SCREENING OF SOME PLANT SPECIES FOR THEIR TOTAL ANTIOXIDANT AND ANTIMICROBIAL ACTIVITIES

A Thesis Submitted to
the Graduate School of Engineering and Sciences of
İzmir Institute of Technology
in Partial Fulfillment of the Requirements for the Degree of

MASTER OF SCIENCES

in Biotechnology and Bioengineering

by
Diren KAÇAR

July 2008
İZMİR

We approve the thesis of **Diren KAÇAR**

_____

Assoc. Prof. Dr. Oğuz BAYRAKTAR
Supervisor

_____

Prof. Dr. Semra ÜLKÜ
Co-supervisor

_____

Assist. Prof. Dr. Çağlar KARAKAYA
Co-supervisor

_____

Assist. Prof. Dr. Figen KOREL
Committee Member

_____

Assist. Prof. Dr. Sait SARGIN
Committee Member

11 July 08
Date

_____

Prof. Dr. Semra ÜLKÜ
Department of Chemical Engineering
İzmir Institute of Technology

_____

Prof. Dr. Hasan BÖKE
Dean of the Graduate School
of Engineering and Science

ACKNOWLEDGEMENT

First of all, I would like to express my deep and sincere gratitude to my advisor , Assoc. Prof. Dr. Oğuz Bayraktar for his suggestions, guidance, encouragement and support throughout this study. His wide knowledge and his logical way of thinking have been great value for me.

I also wish to express my warm and sincere thanks to my co-advisor Professor Dr. Semra Ülkü, Head of the Department of Biotechnology and Bioengineering and to Assist. Prof. Dr. Çağlar Karakaya for their suggestions and advices during my study.

I warmly thank to commitee members of my thesis, Assist. Prof. Dr. Sait Sargın and Assist. Prof. Dr. Figen Korel for their suggestions and evaluations on my study.

I express my special thanks to research assistants Evren Altıok, Çisem Bulut, Seçil Çoban and Dane Ruscuklu for their supports and friendships.

My sincere thanks to my other friends, Kadir Gökavdan, Senar Uçar and Didem Ceyhan for their continued supports and patience.

I wish to extend my final thanks to my family, especially to my parent for their encouragement and supports during my education.

ABSTRACT

SCREENING OF SOME PLANT SPECIES FOR THEIR TOTAL ANTIOXIDANT AND ANTIMICROBIAL ACTIVITIES

In this study aqueous/ethanol extracts of 42 plant species collected from same geographic region (Karaburun/İzmir) were screened for their relative total phenol contents, total antioxidant and antibacterial activities. In the first part of the study, Folin- ciocalteu assay and PCL (Photochemiluminescence) method were performed to detect the total phenol contents and total antioxidant activities of extracts, respectively. It was detected that the *Hypericum empetrifolium* had the highest activities for both water soluble and lipid soluble antioxidants and *Sarcopterium spinosum* has the highest result for total phenol assay as 635.26 GAEqmg/g sample.

In order to detect the antibacterial activities of extracts a preliminary screening study was performed by using disc diffusion method. Out of the 42 plant species tested, 26 species exhibited antibacterial activities by inhibiting one or more microorganisms. Microdilution assays by 96 well plates were applied for the most active species to find out their minimum inhibition concentrations (MICs). The most promising plant species in the study, having the antibacterial activities were determined as *H. empetrifolium*, *P. terebinthus*, *Arbutus unedo*, and *C. parviflorus.*

In this study there is a clear relationship between the analysis results and *S. spinosum* is one of the most noteworthy species in this study showing the highest total phenol content and important biological activities which has never been examined scientifically before. In that manner this study also presents new potential species that can be used as natural raw materials in some related industries.

ÖZET

BAZI BİTKİ TÜRLERİNİN TOPLAM ANTİOKSİDAN VE ANTİMİKROBİYAL AKTİVİTELERİNİN İÇİN TARANMASI

Bu çalışmada, aynı coğrafik bölgeden toplanmış olan 42 bitki özütü, toplam fenol içerikleri, toplam antioksidan ve antibakteriyel aktivitelerinin belirlenmesi amacıyla taranmıştır. Çalışmanın ilk kısmında özütlerin toplam fenol içerikleri ve toplam antioksidan aktiviteleri için sırasıyla Folin-ciocalteu ve PCL (photochemiluminescence) metodları uygulanmıştır. Buna göre, *H. empetrifolium* hem suda hem de yağda çözünen antioksidanlar bakımından en iyi aktiviteyi sergilerken, *S. spinosum* türünün ise test edilen bitkiler arasında en yüksek toplam fenol içeriğine (635.26 GAEqmg/g sample) sahip olduğu belirlenmiştir.

Özütlerin antibakteriyel aktivitelerini belirlemek amacıyla disk difüzyon yöntemi kullanılarak bir ön tarama çalışması gerçekleştirilmiştir. Test edilen 42 bitki türünden 26 tür bir veya birden fazla mikroorganizma üzerine etki ederek bazı antibakteriyel aktiviteler sergilemişlerdir. Sonrasında ise en aktif türler için minimum inhibisyon konsantrasyonlarını bulmak amacıyla 96 çukur plaka ile mikrodilüsyon yöntemi gerçekleştirilmiştir. Çalışmada antibakteriyel aktiviteye sahip en ümit verici türler *H. empetrifolium*, *P. terebinthus*, *Arbutus unedo*, and *C. parviflorus* türleridir.

Bu çalışmada yapılan analiz sonuçları arasında belirgin bir ilişki gözlenmiştir. *S. spinosum*, en yüksek toplam fenol içeriği sunan ve önemli biyolojik aktiviteler sergileyen dikkat çekici türlerden biridir ve daha önce bilimsel olarak incelenmemiştir. Böylelikle bu çalışma ile bazı endüstri dallarında doğal, hammadde olarak kullanılabilecek başta *S. spinosum* olmak üzere yeni potansiyel bitki türleri belirlenmiştir.

TABLE OF CONTENTS

LIST OF FIGURES

Figure **Page**

LIST OF TABLES

Table **Page**

LIST OF ABBREVIATIONS

ABTS	2,2'-azino-bis(3-ethylbenzthiazoline-6-sulphonic acid)
ACL	Antioxidant capacity of lipid soluble compounds
ACW	Antioxidant capacity of water soluble compounds
AST	Antimicrobial susceptibility testing
AUC	Area under curve
BHA	Butylhydroxyanisole
BHT	Butylhydroxytoluene
CA	Catalase
CL	Chemiluminescence
DMSO	Dimethyl sulfoxide
DPPH	Diphenylpicrylhydrazyl
F-C	Folin- Ciocalteu
FRAP	Ferric Reducing Antioxidant Power
GAE	Gallic acid equivalents
GPX	Glutathione peroxide
HAT	Hydrogen Atom Transfer
Hυ	Optical excitation
INT	Iodonitrotetrazolium chloride
LDL	Low–Density Lipoprotein
MIC	Minimum Inhibition Concentrations
OD	Optical density
ORAC	Oxygen radical absorbance capacity
PCL	Photochemiluminescence
PCL	Photochemiluminescence
RNS	Reactive nitrogen species
ROS	Reactive oxygen species
ROS	Reactive oxygen species
SET	Single Electron Transfer
SFE	Supercritical fluid extraction

SOD	Superoxidedismutase
TEAC	Trolox equivalent antioxidant capacity
TRAP	Radical-trapping antioxidant parameter
TLC	Thin layer chromatography

CHAPTER 1

INTRODUCTION

There is an increasing interest in using medicinal and aromatic plants as natural sources in pharmaceutical, food and cosmetic industries all over the world. Bioactive compounds of medicinal plants led them to be used in these industries as botanical drugs, dietary supplements, functional foods and food packaging, etc. Plants also have been used in ethnopharmacy for various diseases such as hypertension, cholesterol, eczema and diarrhoea for centuries and today their scientific validation was provided by identification and isolation of bioactive phytochemicals (Littleton, et al. 2005). Phytochemicals are the secondary metabolites that have several subgroups possessing various bioactivities such as antioxidant, antimicrobial, antivirus, anticancer, etc., (Duffy and Power 2001). Nowadays re-emerging connection between plants and human health especially depends on their antioxidant activities that may delay or reduce the hazardous effects of free radicals. The major causative for the generation of free radicals in food, drugs, and living systems is the oxidation process (Pourmorad, et al. 2006). Free radicals and other reactive oxygen species ($O_2^{\cdot-}$, H_2O_2 , $OH^{\cdot-}$) are released continously during the essential aerobic metabolism as metabolic by-products which are potentially producing damage on biomolecules such as membrane lipids, cellular proteins and DNA which leads to cell death and several diseases (Antolovich, et al. 2002). Most common radical related diseases are atherosclerosis, arthritis, diabetes, cancer and neurodegenerative diseases (Parkinson, Alzheimer and Huntington's disease) and also aging (Pourmorad, et al. 2006). Living systems have their own cellular defence systems including some enzymatic and nonenzymatic systems which protect the functional and structural molecules that are the targets of free radicals (Prior, et al. 2005). They are able to keep the system in the state of equilibrium by controlling the harmful effects of free radicals under normal physiological conditions, but in some cases the equilibrium may be disturbed by some factors that induce the formation of free radicals such as environmental polutants, radiation, chemicals, physical stress and also some endogenous sources including some enzymes and immune system products

(Serafini 2006). Oxidative stress occurs as a result of an overproduction and accumulation of highly reactive compounds (Antolovich, et al. 2002). Dietary antioxidants are the supplements that may delay or reduce the effects of oxidative stress and phenolic compounds are the phytochemicals that are widely present in the plant kingdom exhibiting several bioactivities (King and Young 1999) and can be classified in natural antioxidants that take an important place in our diet which absorb and neutralize free radicals by donating an hydrogen atom from their hydroxyl groups (Boskou, et al. 2006).

Infectious diseases are the primarily threat that account for death worldwide. In the last decades, the clinical efficacy of many synthetic antibiotics is being threated by the emergence of a serious problem which can be defined as multi- drug resistant pathogens (Eldeen, et al. 2005). Multi- drug resistance in both human and plant pathogenic microorganisms has developed due to the indiscriminate usage of commercial antimicrobial drugs that have widely applied in the treatment of infectious diseases. Therefore scientists have tried to discover new antimicrobial substances from various sources including plants. It is known that, now natural products and their derivatives hold more than 50% of all the drugs in clinical usage with one quarter originating from higher plants (Eldeen, et al. 2005).

Turkey has one of the greatest floras in Europe due to its various number of plants also including many endemics (Cetin and Yanikoglu 2006). In this study the purpose was to determine the relative total phenol contents, antioxidant and antimicrobial activities of 42 common plant species from Karaburun/ İzmir. Karaburun has a rich flora which has not been studied by scientific means before. This study also provides an identification of potential, bioactive species that can be used as raw materials for plant derived products in several industries.

CHAPTER 2

LITERATURE REVIEW

2.1. Medicinal Plants

" Let your food be your first medicine" (Hippocrates, 377 BC) was probably the first time that the link was made between nutrition and well-being which emphasizes the importance of functional foods (Carbone 2005). In addition, the practice of medicinal plants is very well known for treating the diseases from ancient times. Even today because of the belief that medicinal plants are safe and effective most of the plant products are being used in local traditional systems of medicine (Dhawan 2003). In developing countries, a report of WHO survey indicates that 80% of the populations rely on mostly traditional medicine for their primary health care needs (Goyal, et al. 2007). Besides, scientific validations of medicinal plants have been ensured by various phytopharmacological studies which evaluate active plant constituents. So today, plants are the important raw materials for pharmacological research and drug developing (Mendonça 2006), and they are also being increasingly used as the complementary or alternative medicine in industrialized countries.

Medicinal plants have considerable importance in international trade and their clinical, pharmaceutical, and economic value is still growing, although this varies widely between countries. Based on current research and financial investments, medicinal plants will, seemingly, continue to play an important role as an health aid. Use of herbal medicines in Asia represents a long history with several applications against various diseases (Draipandiyan, et al. 2006). The practice of traditional medicine is widespread in China, India, Japan, Pakistan, Sri Lanka and Thailand. The countries of the region such as China (30,000 species of higher plants), Indonesia (20,000), India (17,000), Myanmar (14,000), Malaysia (12,000) and Thailand (12,000) have large floras (Ics-Unido. 2006). In China about 40% of the total medicinal consumption depends on traditional medicines. In Thailand, herbal medicines make use of legumes encountered in the *Caesalpiniaceae,* the *Fabaceae*, and the *Mimosaceae*.

The use of medicinal plants like *Eupatorium perfoliatum* in Central America medicinal plants have been widely used (Hoareau, et al. 1999).

However, among the estimated 250,000-400,000 plant species, only 6% have been studied for biological activity, and about 15% have been investigated phytochemically . This shows a need for phyto-pharmacological evaluation of herbal drugs (Goyal, et al. 2007). A vast knowledge of how to use the plants against different illnesses may be taken on a shape in the regions where the use of plants is still of great importance. The medicinal value of plants lies in some chemical substances that produce a definite physiological action on the human body. These phytochemicals are the active constituents that exhibit some biolological activities concerning antioxidant, antimicrobial, antiinflammatory, and anticancer activities, ext. Exploration of the chemical constituents of the plants and pharmacological screening is of great importance which leads for development of novel agents (Goyal, et al. 2007). The most important phytochemicals are alkaloids, flavanoids, tannins and some other phenolic compounds which are abundantly found in plants (Draipandiyan, et al. 2006).

Medicinal plants were the main source of products used to maintain well being until the nineteenth century, when the German chemist Friedrich Wöhler in 1828, attempting to prepare ammonium cyanate from silver cyanide and ammonium chloride, accidentally synthesized urea. This was the first organic synthesis in history and revealed a new area of the synthetic compounds (Mendonça 2006). Today, herbal remedies are back into prominence because of the ineffectiveness of conventional medicines such as antibiotics. The history of modern psychopharmacology is short, and its current concepts are more “pharmaco-centric” than those of most other branches of modern medicine (Husain, et al. 2007). In more recent history, the use of plants as medicines has involved the isolation of active compounds, beginning with the isolation of morphine from opium in the 19th century. Drug discovery from medicinal plants led to isolation of early drugs such as cocaine, codeine, digitoxin, and quinine, in addition to morphine, of which some are still in use (Balunas and Kinghorn 2005). In addition some synthetic medicines has been derived from medicinal herbs are digioxin, aspirin, reserpine, ephedrine, quinine, vincristine, vinblastine, taxol, artemisinin, hypericin and silymarin (Singh 2006).

Rediscovery of the connection between plants and health is responsible for launching a new generation of botanical therapeutics that include plant-derived

pharmaceuticals, multicomponent botanical drugs, dietary supplements, and functional foods. Many of these products will soon complement conventional pharmaceuticals in the treatment, prevention and diagnosis of diseases, while at the same time adding value to agriculture (Raskin, et al. 2002). Today, many plant derived products are being consumed commercially in a rising rate. In Table 2.1 some of the most common botanical diatery supplements are shown.

Table 2.1. Commonly used commercial plant species and their therapeutic useage (Source: Raskin, et al. 2002)

Scientific name	**Common name**	**Active constituents**	**Therapeutic use**
Panax ginseng, L.A.	Ginseng	Ginsenosides, panaxans, sequiterpenes	(Roots) Fatigue and stress, high cholesterol, diabetes,
Ginkgo biloba L.	Ginkgo	Terpene trilactones ginkgolides, flavonol glycosides	(Leaves) Dementia, cognitive decline, mental fatigue
Hypericum perforatum L.	St John's wort	Hyperforin, adhyperforin, hypericin, flavonol glycosides	(Shoots) Mild and moderate, depression, epilepsy
Allium sativum L.	Garlic	Alliins, allicin, ajoens, oligosulfides	(Bulb, oil) Cancer, high cholesterol, diabetes, arteriosclerosis, hypertension, respiratory
Tanacetum parthenium	Feverfew	Sesquiterpene, lactones, canin, artecanin	(Herb) Migraines, inflammation
Hydrastis canadensis L.	Goldenseal	Hydrastine, berberine, canadine	(Rhizome, roots) Diarrhea, respiratory and gastrointestinal infections

2.1.1. Turkish Medicinal Plants

Turkey is very rich in medical and aromatic plants with its large floristic diversity. The estimated number for Turkish flora is 11,000 for specific, and infraspecific taxa of higer plants including 3000 endemic species. Turkey contains 347 species that have commercial values and about 30,000 tons of plants are being exported annually. In Anatolia plants have been commonly used as the source of food, remedy, animal fodder, tinder and some utensils from time immemorial. Although the ethnobotanical experience is being lost with the modernization of society, in some rural areas, people still use traditional medicine for health care (Satıl, et al. 2008, Coskun, et al. 2005). Some important plant species and their ethnobotanical use are shown in Table 2.2.

Table. 2.2. Ethnobotanical use of some Turkish plants

(Source: Sezik, et al. 2001, Tuzlacı and Erol 1999, Tuzlacı and Aymaz 2001)

Plant species	**Ethnobotanical use**
Hypericum perforatum, Urtica dioica, Thymus longicaulis, Salvia tomentosa	Mostly used for the treatment of haemorrhoid, rheumatism, stomach and kidney ailments.
Juniperus oxycedrus	Cold, stomachache
Origanum onites	Stomachache
Teucrium chamaedrys	Goiter
Pictacia terebinthus L. *ssp.*	Diabetes mellitus, decoction, as tea
Alkanna cappadocica	Wound healing, red-colored barks are roasted in butter to obtain ointment and applied on wounds
Brassica oleracea L. *var. capitata*	Ulcer; fresh leaves are ingested
Juniperus oxycedrus L. *ssp.*	Bronchitis;
Quercus libani Olivier	Hemorrhoids
Hypericum perforatum	Wound healing; stomach ache, colitis, intestinal disorders

(Cont. on next page)

Table. 2.2. (cont.) Ethnobotanical use of some Turkish plants

(Source: Sezik, et al. 2001, Tuzlacı, et al. 1999, Tuzlacı, et al. 2001)

Allium cepa L.	Abscess, gastric ulcers
Allium sativum L.	Sunstroke, hemorrhoids, as hypotensive
Urtica dioica L.	Abscess, rheumatic pain, eczema
Teucrium polium L.	Common cold, antipyretic; decoction, as tea for rheumatic pain

Today, developing phytopharmacological industry leads to the examination of some medicinal species for their biological activities in the laboratories and these studies mostly confirm therapetical usage in ethnopharmacy of some species. Table 2.3 summarizes some of the Turkish species examined for their activities and phytochemical groups.

Table 2.3. Scientific evaluation of some Turkish medicinal plants

Scientific name	**Findings of the studies**	**References**
Arbutus unedo	A phytochemical study of the petroleum ether and ethyl acetate extracts of the entire plant of *Arbutus unedo* led to the isolation of a new sterol,	Carcache, et al. 2006
Arbutus unedo	Quercitrin, isoquercitrin, hyperoside and rutin were identified in all leaf samples by means of thin-layer chromatography; the fruits contained only isoquercitrin	Males, et al. 2006
Cistus genus	Flavonoids (quercetin-3-O-methyl ether) was found to be as potent against diabet	Coşkun and Özkan 2005
Capparis sinosa	The methanolic extract of the aerial parts of *Capparis spinosa* yielded the new flavonoid quercetin 3-*O*-w690-a-L-rhamnosyl-60-b-D-glucosylx-b-D-glucoside.	Sharaf, et al. 2004
Hypericum empetrifolium	Antioxidant activity and total phenol assays were performed for three hypericum species. Hypericum empetrifolium exhibited the highest values for both experiments.	Meral, et al . 2004
Lavandula stoechas	Lavender had effective reductive potential, free radical scavenging, superoxide anion radical scavenging, and metal chelating activities at all tested concentrations.	Gülçin, et al. 2004
Pistacia lentiscus	A quantitative determination of a-tocopherol in *Pistacia lentiscus, Pistacia lentiscus* var. chia, and *Pistacia terebinthus*, leaves was established by TLC-densitometry and colorimetry. The highest amount of a-tocopherol was found in *P. lentiscus* var. chia.	Kıvçak and Akay 2005

(Cont. on next page)

Table 2.3. (cont.) Scientific evaluation of some Turkish medicinal plants

Pistacia lentiscus	Total phenol content was determined in a comparison study	Stocker, et al. 2004
Pistacia terebinthus	the most active three fractions in DPPH assay were purified from P.terebinthus to afford a new flavone 60-hydroxyhypolaetin 30-methyl ether .	Topçu, et al. 2007
Pistacia terebinthus	It has a noticable antioxidant activity particularly in the protection of human LDL from oxidation . However the phytopharmacology and phytochemistry of this plant is not known.	Kıvçak and Akay 2005
Quercus infectoria	Quercus infectoria is rich in phenolic acids, flavonoid glycosides, and phenolic volatile oils.	Surveswaran, et al 2007
Solanum nigrum	Antioxidant and total phenol content was determined in a comparison study of 133 Indian plants	Surveswaran, et al. 2007
Teucrium chamaedrys	Strong inhibitory activity was shown by *T. montanum* and *T. chamaedrys* extracts.	Panovska, et al. 2005
Teucrium polium	Tyrosol, caffeic acid, ferulic acid and lutein were identified.	Proestos, et al. 2006
Teucrium polium	Flavanoid and total phenol contents were determined.	Djeridane, et al. 2006
Urtica dioica	*Urtica dioica* had powerful antioxidant activity when compared with standard antioxidants	Gülçin, et al. 2004
Urtica dioica	Flavvonoid content and total phenol content were identified with some other Greek plants	Proestos, et al. 2006
Vitex agnus-castus	Dopaminergic compounds present in *Vitex agnus castus* are clinically the important compounds which improve premenstrual mastodynia and possibly also other symptoms of the premenstrual syndrome.	Wuttke, et al. 2003

2.1.2. Processing of Medicinal Plants

The increasing demand and consumption of medicinal plants induced the large scale production and processing of plant products as row materials for several industries. Processing of medicinal plant products need to follow a standardized quality arrangement. Quality here refers to the product in terms of technical specifications and to the organization of the production process and the continuity of service. The know-how and control of the production process and the coordination of all links are essential for good quality. Monitoring should be done at selected steps in production process (Groot and Roest 2006).

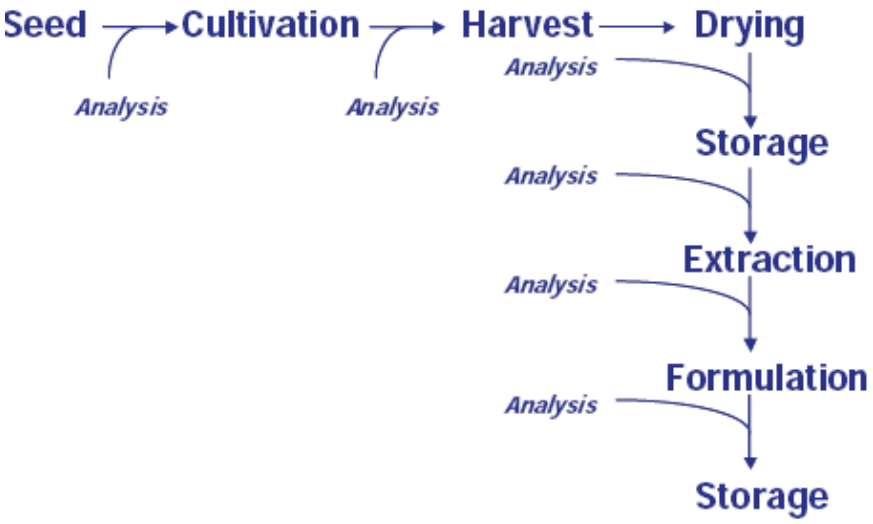

Figure 2.1. Quality control points in the production chain
(Source: Groot and Roest 2006)

2.2. Phytochemicals

The "phyto-" of the word phytochemicals is derived from the Greek word *phyto*, which means plant. Therefore, phytochemicals can be defined as plant chemicals. Phytochemicals are bioactive plant compounds in fruits, vegetables, grains, and other plant foods that play a role of reducing the risk of major chronic diseases. It is estimated that 5000 individual phytochemicals have been identified in fruits, vegetables, and grains, but a large percentage still remain unknown and need to be identified before we can fully understand the health benefits of phytochemicals in whole foods (Liu 2004). There are apperent evidences that bioactive compounds will reduce the risk of many diseases, including chronic diseases such as cardiovascular disease. One example of how bioactive compounds that show how they modify disease risk is illustrated by the large difference in absolute coronary disease mortality rates at a given total cholesterol level observed in the 25-year follow-up of the Seven Countries Study (Kris-Etherton, et al 2004). Epidemiological studies have consistently shown that a high dietary intake of fruits and vegetables as well as whole grains is strongly associated with reduced risk of developing chronic diseases, such as cancer and cardiovascular disease, which are the top 2 causes of death in the United (Liu 2004). Identifying bioactive compounds and seeking their health effects are active areas of scientific surveys. Because of the great number of bioactive compounds and the diversity of likely biological effects, numerous and diverse experimental approaches must be taken to increase our understanding of the biological activities of bioactive compounds. Recognizing the complexity of this biology, sophisticated experimental designs and analytical methodologies must be employed to advance the field. The discovery of novel health effects of bioactive compounds will provide the scientific basis for future efforts to use biotechnology to modify and fortify foods and food components as a means to improve public health (Kris-Etherton, et al. 2004).

Phytochemicals can be classified as carotenoids, phenolics, alkaloids, nitrogen-containing compounds, and organosulfur compounds. The most studied of the phytochemicals are the phenolics and carotenoids (Liu 2004). These groups have also several subgroups and these are demostrated in Figure 2.2.

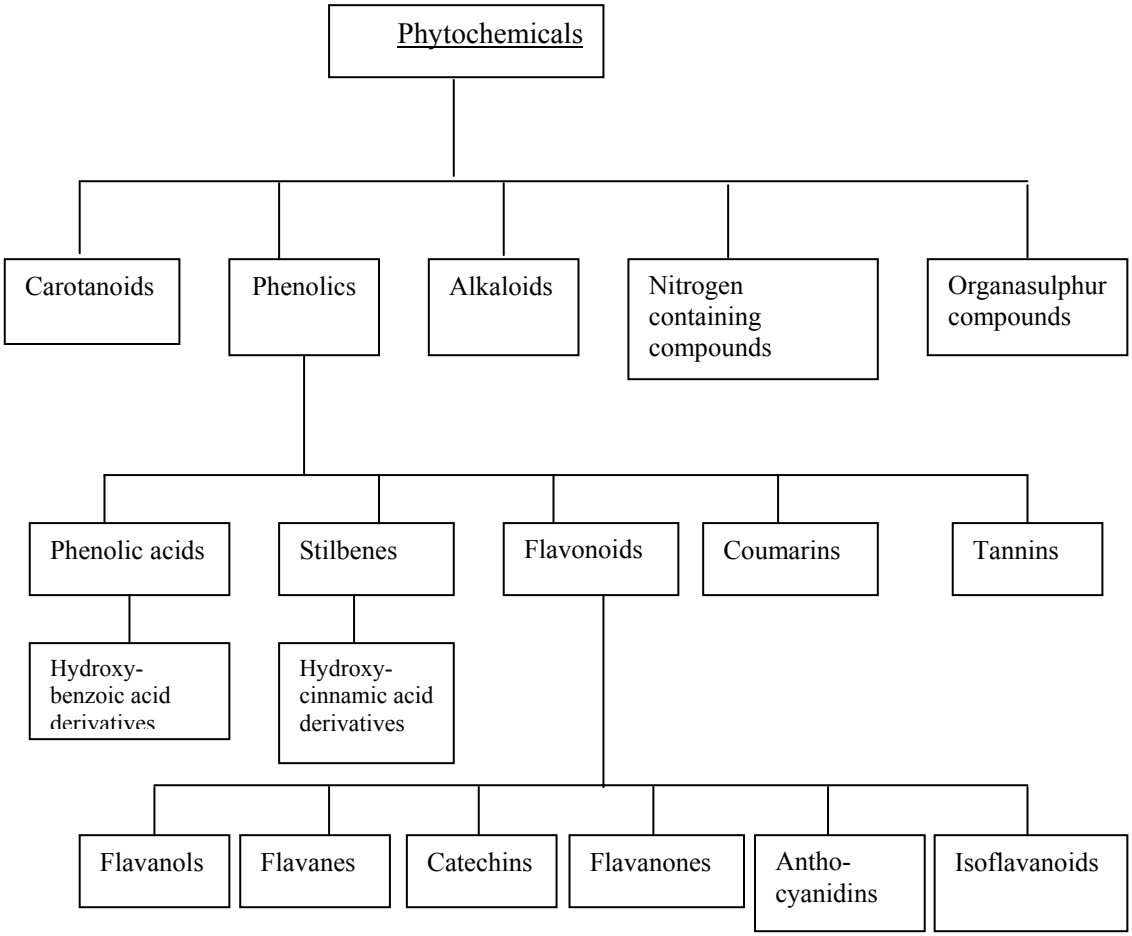

Figure 2.2. Phytochemical groups

(Source: Liu 2004)

2.2.1. Phenolic Constituents in Plants

Among the various pytochemicals as the secondary metabolites of plants, phenolic compounds are the common ones and frequently present in the plant kingdom. Phenolic constituents exhibit several bioactivities such as antimicrobial, antioxidant, antiviral, antiinflammatory. Dietary phenolics that have being researched deeply in the last decades are divided into various subgroups and the major categories of phenolic compounds are flavonoids, phenolic acids, and tannins (King and Young 1999). Some of the other type of phenolics are coumarins, lignans, quinones, and stilbenes (Chai, et al. 2004).

Flavonoids:

Flavonoids are the most important and most studied phenolic phytochemicals that are widely distributed in plants (Chai, et al. 2004). More than 6,400 flavonoid structures were determined in the performed studies (Silva, et al. 2006). Generally they include particular hydroxyl groups with the constitution of ring structures. They have a basic carbon skeleton ($C_6 + C_3 + C_6$). Flavonoids are consist of several subclasses such as; flavones, flavonols, flavanones, flavanonols, chalcones, isoflavonoids, anthocyanins, biflavonoids (Chai, et al. 2004). Flavonoids are basically divided into two groups; anthocyanins and anthoxanthins. Anthocyanins have some colour pigments such as red, blue, and purple. Anthoxanthins possess colorless or white to yellow molecules (flavonols, flavones, isoflavones) (King and Young 1999).

Differences in the generic structure of the heterocycle C ring classify them as flavonols, flavones, flavanols (catechins), flavanones, anthocyanidins, and isoflavonoids (Fig. 2.3). Flavonols (quercetin, kaempferol, and myricetin), flavones (luteolin and apigenin), flavanols (catechin, epicatechin, epigallocatechin, epicatechin gallate, and epigallocatechin gallate), flavanones (naringenin), anthocyanidins, and isoflavonoids (genistein) are common flavonoids in the diet (Liu 2004).

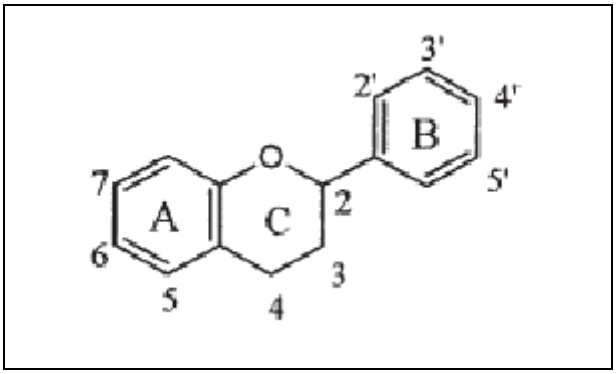

Figure 2.3. The generic structure of flavonoids
(Source: Liu 2004)

Table 2.4. Flavonoids in foods: flavonoid subclasses, compounds, and food sources (Source: Kris-Etherton, et al. 2004)

Subclass	Compounds	Primary food sources
Flavonols	Quercetin, kaempferol, myricetin, isorhamnetin	Onions, apples, teas, berries, olives, bananas, lettuce, plums, red wine
Flavones	Luteolin, apigenin	Apples, celery, celeriac, lemons, parsley, oregano, lettuce, beets
Isoflavones	Genistein, diadzein	Soybeans, legumes
Flavanones	Hesperetin, naringenin, eriodictyol	Oranges, grapefruits, lemons
Anthocyanidins	Cyandin, delphinidin, malvidin, pelargonidin, peonidin, petunidin	Blueberries, raspberries, strawberries, cranberries
Flavan-3-ols	Catechin, gallocatechin, epicatechin, epigallocatechin, epicatechingallate, epigallocatechingallate, theaflavin, theaflavingallate, theaflavindigallate, thearubigins	Green tea, black tea, plums, apples, cranberries
Procyanidins	Polymeric catechins and epicatechins	Cocoa, chocolate, cinnamon, cranberries, pinto beans, kidney beans, hazelnuts, pecans

Flavonoids generally exist as glycosides, nevertheless some of them are found as aglycones. There is an insufficient knowledge about metabolism, extraction and absorption of dietary polyphenols in humans and recovery in the gastrointestinal surface. Furthermore, the hydrolysis of flavonoid glycosides and the reductive metabolism are performed by intestinal microorganisms (Rice-Evans, et al. 1997).

Phenolic acids:

Phenolic acids form another large class of phenolic compounds. Phenolic acids contain two main groups;

1. *Hydroxybenzoic acids* (e.g. gallic acid, *p*-hydroxybenzoic acid, protocatechuic acids, vanillic acids)
2. *Hydroxycinnamic acids* (e.g. ferulic acid, caffeic acid, coumaric acid, chlorogenic acids, cinnamic acids)

Tannins:

Phenolic polymers, commonly known as tannins and they are divided into two general classes:

1. *Hydrolyzable tannins* : They include a central core of polyhydric alcohol such as glucose and hydroxyl groups. They are esterified partially or wholly by gallic acid (gallotannins) or hexahydroxy-diphenic acid (ellagitannins).

2. *Condensed tannins*: They are more common and have more complex structures then the hydrolyzable tannins. They consist of oligomers and polymers of catechins.

In some cases hydrolyzable and condensed tannins are present together in plants, so this kind of tannins can be defined as *complex tannins* (Chai, et al. 2004).

Polyphenolic phytochemicals are ubiquitous in plants, in which they function in various protective roles. A recommended human diet contains significant quantities of polyphenolics, as they have long been assumed to be antioxidants that scavenge excessive, damaging, free radicals arising from normal metabolic processes (Stevenson, et al. 2007). Structural diversity of polyphenolics are a diverse class of plant secondary metabolites. They are characterised structural by the presence of one or more six-carbon aromatic rings and two or more phenolic (i.e.., linked directly to the aromatic ring) hydroxyl groups. Strictly speaking, mono-phenols such as p-coumaric acid are not

polyphenolics , but they share many of their properties and characteristics and are most usefully considered as functional polyphenolics (Stevenson, et al. 2007).

2.3. Extraction

The pharmaceutical definition of extraction may be expressed as the separation of medicinally active portion from plant or animal tissues using selective solvents through standard extraction procedures. Primarily criteria of extraction techniques is separating the soluble and insoluble components and leaving behind only insoluble cellular marc. The extraction products of plants have relatively complex mixtures covering a number of groups of plant metabolites either in liquid form or semi-solid state or after removing the solvent resulting in dried powdered extract**.** Obtaining the therapeutically desired portion of the plant material and the elimination of unwanted material by treatment with a selective solvent is the main purpose of a standardized extraction procedure for medicinal plants. An extract may be further processed through various techniques of fractionation to isolate individual chemical entities such as vincristine, vinblastine, hyoscyamine, hyoscine, pilocarpine, forskolin, codeine, etc., to be used as modern drugs (Ics-Unido 2006). Extraction and characterization of several active phyto-compounds from these green factories have given birth to some high activity profile drugs (Mandal, et al. 2007).

The choice of extraction method, can have an effect on the efficacy of active plant constituents (Shaalan, et al. 2005). The general techniques of extraction of medicinal plants include maceration, infusion, percolation, digestion, decoction, hot continuous extraction (soxhlet), aqueous-alcoholic extraction by fermentation, counter current extraction, microwave assisted extraction, ultrasound extraction (sonication), supercritical fluid extraction (SFE), phytonic extraction (with hydro-flouro-carbon solvents), etc. For the aromatic plants, three types of hydro-distillation techniques (water distillation, steam distillation, steam and water distillation), hydrolytic maceration followed by distillation technique, expression method and enfleurage method (cold fat extraction) may be employed. Some of the latest methods of extraction for aromatic plants include head space trapping technique, solid phase micro-extraction, protoplast extraction technique, micro-distillation, thermo-micro-distillation, and molecular distillation techniques (Ics-Unido 2006). Novel extraction methods including

microwave assisted extraction, supercritical fluid extraction, pressurized solvent extraction have drawn significant research attention in the last decade (Shaalan, et al. 2005). In recent years, the use of microwave for extraction of constituents from plant material has shown tremendous research interest and potential. Conventional techniques for the extraction of active constituents are time and solvent consuming, thermally unsafe and the analysis of numerous constituents in plant material is limited by the extraction step (Shaalan, et al. 2005).

The extraction of essential oil components using solvent at high pressure, or supercritical fluids (SCF), has received much attention in the past several years, especially in food, pharmaceutical and cosmetic industries, because it presents an alternative for conventional processes such as organic solvent extraction and steam distillation (Xiao, et al. 2007). There is also a technique called enzyme assisted extraction. The mechanism for enzyme-assisted extraction is that cell wall degrading enzymes (i.e., glucanases and pectinases) can weaken or break down the cell wall rendering the intracellular materials more accessible for extraction (Li, et al. 2006).

Many valuable natural materials have traditionally been extracted with organic solvents. However, some of the organic solvents are believed to be toxic, and the extraction conditions are often harsh. A simple method using ethanol (a food-grade solvent) instead of methanol for the extraction of phenolic compounds is the preferance frequently in the literature (see Table 2.2) (Li, et al. 2006). The traditional techniques of solvent extraction of plant materials are mostly based on the correct choice of solvents and the use of heat or/and agitation to increase the solubility of the desired compounds and improve the mass transfer. Usually the traditional technique requires longer extraction time thus running a severe risk of thermal degradation for most of the phyto-constituents (Mandal, et al. 2007).

Thus the basic parameters influencing the quality of an extract are: a) the plant part used as starting material, b) the solvent used for extraction, c) the manufacturing process (extraction technology) used with the type of equipment employed, and d) crude-drug: extract ratio (crude drug: extract). The use of the appropriate extraction technology, plant material (nature of the plant material, its origin, degree of processing, moisture content, particle size), manufacturing equipment (type of extraction, filling height, hydrostatic pressure, batch size), extraction method (type of extraction, time of extraction, flow velocity, temperature and pressure) and the solvent (nature of solvent, its concentration and polarity) and good manufacturing practices, will certainly produce

good desired quality of extract. From laboratory scale to pilot scale, all the conditions and parameters, if properly and accurately recorded, one can employ process simulation for successful industrial scale production (Ics-Unido. 2006). In Table 2.2 the basic parameters such as solvent type and concentration, solid liquid ratio and extraction time that have been applied in several studies were summarized. The studies in Table 2.2 cover the screening of some medicinal plants for their antioxidant and antimicrobial properties. It is nearly imposible to optimize extraction parameters for each plant material in screening studies that includes great numbers of plant species. So for screening studies generally a standardized extraction procedure is applied for all samples. Table 2.2 indicates for commonly used parameters in extraction of screening plants. Mostly ethanol and methanol in various concentrations of water are used as solvent in these studies. But ethanol is more advisable because it is much more safer than methanol which has high toxic effects. The solid-liquid ratio is another important parameter in extraction of plant materials and studies indicate that mostly 1/10-50 ratios are used in screening studies.

Table 2.5. Comparison of performed studies related with extraction of phytochemicas

solvent type	solid-liquid ratio (g/ml)	extr. time (min.)	references
methanol	1/3	30	Mosaddik, et al. 2004
methanol	1/20	NA	Chanwitheesuk, et al. 2005
1-)methanol 2-)water+cloroform	NA	180	Tepe, et al. 2006
1)water 2)ethanol	1/20	1-15	Gülçin, et al. 2003
1-)70% acetone 2-)methanol	1-)1/8 2-)1/5	NA	Neergheen, et al. 2005
70% methanol	NA	180	Lee, et al. 2003
80% ethanol	NA	120	Mantle, et al. 2000
aqueous extraction	1/20	10	Vanderjagt, et al. 2002
aqueous extraction	1/10	15	Ljubuncic, et al. 2005
1-)water 2-)60% ethanol	1/8	30	Duffy and Power 2001
1-)methanol 2-)water+chlaroform	NA	360	Sökmen, et al. 1999
1-)methanol 2-)chlaroform	1/10	360	Matkowski, et al. 2006
70% ethanol	1/50	1440	Djeridane, et al. 2006
methanol	1/10	2880	Pourmorad, et al. 2006
80% ethanol	1/10	NA	Boskou, et al. 2006
1-) 90% ethanol 2-)water	NA	NA	Auddy, et al. 2003
water	3/200	30	Katalinic, et al. 2006

2.4. Free Radicals

Oxidation process is the major occurrence that gives rise to free radical formation in food, drugs, and living systems (Pourmorad, et al. 2006). Free radicals and other reactive oxygen species (ROS) are released continously during the essential aerobic metabolism as unwanted metabolic by-products (Mantle, et al. 2000). Structurally unstable free radicals has been defined as a molecular entitiy which retain an unpaired electron and that's the reason of free radicals are mentioned as highly reactive (Madhavi, et al. 1996).

Several facts contribute the formation of free radicals such as environmental pollutants, radiation, chemicals, toxins, deep fried and spicy foods, also physical stress leading to depletion of immune system antioxidants, modifications in gene expression and proteins (Pourmorad, et al. 2006). That's why the free radicals are among the common intracellular DNA modifiers (Ramos, et al. 2003).

Superoxide radical ($O_2^{-\bullet}$), hydroxyl radical ($^{\bullet}OH$) and non-free radical species such as H_2O_2 and singlet oxygen ($^1O_2$) are generated during the oxidative stress and accelerate more than one hundred disorders in humans. Most common radical related diseases are atherosclerosis, arthritis, diabetes, ischemia, central nervous system injury, cancer, AIDS, inflamentation and aging (Pourmorad, et al. 2006, VanderJagt, et al. 2002, Chanwitheesuk, et al. 2005, Chai, et al., 2002). The most reactive forms of active oxygen species are $HO^{\bullet}$ and $^1O_2$.

Comparison of the reactivity: $HO^{\bullet}$ and $^1O_2 > O_2^{\bullet -} > H_2O_2$

Primary factors that contributing prooxidant states in the initiation of chain reactions(Madhavi, et al. 1996):

- Hyperbaric oxygen tension
- Radiation
- Reagents
- Electron transport chain
- Inhibition of antioxidant defence system

2.4.1.Free Radical Chain Reactions

Lipid oxidation process causes complex free radical chain reactions releasing various radicals (Maisuthisakul, et al. 2007). Peroxyradicals and hydroperoxides are the initiators of this chain reaction introduced when unstable free radicals react with molecular oxygen. Free radical chain reaction termed as autoxidation is distinguished in three distinct steps: initiation, propagation and termination.

Initiation :

When an unsaturated lipid contact with oxygen this produces free radicals (Eq. 2.1)

$$RH \rightarrow R^{\bullet} + H^{\bullet} \quad (2.1)$$

$$ROOH \rightarrow RO^{\bullet} + HO^{-} \quad (2.2)$$

$$2ROOH \rightarrow RO^{\bullet} + ROO^{\bullet} + H_2O \quad (2.3)$$

$R^{\bullet}$ = lipid radicals

$RO^{\bullet}$ = alkoxy radicals

$ROO^{\bullet}$ = lipid peroxyradicals

Propagation :

Propagation reactions generate different type of radicals. Previously formed free radicals in the initiation reactions take part in the chain reactions and as a result of consuming of oxygen by lipids new free radical species occurs such as peroxy radicals ($ROO^{\cdot}$) and peroxides (ROOH) .

$$R^{\bullet} + O_2{}^3 \rightarrow ROO^{\bullet} \quad (2.4)$$

$$ROO^{-} + RH \rightarrow ROOH + R^{\bullet} \quad (2.5)$$

ROOH: lipid peroxides

$R^{\bullet}$: lipid radicals

$ROO^{\bullet}$: lipid peroxy radicals

As a result of repated reactions in propagation step, accumulation of hydroperoxides occurs. Lipid peroxy radicals react with other molecules which give rise to lipid hydroperoxides (ROOH) and lipid free radicals $R^{\bullet}$. Lipid hydroperoxides can be also generated enzymatically by the action of lipoxygenase.

Termination:

In the further steps of propagation, the amount of unsaturated lipids (or fatty acids) is reduced and free radicals react with each other, resulting in stable non radical compounds (Madhavi, et al. 1996).

$$R^{\bullet}+R^{\bullet}\rightarrow R\text{-}R \quad (2.6)$$

$$R^{\bullet}+ROO^{\bullet}\rightarrow ROOR \quad (2.7)$$

$$ROO^{\bullet}+ROO^{\bullet}\rightarrow ROOR+O_2 \quad (2.8)$$

In the situation of imbalance between scavenging enzymes and free radical formation, destructive and lethal cellular effects occur (Mantle, et al. 2000). Scavenger systems contain both endogenous defences and diatery antioxidants. Endogenous defences are enzymes such as superoxidedismutase (SOD), catalase (CA), glutathione peroxide (GPX), plus vitamin E, uric acid and serum albumins. Beside these, intaking of dietary antioxidants is quite essential for human health to cope with the degenerative effects of lipid oxidation (Antolovich, et al. 2002). Enzymatic and non-enzymatic antioxidant defence mechanisms convert these life threatening free radicals and reactive oxygen species into non- reactive forms (Dasgupta and De 2007).

2.5. Antioxidants

Antioxidants have several definitions, but the common definition can be expressed as “any substance delay or inhibit oxidation of oxidizable substrate by neutrilizing free radicals” (Antolovich, et al. 2002). Antioxidant activity is an important parameter and it is widely used for characterize and determine the various plant materials such as fruits, vegetables, wine, teas, oils and etc. In the recent years, the

strategy of implementing the diet with antioxidants especially deriving from natural sources, is becoming more and more convincing against oxidative stress damages (Vertuani, et al. 2002). In Table 2.3. some of the natural antioxidant classes are showed. Figure 2.4 shows a general chemical structure of antioxidants.

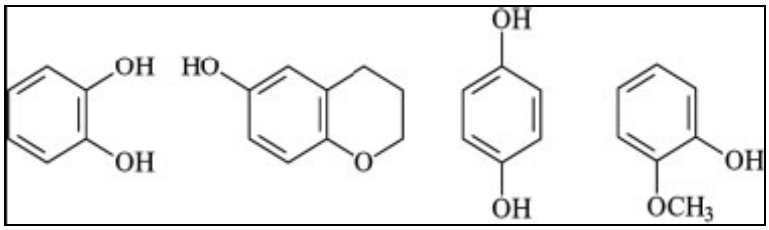

Figure 2.4. Basic chemical structure of antioxidants

(Source: Pokorny 2007**)**

Table 2.6. The most frequently encountered natural antioxidants in plants

(Source: Pokorny 2007)

Antioxidant class	Examples of substances
Phenolic acids	
Hydroxybenzoic acid series	Vanillic acid
Hydroxycinnamic acid series	Ferulic acid, chlorogenic acid
Flavonoids	Quercetin, catechin, rutin
Anthocyanins	Delphinidin
Tannins	Procyanidin, ellagic acid, tannic acid
Lignans	Sesamol
Stilbenes	Resveratrol
Coumarines	*ortho*-Coumarine
Essential oils	*S*-Carvone

Living organisims have their protective systems with a high regenerative ability which protect the structures and the functional molecules of the organisms against hazardous effects of both endogenous radicals and exogeneous radicals generated as a

result of normal metabolic processes. The intracellular mechanisms for suppressing by products of aerobic metabolism include enzymatic mechanisms such as glutathine-peroxidase- glutathione system, superoxide dismutase (SOD) and the catalase (CA) (Matthias, et al.). Besides, the biological systems use different antioxidant sources such as, some large molecules (albumin,ceruloplasmin,ferritin,other proteins), and small molecules (ascorbic acid, glutathione, uric acid ,tocopherol, carotenoids, (poly)phenols) and some hormones (estrogen, angiotensin, melatonin, etc.) (Prior, et al. 2005). Under normal conditions natural protective systems of organisms can cope with the radicals and keep the system in the state of equilibrium by controlling the harmful effects of them, but in some cases this equilibrium may be disturbed by exogeneous factors. The increased accumulation of highly reactive compounds is called oxidative stress (Matthias, et al.). Clasically oxidative stres is described as an imbalance between generation and elimination of ROS (Reactive Oxygen Species) and RNS (Reactive Nitrogen Species) thus goes with many critical diseases and aging (Emerit, et al. 2004).

2.5.1. Antioxidative Mechanism of Action

Individual antioxidants may, in some cases, act by multiple mechanisms depending on the reaction system. Furthermore, antioxidants may respond in a different manner to different radical or oxidant sources due to their different characteristics (Prior, et al. 2005). In that manner antioxidants can be classified in two general groups as primary (chain breaking) antioxidants which delay or inhibit the initiation step by reacting with a lipid radical or inhibit the propagation step by reacting with peroxyl or alkoxyl radicals and as secondary (preventative) antioxidants that retard the rate of oxidation. Antioxidants also divided into two groups according to their origin as natural (e.g. tocopherols, ascorbic acids) and synthetic antioxidants [e.g.,BHT (butylhydroxytoluene), BHA (butylhydroxyanisole)] (Antolovich et al. 2002).

Antioxidants can deactivate radicals by two major mechanisms, HAT (Hydrogen Atom Transfer) and SET (Single Electron Transfer). Antioxidants with the HAT mechanism quench free radicals by hydrogen donation and with SET mechanism antioxidants transfer one electron to reduce any compound, including metals, carbonyls, and radicals (Prior, et al. 2005). These mechanisims are shown with the equations below (Antolovich, et al. 2002).

$$L^{\bullet} + AH \rightarrow LH + A^{\bullet} \quad (2.9)$$

$$LOO^{\bullet} + AH \rightarrow A^{\bullet} + LOOH \quad (2.10)$$

$$LO^{\bullet} + AH \rightarrow A^{\bullet}LOH \quad (2.11)$$

$$LOO^{\bullet} + A^{\bullet} \rightarrow LOOA \quad (2.12)$$

$$LO^{\bullet} + A^{\bullet} \rightarrow LOA \quad (2.13)$$

2.5.2. Antioxidant Activity of Phytochemicals

The primary constituents of phytochemicals that have the ability of contributing total antioxidant capacity of plants are the polyphenols, carotenoids, and traditional antioxidant vitamins such as vitamin C and E (Lako, et al. 2007). Polyphenols are widely present in plant kingdom and possessing significant bioactivities just like the antioxidant activity by adsorbing and neutralizing free radicals (Djeridane, et al. 2006). Polyphenols can be classified in natural antioxidants and take an important place in our diet (Boskou, et al. 2006). Polyphenols are among the most efficient antioxidant molecules owing to the ability of stabilizing and delocalizing the unpaired electron of free radicals by donating an hydrogen atom from their hydroxyl groups. There are many constituents of phenolics retaining potential antioxidant properties such as preventing agents against some critical diseases, independently or in synergetic action (Rice-Evans, et al. 1997, Villano et al. 2004). Among the phenolic compounds , bioflavonoids have important antioxidant activity because of their natural origin and importance as efficient free radical scavengers (Katalinic, et al. 2006, Heim, et al. 2002).

Main function of antioxidants on free radicals is, distrupting the free radical chain reaction or decomposing the lipid peroxides formed into stable end products (Madhavi, et al. 1996). Antioxidants have two basic groups related with their action mechanisms. These two groups are primary and secondary antioxidants. Primary antioxidants help delay or inhibit lipid oxidation as free radical scavengers by donating hydrogen atoms or electrons so more stable products can be achived. Secondary antioxidant activities include several mechanisms such as binding of metal ions, scavenging oxgen, converting hydroperoxides to non-radical species, absorbing UV

radiation or deactivating singlet oxygen and decreasing localized oxygen concentrations (Maisuthisakul, et al. 2007, Tepe, et al. 2006).

For the exact and effective usage of phytochemical antioxidants, lipid oxidation, the action mechanism of antioxidants and some other properties such as synergism and degradation should be known well (Madhavi, et al. 1996).

2.5.3. Methods for Determination of Antioxidant Activity

Methods utilizing HAT reaction mechanisms

ORAC Method: measures antioxidant inhibition of peroxyl radical induced oxidations and thus reflects classical radical chain breaking antioxidant activity by H atom transfer. In the basic assay, the peroxyl radical reacts with a fluorescent probe to form a nonfluorescent product, which can be quantitated easily by fluorescence.

TRAP Method: This method monitors the ability of antioxidant compounds to interfere with the reaction between peroxyl radicals generated by AAPH or ABAP [2,2'- azobis(2-amidinopropane) dihydrochloride] and a target probe.

Chemiluminescence (CL) Method: The fundamental chemistry of CL assays is based on the reaction of radical oxidants with marker compounds to produce excited state species that emit chemiluminescence (chemically induced light). Compounds that react with the initiating radicals inhibit the light production. The most widely used marker is luminol that have extensively used to study radical reactions and is acceptable when single oxidants are being measured (Prior, et al. 2005).

PCL (Photochemiluminescence) Assay: The assay involves the photochemical generation of superoxide $O_2^{\cdot -}$ free radical combined with CL (chemiluminescence) detection. The assay is initiated by optical excitation of a photosensitizer (S), resulting in the generation of the superoxide radical anion.

$$S + h\upsilon + O_2 \rightarrow [\, S^{*}O_2 \,] \rightarrow S^{\bullet +} + O_2^{\bullet -} \qquad (2.14)$$

LDL (Low–Density Lipoprotein) Oxidation: LDL is isolated fresh from blood samples, oxidation is initiated by Cu(II) or AAPH, and peroxidation of the lipid components is followed at 234 nm for conjugated dienes or by peroxide values for lipid hydroperoxides (Prior et al. 2005).

Methods utilizing SET reaction mechanisms

FRAP (Ferric Reducing Antioxidant Power): The reaction measures reduction of ferric to a colored product. The reaction detects compounds with redox potentials of <0.7 V, so FRAP is a reasonable screen for the ability to maintain redox status in cells or tissues.Reducing power appears to be related to the degree of hydroxylation.

Methods utilizing both SET and HAT mechanisms

TEAC or Other ABTS Assays: This method is based on the scavenging ability of antioxidants to the long-life radical anion $ABTS^{\cdot+}$. In this assay, ABTS is oxidized by peroxyl radicals or other oxidants to its radical cation, $ABTS^{\cdot+}$, which is intensely colored, and antioxidant capacity is measured as the ability of test compounds to decrease the color reacting directly with the $ABTS^{\cdot+}$ radical .Results of the compounds are expressed relative to Trolox.

DPPH Assay: DPPH radical is commercially available and does not have to be generated before assay like $ABTS^{\cdot+}$. This assay is based on the measurement of the reducing ability of antioxidants toward DPPH radical by measuring the decrease of its absorbance (Prior, et al. 2005).

The effects of the oxidative stress may be delayed or reduced by taking dietery supplements (Villeponteau, et al. 2000). It is important to determine the antioxidant capacities of dietary antioxidants. Various kinds of methods are being used for measuring antioxidant capacity of substances such as physical, chemical and biochemical generator systems. Most of these methods have quite time consuming procedures up to several hours for a single sample and many substances contain both water-soluble and lipid- soluble antioxidants. However most of the methods have a

single measuring principle that determines only one of the two substance classes (Matthias, et al.)

2.6. Antimicrobial Agents

Antimicrobial agents are the important chemicals that are widely used in modern medical practice thanks to their disease treatment features by eliminating or killing the infecting microorganisms. There are a various number of antimicrobial agents currently available. When selecting for a particular antimicrobial agent, its selective toxicity must be evaluated. Because the antimicrobial agent is desired to exhibit greater toxicity to the infecting pathogens then to the host organism (Atlas, et al. 1995).

Antibiotics are the biochemicals produced by microorganisms from organic chemicals and many antibiotics in current medical use are chemically modified forms of microbial biosynthetic products (Atlas, et al. 1995). Each class of antimicrobial agents represents specific interraction with a particular microorganism by exhibiting a uniqe mode of action (Stephen, et al. 2005). These mechanisms of action depends on the type of microorganism under consideration and mainly related to the bacterial cell structure and the target sites of the microorganism (Mendonça, et al. 2006). Outermost structures of gram positive and negative bacteria possesing some main differences which affects the mechanisms of antimicrobial resistance (Holley and Patel 2005) (See figure 2.5 and 2.6).

2.6.1. Structural Features of Gram Negative Bacteria

The cell wall of gram negative bacteria has a greater complexity of the double membrane which contributes the resistance against antimicrobial agents. The components of the gram negative bacteria cell envelope (see Figure 2.5) are:

- Porins
- Lipopolysaccarides
- Lipoproteins
- Peptidoglycan layer
- Periplasmic space

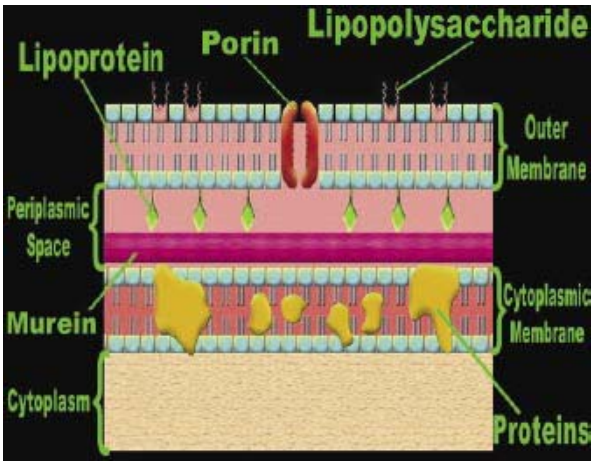

Figure 2.5. Cell wall of gram-negative bacteria
(Source: Stephen, et al. 2005)

Cytoplasmic membrane: The cell is surrounded by the cytoplasmic membrane which contains some proteins and phospholipids. Most of these enzymes are the functional proteins that are associated with cellular metabolism.

Cytoplasm: The cytoplasm contains some organelles, and internal structures.

2.6.2. Structural Features Gram Positive Bacteria

Cell wall : It has a less complex structure with two main components

- Teichoic acids: extended polymers in the peptidoglycan layer
- Peptidoglycan layer: it is much more thicker than that of gram negative bacteria

Cytoplasm: It is similar to that of gram negative bacteria

Structural features of microorganisms contribute the mechanism of resistance (see Figure 2.6) but metabolic diffrences may also be important in the resistance (Holley and Patel 2005). Another key point for the modes of action of antimicrobial agents is the initial electrostatic attraction between cationic agent and the negatively charged components on the outer membrane and leads to the disturbance of the outer membrane structure by permeabilizing it against the other molecules and antimicrobial agents.

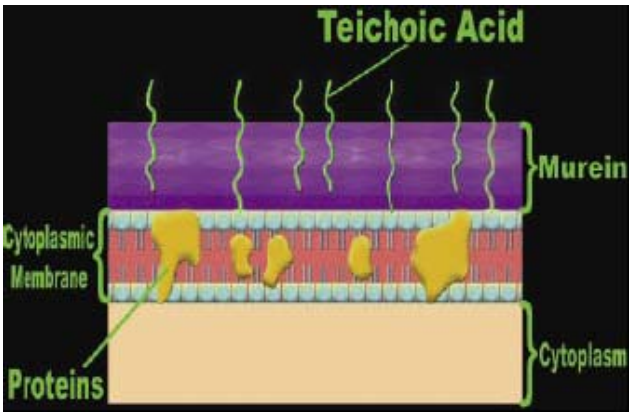

Figure 2.6. Cell wall of gram-positive bacteria
(Source: Stephen, et al. 2005)

2.6.3. Modes of Antimicrobial Action

Antimicrobial agents have different antimicrobial activities that allow them to interfere with cell wall synthesis, inhibit protein synthesis, interfere with nucleic acid synthesis or inhibit a metabolic pathway (See figure 2.7).

Interference with cell wall synthesis: Some antimicrobial agents are responsible for blocking the cell wall synthesis by preventing the peptidoglycan layer synthesis.

Interference with the cytoplasmic membrane : These type of antimicrobial agents play role in distruption and destabilization of the cytoplasmic membrane.

Interference with protein synthesis by binding ribosomal subunits: Antimicrobial agents may bind to the ribosomal subunits including 30S and 50S also bind to 70S initiation complex. They cause protein chain termination and inhibition of protein synthesis.

Interference with DNA synthesis by blocking the enzyme DNA gyrase: Antimicrobial agents may bind to the enzyme gyrase which is responsible for winding and unwinding DNA during the replication that cause releasing of broken DNA strand into cell and leads to cell death (Stephen, et al. 2005).

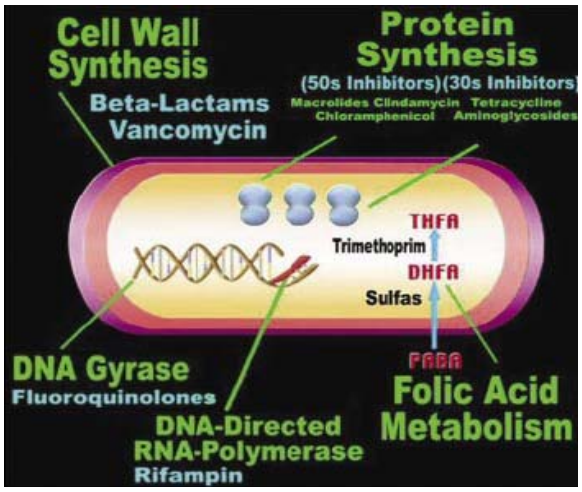

Figure 2.7. Target sites of some antimicrobial agents

(Source: Stephen, et al. 2005)

2.7. Resistance to Antimicrobial Agents

The continous emergence of multiple- drug resistant microbes is a serious problem that today's health care practitioners cope with (Cloutier, et al. 1995). The widespread and sometimes inappropriate use of antimicrobials are the main factors that give rise evolution of antimicrobial resistant bacterial species (Lowy, et al. 2003). The evolution of bacteria toward antimicrobial drug resistance increased rapidly in the last 60 years including the pathogenic species for humans (Courvalin, et al. 2005). So today it is a need to search and discover new, effective antimicrobials by supplying them with novel mechanisms of action and new targets to act on (Cloutier, et al. 1995).

A various number of reasons induce the emergence of antimicrobial resistant bacteria , but the common factors are the genetic mutation or transfer in housekeeping structural or regulatory genes. The resistance to antimicrobial drugs may also inherent to bacteria (Patel and Crank 2005, Courvalin, et al. 2005). The genetic mutations affecting the resistance of bacteria include single point mutations and also multiple step mutations, and the role of genetic transfere in resistance occurs by the plasmid and transposons (Patel, et al. 2005). These plasmids are the extrachromosomal circular DNA pieces that only capable to contain limited number of intact genes but it is important to emphasize that many copies of plasmids can be shared among different bacteria (Cloutier, et al. 1995).

The mechanisms of transfering DNA sequences

Acquired antimicrobial resistance by transfering the DNA has several possible mechanisms. These mechanisms are mostly include transformation, transduction and conjugation mechanisms.

Transformation: The naked DNA is released from one organism and then integrate into the genetic material of another organism by transformation.

Transduction: Bacteriophages are responsible for transduction by DNA sequences transfering from one organism to another.

Conjugation: DNA is transfered by a sex pilus between two organisms (Dargatz et al. 2002).

There are several mechanisms of antimicrobial resistance which are also depend on the type of organism. The basic mechanisms are mentioned below:

Production of enzymes for inactivation of the antibiotic: Beta- lactames, aminoglycoside-modifying enzymes and chloromphenical acetyl transferase are some of the enzymes that provides resistant to the action of antimicrobial drugs.

Decreased antibiotic uptake reduced cell wall permeability to the drug: by alteration of porins in gram-negative bacteria. Altered porin channels in the outer membrane no longer allow the entrance and passage of antibiotic molecules into the cell. Mutations in the porin genes cause the reduced expression or activity of these channels

Altered target receptor and altered biochemical metabolic pathway to bypass the effect of the drug: Mutations in some targets such as PBPSs, ribosomal RNA, DNA gyrase and topoisomerase IV confer antimicrobial resistance.

Active drug export (Efflux pumps): This mechanism is common in both gram positive and gram negative bacteria. Active efflux of antibiotics is mediated by transmembrane

proteins inserted in the cytoplasmic membrane of gram positive and in the outer membrane and periplasm of gram negative organisms (Patel and Crank 2005, Dargatz, et al. 2002, Stephen, et al. 2005, Cloutier, et al. 1995).

2.8. Antimicrobial Activities of Plant Extracts

Even before the discovery of the existance of microbes, it was a common belief that certain plants contained potential healing features which we can characterize them today as antimicrobial principles (Mendonça, et al. 2006). Although historically plants have provided a good source of anti-infective agents in the fight against microbial infections, by the advent of antibiotics in the 1950's the use of plant derivatives as antimicrobials has been ignored (Cowan, et al. 1999, Iwu, et al. 1999). But increased resistance to antibiotics gave rise to demand on finding and developing new antimicrobial agents by focusing on plant derived active principles (Sousa, et al. 2006). Following the industrial revolution, alternative strategies including the emergence of plant derived drugs based on the isolation of plants active constituents and scientific progressing of plant extracts (Iwu, et al. 1999). Several scientific studies have also indicated the importance of new bioactive phytochemicals against multi-drug resistant bacteria (Mendonça, et al. 2006) which are pure molecules and also some of them exhibit much more effective pharmacological activities than their synthetic alternatives (Iwu et al. 1999) Laboratories of the world have documented literally thousands of phytopharmaceutical agents that have important inhibitory effects on all types of microorganisms in vitro (Mendonça, et al. 2006).

Infectious diseases cause approximately one half of all deaths in tropical countries. Phytomedicines derived from natural plant extracts and their intrinsic active principles have shown great promise in the treatment of important infectious diseases including AIDS infections. They act on infections while simultaneously reducing many of the side effects related to the usage of synthetic antimicrobials (Iwu, et al. 1999). The rise in antibiotic resistant microorganisms is also a notable fact. In that manner, the continuous increase in medicinal plant consumption is not an unexpected situation. Some of the Turkish plant species having antimicrobial effects are listed below in Table 2.7.

Table 2.7. Some Turkish medicinal plants with antimicrobial avtivities.

Helichrysum species	contain secondary metabolites of alkaloids, flavanoids, and tannins with antibacterial properties	Dekker, et al. 1983
Campanula Lyrata and *Abies nordmanniana subsp. bornmuelleriana*	were found to be effective on some bacteria	Benli, et al. 2008
Verbascum eriocarpum (flower) , *Stachys cretica* subsp. *Anatolica* (leaf and flower), *Heracleum paphlagonicum* (leaf),	it was demostrated that these species are effective on some bacteria species	Benli, et al. 2007
the seed *of Pimpinella anisum*, and the bark of *Cinnamomum cassia*, the seed of *Juniperus oxycedrus* and the root of *Glycyrrhiza glabra*	showed various antimicrobial effects	Ateş and Ertuğrul 2003
Origanum L.	were found to be effective against all tested microorganisms used in this study in different level,	Dulger, et al. 2005
Quercus infectoria	contains high amounts of tannic acids such as gallic and egallic acids Its tannins are also included in the hydrolyzable tannin groups and consist of polygallol and mdigallolyl derivatives of glucose or polysaccharides	Dıgrak, et al. 1999
Salvia aucheeri	It has externally been used as an antiseptic (especially in the nose and ear), antiflatulent, and stimulant	Dıgrak, et al. 1999
S.cretica	dyspeptic complaint	Sarac and Uğur 2007
T.chamaedrys	appetizing, relief of gastric pain, stomach diseases, diabetes, abdominal pain, dierrhea	Sarac and Uğur 2007
T. polium	appetizing , gastric pain, internal diseases, bronchitis	Sarac and Uğur 2007
Hypericum scabrum and Perganum harmala	showed strong antimicrobial activities against various bacteria	Sokmen, et al. 1999

2.8.1. Mechanisms of Action of Antimicrobial Agents

Phytochemicals that have antimicrobial effects can be divided into several subgroups but the most common ones are represented below.

Simple phenols and phenolic acids

These are the simplest bioactive phytochemicals that have a single substituted phenolic ring. It is thought that their relative toxicity to microorganims is associated with the site(s) and number of hydroxyl groups they have. Also it was shown that increased hydroxylation results in increased toxicity. In addition some authors have observed that more highly oxidized phenols are more inhibitory.

The main mechanism of action of phenolic compounds thought to be related with their toxicity to microorganisms that cause enzyme inhibition through a reaction between oxidized compounds and sulfhydryl groups or by other nonspecific interractions of the proteins and oxidized compounds.

Quinones: Quinones are an important group of phytochemicals that may complex irreversably with nucleophilic aminoacids in proteins. This action leads to inactivation of proteins and loss of funtion. That is why the potential range of quinone antimicrobial effects is great. Their mechanisms of action associate with some targets in the microbial cell including the surface-exposed adhesins, cell wall polypeptides and membrane bound enzymes.

Flavones, flavonoids, and flavonols: They are accepted as the effected antimcrobials against a wide array of microorganis. Their activity is thought to be responsible for interractions with extracellular and soluble proteins also for ability to complex with bacterial cell walls. It was determined that more lipophilic flavonoids may also damage microbial membrane.

Tannins: They contain two subgroups as hydrolyzable and condensed tannins. Their ability to inactivate microbial adhesins, enzymes cell envelope transport proteins is thought to be form their mode of antimicrobial action.

Terpenoids and Essential Oils

These are the secondary metabolites consist of isoprene structure and called terpenes. They have a chemical structure of $C_{10}H_{16}$ and they are characterized as diterpenes, triterpenes and tetraterpenes, also they can take place as hemiterpenes, and sesquiterpenes. Their mode of antimicrobial action is not fully obvious but they are thought to be involved in membrane disruption by the lipophilic compounds (Cowan, et al. 2005). Many of the terpenes exhibit antimicrobial action on a wide range of microorganisms including the gram negative and positive bacteria. They are generally known with their toxic effects on membrane structure and function of microorganisms owing to their lipophilic character that results in membrane expansion, increased membrane fludity and permeability, disturbance of membrane proteins, inhibition of respiration, and alteration of ion transport process (Trombetta, et al. 2005).

Alkaloids

They are the phytochemicals that composed of heterocyclic nitrogen compounds. Morfine is a quite important compound as the first medically useful example of an alkaloid which was isolated in 1805 from the opium popy *Papaver samniforum* (Balunas and Kinghorn 2005). Alkaloids have mechanism of action particularly with the ones have highly aromatic planar quaternary structures such as berberine and harmane is attributed to their ability to intercalate with DNA (Cowan, et al. 2005).

Consequently, plants are the green factories covering thousands of components which have therapeutical potentials. This means that, there is a need for screening plants therapeutically especially for their antimicrobial activities with high througput systems to decrease the time consumed. Several AST (Antimicrobial Susceptibility Tests) are being applied in that manner.

2.9. AST (Antimicrobial Susceptibility Tests)

It is known that, now natural products and their derivatives hold more than 50% of all the drugs in clinical usage with one quarter originating from higher plants . Plant extracts are important materials that have potential antimicrobial agents that confer an antimicrobial defence against microbes in their own environment (Eldeen, et al. 2005). However it is quite difficult to determine the appropriate plant species due to their enormous numbers in plant kingdom. So screening methods for AST are of great important in order to detect phytochemical antimicrobial agents. The currently available screening methods for detection of antimicrobial activity of natural products are classified in three general groups, including bioautographic, diffusion, and dilution methods. The bioautographic and diffusion methods can be described as qualitative techniques by which only the presence or absence of substances with antimicrobial activity can be detected. On the contrary dilution methods are known as quantitive assays since they determine the minimal inhibitory concentration (Valgas, et al. 2007).

There has been much research interest in agar-based testing of disk-diffusion method, and commonly used owing to its relative simplicity and the lack of need for specialized equipment (Scorzoni, et al. 2005). Antimicrobial susceptibility discs are absorbent, paper discs that are impregnated with a specific antimicrobial agent and are usually marked with the drug concentration and the disk code. The test procedure is based on the Kirby-Bauer method. Discs are placed on the surface of an agar plate that is inoculated with the organism to be tested then incubated overnight. Clinically the in-vitro antimicrobial susceptibility is used to determine antimicrobial agent of choice whenever the susceptibility of a bacterial pathogen is unpredictable (Hansen, et al. 1996). The liquid-dilution method also allows detecting of whether a compound or extract has antimicrobial action at a particular concentration. The serial dilution test was reported to give the most reproducible results on the minimal inhibitory concentration (MIC) and was recommended as general standard methodology for the testing of natural products **(**Burrows, et al. 1993, Hansen, et al. 1996). The MIC generally is considered the most basic laboratory measurement of the activity of an antimicrobial agent against an organism. It is defined as the lowest concentration of an antimicrobial agent that is required to inhibit the growth of a particular organism in a well-standardized in vitro susceptibility test. The conventional technique for measuring the MIC involves

exposing the test organism to a series of two-fold dilutions of the antimicrobial agent in a suitable culture media (eg, broth or agar formats) (Jorgensen, et al. 2004). MIC information is quantitative rather than qualitative, it may be useful for dosage determinations as well as antibiotic selection (Burrows, et al. 1993). Determinations of MIC are influenced by numerous variables, including the composition of the test medium, the size of the bacterial inoculum, and the duration of incubation. Also the in-vitro test conditions cannot encompass all factors that can have an influence on in-vivo antimicrobial activity (Burrows, et al.1993).

CHAPTER 3

OBJECTIVES

The primarily aim of this study was to find out new potential plant species that have important biological activities such as antioxidant and antimicrobial activities. In order to determine these activities a comprehensive screening study was carried out for 42 plant species collected from Karaburun region of İzmir. The main goals in this study conceived as follows:

1. Extraction of plant species with a standardized extraction process.

Evaluation of extraction yields for each plant material.

2. Screening for total phenol content which is a biologically active, important group of phytochemicals.

Searching for the correlation between total phenol content and biological activities (antioxidant, antimicrobial).

3. Screening for antioxidant activities concerning lipid soluble and water soluble antioxidant capacities of plant species
4. Screening for antibacterial activities of plant species

4.1. Determination of minimum inhibition concentration of species exhibiting strong antibacterial activities.

CHAPTER 4

EXPERIMENTAL

4.1.Materials

4.1.1. Chemicals

For the assays, DMSO (Dimethyl sulfoxide-99.5%) was obtained from Amersco and gallic acid was purchased from Merck. Methanol (99.7%) and ethanol absolute-chromasol (99.8%) were obtained from Riedel. Folin-ciocalteu reagent was obtained from Sigma, sodium carbonate anhydrus (99.5%) was obtained from Fluka and for the photochem analysis ACW and ACL kits were purchased from Analytik Jena AG. Various growth medias (broth and agar) were used to ensure the cultivation of the microorganisms. Bacto Agar (214010) was obtained from Sinerji, Nutrient Agar (70116) and Nutrient Broth (70122) were purchased from Fluka. Mueller –Hinton Broth (A3751) was purchased from Applichem. Bacto Peptone (211677) was used to dilute the microbial cultures and obtained from Sinergi. Gentamicin and penicillin were used for comparison to evaluate the antimicrobial activities of plant extracts in microdilution assays. Gentamicin (15710) was purchased from Invitrogen and penicillin (Iecilline flakon 400.000 IU) was obtained from a pharmacy. Besides, penicillin G (CTOO43B), gentamicin (CTOO24B), and vancomycin (CTOO58B) are the antibiotic discs used in the disc diffusion assays and purchased from OXOID. Glycerol (15524) was used to prepare the stock cultures and store them in –80 ^{o}C (Revco). Finally INT (Iodonitrotetrazolium chloride) was used as a dye in 96 well plates for visual identification of MICs (Minimum Inhibition Concentrations).

4.1.2. Instuments and Equipments

In order to carry out the microdilution test for MIC values, an instrument called Varioskan (microplate reader) was used and it was purchased from Thermo. Microtiter 96 Well plates were obtained from Thermo. These sterile microplates have clear, flat

bottom and polystyrene properties. Photochem (Analytik Jena) instrument was used to determine the antioxidant capacities of extracts, and UV spectrophotometer (Perkin Elmer) was used for total phenol determination tests.

4.2. Methods

4.2.1. Extraction Procedure

Preparation of plant extracts

Plant species used in this study were collected from Karaburun region in İzmir/Turkey. They were collected in spring (May) and properly prepared for drying process in the same day. Besides, a pattern for each species was taken in order to make a voucher specimen by pressing and drying plant species that enables for long period storing. These species were identified for their scientific names in Ege Foresty Research Institute. All plant species were dried in the same conditions (at room temperature, in a dark and clean place).

A standardized solvent extraction protocol was used for all plant materials. The air dried plant materials were ground in a blender with a particular size to ensure the plant powders in identical size. 10 g of each plant powder was extracted for 2 hrs with 200 ml of 80% (v/v) aqueous ethanol at 35 ^{0}C by a thermo-shaker which is fixed to 180 rpm. Then the samples were centrifuged at 5000 rpm and the supernated parts of the samples were evaporated with a rotary evaporator to remove of the ethanol under reduced pressure at 35^{0}C. The remaining aqueous solutions were lyophilized and the percent (w/w) extraction yields of plant materials were calculated. The crude extracts were kept in refrigerator in glass bottles until the further experiments.

4.2.2. Determination of Relative Antioxidant Activities of Plant species

PCL (Photochemiluminescence) assay was determined by Popov and Lewin (Popov, et al. 1999), was commercialized by Analytik Jena AG (Jena Germany), and is sold as a complete system under the same name PHOTOCHEM. PCL (Photochemiluminescence) method was used to determine the relative antioxidant

activities of plant extracts. Antioxidant capacity of water soluble (ACW) and antioxidant capacity of lipid soluble (ACL) compounds were detected by using a single system on photochem.

4.2.2.1. Mechanism of PCL

In the PCL assay the photochemical generation of free radicals is combined with a sensitive detection method using chemiluminescence (Vertuani, et al. 2004). The reaction is induced by optical excitation (hυ) of the photosensitiser S which results in the generation of the superoxide radical $O^{\bullet}_2{}^-$ which is one of the most dangerous ROS , also accuring in the human body (Prior, et al. 2005).

$$S + h\upsilon + O_2 \rightarrow [\, S^{*}O_2 \,] \rightarrow S^{\bullet +} + O_2{}^{\bullet -} \qquad (4.1)$$

There are two basic kinds of radicals present in the PCL measuring system ; $O_2{}^{\bullet -}$ and luminol radicals (Prior, et al. 2005). Luminol acts as photosensitiser as well as oxygen radical detection reagent (Vertuani, et al. 2002). The antioxidants can be quantified from the changes of the measurement signal caused by them. The antioxidant capacity thus determined is referenced to equivalent concentration units of the standards used (e.g., ascorbic acid, and trolox as a tocopherol equivalent) (Margrit and Tirok 2000).

Standard kits provided by the manufacturer are used to measure hydrophilic and lipophilic antioxidant activity (ACW and ACL kits respectively). Calibrations and measurements for ACW (antioxidant capacity of water soluble compounds) are based on the difference in the lag time between sample and the blank. Measurements for the ACL (antioxidant capacity of lipid soluble compounds) are based on the inhibition of the area under curve (AUC) of the blank by sample (Harrison and Were 2007).

Measuring Kits Principles

The ACW and ACL kits provided by the manufacturer are used to measure hydrophilic and lipophilic antioxidant capacity, respectively, of biological samples (Prior, et al. 2005). Calibration and measurements for ACW are based on the difference in lag time between sample and blank. The ACL calibration and measurements were

based on inhibition (Harrison, et al. 2007). Ascorbic acid and trolox are typically used as calibration reagents for hydrophilic and lipophilic antioxidant capacity respectively (Prior, et al. 2005).

The hydrophilic antioxidant capacity is assayed by means of lag phase (L) in seconds.

$$L = Lo - L_1 \quad (4.2)$$

Where Lo and L_1 are the respective parameters of the blank and sample.

The lipophilic antioxidant capacity is assayed by the degree of PCL inhibition (I), according to the calculation.

$$I = 1 - S/So \quad (4.3)$$

Where So is the integral under the blank curve ans S is the integral under the sample curve.

4.2.2.2. Sample Preparation for PCL

In water soluble fraction antioxidants such as flavonoids, ascorbic, aminoacid, etc., are detected while in the lipid soluble fraction tocopherols, tocotrienols, carotenoids, etc. These antioxidant capacities of fractions depend on their polarities and together give the integral antioxidant capacity as the sum of the separated values for ACW and ACL (Vertuani, et al. 2002).

4.2.2.2.1. Sample Preparation for ACW

Crude plant extracts were firstly dissolved in DMSO as the presolution of 1g extract in 20 ml DMSO. Samples were diluted with deionized water for ACW measurements to keep the concentrations in the calibration range. In the study, measurements were conducted with the standard ACW kit (Analytik Jena) : 1.5 ml of reagent 1 (solvent), 1 ml reagent 2 (water buffer solution pH: 10.5), 25 µl reagent 3 (photosensitizer-luminol) and 1-4 nmol standard (ascorbic acid) solutions.

4.2.2.2.2. Sample Preparation for ACL

The same procedure was carried out for the presolution of crude plant extracts for ACL. In that measurements the ACL kit procedure was employed : 2.3 ml reagent 1 (solvent), 200 µl reagent 2 (water buffer solution pH: 10.5) and 25 µl reagent 3 (photosensitizer-luminol) and 1-4 nmol standard (trolox) solutions were used as described in test kits procedure (Analytik Jena). Samples were diluted with methanol for ACL measurements to obtain the lipophilic compounds and to keep the concentrations in the calibration range.

4.2.3. Determination of Total Phenol Contents

Total phenol content of 42 plant species was determined by using Folin-ciocalteu method with a modification of Lako (Lako, et al. 2007). Crude extracts were dissolved in DMSO in a ratio of 1 g extract in 20 ml DMSO. 500 µl plant extracts or standard (gallic acid) solutions were mixed with 2.5 ml Folin- ciocalteu reagent (1:10 dilution with deionized water) and left to stand 2.5 min at room temperature and then 2 ml of sodium carbonate solution (7.5 % in deionized water) was added. After incubating 1 hr at room temperature in a dark place the absorbances were measured at 725 nm by UV spectrophotometer (Perkin Elmer). Results were expressed as miligrams of gallic acid equivalents (GAE) per gram fresh weight. Calibration curve of gallic acid can be seen in appendix A.

Folin- Ciocalteu (F-C) Method

The F-C assay has for many years been used as a measure of total phenolics in natural products, and the mechanism is an oxidation-reduction reaction. This method was developed in 1927 originated chemical reagents used for tyrosine analysis in which oxidation of phenols by a molybdotungstate reagent yields a colored product with λ_{max} at 745 – 750 nm. The method is simple, sensitive, and precise. Results are expressed as the standard based equivalents such as, gallic acid, catechin, tannic acid, caffeic acid equivalents.

4.2.4. Determination of the Relative Antibacterial Activities of Plant Extracts

4.2.4.1. Disc Diffusion Assays

Disc diffusion Susceptibility Testing (Kirby-Bauer, Method) was performed to carry out the initial screening of relative antimicrobial effects of the 47 plant extracts. The procedure used in this assay is a modification of the Kirby-Bauer test which is commonly used as an antimicrobial susceptibility testing. The detail procedure of the disc diffusion assay was expressed in Appendix B. This method is a simple way of determining the susceptibility of a microorganism to an antimicrobial agent by inoculating an agar plate with the culture and allowing the antimicrobial agent to diffuse into the agar medium. A filter disc impregnated with the agent is applied to the surface of an agar plate containing the microorganism to be tested. The effectiveness of a particular antimicrobial agent is shown by the presence of growth inhibition zones. The zones of inhibition appear as clear areas surronding the disc from which the substances with antimicrobial activity diffused.

The size of the zone may be affected by the density or viscosity of the culture medium, the rate of diffusion of the antimicrobial agent, the concentration of the agent, and the sensitivity of the microorganism to the agent, the interaction between the antimicrobial agent and the medium.

In the disc diffusion assays, three bacteria species were chosen to determine the antimicrobial activities of plant extracts. *E.coli* NRRL B- 3008, *Bacillus subtilis* NRRL B 4378 and *Enterococcus faecium* NRRL-B 2354 were obtained from Agricultural Research Service Culture Collection (USA). *E. coli* is a gram negative bacteria while the other two species are gram positive bacteria.

4.2.4.1.1. Strains and Preparation of Stock Cultures

Some of the strains were purchased as liyofilized powders from suppliers and they were inoculated into appropriate broths and incubated overnight in optimum conditions (time, temperature, shaking, etc.,). In order to prolong usage time, stock cultures and their reserves were prepared in 40% glycerol broths by inoculation of the fresh culture (1:1). Stock cultures were labeled and kept in the -80 ^{o}C for further studies.

4.2.4.1.2. Determination of the Microbial Load in Assays

It is a critical point to ensure the same microbial load each time that is examined in the antimicrobial susceptibility tests (AST) in order to obtain accurate results and comparison. Incubation times before the inoculation of the microorganisms are variable for each strain, but it is advised to inoculate them when microorganisms are in their logarithmic phases. In this study for adjusting the microbial load the incubation and inoculation procedures were kept same and microbial loads were confirmed each time by fixing the OD (optical density) values of cultures inoculated into 96 well plates that is corresponding to a certain numbers of bacteria (CFU/ml) that were determined by colony counting method.

4.2.4.2. Minimum Inhibition Concentrations of Plant Extracts by Micro-dilution Assay

4.2.4.2.1. Definitions of MIC Value

Minimum inhibitory concentration (MIC) is the lowest concentration at which an antimicrobial substance will inhibit a particular microbial growth under specified test conditions (Reeks, et al. 2005). The MIC measured for an antimicrobial compound is the lowest concentration that will prevent growth; it does not necessarily mean that the organisms in the test have been killed, just stoped from growing (increasing in size or number) (Zakaria, et al. 2007).

4.2.4.2.2. Determination of the MIC Values of Plant Extracts

In order to determine the minimum inhibition concentrations (MIC) of the plant extracts; a gram negative (*E. coli*) and two gram positive bacteria (S. aureus and S. epidermidis) were used. Their reference numbers are as follow; *E. coli* NRRL B-3008 [Agricultural Research Service Culture Collection (USA)], *Staphylococcus aureus* ATCC 29213, *Staphylococcus epidermidis* ATCC 12228 (Ege University, Biology Department).

In the present study a serial 2-fold micro-broth dilution method (Kuete et al., 2007) was performed to determine the MICs of plant extracts. For the MIC of any tested extract to have meaning, a standard protocol was followed for each test. Firstly the test samples (crude extracts) were dissolved in dimethylsulfoxide (DMSO) the stock solutions for a concentration of 100mg/ml and prepared daily for each test. Two fold serial dilutions of each extract were carried out , with a final concentration of 50mg/ml by using sterile deionized water. Then 100µl of each extract concentration and 95 µl nutrient broth were added in each well of 96 well microplate and each well inoculated with 5 µl of 6 h incubated bacterial suspensions after standardized by adjusting their optical densities at 420 nm by UV spectrophotometer (Perkin Elmer) to obtain 0.8- 1.2 absorbances corresponding to approximately 10^7 CFU/ml. Negative and positive controls were also carried out for each strain. Negative controls were performed by serial dilutions of DMSO (50%) and the other negative control well consisted of 195 µl of NB (Nutrient broth) and 5 µl of the standard inoculum. Positive control wells consisted of serial dilutions of penicillin (400U) and gentamicin(10mg/ml) antibiotics. The assay plates were incubated at 37 ^{0}C for 24 h and the growth kinetic assays for each strain were performed by dublicate growth curves and observed as turbidity determined by a microplate reader (Varioskan) at 620 nm. MIC results for extracts were reported as mg/mL. These spectrophotometric measurements of MICs were carried out with a standardized protocol of Varioskan multiplate reader (see Table 4.1). After the 24 h incubation, INT was added into each test well in order to ensure visible indication of minimum inhibition concentrations. INT reacts with the metabolites produced by the microorganisms and the wells with the microorganism turn to pink colour.

Table 4.1. Parameters of Varioskan (multiplate reader)

Shaking Parameters	
On time	23:59:59.0
Off time	00:00:00.0
Speed (spm)	120
Diameter (mm)	3
Shaking mode	background
Kineticloop parameters	
Reading number	48
Interval (min)	30
Photometric Assay Parametes	
Wave length	620 nm
Measurement time	100 ms

CHAPTER 5

RESULTS and DISCUSSION

5.1. Extraction of Plant Species

The study was aimed to quantify and compare 47 plant materials of 42 plant species relatively. These plant species are the common species in Karaburun region that may be used for therapetic, nutriceutic and cosmetic claims. The plant codes, scientific names, their common names and extraction yields are present in Table 5.1.

Among the various extraction techniques such as, maceration, hot continuous extraction (soxhlet), microwave assisted extraction, ultrasound extraction (sonication), supercritical fluid extraction (SFE), enzyme assisted extraction and solvent extraction, the solvent extraction technique was prefered in this study.

The choice of extraction method, however, can have an effect on the efficacy of active plant constituents (Shaalan, et al. 2005), there are some other important factors affecting the quality of an extract, such as the plant part used as starting material, the solvent used for extraction, solid- liquid ratio and extraction time. The traditional techniques of solvent extraction of plant materials are mostly based on the correct choice of solvents and the use of heat or/and agitation to increase the solubility of the desired compounds and improve the mass transfer. Usually the traditional technique requires longer extraction time thus running a severe risk of thermal degradation for most of the phyto-constituents (Mandal, et al. 2007).

A preliminary study was carried out in order to display the influence of some parameters used in extraction including solvent concentration, solid liquid ratio and extraction time. Among the 47 plant materials *P. lentiscus* (leaf) was chosen and it's total phenol contents were measured varying with the parameters used. In figure 5.1, 5.2, and in figure 5.3 the result can be seen.

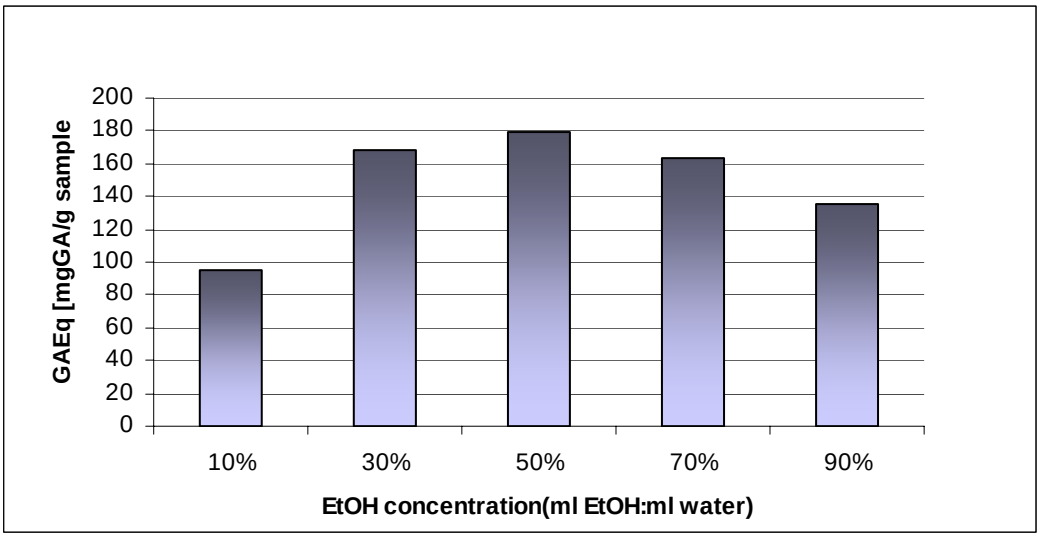

Figure 5.1. Effect of solvent concentration on total phenol content of *P. lentiscus* (1/15 solid liquid ratio, for 2h)

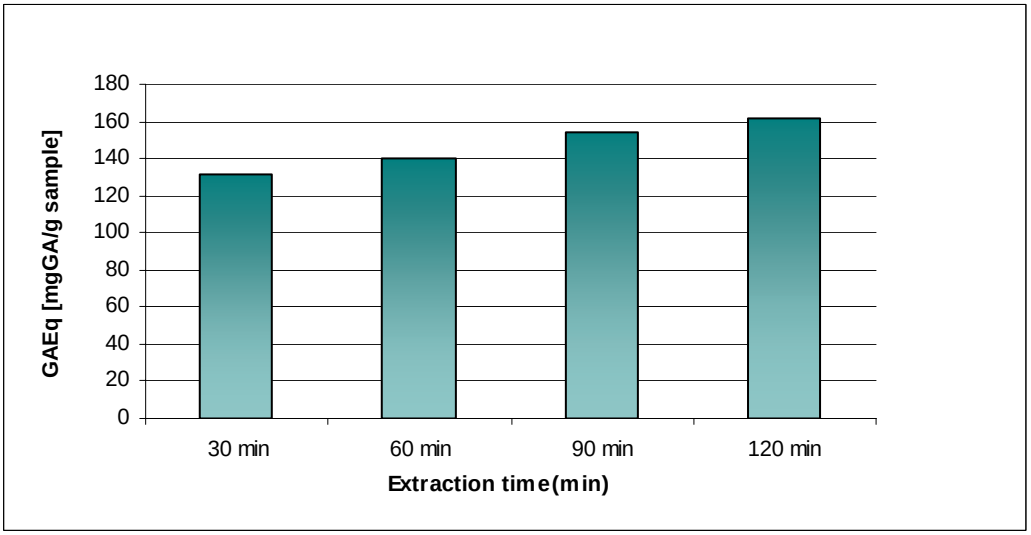

Figure 5.2. Extraction time effect on total phenol content of *P. lentiscus* (at 70% EtOH and with 1/15 solid liquid ratio)

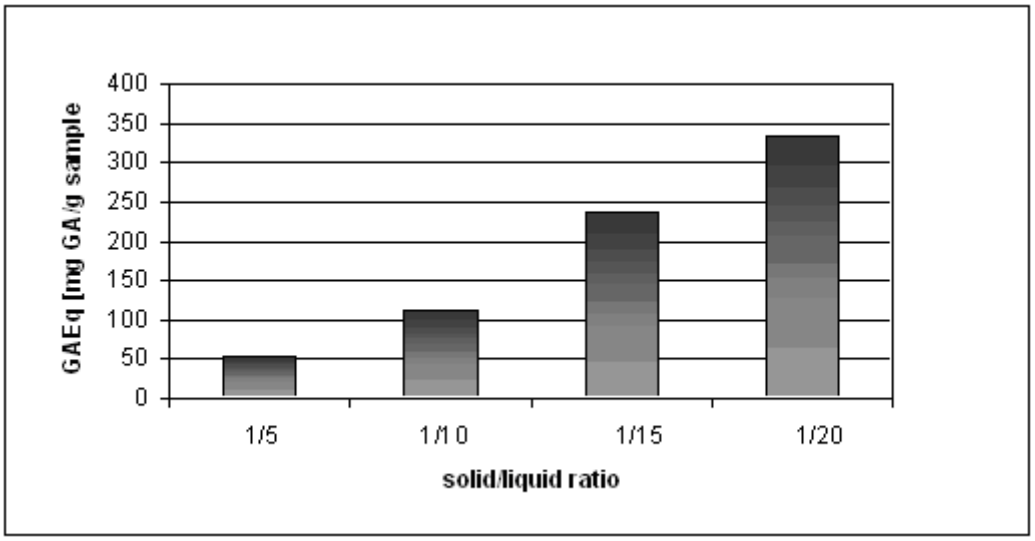

Figure 5.3. The effect of solid/liquid ratio on total phenol content of *P. lentiscus* (at 70% EtOH, for 2h)

These results could show variability for each plant species and plant part being examined. It is important here to emphasize that it is nearly impossible to optimize parameters for all the plants used in bioactivity screening studies, like our study including 47 plant species. But in the literature it has been often applied and advised to use a common standard protocol that enables to obtain as much phytochemicals as possible from plant materials for screening studies. This is achieved by using solvents ranging from water, because of the primary consideration that the type of solvent used since polar solvents will extract polar molecules, and non-polar solvents will extract non-polar molecules. Solvent types can significantly affect the potency of extracted plant compounds (Shaalan, et al. 2005). Many valuable natural materials have traditionally been extracted with organic solvents. However, some of the organic solvents are believed to be toxic, and the extraction conditions are often harsh. A simple method using ethanol instead of methanol for the extraction of phenolic compounds is the preferance frequently in the literature (Li, et al. 2006).

In order to make a reliable comparison, all operations in the extraction procedure of this study were standardized by taking the common processes into account in literature (see Table 2.2). But between different screening studies relative comparisons are more proper then quantitive comparisons due to the different extraction parameters that have been used.

5.1.1. Extraction Yields

As a result of the standardized extraction protocol (with a 1/20 solid liquid ratio, 80% EtOH concentration, for 2h) extraction yields were obtained for each plant material. Extraction yields in this study are ranged between 37.17 % and 1.24 %. According to the results *Hypericum empetrifolium* has the highest extraction yield with 37.17 %. Among the plant materials, some of them with the high extraction yields were demostrated in the figure 5.4. Plant codes in the figures represent plant's scientific names and can be seen in Table 5.1.

Figure 5.4 . Plant materials with the highest extraction yields

Table 5.1. The plant codes, scientific names, plant's common names and extraction yields

Plant codes	Scientific names	Plant part	Common names	Family	Ext. yields
1	*Sarcopterium spinosum*	leaf	Thorny burnet	Rosaceae	28.74
2	*Pistacia terebinthus*	leaf	Cyprus turpentine	Pistaciaceae	29.59
3	*Cistus parviflorus*	leaf	__	Cistaceae	19.05
4	*Arbutus unedo*	leaf	Strawberry tree	Ericaceae	35.81
5	*Quercus coccifera*	leaf	Kermes oak	Fagaceae	7.62
6	*Hypericum empetrifolium*	leaf	St John's worts	Hypericaceae	37.17
7	*Pistacia lentiscus*	leaf	Mastic tree	Pistaciaceae	31.98
8	*Helichrysum pallasii*	flower	Pallas' everlasting	Compositae	8.85
9	*Cercis siliquastrum*	seed	Judas tree	Leguminosae	14.56
10	*Rumex pulcher*	leaf	Fiddle dock	Polygonaceae	18.66
11	*Teucrium chamaedrys*	leaf	Wall germander	Labiatae	27.8
12	*Phillyrea latifolia*	leaf	Phillyrea	Oleaceae	29.91
13	*Quercus infectoria*	leaf	Oak	Fagaceae	2.79
14	*Salvia virgata*	leaf	Meadow sage	Lamiaceae	1.24
15	*Stachys cretica*	leaf	Cretan hedgenettle	Lamiaceae	NA
16	*Anthyllis hermanniae*	leaf	Kidney vetch	Fabaceae	21.37
17	*Lavandula stoechas*	flower	French lavender	Labiatae	14.34
18	*Origanum onites*	leaf	Pot marjoram	Labiatae	19.32
19	*Lavandula stoechas*	leaf	French lavender	Labiatae	21.55
20	*Vitex agnus-castus*	flower	Chaste tree	Verbenaceae	32.04
21	*Vitex agnus-castus*	leaf	Chaste tree	Verbenaceae	23.34
22	*Anchusa azurea*	flower	Anchusa	Boraginaceae	14.55
23	*Teucrium polium*	leaf	Golden germander	Labiatae	11.01
24	*Solanum nigrum*	leaf	Black nightshade	Solanaceae	18.92
25	*Chrysanthemum segetum*	flower	Corn daisy	Compositae	19.41
26	*Smyrnium rotundifolium*	leaf	Perfoliate alexander	Umbelliforae	28.74
27	*Verbascum lychnitis*	flower	White mullein	Scrophulariaceae	1.74
28	*Rhamnus alaternus*	leaf	Italian buckthorn	Rhamnaceae	31.34
29	*Corydothymus capitatus*	leaf	Oregano spanish	Lamiaceae	NA
30	*Alkanna tinctoria*	leaf	alkanet	Boraginaceae	8.72
31	*Stachys cretica*	flower	Cretan hedgenettle	Lamiaceae	NA
32	*Verbascum lydium*	leaf	__	Scrophulariaceae	NA
33	*Capparis spinosa*	leaf	Spiny caper	Capparidaceae	19.72
34	*Rhamnus alaternus*	seed	Italian buckthorn	Rhamnaceae	32.14

(Cont. on next page)

Table 5.1. (cont.) The plant codes, scientific names, plant's common names and extraction yields

35	*Chrozophora tinctoria*	leaf	Giradol	Euphorbiaceae	12.33
36	*Genista acanthoclada*	leaf	__	Fabaceae	15.12
37	*Ballota acetabulosa*	leaf	Greek horehound	Labiatae	12.03
38	*Rumex pulcher*	seed	Fiddle dock	Polygonaceae	8.31
39	*Aristolochia hirta*	leaf	__	Aristolochiaceae	25.05
40	*Smyrnium rotundifolium*	seed	Perfoliate alexander	Umbelliforae	7.18
41	*Onopordum illyricum*	flower	Illyrian cottonthistle	Compositae	NA
42	*Psoralea bituminosa*	leaf	Scurvy pea	Leguminosae	21.89
43	*Eryngium campestre*	leaf	Field eryngo	Umbelliforae	12.99
44	*Allium ampeloprasum*	flower	Broadleaf wild leek	Alliaceae	NA
45	*Echium plantagineum*	leaf	Salvation Jane	Boraginaceae	NA
46	*Carlina corymbosa*	flower	__	Compositae	16.04
47	*Urtica dioica*	flower	Great nettle	Urticaceae	9.75

5.2. Determination of Antioxidant Activity by PCL Assay

Antioxidant capacity of water soluble (ACW) and antioxidant capacity of lipid soluble (ACL) compounds were detected by using a single system on photochem which was determined by Popov and Lewin (Popov and Lewin 1999) and commercialized by Analytik Jena AG (Jena Germany). The mean values of ACW and ACL analysis are presented in Table 5.2 that are expressed as standard (trolox or ascorbic acid) equivalents. The results are mean of triplicate measurements.

Standard kits provided by the manufacturer are used to measure hydrophilic and lipophilic antioxidant activity (ACW and ACL kits respectively). Calibrations and measurements for ACW (antioxidant capacity of water soluble compounds) are based on the difference in the lag time between sample and the blank (Figures 5.6 and 5.8). Measurements for the ACL (antioxidant capacity of lipid soluble compounds) are based on the inhibition of the area under curve (AUC) of the blank by sample (Figures 5.5 and 5.7). Calibration and some important sample measurements of ACW and ACL assays are shown in Figures 5.5, and 5.6 below.

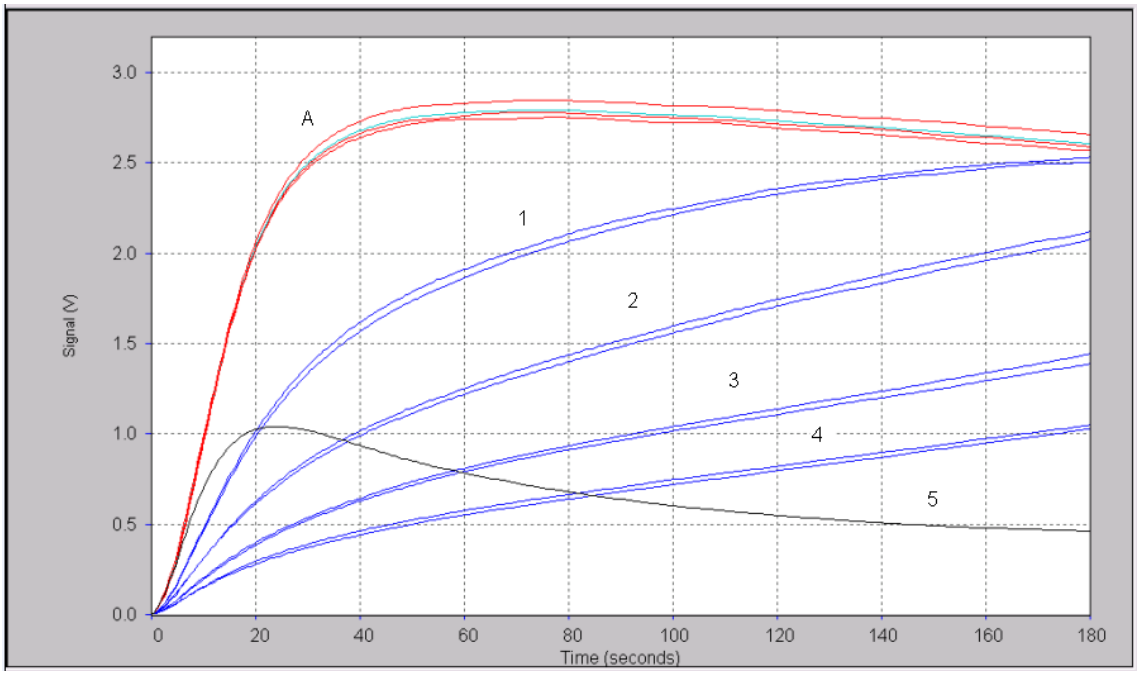

Figure 5.5.Photochem measuremets of trolox calibration , **A**. Blank, **1-4** . different concentrations of trolox ranging 1-4 nmol. **5**. *H. empetrifolium*- example for measurement curve of lipophilic antioxidants activity

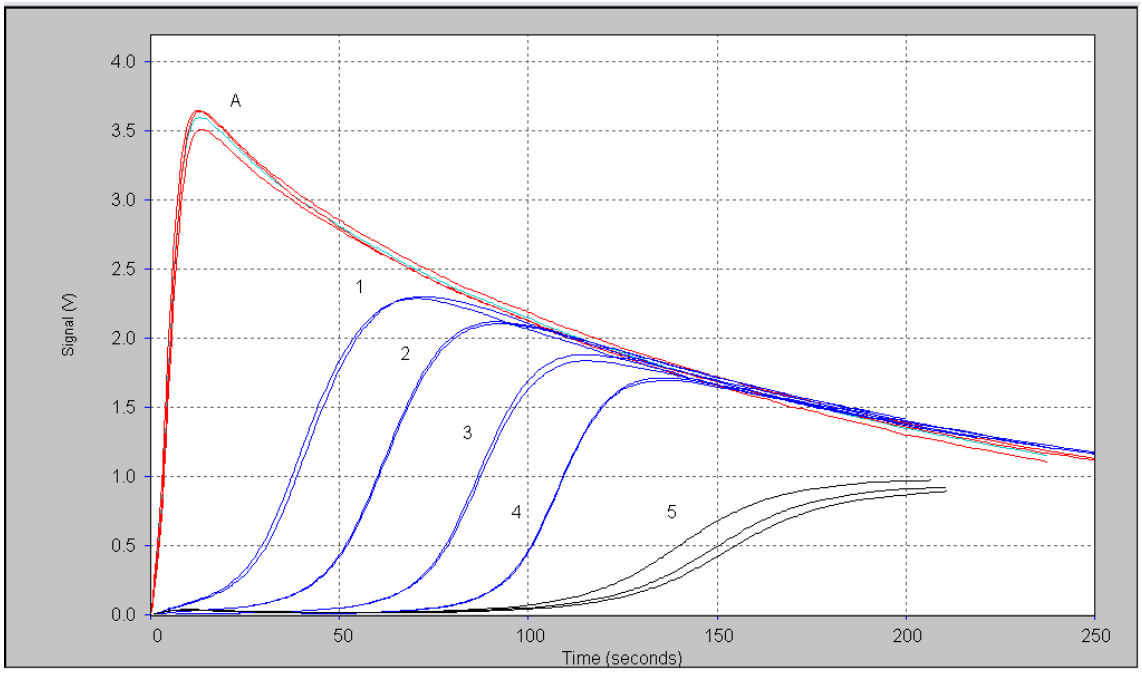

Figure 5.6. Photochem measurements of ascorbic acid calibtarion **A**. Blank, **1-4** . different concentrations of ascorbic acid ranging 1-4 nmol, **5**. *Hypericum empetrifolium* -an example for measurement curve of hydrophilic antioxidants activity

5.2.1. Antioxidant Activities of Water Soluble Antioxidants (ACW)

In the ACW tests the highest water soluble antioxidant activity was observed for *Hypericum empetrifolium* resulted with a potent capacity corresponding to 1866.39 ug/mg of ascorbic acid and that is followed by *Teucrium chamaedrys*, whose strong inhibitory activity against free radicals was shown previously by Panovska (Panovska, et al. 2005). The lowest activities were detected for *Smyrnium rotundifolium* and *Allium ampeloprasum* among 47 plant extracts. In this study *Allium ampeloprasum* gives the lowest values for both ACW and ACL antioxidant analysis. These species has been also indicated with its weak activities in some screening studies (The Local Food Nutraceuticals Consortium-2005). The most promising seven plant species in ACW analysis are shown in Figure 5.9 and the plant codes in figures are corresponding to the scientific plant names which are shown in Table 5.1.

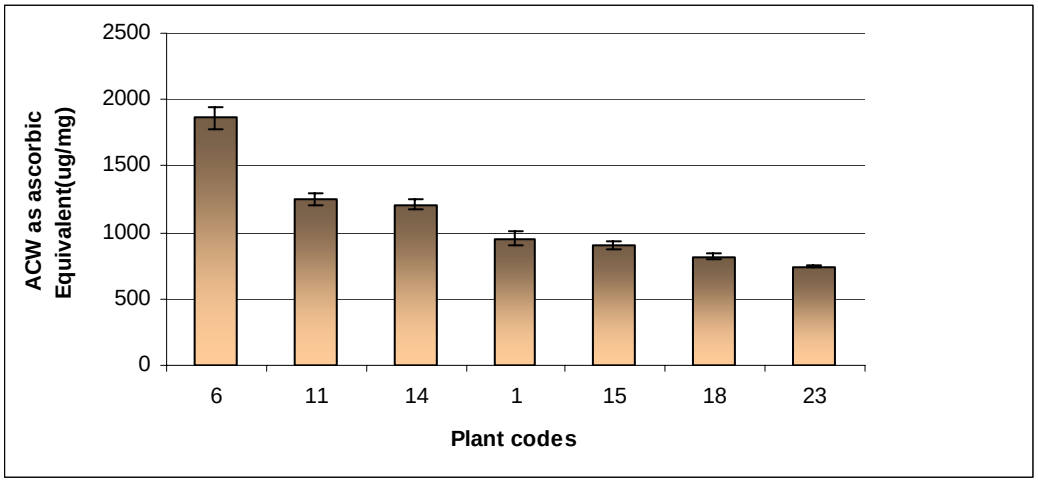

Figure 5.7. The most important seven species in ACW

5.2.2. Antioxidant Activities of Lipid Soluble Antioxidants (ACL)

The highest lipid soluble antioxidant value was detected for *Hypericum empetrifolium* corresponding to 1889.51 ug/mg trolox equivalents (see Table 5.2). It was followed by *Phillyrea latifolia* and *Vitex agnus-castus*. The extracts with the lowest activities are detected in *Smyrnium rotundifolium* and *Allium ampeloprasum*.

Some important plant species in ACL analysis are shown with their codes in Figure 5.10. The codes can be seen in Table 5.1.

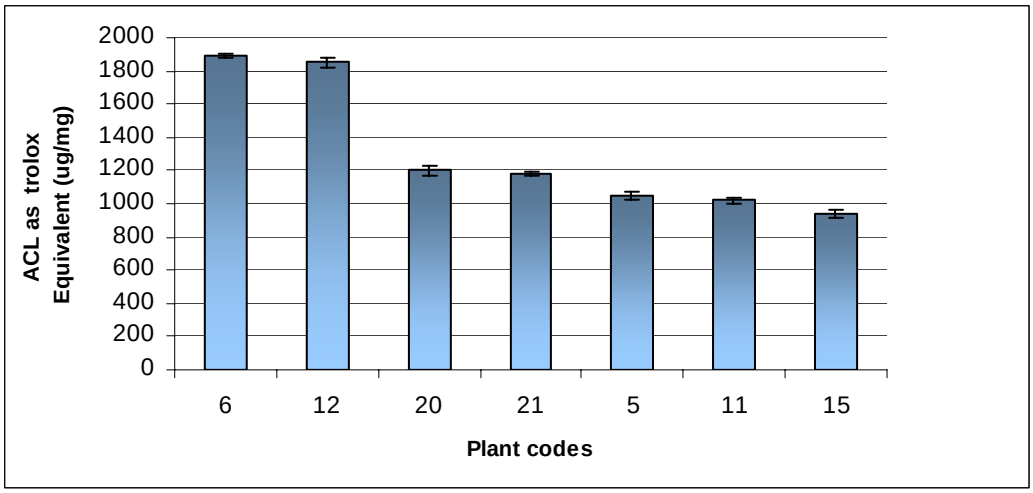

Figure 5.8. The most important species in ACL tests

Determination of water soluble and lipid soluble antioxidant capacities separately in an extract is allows us to evaluate the plant material accurately. Differences in the antioxidant activity ranking in the ACW and ACL test results are expectable through the concentration variability of the lipophilic and hydrophilic antioxidant groups in the same plant extract. However some of the plant extracts demostrated important antioxidant activities in both ACW and ACL tests. These species are *Hypericum empetrifolium*, *Teucrium chamaedrys*, *and Stachys cretica.*

The most active plant species in ACW and ACL tests are partially examined in the literature that also make a confirmation of antioxidant activities of these species. *Hypericum empetrifolium*, and *Sarcopterium spinosum* are important plant species in this study, especially *Sarcopterium spinosum* has not been argued in litrature before. However other Hypericum species were investigated and confirmed for their high antioxidant capacities, *Hypericum empetrifolium* has not been examined in litrature except a research of Meral (Meral and Konyalıoğlu 2004) who showed its strong antioxidant activity, but this species was not evaluated in detail. Strong inhibitory activity of *Teucrium chamaedrys* extracts was shown by Panovska, and phytochemical screening of the this plant extract proved the presence of flavonoids luteolin, apigenin and/or diosmetin. The chemical composition of this extract was evaluated by HPLC and spectrophotometry (Panovska, et al. 2005).

Other active species are also mentioned in studies, for example some phenolic compounds of *Salvia virgata* were identified as benzoates, hydroxycinnamates , and flavonoids (Akkol, et al. 2008). *Quercus coccifera* is another active species in this study that was reported before for its phytochemistry including tannins (Ito, et al. 2002). *Vitex agnus-castus* is an important medicinal plant in ethnopharmacy and some of the bioactive ingredients have been identifiyed and isolated in other studies (Hirobe, et al. 1997) and dopaminergic compounds present in *Vitex agnus castus* are clinically the important compounds which improve premenstrual mastodynia and possibly also other symptoms of the premenstrual syndrome (Wuttke, et al. 2003).

The photochemiluminescence assay provided a simplicity to detect the relative antioxidant activities of 47 plant extracts. This method is a new one with its applications repsesenting so many advantages mentioned below. The PCL assay , which is easy and rapid to performe, presents numerous advantages; it does not require high temparetures to generate radicals and it is more sensitive to measure in a few minutes, the scavenging activity of antioxidants against the superoxide radical which is one of the most dangerous reactive oxygen species (ROS) also accuring in the human body (Vertuani, et al. 2002). The antioxidant status of biological materials can be made quickly, at low cost and environmentally compatible. PCL based methods differ from other procedures for antioxidant evaluation principally because do not require oxidizing reagents for the generation of the radical species. Moreover only two assays (LDL, and PCL) are able to analyse antioxidant activity in the nanomolar range (Vertuani, et al. 2004). In PCL system, ACW and ACL measuring kits allows the user to detect both antioxidant capacities of water soluble compounds and antioxidant capacities of lipid soluble compounds respectively in a single system (Margrit and Tirok 2000). As water soluble fractions antioxidants such as flavanoids, ascorbic acid, aminoacids, etc.., are detected while, tocopherols, tocotrienols, carotenoids, etc, are measured as lipid soluble antioxidant fractions. In our study PCL was prefered for its rapid, sensitive, relatively simple and reproducible measuring system.

5.3. Total Phenol Contents of Plant Extracts

Phenolic compounds are a class of antioxidant agents which act as free radical terminators (Pourmorad, et al. 2006). Phenolic compounds inhibit lipid oxidation by scavenging free radicals, chelating metals, activating antioxidant enzymes, reducing tocopherol radicals and inhibiting enzymes that cause oxidation reactions (Harrison and Were 2007).

Total phenol contents of plant species were analyzed with folin-ciocalteu reagent. The results of this assay were ranged from 635.2 mgGAE/gdw to 27.3 mgGAE /gdw. *Sarcopterium spinosum* is the most interesting one with the highest phenolic content as 635.2 mgGAE/gdw. The highest total phenol contents resulted in *Sarcopterium spinosum*, *Pistacia terebinthus* , *Cistus parviflorus* and the lowest in *Urtica dioica* that is followed by *Carlina corymbosa* and *Echium plantagineum* (see Table 5.2). The most promising plant species in total phenol analysis are demostrated in figure 5.11.

Figure 5.9.Plant species (given with their codes) having the highest total phenol contents in Folin-ciocalteu assays

Several plant species with significant activities have been detected in this study and most of them have a lack of laboratory data on this bioactivities. Especially *Hypericum empetrifolium* and *Sarcopterium spinosum* , *Phillyrea latifolia and Cistus*

parviflorus are the most interesting ones that are needed to investigate for the identification of their bioactive phytochemicals. Phenolic compounds are a class of antioxidant agents which inhibit lipid oxidation by scavenging free radicals, chelating metals, activating antioxidant enzymes and inhibiting enzymes that cause oxidation reactions (Harrison and Were 2007). Some of the authors emphasize an obvious correlation between total polyphenols and some bioactivities such as antioxidant activity (Tawaha, et al. 2007, Bouayed, et al. 2007), but in this present study there is no strong correlation between total phenolics and total antioxidant activity which is also confirmed by some other studies (Dasgupta, et al. 2007). On the other hand this result is giving important suggestions about phenolics are not the only essential compounds that induce the antioxidant activity. It is also giving clues for the presence of substantial amounts of nonphenolic constituents such as carotenoids, alkaloids, vitamins and terpenes that contribute to antioxidant activity (Liu, et al. 2004). However it is also obvious in results that there is a clear relationship between antioxidant capacities and total phenol contents in this study.

Hypericum empetrifolium and *Sarcopterium spinosum* are some of the most promising species in both antioxidant tests and Folin- ciocalteu assays. In the literature, total phenol assay of *H. empetrifolium* was evaluated which agrees with our results by Meral (Meral and Konyalıoğlu 2004). However *Sarcopterium spinosum* is the plant species having the highest total phenol content in this assay has not been examined in the literature before. That makes our study the first survey detected important phenolic content and antioxidant activity of *S. spinosum* in literature.

Other species with the high total phenol contents in our study such as *Arbutus unedo* was mentioned before several times for phenolic constituents including quercitrin, isoquercitrin, hyperoside and rutin detected by TLC (thin-layer chromatography) (Males, et al. 2006). And *Q. coccifera* having the phenolic constituents such as hydrolyzable tannins was investigated by Ito (Ito, et al. 2002).

Pistacia species in our study exhibited important antioxidant activities and high phenol content have been researched for several times and especially, phytochemical constituents of *P. lentiscus* was identified and isolated in several studies.

Table 5.2. Results of Folin-ciocalteu assays and antioxidant assays (ACW and ACL)

Code	Total Phenol Capacity[a]	ACW (water-soluble compounds)[b]	ACL (lipid-soluble compounds)[c]
1	635.2 ± 12.9	954.6± 48.4	789.9 ± 15.3
2	567.6 ± 18.4	684.2 ± 3.9	711.1 ± 17.3
3	552.7 ± 17.2	728.9 ± 90.8	644.7 ± 29.7
4	500.3 ± 34.4	517.1 ± 2.9	867.0 ± 4.4
5	489.6 ± 9.8	693.1 ± 19.9	1053.0 ± 23.7
6	483.9 ± 17.8	1866.4 ±83.9	1889.5 ± 13.8
7	469.4 ± 3.3	403.9 ± 4.3	818.8 ± 23.6
8	419.1 ± 0.8	649.6 ± 26.1	337.2 ± 31.3
9	400.6 ± 11.5	138.5 ± 1.5	768.3 ± 1.8
10	318.5 ± 6.4	144.9 ± 30.8	641.5 ± 12.1
11	300.9 ± 11.1	1249.9 ± 39.5	1020.2 ± 16.5
12	283.2 ± 26.7	736.3± 25.5	1852.6 ± 28.0
13	260.6± 2.1	521.4 ± 16.6	676.1 ± 3.2
14	258.8 ± 2.1	1212.2 ± 36.3	774.1 ± 29.0
15	254.6 ± 2.2	906.4 ± 28.9	937.7 ± 24.4
16	243.8 ± 0.5	102.6 ± 7.8	151.9 ± 3.9
17	228.2 ± 10.9	722.2 ± 30.7	603.2 ± 3.8
18	202.8 ± 1.6	820.6 ± 23.4	738.2 ± 19.3
19	202.8 ± 14.1	739.8 ± 24.3	701.5 ± 9.3
20	192.1 ± 2.9	521.5 ± 22.6	1199.3 ± 33.1
21	175.1± 4.6	417.7 ± 12.9	1181.4 ± 11.8
22	171.2 ± 0.4	128.4 ± 1.4	269.4 ± 1.3
23	167.8 ± 0.7	739.9 ± 7.2	715.6 ± 4.7
24	167.4 ± 2.5	302.5 ± 3.1	314.6 ± 15.3
25	159.3 ± 4.9	255.5 ± 7.9	212.3 ± 8.4
26	157.3 ± 9.2	30.3 ± 0.6	50.9 ± 3.3
27	152.3 ± 4.0	367.2 ± 26.5	547.1 ± 24.0
28	151.2 ± 3.5	93.3 ± 1.2	158.5 ± 4.1
29	148.6 ± 5.2	110.6 ± 12.2	573.3 ± 19.7
30	141.4 ± 6.4	613.1 ± 14.0	750.6 ± 29.3
31	129.2 ± 0.9	257.1 ± 7.9	374.4 ± 23.6
32	125.6 ± 1.4	319.7 ± 17.7	549.6 ± 15.4
33	122.5 ± 3.6	85.8 ± 8.9	224.9 ± 6.8
34	121.8 ± 3.0	33.7 ± 1.5	130.5 ± 2.1
35	121.2 ± 14.7	91.6 ± 0.5	416.7 ± 7.2
36	109.9 ± 1.6	115.9 ± 7.9	267.5 ± 7.5
37	97.9 ± 1.8	387.4 ± 43.0	516.9 ± 25.2
38	88.1 ± 2.3	43.6 ± 0.3	106.8 ± 5.2
39	83.8 ± 1.6	413.8 ± 8.9	587.8 ± 15.6
40	81.1 ± 4.9	152.4 ± 2.0	508.3 ± 9.08
41	68.5 ± 0.9	463.8 ± 16.4	350.5 ± 9.4
42	67.7 ± 0.4	79.1 ± 3.2	118.4 ± 2.6
43	62.2 ± 2.2	217.2 ± 7.2	325.0 ± 28.9
44	60.6 ± 2.6	19.7 ± 0.3	90.9 ± 1.3
45	42.9 ± 2.6	45.1 ± 9.1	193.5 ± 10.7
46	32.2 ± 1.5	52.6 ± 2.1	327.7 ± 2.4
47	27.3 ± 0.7	38.5 ± 3.7	105.8 ± 1.8

[a](GAEq as mg/g sample), as gallic acid equivalents
[b]As ascorbic Equivalent(ug/mg)
[c]As trolox Equivalent (ug/mg)

5.4. Antibacterial Activities of Plant Extracts

5.4.1. Disc Diffusion Assays

In the disc diffusion assays, three bacteria species were chosen to determine the antimicrobial activities of plant extracts. *E.coli* NRRL B- 3008, *Bacillus subtilis* NRRL B 4378 and *Enterococcus faecium* NRRL-B 2354. *E. coli* is a gram negative bacteria while the other two species are gram positive bacteria.

Out of the 42 plant species tested for antibacterial activity 26 plant species showed activities by inhibiting one or more microorganisms. The results of antimicrobial screening of the crude extracts of all species by disc diffusion method are shown in Table 5.4. Among the plants screened, *Cistus parviflorus, Arbutus unedo, Quercus coccifera, Hypericum empetrifolium, Pistacia lentiscus, Cercis siliquastrum, Rumex pulcher* and *Psoralea bituminosa* demostrated promising antibacterial activities against all tested microorganisms. The tested plant extracts were more active against gram-positive bacteria then gram-negative bacteria, depending on the different structural and inherited features of these two groups.

Antibiotic controls were also performed in order to compare the sensitivity of tested microorganisms against antimicrobial agents. Among the antibiotic discs, gentamicin exhibited highly strong antimicrobial activity against bacteria tested (see Table 5.3).

Table 5.3. Results for diameters of antibiotic control zones (in mm)

Microorganisms	**Gentamicin zone**	**Penicillin zone**	**Vancomycin zone**
Escherichia coli (NRRL-B 3008)	22.24 ± 0.11	10.15 ± 0.007	0
Enterococcus faecium (NRRL-B 2354)	11.25 ± 1.08	0	0
Bacillus subtilis (NRRL-B 4378)	25.1 ± 0.14	23.42 ± 0.01	19.66 ± 0.61

As it is seen in Table 5.3 and 5.4 *Escherichia coli* and *Enterococcus faecium* are much more resistant against antibiotic controls and plant extracts than *B.subtilis*. In Figure 5.12 below, antimicrobial effect of antibiotic controls and some species against *E.coli* were demostrated .

A B

Figure 5.10.**A.** plant extract (29) gentamicin, vancomycin, and penicillin controls against *E. coli,* **B**. Some plant extracts (1, 2, 3, 5) and their weak activity on *E. coli*

Disc diffusion assays were performed in duplicate experiments for aech species. Antimicrobial effects of some of the tested samples and their parallels are shown in Figure 5.13. *B. subtilis* exhibited weak resistances against samples and antibiotic controls in disc diffusion assays.

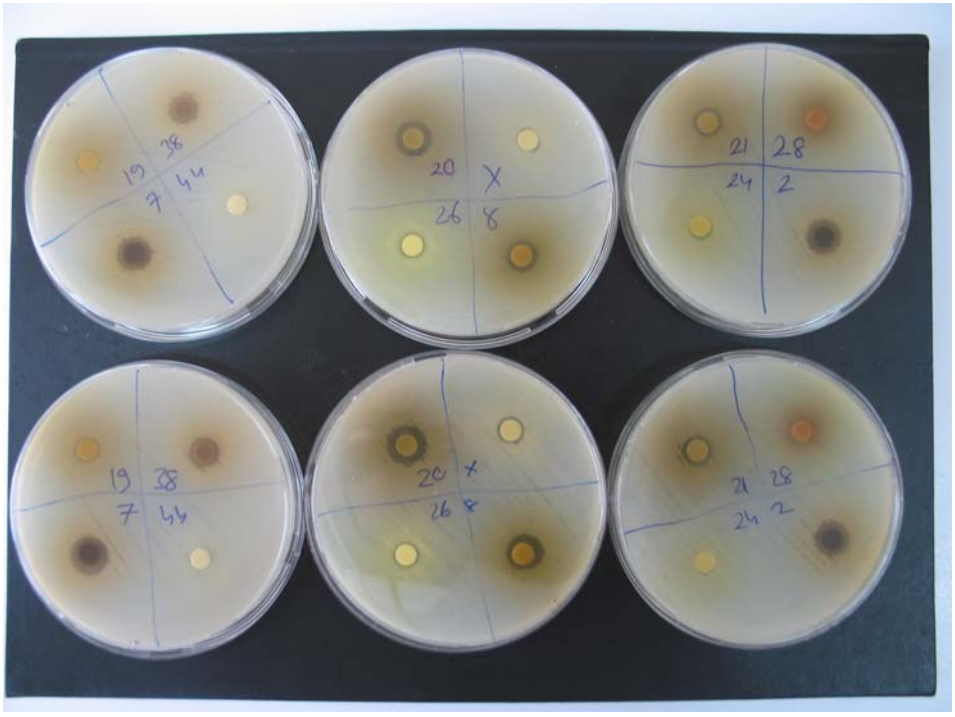

Figure 5.11. Zones of some plant extracts on *B. subtilis*

Table 5.4. Results for disc diffusion testing of 47 plant extracts (expressed in mm)

Plant species	**Plant part**	**E.coli**	**E. faecium**	**B. subtilis**
Sarcopterium spinosum	leaf	8.23 ± 0.421	6.74 ± 1.046	7.33 ± 0.098
Pistacia terebinthus	leaf	0	7.75 ± 0.692	8.37 ± 0.077
Cistus parviflorus	leaf	7.56 ± 0.556	9.59 ± 0.586	9.19 ± 0.155
Arbutus unedo	leaf	6.57 ± 0.806	7.35 ± 0.021	9.75 ± 2.446
Quercus coccifera	leaf	7.48± 0.856	9.69 ± 0.933	11.35 ± 0.070
Hypericum empetrifolium	leaf	7.09 ± 0.042	7.75 ± 0.530	12.30 ± 1.477
Pistacia lentiscus	leaf	7.39 ± 0.042	8.345± 0.106	9.71 ± 0.657
Helichrysum pallasii	flower	0	0	8.58 ± 0.615
Cercis siliquastrum	seed	7.69 ± 0.509	9.73 ± 0.403	9.38 ± 0.070
Rumex pulcher	leaf	0	0	0
Teucrium chamaedrys	leaf	0	13.90 ± 0.700	7.83 ± 0.905
Phillyrea latifolia	leaf	0	0	6.13 ± 0.183
Quercus infectoria	leaf	8.36 ± 0.084	7.22 ± 0.247	7.2 ± 0.141
Salvia virgata	leaf	0	0	8.32 ± 0.106
Stachys cretica	leaf	0	7.06 ± 1.506	6.24 ± 0.34
Anthyllis hermanniae	leaf	0	9.71 ± 3.471	7.29 ± 0.042
Lavandula stoechas	flower	0	6.62 ± 0.530	0
Origanum onites	leaf	0	0	7.24 ± 0.289
Lavandula stoechas	leaf	0	6.29 ± 0.098	0
Vitex agnus-castus	flower	0	7.17 ± 0.169	9.31 ± 0.254
Vitex agnus-castus	leaf	0	6.59 ± 0.841	7.29± 0.007
Anchusa azurea	flower	0	0	0
Teucrium polium	leaf	0	6.56 ± 0.791	6.3 ± 0.141
Solanum nigrum	leaf	0	0	0
Chrysanthemum segetum	flower	7.67 ± 2.085	0	0
Smyrnium rotundifolium	leaf	0	0	0
Verbascum lychnitis	flower	0	0	0
Rhamnus alaternus	leaf	0	7.06 ± 0.014	0
Corydothymus capitatus	leaf	0	0	8.92 ± 0.742
Alkanna tinctoria	leaf	0	0	0
Stachys cretica	flower	0	7.64 ± 0.714	6.57 ± 0.601
Verbascum lydium	leaf	0	0	0
Capparis spinosa	leaf	0	0	0
Rhamnus alaternus	seed	6.54 ± 0.763	6.57 ± 0.813	0
Chrozophora tinctoria	leaf	0	0	6.40 ± 0.063
Genista acanthoclada	leaf	0	0	0
Ballota acetabulosa	leaf	0	0	7.8 ± 0.876
Rumex pulcher	seed	7.76 ± 0.466	9.30 ± 0.219	7.26 ± 0.141
Aristolochia hirta	leaf	0	0	0
Smyrnium rotundifolium	seed	0	0	0
Onopordum illyricum	flower	0	0	0
Psoralea bituminosa	leaf	6.59 ± 0.834	7.36 ± 0.113	16.21 ± 0.261
Eryngium campestre	leaf	0	0	0
Allium ampeloprasum	flower	0	0	0
Echium plantagineum	leaf	0	0	0
Carlina corymbosa	flower	6.23± 0.332	0	0
Urtica dioica	flower	0	0	0

All plant samples that were used in the disc diffusion assays showed bacteriocidal activity except *S. spinosum* extract on *S. aureus*. Bacteriocidal activity is defined as the transparently cleared zones around discs. On the contrary bacteriostatic activity is defined with cleared zones containing micro colonies around the discs (Aboaba, et al. 2006). *S. spinosum* exhibited antibacterial activity with such micro-colonies (see Figure 5.12). "Bacteriostatic" means that the agent prevents the growth of bacteria (i.e., it keeps them in the stationary phase of growth), and "bactericidal" means that it kills bacteria. The clinical definition is even more arbitrary. Most antibacterials are better described as potentially being both bactericidal and bacteriostatic (Pankey et al. 2004).

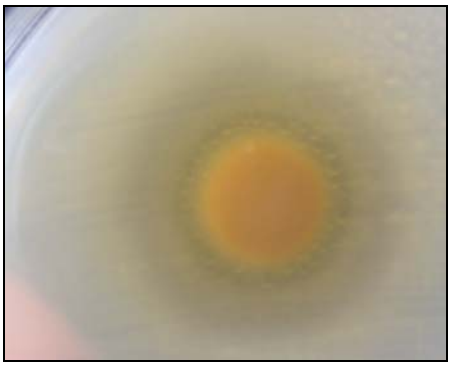

Figure 5.12. *Sarcopterium spinosum* against *S. aureus* and its bacteriostatic activity with micro-colonies around the disc

5.4.2. MIC (Minimum Inhibition Concentration) Assays

In this study eleven plant species that have previously confirmed for their antimicrobial activities by disc diffusion tests in the preliminary experiments were examined for their minimum inhibition concentrations (MICs). These plant species are listed in Table 5.5. In order to determine the MICs serial micro-broth dilution method was performed by using 96 well microtiter plates (Thermo). Dilution methods are known as quantitive, more repeatible and reliable assays when compared with other AST methods **(** Valgas, et al. 2007).

When performing this study a new microplate reader was used called Varioskan (Thermo). Varioskan has several features that facilitate in continous antimicrobial

susceptibility testing. In similar systems there are various problems effecting the reliability of results such as temperature fluctuations and evaporation from microplate wells. This specific instrument enables a single system comprising a spectrometer, spectrofluorometer, incubator, shaker and microplate lid heater which inhibits the evaporation problem (Lampinen, et al. 2005).

According to the results shown in Table 5.6 the most active plant species are *Hypericum empetrifolium, Pistacia lentiscus, Pistacia terebinthus, Arbutus unedo and Cistus parviflorus*. In the literature some of these plant species were indicated for their antimicrobial potentials (Aksoy, et al. 2006, Kordali, et al. 2003). Antimicrobial activities of *Arbutus unedo* and *P. lentiscus* are well represented and essential oils of *C.parviflorus* were examined for the antimicrobial activity in literature. But there has not been a comprehensive study for antimicrobial activities and MICs of *H. empetrifolium* and *P. terebinthus* before.

Table 5.5. List of extracts used in MIC assays

Plant species	Plant part	Extraction yield (%)
Arbutus unedo	Leaf	35.85
Cistus parviflorus	Leaf	19.05
Hypericum empetrifolium	Leaf	37.17
Pistacia lentiscus	Leaf	31.98
Pistacia terebinthus	Leaf	29.59
Psoralea bituminosa	Leaf	21.89
Quercus coccifera	Leaf	7.62
Quercus infectoria	Leaf	2.79
Rumex pulcher	Seed	8.31
Sarcopterium spinosum	Leaf	28.74
Vitex agnus castus	Leaf	23.34

Table 5.6. MIC and disc diffusion results of plant extracts and controls

Plant species	*E. coli*		*S.aureus*		*S.epidermidis*	
	Disc diff.(mm)	MIC (mg/ml)	Disc diff.(mm)	MIC (mg/ml)	Disc diff.(mm)	MIC (mg/ml)
Arbutus unedo	6.57	50	7.65	1.56	7.18	0.78
Cistus parviflorus	7.56	25	7.84	1.56	7.34	0.78
Hypericum empetrifolium	7.09	50	11.05	0.78	13.20	0.78
Pistacia lentiscus	7.39	12.5	9	0.78	7.56	1.56
Pistasia terebinthus	__	50	8.15	1.56	7.45	0.78
Psoralea bituminosa	6.59	25	8.27	>3.125	__	>1.56
Rumex pulcher	7.76	25	9.30	3.125	7.04	>1.56
Sarcopterium spinosum	8.23	>50	10.10	>3.125	7.87	>1.56
Quercus coccifera	7.48	25	11.06	>3.125	10.12	3.125
Quercus infectoria	8.36	25	7.43	>3.125	__	>1.56
Vitex agnus castus	__	50	7.26	>3.125	7.57	>1.56
Positive controls	Disc diff.(mm)	MIC	Disc diff.(mm)	MIC	Disc diff.(mm)	MIC
Penicillin	7.02	2 (IU)	»25	>0.02	»25	>0.02
Gentamicin (μg/ml)	10.25	0.625	11.35	2.5	10.35	100

In the study it was well shown that DMSO used for dissolving extracts has no inhibitory effect on *E.coli*, *S.epidermidis*, and *S.aureus* (Figure 5.14, 5.15 and 5.16). Gentamicin (Figure 5.17, 5.18 and 5.19) and penicillin were tested to evalute the resistance of the strains. According to test results, *E.coli* NRRL B 3008 is an evidently much more resistant strain when compared with the other two species against control antibiotics and plant extracts. This is not a supprising result if we consider that *E.coli* is a gram negative bacteria. It is important here to emphasize that gentamicin has a MIC of 100 μg/ml (0.1 mg/ml) for *S. epidermidis*, while *Hypericum empetrifolium, Pistacia terebinthus, Arbutus unedo and Cistus parviflorus* extracts have MICs of 0.78 mg/ml which can be accepted as an alternative dose for gentamicin.

The entry of antibiotics as well as other complex molecules into gram-negative bacteria requires a pathway through the lipopolysaccharide outer membrane. This pathway is provided by protein channels called porins. The ability of molecules to pass through these channels is influenced by their size, shape, and electrical charge. It has been demonstrated that porins serve as major entry gates for antibacterial compounds in

these organisms. These membrane proteins were originally thought to be exclusively responsible for the inherently higher resistance of gram-negative bacteria to antibacterials. Decreased entry of antibiotic into the bacterial cell is not important in gram-positive bacteria because they lack a lipopolysaccharide outer membrane. Although the peptidoglycan layer of gram-positive bacteria is thicker than that of gram-negative bacteria, it does not pose a significant barrier to antibiotic entry (O'Shea and Moser 2007).

In the control tests, DMSO was showed that has no inhibitory effect on any of the bacteria tested. The positive and negative control figures were presented in the figures below.

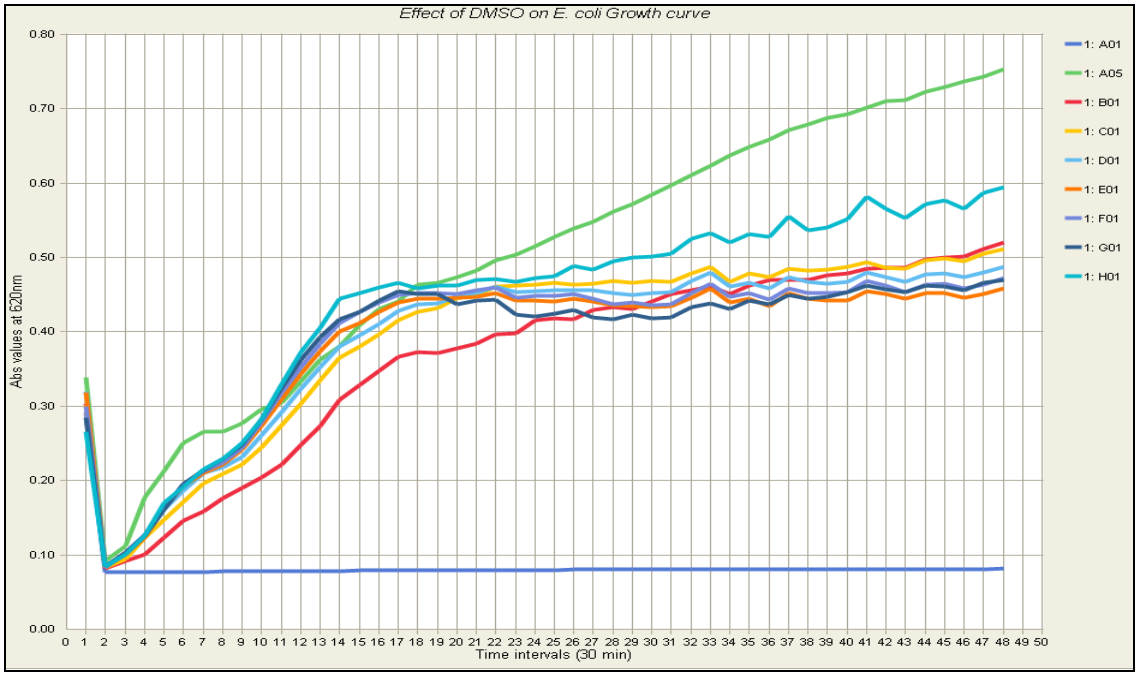

Figure 5.13. A01:blank, A05:negative control (195µl nutrient broth+ 5µl inoculum), B01:50 %DMSO , C01:25% DMSO, D01:12.5 % DMSO , E01:6.25 % DMSO, F01:3.125 % DMSO , G01:1.56 % DMSO , H01:0.78 %DMSO

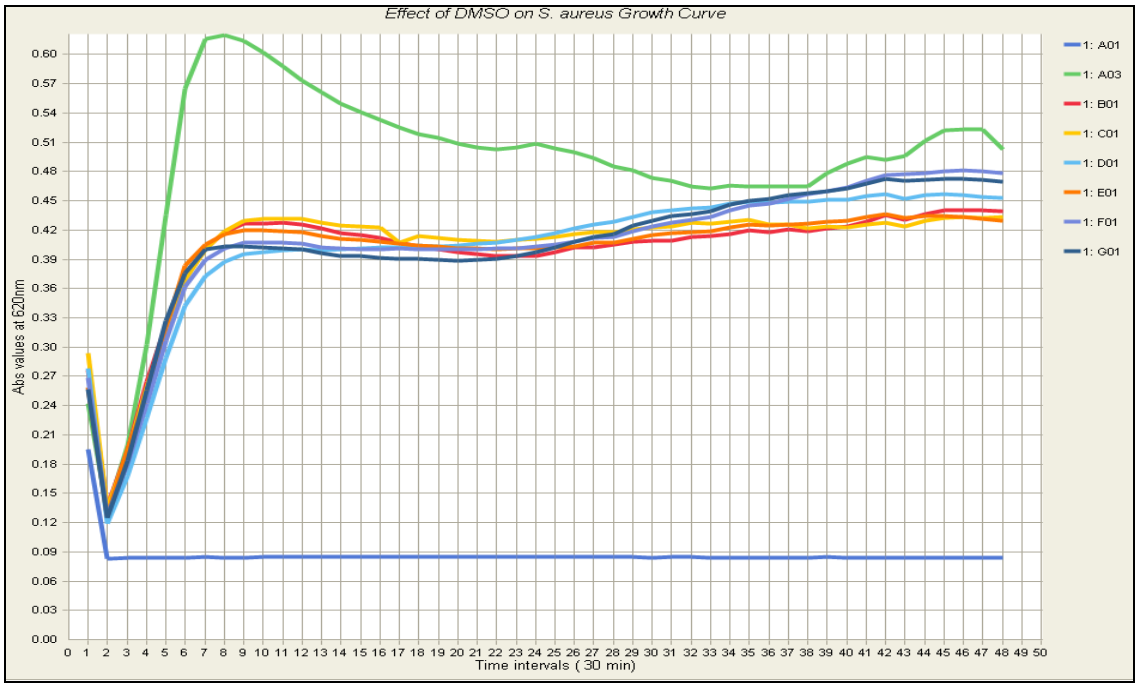

Figure 5.14. A01:blank, A03:negative control (195µl nutrient broth+ 5µl inoculum), B01:50 %DMSO , C01:25% DMSO, D01:12.5 % DMSO , E01:6.25 % DMSO, F01:3.125 % DMSO , G01:1.56 % DMSO , H01:0.78 %DMSO

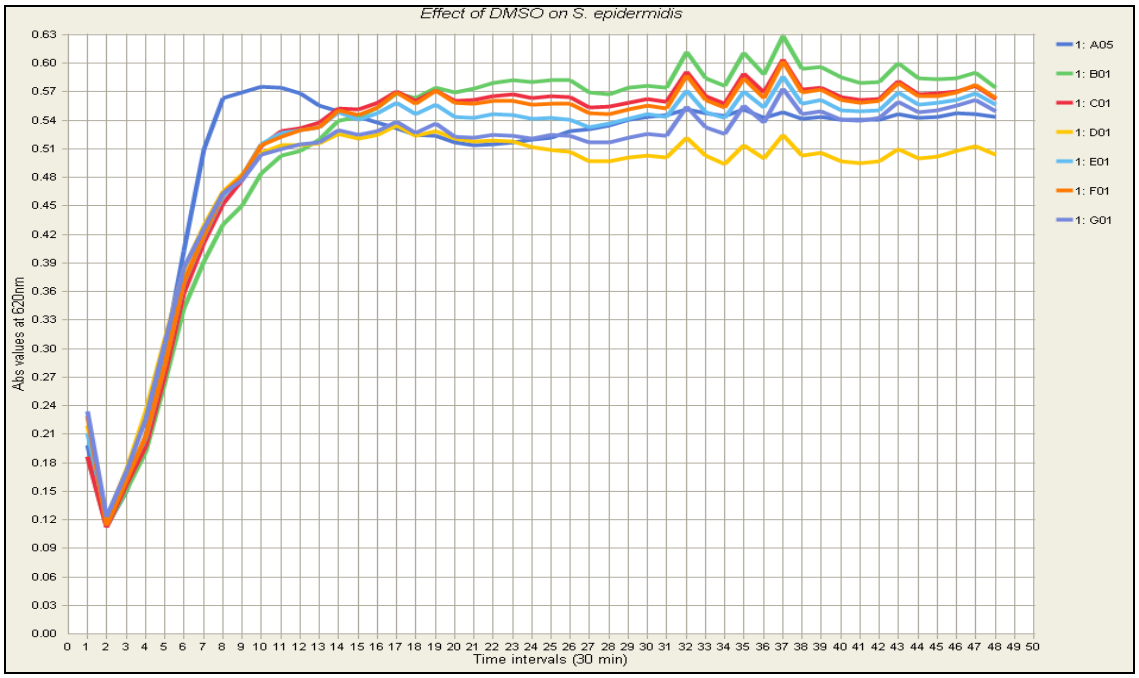

Figure5.15. A05:negative control (195µl nutrient broth+ 5µl inoculum), B01:50 %DMSO , C01:25% DMSO, D01:12.5 % DMSO , E01:6.25 % DMSO, F01:3.125 % DMSO , G01:1.56 % DMSO

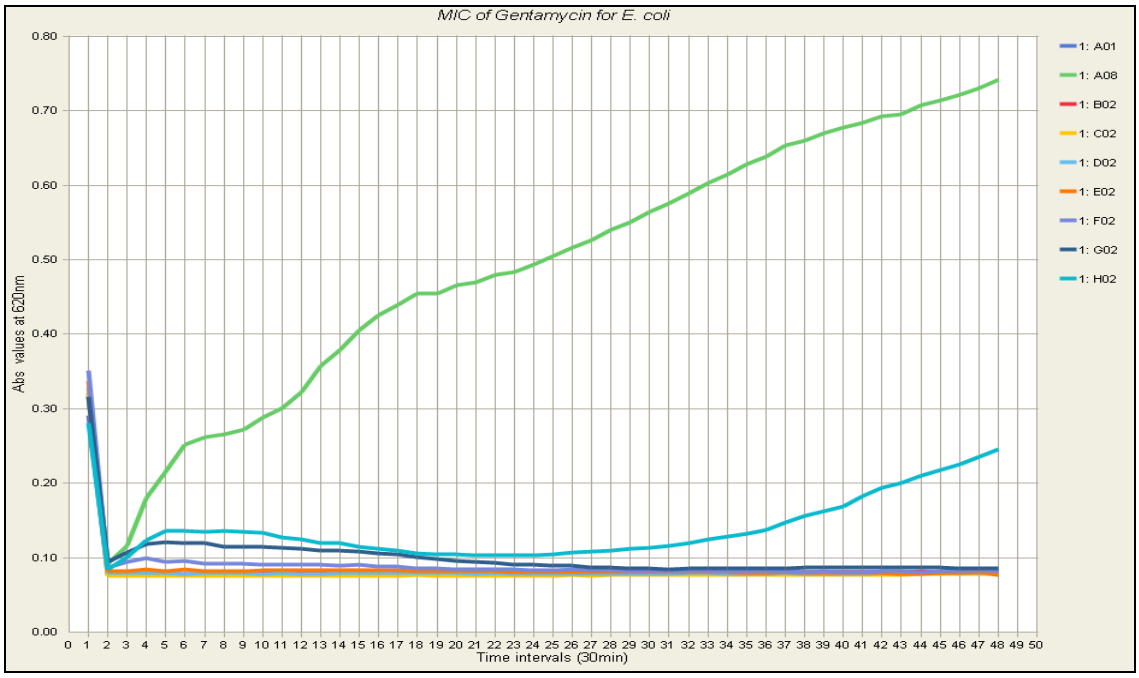

Figure 5.16. A01:blank, A08:negative control (195µl nutrient broth+ 5µl inoculum), B02:10µg/ml, C02:5 µg /ml ,D02:2.5 µg /ml , E02: 1.25 µg /ml, F02:0.625 µg /ml , G02:0.312 µg /ml , H02:0.156 µg /ml

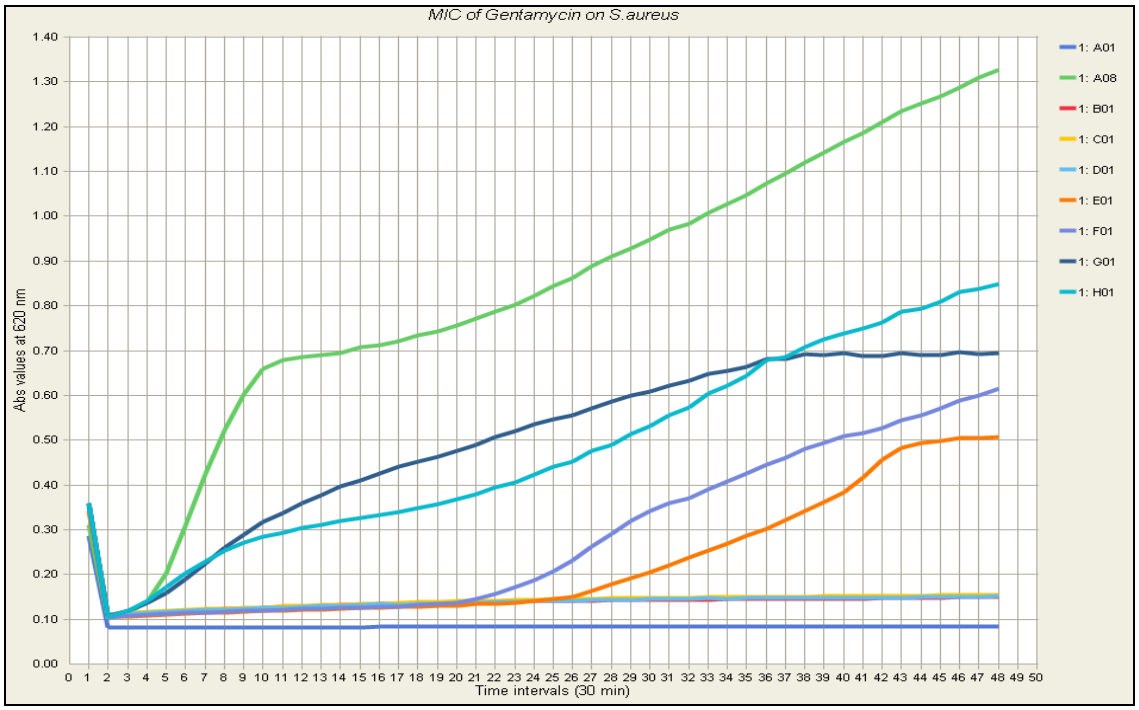

Figure 5.17. A01:blank, A08:negative control (195µl nutrient broth+ 5µl inoculum), B01: 10µg/ml, C01: 5 µg /ml ,D01: 2.5 µg /ml , E01: 1.25 µg /ml , F01: 0.625 µg /ml , G01: 0.312 µg /ml , H01: 0.156 µg /ml

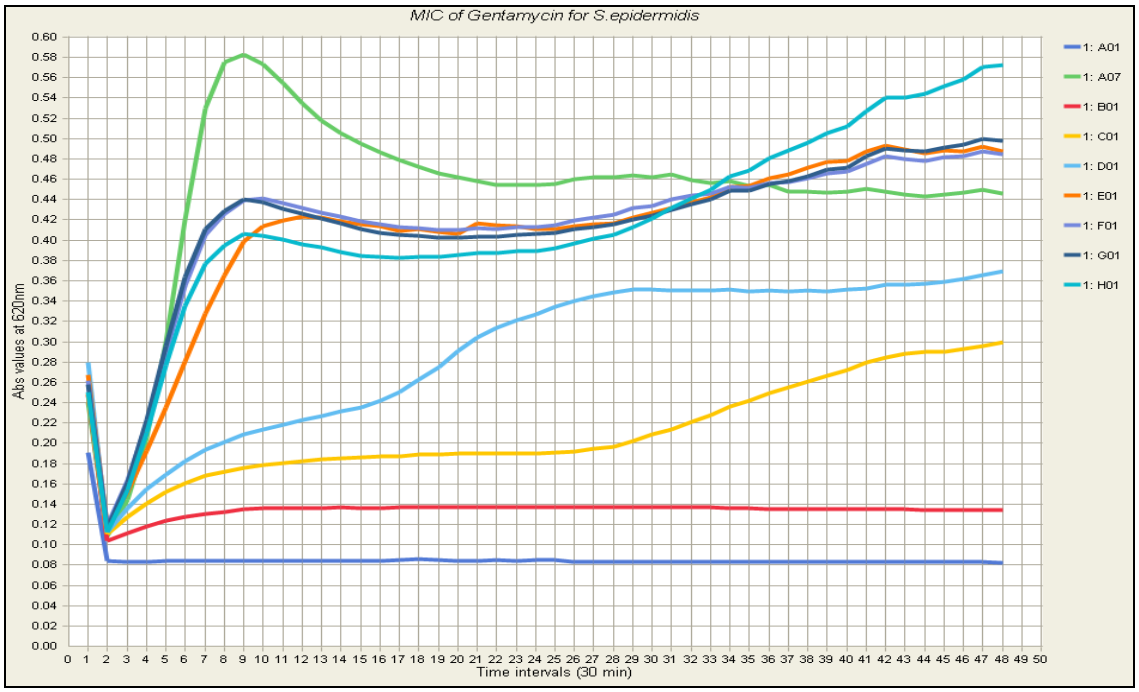

Figure 5.18. A01:blank, A07:negative control (195µl nutrient broth+ 5µl inoculum), B01: 100µg/ml, C01:10 µg /ml, D01:5µg /ml , E01: 2.5 µg /ml, F01: 1.25 µg /ml, G01: 0.625 µg /ml, H01: 0.312 µg /ml

However the whole MIC graphs were ploted by the software of the multiplate reader (Varioskan), increases in the starting points of OD values occured in the graphs of the wells containing the initial dilutions of extracts. This situation is based on the dark colours of plant extracs in the wells. So it was better to fix the growth curves starting from the same OD value (0 nm) by ploting them in excel programe. Some of the important species, their effects on bacterial growths and their MIC values are shown in Figures 5.20, 5.21, 5.22, 5.23, 5.24 and 5.25 below. In order to confirm the test results a visible indication for MICs was performed by using INT (Iodonitrotetrazolium chloride), a dye which reacts with the metabolic products of the microorganisms and forms a colour change in the wells as pink. This colour change indicates the presence of microbial growth in well. These visible confirmations for active plant species are shown beside the growth curve figures below. In these figures antibacterial effects of the most active species (*P. lentiscus*, *P. terebinthus*, *H. empetrifolium*, and *C. parviflorus*) were showed.

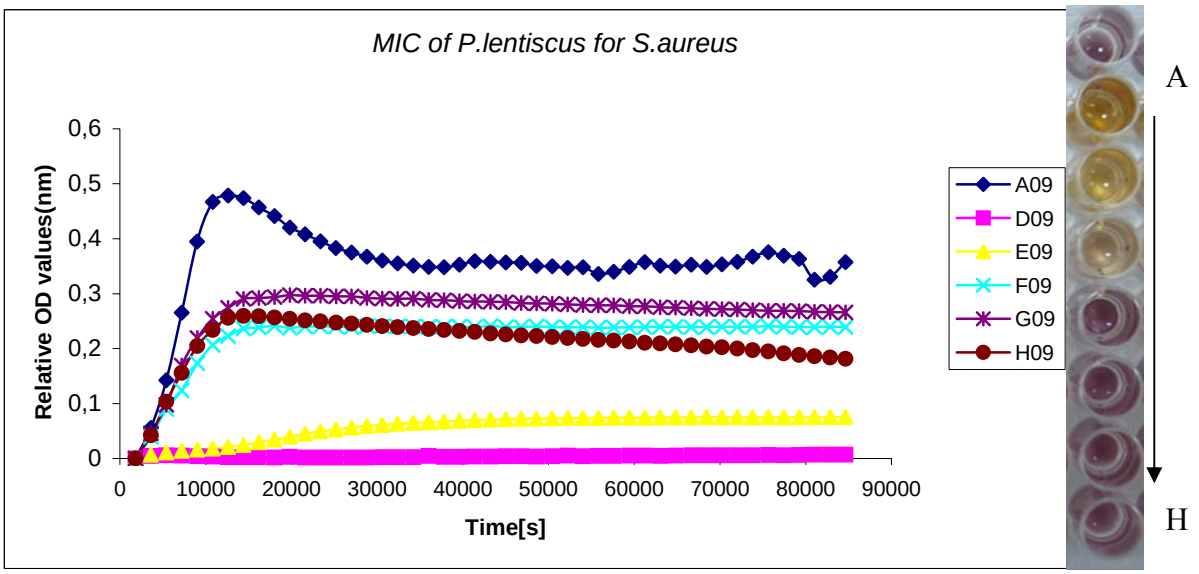

Figure 5.19. A09:negative control (195µl nutrient broth+ 5µl inoculum), D09:0.78 mg/ml , E09: 0.39mg/ml , F09:0.195 mg/ml , G09:0.097 mg/ml

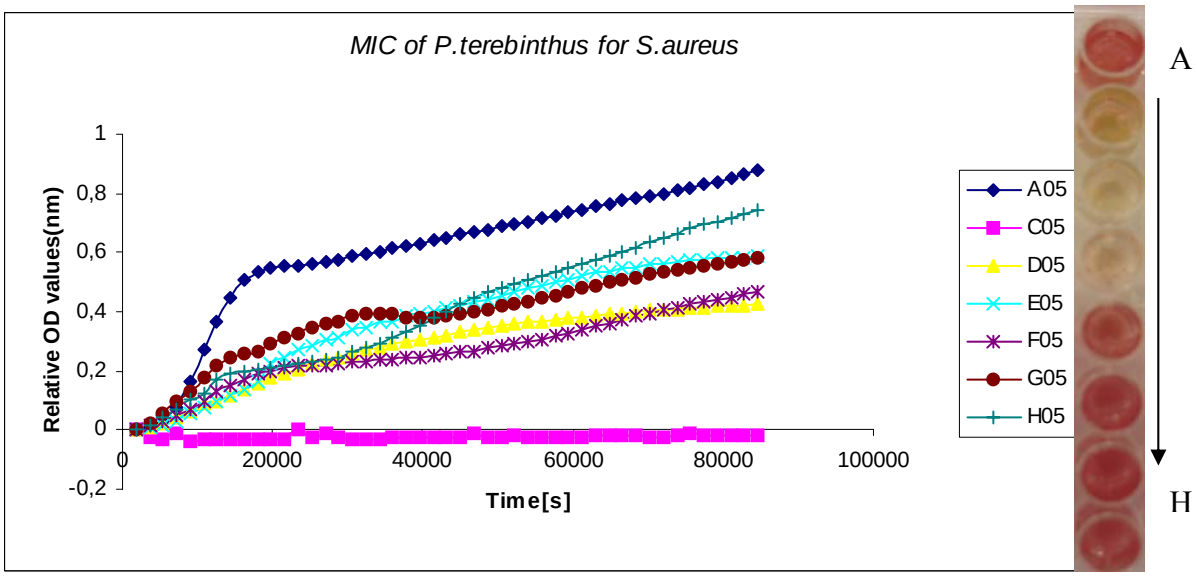

Figure 5.20.A05:negative control (195µl nutrient broth+ 5µl inoculum), C05:1.56 mg/ml, D05:0.78 mg/ml , E05: 0.39mg/ml , F05:0.195 mg/ml , G05:0.097 mg/ml, H05: 0.048 mg/ml

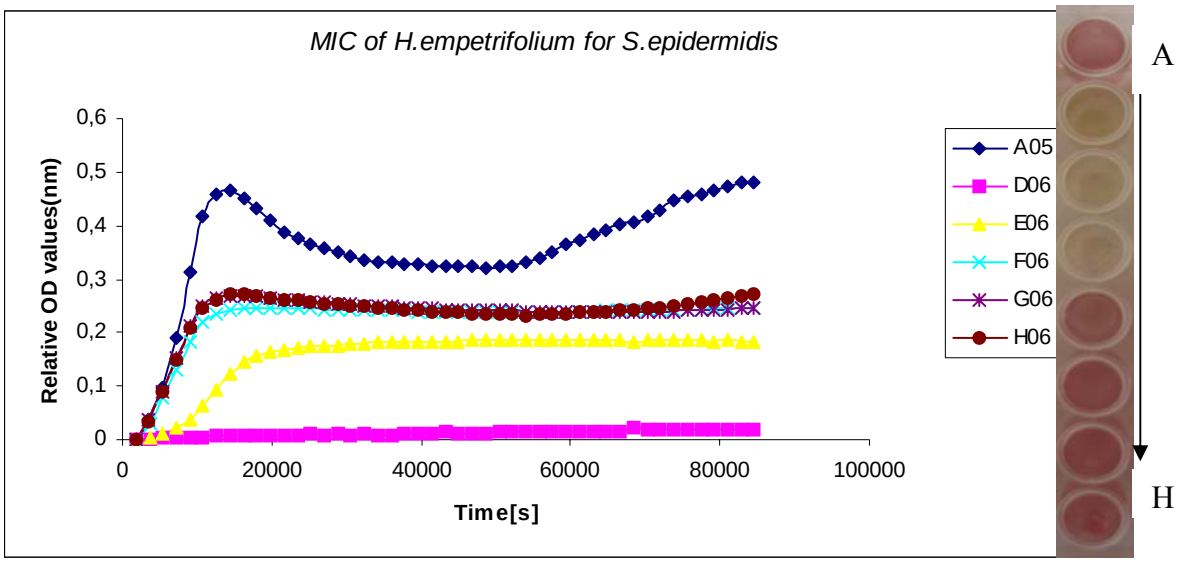

Figure 5.21. A05:negative control (195µl nutrient broth+ 5µl inoculum), D06:0.78 mg/ml , E06: 0.39mg/ml , F06:0.195 mg/ml , G06:0.097 mg/ml, H06: 0.048 mg/ml

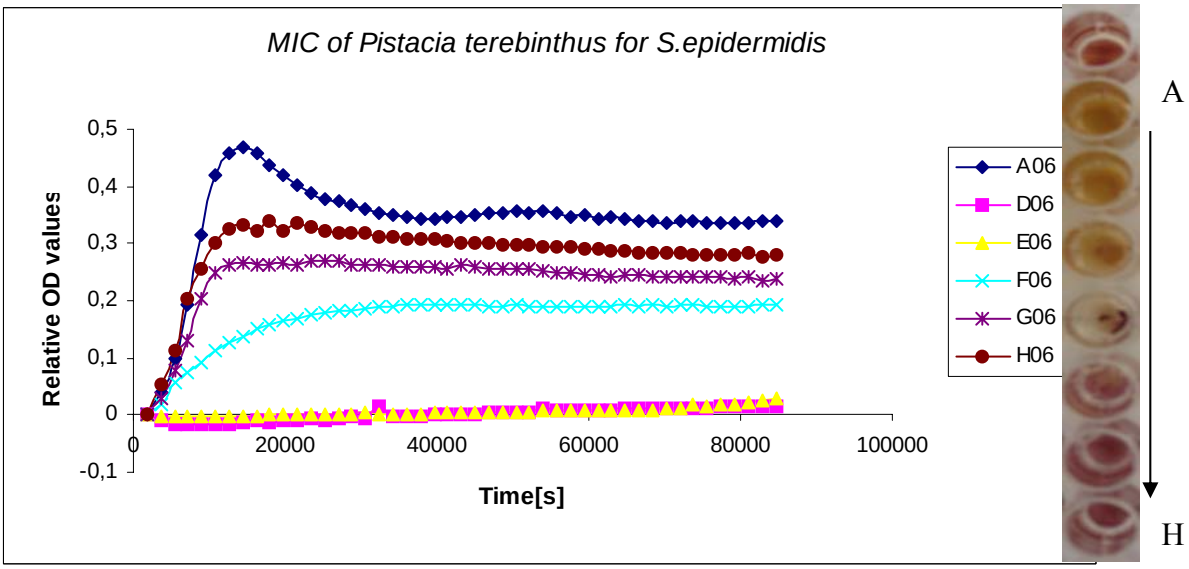

Figure 5.22.A06:negative control (195µl nutrient broth+ 5µl inoculum), D06:1.56 mg/ml , E06: 0.78mg/ml , F06:0.39 mg/ml , G06:0.195 mg/ml, H06: 0.097 mg/ml

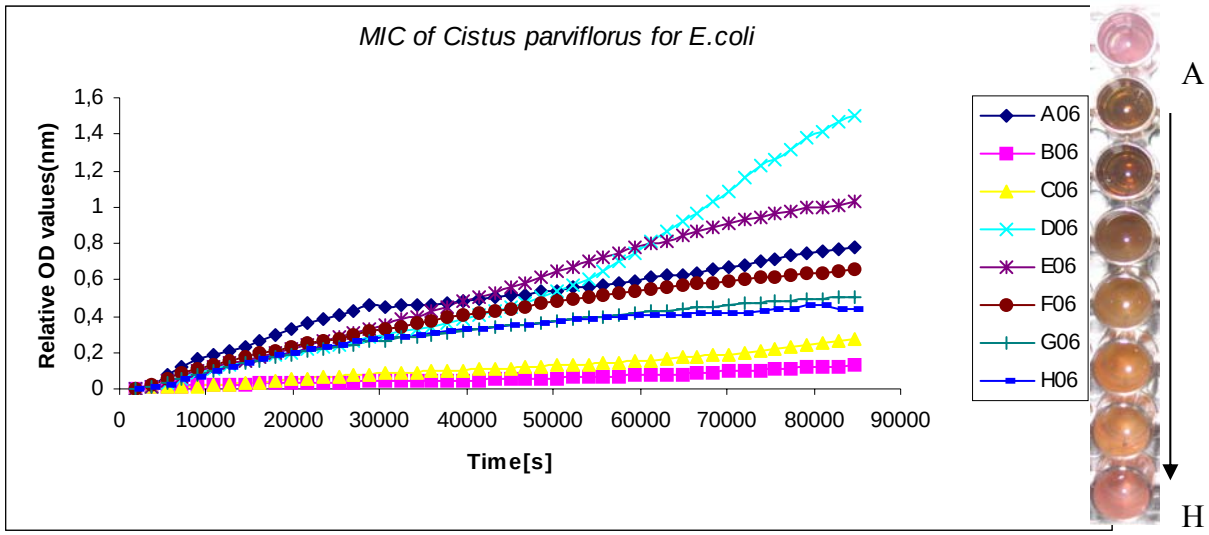

Figure 5.23. A06:negative control(195µl nutrient broth+ 5µl inoculum), B06:50mg/ml, C06:25 mg/ml ,D06: 12.5 mg/ml , E06: 6.25 mg/ml , F06: 3.125 mg/ml, G06: 1.56 mg/ml, H06: 0.78 mg/ml

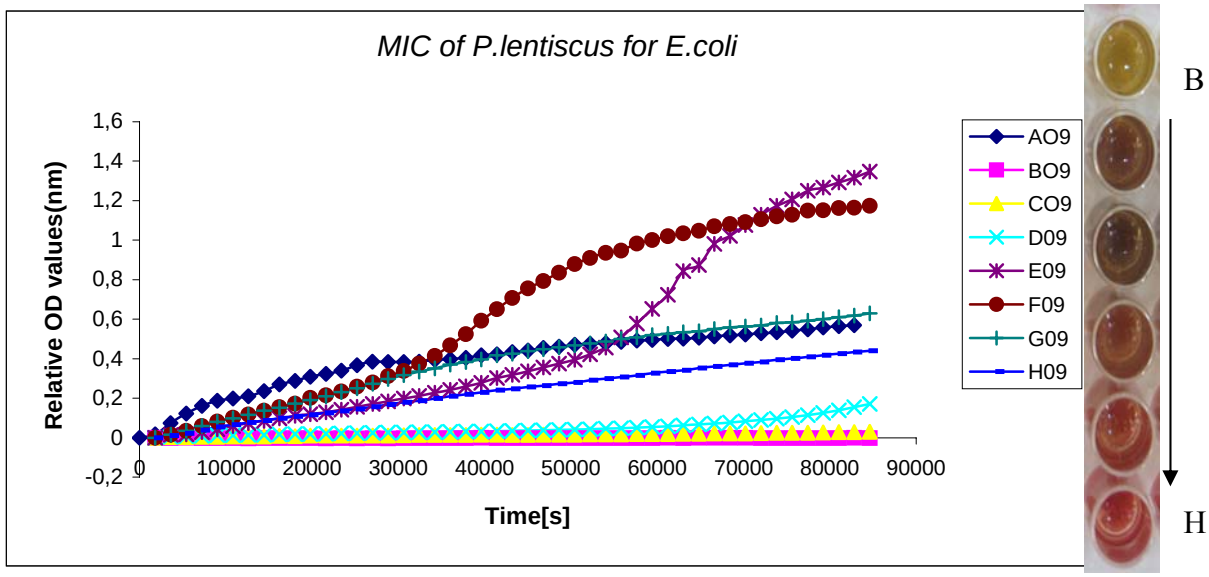

Figure 5.24. A09:negative control (195µl nutrient broth+ 5µl inoculum), B09:50 mg/ml, C09:25 mg/ml , D09:12.5 mg/ml , E09:6.25 mg/ml , F09:3.125 mg/ml, G09:1.56 mg/ml , H09:0.78 mg/ml

It is important to evaluate these active plants with their chemical constituents. Phytochemical analysis in literature showed that *Arbutus unedo* extracts contained flavonol glycosides and tannins (Pamukcuoğlu, et al. 2003) and its leaves contain tannin level of 37% besides arbutin (as glycoside), arbutoflavoneols, quercitrin, and rutin (Ayaz, et al. 2000, Males, et al. 2006).

Pistacia species are extensively used in folk medicine for the treatment of throat infections and ulcer. It is known that α-Tocopherol (vitamin E) is naturally occuring in Pistacia leaves (Kıvcak and Akay 2005). Pistacia species have antimicrobial potential besides antioxidant and anti-inflammatory activities particularly due to flavonoids, and other phenolic constituents (Topcu, et al. 2007). Antimicrobial effects of *H. empetrifolium* extracts are not investigated before except a study detected its antimicrobial potential with disc diffusion method by Meral (2002). It is a health promising plant species against infectios diseases and has not been fully investigated. *C. parviflorus* has been researched in detail for its antimicrobial activities of volatile constituents, but still not extensively examined for its leaf extracts.

CHAPTER 6

CONCLUSION

Medicinal plants are the important natural raw materials in food, cosmetic, and pharmaceutical industries due to their several biological activities such as, antioxidant, antimicrobial, anticancer, and antiinflammatory, etc. In this study 42 plant species collected from Karaburun region of İzmir were examined with several analysis in order to determine their relative total antioxidant and antibacterial activities and also for their total phenol contents.

This thesis mainly comprises the screening studies of some biological activities but the first step of this study was the preparation and extraction of the collected plant species. Extraction yields for each species were obtained after a standardized solvent extraction protocol. The most efficient extraction yield was obtained for *Hypericum empetrifolium* as 37.17 %, and followed with *Arbutus unedo* (35.81 %) and *Vitex agnus castus* (32.04 %) species.

After the extraction process, biochemical analysis were performed for each plant extract. Firstly, the total phenol contents of extracts were determined by Folin-ciocalteu method and according to results, *Sarcopterium spinosum* exhibited the highest phenol content as 635.26 GAEqmg/g sample. The other species with the high phenol contents are *P.terebinthus* (567.6 GAEqmg/g sample) and *C. parviflorus* (552.7 GAEqmg/g sample).

Secondly, relative total antioxidant activities were determined by using the photochem antioxidant analyzer which can detect the lipid soluble and water soluble antioxidant compounds of an extract seperately in a single system. According to the results *Hypericum empetrifolium* showed highest antioxidant activities for both lipid soluble and water soluble compounds with 1889 µg trolox/mg sample and 1866.39 µg asc.acid/mg sample respectively. The other important species for the antioxidant capacities are *Teucrium chamaedrys* and *Salvia virgata* for water soluble antioxidants and *Phillyrea latifolia*, *V. agnus castus* for lipid soluble antioxidants.

In the next step the antibacterial activities of plant extracts were evaluated by using disc diffusion assays and microdilution assays. In order to detect the plant species having the antibacterial properties, a preliminary screening study was performed by

using disc diffusion assay. In the disc diffusion assays *E. coli*, *Bacillus subtilis*, and *Enterococcus faecium* were used. The disc diffusion test results showed that, out of the 42 plant species tested, 26 species exhibited antibacterial activities by inhibiting one or more microorganisms. Among the plants screened 9 species showed promising antibacterial activities against all tested microorganisms.

Finally the microdilution tests were performed by 96 well plates for some selected species, especially, the ones that are previously confirmed for their antibacterial activities by disc diffusion assays. These species were also compared with some negative and positive controls. The best efficient MICs values were determined for *H. empetrifolium*, *P. terebinthus*, *Arbutus unedo*, and *C. Parviflorus.*

In conclusion, some potential species were identified for their antioxidant, antibacterial, and total phenol contents in particular experimental conditions. It is difficult to make a quantitive comparison with other screening studies for the species in this study but relative comparisons of some studies confirm our results for some species. *Sarcopterium spinosum* exhibited the highest phenol content and important antioxidant and antibacterial activities in this study. This species has never been examined scientifically before and in that manner it is quite important as a new natural source for some related industries.

In the study there is a clear relationship between the analysis results. Especially between the total phenol content results and antibacterial activity results have an obvious relationship. Besides the species with the most efficient extraction yields also exhibited the highest antibacterial and antioxidant activities and total phenol contents.
This study presented new potential species that can be used as raw materials in some related industries and also also presented the first scientific evaluation of Karaburun flora of İzmir.

REFERENCES

Aboaba, O.O., S.I. Smith., F.O. Olude. 2006. Antibacterial effect of edible plant extract on *Escherichia coli* 0157:H7. *Pakistan Journal of Nutrition* 5: 325-327.

Akkol, E.K., F. Goger., M. Kosar., K.H.C. Baser. 2008. Phenolic composition and biological activities of *Salvia halophila* and *Salvia virgata* from Turkey. *Food Chemistry* 108: 942–949.

Aksoy, A., N. Duran., K. Köksal. 2006. In vitro and in vivo antimicrobial effects of mastic chewing gum against Streptococcus mutans and mutans streptococci. *Archives of Oral Biology* 51:476-481.

Antolovich, M., P.D. Prenzler., E. Patsalides., S. Mcdonal., K. Robards. 2002. Methods for testing antioxidant activity. *The Analyst* 127:183-198.

Ateş, D.A. and Ö.T. Ertuğru. 2003. Antimicrobial activities of various medicinal and commercial plant extracts. *Turk J Biol* . 27:157-162

Atlas, R. M., A.E. Brown., L.C. Parks. eds. 1995. Experimental microbiology-laboratory manual. edited by Mosby-Year Book, Inc.

Auddy, B., M. Ferreira., F. Blasina., L. Lafon., F. Arredondo., F. Dajas., P.C. Tripatri., T. Seal., B. Mukherjee. 2003. Screening of antioxidant activity of three Indian medicinal plants, traditionally used for the management of neurodegenerative diseases. *Journal of Ethnopharmacolog* 84:131-138.

Ayaz, F. A., M. Kucukislamoglu., M.R. Sugar. 2000. Non-volatile and phenolic acids composition of strawberry tree (*Arbutus unedo* L. var. *ellipsoidea*) Fruits. *Journal of Food Composition and Analysis* 13:171-177.

Balunas, M.J. and A. Kinghorn. 2005. Drug discovery from medicinal plants. *Life Sciences* 78: 431-441.

Benli, M., K. Güney., U. Bingöl., F. Geven., N. Yiğit. 2007. Antimicrobial activity of some endemic plant species from Turkey. *African Journal of Biotechnology* 6 (15): 1774-1778.

Benli, M., U. Bingol., F. Geven., K. Guney., N. Yigit. 2008. An investigation on the antimicrobial activity of some endemic plant species from Turkey. *African Journal of Biotechnology* 7:001-005.

Boskou, G., F.N. Salta., S. Chrysostomou., A. Mylona., A. Chiou., N.K. Andrikopoulos. 2006. Antioxidant capacity and phenoic profile of table olives from Greek market. *Food Chemistry* 94:558-564.

Bouayed , J., K. Piri., H. Rammal., A. Dicko., F. Desor., C. Younos., R. Soulimani. 2007. Comparative evaluation of the antioxidant potential of some Iranian medicinal plants. *Food Chemistry* 104: 364–368

Carcache, E. J., M. Cuendet., E.J. Park., B.N. Su., J.F. Rivero., N.M. Farnsworth., J.M. Pezzuto., A.D. Kinghorn. 2006. Potential cancer chemopreventive agents from *Arbutus unedo*. *Natural Product Research* 20: 327-334.

Carbone. M. 2005. Stirring it up with some good , old, common sense. *Footprints* 1.

Cetin, H. and A. Yanikoglu. 2006. A study of the larvicidal activity of *Origanum* (Labiatae) species from southwest Turkey. *Journal of Vector Ecology* 31 :No.1

Chai, C.W., S.C. Kim., S.S. Hwang., B.K. Chai., H.J. Ahn., M.Y. Lee., S.H. Park., S.K. Kim. 2002. Antioxidant activity and free radical scavenging capacity between Korean medicinal plants and flavonoids by assay-guided comparison. *Plant Science* 163:1161-1168.

Chanwitheesuk, A., A. Teerawutgulrag., N. Rakariyatham. 2005. Screening of antioxidant activity and antioxidant compounds of some edible plants of Thailand. *Food Chemistry* 92:491-497.

Cloutier, M.J. 1995. Antibiotics: Mechanisms of action and the acquisition of resistance-when magic bullets lose their magic. *American Journal of Pharmaceutical Education* 59:167-172.

Coskun, M. And A.M.G. Özkan. 2005. Global phytochemistry: The Turkish frame. *Phytochemistry* 66:956–960

Courvalin, P. 2005. Antimicrobial Drug Resistance: "Prediction is very difficult, especially about the future" *Emerging Infectious Diseases* 11.

Cowan, M.M. 1999. Plant products as antimicrobial agents. *Clinical Microbiology Reviews* 12:564-582.

Dargatz, J.L.T., D. Dargatz., P.S. Morley. 2002. Antimicrobial resistance: what's the big deal? Importance of antimicrobial resistance to the equine practitioner. *Medicine* 48:138-144.

Dasgupta, N. and B. De. 2007. Antioxidant activity of some leafy vegetables of India:a comparative study. *Food Chemistry* 101:471-474.

Dekker, T.G., T.G. Fourie., F.O. Snyckers., C.J. Van der Schyf. 1983. Studies of south African medicinal plants , caspitin, a new phloroglucinal derivative with antimicrobial properties from *Helichrysum caespititum* South Africa. *Journal of Chem* 36(4):114-116

Dhawan, B.N. 2003. WHO monographs on selected medicinal plants. *Indian J Med.* 3:97-98

Dıgrak, M., A. Ilçım., M.H. Alma., S. Sen. 1999. Antimicrobial activites of the extracts of various plants (valex, mimosa bark, gallnut powders, Salvia sp. and Phlomis sp.). Tr. *J. of Biology* 23:241-248

Djeridane, A., M. Yousfi., B. Nadjemi., D. Boutassouna., P. Stocker., N. Vidal. 2006. Antioxidant activity of some Algerian medicinal plant extracts containing phenolic compounds. *Food Chemistry* 97:654-660.

Duffy, C.F. and R.F. Power. 2001. Antioxidant and antimicrobial properties of some Chinese plant extracts. *International Journal of Antimicrobial Agents.* 17:527-529.

Dulger, B. 2005. An investigation on antimicrobial activity of endemic *Origanum solymycum* and *Origanum bilgeri* from Turkey. *Afr. J. Trad.* 2 (3): 259 -263

Duraipandiyan, V., M. Ayyanar, S. Ignacimuthu. 2006. Antimicrobial activity of some ethnomedicinal plants used by Paliyar tribe from Tamil Nadu, India. *BMC Complementary and Alternative Medicine*. 6:35 doi:10.1186/1472-6882-6-35

Eldeen, I.M.S., E.E. Elgorashi., J.V. Staden. 2005. Antibacterial, anti-inflammatory, anti-cholinesterase and mutagenic effects of extracts obtained from some trees used in South African traditional medicine. *Journal of Ethnopharmacology* 102:457–464

Emerit, J., M. Edeas., Bricaire. 2004 Neurodegenerative diseases and oxidative stress. *Biomedicine&Pharmacotherapy* 58:39-46.

Groot, M.J. and J.V.D. Roest. 2006. Quality control in the production chain of herbal products. *Medicinal and Aromatic Plants* 253-260.

Goyal, B.R., R.K. Goyal., A.A. Mehta. 2007. Phyto-pharmacology of *Achyranthes aspera* : A Review. *Pharmacognosy Reviews* 1

Gülçın, İ., M. Oktay, E. Kireçci., Ö.İ. Küfrevioğlu. 2003. Screening of antioxidant and antimicrobial activities of Anise (*Pimpirella anisum* L.) seed extracts. *Food Chemistry*.83:371-382.

Gülçin, İ., O.I. Küfrevioğlu., M. Oktay., M.E. Büyükokuroğlu. 2004. Antioxidant, antimicrobial, antiulcer and analgesic activities of nettle (*Urtica dioica* L.) *Journal of Ethnopharmacology* 90:205-215.

Hansen, S.L.1996. Review criteria for assessment of antimicrobial susceptibility test discs. *Food and Drug Administration Center for Devices and Radiological Health* 301: 594-2096.

Harrison, K. and L.M. Were. 2007. Effect of gamma irradiation on total phenolic content yield and antioxidant capacity of almond skin extracts. *Food Chemistry* 102:932-937.

Heim, K.E., A.R. Tagliaferro., D.J. Bobilya. 2002. Flavonoid antioxidants: chemistry, metabolism and structue-activity relationships. *The Journal of Nutritional Biochemistry* 13:572-584.

Hirobe, C., Z.S. Qiao., K. Takeya., H. Itokawa. 1997. Cytotoxic flavonoids from *Vitex agnus – castus*. *Phytochemistry* 46: 521-524.

Hoareau, L. and E.J. DaSilva. 1999. Medicinal plants: a re-emerging health aid. *EJB Electronic Journal of Biotechnology* 2: 0717-3458

Holley, A. R. and D. Patel. 2005. Improvement in shelf-life and safety of perishable foods by plant essential oils and smoke antimicrobials. *Food Microbiology* 22: 273-292.

Husain, G.M., D. Mishra., P.N. Singh., C.V. Rao., V. Kumar. 2007. Ethnopharmacological review of native traditional medicinal plants for brain disorders. *Pharmacognosy Reviews* 1.

Ics-Unido. 2006. South-East Asian (SEA) regional workshop on ″extraction technologies for medicinal and aromatic plants. *International Centre for Science and High Technology*.

Ito, H., K. Yamaguchi., T.H. Kim., S. Khennouf., K. Gharzouli., T. Yoshida. 2002. Dimeric and trimeric hydrolyzable tannins from *Quercus coccifera* and *Quercus suber. J. Nat. Prod*. 65: 339-345

Iwu, M.M., A.R. Duncan., C.O. Okunji. 1999. New antimicrobials of plant origin *Perspectives on new crops and new uses*.

Katalinic, V., M. Milos., T. Kulisic., M. Jukic. 2006. Screening of 70 medicinal plant extracts for antioxidant capacity and total phenols. *Food Chemistry* 94:550-557.

Kıvcak, B. and A. Akay. 2005. Quantitative determination of a-tocopherol in *Pistacia lentiscus*, *Pistacia lentiscus* var. chia, and *Pistacia terebinthus* by TLC-densitometry and colorimetry. *Fitoterapia* 76: 62–66

King, A.G. and G. Young. 1999. Characteristics and occurrence of phenolic phytochemicals. *Journal of the American Dietetic Association* 99:213-218.

Kordali, S., A. Cakir., H. Zengin., M.E. Duru. 2003. Antifungal activities of the leaves of three *Pistacia* species grown in Turkey. *Fitoterapia* 74:164–167.

Kris-Etherton, P.M., M. Lefevre., G.R. Beecher., M.D. Gross., C.L. Keen., T.D. Etherton. 2004. Bioactive compounds in nutrition and health-research methodologies for establishing biological function: the antioxidant and anti-inflammatory effects of flavonoids on atherosclerosis annu. *Rev. Nutr*. 24:511–38

Kuete,V., R. Metunob., B. Ngamenib., A.M. Tsafack., F. Ngandeuc., G.W. Fotso., M. Bezabih., F-X. Etoa., B.T. Ngadjui., B.M. Abegaz., V.P. Benga. 2007. Antimicrobial activity of the methanolic extracts and compounds from Treculia obovoidea (Moraceae).*Journal of Ethnopharmacology* 112 :531–536

Lako, J., Trenerry, V.C., M. Wahlqvist., N. Wattanapenpaiboon., S. Sotheeswaran., R. Premier. 2007. Phytochemical flavonols, carotenoids, and the antioxidant properties of a wide selection of fijian fruit, vegetables and other readily available foods. *Food Chemistry* 101:1727-1741.

Lampinen, J. 2005. Continous antimicrobial susceptibility testing in drug discovery. *Drug Plus International*.

Lee, S.E., H.J. Hwang., H.S. Jeang., J.H. Kim. 2003. Screening of medicinal plant extracts for antioxidant activity. *Life Sciences* 73:167-179.

Li, B.B., B. Smith., M.M. Hossain. 2006. Extraction of phenolics from citrus peels I. Solvent extraction method. *Separation and Purification Technology* 48:182–188,(a).

Li, B.B., B. Smith., M.M. Hossain. 2006. Extraction of phenolics from citrus peels II. Enzyme-assisted extraction method. *Separation and Purification Technology* 48:189–196, (b).

Liu, R.H. 2004. Potential synergy of phytochemicals in cancer prevention: mechanism of action. *J. Nutr.* 134: 3479S–3485S.

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

Ljubuncic, P., H. Azaizeh., I. Portnaya., U. Cogan., O. Said., K.A. Saleh., A. Bomzon. 2005. Antioxidant activity and cytotoxicity of eight plant used in traditional Arab medicine in Israil. *Journal of Ethnopharmacology* 99:43-47.

Lowy, F. D.2003. Antimicrobial resistance: the example of Staphylococcus aureus. *Science in medicine* 111:1265–1273.

Madhavi, D.L., A.D. Kulkarni., S.S. Nimbalkar., S.J. Jadhav. eds. 1996. Lipid oxidation in biological and food systems' in food antioxidants, edited by Madhavi, D.L., Deshpande, S.S., Salunkhe, D.K. (Marcel Dekker, Inc: New york).

Maisuthisakul, P., M. Suttajit., R. Pongsawatmanit. 2007. Assessment of phenolic content and free radical –scavenging capacity of some Thai indigenous plants. *Food Chemistry* 100: 1409-1418.

Males, Z., M. Plazibat., V.B. Vundac., I. Zuntar. 2006. Qualitative and quantitative analysis of flavonoids of the strawberry tree – *Arbutus unedo* L. (*Ericaceae*). *Acta pharm*. 56: 245-250.

Mandal, V., Y. Mohan., S. Hemalatha. 2007. Microwave Assisted Extraction – An ınnovative and promising extraction tool for medicinal plant research. *Pharmacognosy Reviews* 1.

Mantle, D., F. Eddeb., A.T. Pickering. 2000. Comparison of relative antioxidant activities of British medicinal plant species in vitro. *Journal of Ethnopharmacology* 72:47-51.

Margrit, K. J. and S. Tirok. 2000. Novel Food: Determination of antioxidants in food analysis *Analytik Jena AG.*

Matkowski, A. and M. Piotrowska. 2006. Antioxidant and free radical scavenging activities of some medicinal plants from the Lamiaceae. *Fitoterapia* 77:346-353.

Matthias, R. Trend in Bioanalysis: Analysis of antioxidant by chemiluminescence. *Analytik Jena AG*.

Mendonça, R.R. 2006. Bioactive phytocompounds: new approaches in the phytosciences. *Modern Phytomedicine* 23:457-478

Meral, G. E. and S. Konyalıoğlu. 2004. Üç Hypericum L. türünün antioxidan etkilerini incelenmesi. bitkisel ilaç hammaddeleri toplantısı, Bildiriler. 36:567-580

Mosaddik, M.A., L. Banbury., P. Forster., R. Bocth., J. Mark., D. Laech., P.G. Waterman. 2004. Screening of some Australian flacourtiaceae species for in vitroantioxidant , cytotoxic and antimicrobial activity. *Phytomedicine* 11:461-466.

Neergheen, V.S., M.A. Socbrattee., T. Bahorun., O. Aruoma. 2005. Characterization of the phenolic constituents in Mauritian endemic plants as determinants of their antioxidant activities in vitro, *Journal of Plant Physiology*.

O'Shea, R. and H.E. Moser. 2007. Physicochemical properties of antibacterial compounds: implications for drug discovery. *J. Med. Chem.* 26: 234-238

Pamukcuoglu, A., B. Kıvcak., M. Basa., T. Mert. 2003. Antioxidant activity of *Arbutus unedo* leaves. *Fitoterapia* 74:597–599.

Pankey, G.A. and L.D. Sabath. 2004. Clinical relevance of bacteriostatic versus bactericidal mechanisms of action in the treatment of gram- positive bacterial infections. *Clinical Infectious Diseases* 38: 864–70

Panovska, T. K., S. Kulevanova., M. Stefova. 2005. *In vitro* antioxidant activity of some *Teucrium* species *(Lamiaceae). Acta Pharm*. 55:207-214.

Patel, G.P. and C.W. Crank. 2005. Antimicrobial resistance in gram-positive and gram-negative bacteria

Pokorny, J. 2007. Are natural antioxidants better – and safer – than synthetic antioxidants? *Eur. J. Lipid Sci. Technol.* 109:629–642

Popov, I. and G. lewin. 1999. Antioxidative homeostasis:charachterization by means of chemiluminescent technique. *Methods in Enzymology* 36:177-87.

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

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

Proestos, C., D. Sereli., M. Komaitis. 2006. Determination of phenolic compounds in aromatic plants by RP-HPLC and GC-MS. *Food Chemistry* 95:44-52.

Ramos, A., A. Visozo., J. Piloto., A. Garcia., C.A. Rodrigez., R. Rivero. 2003. Screening of antimutagenicity via antioxidant activity in Cuban medicinal plants. *Journal of Ethnopharmacology* 87:241-246.

Raskin, I., D.M. Ribnicky., S. Komarnytsky., N. Ilic., A. Poulev., N. Borisjuk., A. Brinker., D.A. Moreno., C. Ripoll., N. Yakoby., J. M.O'Neal., T. Cornwell., I. Pastor., B. Fridlender. 2002. Plants and human health in the twenty-first century *Trends in Biotechnology* 20: No.12

Reeks, B.Y., F.R. Champlin., D.B. Paulsen., D.W. Scruggs., M.L. Lawrence. 2005. Effects of sub-minimum inhibitory concentration antibiotic levels and temperature on growth kinetics and outer membrane protein expression in Mannheimia haemolytica and Haemophilus somnus. *The Canadian Journal of Veterinary Research* 69:1–10.

Rice-Evans, C.A., N.J. Miller., G. Paganga. 1997. Antioxidant properties of phenolic compouns. *Trends in Plant Science Reviews*. 2:1360-1385.

Sarac, N. and A. Ugur. 2007. Antimicrobial activities and usage in folkloric medicine of some Lamiaceae species growing in Mugla, Turkey. *EurAsia J BioSci*. 4:28-37

Satıl, F., T. Dirmenci., G.Tümen., T. Yusuf. 2008. Commercial and ethnic uses of *Satureja* (sivri kekik) species in Turkey. *Ekoloji* 67:1-7.

Scorzoni, L., T.L. Benaducci., A.M.F. Almeida., D.H.S. Silva., V.S. Bolzani., M.J.S. Mendes-Giannini. 2007. Comparative study of disk diffusion and microdilution methods for evaluation of antifungal activity of natural compounds against medical yeasts Candida spp and Cryptococcus sp. *Rev. Ciênc. Farm. Básica Apl*. 28:25-34.

Serafini, M. 2006. The role of antioxidants in disease prevention. *Nutrition and coexisting disease* 34:533-535.

Sezik, E., E. Yeşilada., G. Honda., Y. Takaishi., Y. Takeda., T. Tanaka. 2001. Traditional medicine in Turkey X. Folk medicine in Central Anatolia. *Journal of Ethnopharmacology* 75: 95–115.

Shaalan, E. A.-S., D. Canyonb., M.W.F. Younesc., H. Abdel-Wahaba., A.-H. Mansour. 2005. A review of botanical phytochemicals with mosquitocidal potential. *Environment International* 31:1149 – 1166.

Sharaf, M., M.A.E. Ansari., N.A.M. Saleh. 2000. Quercetin triglycoside from *Capparis spinosa*. *Fitoterapia* 71:46-49.

Singh, A.P. 2006. Distribution of Steroid like Compounds in Plant Flora. *Pharmacognosy Magazine* Vol 2:87-89.

Sokmen, A., B.M. Jones. M. Erturk. 1999. The in vitro antibacterial activity of Turkish medicinal plants. *Journal of Ethnopharmacology* 67:79-86.

Sousa, A., I.C.F.R. Ferreira., Calhelha, R.P.B. Andrade., P. Valentao., R. Seabra., L. Estevinho., A. Bento., J. A. Pereiraa. 2006. Phenolics and antimicrobial activity of traditional stoned table olives 'alcaparra'. *Bioorganic & Medicinal Chemistry* 14:8533-8538.

Stephen, J., J. Cavalieri, Ronald., S. Harbeck, Yvette., H. O. McCarter, José. 2005. Manual of antimicrobial susceptibility testing.

Stevenson, D. E. and R. D. Hurst. 2007. Polyphenolic phytochemicals – just antioxidants or much more? *Cell. Mol. Life Sci*. 64: 2900 – 2916.

Stocker, P ., M. Yousfi., O. Djerridane., J. Perrier., R. Amziani., S.E. Boustani., A. Moulin. 2004. Effect of flavonoids from various Mediterranean plants on enzymatic activity of intestinal carboxylesterase. *Biochimie* Vol .86:919-925.

Surveswaran, S., Y.Z. Cai., H. Corke., M. Sun. 2007. Systematic evaluation of natural phenolic antioxidants from 133 Indian medicinal plants. *Food Chemistry* 102: 938-953.

Tawaha, K., F.Q. Alali., M. Gharaibeh., M. Mohammad., T El-Elimat. 2007. Antioxidant activity and total phenolic content of selected Jordanian plant species. *Food Chemistry* 104: 1372–1378.

Tepe, B., M. Somken., H.A. Akpulat., A. Somken. 2006. Screening of the antioxidant potentials of Six S*alvia* species from Turkey. *Food Chemistry* 95:200-204.

The Local Food-Nutraceuticals Consortium. 2005. Understanding local Mediterranean diets: A multidisciplinary pharmacological and ethnobotanical approach. *Pharmacological Research* 52:353-366.

Trombetta, D., F. Castelli., M.G. Sarpietro., V. Venuti., M. Cristani., C. Daniele., A. Saija., G. Mazzanti., G. Bisignano1. 2005. Mechanisms of antibacterial action of three monoterpenes. *Antimicrobial Agents and Chemotheraphy* 49:2474-2478.

Topcu, G., M. Ay., A. Bilici., C. Sarikurkcu., M. Öztürk., A. Ulubelen. 2007. A new flavone from antioxidant extracts of Pistacia terebinthus. *Food Chemistry* 103:816–822.

Tuzlacı, E. and M.K. Erol. 1999. Turkish folk medicinal plants. Part II: Eğirdir (Isparta). *Fitoterapia* 70: 593-610.

Tuzlacı, E. and P.E. Aymaz. 2001. Turkish folk medicinal plants, Part IV: Gönen (Balıkesir). *Fitoterapia* 72: 323-343.

Valgas, C., S.M. Souza., E.F.A. Smânia., A.Smânia. 2007. Screening methods to determine antibacterial activity of natural products. *Brazilian Journal of Microbiology* 38:369-380.

VanderJagt, T.J., R. Ghattas., D.J. VanderJagt., M. Crossey., R.H. Glew. 2002. Comparison of the total antioxidant content of 30 widely used medicinal plants of New Mexico. *Life Sciences* 70:1035-1040.

Vertuani, S., E. Braccioli., V. Buzzoni., S. Manfredini. 2002. Antioxidant capacity of *Adansonia digitata* fruit pulp and leaves. *Acta Phytotherapeutica* 26:654-666

Vertuani, S., E. Bosca., E. Braccioli., Manfredini. 2004. Water soluble antioxidant capacity of different teas. *Nutra Foods* 3 (2):05-11.

Villano, D., M.S. Fernandez-Pachon., A.M. Troncosa., M.C. Garcia-Parilla. 2004. The antioxidant activity of wines determined by the $ABTS^{\bullet+}$ method : Influence of sample dilution and time. *Talanta* 64:501-509.

Villeponteau, B., R. Cockrell., J. Feng. 2000. Nutraceutical interventions may delay aging and the age-related diseases. *Experimental Gerantology* 35:1405-1417.

Wuttke, W., H. Jarry., V. Christoffel., B. Spengler., D. Seidlová. 2003. Chaste tree (*Vitex agnus-castus*) –pharmacology and clinical indications. *Phytomedicine* 10:348-357.

Xiao, J.B., J.W. Chen., M. Xu. 2007. Supercritical fluid CO2 extraction of essential oil from *Marchantia convoluta*: global yields and extract chemical composition. *Electronic Journal of Biotechnology* 10:141- 148.

Zakaria, Z., S. Sreenivasan., M. Mohamad. 2007. Antimicrobial activity of piper ribesoides root extract against Staphylococcus Aureus. *Journal of Applied Biological Sciences* 3:87-90.

APPENDIX A

CALIBRATION CURVE OF GALLIC ACID

Calibration Curve of gallic acid standard

0.5 mg/ml stock standard of gallic acid was prepared by firstly dissolving 250 mg of dry gallic acid in 10 ml of ethanol and then diluting to 500 ml with distilled water. The solution were kept in the 4 C^0. The standard concetrations that were prepared for calibration curve are 0.02-0.03-0.04-0.05 and 0.06 mg/ml.

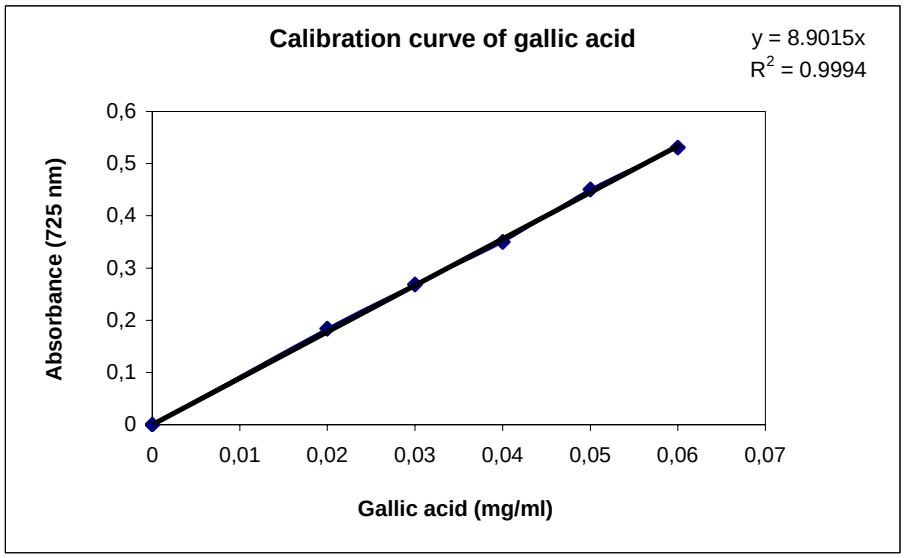

Figure A1. Calibration curve of gallic acid standard which is used for expression of total phenol contents as gallic acid equivalents.

Calculation of total phenol contents in gallic acid equivalent

" GAEq(mg GA/g sample)=[A*DF*Vsolv(mL)]/[slope of cal.curve*sample amount(g)] "

In this equation:
GA: gallic acid
A: absorbance of working solution
V:solvent volume for dissolving extract
Sample amount: weighted extract
DF: dilution factor

APPENDIX B

THE PROCEDURE OF STANDARD DISC DIFFUSION METHOD

1. Fresh cultures were prepared daily in 8 ml broth by transfering one loop of stock bacteria which are kept in –80 ^{0}C.
2. These cultures incubated for 18 h and subcultures were obtained by transfering 30 µl from this 18 h incubated cultures to fresh broth (8 ml).
3. Experiments were performed with this daily prepared subcultures which are standardized for inoculation on agar surface corresponding to certain numbers of CFU/ ml. Log. phases of growth curves were taken into account to reach approximate inoculation numbers also the standardized inoculums were confirmed by measuring OD values.
4. 100 µl of bacteria culture during the lag phase were inoculated onto agar surface. The agar dept adjusted to 25ml for each plate
5. Inoculated culture was dispersed by streaking the steril swab over the entire steril agar surface by rotating the plate 60^o each time to ensure the inoculum uniformly spread.

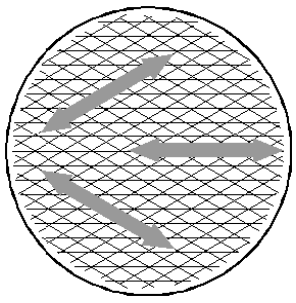

Figure B1. Uniform inoculation of culture onto agar surface

6. The inoculated plates were allowed to sit for 5-10 minutes to let the broth absorp into agar.

7. Sterile blank discs were applied by soaking them into sterilized extracts by using 0.45 µm filters.
8. The concentration of extract solutions was determined as 1g extract/10ml DMSO for each plant extracts.
9. Only 4 discs were placed on each plate and then gently pressed to ensure contact with the agar surface.
10. Plates were incubated for 24h at 37 ^{0}C .
11. After 24h the zone diameters were measured by using a compass and results were expressed as mm.
12. Assays for disc diffusion were performed twice.
13. After all steps plates were sterilized and discarded properly as biohazard material.

Some notifications have been declared by the authors about the factors that affect the results of the disc diffusion method are as follows:

- MHA (Muller Hinton Agar) and NB are recommended mostly for disc diffusion test due to their results in good batch-to batch reproducibility; they result in satisfactory growth of most bacterial pathogens; and a large amount of data has been collected with these mediums.
- The agar medium should be PH 7.2 to PH 7.4 at room temperature.
- The surface should be moist but without droplet of moisture.
- The antibiotic and blank discs should be maintained at 4 ^{0}C –8 ^{0}C and allowed to warm to room temperature before use.
- Inoculation density of microorganisms should be standardized with specific number of cells. This could be achived by the followings:

  ♦ by standard plate count method

  ♦ by turbidity measurement with spectrophotometric method

  ♦ by Mc-Farland reagent (adjusting turbidity by compering the 0.5 Mcfarland solution.)

- The dept of the agar should be standardized (mostly 25ml for each plate)

- Application of extract concentration should be standardized.
- A well distruption of inoculum should be ensured by a steril swap and streak the agar surface from edge to edge and from top to bottom.
- Incubation time and tempereture need to be determined for each microorganism

Procedure for Determination of the Growth Curves of Microorganisms by Colony Counting Method

1. Microorganisms were firstly picked up from the stock cultures which are kept at - 80 ^{0}C. Only one loop of bacteria was inoculated to the growth media (8 ml Broth).

2. This subculture was incubated for 24 h at 37 ^{0}C in a dark place. Incubation by shaking was performed only if it is necessary for the aerobic or facultative aerobic microorganisms. This first subculture was checked for the purity by streak plate method.

3. In the following day 1ml of 24 h subculture was inoculated into the 100 ml of broth for the enumeration of organisms by using spread plate method.

4. Inoculations for spread plates were performed in 2 hours time intervals during the 24 hours. 100µl of bacteria from the second subculture were inoculated to the solid surface of the agar plates.

5. Serial dilutions between10^{-1} and 10^{-9} of the second subculture were inoculated for enumerations.

6. Inoculated agar plates were incubated for 24 h at 37 ^{0}C. After the 24 h incubation colony numbers were counted. Only the numbers of which are between 30 and 300 were evaluated in the CFU/ml calculations (CFU: Colony Forming Unit).

Calculation of CFU/ml

The calculation is performed thus:

C.F.U/mL original sample = C.F.U./plate x (1/mL aliquot plated) x dilution factor

For example:

32 colonies on plate x 1/0.1 mL (aliquot) x 10^6 (dilution factor) = 32 x 10^7 CFU/ mL

Growth curves of some species in this study which were determined by colony counting method are shown in Figure B2 and Figure B3.

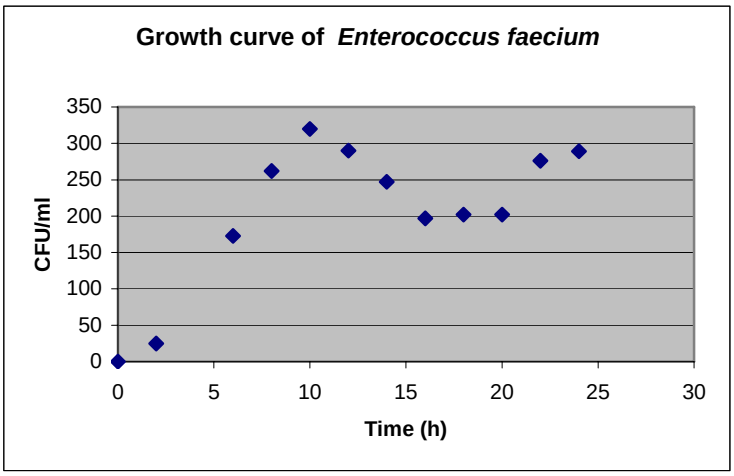

Figure B2. The curve for 24 h growth of *E. coli*

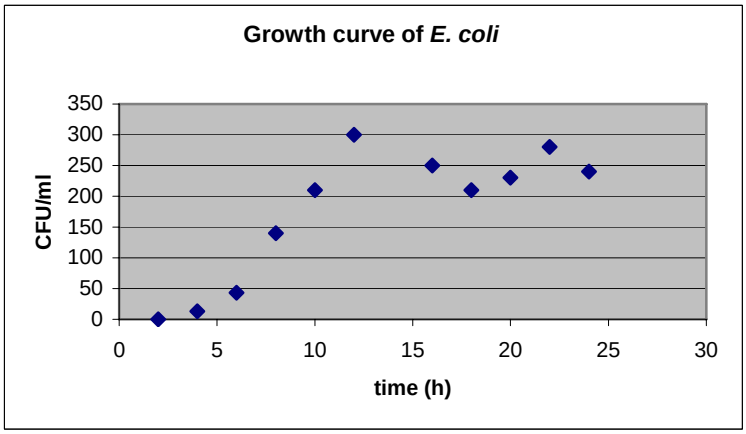

Figure B3.The curve for 24h growth of *Enterococcus fecium*