INVESTIGATION OF ANTIOXIDANT ACTIVITIES OF FRUIT JUICES AND HERBAL TEAS AND THEIR ANTIMICROBIAL EFFECTS ON PROTEUS MIRABILIS

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INVESTIGATION OF ANTIOXIDANT ACTIVITIES OF FRUIT JUICES AND HERBAL TEAS AND THEIR ANTIMICROBIAL EFFECTS ON PROTEUS MIRABILIS

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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 the this work.

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ABSTRACT

INVESTIGATION OF ANTIOXIDANT ACTIVITIES OF FRUIT JUICES AND HERBAL TEAS AND THEIR ANTIMICROBIAL EFFECTS ON PROTEUS MIRABILIS

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Herbal teas and fruit juices used in our regular diet may have importance in the protective treatment of some infectious diseases. In this study, selected dietary beverages were investigated for their antioxidant capacities and antimicrobial activities against *Proteus mirabilis*, a well known bacteria in urinary tract infections.

Herbal teas; sage (*Salvia fruticosa Mill*), anise (*Pimpinella anisum L.)*, rosehip (*Rosa canina L.)*, camomile (*Anthemis arvensis L.)* and fruit juices; grape (*Vitis vinifera L.)*, orange (*Citrus sinensis L*.), peach (*Prunus persica L*.), and pomegranate (*Punica granatum L.)* were chosen as samples of regular diets. Selected fruit juices and aqueous infusion tea extracts, lyophilised to dryness, were used throughout this study. Antioxidant

capacities of the extracts were carried out by using 2,2'-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid) radical scavenging (ABTS) and 2,2-diphenyl-1-picrylhydrazyl radical scavenging (DPPH) methods along with the determination of total phenolic compounds in the extracts.

Antimicrobial activities of extracts were determined by disc diffusion test, minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) methods.

Among the herbal teas, sage infusion extract has displayed the highest radical scavenging capacity with ABTS EC_{50} value of 5.152 mg/mL, DPPH EC_{50} value of 0.072 mg/mL and with its high phenolic content of 0.411 mg/mg gallic acid equivalence. Among the fruit juices pomegranate has revealed significantly high DPPH EC_{50} and TEAC values 0.924 mg/mL and 0.552 mmol/g, respectively. Peach juice has been found with the highest total phenolic amount of 0.067 mg/mg gallic acid equivalent.

Antimicrobial activities of herbal teas were correlating with antioxidant capacity studies, whereas sage infusion tea extract exhibited 3 mg/mL of minimum inhibitory concentration (MIC) and 6 mg/mL of minimum bactericidal concentration (MBC). Rosehip was also found as an effective antimicrobial agent with a minimum inhibitory concentration value of 3 mg/mL. In the meantime, there was no significant difference in the zone inhibition of herbal tea infusion extracts. In case of fruit juices grape and pomegranate may be effective antimicrobials in *P. mirabilis* infections with 0.75 mg/mL MIC and 6 mg/mL MBC, respectively at the same time both juices revealed significantly high inhibition zones with 11 mm.

Keywords: *Proteus mirabilis*, urinary tract infection, extraction, antioxidant, antimicrobial activity

ÖZ

MEYVE SUYU VE BİTKİ ÇAYLARININ ANTİOKSİDAN AKTİVİTELERİNİN İNCELENMESİ VE PROTEUS MİRABİLİS ÜZERİNE ANTİMİKROBİYAL ETKİLERİ

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Günlük diyette kullanılan bitki çayları ve meyve suları, bazı enfeksiyon hastalıklarının koruyucu tedavisinde önem arz edebilmektedir. Bu çalışmada, bazı diyetsel içeceklerin antioksidan kapasiteleri ve idrar yolları enfeksiyonuna yol açtığı bilinen bir patojen olan *Proteus mirabilis* üzerine antimikrobiyal etkileri araştırılmıştır.

Adaçayı, anason, kuşburnu, papatya gibi bitki çayları ve üzüm, portakal, şeftali, nar gibi meyve suları günlük diyetlerden örnekler olarak seçilmiştir. Seçilmiş olan, liyofilizasyonla elde edilmiş meyve suları ve sulu demlenmiş çay özütleri bu çalışma boyunca kullanılmıştır. Özütlerin antioksidan kapasiteleri 2,2’-azinobis-(3-etilbenzotiyazolin-6-sülfonik asit) radikali

yakalama (ABTS) ve 2,2-difenil-1-pikrilhidrazil radikali yakalama (DPPH) metodları ile toplam fenolik madde tayini ile çalışılmıştır. Özütlerin antimikrobiyal aktiviteleri disk difüzyon testi, minimum inhibe edici konsantrasyon (MİK) ve minimum bakterisidal konsantrasyon (MBK) metodları kullanılarak incelenmiştir.

Bitki çaylarından, 0.411 mg/mg lık gallik asit eşleniğiyle yüksek fenolik madde miktarına sahip olan, demlenmiş adaçayı özütü 5.152 mg/mL lik ABTS EC_{50} değeri ve 0.072 mg/mL lik DPPH EC_{50} değeri ile en yüksek radikal yakalama kapasitesi göstermiştir. Meyve sularından nar suyu sırasıyla 0.924 mg/mL ve 0.552 mmol/g lık DPPH EC_{50} ve TEAC değerleriyle dikkate alınır bir aktivite göstermiştir. Şeftali suyunun 0.067 mg/mg lık gallik asite eş değer yüksek toplam fenolik içeriğine sahip olduğu bulunmuştur.

Adaçayı demleme özütü, göstermiş olduğu 3 mg/mL lik minimum inhibe edici konsantrasyonu ve 6 mg/mL lik minimum bakteri öldürücü konsantrasyonu ile bitki çaylarının antimikrobiyal aktivitelerinin antioksidan kapasiteleriyle uyumlu olduğunu sonucunu vermiştir. Kuşburnu demleme çay özütü de 3 mg/mL lik minimum inhibe edici konsantrasyonu ile etkili bir antimikrobiyal olarak belirlenmiştir. Aynı zamanda, bitki çayı demleme özütlerinin inhibe ettikleri alanlar arasında dikkate değer bir farklılık gözlenmemiştir. Meyve sularından üzüm ve nar sularının 0.75 mg/mL MİK ve 6 mg/mL MBC değerleri ve meyve suları içinde en yüksek oldugu belirlenen 11 mm lik inhibe ettikleri alanlarla P. mirabilis enfeksiyonlarında etkili olabilecekleri belirlenmiştir.

Anahtar Kelimeler: *Proteus mirabilis*, idrar yolu enfeksiyonu, özütleme, antioksidan, antimikrobiyal aktivite

To my family

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LIST OF ABBREVIATIONS

mg Milligram

mL Milliliter

µL Microliter

mm Millimeter

nm Nanometer

HPLC High pressure liquid chromatography

UTI Urinary tract infections

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

DPPH 2,2-diphenyl-1-picrylhydrazyl

TP Total Phenolics

TEAC Trolox equivalent antioxidant capacity

GAE Gallic acid equivalent

CFU Colony forming unit

MIC Minimum inhibitory concentration

MBC Minimum bactericidal concentration

a.u. Absorbance unit

DW Dry weight

CHAPTER 1

INTRODUCTION

1.1. Antioxidants

Antioxidants are molecules capable of inhibiting the oxidation of other molecules. Antioxidants has a big role in the maintenance of human health and prevention and treatment of diseases (Niki, 2010).

1.2 Plant phenols

A phenol is a compound that contains -OH group attached to a benzene ring. Plants are source of polyphenols that exhibit a wide range of biological effects as a consequence of their antioxidant properties. Flavonoids are important phenol groups and most attention has been given to this group.

Fruits, vegetables and herbs contain many compounds with antioxidant activity. Several plants have been studied as sources of good natural antioxidants. (Hagerman, 1998).

1.3 Importance of medicinal plants in microbial infections

Plants, which contain natural chemicals and have healing characteristics, are gifts to people by nature; and throughout history, they were used as medical means by almost every society. Apart from medical effects of plants, they also can be used as models for research areas in which new antibacterial drugs can be developed.

Reports of World Health Organization (WHO) show that 3.3 billion people in under-developed and developing countries are benefiting from medicinal plants as treatment tools. In the recent years medicinal plants, assuming with minimal side effects, are being investigated regarding their microbiological and pharmacological properties.

Plants included in our daily diet can also be thought as functional foods. In this study, therefore fruit juices and herbal teas that are commonly used in Turkish diet will be investigated for their antimicrobial effects *against P. mirabilis* besides their antioxidant properties.

1.4 Herbal infusion teas and fruit juices in daily diets

Fruits and herbs are important sources of chemicals such as ascorbic acid (orange, rosehip,…), resveratrol (grape), steroid estrogen and pectin (pomegranate). In this study daily consumed fruit juices and herbal teas were chosen as samples because of their nutritional values and valuable chemicals.

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.*

1.5 *Proteus mirabilis*

Proteus mirabilis (P. mirabilis) is a Gram-negative and facultatively anaerobic bacterium (shown in Figure 1.1). It shows urease activity and causes nearly 90% of human “Proteus” infections (Jones, 1990). *P. mirabilis* is a common cause of urinary tract infection (UTI) in patients, with urinary tract abnormalities, those catheterized during hospitalization (Jabalameli, 2005).

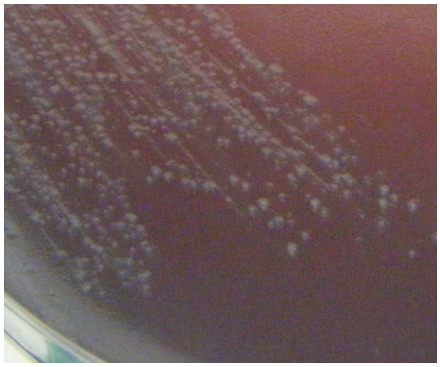


Figure 1.1 *Proteus mirabilis* colonies

Proteus mirabilis is an opportunistic pathogen causes acute or chronic urinary tract diseases, the progress of cytotoxicity manifested by inflammation (Murphy, 1999; Mobley and Warren, 1987).

In 1885, Hauser described the genus *Proteus* and the species *P. mirabilis* (O’hara, C.M.; 2000).

Scientific classification of *Proteus mirabilis* is,

Kingdom: Bacteria
Phylum: Proteobacteria
Class: Gamma Proteobacteria
Order: Enterobacteriales
Family: Enterobacteriaceae
Genus: *Proteus*
Species: *P. mirabilis*

1.6 Urinary Tract

The urinary tract consists of the organs kidneys, ureters, bladder, and urethra that produce, store, and get rid of urine.

1.7 Urinary tract infections:

Urinary tract infections (UTI) is defined as the presence of bacteria in urine with symptoms of infection. It was reported that nearly half of all adult women suffer from UTI at least once in their lifetime. Chronic infections in the upper urinary tract is a major cause of renal disease (Bergsten, 2005; Wael, 2008).

Symptoms of UTI are:

- pain or burning during urination
- need to urinate too often, even right after the bladder is emptied
- blood in the urine
- cloudy urine
- a foul odor to the urine
- urinary incontinence
- fever and chills
- nausea and vomiting
- pain, cramps or heaviness in the lower abdomen, pelvic area, or in the back below the ribs

Urinary tract infections may also fail the symptoms. This condition, that is “asymptomatic bacteriuria”, is known to heal without a treatment. It is especially, common in pregnant women manifesting itself in pre-term labor if not treated. In children, it appears with high fever without other symptoms (UCLA Health System).

The incidence of urinary tract infections is much higher in adult women due to anatomical and physiological reasons . However due to the urinary tract lesion formation in males and children, it is more dangerous and must be treated immediately to suppress the infection and prevent spreading. The patient's background also helps to categorize UTIs according to age, type of urinary tract lesion(s), and patients with suppressed immune system, especially in diabetes and pregnancy. A wide spectrum of treatment can be ranging from a single-dose antibiotic treatment of simple cystitis in young females, to rescue nephrectomy for pyonephrosis in diabetic patients with septic shock (Meyrier, 2003).

The following also increase the chance of developing UTI:

- Diabetes
- Advanced age (especially people in nursing homes)
- Problems emptying the bladder (urinary retention) because of brain or nerve disorders
- A tube called a urinary catheter inserted into urinary tract
- Bowel incontinence
- Enlarged prostate, narrowed urethra, or anything that blocks the flow of urine
- Kidney stones
- Staying still (immobile) for a long period of time (for example, while recovering from a hip fracture)
- Pregnancy

Urinary tract infection is the most common cause of sepsis in elderly people. Patients diagnosed with urosepsis should be treated with a suitable antibiotic, prefarably with a broad-spectrum antibiotic immediately. Unfortunately, misuse of broad-spectrum antibiotics can cause an increasing resistance to antibiotics as a big problem in the antibiotic treatment (Tal, 2005).

1.8 Principal pathogens of urinary tract infections

The most common causes of UTI are *E. coli, Proteus mirabilis, Klebsiella pneumoniae, Enterococcus* sp., *Pseudomonas aeruginosa*. In rare cases *Candida albicans* can cause UTI such as in diabetic patients. Frequency of incidence in urinary tract infections caused by bacterial strains is given in Table 1.1.

Table 1.1 Bacterial strains that are responsible for developing UTI, and their prevalence (Vosti, 2007).

Bacterial Strain	Prevalence %
Escherichia coli	71%-79%
Proteus mirabilis	1.1%-9.7 %
Klebsiella	-
Enterobacter	1.0%-9.2%
Enterococcus	1.0%-3.2%
Staphylococcus saprophyticus	3%-7%
Other species	2%-6%

1.9 Antibiotics used in treatment of urinary tract infections

A suitable antibiotic for UTI must have bactericidal characteristics with following pharmacological specifications:

1) rapid absorption and fast peaking in serum concentration

2) with high concentrations in the renal or prostate tissue and renal excretion

3) broad bacterial spectrum to increase the chance of effectiveness.

Antibiotic should also be easily accessible, low cost, tolerability by most of the patients (Meyrier, 2003).

It is reported that *Proteus mirabilis* is resistant to tetracycline and nitrofurantoin and susceptible to ampicillin, amoxicillin, piperacillin, cefazolin, cephoxitin, cefuroxime, cephotaxim, cephtazidim, cephtriaxon, ceftizoxim, cephepim, amikacin, gentamicin, tobramycin, imipenem, ciprofloxacin, and co-trimoxazole (Kurtoglu, 2008).

1.10 Antimicrobial tests

1.10.1 Disc diffusion test: the Kirby-Bauer method

Disc diffusion test, is one of the most common methods, used to determine bacterial sensitivity and resistance to antimicrobial agents, based on measurement of growth inhibition zones of antimicrobial agents adding on empty filter discs as indicated in Figure 1.4. Bacteria are standardized monitoring optical densities, by spreading them on the agar in their early stages of growth. This method provides equal susceptibility of all cells to antimicrobial agent. Antimicrobial discs should also be standardized. The amount of antimicrobial agent on each disc should be the same (Pollack, 2005).

The measurement is based on the size of the clearance zone of inhibition surrounding each disc. These clearance zones are measured in millimetres (mm), and difference in size of 2 to 3 mm means susceptibility or sensitivity to the antimicrobial agent, or zero clearance describes ineffectiveness or resistance, as sketched in Figure 1.2. The zone of inhibition that is between susceptible and resistant is called as intermediate (Pollack, 2005).

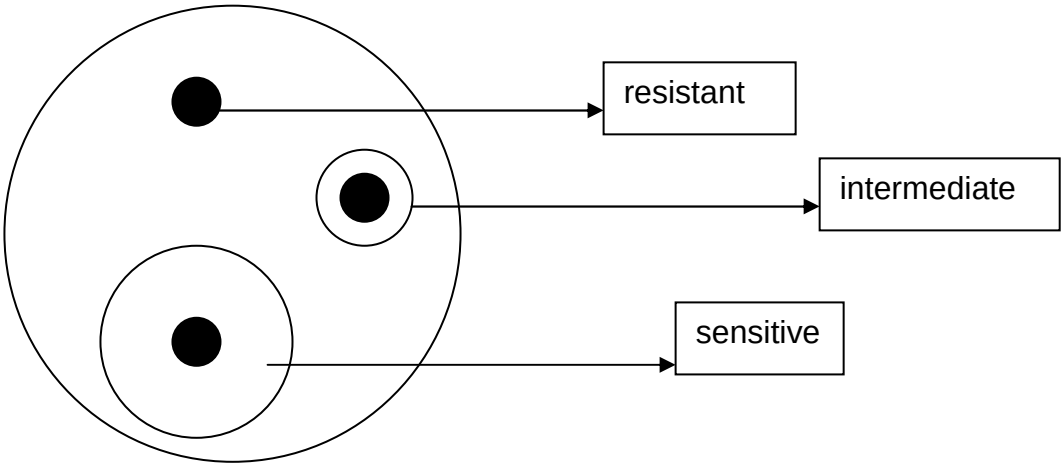


Figure 1.2 Representation of disc diffusion test; agar plate and discs

1.10.2 Minimum inhibitory concentration

The effectiveness of an antimicrobial agent is described in terms of its minimum inhibitory concentration (MIC), the lowest concentration of the compound capable of inhibiting the growth of the bacteria of interest (Mann, 1997).

The most commonly used techniques are agar dilution and broth dilution methods to determine the minimum inhibitory concentration (MIC) of antimicrobial agents that inhibit the growth (bacteriostatic activity) of bacteria (Wiegand, 2008).

1.10.3 Minimum bactericidal concentration

Minimum bactericidal concentration (MBC) is defined as the lowest concentration of an antimicrobial that kills the bacteria (Andrews, 2001). The minimum bactericidal concentration of an antimicrobial agent to kill the bacteria is more important rather than inhibition of the growth. As an adaptation of the agar dilution method, micro agar dilution method is used to determine MBC in 96 micro-well plates (Schwalbe, 2007).

1.11 Scope of the work:

Plants included in our daily diet can be consumed for protection from lots of diseases. In this study, fruit juices and herbal teas that are commonly used in Turkish diet will be investigated for their antioxidant properties and their antimicrobial effects *against P. mirabilis* as a new alternative to antibiotics, which gained resistance throuhgout the years, used in urinary tract infections.

CHAPTER 2

MATERIALS AND METHODS

2.1 MATERIALS

2.1.1 Chemicals

Proteus mirabilis used for antimicrobial assays was obtained from Refik Saydam Hygiene Center (code: RSKK 737).

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

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

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

Powder cell culture tested grade of Gentamicin was purchased from Sigma-Aldrich (Germany).

Thermo Scientific Finnpipettes (12 multichannel 5-50 µL, 12 multichannel 100-300 µL, and 8 multichannel 5-50 µL) were used during the experimental research.

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

Milli-Q system (Milli-pore, Bedford, MA, USA) was used to get ultrapure water (18.2 M ohm.cm)

Distilled water was obtained from Milli-pore system (>1 M ohm.cm)

Milli-pore sterility filters, 0.4 µm, were used for sterilization of solutions.

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

Tea bags and fruits which had been bought from local markets. Fresh rosehips from *Rosa canina* fruits (from) had been dried and ground 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. Grape (*Vitis vinifera*), orange (*Citrus sinensis*), peach (*Prunus persica*), and pomegranate (*Punica granatum*) had been bought freshly from local markets. Herbal tea species were identified in Biology Department of METU.

2.1.2 Apparatus

For antimicrobial assays Class II Safety Cabinet (ESCO, Thailand) was used.

Cary 50 Bio UV-VIS spectrophotometer (Varian, USA) was used for spectrophometric analyses.

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

Nuve EN-500 incubator

Optic Ivymen System (incubator and shaker)

Bandelin Sonorex (ultrasonic bath)

Rotary evaporator (Heidolph Laborota 4000)

Lyophilizator (Heto-Holten Model Maxi-Dry Lyo) in the Central Lab of Biological Sciences Department , METU.

2.2 METHODS

2.2.1 Preparation of the plant extracts

Fruit juice and herbal tea extracts were prepared and kept at 4 ℃ in the dark, in our laboratory. Juices of pomegranate, peach, orange and grapes were grouped as the fruit juices and tea extracts including sage, anise, rosehip, and camomile were grouped as herbal teas.

Commercial tea-bags of sage, anise and camomile were opened and weighed a number of them gathered for 40 g of each. Fresh rosehip fruits were washed and dried at room temperature. Infusion was performed at two steps. 40 grams of each tea sample was let to infuse in 480 mL boiled distilled water for about one hour standing at constant room temperature (25℃). Then, infused tea solution were incubated at 37 ℃ for 24 hours, using rocking-incubator at 180 rpm, in order to increase the yield. After incubation, the tea infusion solutions were filtered through filter paper and filtrates were lyophilized to dryness. The lyophilized aqueous tea extracts were kept at 4 ℃ in the dark until they were used.

Individually, 474.9 g of grapes, 1241.8 g of orange, 655 g of peach and 1665.5 g of pomegranate arils were blended using a commercial food processor to seperate juice from the pulp. Then, juices were filtered through filter paper and filtrates were lyophilized to dryness and refrigerated (4 ℃) until they were used.

2.2.2 Preparation of the microbial strains and stocks

Proteus mirabilis (737 RSKK code, from Pasteur Institute) strain was bought from Refik Saydam Hygiene Center in Ankara. The pellet of *Proteus mirabilis* was suspended in 1.0 mL of Luria Broth (LB) and streaked onto ready to use agar plate containing sheep blood medium, then incubated for 24 hours at 37 ^{o}C for short term bacterial stocks, as photographed in Figure 2.1. Then stock plates were stored at 4 ^{o}C room and stocks were renewed each week to maintain the freshness.

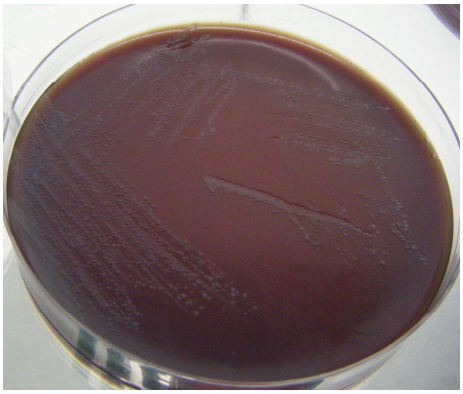


Figure 2.1 The microbial strain of *Proteus mirabilis*, which was used in microbiological studies was bought from Refik Saydam Hygiene Center and transferred to agar medium incubated at 37 ℃ for 24 hours.

For long term storage 3 to 5 of the isolated colonies with similar spherical shapes, from previously described agar stocks were transferred and suspended a 1.0 mL solution of LB. 100 µL of that suspension then was inoculated into 100 mL of a new solution of LB medium incubated at 37 ^{o}C, at 180 rpm, for 24 hours. At the end of 24 hours, absorbance was monitored at 570 nm and carefully diluted with LB broth to decrease its absorbance to a 0.6 absorbance unit (a.u.). Finally, a 100 µL of the bacterial suspension (with 0.6 a.u.) was mixed with a sterilized pre-chilled 20% (v/v) of 2 mL of glycerol and 8 mL of distilled water. Glycerol-bacterial solution was seperated into eppendorphs as 1 mL of aliquots for long term storage at -80 ^{o}C freezer.

2.2.3 Bacterial growth curve

2.2.3.1 Determination of OD_{570} values versus growth time

Proteus mirabilis from the previously described short term stocks were inoculated to 100 mL of Luria Broth (LB) medium, then incubated at 37 °C at 180 rpm for 24 hours. After monitoring absorbance of the bacteria at 570 nm, bacterial broth solution was diluted with LB broth to reduce the final absorbance to a 0.03 a.u. Then this bacterial solution was let to grow at 37 °C in the shaker and at every couple of hours absorbance was monitored at Cary Bio spectrophotometer, by taking samples, and sampling continued up to 24 hours. Dilutions were applied up to 10x as necessary with LB broth. Observations were plotted for the OD_{570} versus time (hr). 1.5 mL of LB broth was used as blank at spectrophotometric measurements.

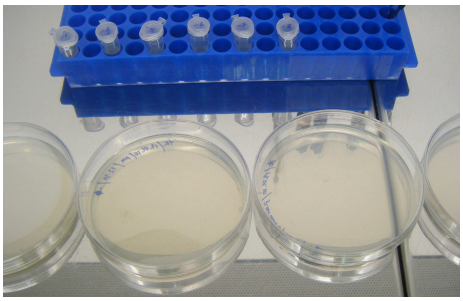
2.2.3.2 Estimation of colony numbers of bacteria versus growth time

LB agar plates were prepared by pouring sterilized LB agar as a 3 mm thick layer into 9 mm petri plates and they were cooled. Bacteria growing at 37 °C sampled at every 4 hour intervals were spread on LB agar plates using dilutions of 10^{-5}, 10^{-6}, 10^{-7}, 10^{-8} (Figure 2.2.a) and incubated at 37 ^{o}C (Figure 2.2.b), approximately 12-16 hours until colonies became visible for colony count (Figure 2.2.c). Colony numbers of bacteria on LB agar plates were estimated by counting the visible bacterial colonies and colony numbers were plotted against time.

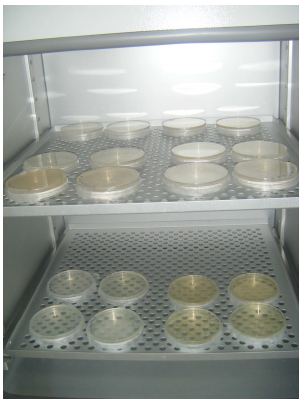
2.2.3.3 Estimation of colony forming unit

Numeric estimation of the bacterial colonies against OD_{570} values which were deduced from previous sections, will be defined as colony forming unit (CFU), is a crucial information in the antimicrobial studies. CFU is a measure of viable number of bacterial colonies and it is related to the optical density. Colony forming unit (CFU/mL) of bacteria is used to standardize the number of colonies available in the antimicrobial studies.

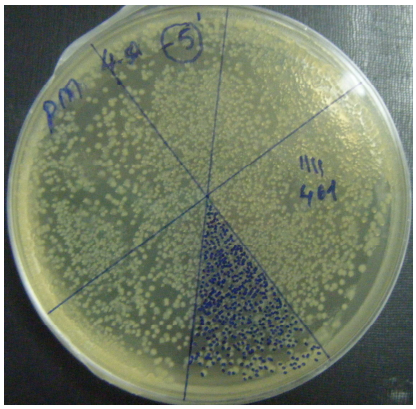
Entegrating the two plots of OD_{570} versus growth time and bacterial number of colonies versus growth time, colony numbers of bacteria versus OD_{570} was obtained. Using the resultant plot, trendline equation would lead to the estimation of CFU/mL. Hence, one can approximate the colony forming unit per mL for a given OD_{570} value.



(a)



(b)



(c)

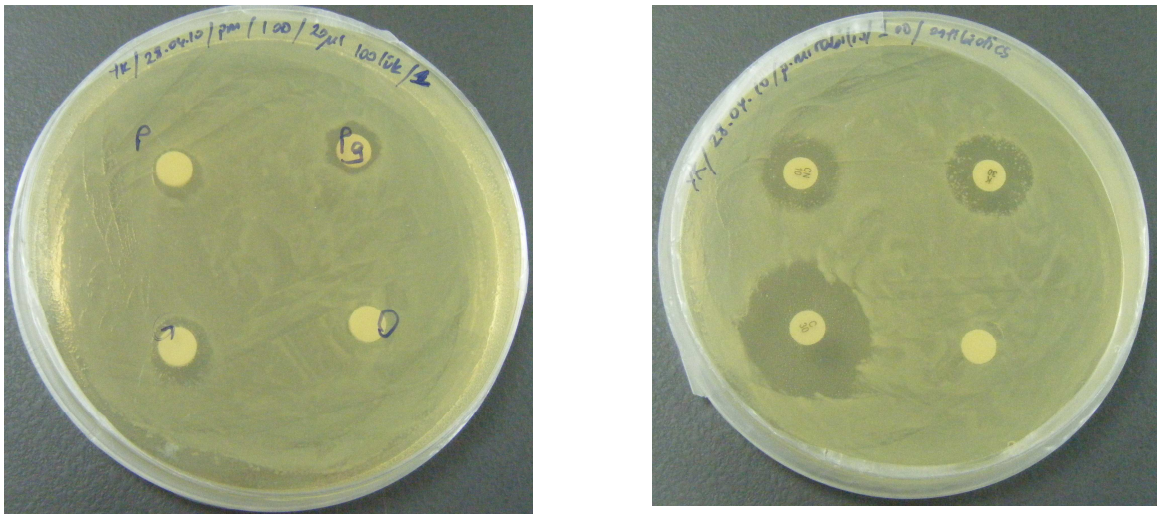
Figure 2.2 Pictures of bacterial growth experiments: (a) Various dilutions of P.mirabilis medium were spread onto LB agar. (b) Spread agar plates were incubated for 12-16 hours, at 37 ^{o}C. (c) Colony count of *P. mirabilis*.

2.2.4 Antibacterial activity tests

All of the antimicrobial activity studies were carried out at aseptique conditions in order to prevent the risk of contamination along with protection of our own health.

2.2.4.1 Kirby-Bauer disc diffusion method

LB agars were prepared according to the given directions and poured into 9 mm plates as a 3 mm thick layer. Plates were cooled and kept in cold room (4 ^{o}C). A 100 µL of inoculated bacteria with 1.0 OD_{570} (2x10^{9} CFU/mL) were spread over the agar surface at aseptique conditions. Then empty discs and standard antibiotic discs as control (Gentamicin-10 mcg and Kanamycin-30 mcg) were placed on agar surface. Lyophilised fruit juices and herbal tea infusion extracts in methanol (120 mg/mL) were added as 20 µL to empty filter discs, and then they were kept in incubator at 37 ^{o}C for 16 hours. Zone diameters were measured in millimeters as displayed in Figure 2.3. Water and methanol were used as control.



(a) **(b)**

Figure 2.3 Inhibition zone diameters of fruit juice extracts after 16 hours incubation period (a), inhibition zones of ultrapure water and antibiotics which are 10 mcg gentamicin, 30 mcg kanamycin and 30 mcg chloramphenicol (b).

2.2.4.2 Minimum inhibitory concentrations

2.2.4.2.1 Solvent Effects

In the determination of minimum inhibitory concentration (MIC) of solvents 96-well plates were used. The first well of each row contained 20 µL of the solvent added into 175 mL of LB broth (10% solvent in LB broth). Then, the solution in the first well was used to prepare ½ serial dilutions of solvent (well 2 to 10) by transferring half of the solution to the next well. Column 11 and 12 were used for sterility control (100 µL of LB only) and growth control (95 mL of LB medium and bacteria), respectively.

Ethanol (EtOH), methanol (MeOH), dimethylsulfoxide (DMSO), ethyl acetate (EtOAc), acetonitrile, cyclohexane, acetone, and double distilled water (ddH_2O) were examined as the choice of solvents in respective orders as displayed in Table 2.1. A 5 µL of the inoculated bacterial suspension at 0.05 OD_{570} (10^7 CFU/mL) was added to each well except the column for sterility control. In sterility control column of wells there was only a 100 µl of broth, in growth control column of wells there was 95 µL of broth and 5 µL of inoculated bacteria. After 24 hours incubation at 37 ^oC, bacterial growth was observed as if there was bacterial growth or not and minimum inhibitory concentrations of the solvents were determined.

Table 2.1 5 µl of *P.mirabilis* with 0.05 absorbance unit was added to each well and total volume was adjusted to 100 µl. All of the wells were prepared with respected solvents with same broth.

		9.75%	4.88%	2.44%	1.22%	0.61%	0.31%	0.16%	0.08%	0.04%	0.02%	sterility control (broth only)	growth control (broth and bacteria)
		1	2	3	4	5	6	7	8	9	10	11	12
EtOH	A												
MeOH	B	20 ul MeOH, 175 ul broth	95 ul broth 50 % of well-1	95 ul broth 50% of well-2	95 ul broth 50% of well-3	95 ul broth 50% of well-4	95 ul broth 50% of well-5	95 ul broth 50% of well-6	95 ul broth 50% of well-7	95 ul broth 50% of well-8	50% of well-9	100 ul broth	95 ul broth
DMSO	C												
EtOAc	D												
Acetonitrile	E												
Cyclohexane	F												
ddH2O	H												

2.2.4.2.2 Stock solutions of plant extracts

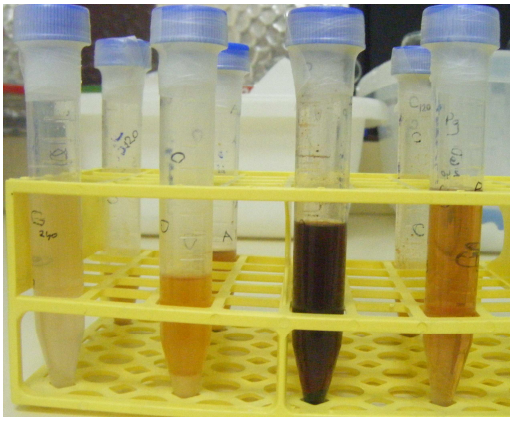


Figure 2.4 Stock concentrations of tea infusion and fruit juice extracts.

All the stock solutions of tea infusion and fruit juice extracts (Figure 2.4) were prepared in methanol in concentrations as 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)
Sage	120
Anise	240
Rosehip	240
Camomile	120
Grape	240
Orange	240
Peach	240
Pomegranate	240

2.2.4.2.3 Minimum inhibitory concentration determination by micro broth dilution method

Minimum inhibitory concentration (MIC) experiments were carried out according to the “Nature Protocols”, micro broth dilution method. First column of 96-wells were arranged with a 175 µl of LB broth and 20 µl of the stock concentration of plant extract as shown in Table 2.1. A 95 µl of LB broth was put into 2nd through 8th column of wells arranged as in Figure 2.5. Two fold dilutions were applied to the wells starting from the first column of wells by using multichannel pipettes.

Bacteria was prepared for the inhibitory concentration studies by taking from the stationary stage of the growth, was diluted with LB broth upto 0.05 a.u. at OD_{570} to observe the inhibitory effect. Then, 5 µL of diluted bacteria was inoculated ($5x10^5$ bacterial cells of *Proteus mirabilis*) to all of the columns except sterility control columns (9th and 11th).

	1	2	3	4	5	6	7	8	9- sterility control	10- growth control	11-antibiotic sterility	12- antibiotic growth control
Sage												
Anise												
Rosehip												
Camomile												
Grape												
Orange												
Peach												
Pomegranate												

Figure 2.5 96-well plate design in MIC and MBC experiments.

Sterility control including only 100 µL of LB broth was placed in 9th well of 96-well plate to control the sterility of broth medium. In order to control the bacterial growth, 10th well was labeled as growth control, included 95 µL of LB broth and 5 µL of bacterial inoculation. Antibiotic sterility control contained 5 µL of antibiotic solution (gentamicin) and 95 µL of broth in the 11th well, in order to observe if there is a bacterial contamination from environmental effects . The last one was antibiotic growth control and 90 µL of of LB broth, 5 µL of gentamicin antibiotic and 5 µL of bacterial inoculum were added, in order to check the sensitivity of the bacteria to the selected antibiotic.

Gentamicin was the choice of antibiotic which has been widely used in urinary tract infections. A 10 mg/mL stock solution of antibiotic was prepared with ultra pure, sterilized water.

After completion of the MIC set 96-well plates were incubated at 37℃ for 16 hours and absorbancewas monitored at ELISA plate reader at 570 nm.

2.2.4.2.4 Minimum bactericidal concentration determination by micro agar dilution method

Micro agar dilution method was applied to determine minimum bactericidal concentration (MBC). After conducting micro broth dilution (MIC) experiments in a 96-well plate as described in the section 2.2.5.2.2; a 10 µL of solution, from the previous MIC 96-well plate, was transferred by using a 12 multi-channel pippette (5-50 µL) to a new 96-well plate containing 100 µL of LB agar as displayed in Figure 2.6. Minimum bactericidal concentration was determined as the lowest concentration of extract where no visible bacteria was observed on the agar surface. ELISA plate reader was used to obtain the exact absorbance in each set of micro broth dilution wells where there is no visible bacteria. All experiments were performed in triplicates.

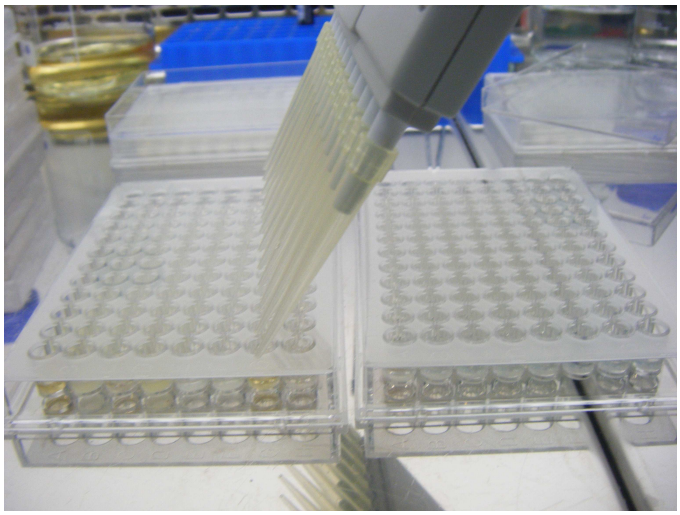


Figure 2.6 Transferring of 10 µl of medium from MIC wells to MBC wells.

2.2.5 Antioxidant activity tests

2.2.5.1 ABTS method

2,2'-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) method was performed according to Re et. al (1999).

ABTS (displayed in Figure 2.7) was dissolved in ethanol to prepare a 20 mL stock solution of 7 mM. ABTS radical cation ($ABTS^{\bullet+}$) was produced by reacting ABTS solution with potassium persulfate at 2.45 mM final concentration in the stock solution. The mixture then allowed to stand in the dark at room temperature for 12–16 h before using.

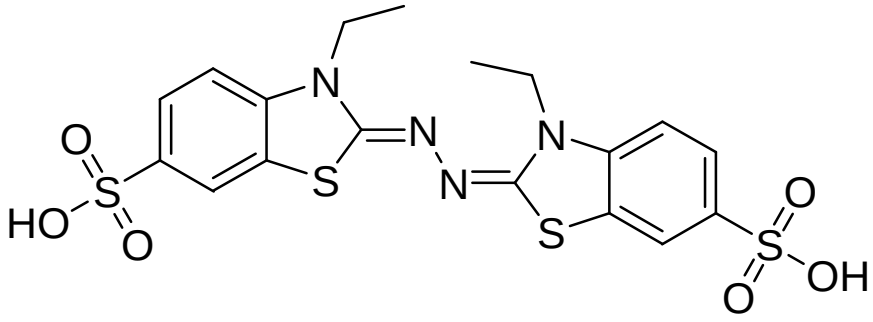


Figure 2.7 2,2'-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid)

A working solution of $ABTS^{\bullet+}$ was prepared by diluting stock solution with 120 mL of methanol to an approximate absorbance unit of 0.70 (±0.02) at 734 nm. Different calculated volumes of 1.0 mg/mL tea infusion and fruit juice extract solutions were added to 2 mL of ABTS solution and mixed (Final concentrations were calculated). Absorbance was monitored at Cary Bio single beam spectrophotometer at 734 nm, after the initial mixing time recorded in every minute from 1^{st} to 6^{th}. In ABTS method 6th minute results were required since the reaction was stabilized at 6^{th} minute and there was no change in the percent inhibition after 6^{th} minute.

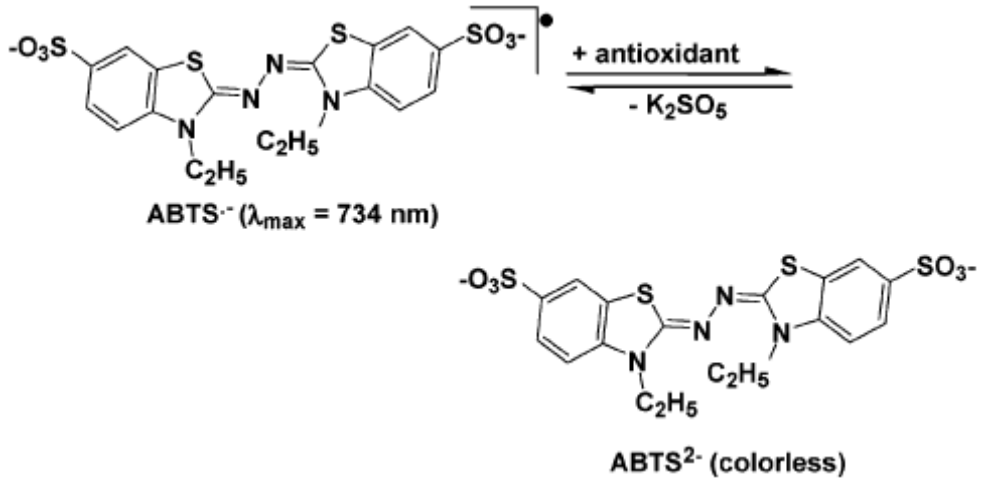


Figure 2.8 Reaction between $ABTS^{\bullet+}$ radical and antioxidant (Apak, 2007).

Trolox was used as a standard in 5, 10, 15, 20 µM concentrations to calculate the trolox equivalent antioxidant capacity (TEAC) values. TEAC values were calculated by dividing the slope of extract concentration vs percent inhibition graph to that of trolox standard curve. The results were divided by dilution factor (120) due to the dilution of stock solution. Trolox standard curve was plotted to use it as reference.

All determinations were carried out three times, and in duplicate. The percent inhibition was calculated at 734 nm absorbance and plotted as a function of the concentration of extracts. Percent inhibition was calculated as;

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

where A_0 is the absorbance of the ABTS solution and methanol, A_1 is the absorbance of the ABTS in the presence of extracts at various concentrations.

2.2.5.2 DPPH method

2,2-diphenyl-1-picrylhydrazyl (DPPH, Figure 2.9) and ethanol solution was prepared in the concentration of 0.05 mg/mL. It gives nearly 1.4 unit of absorbance at 517 nm. Various concentrations of the extracts were prepared with methanol, and 100 µL of the extract concentrations were added to 1.4 mL of DPPH solution. Then the absorbance was recorded at 517 nm after 20 minute of incubation, as displayed in Figure 2.10.

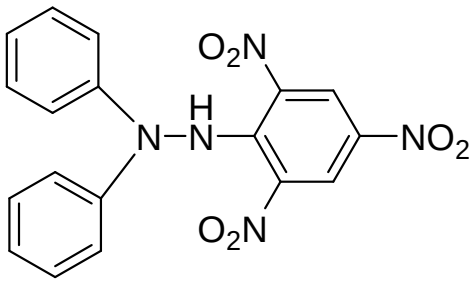


Figure 2.9 2,2-diphenyl-1-picrylhydrazyl (DPPH)

Blank sample contained the 100 µL of methanol and 1.4 mL of DPPH solution. These experiments were carried out 3 times in duplicates.

Figure 2.10 Absorbance of the samples (extract and DPPH) were read after 20 min of incubation period, at 517 nm.

Radical scavenging activities of the extracts were calculated according to the formula,

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

where A_0 is the absorbance of the blank sample (DPPH solution with 100 µL of methanol) and A_1 is the absorbance of the DPPH solution with the extract concentrations that are dissolved in methanol.

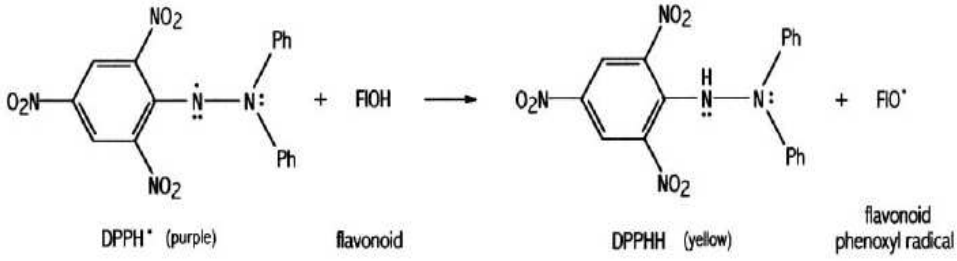


Figure 2.11 Scavenging of DPPH (2,2-Diphenyl-1-picrylhydrazyl) free radical by a flavonoid (Dragan, 2003).

According to the results, RSA% vs final concentrations of the extracts (mg/ml) was plotted and EC_{50} (50 % effective concentration) values were calculated. Statistical analysis were done according to Minitab Release 14 software.

2.2.5.3 Total phenolic content

Total phenolic content determination was done according to the Singleton and Rossi (1963). 100 µl of each extract solution prepared in methanol at various concentrations were mixed with 100 µl of 50 % Folin–Ciocalteu's phenol reagent and they were vortexed vigorously. Then, 2 mL of aqueous solution of 2 % Na_2CO_3 was added into the test tubes in equal time intervals to stop the reaction and vortexed again.

Blank solution was prepared by putting 2 mL of Na_2CO_3, 100 µl of Folin reagent and 100 µl of methanol instead of extract.

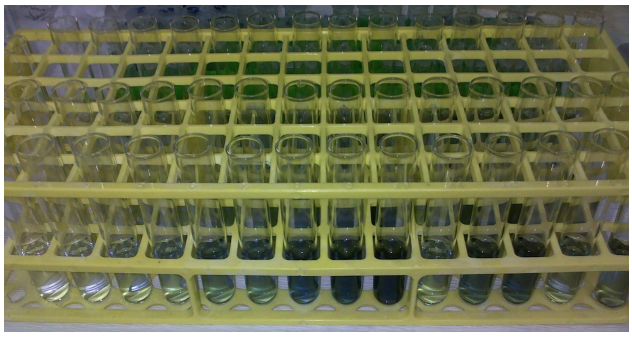


Figure 2.12 Total phenolic content experiments were done in triplicates, and absorbance of the mixtures at 750 nm were recorded.

Gallic acid was used as standard at the concentrations in the range of 0.05-0.3 mg/mL, to construct a standard curve in order to calculate total phenol concentration of extracts as gallic acid aquivalence (GAE).

After 30 min of incubation time, absorbance of the mixtures was recorded at Cary Bio single beam spectrophotometer at 750 nm, as displayed in Figure 2.12. Absorbance of the background mixture was also read and its affect was omitted.

Results were calculated by using the equation of the gallic acid standard curve and they were recorded as milligrams of total phenolics (TP) being contained in milligrams of extract as the gallic acid equivalents (GAE).

2.2.3 Statistical analysis

All results are expressed as mean of ± standard deviation (SD). Differences between data were determined using student *t*-test and noted to be significantly different where $p<0.02$.

CHAPTER 3

RESULTS AND DISCUSSION

3.1 Antimicrobial activity of extracts on *Proteus mirabilis*

Antimicrobial activity of the daily consumed teas and fruit juices are investigated against *Proteus mirabilis* which was not reported before. Optimizations were designed as determination of growth curve of the bacteria at 37 ^{o}C and the solvent effect on the growth.

3.1.1 Bacterial growth curve

The estimation of the number of colonies of bacteria is very important in the antimicrobial studies. Colony forming unit (CFU/mL) of bacteria should be known to standardize the number of colonies that are used in the antimicrobial experiments. CFU is a measure of viable bacterial colony numbers and it is related to the optical density of the bacteria.

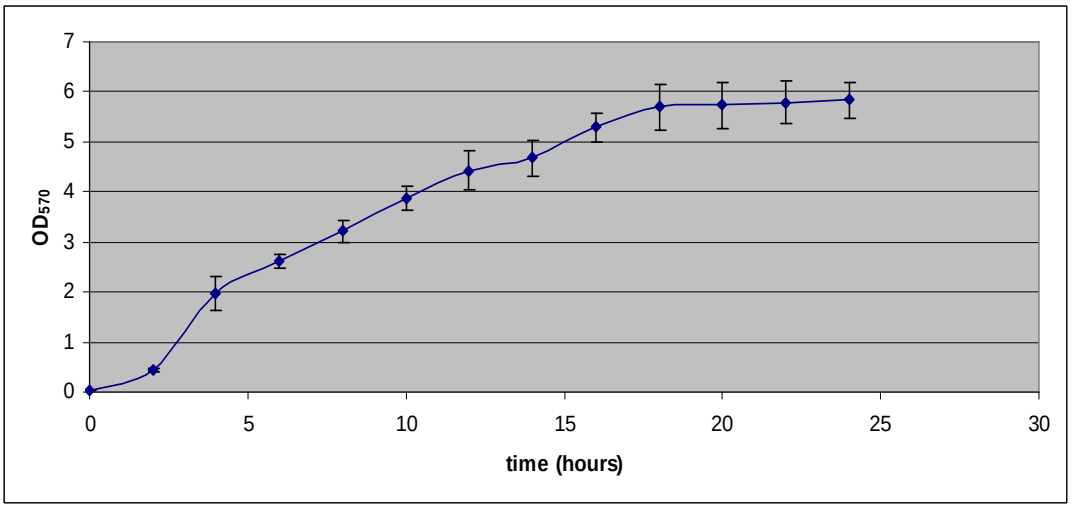


Figure 3.1 Determination of OD_{570} values versus growth time (every couple of hours intervals) starting with bacterial OD_{570} value of 0.03 at zero growth time. Dilutions were applied up to 10x as necessary. (Results are the mean of three independent experiments in duplicates).

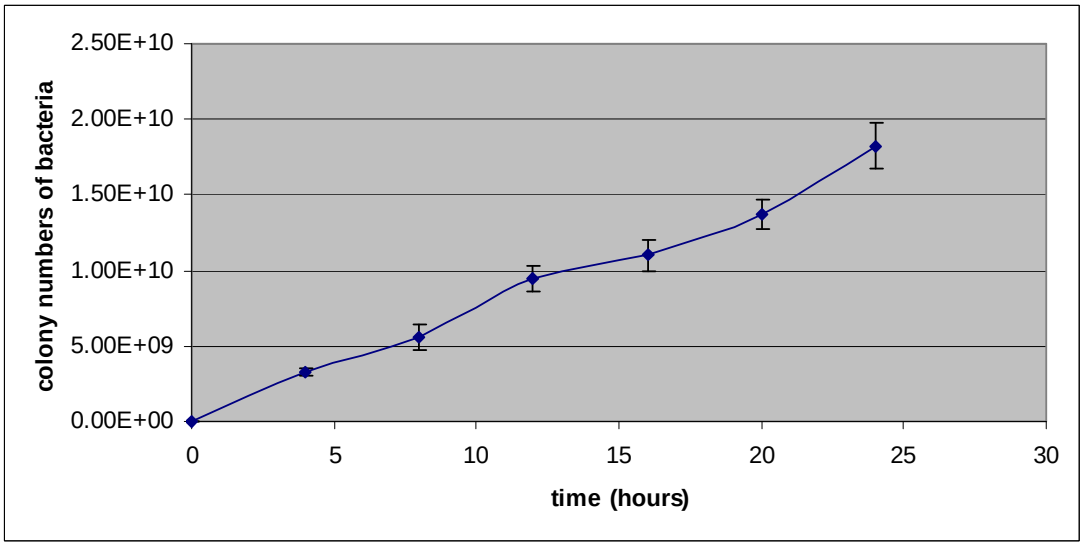


Figure 3.2 Estimation of colony numbers of bacteria versus growth time. Bacterial samples at every 4 hour intervals (from Figure 3.1) were spread on LB agar plates using various dilutions, incubated at 37 ^{o}C, up to 16 hours until colonies become visible (Results are the mean of three independent experiments in duplicates).

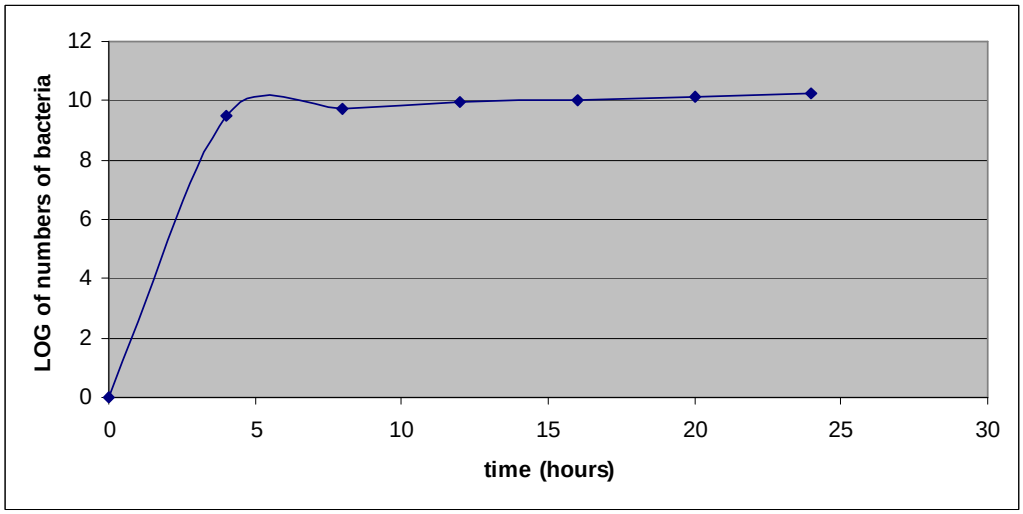


Figure 3.3 Logarithmic base of colony forming bacteria count versus growth time intervals (an initial bacterial OD_{570} value of 0.03) (Results are the mean of three independent experiments in duplicates).

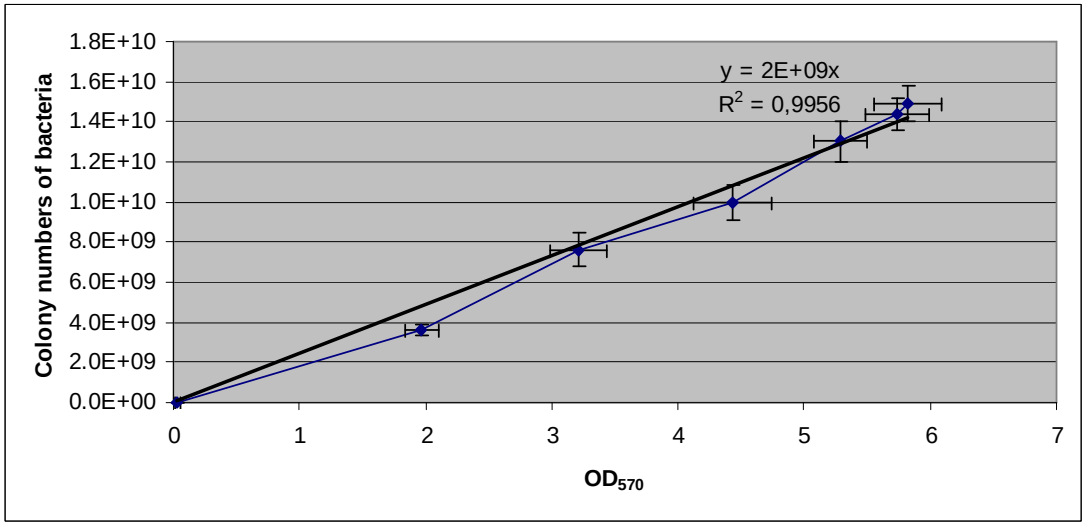


Figure 3.4 Estimation of colony forming unit per mL. Colony numbers of bacteria versus absorbance unit at 570 nm were rearranged using Figures 3.1 and 3.2. Resultant trendline equation was used to approximate the colony forming unit per mL count for a given OD_{570} value (e.g: 1 OD_{570} = $2x10^9$ CFU/mL). Data was collected as mean of three independent experiment in duplicates.

As displayed in Figure 3.4 the trendline equation is used to standardize the colony numbers as Colony Forming Units (CFU) at given absorbance unit (at 570nm). As a result, trendline (**$y=2*10^9x$**; where x is the absorbance unit at 570 nm, and y is the CFU) is interpreted as for 1mL of inoculum of bacteria having 1.0 units of OD_{570} would be resultant of $2x10^9$ CFU/mL for *Proteus mirabilis*. It is important to standardize the antimicrobial experiments using the same number of bacteria.

3.1.2 Preparation of extract solutions

Since we knew the effect of solvents on *Proteus mirabilis*, we chose methanol as solvent to prepare our extract solutions at varying concentrations. Our stock solutions were as seen in the Table 2.1.
Percent yield results of tea infusion extracts and fruit juices were found as in Table 3.1 and Table 3.2.

Table 3.1 Extraction data of tea infusion extracts.

Tea infusions	Sage	Anise	Rosehip	Camomile
Total weight (g)	40	40	40	40
Infusion volume (mL)	480	480	480	480
Total extract (g)	6.20	8.00	22.20	23.70
Yield (%)	15.50	20.00	55.50	59.25

Table 3.2 Extraction data of fruit juices.

Fruit Juices	Grape	Orange	Peach	Pomegranate
Total weight (g)	474.9	1241.8	655.00	1665.5
Juice (mL)	371.00	524.00	452.00	407.00
Total extract (g)	42.40	51.60	52.00	59.20
Yield (%)	11.43	9.85	11.50	14.55

3.1.3 Minimum inhibitory concentration

Minimum inhibitory concentrations were determined by using 96-well plates, as described in the methods part 2.3.2. All of the experiments were done in triplicates. Concentration of *Proteus mirabilis* was 0.05 OD_{570} (approximately $5*10^5$ bacteria) added in 5 µL to each 96-wells throughout these experiments.

3.1.3.1 Solvent effects

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

In order to find out the solvent with minimum inhibitory effect against *Proteus mirabilis,* 20 µL of each respective solvent, 175 µL of Luria Broth, and 5 µL of bacteria with 0.05 OD_{570} were added to the wells, as shown in Table 2.2.
The results of solvent effect experiment was given in Table 3.3. It was found that ethanol, methanol, dimethylsulfoxide and acetonitrile have only displayed the growth inhibitory effect (MIC) for concentrations of 10 % against *Proteus mirabilis.* All other solvents have displayed no growth inhibitory effect when used up to 10 %. Methanol was the best solvent choice, with minimum inhibitory effect and for the extract dissolution purposes in order to prepare as high extract concentrations as possible. Throughout the experiments methanol was used as a solvent in 5 % concentrations in the wells.

Table 3.3 Determination of minimum inhibitory concentrations for various solvents. 10 µL of each respective solvent, 85 µL of Luria Broth, and 5 µL of *P.mirabilis* with 0.05 absorbance unit was added to each well and total volume was adjusted to 100 ul. All of the wells were prepared with respected solvents with same broth.

		9.75%	4.88%	2.44%	1.22%	0.61%	0.31%	0.16%	0.08%	0.04%	0.02%	sterility control (broth only)	growth control (broth and bacteria)
		1	2	3	4	5	6	7	8	9	10	11	12
EtOH	A	x	√	√	√	√	√	√	√	√	√	x	√
MeOH	B	x	√	√	√	√	√	√	√	√	√	x	√
DMSO	C	x	√	√	√	√	√	√	√	√	√	x	√
EtOAc	D	√	√	√	√	√	√	√	√	√	√	x	√
Acetonitrile	E	x	√	√	√	√	√	√	√	√	√	x	√
Cyclohexane	F	√	√	√	√	√	√	√	√	√	√	x	√
Acetone	G	√	√	√	√	√	√	√	√	√	√	x	√
ddH2O	H	√	√	√	√	√	√	√	√	√	√	x	√

3.1.3.2 Minimum inhibitory concentration of extracts

Herbal teas and fruit juices often consumed in our daily diet were investigated for their growth inhibitory effect against *Proteus mirabilis* which is a dangerous pathogen for urinary tract infections.

3.1.3.2.1 Minimum inhibitory concentration of tea infusions

Antimicrobial activities of tea infusions (sage, anise, rosehip and camomile) were studied against *Proteus mirabilis*. Micro broth dilution method was used as described in section 2.3.4 to determine minimum inhibitory concentrations. Final extract concentrations in the wells were in the range of 0.047-12.0 mg/mL. According to the growth curve of *Proteus mirabilis* in Figure 3.4, we added bacterial inoculation with 0.05 OD_{570} which means there were $5x10^5$ bacterial cells in each well.

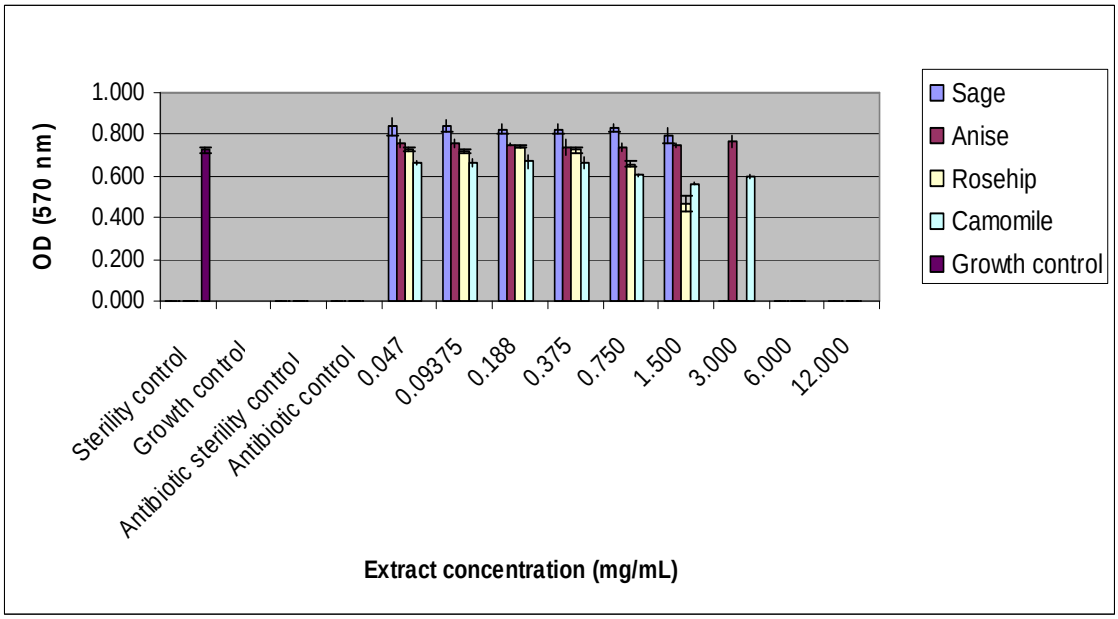


Figure 3.5 Determination of minimum inhibitory concentrations of tea infusion extracts with 16 hours incubation against *Proteus mirabilis*, at 570 nm.

Sterility control: Test the experiments if they were performed in aseptique conditions in the presence of broth only.
Growth control: Test of bacterial growth in the presence of only broth and bacteria.
Antibiotic sterility control: Sterility of the Gentamicin in the absence of bacteria.
Antibiotic control: 5 µL of Gentamicin was used as antibiotic in the concentration of 10 mg/mL in the presence of *P. mirabilis* of 10^9 CFU/mL.
Each data was obtained by taken the mean of three independent experiments in triplicates.

After 16 hours incubation, OD values of the wells were monitored at 570 nm. Minimum inhibitory concentration ideally must be obtained with zero absorbance as a result of no bacterial growth.

Tea infusion extract of sage displayed some bacterial growth for the extract concentrations from 0.047 to 1.5 mg/mL, however at and above 3.0 mg/mL concentration, inhibition against bacterial growth was observed where optical density was read as zero absorbance unit at 570 nm. So, 3.0 mg/mL of tea infusion extract of sage is considered as the minimum inhibitory concentration against *Proteus mirabilis*. Tea infusion extract of rosehip also presented its growth inhibitory effect at the same concentration with tea infusion extract of sage.

MIC values for the infusion extracts of anise and camomile were observed as 6 mg/mL which are less effective than the extracts of sage and rosehip.

Hammer et al. studied antimicrobial activities of 52 plant oils and extracts against *Acinetobacter baumanii*, *Aeromonas veronii* biogroup *sobria*, *Candida albicans, Enterococcus faecalis, Escherichia coli*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, *Salmonella enterica* subsp. *enterica* serotype *typhimurium, Serratia marcescens* and *Staphylococcus aureus*, using an agar dilution method. They have reported that rosewood, coriander, palmarosa, tea tree, niaouli, peppermint, spearmint, sage and marjoram inhibited all organisms except *Ps. aeruginosa* at ≤2·0% (v/v) (Hammer, 1999).

Tepe et al. reported the antimicrobial capacities of the essential oils from *P. Anisetum* against *Streptococcus pneumoniae* by microbroth dilution method. They have given the MIC result of *P. Anisetum* as 18 mg/mL (Tepe, 2006).

3.1.3.2.2 Minimum inhibitory concentration of fruit juices

Antimicrobial activities of highly consumed selection of fruit juice extracts (grape, orange, peach, pomegranate) were studied against *Proteus mirabilis*. Micro broth dilution method was used as described in section 2.3.4 to determine minimum inhibitory concentrations. Final extract concentrations used in the 96-well preparations were in the range of 0.094 to 12.0 mg/mL. In each well *Proteus mirabilis* was added as 5 µL with 0.05 OD_{570} ($5x10^5$ CFU/mL).

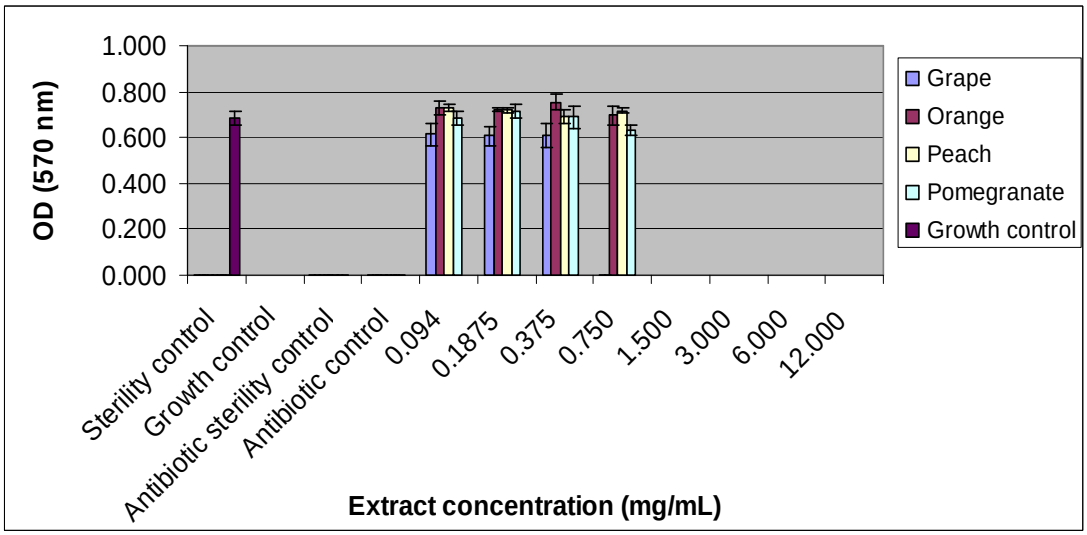


Figure 3.6 Determination of minimum inhibitory concentrations of fruit juice extracts with 16 hours incubation against *Proteus mirabilis*, at 570 nm.

Sterility control: Test the experiments if they were performed in aseptique conditions in the presence of broth only.
Growth control: Test of bacterial growth in the presence of only broth and bacteria.
Antibiotic sterility control: Sterility of the Gentamicin in the absence of bacteria.
Antibiotic control: 5 µL of Gentamicin was used as antibiotic in the concentration of 10 mg/mL in the presence of *P. mirabilis* of 10^9 CFU/mL.
Each data was obtained by taken the mean of three independent experiments in triplicates.

Minimum inhibitory concentration results of fruit juice extracts shown in the Figure 3.6, were found as 0.750 mg/mL for grape juice extract and 1.5 mg/mL for orange, peach and pomegranate juice extracts. Therefore, grape extract was observed as the most effective bacterial inhibitor against *P.mirabilis* among the selected fruit juice extracts.

Brown et al. studied antibacterial effects of grape extracts on *Helicobacter pylori* by agar dilution, using confocal laser scanning microscopy, and cell proliferation assays following treatment with various grape extracts. Muscadine grape skin extract was found as most effective with MIC range from 256 to 512 µg/mL, followed by muscadine seed with MIC range from 256 to 1,024 µg/mL and synergic effect of skin and seed extracts together with MIC range from 512 to 1,024 µg/mL (Brown, 2009).

Gould et al. reported that pomegranate rind extracts had MIC between 25.0–12.5 mg/mL against clinical isolates of *S. aureus*, MRSA and PVL positive CA-MSSA (Gould, 2009).

Another group from Turkey studied antimicrobial activity of six pomegranate (*Punica granatum* L.) varieties in comparison to phytonutrition values. MIC and agar diffusion methods weer applied against *Bacillus megaterium* DSM 32, *Pseudomonas aeruginosa*, *Staphylococcus aureus*, *Corynebacterium xerosis, Escherichia coli*, *Enterococcus faecalis*, *Micrococcus luteus,* and three fungi *Kluvyeromyces marxianus*, *Rhodotorula rubra*, *Candida albicans*. They have found the pomegranate aril extracts had antimicrobial effect on all microorganisms, giving inhibition zones ranging in size from 13 to 26 mm. The MIC values for pomegranate extracts were found between 30 and >90 µg/mL (Duman, 2009). Results by Duman et al. have revealed an effective antimicrobial potential for Punica granatum varieties.

Another pomegranate study was done by Panichayupakaranant et al. on antibacterial, anti-inflammatory and anti-allergic activities of rind extract. Bacteriostatic effect against *Propionibacterium acnes* was found with a MIC of 15.6 µg/mL, and against *Staphylococcus aureus* and *Staphylococcus epidermidis*, with MICs of 7.8–15.6 µg/mL (Panichayupakaranant, 2010).

3.1.4 Minimum bactericidal concentration of extracts

Bactericidal effect is always more important than the growth inhibition once the bacterial infection has started. Therefore, bactericidal effects of daily consumed herbal teas and fruit juices were important to examine against *Proteus mirabilis*. Bactericidal dosage of the extracts were determined from their minimum inhibitory concentration experiments. In the assay medium, absence of visible bacterial growth shows the minimum inhibitory concentration has been reached however it does not mean all the bacteria has been killed. It may be due to the bacteriostatic effect of the antimicrobial agent or due to the reduction of growth rate of the bacteria. Nevertheless, MBC test is crucial, to make sure all the bacteria has been killed for the sake of treatment.

3.1.4.1 Minimum bactericidal concentration of tea infusion extracts

Micro agar dilution method applied to the assay medium of MIC test by transferring medium from MIC to MBC wells as described in section 2.3.5.
Sage, anise, rosehip and camomile were selected as herbal tea infusion extractions.

To determine the minimum bactericidal concentration of the tea infusion extracts, 10 µL of MIC medium was transferred to a new MBC 96-well plate with a 100 µL of LB agar in each well were incubated together for 16 hours. At the end of the 16 hours incubation 96-well plates were tested for their absorption at 570 nm using ELISA plate reader.

Bactericidal effect of the tea infusion extracts were presented in the Figure 3.7. Minimum bactericidal concentration results were found to be 6 mg/mL for sage extract although 12 mg/mL for anise, rosehip and camomile tea infusion extracts.

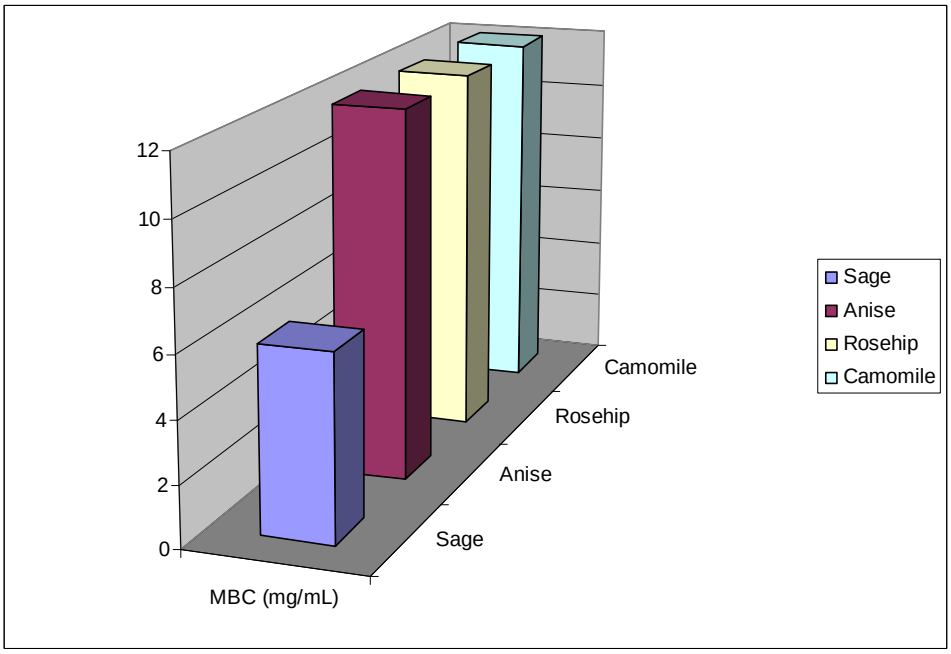


Figure 3.7 Minimum bactericidal concentrations (mg/mL) for tea infusion extracts against *Proteus mirabilis* at 570 nm. Each data was obtained by taken the mean of three independent experiments in triplicates.

Antimicrobial effects of essential oils were reported on pathogens that contaminate raw vegetables in organic Swiss chards. Highest antimicrobial activities were obtained for the essential oils of *Eucalyptus globules, Melaleuca alternifolia, Pimpinella anisum and Syzygium aromaticum* with MBC values in the range of 0.093–1.5 mL/100 mL (Ponce, 2003).

Aqueous extracts of *Cocos nucifera* (husk fiber), *Ziziphus joazeiro* (inner bark), *Caesalpinia pyramidalis* (leaves), and alcoholic extract of *Aristolochia cymbifera* (rhizomes) were examined against oral bacteria *Prevotella intermedia, Porphyromonas gingivalis, Fusobacterium nucleatum, Streptococcus mutans* and *Lactobacillus casei* by Alviano et al. Highest bactericidal effect was reported for *A. cymbifera* extract against all the bacteria tested. C. nucifera, Z. joazeiro and C. pyramidalis extracts were also found to be effective bactericidals (Alviano, 2008).

3.1.4.2 Minimum bactericidal concentration of fruit juices

Fruit juices of grape, orange, peach and pomegranate were chosen for the minimum bactericidal effects on *Proteus mirabilis*. Micro agar dilution method was used as described in section 2.3.5. to determine minimum bactericidal concentrations (MBC) of fruit juice extracts.

MBC results of selected fruit juice extracts as shown in the Figure 3.8, were found to be 6 mg/mL for pomegranate and 12 mg/mL for grape, orange and peach extracts.

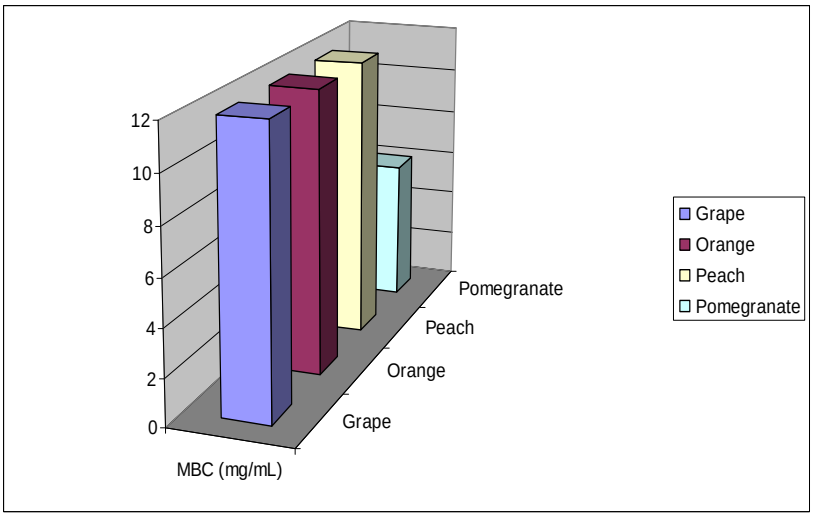


Figure 3.8 Minimum bactericidal concentrations (mg/mL) for fruit juice extracts against *Proteus mirabilis* at 570 nm. Each data was obtained by taken the mean of three independent experiments in triplicates.

Antimicrobial effects of the grape seed extracts (GSE) were throughly studied by Furiga et al. Besides antimicrobial, antioxidant activities were also studied for the grape seeds having rich polyphenolic compounds. Two oral anaerobes associated with periodontal diseases, *P. gingivalis* and *F. Nucleatum,* were studied against by grape seed extracts. As a result, minimum bactericidal concentrations of GSE against the two bacteria were the same, with a value of 8000 µg/mL suggesting GSE may be used in oral hygiene to prevent periodontitis (Furiga, 2009).

The antioxidant and antimicrobial activities of some grape cultivars, such as Colorino, Sangiovese and Cabernet Sauvignon, against *H.pylori* clinical isolates were studied by Santucci et al. As a result of this research, Colorino extract was found as the most powerful antibacterial with its 1.35 mg/mL minimum bactericidal concentration (Santucci, 2009).

Panichayupakaranant et al. have also reported for pomegranate rind extract were effective bactericidals against *Propionibacterium acnes* with a MBC of >1000 µg/mL, *Staphylococcus aureus* with MBCs of 500-1000 µg/mL and *Staphylococcus epidermidis* with MBC of 250 µg/mL (Panichayupakaranant, 2010).

3.1.5 Antimicrobial activity of extracts by disc diffusion test

Kirby-Bauer method was used as described in the section 2.2.4.1 to determine antimicrobial activity of the extracts.

3.1.5.1 Antimicrobial activity of tea infusions by disc diffusion test

100 µL of bacteria grown upto 1.0 absorbance unit (approximately $2x10^8$ bacteria) at 570 nm, were spread onto LB agar plates with placement of empty fitler discs. Then 20 µL of 120 mg/mL concentrations of the tea infusion extracts were applied on empty filter discs. Plates then were incubated at 37 ^oC for 16 hours. After the incubation period, inhibition diameter zones were measured in millimeters.

Inhibition zones for sage, anise, rosehip, and camomile extracts were 7 mm, 7 mm, 8 mm and 8 mm, respectively as displayed in Figure 3.9.

Highest antimicrobial activity was observed with the rosehip and camomile tea infusion extracts with 8 mm inhibition zones.

Standard antibiotic discs of Gentamicin and Kanamycin had inhibition zone diameters of 16 mm and 17 mm, respectively.

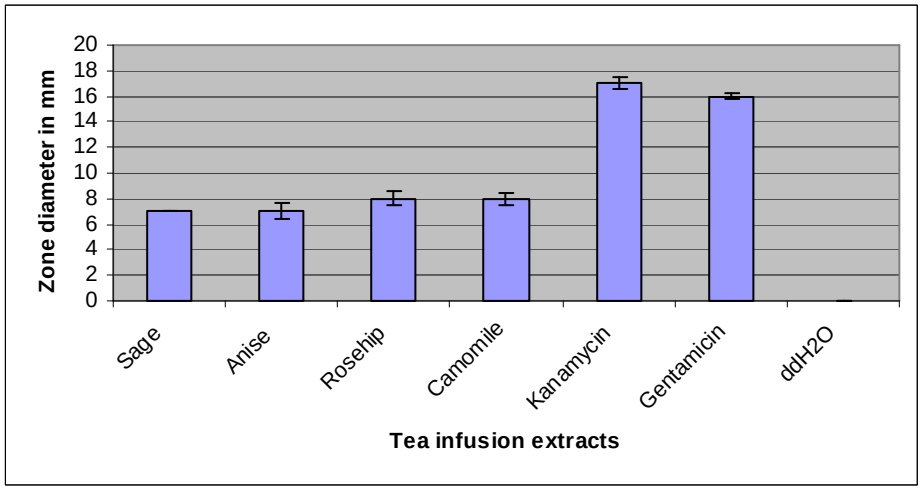


Figure 3.9 Antimicrobial activities of tea infusion extracts by disk diffusion method. Diameter of inhibition zone (mm) includes disc diameter of 6 mm.
Each data was collected by means of three indepent experiments in duplicates.

3.1.5.2 Antimicrobial activity of fruit juices by disc diffusion test

Antimicrobial activity by disc diffusion test were determined by pouring at least 3 mm thickness of LB agar on 9 mm petri plates and placing enough number of empty filter discs according to Kirby-Bauer method as described in the section 2.3.3. Disc diffusion test was determined by the application of the fruit juice extracts using 20 µL of 120 mg/mL concentrations on empty filter discs. Prepared petri plates with discs were incubated at 37 ^{o}C for 16 hours. After incubation period, inhibition diameter zones were measured in millimeters.

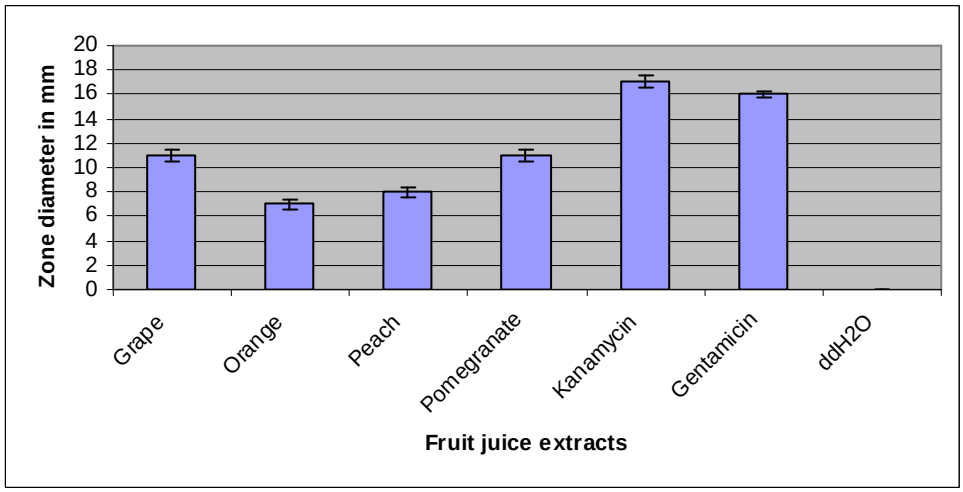


Figure 3.10 Antimicrobial activities of fruit juice extracts by disk diffusion method. Diameter of inhibition zone (mm) includes disc diameter of 6 mm.
Each data was collected by means of three indepent experiments in duplicates.

Inhibition zones for grape, orange, peach and pomegranate extracts were 11 mm, 7 mm, 8 mm and 11 mm, respectively as displayed in Figure 3.10.

Highest antimicrobial activity was observed with the grape and pomegranate fruit juice extracts with 11 mm inhibition zones.

Standard antibiotic discs of Gentamicin and Kanamycin displayed inhibition zone diameters of 16 mm and 17 mm, respectively.

3.2 Determination of antioxidant activities

3.2.1 ABTS method

Radical scavenging activities of the extracts were studied by using 2,2'-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) method which was done according to Re et al., as described in section 2.4.1. Results were calculated according to the trolox equivalence antioxidant capacity (TEAC), a known antioxidant standard trolox standard curve was plotted as shown in Figure 3.11.

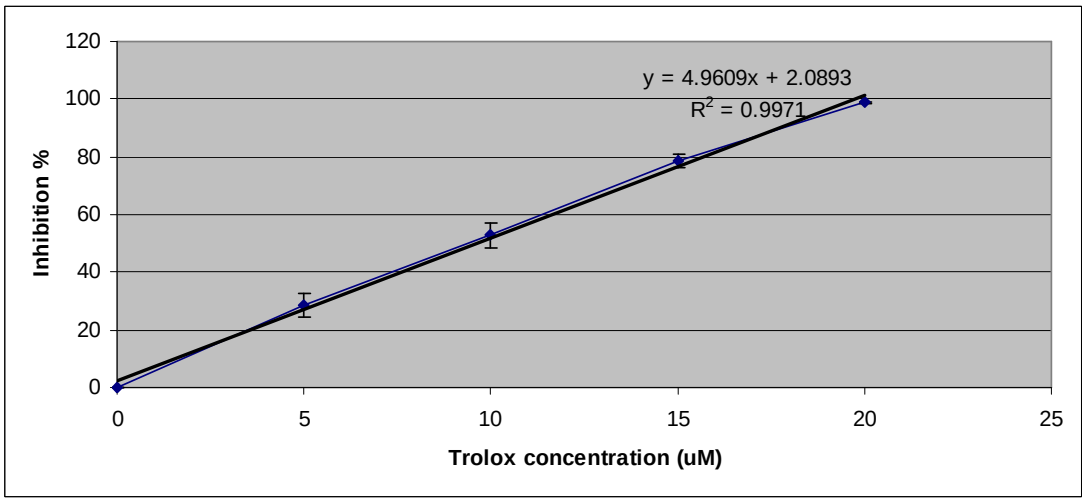


Figure 3.11 Trolox standard curve to calculate trolox equivalent antioxidant capacities of the extracts (at 734 nm).
Each data were calculated by means of three independent experiments in duplicates (n=6).

3.2.1.1 Determination of free radical scavenging capacities of tea infusion extracts by ABTS method

Trolox equivalent antioxidant capacity (TEAC) values of tea infusion extracts are given in Table 3.4. The higher the TEAC value means the higher the antioxidant capacity. Therefore, sage extract was found as having the highest ABTS radical scavenging activity with TEAC value of 5.152 mmol/g among other selected tea infusion extracts. Tea infusion extracts of rosehip, camomile and anise had TEAC values of 1.362, 0.666, 0.342 mmol/g; respectively with a decreasing order.

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

Extract	*TEAC Value (mmol/g) ± SD	**Inhibition % ± SD
Sage	5.152 ± 0.065	81.980 ± 1.659
Anise	0.342± 0.011	85.716 ± 1.776
Rosehip	1.362 ± 0.043	86.093 ± 1.082
Camomile	0.666 ± 0.021	91.942 ± 0.573

TEAC value: Radical scavenging activity mmol equivalents of trolox/g of extract (DW)
*Mean of three independent experiment in duplicates
**Scavenging capacities at 6th min

By using ABTS method, Chohan et al. examined the antioxidant capacity of some edible herbs by exposing them to various cooking and storage procedures. TEAC values for the extracts of sage and ginger were calculated as 625±0.5 µmol/g and 4.6±0.5 µmol/g respectively (Chohan, 2008).

Some medicinal and aromatic plant extracts were screened by Miliauskas et al. for their radical scavenging activity. Among the screened medicinal plant extracts, *G. macrorrhizum* and *P. fruticosa* were most active. *S. officinalis* extracts have revealed 88.57 % scavenging (Miliauskas, 2004) similar to the results in this study which was 82 %.

3.2.1.2 Determination of antioxidant capacities of fruit juices by ABTS method

Radical scavenging activities of fruit juice extracts were investigated according to ABTS method. The results of trolox equivalent antioxidant capacities (TEAC) in mmol trolox equivalent per gram of fruit juice extracts are shown in Table 3.5. Pomegranate was found as having the highest TEAC value of 0.552. with a higher radical scavenging capacity among fruit juice extracts. Fruit juice extracts of peach, orange, and grape had TEAC values as 0.492, 0.258, 0.210; respectively with a decreasing order.

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

Extract	*TEAC Value (mmol/g) ± SD	**Inhibition % ± SD
Grape	0.210 ± 0.018	64,616 ± 0,599
Orange	0.258 ± 0.020	79,485 ± 1,987
Peach	0.492 ± 0.041	97,644 ± 0,246
Pomegranate	0.552 ± 0.056	98,781 ± 0,134

TEAC value: Radical scavenging activity mmol equivalents of trolox/g of extract (DW)
*Mean of triplicate trials
**Scavenging capacities at 6th min

Depending on the ABTS method, in 2009, Furiga et al. asserted that the extracts of grape seeds in regard to their antioxidant characteristics were more valuable than vitamin C and E comparison to Trolox, which was commonly used as an antioxidant. This research indicated that TEAC value of grape seeds extracts was 7.01 micromolar per microgram (Furiga, 2009).

According to a recent study through ABTS method, Breksa et al. ascertain that 16 kinds of grapes have showed antioxidant activity from 7.7 to 60.9 µmol conjugated to Trolox (Breksa, 2010).

3.2.2 Determination of antioxidant capacities of extracts by DPPH method

Antioxidant capacities of the tea infusion and fruit juice extracts were studied by using DPPH radical scavenging method.

3.2.2.1 Determination of radical scavenging activities of tea infusion extracts by DPPH method

2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging activities of tea infusion extracts were investigated as described in section 2.4.2. Results are given in Figure 3.12 and Table 3.6.

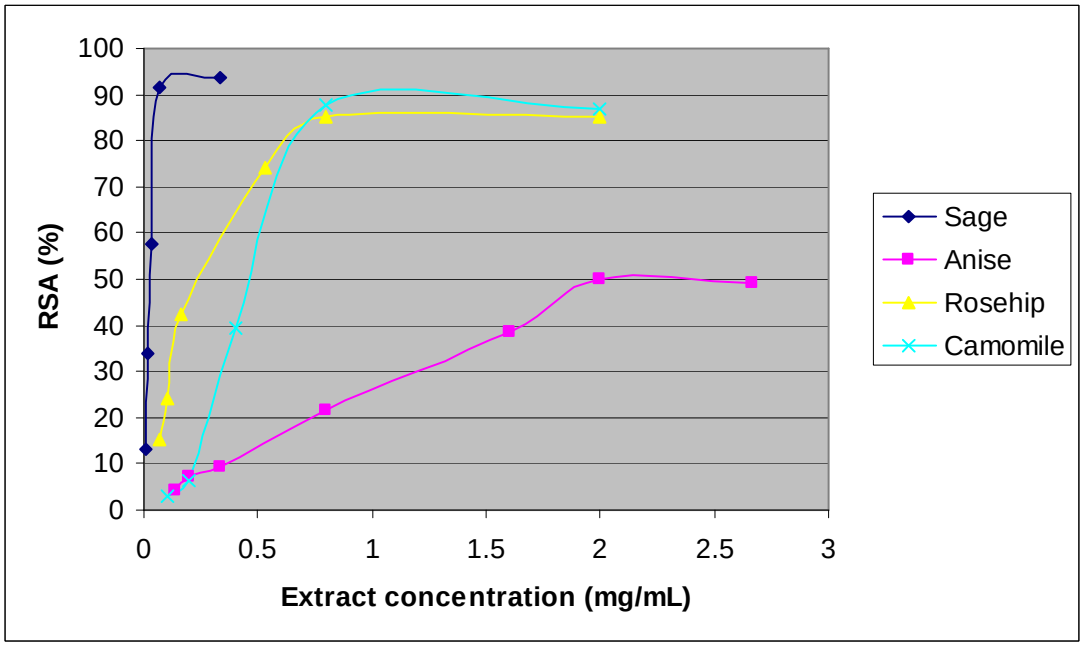


Figure 3.12 DPPH radical scavenging activity in percent versus extract concentrations (mg/mL) of tea infusion extracts. DPPH radical scavenging activities were measured at 20th minute of incubation period, at 517 nm.

Among selected tea infusion extracts sage extract has revealed the most effective radical scavenging with an EC_{50} value of 0.072 mg/mL.

Table 3.6 Fifty percent effective concentrations for DPPH radical scavenging activities and total phenolic contents of selected tea infusion extracts.

Tea infusions	***DPPH RSA EC_{50} (mg/mL)**
Sage	0.072 ± 0.001
Anise	2.024 ± 0.015
Rosehip	0.530 ± 0.004
Camomile	0.782 ± 0.008
Quercetin	0.087 ± 0.002

DPPH RSA EC_{50} : Effective concentration of plant extracts for 50 % of DPPH radical scavenging activity

* Mean of triplicate trials

Using “total polyphenol and flavonoid contents, DPPH radical scavenging, hydroxyl radical scavenging, nitrite scavenging activity and antimicrobial activity”, Chung evaluated that off four various solvents, methanol star anise extract has the highest radical scavenging activity with the rate as 89.02% (Chung, 2009).

Taking rosa mosqueta roseship seeds as a sample, in 2006 Franco et al. made a research upon the antioxidant activities of herbal oils and fusible compounds exposed from the mentioned plant. Resulted from this study it is claimed that approximately 80% of ethanol, 52.2% of methanol, and 41% of water extracts revealed DPPH inhibition (Franco, 2006).

Free radical scavenging activities and protective effects on oxidative cardiac cell injury of *Salvia brachyantha* have been studied by Esmaeili et al. and DPPH (IC_{50}) value of the extract was given as 46.72 ± 2.14 (µg/mL) (Esmaeili, 2010).

3.2.2.2 Determination of radical scavenging activities of fruit juice extracts by DPPH method

DPPH radical scavenging activities of fruit juice extracts were tested as described in section 2.4.2. Results were given in Figure 3.13 and Table 3.7.

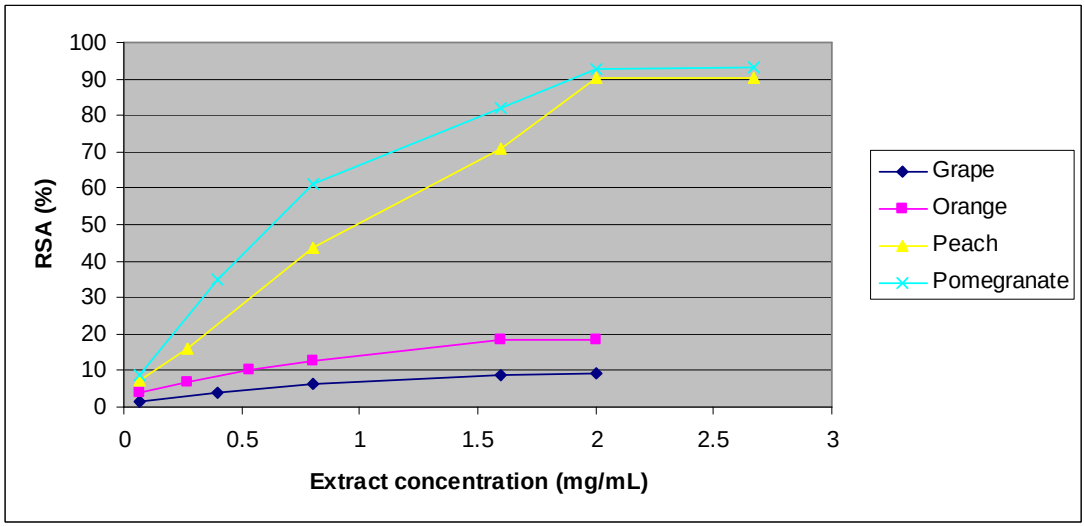


Figure 3.13 DPPH radical scavenging activity in percent versus extract concentrations (mg/mL) of fruit juice extracts. DPPH radical scavenging activities were measured at 20th minute of incubation period, at 517 nm.

Pomegranate and peach were found as most effective in radical scavenging with EC_{50} values of 0,924 and 1.148 mg/mL, respectively. Pomegranate fruit juice extract was much more effective in radical scavenging than peach.

Table 3.7 Fifty percent effective concentrations for DPPH radical scavenging activities and total phenolic contents of selected fruit juice extracts.

Fruit Juices	***DPPH RSA EC_{50} (mg/mL)**
Grape	NA
Orange	NA
Peach	1.148 ± 0.034
Pomegranate	0.924 ± 0.014
Quercetin	0.087± 0.002

DPPH RSA EC_{50} : Effective concentration of plant extracts for 50 % of DPPH radical scavenging activity
NA: not applicable
Mean of triplicate trials

Phenolic compositions and antioxidant capacities of orange juice and orange wine from Kozan, Turkey, studied by Kelebek et al. Orange juice has revealed as higher radical scavenger than orange wine with EC_{50} values of 0.31 mg/mL and 0.46 mg/mL, respectively (Kelebek, 2009).

In a recent study, Lee et al. compared chemical compositions and radical scavenging activities of peach extracts from six different cultivation areas. It was concluded that DPPH (1,1-diphenyl-2-picrylhydrazyl) radical scavenging activities of the peach extracts were as in order of Turkey > Uzbekistan> > Spain > Iran >Taiwan (Lee, 2009).

Antioxidant capacities of pomegranate juices from eight cultivars were examined with four methods by Çam et al. DPPH, ABTS, and b-carotene-linoleate methods were applied to the extracts and efficient concentration (EC_{50}) of cultivar 8 Izmir was 29.8 ± 2.9 ml of pomegranate juice/g of DPPH (Çam, 2009).

3.2.3 Determination of total phenolic content of extracts

Determination of total phenolic content was done as described in section 2.4.3. Gallic Acid (GA) standard curve was plotted as in the Figure 3.14.

Since the most problematic interference in total phenolic content procedure may be sugar and can be found at very high levels in fruits, the interference of sugar may be corrected by conducting the analysis of the standards with the same level of sugar as the sample, but this is a complex issue because different sugars yield different intereferences. In this study, the results of total phenolic content of fruit juices were apparent phenol content with invert sugar content.

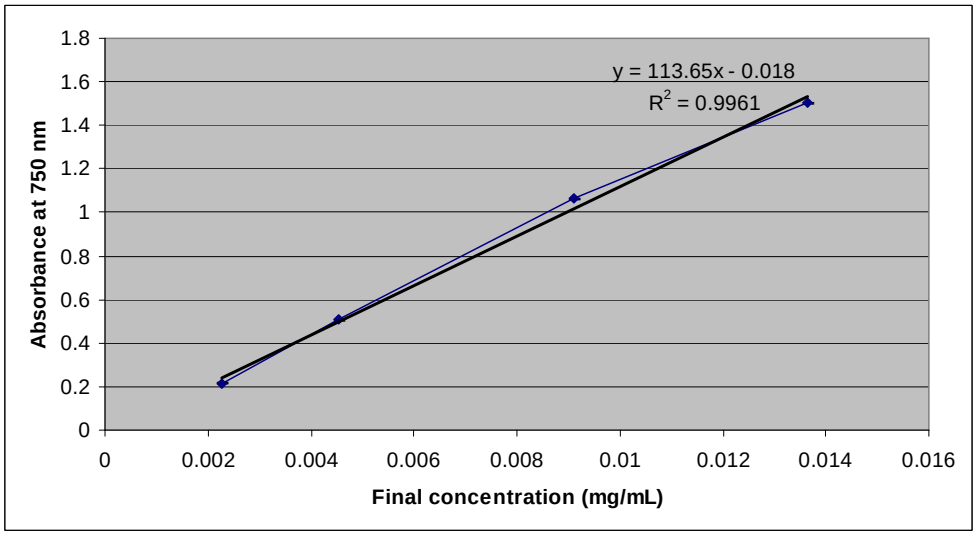


Figure 3.14 Gallic acid standard curve to calculate total phenolic content of extracts in terms of gallic acid equivalence.
Each data was collected by means of two indepent experiments in triplicates.

3.2.3.1 Determination of total phenolic content of tea infusion extracts

In the tea infusion extracts of selected herbs, amount of phenolic compounds were searched in order to correlate antioxidant activities of the extracts. Sage extract has revealed the highest amount of 0.411 mg gallic acid equivalence (GAE) in 1 mg of extract as can be seen in Table 3.8. Sage was also found as most effective radical scavenger with an EC_{50} value of 0.072 among selected herbal tea extracts correlating with its high phenolic content.

Table 3.8 Total phenolic content results of tea infusion extracts.

Tea infusions	***TP GAE (mg/mg)**
Sage	0.411 ± 0.0052
Anise	0.040 ± 0.0017
Rosehip	0.101 ± 0.0005
Camomile	0.038 ± 0.0004

TP GAE : Total phenolic content mg equivalents of gallic acid/mg of plant extract

*Mean of triplicate trials

In 2004, Miliauskasa et al. studied total phenolic compounds in sage (*S. officinalis*) and total phenolic content of sage was found as 22.6 mg of GAE per g of plant extract (Miliauskasa, 2004).

Yoo *et al.* reported total phenolic content of *Rosa rubiginosa* as 818.5 mg GAE per 100 g of extract and total flavonoid content as 400.5 mg catechin equivalents per 100 g (Yoo, 2008).

3.2.3.2 Determination of total phenolic content of fruit juices

Total phenolic contents of fruit juice extracts were investigated and peach extract has displayed the highest phenolic content with a TP GAE value of 0.067 as gallic acid equivalent in mg per mg of extract as can be seen in Table 3.9. Total phenolic content of grape, orange and pomegranate extracts were 0.006, 0.024, and 0.033 as mg of gallic acid equivalence in mg of extract, respectively.

Table 3.9 Total phenolic content results of fruit juice extracts.

Fruit Juices	**TP GAE (mg/mg)**
Grape	0.006 ± 0.0005
Orange	0.024 ± 0.0011
Peach	0.067 ± 0.0003
Pomegranate	0.033 ± 0.0009

TP GAE : Total phenolic contents mg equivalents of gallic acid/mg of plant extract

*Mean of triplicate trials

Total phenolic content (TPC) of pomegranate was studied by Çam et al. and it was found between the range of 208.3–343.6 mg catechin equivalents (Çam, 2009).

ABTS, DPPH, TP, MIC, MBC and disk diffusion test and their results for all selected fruit juice and tea infusion extracts are shown in Table 3.10 for comparison.

Table 3.10 Comparison of DPPH EC_{50} (mg/mL), TP GAE (mg/mg), MIC (mg/mL), MBC (mg/mL) and Disk diffusion test (mm) results for all tested herbal tea infusion and fruit juice dried extracts.

	TEAC Value* (mmol/g)	DPPH EC_{50}* (mg/mL)	TP GAE** (mg/mg)	MIC** (mg/mL)	MBC** (mg/mL)	Disc diffusion* (mm)
Sage	5.152 ± 0.065	0.072 ± 0.001	0.411 ± 0.0052	3	6	7 ± 0.00
Anise	0.342± 0.011	2.024 ± 0.015	0.040 ± 0.0017	6	12	7 ± 0.63
Rosehip	1.362 ± 0.043	0.530 ± 0.004	0.101 ± 0.0005	3	12	8 ± 0.52
Camomile	0.666 ± 0.021	0.782 ± 0.008	0.038 ± 0.0004	6	12	8 ± 0.49
Grape	0.210 ± 0.018	NA	0.006 ± 0.0005	0.75	12	11 ± 0.52
Orange	0.258 ± 0.020	NA	0.024 ± 0.0011	1.5	12	7 ± 0.41
Peach	0.492 ± 0.041	1.148 ± 0.034	0.067 ± 0.0003	1.5	12	8 ± 0.41
Pomegranate	0.552 ± 0.056	0.924 ± 0.014	0.033 ± 0.0009	1.5	6	11 ± 0.52
Quercetin	ND	0.087± 0.002	ND	ND	ND	ND
Gentamicin	ND	ND	ND	ND	ND	16 ± 0.00

TEAC value: Radical scavenging activity mmol equivalents of trolox/g of extract (DW)
DPPH RSA EC_{50} : Effective concentration of plant extracts for 50 % of DPPH radical scavenging activity
TP GAE : Total phenolic contents mg equivalents of gallic acid/mg of plant extract
Diameter of inhibition zone (mm) including disk diameter of 6 mm.
NA: not applicable, ND: not determined, * Mean of triple trials in duplicates, **Mean of triple trials in triplicates

CHAPTER 4

CONCLUSION

All of the antioxidant and antimicrobial methods that were used in this study were summarised in Table 3.8. As a conclusion, sage extract was found with the highest ABTS radical scavenging activity and phenolic content as well as with the highest DPPH radical scavenging capacity, among tea infusion extracts. Rosehip also showed second high antioxidant capacity with its high phenolic content.

Pomegranate has been found as most effective in ABTS radical scavenging with its high TEAC value among fruit juice extracts. Peach showed second high ABTS radical scavenging such as in DPPH. Total phenolic content of these two fruit juice extracts were not correlated with their antioxidant capacities.

Most effective inhibition with minimum inhibitory concentration method was observed by sage and rosehip extracts in the same amount. However bactericidal concentration of sage was much more effective than anise. Sage extract was much more effective against *Proteus mirabilis* as observed from MBC values of the extracts, since it is more important killing of bacteria than inhibition.

Grape extract has been observed having the most effective inhibition with its low MIC concentration correlating with its high inhibiton zone diameter against *Proteus mirabilis.* Minimum bactericidal concentration of pomegranate extract was the second best among fruit juice extracts and it also showed high inhibition zone diameter in disc diffusion test against *Proteus mirabilis*.

In disc diffusion test study, 10 μg of gentamicin disc, a commonly used antibiotic in urinary tract infections has shown a 16 mm of zone clearance on the inhibition of *Proteus mirabilis*, when 2.4 miligrams of grape extract was loaded it has revealed a 11 mm of zone clearance. Meanwhile, 39.10 mg of grape juice is expected to inhibit the 16 mm of bacterial zone as gentamicin, besides without resistance. Similarly, 95.08 mg of pomegranate juice may inhibit the same zone as gentamicin. So, in conclusion these fruit juices would be anew alternative to urinary tract infections instead of antibiotics.

At the beginning stage of urinary tract infections one could prevent the progress of *Proteus mirabilis* infection by consuming daily infusion teas sage, anise, rosehip and camomile in 35.40, 27.45, 8.60 and 8.10 miligrams of respective tea bags. Similarly, the consumption of 39.10 mg of grape juice, 160.80 mg of orange juice, 60.50 mg of peach juice and 85.08 mg of pomegranate juice would be effective at the beginning of the infection.

The antimicrobial effects of active chemicals obtained by chemical methods might be important for producing new antibiotics in the future. The contribution of naturally gained antibiotics to medical industry of our country and other countries of the world is very important. Getting under control of bacterial infections before its progress, by consuming fruit juices and herbal teas daily, is also important not only for protecting human health but also for national economy. Therefore, daily consumed fruit juices and herbal teas which were studied in this study may be used for urinary tract infections that *P. mirabilis* causes.

REFERENCES

Alviano W. S., Alviano D. S., Diniz C. G., Antoniolli A. R., Alviano C. S., Farias L. M., Carvalho M. A. R., Souza M. M.G., Bolognese A. M. (2008). In vitro antioxidant potential of medicinal plant extracts and their activities against oral bacteria based on Brazilian folk medicine; *Archives of Oral Biology*, 53: 545 – 552.

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

Bergsten G., Wullt B., Svanborg C., (2005). Escherichia coli, fimbriae, bacterial persistence and host response induction in the human urinary tract; *International Journal of Medical Microbiology*, 295; 487–502.

Breksa A. P., Takeoka G. R., Hidalgo M. B., Vilches A., Vasse J., Ramming D. W. (2010). Antioxidant activity and phenolic content of 16 raisin grape (Vitis vinifera L.) cultivars and selections; *Food Chemistry* 121: 740–745.

Brown J. C., Huang G., Haley-Zitlin V., Jiang X. (2009). Antibacterial Effects of Grape Extracts on *Helicobacter pylori*; *Applied and Environmental Microbiology*, p. 848–852 Vol. 75.

Chohan M., Forster-Wilkins G., Opara E. I. (2008). Determination of the Antioxidant Capacity of Culinary Herbs Subjected to Various Cooking and Storage Processes
Using the ABTS*+ Radical Cation Assay; *Plant Foods Hum Nutr*; 63:47–52.

Chung H-J. (2009). Evaluation of the Biological Activity of Extracts from Star-Anise (Illicium verum); *J Food Sci Nutr Vol 14, p 195～200.*

Çama M., Hısıl Y., Durmaz G. (2009). Classification of eight pomegranate juices based on antioxidant capacity measured by four methods; Food Chemistry 112: 721–726.

Duman A. D., Ozgen M., Dayisoylu K. S., Erbil N., Durgac C. (2009). Antimicrobial Activity of Six Pomegranate (*Punica granatum* L.) Varieties and Their Relation to Some of Their Pomological and Phytonutrient Characteristics*, Molecules* 2009*, 14,* 1808-1817.

Dragan A., Dusanka D.A., Drago B., Nenad T. (2003). Structure radical scavenging activity relationships of flavonoids. *Croatica Chemica Acta* 76, (1), 55-61.

Esmaeili M. A., Sonboli A. (2010). Antioxidant, free radical scavenging activities of Salvia brachyantha and its protective effect against oxidative cardiac cell injury; *Food and Chemical Toxicology* 48: 846–853.

Franco D., Pinelo M., Sineiro J., Nu´n˜ez M. J. (2007). Processing of Rosa rubiginosa: Extraction of oil and antioxidant substances; *Bioresource Technology* 98:3506–3512.

Furiga A., Lonvaud-Funel A., Badet C. (2009). In vitro study of antioxidant capacity and antibacterial activity on oral anaerobes of a grape seed extract; *Food Chemistry* 113: 1037–1040.

Gould S. WJ, Fielder M. D., Kelly A. F., Naughton D. P. (2009). Anti-microbial activities of pomegranate rind extracts: enhancement by cupric

sulphate against clinical isolates of *S. aureus*, MRSA and PVL positive CA-MSSA; *BMC Complementary and Alternative Medicine* 2009, 9:23, doi:10.1186/1472-6882-9-23.

Hagerman, A. E., Riedl, K. M., Jones, G. A., Sovik, K. N., Ritchard, N. T., Hartzfeld, P. W., & Riechel, T. L. (1998). High molecular weight plant polyphenolics (tannins) as biological antioxidants. Journal of Agricultural and Food Chemistry, 46, 1887±1892.

Hammer K.A., Carson C.F., Riley T.V. (1999). Antimicrobial activity of essential oils and other plant extracts; *Journal of Applied Microbiology* 1999, 86, 985–990

Jabalameli F., Malekzadeh F., Mirsalehian A. (2005). Effects of antibiotics on adhesion and invasion of *Proteus mirabilis*; *Acta Medica Iranica*, 43(1): 55-59.

Jones D. B., Lockatell C. V., Johnson D. E., Warren J. W., Mobley H. L.T. (1990). Construction of a Urease-Negative Mutant of Proteus mirabilis: Analysis of Virulence in a Mouse Model of Ascending Urinary Tract Infection; *Infection and Immunity*, Apr. 1990, p. 1120-1123

Kelebek H., Selli S., Canbas A., Cabaroglu T. (2009). HPLC determination of organic acids, sugars, phenolic compositions and antioxidant capacity of orange juice and orange wine made from a Turkish cv. Kozan; *Microchemical Journal* 91: 187–192.

Kurtoğlu M.G., Bozkurt H., Güdücüoğlu H., Bayram Y., Berktaş M.. (2008). Klinik örneklerden izole edilen *Proteus mirabilis* suşlarının antimikrobial ajanlara duyarlılıkları; *Genel Tıp Dergisi*, 18(1):23-26.

Lee H. J., Cho H. J., Lee H., Kwon K. H., Hong Y. H., Kim S., Suh H. J. (2009). Comparison of Chemical Composition and Radical Scavenging Activity of Pomegranate Extracts from Different Growing Areas; *Journal of Food Sci Nutr*., *Vol 14, p 214 ~ 219.*

Mann C. M., Markham C.L. (1997). A new method for determining the minimum inhibitory concentration of essential oils; *Journal of Applied Microbiology* 1998, 84, 538–544

Meyrier A., (2003). Urinary Tract Infections, Chapter-7 [online], www.kidneyatlas.org/book2/adk2_07.pdf [searching date: 15.06.2010]

Miliauskasa G., Venskutonis P. R., van Beek T. A. (2004). Screening of radical scavenging activity of some medicinal and aromatic plant extracts; *Food Chemistry*, 85: 231–237.

Murphy, C. A. ; Belas, R. (1999). Genomic rearrangements in the flagellin genes of *Proteus mirabilis*; *Molecular Microbiology* 31(2), 679–690.

Mobley H. L. T.; Warren J. W., 1996, Urinary Tract Infections: Molecular Pathogenesis and Clinical Management*; American Society for Microbiology Press, USA*, 1-55581-093-4.

Niki E. (2010). Assessment of antioxidant capacity in vitro and in vivo; Free Radical Biology & Medicine 49 (2010) 503–515.

O'hara, C.M.; Brenner, F.; Miller, J.M. (2000). Classification, Identification, and Clinical Significance of *Proteus*, *Providencia*, and *Morganella; Clinical Microbiology Reviews,* 0893-8512/00/04.0010 Oct. 2000, p. 534–546.

Panichayupakaranant P. , Tewtrakul S., Yuenyongsawad S. (2010). Antibacterial, anti-inflammatory and anti-allergic activities of standardised pomegranate rind extract; *Food Chemistry* 123: 400–403.

Pollack, R.A.; Findlay, L.; Mondschein,W.; Modesto, R.R. (2005). "Microbial Sensitivity Testing", *Laboratory Exercises in Microbiology,* 2nd Edition.; J.Wiley and Sons, Inc., USA, 93-105.

Poncea A.G., Fritzb R., Valleb C., Rourac S.I. (2003). Antimicrobial activity of essential oils on the native microflora of organic Swiss chard; *Lebensm.-Wiss. u.-Technol*., 36: 679–684.

Santucci A., Bonechi C., D'Addario C., Rossi C., Braconi D., Bernardini G., Salvini L., Figura N., Martini S. (2009).Grape and wine extracts have antibacterial activity against Helicobacter pylori clinical isolates; *J Chemother*; 21(5):507-13.

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

Tal S., Guller V., Levi S., Bardenstein R., Berger D., Gurevich I., Gurevich A., (2005). Profile and prognosis of febrile elderly patients with bacteremic urinary tract infection; *Journal of Infection*, 50, 296–305.

Tepe B., Akpulat H. A., Sokmen M., Daferera D., Yumrutas O., Aydin E., Polissiou M., Sokmen A. (2006). Screening of the antioxidative and antimicrobial properties of the essential oils of *Pimpinella anisetum* and *Pimpinella flabellifolia* from Turkey; *Food Chemistry*, 97: 719–724.

Vosti L. K. (2007). A prospective, longitudinal study of the behavior of serologically classified isolates of *Escherichia coli* in women with recurrent urinary tract infections, Journal of Infection 55, 8-18.

Vrcˇek I. V., Bojic M., Zˇuntar I., Mendaš G., Medic´-Šaric´ M. (2011). Phenol content, antioxidant activity and metal composition of Croatian wines deriving from organically and conventionally grown grapes, *Food Chemistry* 124: 354–361.

Wael M, Ghoush A., (2008). Screening Test for Detection of Urinary Tract Infections: Evaluation of the Urinary Leukocyte Esterase Dipstick Test; *TAF Preventive Medicine Bulletin,* 7(3).

Yoo K. M., Lee C. H., Lee H., Moon B., Lee C. Y. (2008). Relative antioxidant and cytoprotective activities of common herbs. *Food Chemistry*, 106: 929–936.

(n.d.). Retrieved 26/07/ 2010, from UCLA Health System: http://www.uclahealth.org/homepage_site.cfm?id=11