decreasing antimicrobial activity. 20 µL of plant extracts with a concentration of 60 mg/mL were applied to the filter disc, so that one can compare the effectiveness of plant extracts with respect to common antibiotics.

Diameter of inhibition zone formed by standard penicillin disc containing 10 µg antibiotic was measured as 11 mm. *V. vinifera*, third active plant extract against *S. aureus,* also showed an antimicrobial activity equal to penicillin. The diameter of inhibition zone of *V. vinifera* was found to be 11 mm. This means that 10.5 mL of grape juice had the same effect with 10 µg penicillin.

To sum up, fruits such as *P. granatum*, *V. vinifera* and herbal teas like *S. fruticosa*, *P. anisum* which are widely used in regular diet, can be useful natural sources for inhibiting and/or killing the food-borne pathogen, *Staphylococcus aureus.*

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