DEVELOPMENT OF NATURAL COMPOUND-LOADED NANOFIBERS BY ELECTROSPINNING

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Ali Bora BALTA

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We approve the thesis of **Ali Bora BALTA**

Assoc. Prof. Dr. Oğuz BAYRAKTAR
Supervisor

Assist. Prof. Dr. Güldemet BAŞAL
Co-Advisor

Assoc. Prof. Dr. Figen KOREL
Committee Member

Assist. Prof. Dr. Ayşegül BATIGÜN
Committee Member

12 July 2010

Assoc. Prof. Dr. Ahmet KOÇ
Head of the Department of
Biotechnology and Bioengineering

Assoc. Prof. Dr. Talat YALÇIN
Dean of the Graduate School of
Engineering and Sciences

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ABSTRACT

DEVELOPMENT OF NATURAL COMPOUND-LOADED NANOFIBERS BY ELECTROSPINNING

In this study, the crude silk which is obtained from silkworm was turned into a silk solution after a serial procedure. Then, regenerated silk (foam) was obtained. The regenerated silk was dissolved in formic acid and polymer solution was prepared. After, nanofibers were produced by electrospinning. On the other hand, the content and antimicrobial activities of some plants were analyzed. Then all three forms of silk was absorbed with the olive leaf extract and extract desorption tests were done.

As the first step of the study, the analyses of extracts which were obtained from four different plants were done. For this, total phenolic content and antioxidant capacity were found. In addition, minimum inhibition concentration (MIC) test and disc diffusion test were made for all extracts to *Escherichia coli, Staphylococcus epidermidis, Staphylococcus aureus, Bacillus subtilis, Pseudomonas aeruginosa* bacteria and *Candida albicans* fungi in order to determine their antimicrobial activity.

While producing nanofibers from silk polymer with electrospinning method, different parameters such as concentration, voltage and distance were examined. Morphological characterization of nanofibers was done by scanning electron microscope (SEM). According to the results, the nanofiber with an optimum value which has a suitable diameter and structure was selected. With this nanofiber, the absorption and desorption tests of natural compound were made. The results were obtained by High pressure liquid chromatography (HPLC). Same adsorption and desorption tests were done also with the microfiber silk and regenerated silk (foam). As a result, it was shown by the controlled experiments that nanofibers were better for adsorption and desorption of natural compound when compared to microfiber silk and regenerated silk.

In conclusion, nano-sized silk fibroin structures can be adsorbed with natural compounds in order to gain functionality. Using this kind of biofunctional products as medical textile and wound dressing material will be more advantageous when compared to current wound dressing materials.

ÖZET

ELEKTROEĞİRME YÖNTEMİ İLE DOĞAL BİLEŞİK YÜKLÜ NANOLİFLERİN GELİŞTİRİLMESİ

Bu çalışmada, ipek böceğinden elde edilen ham ipek, bir seri işlemden geçtikten sonra sulu ipek çözeltisi haline getirilmiştir. Daha sonra rejenere ipek (köpük) elde edilmiştir. Elde edilen bu rejenere ipek formik asit içerisinde çözülmüş ve polimer çözeltisi hazırlanarak elektro lif çekim yöntemi ile nano boyutta lifler (nanofiber) elde edilmiştir. Diğer bir taraftan, bazı bitkiler antimikrobiyallik ve içerik açısından incelenmiştir. Zeytin yaprağı özütü, ipeğin elde edilen üç formuna da emdirilmiş ve salım testleri yapılmıştır.

Çalışmanın ilk aşamasında, ekstarksiyon yöntemi ile bitkilerin özütleri elde edilmiş ve toplam fenol ile toplam antioxidant kapasiteleri bulunmuştur. Ayrıca her bir özüt için minimum inhibisyon konsantrasyonu bulunmuştur. Disk difüzyon yöntemi ile *Escherichia coli, Staphylococcus epidermidis, Staphylococcus aureus, Bacillus subtilis, Pseudomonas aeruginosa* bakterileri ve *Candida albicans* mantarına karşı antimikrobiyal değerler saptanmıştır.

Elektro lif çekim yoluyla ipek polimer çözeltisinden nanolifler üretilirken, başta konsantrasyonun etkisi olmak üzere uygulanan yüksek voltaj değeri ve mesafe parametreleri incelenmiştir. Bu şekilde elde edilen nanolifler, taramalı elektron mikroskobu (SEM) ile morfolojik olarak karakterize edilmiştir. Elde edilen sonuçlar doğrultusunda, optimum değerdeki, uygun çap ve yapıya sahip nanolif seçilmiştir. Seçilen bu nanolif ile doğal bileşiğin yükleme ve salım testleri yapılmış olup sonuçlar yüksek performans sıvı kromotografisi (HPLC) ile elde edilmiştir. Aynı yükleme ve salım testleri, ipeğin mikrolif yapısı ve rejenere formundaki (köpük) yapısı ile de yapılmıştır. Bütün bu elde edilen sonuçlar neticesinde nanoliflerin, ipek mikrolif ve köpüklere oranla daha fazla aktif bileşeni tuttuğu ve aynı şekilde daha fazla miktarda ortama salım yaptığı, yapılan kontrollü deneyler ile gösterilmiştir.

Sonuç olarak, nano boyuttaki ipek lif yapılarına, doğal bileşikler yüklenerek, fonksiyonellik kazandırılabilir. Böylece, medikal tekstil ve yara örtü malzemesi olarak bu tür biyofonksiyonel ürünlerin kullanılması, mevcut yara örtülerine göre büyük avantaj sağlayacaktır.

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3D	Three-dimensional
$ABTS^+$	2,2'-Azinobis 3-Ethylbenzothiazoline-6-Sulphonic Acid
AFM	Atomic Force Microscopy
B.subtilis	*Bacillus subtilis*
C_i	Initial concentration
C.albicans	*Candida albicans*
d	Distance (between needle and collector)
E.coli	*Escherichia coli*
F-C	Folic-Ciocalteu
GAE	Gallic acid equivalent
GC	Gas Chromotography
GEN	Gentamicin (antibiotic)
HPLC	High Performance Liquid Chromotography
kV	Kilovolt, One thousand (10^3) volts
L.ori	*Liquidambar orientalis*
MF	Micro Fiber
MIC	Minimum Inhibition Concentration
Mm	Micrometer (Micron)
NF	Nano Fiber
nm	Nanometer
OD	Optic Density
OLE	Olive Leaf Extract
ole	Oleuropein
P.aeruginosa	*Pseudomonas aeruginosa*
PEN	Penicillin (antibiotic)
RSF	Regenerated Silk Fibroin
TEAC	Trolox equivalent antioxidant capacity
S.epidermidis	*Staphylococcus epidermidis*
S.aureus	*Staphylococcus aureus*
SEM	Scanning Electron Microscope
SF	Silk Fibroin

STR	Streptomycin (antibiotic)
TEM	Transmission Electron Microscope
VAN	Vancomycin (antibiotic)
Z. jujuba	*Ziziphus jujuba*

CHAPTER 1

INTRODUCTION

Nanotechnology is an interdisciplinary science and can be defined as the engineering of functional systems at the molecular scale. In other words, nanotechnology is the technology of creating nanoparticles and materials or manufacturing devices which have sizes within the range of 1 to 100 nanometers (nm). The term of nanotechnology was first used by Norio Taniguchi from Tokyo Science University (Taniguchi, 1974). The word of 'fiber' can be used in different meaning according to viewpoint. For instance, anatomists identify fibers as any of the filaments constituting the extracellular matrix of connective tissue or longitudinal cell cluster such as muscle fiber and nerve fiber. Botanists understand fiber term with elongated, thick-walled cells that gives strength and support to plant tissue. The textile industry views fibers as synthetic or natural flament such as nylon, cotton or silk. In this thesis, the word of fiber will be used with nano prefix (nanofiber) so as to define elongated, uniform, threadlike object or structure. Nanofibers are nano-scales, three-dimensional (3D) structures and the distribution of diameters ranging between 10 to 500 nanometers.

Nanofibers can be produced by various techniques such as drawing, template synthesis, phase separation, self assembly and electrospinning. Among them, electrospinning is the most efficient and multifunctional method in order to produce nanofibers. As in every method, there are some critical and significant parameters in electrospinning process (Greiner et al., 2007). By changing these parameters, we can obtain different structures and features of nanofibers (Zong et al., 2002). Beside this, nanofibers can be used for many industrial area such as medical, textile, protective material, energy, agriculture and filtration (Burger et al., 2006; Jian et al., 2008). In addition to all, nanofibers that are fabricated with electrospinning method can be gained functionality with addition of inorganic and organic supplies (An et al., 2009). The recent and the most important phenomena is using natural compounds as an organic material. In this manner, we can produce completely biodegradable, biocompatible and non-toxic functional nanofibers. In this context, these kind of nanofibers are seen as a novel candidate and a shining star for many medical applications and tissue engineering.

There is an intense concern about antibacterial natural compounds. Especially, plant-derived components are commonly used for many industries all over the world

(Mason et al., 2002). They are known as plant secondary metabolites and have antioxidative, antimicrobial and anti-inflammatory properties (Sun et al., 2002). These components are very effective and some of them have extremely useful features. Such components are using for pharmaceutical preparations, cosmetic products, food supplements and therapy investigation so as to find new treatment methods. Today, scientist try to isolate this bioactive components because this phytochemicals are being used in a great deal of area and have a good potential for medical applications (Altiok et al., 2007).

Silk is a polyamino acid based protein which is produced by silkworms in order to protect themselves during their metamorphosis. For centuries, humans have harvested silk coccons so as to produce textile manufacturing. Silk has a great deal of characteristic properties like luster, moisture absorbance and strength (Hardy et al., 2008). Silk fibroin is a good candidate in many biotechnological application, for instance; medical textile, drug delivery and tissue scaffolding.

Since ancient times, many plants and their parts such as leaves, seeds, roots and flowers are being used in various application, especially for therapeutic usage (Williamson et al., 1996), because plants and their compounds are really significant for improving many diseases. So, in this study, four different plant species were used that are collected from different location in Aegean region and their contents were investigated. These plants are; *Olea europaea* (Olive leaf), *Liquidambar orientalis* (Turkish sweetgum), *Ziziphus jujuba* (Lotus) and *Juniperus communis* (Juniper). The crude extract which is from these plants are tested against several bacteria and fungi in order to find out their antimicrobial activity. Among them, olive leaf was chosen in order to incorporete with nanofibers.

The olive (*Olea europaea*) is a species of small tree native to coastal areas of the eastern Mediterranean region. The olive tree is one of oldest cultivated trees and the olive leaf has been used medicinally throughout history. The leaves of this plant are used for centuries as a therapeutic and many researchers emphasize that there are a great deal of useful phenolic compounds in olive leaf (Garcia et al., 2000). Also, extract which is obtained from olive leaf is one of the most effective natural compound in order to destroy microorganism and free radicals that cause diseases and adverse effect on human health (Sudjana et al., 2009).

Adsorption is one of the best technique in order to load olive leaf polyphenols on to nanofibers. Because, for impregnation, this method is efficient and convenient, also can be applied for small and large scales. Many researchers have been studying the

potential of polyphenols with silk fibroin (Altiok et al., 2007). Especialy, the relationship between the components in olive leaf and silk fibroin has been examined recently (Baycin et al., 2007) and very satisfactory results were obtained. Due to this reason, olive leaf extract (OLE) was chosen for adsorption and desorption assays.

Beside being a very new topic, antimicrobial nanofibers can be obtained by adding some antimicrobial agents (Son et al., 2006). In order to gain antimicrobial property to nanofiber, silver particles are used but nowadays some natural biopolymers have being used for this purpose such as chitosan or poly ethyleneoxide complex (Ignatova et al., 2007; Ginner et al., 2008). To our knowledge, there is no such study that loading natural compound on to nanofibers. So, these kind of materials can be used as a wound dressing and protective material for medical applications.

The first aim of this study was investigate the four plant species and determine their total phenol contents and antioxidant capacities. Beside these, their antimicrobial activities were determined with standart disc diffusion method and minimum inhibition concentration assay against several bacteria and fungi. On the other hand, silk fibroin nanofibers were produced via electrospinning method. By changing some critical parameters, nanofibers were obtained with different diameters. Moreover, adsorption and desorption experiments were performed with olive leaf extract and silk fibroin nanofibers (NF). In addition to this, the performances of silk fibroin microfiber (MF) and regenerated silk fibroin (RSF) in terms of adsorb the polyphenols were tested and compared with those of nanofibers. Consequently, very satisfactory results were obtained. As a result, it was clearly seen that such a kind of rich contents natural compounds can be loaded with fibers. Another useful feature is both silk fibroin and plant-derived bioactive components are natural. The combinations of them are biocompatable and biodegredable so, in this manner, these products are being a shining star and the candidate for futures wound dressing and tissue engineering materials.

CHAPTER 2

LITERATURE REVIEW

2.1. Antibacterial Natural Compounds

It is estimated that there are more than 400.000 species of plants on earth (Borris, 1996). A relatively small percentage of these are used as foods by both humans and other animal species. It is possible that there are many potential plants can be used for medicinal purposes (Moerman, 1996). Before starting to examine the antimicrobial natural compounds, I would like to give brief historical information about the medical plants. Herbal were utilized since ancient times to prevent and reverse infectious diseases. Hippocrates burned aromatic plants to help combat the epidemic of plaque in Athens. The Bible described approximately 30 plants which have a healing power. Asian cultures were also busy compiling their own pharmacopoeia, a kind of book which reported procedures for the preparation of medicines and information about herbal remedies. On the other part, in the western regions, the Renaissance years saw a revival of ancient medicine, which was built largely on plant medicinals. The use of plant extracts, as well as other alternative forms of medical treatments, is enjoying great popularity in the late 1990s (Eisenberg et al., 1993).

A natural product is a chemical compound or substance produced by a living organism found in nature that usually has a pharmacological or biological activity for use in pharmaceutical drug discovery and drug design. In particular, these compounds are important in the treatment of life threatening conditions (Newman et al., 2007). Natural compounds have been investigated and screening by scientist in order to find alternative antimicrobial agents instead of synthetic antibiotics (Silver et al., 1990).

The overuse of antibiotics have raised concern by health officials because of the rise of antibiotic resistant strains of bacteria. Many plant products such as coffee, tea, spices and herbs have been shown to contain compounds that act as antimicrobial agents. (Cowan, 1999). Antimicrobial action can be tested by culturing bacteria in the presence of an extract that considered the effect of antimicrobial property.

Major groups of antimicrobial compounds from plants have an almost limitless ability to synthesize aromatic substances, most of which are phenols or their oxygen

substituted derivatives (Tringali, 2001). Most are secondary metabolites, of which at least 12.000 have been isolated, a number estimated to be less than 10% of the total (Cowan, 1999). In many cases, these substances serve as plant defense mechanisms against predation by microorganisms, insects, and herbivores. For instance, terpenoids give plants their odors. Quinones and tannins are in charge of plant pigment. On the other hand, polyphenolic compounds have an essential role and responsible for the defence mechanism of plants (Beart et al., 2001). Useful antimicrobial phytochemicals can be divided into several categories. The main category is phenolics and polyphenols which composed of phenols, phenolic acids, quinones, flavones, flavonoids, flavonols, tannins and coumarins. Other groups are terpenoids, essential oils, alkoloids, lectins and polypeptides (Grusak et al., 2002). Throughout the entire life of the plant, these natural compounds are playing a critical role and they posses health-beneficial components for human life.

Antimicrobial research is goings on the discovery and development of novel chemical structures such as therapeutic antimicrobial agents. The continuing problem of development of resistance to existing antibacterial agents and the dearth of good antifungal agents motivates this effort toward innovation. A broad screen of chemical entities can be undertaken, dedicated to the discovery of novel antimicrobial inhibitors. A major part of the endeavor is in the historically productive area of natural product screening. To make the best use of natural product resources for the discovery of novel antibiotics, a balance is struck between screening for inhibitors of rationally chosen targets for which clinically useful inhibitors are not yet available, and screening more broadly to ensure that rare activities of unanticipated mode-of-action are not missed (Silver et al., 1990).

2.2. Plant Polyphenols and Antioxidants

Antioxidants can be defined as an active compounds which have reduce the harmful effects caused by free radicals or could delay some mechanisms. Actually, living organisms have their own defence mechanism against free radicals or hazardous components. But some external factors such as air pollution, radiation, chemicals, physical stress, tissue damages that results from injury and some internal factors like enzymes, tissue dysfunctions, immune system products could be increse the free radicals to form. So, the intracellular concentration of free radical amounts may be rised

in a critical levels. These free radicals attact the living tissues and destroy their structures. At this point, polyphenols and antioxidants sacrifice themselves instead of our cells. For centuries, its known that some plant extracts have a powerfull effect in order to improve wounds and tissue damages. These extract contain the natural compounds which possess different biological activity such as antioxidant, antimicrobial and anticancer (Valko et al., 2006; Collins, 2005).

Phenolic compounds are playing an essential role because they possess natural antioxidant property. In order to constitute protection mechanism against microorgansms, harmfull ultraviolet (UV) lights and other threats, these components are synthesized by plants. Antioxidants are inserted into foods so as to preserve the content. Sometimes, toxic residuals or taste disturbance may occure due to oxidation. Hence, antioxidants are very important for conserve foods. Since these components satisfactorly have high antioxidant activity, plant extracts constitute a natural alternative compared to synthetics (Floch et al., 1998)

2.3. Plant Species Used in the Study

As we mantion in previous sections, many plants are rich in phenolics and antioxidant activity. The first aim of this study is, investigated some regional plants and determine their total phenol and antoxidant capacities. Beside this, their antimicrobial activities will be examined. Plants to be analyzed are given below as a subtitles.

2.3.1. Olive Leaf (*Olea europaea*)

The content of olive leaves may exhibit different properties accroding to the species of olive tree, maturity of leaf or harvest period but it is known that the major factor is genetical features. However, young leaves are higher amount of oleuropein content than mature leaves and this situation is also acceptable for leaves harvested in spring compared to autumn. According to the studies, oleuropein and hydroxityrosols were determined as an essential phenolic components in olive leaf extract (Altiok et al., 2008; Pereira et al., 2007). Althought, oleuropein is the main phenolic component in olive leaf extract, the antioxidant capacity of pure oleuropein is limited. In any case, antioxidant capacity of olive leaf extract shown a powerful effect when compared to

another strong compound hydroxityrosols. For some researchers, this phenomena is explained in two ways;

- Oleuropein is dismantled into hydroxityrosols and increase the antioxidant capacity
- Phenolic compounds in extract show a synergytic effect

It was reported that main phenolic compounds present in olive leaves are oleuropein, hydroxityrosols, rutin, verbaskosit, apigenin-7-glucoside, luteolin-7-glucoside, tyrosol, vanilic acid, diosmetin-7-glucoside, caffeic acid, luteolin, diosmetin, vanillin, and catechins (Erbay et al., 2008).

If we take into account all these utilities and look at the studies about the incidence of olive leaf extract, it is obviously seen that olive leaf extract is used in a wide range of applications especially for medical purpose. Table 2.1., summarize the using area of olive leaf extract.

Table 2.1. Olive Leaf Extract for Medicinal Usage

Reference(s)	**Effect of Olive Leaf Extract**
Garcia et al., 2002	Anti-carcinogenic
Andreadou et al., 2007	Cardiovascular
Moccettil et al., 2008	Lowering Blood Pressure
Eidi et al., 2008	Antidiabetic
Sudjana et al., 2008	Antimicrobial
Ferreira et al., 2006	Antioxidant
Puel et al., 2006	Bone health
Giamarellos et al., 2006	Immune System Booster
Kendall., 2009	Body Resistance and Defence System

2.3.2. Turkish Sweetgum (*Liquidambar orientalis*)

Turkish sweetgum with another name Anatolian sığla tree comes from the *Altingiaceae* family and exist only the limited location of Turkey. These perennial plants with a high tall are only distributed Fetiye, Muğla and Köyceğiz (Öztürk et al., 2008). Although the species spread into the northern part of Anatolia in the past, its distribution is now restricted to the Mediterranean. The scientific name of the tree, *Liquidambar* is a combination of the latin and arabic words 'Liquidus' and 'Amber', meaning 'fragrant liquid'. In medical applications this 'fragrant liquid' is used for the treatment of respiratory diseases such as asthma and bronchitis. Also, it has an essential role in dermatology, especially in order to heal dermal wounds (Efe et al., 2000).

Oriental sweet gum is important both as a relic and endemic species. Trees must be protected because it has an economically important, due to its natural balsam producing ability which is rare among forest trees. It was stated that balsams contain profitable compounds and have a powerful effect (Hovaneissian et al., 2008). Since research and breeding focus on the oil or balam production properties. Sweet gum oil is used in medicine, chemical and cosmetic industries.

Studies about the component of *Liquidambar orientalis* are made in previous years. Active compounds, especially essential oils in sweetgum was determined by Gas chromotograpy (GC) and six main component indicated in plant balsam which are styrene, phenylpropl alcohol, cinnamyl alcohol, cinnamic acid, phenylpropyl cinnamate and styracin. Among them, phenylpropyl cinnamate and styrcin persentages are 7.5% and 20% respectively (Hafızoglu, 1982). According to the recent GC study of oriental sweetgum, eleven main component was determined which two of them are consist of hydrocinnamyl alcohol and trans-cinnamyl alcohol with very high value (Lee et al., 2009). The Components in *Liquidambar orientalis* are given in Table 2.2.

Although there is very little genetic knowledge available of this species, there are some ongoing research so as to obtain information about this species. Inhibitory effects of *Liquidambar orientalis* against several bacteria were indicated by some reserachers and quite effective results were obtained. The results showed that, *Liquidambar orientalis* crude extract has antibacterial activity against many bateria at concentration ranging between 10% to 0.2%. (Sagdıc et al., 2005). Antifngal activity of extract was also studied and represent a satisfactory inference (Lee et al., 2009).

Table 2.2. Components in *Liquidambar orientalis*

Component in *Liquidambar orientalis*	**Persentages (%)**
β-pinene	0.15
1-phenyl-1-ethanol	0.17
Acetophenone	0.19
Trans-cinnamyl Aldehyde	0.24
Benzaldehyde	0.47
α-pinene	1.02
Benzyl Alcohol	1.22
Styrene	1.56
β-caryophllene	3.60
Hydrocinnamyl Alcohol	41.13
Trans-cinnamyl Alcohol	45.07

2.3.3. Lotus (*Ziziphus jujuba*)

When we give a general information about this plant, *ziziphus jujuba* is an interesting deciduous tree with spiny, gnarled branches and an open, irregular form. It comes from the member of *Rhamnaceae* family and reach a high approximately 5 to 10 meters (Gilman et al., 1994). Especially in eastern region, it calls 'Chinese Jujube' or 'Red Date'. It shows wide range of distribution from China to southeaster Europe. In Turkey, natural *Ziziphus jujuba* forest are distributed in Denizli region and researchers indicated that there is a various type of Ziziphus genotypes are living in the region (Ecevit et al., 2007). The size of leaves are small and colour of leaves are green. The flowers are small with five inconspicuous yellowish green petals. The fruits are edible and dark red seems oleaster fruit. Jujube is both a delicious fruit and an effective herbal remedy. It aids weight gain, improves muscular strength and increases stamina. In Chinese medicine it is prescribed as a tonic to strengthen liver function (Chevallier, 1996).

The fractination of *Zizihus jujuba* was determined by Thin Layer Chromotography (TLC). Datas indicated that the active compounds in plant consist of a triterpenes, saponines and glycosides (Kennedy et al., 1980). However, Recent studies indicated that alkaloids, flavonoids, sterols, tannins and saponin have been isolated and chemically idendified from the different species of the genus *Ziziphus* plant (Cheng et al., 2000; Croueour et al., 2002). Likewise, saponins and fatty acids are examined in a detailed with High Performance Liquid Chromotography (HPLC). Some essential compounds such ascorbic acid, aslauric acid, palmitoleic acid, linoleic acid, palmitic acid, oleic acid, stearic acid, arachidic acid and docosanoic acids are found in a significant amount. Among them, linoleic acid is the most abundant one. (Zhao et al., 2006).

On the other hand, *Ziziphus jujuba* has significant levels of antioxidant activity and scavenging effect on free radicals (Li et al., 2005). Beside these, it is used traditionally as tonic and sometimes as sedative and anxiolytic. Some studies also show that extracts obtained from plant's parts are drived for anticancer, antifungal, antibacterial, antiulcer, anti-inflammatory, antinephritic, imunositimulant and wound healing treatments (Mahajan et al., 2009). Table 2.3., summarize the Medical applications and benefits of *Ziziphus jujuba* plant.

Table 2.3. Medical Applications of *Ziziphus jujuba* Plant

Reference(s)	**Part of Plant**	**Active Components**	**Medical Applications**
Che, 1985 Bown, 1995	Seeds	Saponins, Triterpenes, Flavanoids and Alkoloids	Insomnia, Nervous exhaustion, Excessive perspiration,
Duke et al., 1985 Grieve, 1984 Mukhtar et al., 2004	Roots	Saponins, Triterpenes, Glycosides	Dyspepsia, Fever, Gout, Rheumatism, Wounds, Ulcers
Chopra et al., 1986 Grieve, 1984 Mahajan et al., 2009	Leaves	Alkoloids, Flavanoids, Sterols	Nervous diseases, Stem the bleeding, Growth of hair, Burns, Skin eruptions
Che, 1985 Duke et al., 1985	Fruits	Saponins, Triterpenoids, Alkoloids	Painkiller, Anticancer, Pectoral treatments, Antipyretic, Sedative

2.3.4. Juniper (*Juniperus communis*)

Juniperus communis, commonly called Juniper, is a species in the family Cupressaceae. The distribution area of this plant ranging from the Northern Hemisphere to the Arctic south. It is a shrub or small tree because it shows a wide range of distributon and according to the lacation the high of plants are changing between 1 to 10 meter tall. Common Juniper has needle-like leaves in whorls of three. The leaves are green, with a single white stomatal band on the inner surface. The seed are spherical and about, berry-like with a waxy coating and the diameter sizes are about 6 milimeters. Seeds are rich in essential oil, organic acids, resin, glucose and sucrose.

Juniper fruits are commonly used in herbal medicine as a household remedy and also in some commercial preparations. They are especially useful in the treatment of digestive disorders plus kidney and bladder problems (Grieve, 1984). The fully ripe fruits are strongly antiseptic, aromatic, carminative, diaphoretic, strongly diuretic, rubefacient, stomachic and tonic. Externally, it is applied as a diluted essential oil, having a slightly warming effect upon the skin and is thought to promote the removal of waste products from underlying tissues. When made into an ointment, they are applied to exposed wounds and prevent irritation by flies (Launert, 1981; Chiej, 1984; Chopra, 1986; Chevallier, 1996).

Although there is less study about extraction and fractination of crude seeds, in the literature we can see the examinations about essential oil of juniper plant. According to the GC/MS studies, the main compounds in the oil are α-pinene, β-pinene, sabinene, limoneneand mircene. Among them, α-pinene is the highest percentage compared to others (Pepeljnjak et al., 2005). Also, same study shows that juiper essential oil has an antimicrobial activity against in a wide range of microorganism including yeast anf fungi. Another recent study indicate that the leaves of *Juniperus communis* represent a powerful effect and posses affluent phenolic compounds. The major photochemicals present juniper leaves are alkaloids, phenolics, flavonoids, tannins and terpenids (Kumar et al., 2010).

2.4. Microorganisms and Their Properties

2.4.1. *Escherichia coli* (NRRL B-3008)

Escherichia coli (E.coli) is a rod shaped, Gram-negative bacterium which colonizes in the lower intestine of animals but also survives when released into the environment with an optimum temperature of 37 ^{o}C. They are facultative anaerobes that grow by aerobic respiration or by fermentation. The harmless strains are inhabitant of the gut flora, with a mutual relationship with the host. *E. coli* provides the prevention of overgrowth of pathogenic bacteria and production of vitamin K_2. On the other hand, *E. coli* is an opportunistic pathogen which can cause infection in people with reduced immunity. Also harmful strains of *E. coli* are able to infect human even with healthy immune system. Firstly, *E. coli* colonize at the mucosal site; then escape from immune response and damage the host; causing urinary tract infection. Those infections should be defeated by proper antibiotic treatment (Kaper et al., 2004).

2.4.2. *Staphylococcus epidermidis* (ATCC 12228)

Staphylococcus epidermidis (*S. epidermidis*) is a Gram-positive, coccus shaped bacterium. They are facultative anaerobes that grow by aerobic respiration or by fermentation. This bacterial strain is a part of the human skin surface bacterial flora as a normal inhabitant which does not cause infections in normal conditions. On the other hand, *S. epidermidis* is an opportunistic human pathogen as the cause of wound infections. Such infections occur when microbial growth occurs not only on skin surface but also in the deeper tissues because of the insertion of surgical implants. This microorganism and its species are especially the most common reason of hospital acquired infection and cause one of the major public health issues. *S. epidermidis* becomes problem by forming bio-film structure on surgical implants. A bio-film is combined group of microorganisms in which cells adhere to each other and to a surface. These cells are embedded in an extra cellular polymeric substance. Bio-films cause unsatisfactory diffusion of antibiotics which are used to overcome the infection. This provides *S. epidermidis* strains to be resistant for antibiotics (Vuong et al., 2002).

2.4.3. *Staphylococcus aureus* (ATCC 29213)

Staphylococcus aureus (*S. aureus*) is a Gram-positive, coccus shaped bacterium that belongs to genus *Staphylococcus.* They are facultative anaerobes that grow by aerobic respiration or by fermentation. *S. aureus* is part of the human skin surface flora and mucosal surface flora of nose. 20% of the human population carries *S. aureus* as a commensally. On the other hand, by the overgrowth of *S. aureus* different diseases may appear in human. It can cause skin infections, most importantly the surgical wound infections or toxic mediated diseases. Especially the antibiotic resistant strains of *S. aureus* may cause major public health problems (Miller et al., 2009).

2.4.4. *Bacillus subtilis* (NRRL B-4378)

Bacillus subtilis (*B. subtilis*) is a rod-shaped Gram-positive bacterium that belongs to the genus *Bacillus*. They are naturally inhabitant of soil and vegetation. These bacteria can form endospore due to harsh conditions such as starvation and chemical or oxidative stress. *Bacillus subtilis* are non-pathogenic. They are able to contaminate food, but this spoilage rarely results with food poisoning. Beside this, *B. subtilis* may exist on a dermal wound and may cause dermatologically hazardous effect to the human health (Kobayashi et al., 2002).

2.4.5. *Pseudomonas aeruginosa* (ATCC 27853)

Pseudomonas aeruginosa (*P. aeruginosa*) is a rod-shaped Gram-negative bacterium with one flagellum. It has a wide range nutritional adaptability. Due to metabolic requirements it is capable of growth in aerobic conditions but it can also survive in anaerobic conditions with a limited potential. It is found in natural environments such as soil, water and skin flora. *P. aeruginosa* is an opportunistic pathogen which causes infection in the pulmonary tract, urinary tract, burns, wounds and blood stream. These infections generally occur in people with reduced immunity. Some epidermal infections can be the consequence of *P. aeruginosa* which enters the body through the skin from an open wound. *P. aeruginosa* is the most wide-spread

cause of burn injury infections. It arrives to inner tissues through the burns. Firstly, it adheres to surface via its flagellum. After arriving the suitable number, it releases its virulence factors and cause tissue damage. Microbial growth of *P. aeruginosa* can increase also in the mucosal surface of the lungs in patients with cystic fibrosis. It causes infection in the lungs due to bio-film formation. Generally the multiply antibiotic resistant strains of *P. aeruginosa* are the major problem which causes hospital acquired infection (Iversen et al., 2008).

2.4.6. *Candida albicans* (ATCC 64548)

Candida albicans (*C. albicans*) is a diploid fungus. Normally, 80% of the human population is habitat for *C. albicans* which lives in mouth and gastrointestinal tract without any harmful effect. It is an opportunistic pathogen which causes infection in oral and mucosal surfaces. On the other hand, by the overgrowth of *S. aureus* diseases such as candidiasis may appear in people with reduced immunity. Those infections in the skin and mucosal surfaces may be severe and fatal without proper treatment (McCullough et al., 1996; Arias et al., 2004).

2.5. Why this Microorganisms are Used?

In this study, microorganism which mentioned the previous topic are important because, these microorganisms exist especially in dermal wounds. *S. epidermidis* and *S. aureus* is the most abundant species present on epidermal tissues. On the other hand, *E. coli* is the pathogenic microorganism and it can be exist on inflammation or injury regions. Although *B. subtilis* is non pathogenic, it can also survive on wounds. *P. aeruginosa* is one of the most important pathogenic microorganism. This microorganism is opportunistic pathogen and may cause serious damage on human health. Espacially, when dermal tissues are injured, this pathogen organism immediately placed on wound if exist in the close environment. *C. albicans* is the most dangerous microorganism among all. Beside other negative side effect, this fungus can cause serial dermal inflammation especially on foot and hand fingers. For years many study have been done in order to understand the mechanism of this fungus (Molero et al., 1998; Cutler, 1991). So, the inhibition of this fungus is important for this study.

2.6. Extraction

For centuries, human being used plants for therapeutic purpose (Raskin et al., 2002). Each herb has a various type of phytochemical agent and these compounds offer them a fascinating utilities. When people discover the miracle of plants, they wanted to use them in a different areas. Many civilizations crush and grind some part of plants and transform them into ointment or cream form. In this way, they can easily apply these preparats on their dermal tissues or wounds. Later on, people tried to gain functionality and mix them with other herbs which believed to be beneficial. So, many samples were obtained with different features. These magnificent properties results from the phytochemical compounds in the plants (Cowan, 1999). Scientist focusing on these issues tried to find compounds and concentrated in the direction of reveal their mechanisms (Deverall, 2006). In later years, researchers have emphasized to isolate the essential components of plants (Loomis et al., 2001; Sticher, 2007). In order to perform this phenomena, different solvents and liquids were used (Eloff, 1998). At this point, they used their tissues such as seeds, roots, flowers, fruits and leaves and extraction was appeared in this way. So, the defination of extraction can be expressed; extractions is a technique which is allow us to isolate the bioactive compounds from the plant tissues. Actually, this process consist of a bit complicated operation and needs to be optimized so as to obtain maximum extraction yield with a minimum cost. Not only extraction yield but also obtain the desired extract is important. Hence, many studies was performed in order to accomplish this fact . There are some extraction techniques used for acquire the crude extract that rich in terms of phenolic and antioxidant. Ultrasound extraction, supercritical extraction, Solid-solid extraction and solid-liquid extractions can be given as an example (Rodrigues et al., 2007; Reverchon et al., 2006; Kaufmann et al., 2002).

2.7. Adsorption of Plant Polyphenols

There are some studies about adsorption of polyphenols on to silk fibroin. According to the literature, Baycın et al. showed that olive leaf polyphenols can be adsorbed onto silk fibroin. It is understood here that the polyphenols in olive leaf have a tendency so as to adsorb silk fibroin (Baycın et al., 2007). They stated that 96 mg active compound (oleuropein) can be adsorb onto silk fibroin.

As far as we know from the literature, there is no study about adsorption of polyphenols onto nanofibers. A part of study, consist of adsorption olive leaf crude extract polyphenols onto silk fibroin nanofibers. This application very important because nanofibers have some beneficial properties that high surface area, biodegredable and biocompatible. If this novel nanofibers loaded with crude extract are a candidate for wound healing material, they should be absorb and desorb this bio active compounds.

2.8. Silk Fibroin

Silk has been used in textile production for centuries. This protein is synthesized by silkworms call '*Bombyx mori*' and produce coccons to protect themselves. Because silkworms have to evolve metamorphosis, they fabricate these polyamino acid based silk protein into moths (Sehnal et al., 2008). This non-degradabla sutructure has exhibit an excellent tensile mechanicl properties and people have harvested silk fibers from these cocoons (Hardy et al., 2008). Without any external treatment, silk is consist of two main component; sericin and fibroin. However, there is a protein coating surrounding these two structure (Figure 2.1.). Fibroin is the structural fibrous protein and encircled by sericin which is water-solube and glue-like protein bind fibroins together (Altman et al., 2003).

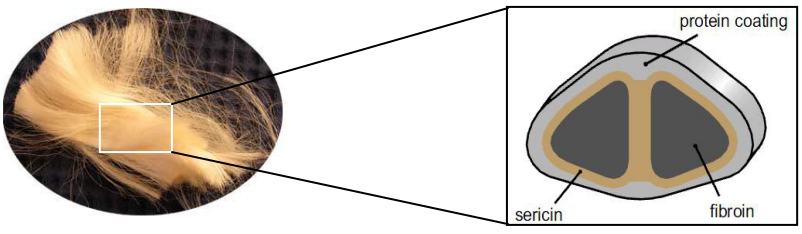


Figure 2.1. Schematic representation of silk protein

Biochemistry refers to four distinct aspects of a protein structure which are primary structure, secondary structure, tertiary structure and quaternary structure. The structure of silk fibroin is secondary structure and common secondary structures are α-helix, β-sheet and β-turn are given in figure 2.2. (Hardy et al., 2008). Silks are generally composted of antiparallel β-sheet structures due to the dominance of hydrophobic domains consisting of short side chain amino acids in the primary sequence (Regina et al., 1999) . The amino acid composition of silk fibroin from consists primarily of glycine, alanine and serine (Vepari et al., 2007).

Figure 2.2. Common secondary structures of silk fibroin

2.9. Biotechnological Applications of Silk Fibroin

Silk Fibroin is a good candidate for biotechnological materials and biomedical applications. This natural macromolecular material has recently been introduced into the field of tissue engineering and nanomedical textile. Bescause silk fibroin is a biocompatible and biodegredabe, it is used in many industrial area such as cosmetics, pharmacological agent and food additive. Beside these, silk also have some positive health effects on to reducing cholesterol level and blood pressure, condense blood, strengthens the immune system and protect liver.

Recently, interest has been concentrating and increasing on the use of several processed forms of the solubilized silk fibroin in biotechnological materials and biomedical applications. Silk solutions have been used to form a variety of biomaterials, such as gels, sponges, films and fibers for medical applications. Several primary cells and cell lines have been successfully grown on different silk biomaterials to demonstrate a range of biological outcomes. Silk biomaterials are biocompatible when studied in vitro and in vivo. Silk scaffolds have been successfully used in wound healing and in tissue engineering of bone, cartilage, tendon and ligament tissues.

Aqueous fibroin solution, which is also called regenerated silk fibroin (RSF), is environment friendly and it has been used in various applications such as in the

development of biosensors, drug coating materials, membranes for controlled drug delivery systems and scaffolds for tissue engineering. RSF is of interest since it represents a good starting material for the preparation of different forms of materials like gels, powders, films, foams and fibers diameter ranging from macroscale to nanoscale. All these structures can be produced or fabricated by the application of various processing techniques. According to the literature, silk forms and applications in biomedical area are given in table 2.4.

Table 2.4. Silk forms and Applications in biomedical area

Reference(s)	Morphologic Form	Application	
Kim et al., 2005	Sponge	Bone Tissue Engineering	Tissue Engineering and Scafolding
Vepari et al., 2007	Film		
Li et al., 2006	Non-woven		
Wang et al., 2006	Sponge	Cartilage Tissue Engineering	
Vepari et al., 2007	Fiber	Tendon Tissue Engineering	
Hu et al., 2006	Film	Hepatic Tissue Engineering	
Unger et al., 2004	Non-woven	Endothelial and Blood Vessels	

(continued on next page)

Table 2.4. (cont.)

Reference(s)	Morphologic Form	Application	
Vepari et al., 2007	Film	Wound Dressing	Medical Textile and Healing
Yeo et al., 2000	Sponge		
Schneider et a., 2009	Non-woven		
Reddy, 2009	Fiber		
Min et al., 2004	Nanofiber		
Bayraktar et al., 2005	Film	Drug Release	Medicine
Wang et al., 2007	Microcapsules	Drug Coating	
Megeed et al., 2002	Poymer	Drug Delivery	

2.10. Nanofiber Fabrication Techniques

Nanofibers can be produced by various techniques such as drawing, template synthesis, phase separation, self assembly and electrospinning (Table 2.5.) Among them, electrospinning is the most efficient and multifunctional methot in order to fabricate nanofibers. As in every method, there are some critical and significant parameters in electrospinning process (Greiner et al., 2007). By changing these conditions, we can obtain different structures and features of nanofibers (Zong et al., 2002). Beside this, nanofibers can be used for many industrial area such as medical (Agarwal et al., 2008), textile, protective material, energy, agriculture and filtration (Burger et al., 2006; Jian et al., 2008; Bijorge et al., 2009). In addition to all, nanofibers that are fabricated with electrospinning method can be gained functionality with addition of inorganic and organic supplies (An et al., 2009).

2.11. Advantages and Disadvantages of Fabrication Techniques

When we compare electrospinning technique with others, it is clear that there are many advantages. The most important benefit is that electrospinning is not only used in lab scale but also can be applied for industrial applications Table 2.6. shows the advantages and disadvantages of fabrication techniques.

Table 2.5. Nanofiber Fabrication Techniques

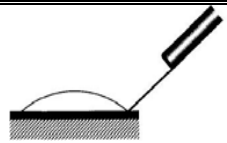
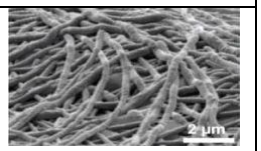
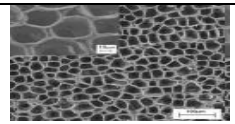
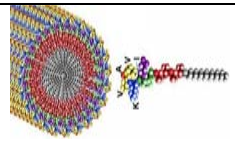
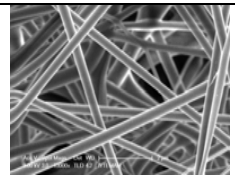
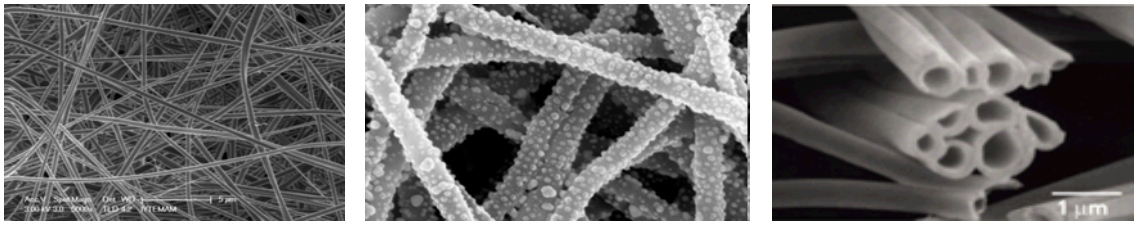
Reference(s)	Techniques	Process	Scaling	Process Conveniences	Control Fiber Dimension	Diameter and Length	Image and Schematic
Ondarcuhu et al., 1998	Drawing	Laboratory	X	✓	X	20 - 100nm 10μm to mm	
Feng, 2002	Template Synthesis	Laboratory	X	✓	✓	100nm 10 μm	
Ma et al., 1999	Phase Separation	Laboratory	X	✓	X	50 - 500nm Porous or Net	
Hartgerink et al., 2001	Self-Assembly	Laboratory	X	X	X	✓	
Reneker et al., 2000 Yang et al., 2005	Electrospinning	Laboratory and Industry	✓	✓	✓	3 - 1000nm Infinitive	

Table 2.6. Advantages and Disadvantages of Fabrication Techniques

Process	Advantages	Disadvantages
Drawing	No special equipment is needed	Discontiuous process Can not be scaled
Template Synthesis	Different fiber diameters can be obtained by using different templates	Non-uniform size distirbution Organic solvents required Difficult full interconnectivity
Phase Separation	Minimum equipment requiremen Suitable with a range of biomaterials Can directly fabricate	Organic solvents required Skinning effect on surface of scaffold
Self-Assembly	Good for obtaining nanoscale nanofibers Typically perform in water Bioactive functionality	Complex process Expensive to manufacture Weak mechanical properties
Electrospinning	In expensive method to produe long nanofiber Wide range of polymers can be used Excellent cell & tissue compatability Extensive surface area & large pores	Organic solvents often required Jet instability Sometimes poor mechanical properties

2.12. Electrospinning Process

Before starting to explain process, briefly I would like to give some important points. Electrospinning is one of the most versatil method to fabricate nano-scale fibers. It can be applied almost every soluble or fusible polymers but mainly melt polymer solutions are used. With this technique, continuous and uniform fibers can be obtained diameters ranging from 10 to 500nm. Recently, scientist try to gain functionality to nanofibers with mixing some organic or inorganic compounds into polymer solution (Liang et al., 2007; Yoon et al., 2008). In figure 2.3., different structures of nanofibers can be seen.



(a) (b) (c)

Figure 2.3. Different Structures of Nanofibers (a)Natural Compound Loaded SF Nanofibers, (b)Catalysis Loaded Nanofiber, (c)Hollow Nanofibers (Source: Li et al., 2004)

The standard laboratory setup for electrospinning generally consists of syringe, high voltage power supply, syringe pump and grounded collector plate. According to the application or usages of nanofibers, these tools can be modified. For example, instead of flat collector plate, high RPM rotating drum can be used in order to fabricate linear, uniform nanofibes. These kinds of nanofibers are especially used for tissue engineering or medical textile applications (Khil et al., 2004). Also two syringe pump and syringe can be used (co-axial electrospinning) so as to produce hollow or core-shell nanofibers (Moghe et al., 2008).

Polymer solution or melt is loaded into the syringe and placed to the syringe pump. After that, high voltage power supply is connected to the syringe needle. Grounded plate is placed in the desired range. The standard setup is shown in figure 2.4.

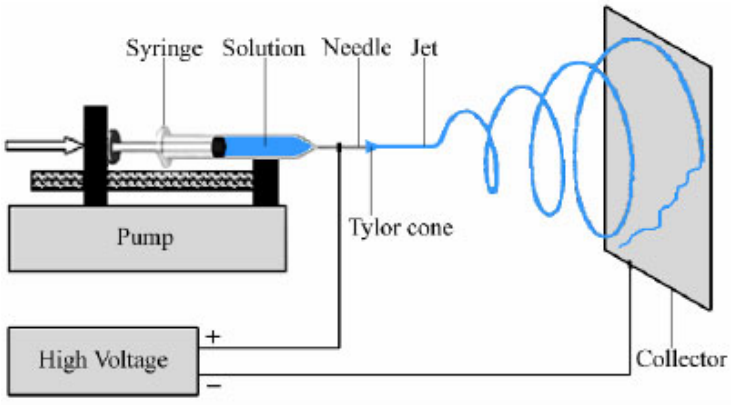


Figure 2.4. General view of Electrospinning Setup

When voltage is applied to the needle, flowing polymer is stretched and extruded from the needle. Before streching, the droplet structure formed at the tip of the syringe called ‘’Taylor cone’’ and this formation is very critical in order to start electrospinning. At this stage, polymer droplet begin to form nanofiber formation. That is to say, if the molecular cohesion of the material is sufficiently high, charged liquid jet is formed and elongated continuously by electrostatic repulsion untill it is deposited on the grounded collector. The elongation by bending instability results in production of uniform fibers with nanometer scales. Figure 2.5., shows the critical points and details of electrospinning moment.

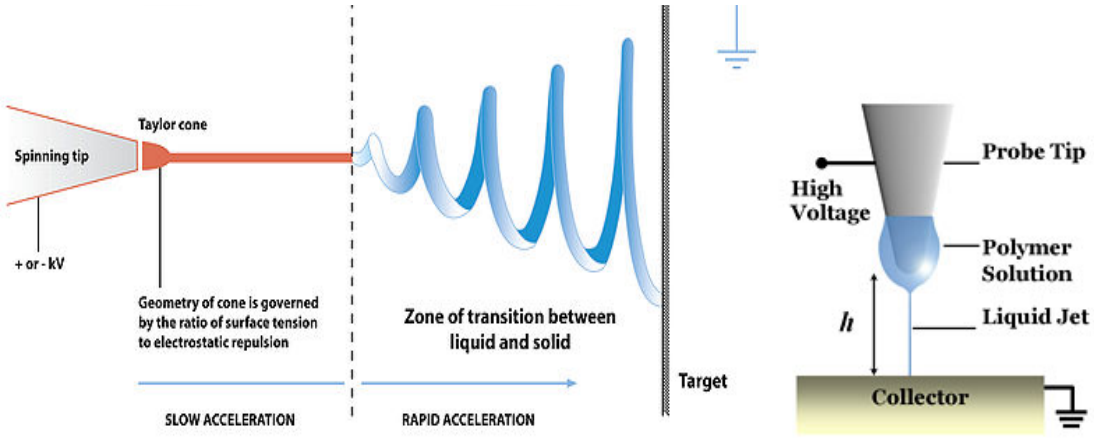


Figure 2.5. Critical Points and Details of Electrospinning Moment

2.13. Parameters Affecting Electrospinning Process

There are some parameters affecting the nanofiber structure. In general, these parameters can be considered under two group; Polymer solution and process parameters.

2.13.1. Polymer Solution Parameters

Many parameters about the polymer can be changed according to the desired nanofiber formation. There are some polymer parameters that affect the electrospinning conditions and nanofibers. These parameters are concentration, molecular weight, solution viscosity, surface tension, conductivity of polymer solution, dielectric effect of solvent and pH value of polymer. However, some of the parameters are directly affects the progress. Primarily concentration and molecular weigth have the critical role, accordingly viscosity is also important. Although all these essential parameters are in optimum value, polymer is not form into taylor cone and electrospinning phenomena may not occure. At this point, polymer surface tension and conductivity play an essential role. Polymer parameters that affecting the electrospinning process are given as a subtitles.

2.13.1.1. Concentration and Viscosity

There is a strong correlation between concentration and viscosity. The molecular weight of the polymer represents the length of the polymer chain, which in turn have an effect on the viscosity of the solution since the polymer length will determine the amount of entanglement of the polymer chains in the solvent. Another way to increase the viscosity of the solution is to increase the polymer concentration. Similar to increasing the molecular weight, an increased in the concentration will result in greater polymer chain entanglements within the solution which is necessary to maintain the continuity of the jet during electrospinning.

The polymer chain entanglements were found to have a significant impact on whether the electrospinning jet breaks up into small droplets or whether resultant electrospun fibers contain beads (Shenoy et al., 2005).

Although a minimum amount of polymer chain entanglements, viscosity is necessary for electrospinning. A viscosity that is too high will make it very difficult to pump the solution through the syringe needle (Kameoka et al., 2003). Moreover, when the viscosity is too high, the solution may dries at the tip of the needle before electrospinning can be initiated (Zhong et al., 2002). Many experiments have shown that a minimum viscosity for each polymer solution is required to yield fibers without beads (Megelski et al., 2002; Fong et al., 1999). At a low viscosity, it is common to find beads along the fibers deposited on the collection plate. When the viscosity increases, there is a gradual change in the shape of the beads from spherical to spindle-like until a smooth fiber is obtained (Fong et al., 1999; Mit-uppatham et al., 2004).

At a lower viscosity, the higher amount of solvent molecules and fewer chain entanglements will mean that surface tension has a dominant influence along the electrospinning jet causing beads to form along the fiber. When the viscosity is increased which means that there is a higher amount of polymer chains entanglement in the solution, the charges on the electrospinning jet will be able to fully stretch the solution with the solvent molecules distributed among the polymer chains. With increased viscosity, the diameter of the fiber also increases (Baumgarten, 1971; Jarusuwannapoom et al., 2005; Demir et al., 2002; Megelski et al., 2002). This is probably due to the greater resistance of the solution to be stretched by the charges on the jet (Jarusuwannapoom et al., 2005).

Another effect of higher concentration is seen by a smaller deposition area. Increased concentration means that the viscosity of the solution is strong enough to discourage the bending instability to set in for a longer distance as it emerges from the tip of the needle. As a result, the jet path is reduced and the bending instability spreads over a smaller area (Mituppatham et al., 2004). This reduced jet path also means that there is less stretching of the solution resulting in a larger fiber diameter. Although viscosity has an important role in the formation of smooth fibers, it may not determine the concentration at which fibers are formed during electrospinning (He et al., 2008).

2.13.1.2. Molecular Weight

One of the factors that affect the viscosity of the solution is the molecular weight of the polymer. Generally, when a polymer of higher molecular weight is dissolved in a solvent, its viscosity will be higher than solution of the same polymer but of a lower molecular weight. One of the conditions necessary for electrospinning to occur where fibers are formed is that the solution must consists of polymer of sufficient molecular weight and the solution must be of sufficient viscosity. As the jet leaves the needle tip during electrospinning, the polymer solution is stretched as it travels towards the collection plate. During the stretching of the polymer solution, it is the entanglement of the molecule chains that prevents the electrically driven jet from breaking up thus maintaining a continuous solution jet. As a result, monomeric polymer solution does not form fibers formation when electrosppinnig occure (Buchko et al., 2009).

2.13.1.3. Surface Tension

The initiation of electrospinning requires the charged solution to overcome its surface tension. However, as the jet travels towards the collection plate, the surface tension may cause the formation of beads along the jet. Surface tension has the effect of decreasing the surface area per unit mass of a fluid. In this case, when there is a high concentration of free solvent molecules, there is a greater tendency for the solvent molecules to congregate and adopt a spherical shape due to surface tension. A higher viscosity will means that there is greater interaction between the solvent and polymer molecules thus when the solution is stretched under the influence of the charges, the solvent molecules will tend to spread over the entangled polymer molecules thus reducing the tendency for the solvent molecules to come together under the influence of surface tension. Solvent such as ethanol has a low surface tension thus it can be added to encourage the formation of smooth fibers (Fong et al., 1999). Another way to reduce the surface tension is to add surfactant to the solution.

2.13.1.4. Conductivity of Polymer and Solution

Electrospinning involves stretching of the solution caused by repulsion of the charges at its surface. Thus if the conductivity of the solution is increased, more charges can be carried by the electrospinning jet. The conductivity of the solution can be increased by the addition of ions. Moreover, most drugs and proteins form ions when dissolved in water. As previously mentioned, beads formation will occur if the solution is not fully stretched. Therefore, when a small amount of salt or polyelectrolyte is added to the solution, the increased charges carried by the solution will increase the stretching of the solution. As a result, smooth fibers are formed which may otherwise yield beaded fibers (Zhong et al., 2002)

2.13.1.5. Dielectric Effect of Solvent

The dielectric constant of a solvent has a significant influence on electrospinning. Generally, a solution with a greater dielectric property reduces the beads formation and the diameter of the resultant electrospun fiber (Son et al., 2004). Solvents such as N,N-Dimethylformamide (DMF) may added to a solution to increase its dielectric property to improve the fiber morphology (Lee et., al. 2003). The bending instability of the electrospinning jet also increases with higher dielectric constant. If a solvent of a higher dielectric constant is added to a solution to improve the electrospinnability of the solution. The interaction between the mixtures such as the solubility of the polymer will also have an impact on the morphology of the resultant fibers. (Wannatong et al., 2004).

2.13.2. Process Parameters

Beside polymer solution parameters, also process parameters have an important role. As seen in figure 6, there are some devices using in electrospinning setup. Each device has its own task and affect the electrospinning process. The process parameters are applied voltage (usually between 5 to 30 kV), distance between capillary and collector, flow rate of polymer solution and motion of target screen. However, ambient

temperature and humidity can affect both polymer and process condtions. From this point of view, applied voltage, flow rate and distance have been a critical role.

2.13.2.1. Applied Voltage

A crucial element in electrospinning is the application of a high voltage to the solution.. The high voltage will induce the necessary charges on the solution and together with the external electric field, will initiate the electrospinning process when the electrostatic force in the solution overcomes the surface tension of the solution. Generally, both high negative or positive voltage of more than 6kV is able to cause the solution drop at the tip of the needle to distort into the shape of a Taylor Cone during jet initiation (Taylor, 1964). Depending on the feedrate of the solution, a higher voltage may be required so that the Taylor Cone is stable. The columbic repulsive force in the jet will then stretch the viscoelastic solution. If the applied voltage is higher, the greater amount of charges will cause the jet to accelerate faster and more volume of solution will be drawn from the tip of the needle. This may result in a smaller and less stable Taylor Cone (Zhong et al., 2002). When the drawing of the solution to the collection plate is faster than the supply from the source, the Taylor Cone may recede into the needle (Deitzel et al., 2001).

As both the voltage supplied and the resultant electric field have an influence in the stretching and the acceleration of the jet, they will have an influence on the morphology of the fibers obtained. In most cases, a higher voltage will lead to greater stretching of the solution due to the greater columbic forces in the jet as well as the stronger electric field. These have the effect of reducing the diameter of the fibers (Megelski et al., 2002) and also encourage faster solvent evaporation to yield drier fibers (Pawlowski et al., 2003). When a solution of lower viscosity is used, a higher voltage may favor the formation of secondary jets during electrospinning. This has the effect of reducing the fiber diameter (Demir et al., 2002).

2.13.2.2. Distance

Varying the distance between the tip and the collector will have a direct influence the electric field strength. For independent fibers to form, the electrospinning jet must be allowed time for most of the solvents to be evaporated. When the distance between the tip and the collector is reduced, the jet will have a shorter distance to travel before it reaches the collector plate. Moreover, the electric field strength will also increase at the same time and this will increase the acceleration of the jet to the collector.

As a result, there may not have enough time for the solvents to evaporate when it hits the collector. When the distance is too low, excess solvent may cause the fibers to merge where they contact to form junctions resulting in inter and intra layer bonding. Decreasing the distance has the same effect as increasing the voltage supplied and this will cause an increased in the field strength. As mentioned earlier, if the field strength is too high, the increased instability of the jet may encourage beads formation (Zhong et al., 2002).

However, if the distance is such that the field strength is at an optimal value, there is less beads formed as the electrostatic field provides sufficient stretching force to the jet (Jarusuwannapoom et al., 2005). In other circumstances, increasing the distance results in a decrease in the average fiber diameter (Ayutsede et al., 2005). When the distance is too large, no fibers are deposited on the collector (Zhao et al., 2004). Therefore, it seems that there is an optimal electrostatic field strength below which the stretching of the solution will decrease resulting in increased fiber diameters.

2.13.2.3. Flow Rate

The feedrate will determine the amount of solution available for electrospinning. For a given voltage, there is a corresponding feedrate if a stable Taylor cone is to be maintained. When the feedrate is increased, there is a corresponding increase in the fiber diameter or beads size. However, there is a limit to the increase in the diameter of the fiber due to higher feedrate (Rutledge et al., 2000). If the feedrate is at the same rate which the solution is carried away by the electrospinning jet, there must be a corresponding increased in charges when the feedrate is increased. Thus there is a

corresponding increased in the stretching of the solution which counters the increased diameter due to increased volume Due to the greater volume of solution drawn from the needle tip, the jet will takes a longer time to dry. As a result, the solvents in the deposited fibers may not have enough time to evaporate. The residual solvents may cause the fibers to fuse together where they make contact forming webs. A lower feedrate is more desirable as the solvent will have more time for evaporation (Yuan et al., 2004).

2.13.2.4. Geometry of Spinneret

The internal diameter of the needle or the pipette orifice has a certain effect on the electrospinning process. A smaller internal diameter was found to reduce the clogging as well as the amount of beads on the electrospun fibers (Mo et al., 2004). The reduction in the clogging could be due to less exposure of the solution to the atmosphere during electrospinning. Decrease in the internal diameter of the orifice was also found to cause a reduction in the diameter of the electrospun fibers. When the size of the droplet at the tip of the orifice is decreased, such as in the case of a smaller internal diameter of the orifice, the surface tension of the droplet increases. For the same voltage supplied, a greater columbic force is required to cause jet initiation. As a result, the acceleration of the jet decreases and this allows more time for the solution to be stretched and elongated before it is collected. However, if the diameter of the orifice is too small, it may not be possible to extrude a droplet of solution at the tip of the orifice (Zhao et al., 2004).

2.13.2.5. Temperature

The temperature of the solution has both the effect of increasing its evaporation rate and reducing the viscosity of the polymer solution. When polyurethane is electrospun at a higher temperature, the fibers produced have a more uniform diameter (Demir et al., 2002). This may be due to the lower viscosity of the solution and greater solubility of the polymer in the solvent which allows more even stretching of the solution. With a lower viscosity, the Columbic forces are able to exert a greater stretching force on the solution thus resulting in fibers of smaller diameter (Mit-

uppatham et al., 2004). Increased polymer molecules mobility due to increased temperature also allows the Columbic force to stretch the solution further. However, in cases where biological substances such as enzymes and proteins are added to the solution for electrospinning, the use of high temperature may cause the substance to lose its functionality.

2.14. Biopolymers for Electrospinning

Many bipolymers have been fabricated into nanofiber formation by electrospinning method. Not only polymer solutions but also their blends have been processed into nanofiber. Especially, in recent years many researchers have been ivestigated blends and co-polymers in order to fabricate biofunctional nanofibers (Schneider et al., 2008). Widely used polymers for electrospinning given in Table 2.7.

Table 2.7. Biopolymers for Electrospinning

Reference(s)	**Biopolymer**	**Solvent**
Matthews et al., 2002	Collagen	Hexafluoroisopropyl Alcohol
Xie et al., 2003	Lipase	Bis-tris-propane buffer
Wnek et al., 2003	Fibrinogen	Hexafluoroisopropyl alcohol
Kim et al., 2003	Silk Fibroin	Formic acid
Xie et al., 2003	Lipase	Bis-tris-propane buffer
Park et al., 2006	Chitin	Hexafluoroisopropyl alcohol
Ohkawa et al., 2004	Chitosan	Acetic acid
Frenot et al., 2007	Cellulose	N,N-dimethylacetamide
Zhang et al., 2005	Gelatin	2,2,2-trifluoroethanol

2.15. Electrospinning of Silk Fibroin

Proteins based natural silk fiber have outstanding mechanical properties. This makes it an interesting candidate for application in biomedical field where mechanical property is important. It is possible to electrospin silk to obtain fibers with average diameter less than 500nm (Ohgo et al., 2003). Electrospun fibers from silk fibroin were found to promote cell adhesion and proliferation (Min et al., 2004). Silk fibroin itself has several advantages biological properties such as good biocompatibility, good oxygen and water vapor permeability, biodegradability and minimal inflammatory reaction (Sakabe et al., 1989). The high surface area to volume ratio of the electrospun fiber also encourages cell attachment, growth and proliferation (Schneider et al., 2009). In the literature, there are some studies about process, characterization, morphology, mechanical and structure properties of silk fibroin nanofibers fabricated via electrospinning. Table 2.8. summarize these studies.

Table 2.8. Electrospinning of Silk Fibroin – Process, Characterization, Morphology, Mechanical and Structure Properties

Reference(s)	Solvent	Concentration	Parameters	Characterization	Objective(s)
Sukigara et al., 2003	Formic acid	5 – 20 wt%	Voltage: 2 - 5 kV, Distance: 5 - 10 cm	SEM	Processing parameters, Geometric properties
Wang et al., 2004	Water	8 wt%	Voltage: 12.5 kV, Distance: 21 cm	SEM, FTIR, AFM, XRD, DSC	Determination the Mechanical properties
Min et al., 2004	Formic acid	8 wt%	Voltage: 15 kV, Distance: 7 cm	SEM, IR	Formation of SF matrices with different texture
Sukigara et al., 2004	Formic acid	12 - 20 wt%	Voltage: 10 - 28 kV, Distance: 5 – 7 cm	SEM	Process Optimization, Empirical Modelling
Ayutsede et al., 2005	Formic acid	9 – 12 wt%	Voltage: 15 - 30 kV, Distance: 5 - 10 cm	RS, FTIR, SEM, XRD	Characterization of SF Non-woven Mats
Wang et al., 2005	Water	17 – 39 wt%	Voltage: 20 - 40 kV, Distance: 11 cm	SEM, XRD	Structure of SF Nanofibers
Chen et al., 2006	Water	28 – 37 wt%	Voltage: 12 - 20 kV, Distance: 18 cm	SEM, FTIR, DSC	Non-woven Mats from Aqueous SF Solution
Zhu et al., 2007	Water	20 - 38 wt%	Voltage: 20 - 40 kV, Distance: 10 - 20 cm	SEM, DSC, RS	Morphology and Structure of SF mats with Adjusting pH

2.16. Applications and Future Aspects

Up to now, we have seen how nonofibers are obtaind via electrospinning method. As we have stated from time to time, nanofibers can be used for many applications. Much effort has been applied to electrospun polymer nanofibers to comprehend the fundamental phenomena of fabrication process as well as the physical and chemical properties from the material science viewpoint. Figure 2.8. shows that 60% of electrospun polymer nanofiber researches have been devoted to the studies of their processing and characterization. Consequently, nanofiber researchers are able to flexibly control the fiber morphology, fiber diameter and patterning of fiber deposition. These developments have provided much impetus to the dream of realizing the potential applications of nanofibers. As seen in Figure 2.6. major possible applications are categorized into Bioengineering, Environmental Engineering & Biotechnology, Energy & Electronics and Defense & Security. In all areas, the demand of new novel materials has been aspired and electrospun polymer nanofibers could make the new wave of material research. In this final section of the literature review, potential applications of electrospun nanofibers and used areas are introduced and the research trend of each topic is briefly analyzed.

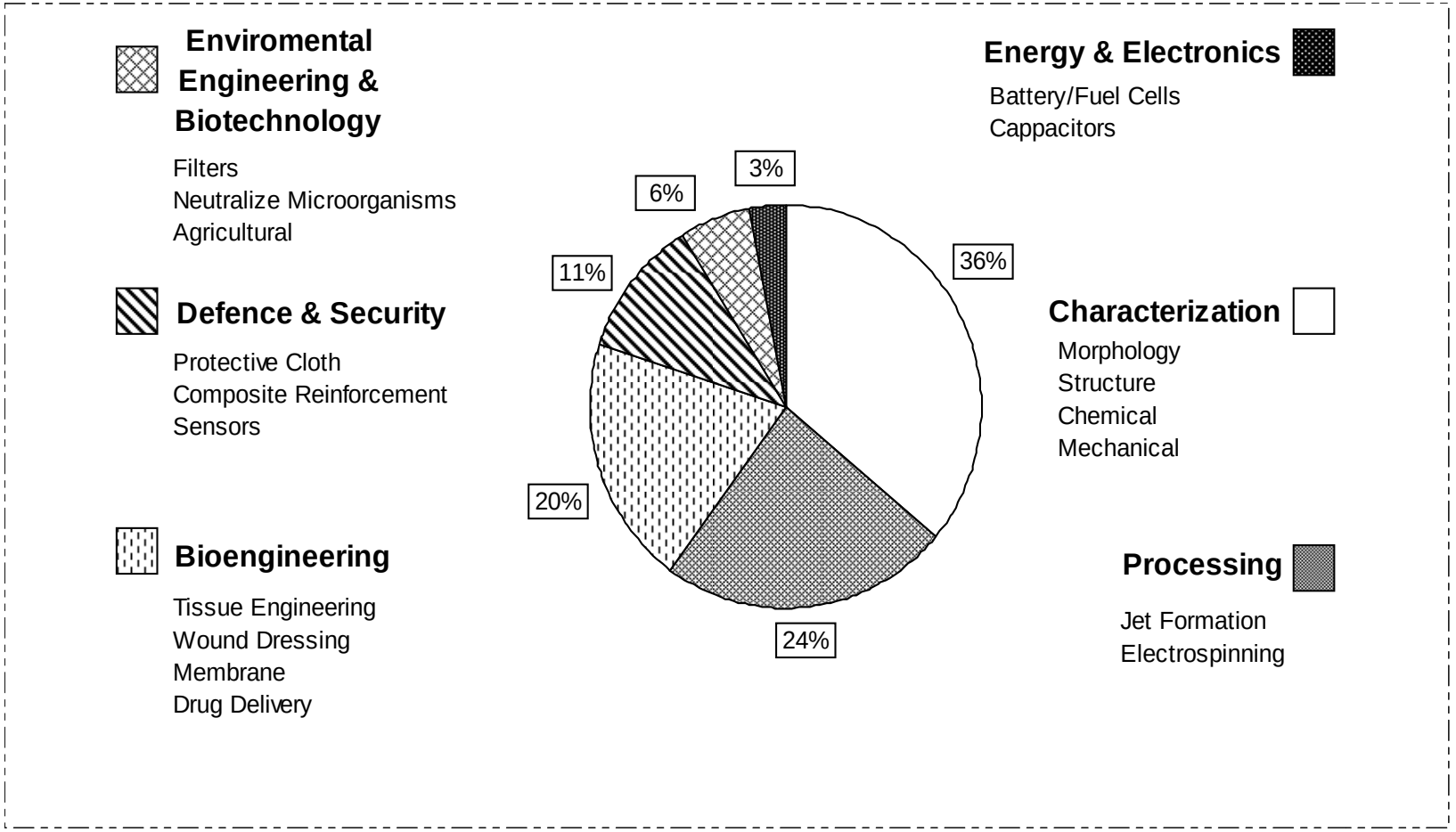


Figure 2.6. Research Category of Electrospun Nanofibers

2.16.1. Medical Applications and Tissue Engineering

Nanostructured polymer systems of natural or synthetic origin have a multitude of possible applications in medicine and pharmacy (in the form of nanofibers, hollow nanofibers, core– shell nanofibers, nanotubes, or nanorods). A main reason for this wide applicability is that the nanoscale is particularly relevant for biological systems, because proteins, viruses, and bacteria have dimensions on this order. Many viruses have the shape of a nanotube. In the following, some current activities are given in the use of nanosystems in tissue engineering, wound dressing and wound healing, scaffold fabrication, artificial organ components, implants material, transport and release of drugs, tumor and cancer therapies and medical textile materials (Agarwal et al., 2008).

Tissue engineering and scaffold fabrication: Tissue engineering is one of the most exciting interdisciplinary and multidisciplinary research areas today, and there has been exponential growth in the number of research publications in this area in recent years. It involves the use of living cells, manipulated through their extracellular environment or genetically to develop biological substitutes for implantation into the body and to foster remodeling of tissues in some active manners (Nerem et al., 1995). Tissue engineering, also called regenerative medicine is an interdisciplinary field involving knowledge from medicine, biology, engineering and materials science fields. Tissue engineering makes use of scaffolds to provide support for cells to regenerate new extra cellular matrix (ECM) which has been destroyed by disease, injury or congenital defects without stimulating any immune response. Natural ECM separates different tissues, forms a supportive meshwork around cells, and provides anchorage to the cells. It is made up of proteins and glycosaminoglycans (GAGs) which are carbohydrate polymers.

Electrospinning generates loosely connected 3D porous mats with high porosity and high surface area which can mimic ECM structure and therefore makes itself andexcellent candidate for use in tissue engineering. The requirements for a material to be used for tissue engineering purpose are biocompatibility, and biodegradability, as the scaffold should degrade with time and be replaced with newly regenerated tissues. Also, the scaffold architecture is very important and affects cell binding as seen in figure 2.7. (Stevens et al., 2005).

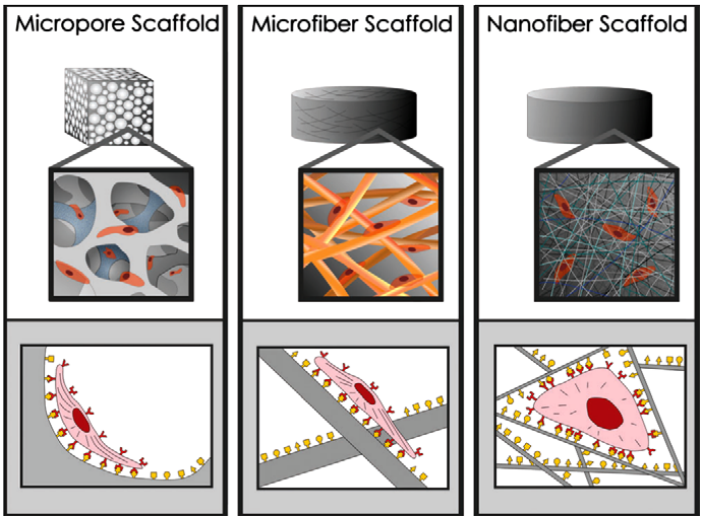


Figure 2.7. Scaffold architecture affects cell binding and spreading

The cells binding to scaffolds with microscale architectures flatten and spread as if cultured on flat surfaces. The scaffolds with nanoscale architectures have bigger surface area for absorbing proteins and present more binding sites to cell membrane receptors. The adsorbed proteins further can change to provide an edge over microscale architectures for tissue generation applications. It is clear that scaffolds mimicking the architecture of the extracellular matrix should offer great advantages for tissue engineering. The extracellular matrix surrounds the cells in tissues and mechanically supports them (Tan et al., 2001). This matrix has a structure consisting of a three-dimensional fiber network, which is formed hierarchically by nanoscale multifilaments. An ideal scaffold should replicate the structure and function of the natural extracellular matrix as closely as possible, until the seeded cells have formed a new matrix.

Scaffolds fabricated from electrospun nanofibers have several clear advantages. However, considerable room for optimization remains with respect to architecture, surface properties, biodegradability, porosity, and mechanical properties, and also with respect to the seeding of cells in the threedimensional space and the supply of nutrients to the cells. It is often observed that the cells preferentially grow on the surfaces or that they initially adhere to the carrier fibers, but then detach after differentiation.

Wound dressing and healing: Wound dressing is a therapy to repair the skin damaged by ambustion and injury. So far electrospun nanofibrous membrane exhibited the potential in wound dressing field. The membrane attained uniform adherence at wet wound surface without any fluid accumulation (Bhattarai et al., 2004). Electrospun nanofiber membrane is a good wound dressing candidate because of its unique properties. Wound dressing with electrospun nanofibrous membrane can meet the requirements such as higher gas permeation and protection of wound from infection and dehydration. The goal of wound dressing is the production of an ideal structure, which gives higher porosity and good barrier. To reach this goal, wound dressing materials must be selected carefully and the structure must be controlled to confirm that it has good barrier properties and oxygen permeability. The rate of epithelialization was increased and the dermis was well organized in electrospun nanofibrous membrane and provided a good support for wound healing (Khil et al., 2003). This wound dressing showed controlled evaporative water loss, excellent oxygen permeability and promoted fluid drainage ability due to the nanofibers with high porosity.

Transport and release of drugs: Generally, patients suffering from diseases take drug orally. Although drug is delivered to the damaged site, the amount of delivered drug decreases against initial drug dose as drugs also spread to the healthy site through digestive organs. Therefore, patients occasionally need to take excessive amount of drug for several times, which may induce undesirable side effect. It is considered that the ideal drug dose is to deliver the minimum required amount of drug to the disease site. Furthermore, those minimized amount of drugs must be effectively absorbed at the disease site. Generally, drug uptake into human body is faster with the smaller size of the drug and its coating material. Hence, drug delivery system has been developed using polymeric materials in the form of nano or micro particles, hydrogels and micelle (Kim et al., 2004). Although those polymeric drug delivery materials improved therapeutic effect and reduced side effect, there is still need to address how to precisely control the drug releasing rate. Based on such background, researchers have recently focused on the usage of polymer nanofiber membranes which encapsulate medical drugs instead of conventional polymeric materials.

In in vitro experiments on the release kinetics of functional molecules, it was possible to follow their release from fibers into the environment (by fluorescence microscopy). However, the experiments demonstrated that the release often occurs as a burst, in a process that is definitely nonlinear with respect to time. The release kinetics,

including the linearity of the release over time and the release time period, can be influenced by the use of core-shell fibers, in which the core immobilizes the drugs and the shell controls their diffusion out of the fibers (Greiner et al., 2006). In addition to low-molecularweight drugs, macromolecules such as proteins, enzymes, growth factors, and DNA are also of interest for incorporation in transport and release systems.

2.16.2. Textile Manufacturing

In recent years, linear, 2-dimensional and 3-dimensional textile fabrics have been used beyond traditional apparels and found applications ranging from medical, chemical separation to composite reinforcements and chemical protection. This is due to the unique combination of light weight, flexibility, permeability, strength and toughness of textiles. However, before fibers can be made into textile, they have to be made into the form of continuous yarns. This has posed a great difficulty for electrospun fibers as they lack sufficient strength to withstand traditionaltextile performing process. It is also a challenge to control the electrospinning process precisely to obtain yarns with different architecture as most methods of electrospinning were only able to obtain either random fiber mesh or aligned fiber. Nevertheless, the ability to produce yarns made of nanofibers are highly attractive, there are several methods that attempts to address the issue.

Nonwovens composed of nanofibers can be used in combination with conventional textiles to modify the properties of the textiles. The objective can be to increase the wind resistance, to regulate the water-vapor permeability, to optimize the thermal insulation behavior, or to give the textile a specific functionality such as the lotus effect, aerosol filtering, or protection against chemical or biological hazards (Figure 2.8). In recent years, nanofibers are being widely used in textile manufacturing. Sport apparels, sport shoes, climbing materials, rainwears, wind proof, water resistants, antibacterial clothes, outerwears garments and baby diapers can be given as an example of textile manufacturing applications (Rozek et al., 2008).

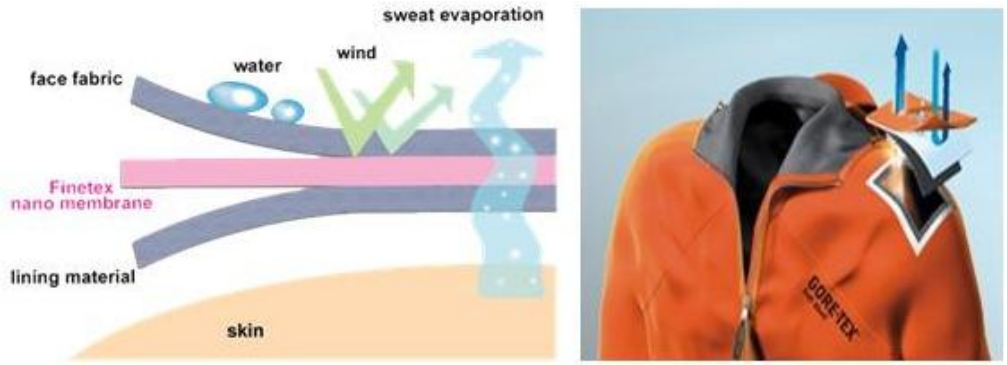


Figure 2.8. Biofunctional nanofibers using in textile applications

2.16.3. Chemical and Biological Protective Cloth Applications

These days, the worldwide threat of chemical and biological agents by hostile military and terrorist groups has been growing. There is a grave concern in the development of reliable and stronger mechanisms of defense. Upholding the motto of "prevention is better than cure" is very essential in the field of national security, where even the smallest compromise cannot be made as it would lead to disastrous effects not only to an individual but to the community and more so to the country as a whole. Hence attention is drawn to develop protective systems to fight these chemical and biological weapons of mass destruction. With all these advancements in artillery, soldier survivability has considerably reduced. Chemical and biological weapons can be used across a wide spectrum of warfare, from acts of assassination and small-scale terrorism to various tactical and operational situations, both defensive and offensive, including strategic population attacks. The technical and economic barriers to development and weaponization have decreased.

There exists a great potential in the polymer nanofibers for use as aerosol filters in face masks and in protective clothing against chemical and biological warfare agents (Gibson et al., 1999; Schreuder et al., 2002). Polymeric nanofibers have been studied as a carrier for active chemistry that may allow for improvements in chemical protective properties. The nanofibers can provide a huge surface area to be functionalized with chemical groups, which are reactive with toxic gases and chemicals. Graham mixed polyoxometallate, a catalyst for the oxidative degradation of sulfur mustard (a chemical weapon agent) with the Polyurethane (PU) solution and electrospun the mixture into nanofibers. The capability of the catalyst in the electrospun nanofiber was found even

higher than the catalyst alone (Graham et al., 2003). Acatay synthesized an antibacterial agent containing perfluorinated terpolymer, poly(vinylbenzyldimethylcocoammonium chloride - MMA - perfluoro alkyl ethyl acrylate). This terpolymer was electrospun into a fluffy nanofiber mesh with fiber diameter as low as 40nm. The nanofiber was immersed into liquid solutions containing *Escherichia Coli* to test the antibacterial ability. Although the antibacterial activity in liquid solution was detected to be weak due to its low surface tension, the material may be used in air filtration applications (Acatay et al., 2003).

Two of the most common classes of chemical warfare agents include the nerve agents and the blister agents. The motivation of develop a novel protective cloth using electrospun polymer nanofiber is to impart a functional group into(or onto) the nanofiber surface in such a way that when the gases come into contact, by chemical reaction on the surface of the nanofibers, their active groups get deactivated into relatively harmless by products. Additionally these nanofibers would have sufficient resistance to block the bacterial contaminants such as Bacillus Anthracis by virtue of small pore size. Briefly, chemical and biological protective materials can be considered such as sound absorption materials, protective clothing's against chemical agents, magnetic protection, thermal protection, protective clothing's against biological warfare agents and sensor applications for detecting (Graham et al., 2004). Figure 2.9. shows the multilayer nanofiber conformation. With this functional structure, foreign agents can not pass through the material and reach the skin but vapour can evaporate from the skin.

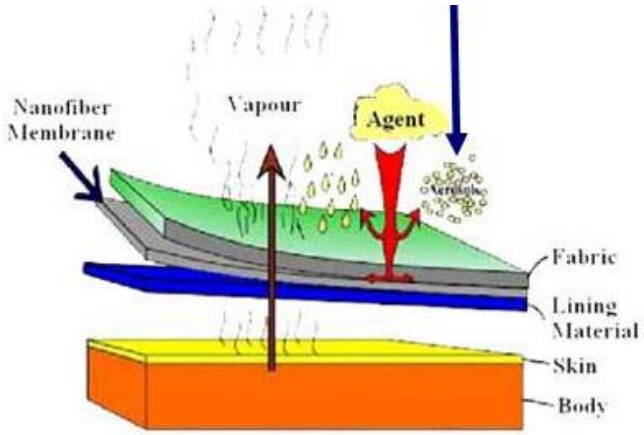


Figure 2.9. Multilayer nanofiber conformation using in protective cloth applications

2.16.4. Catalysts and Sensors Applications

In chemistry and biology, a carrier for catalyst is used to preserve high catalysis activity, increase the stability and life of the catalyst, and simplify the reaction process. An inert porous material with a large surface area and high permeability to reactants could be a promising candidate for efficient catalyst carriers. Using an electrospun nanofiber mat as catalyst carrier, the extremely large surface could provide a huge number of active sites, thus enhancing the catalytic capability. The well-interconnected small pores in the nanofiber mat warrant effective interactions between the reactant and catalyst, which is valuable for continuous-flow chemical reactions or biological processes. Also, the catalyst can be grafted onto the electrospun nanofiber surface via surface coating or surface modification (Jian et al., 2008).

Sensor means devices that respond to physical or chemical stimulus such as biomolecules concentration, gas concentration, thermal energy, electromagnetic energy, acoustic energy, pressure, magnetism, or motion, by producing an easily detectable and measurable signal, usually electrical or optical. (Wang et al., 2002). Electronspun nanofibrous materials have been investigated for use in nanosensor applications, taking advantage of their high surface area and good transport properties needed for efficient molecule detection (Liu et al., 2004; Ding et al., 2004). High surface area is one of the most desired parameters for the sensitivity of conductimetric sensor film. Conductimetric sensor based on semiconducting oxides is a kind of low cost detectors for reductive gas. The operating principle of these devices is associated primarily with the adsorption of the gas molecules on the surface of semiconducting oxides inducing electric charge transport between the two materials, that changes the resistance of the oxide. The structure configuration of the metal oxide materials is one of the key parameters controlling the gas senescing process. Now there is an increasing trend in chemical sensing to utilize nanostructured materials as gas sensing elements because the high surface areas and the unique structure features are expected to promote the sensitivity of the metal oxide to the gaseous component. Electrospun fibers may have potential as a surface for enzymes to be immobilized on. These enzymes could be used to break down toxic chemicals in the environment, among other things (Dersch et al., 2005).

2.16.5. Energy and Electrical Applications

High surface areas combined with the flow of ion-conducting electrolytes through electrically conducting nanofiber mats make electrospun materials attractive as electrodes for batteries or electrochemical supercapacitor applications. Electrospun materials investigated for this purpose include carbon nanofibers (Kim et al., 2003).
In recent years, batteries, photovoltaic cells, solar cells, polymer cells, polymer electrolytes and membrane fuel cells are being investigated by many researchers and become a popular in this area (Dersch et al., 2005). In most batteries, porous structure is an essential requirement. A sponge-like electrode will have high discharge
current and capacity, and a porous separator between the electrodes can effectively stop the short circuit, but allow the exchange of ions freely. Solid electrolytes used in portable batteries, such as lithium ion battery, are typically composed of a gel or porous host to retain the liquid electrolyte inside (Arora et al., 2004). To have high ion conductivity, the host material, also called separator, should have high permeability to ions. A porous membrane with well interconnected pores, suitable mechanical strength and high electrochemical stability could be a potential candidate.

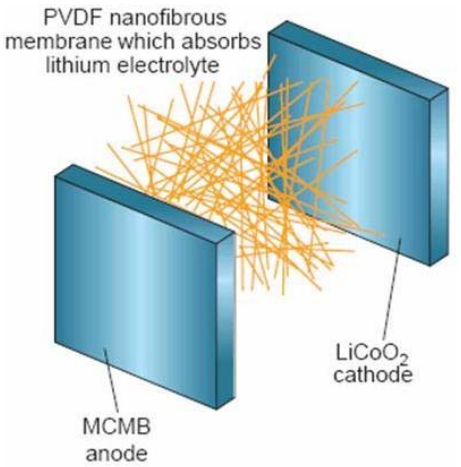


Figure 2.10. PVdF nanofibrous lithium battery application

PVdF electrospun nanofiber membrane has been investigated as separator for lithium battery application (Figure 2.10) (Choi et al., 2003). The PVdF nanofiber membrane showed high uptake to electrolyte solution and high ion conductivity. The fibrous electrolyte also had high electrochemical stability of more than 5 volt. The prototype cell (MCMB/PVdF based electrolyte/LiCoO2) exhibited a very stable charge-discharge behavior (Kim et al., 2004). When a thin layer of polyethylene (PE) was plasma polymerized onto the PVdF nanofiber surface, a role of shutter by melting of the PE layer grafted was rendered to the nanofiber membrane, which improved the safety of battery (Choi et al 2004). It was also found that the formation of interconnected web structure via heat treatment improved both the mechanical properties and dimensional stability of nanofiber membranes (Gao et al., 2006).

2.16.6. Filtration and Membrane Applications

In industrial factory, working office and hygienic surgical operation room, air purification is essential requirement to protect people and precision equipment. Filter media is utilized to purify air which contains solid particles (virus, mine dust and polens) and liquid particles (smog, evaporated water and chemical solvents). Figure 2.11 shows the SEM image of silk fibroin nanofiber. It is clearly seen that there is a polen particules on the fiber surface and there is a huge difference between nanofiber pore size and polen diameter. The nanofiber seen in figure 2.11. was the same fiber that produced and used in this thesis.

Nanofibers are already used extensively in air filters. Conventional air filters consist of paper mats composed of fibers with diameters in the micrometer range. They function by trapping particles floating in the air deep inside the filter, which means that the whole filter material is involved in the retention of the particle. As the number of particles trapped in the filter material increases, the pressure drop across the filter also increases, and above a certain limit, the filter can no longer be used. The filter is usually partially cleaned with a pressure blast, whereby the particles are pushed out of the filter and collected. With each cleaning process, a higher pressure drop remains, and eventually the filter must be replaced (Greiner et al., 2007).

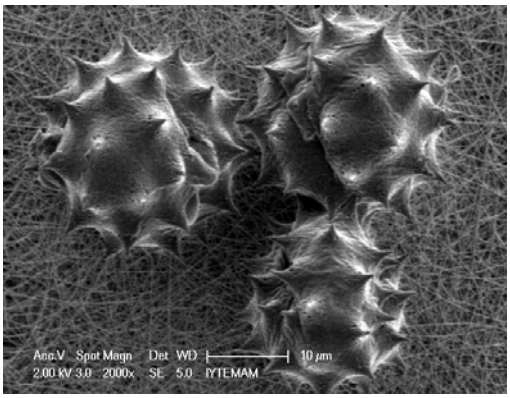


Figure 2.11. Silk fibroin nanofiber and polen particules

So far, High Efficiency Particulate Air (HEPA) filter made of non-woven glass fiber mesh has been utilized to capture particles and 300nm size particles can be excluded with 99.97% filter efficiency (Maus et al., 1997; Kemp et al., 2001). However, mesh pore size must be small or thick mesh is required to remove ultra fine particles, which means that a filtration fun needs to blow the air with high pressure. In contrast, air blowing with lower pressure leads to poor ventilation through a filter media. This kind of property is called "pressure drop" and lower pressure drop is required to an excellent filter media. Filters have been widely used in both households and industry for removing substances from air or liquid. Filters for environment protection are used to remove pollutants from air or water. In military, they are used in uniform garments and isolating bags to decontaminate aerosol dusts, bacteria and even virus, while maintaining permeability to moisture vapour for comfort. Respirator is another example that requires an efficient filtration function. Similar function is also needed for some fabrics used in the medical area (Jian et al., 2008).

One main interest of electrospun polymer nanofiber nonwoven mesh is for affinity membrane applications. Affinity membrane is membrane which bases its separation on the selectivity of the membrane to ‘capture’ molecules, by immobilizing specific ligands onto the membrane surface. For the nanofiber mesh to be used as affinity membrane, specific ligand molecules need to be immobilized on the nanofiber surface. The simplest method to introduce ligands on nanofiber surface is to directly mixing the ligand molecules into the polymer solution and then electrospin the polymer solution. Some researchers have been made to incorporate chemically modified compounds onto the surface of the nanofiber to target potential applications in organic waste treatment for water purification (Kaur et al., 2004).

On such background, electrospun nanofiber membranes have gained the large potential such as high efficient filters, air, pollen, oil, fuel filters for automotive industry, filters for beverage, pharmacy and medical applications (Gopal et al., 2006). On the other hand, nanofibers can be applied in agricultural applications such as protecting plants against greenhouse effect, plant covering, agricultural protection and protect plants from cold circumstances (Huang et al., 2003). It was estimated that future filtration and agricultural market would be up to $700billion by the year 2020 (Suthar et al., 2001).

Each potential application of electrospun polymer nanofibers has been briefly introduced. We can realize that the area of potential applications is very broad including bioengineering, tissue engineering, material engineering, electrical engineering and chemical engineering etc. In the field of tissue engineering and wound dressing, the cell attachment and proliferation on nano fibrous scaffolds have been investigated *in vitro* and now the research trend is heading to animal study of nano fibrous scaffolds with seeded cells. In near future, biomedical therapy using nano fibrous tissue scaffolds may be close to the practical level. The research status of electrospun polymer nanofibers has just finished analyzing the fundamental processing and, physical and chemical properties of nanofibers.

In this respect, it can be said that the application researches of nanoflbers have just started. In other research fields, high research competition will be estimated due to the surrounding world affairs of this century such as terrorism attacks and the rapid economical growth of undeveloped countries. In order to encourage the application researches of electrospun nanoflbers, the cooperation of researchers from various scientific fields is important. The current authors look forward to seeing the bright future of people's daily life by the novel application researches of nanofibers. Figure 2.12. shows the general application areas of nanofibers.

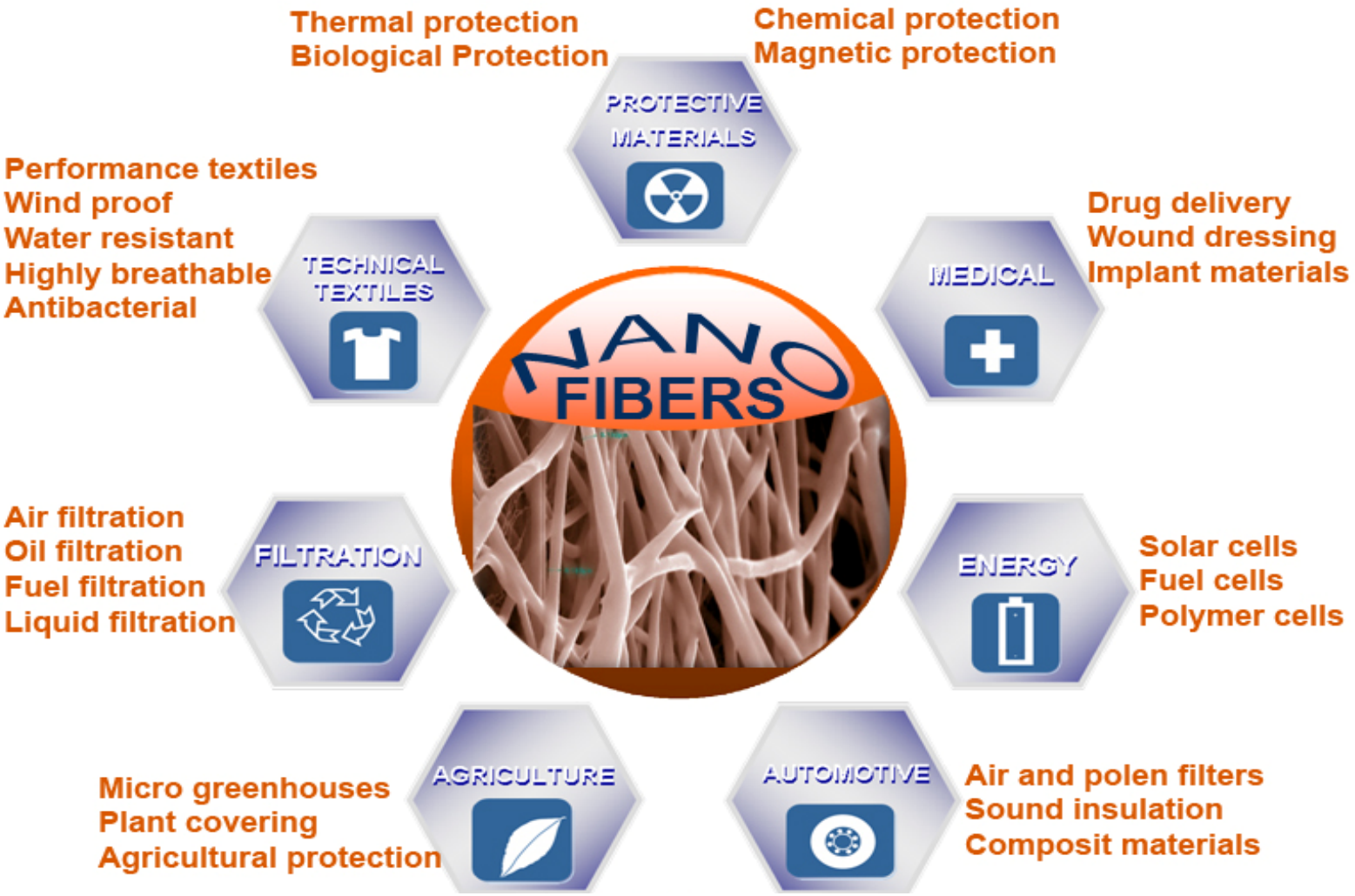


Figure 2.12. The general application areas of nanofiber

CHAPTER 3

OBJECTIVES

The main aim of this study is to understand and investigate the preparation of nanofibers from silk fibroin by electrospinning method. Beside this, determination of antibacterial activity of silk fibroin nanofibers incorporated with natural compounds (Olive leaf extract) will investigate. The goals of this study can be summarized as follows;

- ✓ To obtain the extracts of plant species with a standardized extraction process
- ✓ To determine the extraction yields of plant materials, total phenol contents and antioxidant capacities of extracts
- ✓ To determine antimicrobial activities of plant extracts with standart Disc Diffusion and Minimum Inhibition Concentration (MIC) assays

On the other hand;

- ✓ To produce silk fibroin nanofibers via electrospinning method
- ✓ To examine the parameters (biopolymer concentration, applied voltage and distance) affecting the morphology of silk fibroin nanofibers
- ✓ To investigate the olive leaf extract loaded silk fibroin nanofibers
- ✓ To obtain the extracts of plant species having antimicrobial activities to be incorporated with silk nanofibers in future

CHAPTER 4

EXPERIMENTAL STUDY

4.1. Materials

4.1.1. Plant Materials and Chemicals

New harvested fresh green olive leaves (*Olea europaea*) and Juniper seeds (*Juniperus communis*) were collected from the trees which are grown in Izmir Institute of Technology campus (Urla) and Karaburun region respectively. Turkish sweetgum (*Liquidambar orientalis*) leaves were collected from natural sweetgum forests distributed in Muğla and lotus (*Ziziphus jujuba*) leaves were collected from Ege University botanic garden.

In all extraction experiments analytical grade ehtanol (C_2H_5OH) was used and purchased from Merck (Germany). Folin-ciocalteu was used in order to determine total phenol content obtained from Sigma (USA). Sodium carbonate anhydrus (99.5%) was obtained from Fluka (Switzerland). Trolox (6-hydroxy-2,5,7,8,-tetramethylchroman-2-carboxylic acid), ABTS (2,2'-azinobis(3-ethylbenzothiazoline-6-sulphonic acid) reagents and potassium persulfate ($K_2O_8H_8$) from Fluka (Germany) were used for antioxidant analysis.

Glycerol was used to prepare the stock cultures and stored in - 80°C. Nutrient broth and Nutrient agar were purchased from Fluka and used for bacterial reproduction. Penicillin, Gentamycin and Ampicillin antibiotics purchased from pharmacy were used for comparison to evaluate the antimicrobial activities of plant extracts in microdilution assays. Penicillin G (CTOO43B), Gentamicin (CTOO24B), Vancomycin (CTOO58B) and Streptomycin are the antibiotic discs used in the standart disc diffusion assays and purchased from OXOID.

Silk Fibroin (SF) was obtained in raw form from Bursa Institute for silkworm Research (Bursa, Turkey). In order to remove sericin, silks were boiled in sodium carbonate (99.5+ %) solution purchased from Aldrich (Germany). Sodium sulfide hydrate provided by Fluka (Switzerland) and sulfuric acid (98+ %) from Merck

(Germany) are used in the preparation of dialysis tubing (MW Cut-off: 12–14 kDa) Sigma (USA). Calcium chloride-2-hydrate ($CaCl_2.2H_2O$) was supplied from Riedel-de Haën (Germany) and used for preparation the aqueous silk fibroin solution. Formic acid (HCOOH) was purchased from Merck (Germany). HPLC grade acetonitrile from Sigma-Aldrich (Germany) and HPLC grade acetic acid from Merck (Germany) were used as a mobile phase. Ultra pure water was used during all experiment.

4.1.2. Instruments and Equipments

All plant materials were dried in Memmert UFP 800TS oven. After grinding, extractions were performed in Thermo MaxQ-4000 benchtop orbital shaker. Evaporations were carried out with Heidolph laborata 4001 and aqueous solutions were frozen. Telstar cryodos-50 freeze drier was used in order to remove water, then dry crude extracts were obtained. Phenolic compounds of olive leaf crude extract was analysed with HPLC Agilent Technologies 1100series. The determination of polyphenolic compounds before and after adsorption perform also perform the same device. Multiskan UV spectrophotometer from Perkin Elmer was used for the determination of total phenol contents and antioxidant capacities of extracts.

Electrospinning setup consist of High Voltage Power Supply, iseg T1CP 300 and Syringe pump, Newera NE1000. The Syringes and needles that used during fabrication of nanofibers were purchased from medical suppliers. While the preparation of silk fibron aqueous solution, circular shaking water bath was used. Heidolph MR magnetic heater and stirrer were used in all polymer solution preparations. Phillips XL-30S FEG Scanning Electron Microscope was used for visualization and characterization of fibers. While determination the minimum inhibition concentrations of crude extract, Varioskan flash multiplate reader from Thermo was used. However, Minimum Inhibition Concentration test performed in Microtiter 96 well plates which are sterile, clear and flat was obtained from Thermo. Bacterological medium, pens, glass tools, tubes and ultra pure water which are used during the microbiologic studies have been sterilized Hirayama HVE-50 Autoclave. Nuve EN055 series incubator was used in order to overnight incubation of bacteria and fungi. All microbial strains were stored at -80°C in Revco Ulti1786 series refrigerator. Ultra pure water used in all experiments obtained from Sartorius – Arium 611VF water sanitizer.

4.2. Methods

The methods developed and applied in this study can be divided into three major groups. The first group is extraction of plants which are mentioned in the previous part and appointed the yield values of extract. Then, their total phenol contents and antioxidant capacities were determined. Eventually, antimicrobial activities and minimum inhibition concentrations agains microorganisms of these extract were found with standart disc diffusion method and minimum inhibition concentration assay, respectively.

The second group is the crucial part of this study. This part include the fabrication of silk fibroin nanofibers via electrospinning technique. So, nanofibers diameters ranging from 60 to 180 nanometers with a high surface area were obtained. However, three main parameters (concentration, voltage, distance) were invastigated and optimized. Accordingly, nanofibers were acquired with a wide range of morphological structure. Among them, the best one was chosen which has desired diameter, uniform and no bead conformation in order to load natural compound.

The third and the last group is adsorption of phenolic compounds onto nanofiber. To do this, olive leaf crude extract was chosen as a natural compound. Because, to our knowledge, as we remember from the literature , silk fibroin is a good candidate so as to adsorb phenolic compounds in olive leaf crude extract. After that, desorption of these compounds from nanofiber were investigated. On the other hand, silk fibroin microfibers and regenerated silk fibroin (foam formation) was also tried. Depending the surface areas, their adsorption and desorption amount were compared as a natural compound(mg)/Silk fibroin microfiber, foam and nanofiber (gr).

The schematic representation of the strategy and methods followed during the study is shown in Figure 4.1.

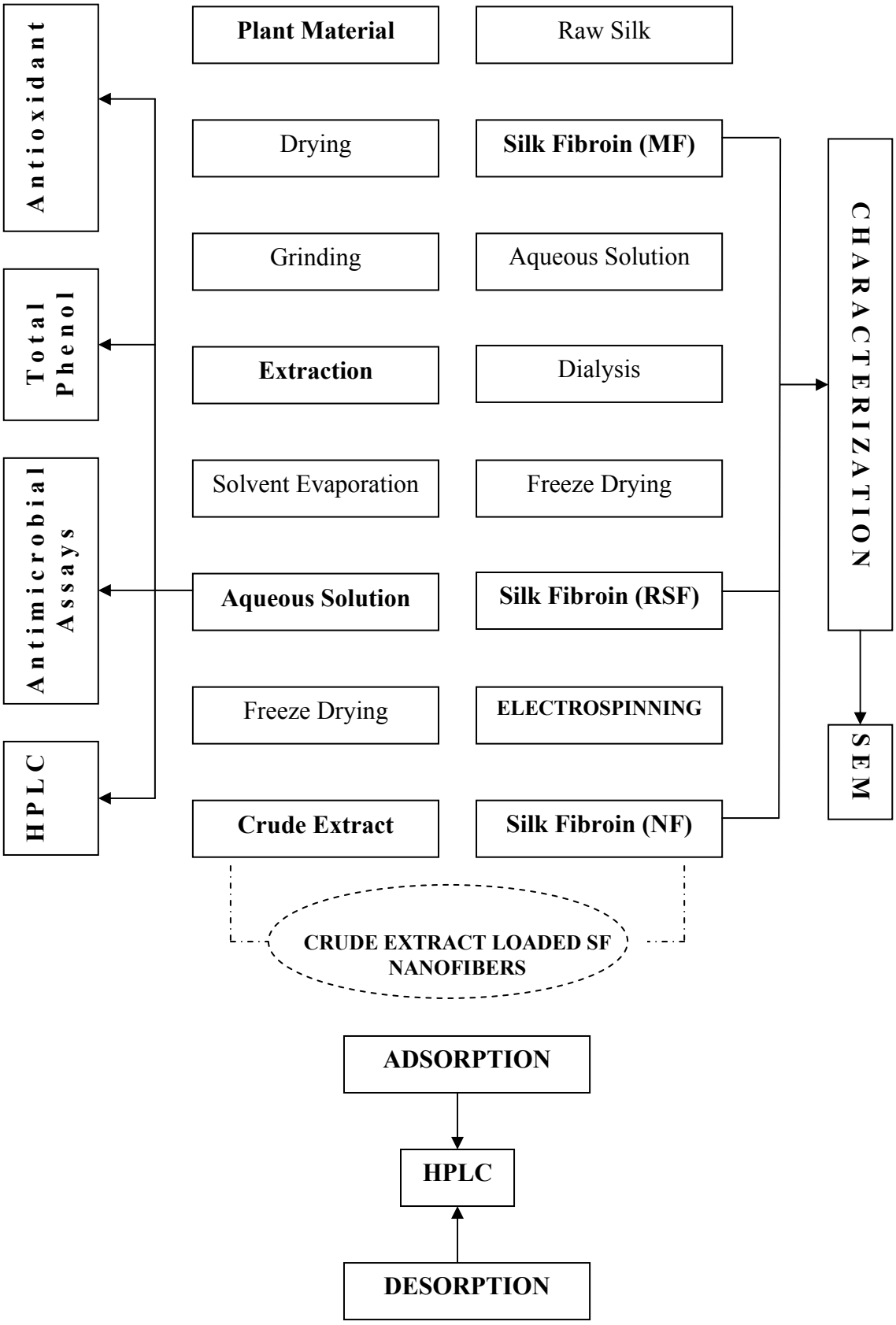


Figure 4.1. The schematic representation of the strategy and methods

4.2.1. Extraction of Plant Materials

All plant species leaves were washed with deionized water and dried in same conditions at 35 °C for 3 days. Dried leaves were crushed and grounded with grinder. After pulverization, the particle size of the leaves are about 70-140μm. 10 g of each plant powder was extracted for 2 hours with 200 ml of 70% (v/v) aqueous ethanol solution. Extraction was performed at 35 °C by a thermo-shaker which is fix to, 180rpm without light. Then the samples were centrifuged at 5000 rpm and the parts of the samples were evaporated with a rotary evaporator in order to remove of the ethanol under low pressure at 35 °C. After evaporation, aqueous extract solutions were kept overnight at +4 °C so as to precipitate chlorophyll and impurities. The remaining aqueous solutions were frozen and lyophilized 3 days. After lyophilization, the percent (w/w) extraction yields of plant materials were calculated. The crude extracts were put in a glass bottles and kept in dark, cool, dry place in order to use for experiments.

4.2.2. Determination of Total Phenol Contents

Total phenol content of 4 plant species was determined by using Folin-ciocalteu method which was optimized and calibrated in our laboratory. Plant extracts were dissolved in ultrapure water in a ratio of 0.05 g extract in 1 ml water. On the other hand, Folin-ciocalteu reagent was diluted with deionized water in a ratio of 1:10 as a stock. 500 μl plant extract was mixed with 2.5 ml Folin-ciocalteu reagent and left to stand 2.5 min at room temperature. Then, 2 ml of sodium carbonate solution was added that prepared 7.5 % in deionized water previously. After incubating 1 hr at room temperature in a dark place, the absorbances were measured at 725 nm by UV spectrophotometer. Results were expressed as mg of gallic acid equivalents (GAE)/gr weight.

4.2.3. Determination of Antioxidant Activity

In order to determine antioxidant activity aqueous ABTS solution was used. 14mM ABTS [light blue] and 4.9mM potassium persulfate ($K_2S_2O_8$) was mixed in a ratio of 1:1 and stand for 16 hours in a dark place at ambient temperature. After the reaction completed, solution forms $ABTS^+$ and its color turns into dark blue. While performing the experiment, first crude extract was dissolved in a solvent (water was used for all extract) and concentration is written down because the initial concentration was noted because the initial concentration value was used in order to calculate the antioxidant activity at the end of the experiment. On the other hand, $ABTS^+$ solution was diluted with ethanol and absorbance was adjusted 0.7 (±0.03) at 734 nm. 10µl dissolved crude extract was added to 2 ml of $ABTS^+$ solution. Six kinetic readings were performed and absorbance was measured at every minute during six minutes with UV-Visible Spectrophotometer. All samples should be analysed at least three times at different concentration (10, 20, 30 µl). The percentage inhibition of absorbance at 734 nm was calculated and the trolox equivalent antioxidant capacity (TEAC) value was determined. Results were expressed as TEAC (mmol/g sample).

4.2.4. Determination of Antimicrobial Activity with Disc Diffusion

Sterile cultures were prepared daily in 8 ml broth by transfering one loop of stock bacteria which are kept in –80 °C. These cultures incubated for 18 hours and subcultures were obtained by transfering 80 µl from this 18 hour-incubated cultures to fresh broth (8 ml). 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. In this study, six hours subcultures were taken in all experiments so as to use the same number of bacteria ($6x10^7$ CFU/ml). 100 µl of bacteria culture (from 6 hours subculture) were inoculated onto agar surface. The agar depth was adjusted to 25ml for each plate. Inoculated culture was dispersed by streaking the steril swab over the entire steril agar surface by rotating the plate 60° each time to ensure the inoculum uniformly spread. The inoculated plates were allowed to sit for 5-10 minutes to let the broth

absorp into agar. On the other hand, sterile blank discs were applied by soaking into sterilized extracts before 6 hours. The concentration of extract solutions was determined as 100mg extract/ml sterile ultrapure water for each plant extracts. During 6 hours, blank discs are absorb the extract by shaking at low speed in ambient temperature. Only 4 discs were placed on each plate and then gently pressed to ensure contact with the agar surface. Plates were incubated for 24 hours at 37 °C . After 24 hours the inhibition zone diameters were measured by using a compass and results were expressed as milimeter (mm).

4.2.5. Determination of Minimum Inhibition Concentrations (MIC)

In order to determine the Minimum Inhibition Concentration (MIC) of the plant extracts; *Escherichia coli* and *Pseudomonas aeruginosa* were used as a gram negative bacteria. *Staphylococcus epidermidis*, *Staphylococcus aureus* and *Bacillus subtilis* were used as a gram positive bacteria. *Candida albicans* was used as a fungus (a form of yeast). First of all, crude extracts were dissolved in sterile deionized water for a concentration of 100mg/ml. Serial dilutions of each extract were carried out with a final concentration of 0.4mg/ml by using sterile deionized water. 100µl of each extract concentration and 95µl nutrient broth were added in each well of 96 well microplate. Each well inoculated with 5µl of 6 hours incubated bacterial subculture suspensions. This value was standardized by adjusting their optical densities at 640 nm by UV spectrophotometer. In all experiments, negative, positive and blank controls were carried out for each strain. Negative control well carried out 195µl of nutrient broth and 5µl of 6 hours bacterial suspension. Positive controls were performed by using antibiotics. Blank control is consisted of only 200µl nutrient broth.

Penicillin, Gentamycin and Ampicillin were used as a positive control. Initial concentration of antibiotics are 1000µg/ml. Serial dilutions of each antibiotic were carried out with a final concentration of 0.2µg/ml by using sterile deionized water. 100µl of each antibiotic concentration and 95µl nutrient broth were added in each well of 96 well microplate. Each well inoculated with 5µl of 6 hours incubated bacterial subculture suspensions. The assay plate were incubated at 37°C for 24 hours and growth kinetic assays for each strain were performed by growth curves. MIC values of each extract and antibiotics were determined by a Varioskan microplate reader at 640 nm.

MIC results were reported as mg/ml for extracts and μg/ml for antibiotics. These spectrophotometric measurements of MIC values were carried out with a standardized protocol of Varioskan multiplate reader.

4.2.6. Parent Materials for Preparation of Silk Fibroin Nanofibers

From the raw silk fibroin with sericin to uniform and smooth silk fibroin nanofiber, there are some steps and pretreatments that should be done. In some studies, silk fibroin microfibers were directly fabricated into nanofiber without any preliminary preparation. If the polymer solution prepared from the silk microfibers, some problems may be occured such as precipitation, gelation, heterogeneous polymer solution and finally non uniform nanofibers with high diameter. In this study, raw silk microfibers were pre-processed in order to obtain homogeneous polymer solution and uniform nano-scales nanofibers. The main steps for preparation of silk fibroin nanofiber are given below as a subtitles.

4.2.6.1. Preparation of Degummed Silk Fibroin

As we remember from the properties of silk, raw silk obtained from cocoons has not sericin free, there is a glue-like, sticky sericin cover the fibroins. First, we need to get away with sericin. 10 grams of raw silk washed in water to remove dusts or impurities and was boiled in 500 ml (0.05%) sodium carbonate solution. Silk is boiled in this solution for 30 minutes and this process repeated three times. After boiling, silk fibroin is washed with distilled water and left to dry in ambient conditions. This pre-treatment step calls ‘’degumming’’.

4.2.6.2. Preparation of Silk Fibroin Aqueous Solution

For one dialysis tube; 14.7 gr Calcium chloride-2-hydrate, 11.6 ml Ethanol (EtOH) and 10.8 ml ultra pure water was put in a schott bottle and mixed properly. The important thing is to dissolve calcium chloride-2-hydrate homogeneously. On the other hand, before prepared 1.2 gr degummed silks were put into this solution and shaked for

2 hours at 150 rpm at 78 °C in a heated water bath. Here, the crucial thing is preparing 1.2 gr silk because silks must be cutted into small pieces before put into solution. At the end of the 2 hours, silk fibroin turns into aqueous form, bottles are taken from the water bath and expected to come to room temperature. Finally, aqueous silk fibroin solution was poured into dialysis tube and end of the tube is tightly bound. Tubes put in distilled water and dialysed three days. Dialysis water should be refrashed every six hours.

4.2.6.3. Preparation of Dialysis Tubes

As mentioned in previous section, while preparing the aqueous silk fibroin solution, dialysis tube was used. First, tubes were cut in desired length and washed under water for 3-4 hours in order to get rid of glycerine covering the tubes. Then these steps were followed; In order to remove sulfide compound, tubes were boiled in sodium sulfide solution (0.3% w/v) for 1 minute at 80°C. Then, kept in distilled water for 2 minutes at 60°C. Later tubes were put in sulphuric acid solution (0.2% v/v) for 1 minute at 30°C. And last step, tubes were kept in distilled water for 2 minutes at 60°C however, last step was repeated at least 3 times. Eventually tubes were taken from the water and left to dry in ambient conditions.

4.2.6.4. Preparation of Regenerated Silk Fibroin

Aqueous silk fibroin solution obtained after dialysis was taken at the end of three days and filtered through a filter paper with a large pores. Collected solution was poured in a freeze drier bottle and left it to frozen. Later on, this frozen solution attached to the freeze drier and stand for 5 days in order to obtain completely dried product. At the end of this process, silk fibroin in the form of foam was obtained and called ‘Regenerated Silk Fibroin (RSF)’.

4.2.6.5. Preparation of Silk Fibroin Polymer Solution

Before performing electrospinning and fabricating the nanofibers, the last step is preparation of polymer solution. To prepare the solution Regenerated silk fibroin (RSF)

and formic acid (98%) was used. RSF was cut into small pieces, desired concentration was determined and mixed with formic acid. Here, the significant point is adding RSF pieces into formic acid in a slow rate because undesirable results could be obtained, for instance gelation may occur and we could not obtain homogeneous polymer solution. To overcome this problem, polymer solution should be stirred over night with adding RSF's gradually.

4.2.7. Preparation of Silk Fibroin Nanofibers

Up to this part, preparation of polymer solution and other preliminary stages were mentioned and explained before electrospinning. Now, fabrication the silk fibroin nanofibers with different parameters will be emphasized. As we remember from the experimental setup of electrospinning, basically, high voltage power supply and syringe pump were used during all experiment. Of course in adition to this, glass syringe and needle were managed. Aluminum plate covered by aluminum foil was used as a collector and connected to the ground. Silk fibroin polymer solution was loaded into glass syringe and placed to the syringe pump. The distance between syringe needle and collector place was measured. Afterward, high voltage was applied to the syringe needle. After making sure everything is ready, one by one syringe pump and high voltage power were switched on in order. So, electrospinning was started and poymer solution was begun to form nanofiber. After few minutes, aluminum foil coated with nanofiber observed as white color. The black color background sheet was placed just behind the electrospinning region where electrospinning process takes place.

In this study, in order to fabricate uniform nanofibers which have different morphologies, some parameters were changed. As mentioned earlier, there were three main parameters which play an essential role for the formation of nanofibers. These were concentration of polymer solution, applied voltage and distance between cappilary and grounded plate. By changing these parameters, different type of nanofibers were fabricated. Five different polymer concentration (60-80-100-120-150 mg/ml), three distinct voltage (17-20-23 kV) and two alternative distance (10-15 cm) were experienced.

4.2.8. Characterization of Silk Fibroin Fibers

By using Scanning Electron Microscope (SEM), all silk fibroin fiber formations were visualized. SEM allow us to display fiber formations with micro and nano scales. SEM has a back-scattering dedector (BS Dedector) and by using BS dedector, three dimensional (3-D) topographic images were obtained. Image-J 1.32j visualization and scaling software was used for measure the diameters of fibers. Diameters of microfiber, RSF and nanofiber were compared and results were exressed as nanometer (nm).

4.2.9. Determination of Pholyphenolic Compounds of with HPLC

At the beginning of the study, four different plant examined and among them olive leaf crude extract was chosen. Before determination the total phenol content and antioxidant capacity of olive leaf crude extract, High Performance Liquid Chromatography (HPLC) analysis was performed in order to quantify and qualify the phenolics in the olive leaf extract. Samples were filtered through 0.45 μm membrane before injection to HPLC. The operating conditions of HPLC optimized in our laboratory are given in Table 4.1.

Table 4.1. Operating conditions and properties of High Performance Liquid Chromatography

Units and Parameters	**Conditions and Numeric Values**
Column	LiChrospher 100, C18 analytic column
Column length	25 cm
Column diameter	4 mm
Particle size	5 μm
Mobile Phase(s)	Mobile phase A: 2.5%acetic acid solution
	Mobile phase B: 100% acetonitrile
Flow rate	1 ml/min
Temperature	30°C
Detector	Dode Array Dedector (DAD)
Absorbance	280 nm

4.2.10. Adsorption and Desorption of Polyphenols

The interaction between silk fibroin and olive leaf polyphenols have been invastigated in our laboratory for the last several years. One of the most important part of this study was the adsorption of olive leaf polyphenols onto nanofiber. On the other hand, degummed silk fibroin microfibers and regenerated silkfibroin was also used for adsorption studies. HPLC was used in all steps to determine the adsorbed oleuropein amount. At the end, the adsorption amounts of these three structure were compared and results were expressed mg oleuropein/gr silk material. The general process steps for silk fibroin nanofiber studies are given below (Figure 4.2);

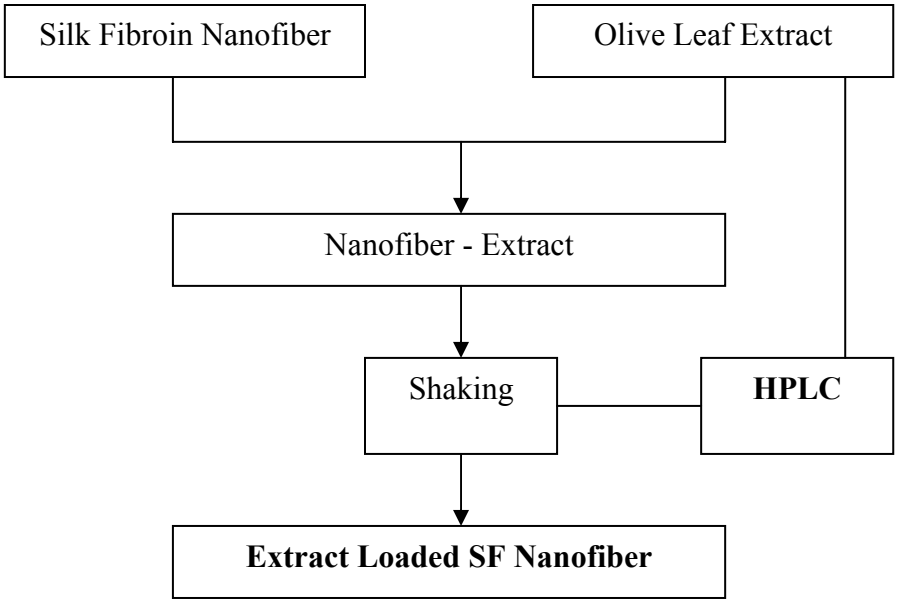


Figure 4.2. Extract Loading steps

- First, olive leaf crude extract was dissolved in ultra pure water. The initial concentration (C_i) of extract was set as 100mg/ml.
- Before incorporating silk fibroin nanofiber with crude extract, HPLC analysis of crude extract was performed in order to find initial oleuropein content.
- After analyzing initial crude extract content, 1 gr nanofiber was put into 10 ml (C_i=100mg/ml) extract solution.
- Nanofibers and extract were shaked at 150 rpm, 25 °C for 6 hours.
- Every hour, 20 µl sample was taken and analyzed with HPLC.
- At the end, 6-hour data were obtained and based on the HPLC chromatogram, adsorbed amount of oleuropein was found.

After adsorption, polyphenolic compound loaded nanofibers were obtained. To our knowledge and studies, phenolics in olive leaf extract have affinities towards surface of silk fibroin fibers. Especially major active compound, oleuropein could be adsorbed on the silk fibroin fibers. The desorption of polyphenols from the silk fibroin nanofiber in ultra pure water were determined via HPLC analysis. Degummed silk fibroin microfibers and regenerated silkfibroin was also used for desorption studies. At the end, the desorbed oleuropein amounts from these three structure were compared and results

were expressed mg oleuropein/gr silk material. The general desorption experimental steps for extract loaded silk fibroin nanofiber are given below (Figure 4.3);

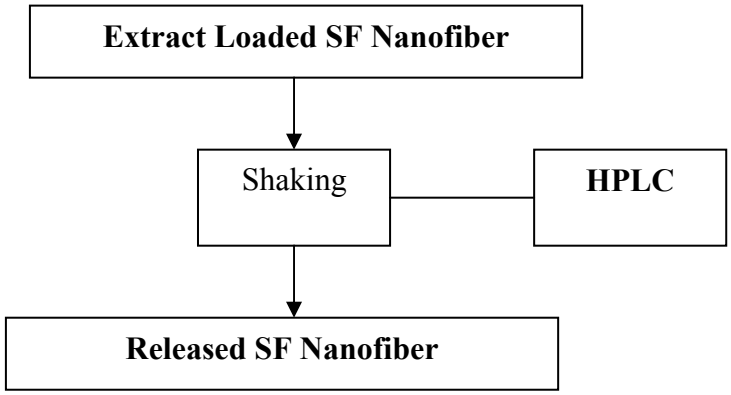


Figure 4.3. Extract Desorption steps

- First, silk fibroin nanofibers were removed from the extract solution
- Silk fibroin nanofibers were put into 10 ml water medium (Ci_{medium}=0mg/ml).
- Nanofibers were shaked at 150 rpm at 25°C for 6 hours.
- Every hour, 20 µl sample was taken and analyzed with HPLC
- At the end, 6-hour data were obtained and based on the HPLC chromatogram, desorbed amount of oleuropein was determined.

CHAPTER 5

RESULTS AND DISCUSSION

5.1. Extraction Yields of Plants Material

As a result of the standardized extraction protocol, extraction yields were obtained for each plant material. Extraction yields are ranged between 12.4 % and 24.7 %. According to the results, *Liquidambar orientalis* has the highest extraction yield with 24.17 %. The extraction yields of *Ziziphus jujuba* 21.5 %, *Olea europaea* 15.6 % and *Juniperus communis* 12.4 % is shown in table 5.1, respectively.

Table 5.1. Extraction Yields of Plants

Plant and Codes		**Part of Plant**	**Extraction Yield (%)**
Liquidambar orientalis	**(A)**	Leaves	24.7
Ziziphus jujuba	**(B)**	Leaves	21.5
Olea europaea	**(C)**	Leaves	15.6
Juniperus communis	**(D)**	Seeds	12.4

These results were calculated on the basis of initial weight and expressed as a percentage. For each plant material, 10 gram dry powder was used and after extraction procedure, dry crude extracts were obtained. These extracts were weighted and the values were converted into percentages. So, figure 5.1. shows the graphical representation of extraction yields of plants.

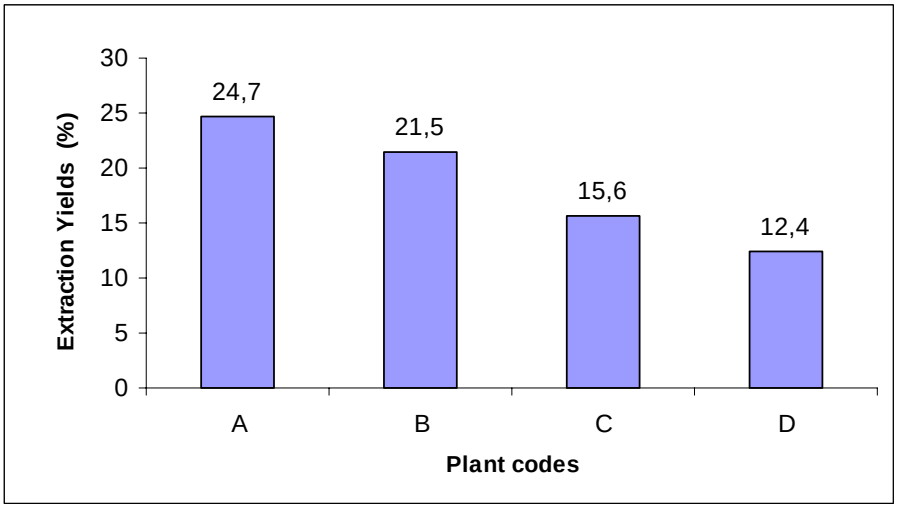


Figure 5.1. Graphical Representation of extraction yields

5.2. Total Phenol Contents of Plants

Phenolic compounds are a class of antioxidant agents which act as free radical terminators. Phenolic compounds inhibit lipid oxidation by scavenging free radicals, chelating metals, activating antioxidant enzymes, reducing tocopherol radicals and inhibiting enzymes that cause oxidation reactions

Total phenol contents of plant species were analyzed with folin-ciocalteu Reagent and obtained for each plant material. Total phenol contents are ranged between 0.372 and 0.032 mgGA/g sample. The most promising plant species in total phenol analysis is *Liquidambar orientalis*. According to the results, *Liquidambar orientalis* has the highest phenolic compounds with 0.372 GAEq. Total phenol contents of *Ziziphus jujuba* 0.210 GAEq, *Olea europaea* 0.159 GAEq and *Juniperus communis* 0.032 GAEq are shown in table 5.2, respectively.

Table 5.2. Total Phenol Contents of Plants

Plant and Codes		Part of Plant	Total Phenol Content GAEq [mgGA/g sample]
Liquidambar orientalis	**(A)**	Leaves	0.372
Ziziphus jujuba	**(B)**	Leaves	0.210
Olea europaea	**(C)**	Leaves	0.159
Juniperus communis	**(D)**	Seeds	0.032

Four plant species with significant activities have been detected in this study and some of them have a lack of laboratory data on this bioactivities. Especially, *Liquidambar orientalis* and *Ziziphus jujuba* 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. For some plant species, the correlation can be seen between total polyphenols and some bioactivities such as antioxidant activity. In this study, we also see that there is a strong correlation between total phenolics and total antioxidant activity. Figure 5.2. shows the graphical representation of total phenol contents of plants. Calibration curve of total phenol content can be seen in Appendix A.

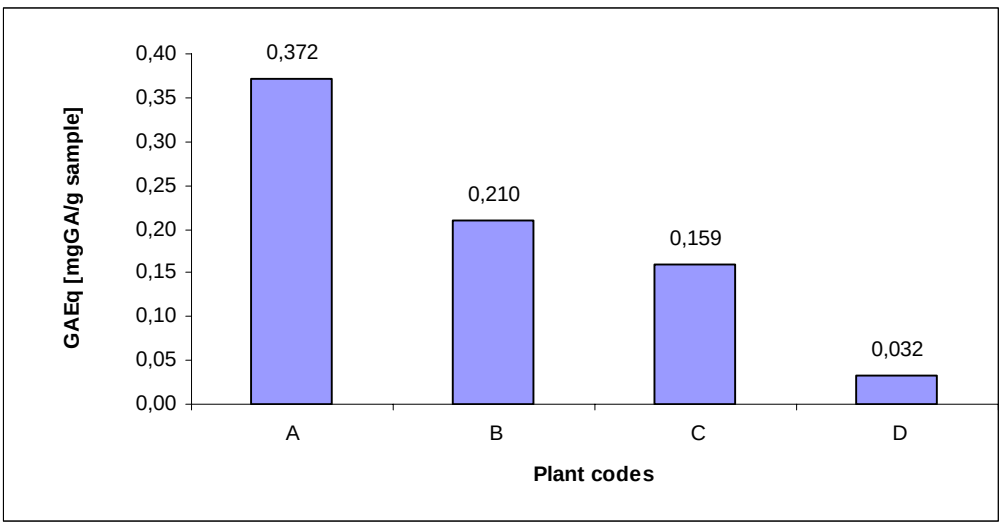


Figure 5.2. Graphical Representation of Total Phenol Contents

5.3. Antioxidant Capacities of Plants

Antioxidant capacities of plant species were determined with ABTS assay and obtained for each plant material. Antioxidant activities were ranged between 8.009 and 0.527 TEAC [mmol/g sample]. According to the results, *Liquidambar orientalis* has the highest antioxidant capacity with 8.009 TEAC [mmol/g sample]. Antioxidant activities of *Ziziphus jujuba* 2.609 TEAC, *Olea europaea* 1.387 TEAC and *Juniperus communis* 0.527 TEAC are shown in table 5.3, respectively.

Table 5.3. Antioxidant Activities of Plants

Plant and Codes		**Part of Plant**	**Antioxidant Capacity TEAC [mmol/g sample]**
Liquidambar orientalis	**(A)**	Leaves	8.009
Ziziphus jujuba	**(B)**	Leaves	2.609
Olea europaea	**(C)**	Leaves	1.387
Juniperus communis	**(D)**	Seeds	0.527

Among these four plant species, *Liquidambar orientalis* shows the significant antioxidant activity compared to the others. 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. However it is also obvious in results that there is a clear relationship between antioxidant capacities and total phenol contents for these species. Figure 5.3. shows the graphical representation of antioxidant activities of plants. Calibration curve of antioxidant capacity and calculations can be seen in Appendix A.

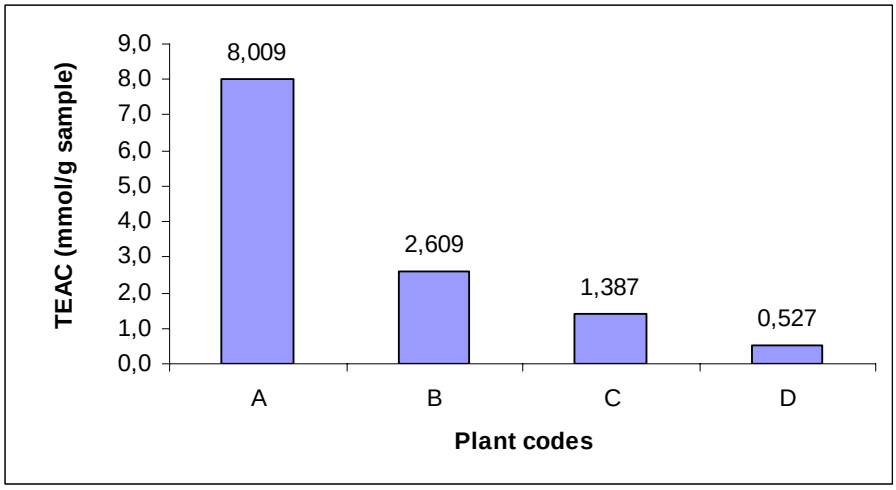


Figure 5.3. Graphical Representation of Antioxidant Activity

5.4. Antimicrobial Activities of Plants and Antibiotics

5.4.1. Disc Diffusion Results

In the disc diffusion assays, six microorganism species were chosen to determine the antimicrobial activities of plant extracts. *Escherichia coli* and *Pseudomonas aeruginosa* were used as a gram negative bacteria. *Staphylococcus epidermidis*, *Staphylococcus aureus* and *Bacillus subtilis* were used as a gram positive bacteria. On the other hand, *Candida albicans* was used as a fungus.

Disc diffusion results were obtained for each plant material. Inhibition zones are ranged between 20.5 and 7.8 mm. According to the results, *Liquidambar orientalis* has the highest inhibition activity with 20.5 and 19.2 against P. aeruginosa and E. coli respectively. Second powerful plant in terms of antimicrobial activity is *Olea europaea* and it shows the strong activity against E.coli which the diameter of inhibition zone is 13.6 mm. *Ziziphus jujuba* and *Juniperus communis* are showed approximately the same inhibition activity. The results of antimicrobial screening of the crude extracts of all species by disc diffusion method are shown in Table 5.4.

Table 5.4. Disc diffusion zone diameters of plants against microorganisms

Microorganisms	**Plants**			
	A	**B**	**C**	**D**
Escherichia coli	19.2	9.6	13.6	8.5
Pseudomonas aeruginosa	20.5	9.8	9.6	7.8
Candida albicans	14.5	9.4	9.1	7.5
Staphylococcus epidermidis	14	14	13	10.6
Staphylococcus aureus	15.3	10.1	9.7	11.2
Bacillus subtilis	16.9	9	9.6	10.4

*Disc diffusion values were expressed as milimeter (mm)

Plant codes: (A) *Liquidambar orientalis*; **(B)** *Ziziphus jujuba*; **(C)** *Olea europaea*; **(D)** *Juniperus communis*

Although, some plant extracts inhibited one microorganism, some of them are effect more than one microorganisms. For example, *Liquidambar orientalis* inhibited all microorganisms including *Candida albicans*. Among the plants , *Liquidambar orientalis*, *Ziziphus jujuba* and *Olea europaea* 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. Growth curves of the microorganisms can be see in Appendix B.

Antibiotic controls were also performed in order to compare the sensitivity of tested microorganisms against antimicrobial agents. Inhibition zones are ranged between 21.1 and 9 mm. According to the results, penicillin has the highest inhibition activity with 40 mm against *S. aureus* but it did not influenced *P. aeruginosa* and *C. albicans*. Table 5.5. shows the inhibition zones of antibiotics against microorganisms. Growth curves of microorganisms can be seen in Appendix B. Disc diffusion images of antibiotics can be seen in Appendix D.

Table 5.5. Disc diffusion zone diameters of antibiotics against microorganisms

Microorganisms	**Antibiotics**			
	Streptomycin (STR)	**Gentamicin (GEN)**	**Vancomicin (VAN)**	**Penicillin (PEN)**
Escherichia coli	16	14	10	7.2
Pseudomonas aeruginosa	17	15.5	No Inhibition	No inhibition
Candida albicans	No Inhibition	No Inhibition	No Inhibition	No Inhibition
Staphylococcus epidermidis	9.5	9	16	21.1
Staphylococcus aureus	13	10	14.5	40
Bacillus subtilis	19	16.5	18	20.6

*Disc diffusion values were expressed as milimeter (mm)

Disc diffusion assays were performed in duplicate experiments for aech species. Disc diffusion images of plants can be seen in Appendix C. *Bacillus subtilis* exhibited weak resistances against samples and antibiotic controls in disc diffusion assays.

All plant samples that were used in the disc diffusion assays showed bacteriocidal activity. 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. ‘Bacteriostatic’ means that the agent prevents the growth of bacteria (it keeps them in the stationary phase of growth). On the other hand, ‘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.

5.4.2. Minimum Inhibition Concentration Values

In this study four 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 (MIC). In order to determine the MIC, serial micro broth dilution method was performed by using Thermo 96 well

microtiter plates. Dilution methods are known as quantitive, more repeatible and reliable assays when compared with other methods. When performing this study a new microplate reader was used called Thermo Varioskan. 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.

Minimum inhibition concentration results were obtained for each plant material. Inhibition concentration values are ranged between 0.4 and 100 mg/ml. According to the results, *Liquidambar orientalis* has the impressive minimum inhibition activity with 0.4 and 0.8 mg/ml against *S. aureus* and *S. epidermidis*, *B. subtilis* respectively. Second powerful plant in terms of antimicrobial activity is *Juniperus communis* and it shows the strong activity against *S. epideridis*, *S.aureus* and *B. subtilis* which the minimum inhibition concentration value is 3.125 mg/ml. *Ziziphus jujuba* and *Olea europaea* are showed approximately the same inhibition concentration value. Table 5.6. shows the minimum inhibition concentration values of plants against microorganisms. Growth curves of microorganisms can be see in Appendix B. Minimum inhibition concentration charts of plants can be seen in Appendix E.

Table 5.6. Minimum inhibition concentration (MIC) values of plants against microorganisms

Microorganisms	**Plants**			
	A	**B**	**C**	**D**
Escherichia coli	1.56	12.5	6.25	12.5
Pseudomonas aeruginosa	1.56	25	50	50
Candida albicans	6.25	50	100	25
Staphylococcus epidermidis	0.8	3.125	25	3.125
Staphylococcus aureus	0.4	6.25	25	3.125
Bacillus subtilis	0.8	12.5	12.5	3.125

*Minimum Inhibition Concentration values were expressed as mg/ml

Plant codes: (A) *Liquidambar orientalis*; **(B)** *Ziziphus jujuba*; **(C)** *Olea europaea*; **(D)** *Juniperus communis*

Minimum inhibition concentration results were obtained for each antibiotic. Inhibition concentration values are ranged between 0.4 and 1250 μg/ml. According to the results, gentamycin and ampicillin have the highest inhibition activity but ampicillin did not inhibit *P. aeruginosa*. Minimum inhibition concentrations were determined for each antibiotic in order to compared with the plants MIC values. Table 5.7. shows the Minimum inhibition concentration values of antibiotics against microorganisms. Growth curves of microorganisms can be see in Appendix B. Minimum inhibition concentration graphs of antibiotics can be seen in Appendix F.

Table 5.7. Minimum inhibition concentration (MIC) values of antibiotics against microorganisms

Microorganisms	**Antibiotics***		
	Penicillin**	**Gentamycin**	**Ampicillin**
Escherichia coli	1250 (1500IU)	3.125	6.25
Pseudomonas aeruginosa	No Inhibition	3.125	No Inhibition
Staphylococcus epidermidis	50 (60IU)	12.5	0.8
Staphylococcus aureus	0.4 (0.48IU)	3.125	0.4
Bacillus subtilis	50 (60IU)	0.4	1.56

*Minimum Inhibition Concentration values were expressed as µg/ml
**1200IU Penicillin = 1000µg/ml

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.

However the whole MIC graphs were ploted by the software of the multiplate reader, 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 by ploting them in excel programe. Minimum inhibition concentration charts of plants can be seen in Appendix E.

5.5. Fabrication of Silk Fibroin Nanofiber

Silk fibroin nanofibers which were fabricated vie electrospinning were viwed under Scanning Electron Microscope (SEM). Images was scaled by using software 'ImageJ 1.32 visualization and scaling'. From each SEM image, fiber diameters were scaled (approximately 10 times) and average diameters were obtained. As mentioned from the experimental part, two different distance (10, 15 cm), three distinct voltage (17, 20, 23 kV) and five varied concentration (60, 80, 100, 120, 150 mg/ml) were studied. Table 5.8. showed the average fiber diameters of each parameter.

Table 5.8. Average Fiber Diameters (nm) of each Parameter

		Concentration (mg/ml)				
Distance (cm)	**Voltage (kV)**	**60**	**80**	**100**	**120**	**150**
10	**17**	32	63	118	156	376
	20	28	52	92	147	328
	23	26	45	85	140	318
15	**17**	28	45	95	123	294
	20	25	43	75	117	268
	23	24	40	73	115	264

*Fiber diameter values on the table were expressed as nanometer (nm)

First of all, it is clearly seen that the diameter distributions are ranging from 24 to 376 nanometer. By changing parameters, wide range of nanofiber thicknesses were obtained. If the concentration of polymer solution is increase, the diameter of nanofiber is increase too. Concentration versus fiber diameter graphs are shown in figure 5.4. and 5.5. So, our datas correlated with the literature. On the other hand, the diameters of nanofibers were decreased by increasing the distance between syringe needle and collector plate. Effect of distance can be seen in figure 5.6. Another event is effect of voltage on the diameter of nanofibers. When applied voltage was increased, the diameter of nanofibers were decreased too.

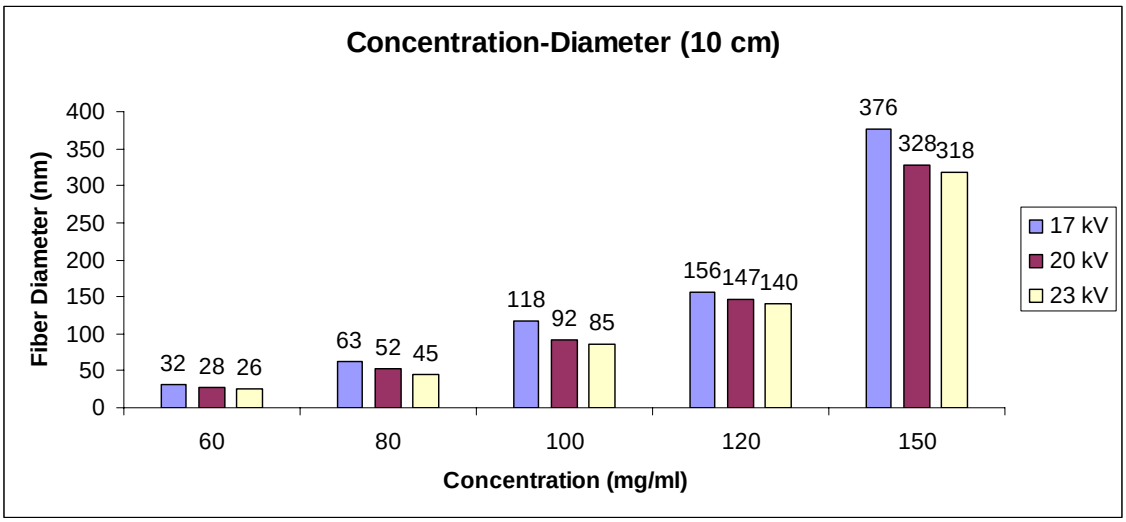


Figure 5.4. Concentration versus Fiber Diameter (d=10cm)

When we look at the figure 5.4., the effect of silk fibroin polymer concentration onto fiber diameter can be seen obviously after 120 mg/ml. Also, there is an apparent increase going from 60 to 120 mg/ml but the absolute thickness differences is seen at the concentration of 150 mg/ml. The same effect can be seen in figure 5.5. Here, the same experiments were performed at the distance 15 cm between tip and the collector plate. If we evaluate each concentration, the colored columns are represent the applied voltage. There is no significant effect of applied voltage onto silk fibroin nanofiber diameters but we can say that when we increase the applied voltage, fiber diameters decrease

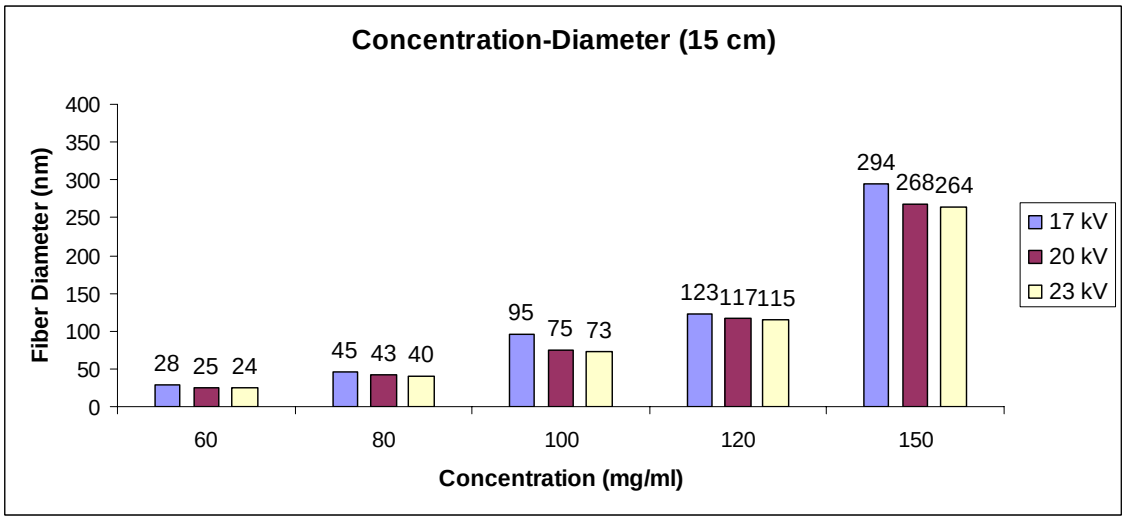


Figure 5.5. Concentration versus Fiber Diameter (d=15cm)

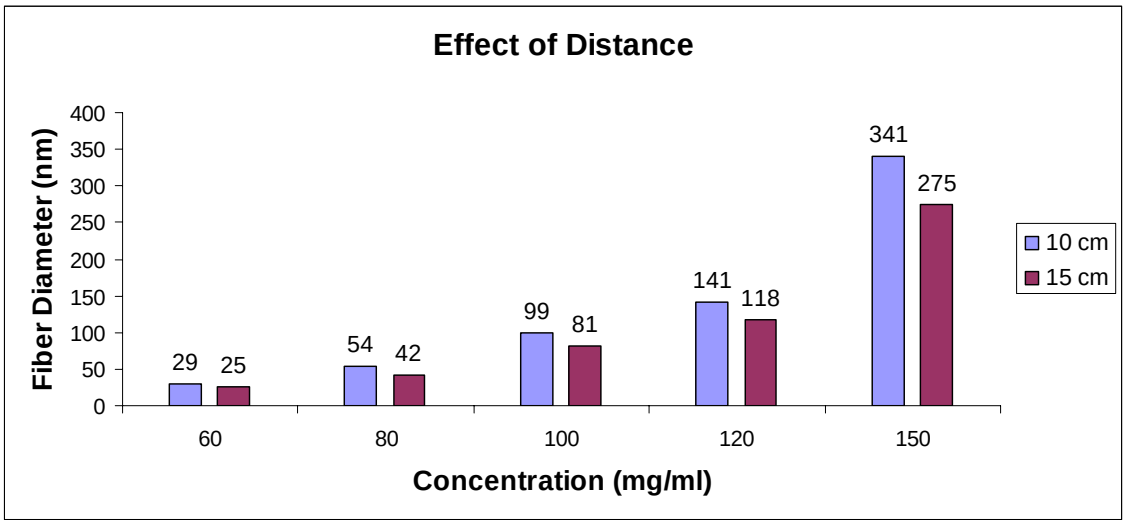


Figure 5.6. Effect of Distance on Nanofiber Diameter

Figure 5.7. and 5.8. showed the Scanning Electron Microscope images of silk fibroin nanofibers from the distance 10 and 15 cm, respectively. Here, it is seen more clearly that the morphology of nanofibers are varied by changing the parameters. Bead formation could be seen where the polymer concentration was 60 mg/ml. By increasing the polymer concentration, beads structures were lost and turned into uniform nanofibers with no-bead conformation.

Effect of applied voltage can be absolutely seen in figure 5.7. Where the polymer concentration was 80 mg/ml and applied voltage was 17 and 20 kV, bead formation was seen, but when the applied voltage increased 23 kV, beeds were disappeared. The same phenomena is seen in figure 5.8. By increasing voltage from 17 kV to 20 kV where the concentration was 80 mg/ml, beads formations were lost.

Among these nanofiber conformations, one of them was chosen in order to load olive leaf extract. Selected characteristics of silk fibroin nanofiber are given below;

- Concentration : 100 mg/ml
- Applied voltage: 20 kV
- Distance: 10 cm

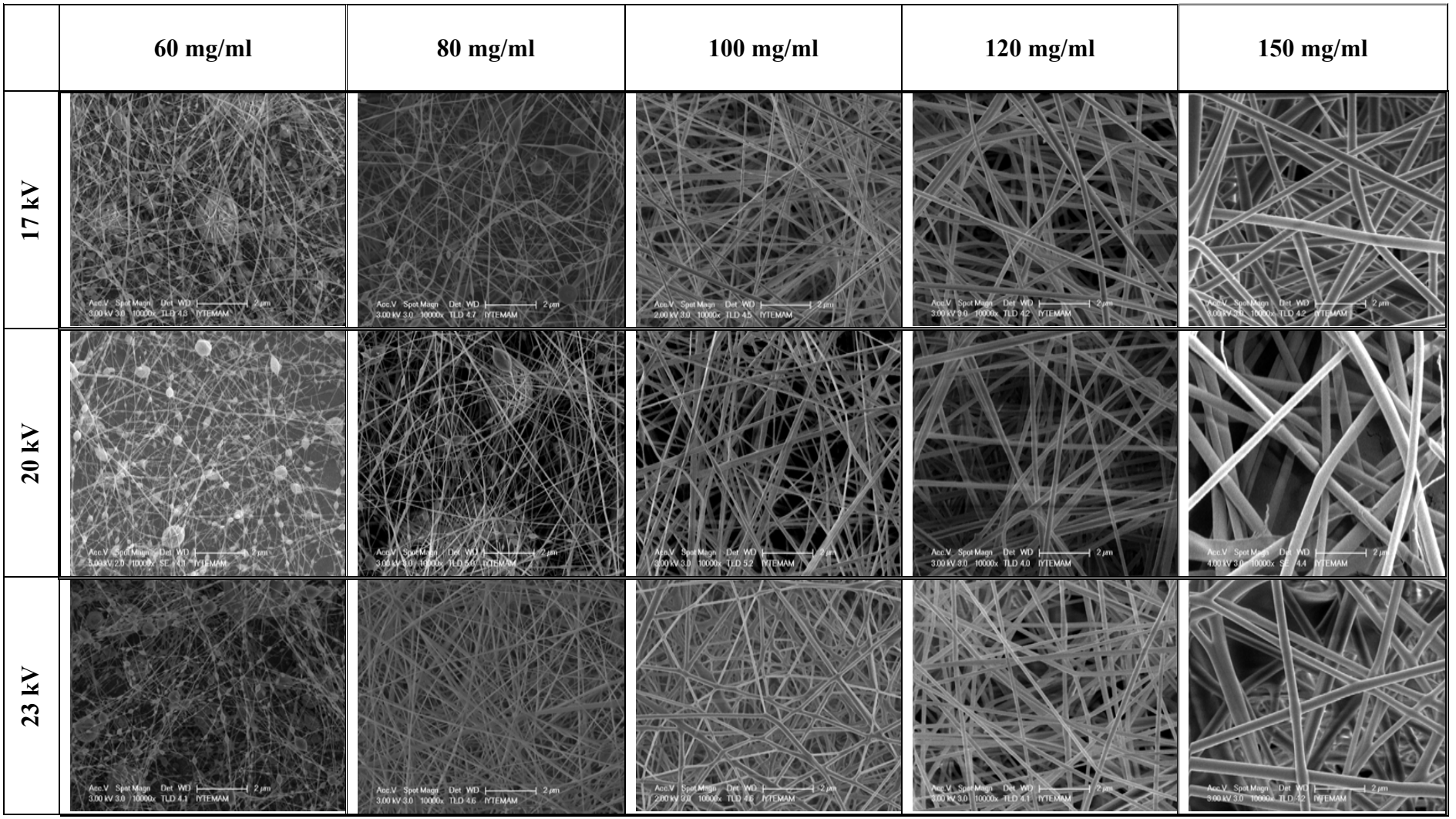


Figure 5.7. SEM image of nanofibers – Distance 10cm

Polymer nanofibers whose diameter is 24nm ~ 326nm, are observed at around 10.000x magnification with 10 ~ 20kV acceleration voltage under SEM. Basically, each diameter of nanofibers is examined using image analyzer and average fiber diameter and fiber distribution are determined. Here, it must be noted that if fiber observation is conducted at extremely high magnification, such as above 10.000x magnification, fiber damage by energetic impinging of electrons takes place. It is known that a significant temperature rise (tens of degrees) occurs when a material surface is bombarded by an energetic electron beam (Campbell et al., 2000). Hence, when ultra-fine nanofibers with less than 100nm diameter are observed, the accuracy of measured value is doubtful. If biodegradable polymer nanofibers and biomimetic collagen nanofibers with poor heat resistance are observed, precaution must be taken to prevent fiber damage.

Another important concern in observing ultra-fine nanofibers is the thickness of the conductive gold coating. Generally, the thickness of gold coating is around 25nm. If the ultra-fine nanofibers are examined under SEM, coating thickness interrupts the accuracy of diameter measurement.

Pores play an important role in determining the physical and chemical properties of porous substrates and have a deterministic effect on the performance of membranes, catalysts and adsorbents. To design electrospun nanofibrous substrates for such applications, it is necessary to analyze the pore size, its distribution and porosity. In electrospun nanofibrous substrates, two types of pores can be identified; one of them is pores on/within each fiber and other is pores (between fibers) on a nanofibrous membrane. Pore size can be measured by direct or indirect methods. Direct methods involve the use of techniques such as electron microscopy (SEM and TEM) or atomic force microscopy (AFM). Both these techniques can provide detailed and magnified images of the electrospun nanofibers from which pore size and its distribution can be determined. Indirect methods include bubble point measurements, solute retention challenge, molecular resolution porosimetry, extrusion porosimetry and instrusion porosimetry.

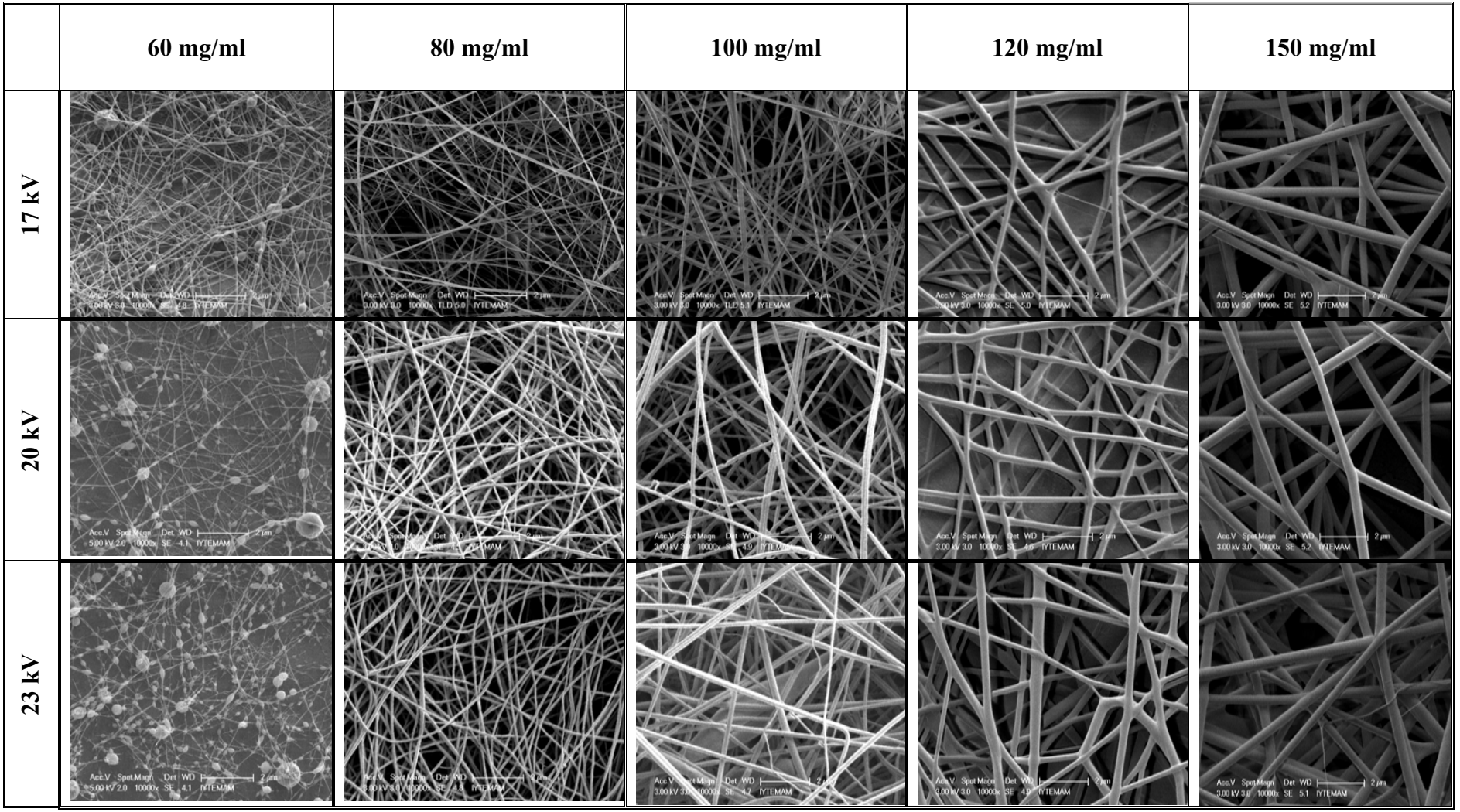


Figure 5.8. SEM image of nanofibers – Distance 15cm

5.6. Morphological Characterization of Silk Fibroin Types

At this part of the study, three different forms of silk fibroin were studied in terms of morphology. Beside this, the essential part of study was performed with these structures; 'Loading olive leaf crude extract as a natural compound'. In figure 5.9. silk fibroin structures and their properties can be seen. First image is silk fibroin microfiber which was obtained at the beginning of process. Briefly, as mentioned earlier, raw silk fibroin passed through pre-treatment and silk fibroin microfibers (MF) were obtained. After this part, microfibers were dissolved in $CaCl_2.2H_2O/EtOH/H_2O$ solution and obtained aqueous silk fibroin solution. After dialysis and freeze dried, regenerated silk fibroin (RSF) was acquired. This form seems like a foam and can be seen from the second images in figure 5.9. Finally, RSF was dissolved in formic acid and from the prepared polymer solution, silk fibroin nanofibers (NF) was fabricated via electrospinning. The last image in figure 5.9 showed the silk fibroin nanofiber (80mg/ml, 20kV, 10 cm).

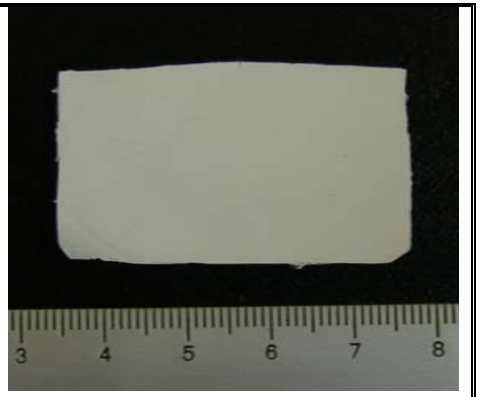
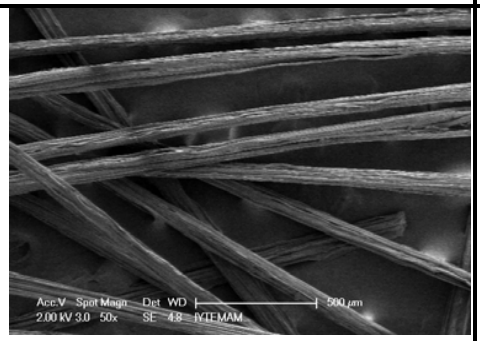
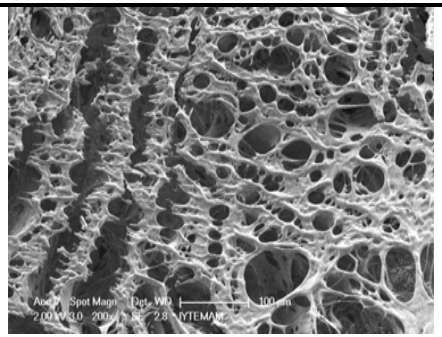
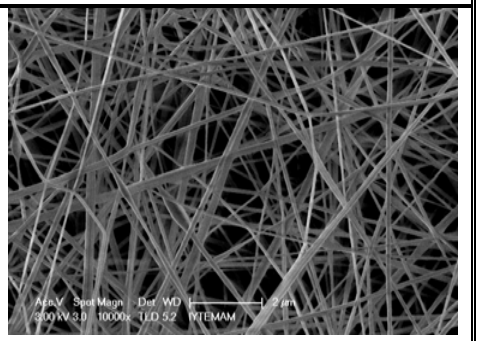
Macro images			
SEM images			
Characteristics & Diameters	Silk Fibroin Microfiber (**MF**) Diameter Distribution: 70-90 micron (μm) Average Fiber Diameter: 80 μm	Regeneated Silk Fibroin (**RSF**) Diameter Distribution: 5-15 micron (μm) Average Fiber Diameter: 10 μm	Silk Fibroin Nanofiber (**NF**) Diameter Distribution: **60-124 nanometer (nm)** Average Fiber Diameter: 92 nm

Figure 5.9. Silk fibroin structures and their properties

5.7. HPLC Analysis of *Olea europaea* Content

Olive leaf extract (OLE) was chosen in order to incorporate with the silk fibroin nanofiber. OLE was chosen because as mentioned earlier, the content of olive leaf is well known and HPLC metods were optimized by our research group. We have several ongoing studies about the silk fibroin and natural compounds. To our knowledge, there is no such study about loading natural compound on silk fibroin nanofiber or any other type of fiber in nano-scale.

Olive leaf is rich in terms of phenolic content and oleuropein (ole) is the most abundant compound in olive leaf. So, while loading OLE onto silk fibroin nanofiber, HPLC analysis were performed in order to determine the adsorption amount. Experiments were done as explained in experimental chapter. Primarily, the initial concentration of oleuropein content in OLE was analysed. Initial content was found as 2.517 mg oleuropein/ml. The HPLC chromatogram of olive leaf extract can be seen in figure 5.10. As seen from the chromatogram, oleuropein was detected in the twenty-first minute and the dominant compound among other phenolics. Calibration curve of oleuropein can be seen in Appendix A.

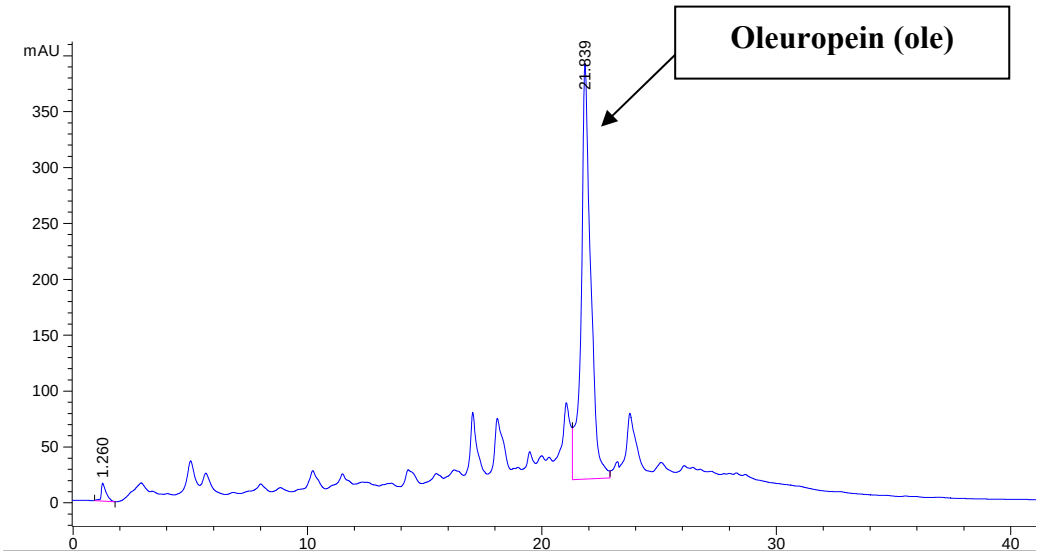


Figure 5.10. The HPLC chromatogram of olive leaf extract

5.8. Adsorption of Polyphenols

Loading experiments were performed for each form of silk fibroin. The data obtained were compared with each others and found very good results. As seen from the table 5.9., initial concentration on fiber is zero for each. (Ci_{fiber}= 0mg oleuropein/gr fiber). According to the results, if we examine every one individually, it is clearly seen that silk fibroin nanofiber (NF) was absorbed a great amount of oleuropein compared to silk fibroin microfiber (MF) and regenerated silk fibroin (RSF). The absorb capacity of silk MF is determined 104.92 mg oleuropein per gram silk MF. This result found from the HPLC analysis. The same situation can be seen for RSF. However, RSF was absorbed 163.07 mg oleuropein per gr RSF at the end of sixth hour. Silk fibroin nanofiber (NF) gave the best results. Silk fibroin nanofiber was absorbed 228.34mg oleuropein per gr silk fibroin NF. The results were given in table 5.9.

Table 5.9. Adsorption of oleuropein onto Silk fibroin fibers

Minutes	SF Microfiber (mg ole/gr MF)	RSF (mg ole/gr RSF)	SF Nanofiber (mg ole/gr NF)
0	0.00	0.00	0.00
60	85.37	119.11	159.03
120	96.51	142.70	205.40
180	101.01	153.43	217.19
240	103.06	160.51	222.54
300	104.92	163.07	228.34

*Adsorption amounts on the table were expressed as mg oleuropein(ole)/gr microfiber(MF), regenerated silk fibroin(RSF) and nanofiber(NF)

Figure 5.11. showed that the general adsorption profile of oleuropein amount on silk fibroin MF, RSF and NF. Likewise, it is clearly seen from the graph that silk fibroin nanofiber was absorbed a maximum yield of oleuropein. The reason for this phenomena is related to high surface area of nanofiber. Silk fibroin nanofibers have a great deal of surface area when compared to others. On the other hand, HPLC chromatogram indicated that the oleuropein amount in the medium decreased at the end of the 6-hour compared to the initial oleuropein concentration in the medium. (Figure 5.12)

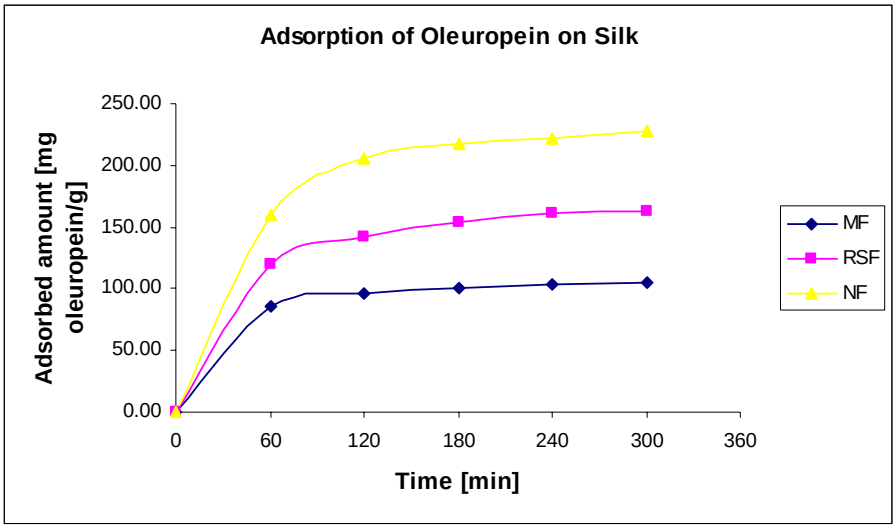


Figure 5.11. Adsorption of oleuropein on silk fibroin microfiber(MF), regenerated silk fibroin(RSF) and nanofiber(NF)

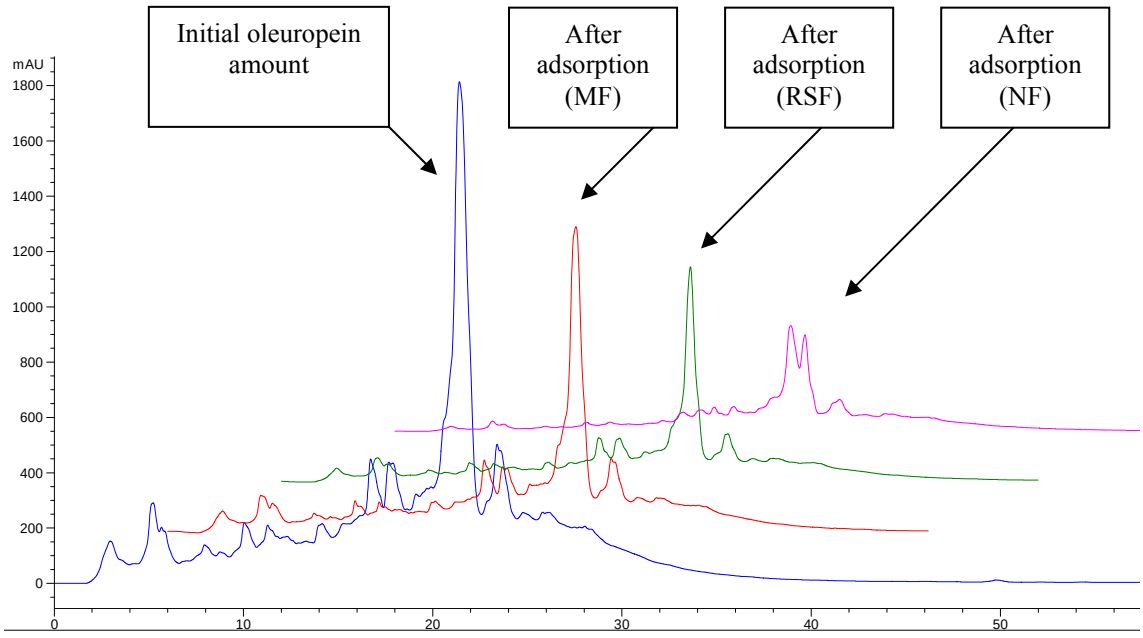


Figure 5.12. HPLC chromatogram - Decreased oleuropein amount in the medium

5.9. Desorption of Polyphenols

Desorption experiments were performed for each form of silk fibroin. The data obtained were compared with each others and found satisfactory results. As seen from the table 5.10., initial concentrations of fibers are different. Because as remembered from the absorbed values, silk fibers were absorbed different amount of oleuropein ranging from 104.92 to 228.34. So, this values are the initial values (Ci_{fiber}) for desorption experiment. During desorption, every hour sample was taken from the medium and analyzed via HPLC. The initial concentration of medium was zero (Ci_{medium}= 0 mg). At the end of six hour, datas were compared and very successful results were obtained. The oleuropein amount on the silk fibroin microfiber was decrease from 104.92 to 14.61 mg oleuropein per gram silk MF. However, the oleuropein amount on the RSF was decrease from 163.07 to 14.2 mg oleuropein per gram RSF. On the other hand, the oleuropein amount on the silk fibroin NF was decrease from 228.34 to 7.6 mg oleuropein per gram silk fibroin NF. If we examine every one individually, it is clearly seen that silk fibroin nanofiber (NF) was released a maximum amount of oleuropein (96.67%). However silk fibroin microfiber (MF) and regenerated silk fibroin (RSF) also released oleuropein in a medium with 86.08 and 91.29 %, respectively (Figure 5.13).

Table 5.10. Released of oleuropein from Silk fibroin fibers

Minutes	SF Microfiber (mg ole/gr MF)	RSF (mg ole/gr RSF)	SF Nanofiber (mg ole/gr NF)
0	104.92	163.07	228.34
60	35.27	99.08	119.56
120	17.97	57.81	70.52
180	14.97	38.56	34.63
240	14.85	22.57	10.41
300	14.61	14.20	7.60

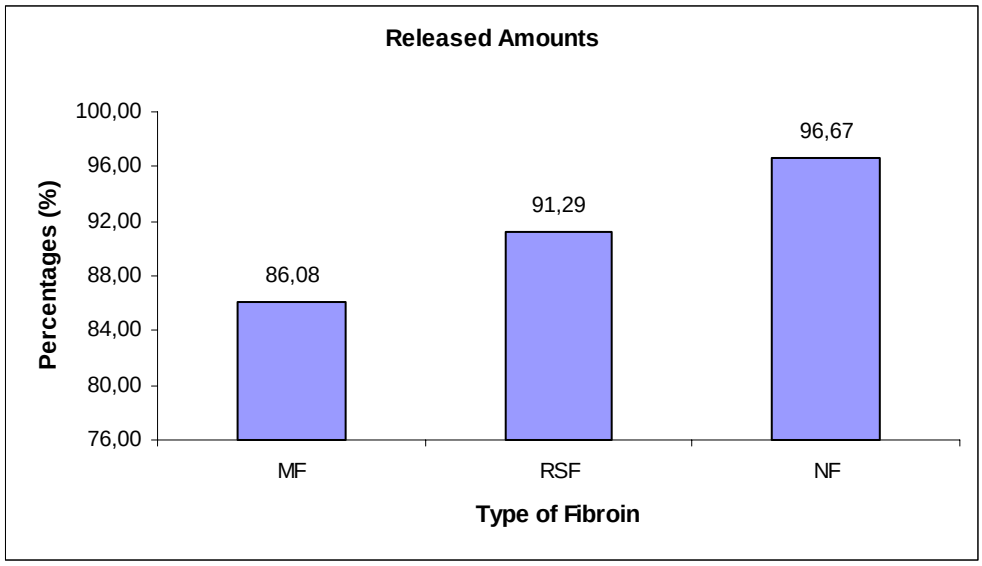


Figure 5.13. Released amounts of oleuropein in a medium

Figure 5.14. showed that the general desorption profile of oleuropein amount from silk fibroin MF, RSF and NF. Likewise, it is clearly seen from the graph that each fiber was desorb almost all oleuropein amount which absorbed earlier. On the other hand, HPLC chromatogram indicated that the oleuropein amount in the medium increase at the end of the 6-hour compared to the initial oleuropein concentration (Ci_{medium}= 0 mg) in the medium (Figure 5.15).

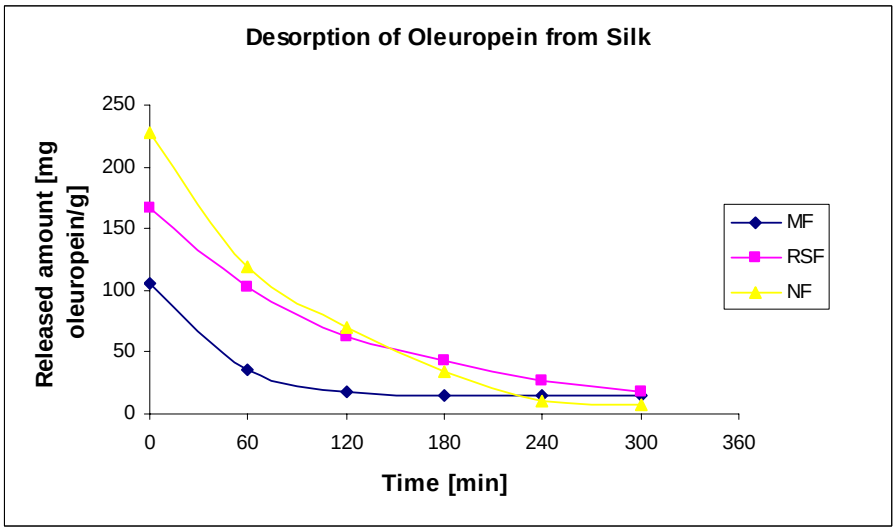


Figure 5.14. Desorption of oleuropein from silk fibroin microfiber(MF), regenerated silk fibroin(RSF) and nanofiber(NF)

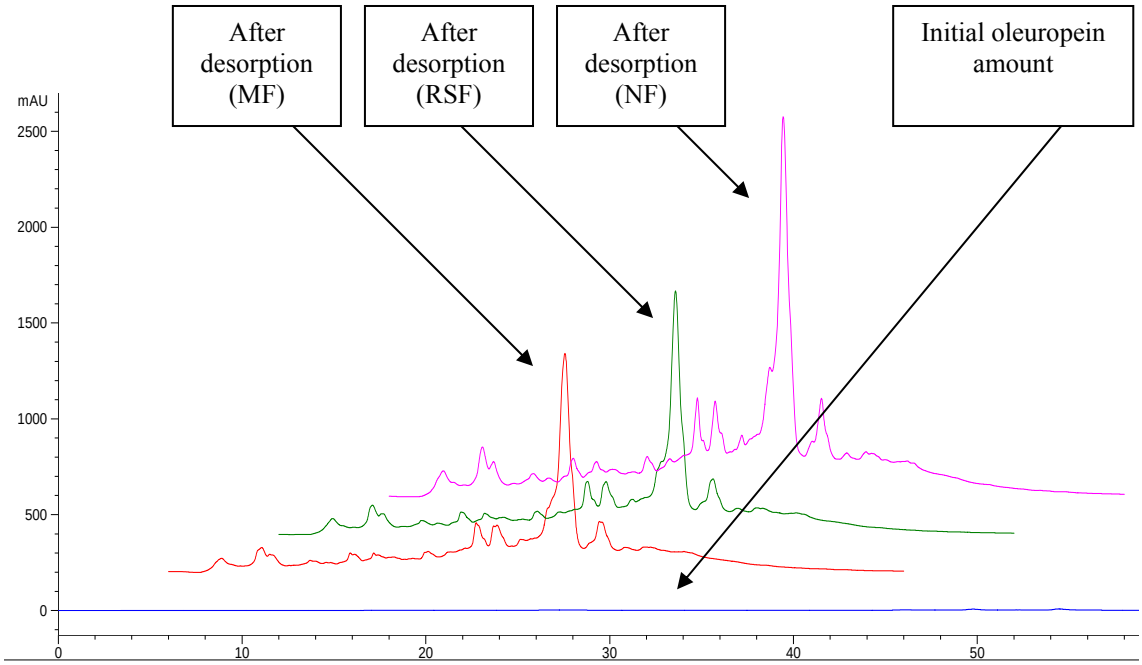


Figure 5.15. HPLC chromatogram - Increased oleuropein amount in the medium

As it can be seen from the HPLC chromotogram, silk fibroin microfiber, regenerated silk fibroin and silk fibroin nanofiber showed very satisfactory release profile. The bioactive plant components which are loaded onto the fiber surface were released to the medium. So, these kind of biofunctional nanofibers include bioactive plant compounds are potential candidate for new generation medical applications.

CHAPTER 6

CONCLUSION

The prime intention of this thesis is the development of natural compound loaded silk fibroin based nanofibers. In order to success this aim, first silk fibroin was passed through a number of pretreatment such as degumming to obtain silk microfiber (MF), dissolving in a $CaCl_2.2H_2O/EtOH/H_2O$ solution in order to obtain regenerated silk fibroin (RSF) and lastly dissolved in formic acid in order to prepared silk fibroin polymer solution. Then, this polymer solution was fabricated into nanofiber via electrospinning method. Concentration, applied voltage and distance parameters were changed and silk fibroin nanofibers were fabricated with different morphology. By increasing the polymer concentration, fiber diameters increased and uniform nanofibers were obtained without bead structure. The diameters ranged between 24 and 326 nanometers (nm) and by increasing the distance beween tip and collector, diameters of nanofibers decreased. Also, applied voltage effect the formation of nanofibers. Where the polymer concentration is 80 mg/ml, nanofibers were in bead conformation, but by increasing the applied voltage from 17 kV to 23 kV, nanofibers were fabricated with no-bead, uniform conformation. After nanofibers were produced, they were visualized via SEM and scaled by using software. Among them, the optimum conformation was chosen in order to load active compound (olive leaf crude extract).

In this study four plant species collected from the different parts of aegean region were examined with several analysis in order to determine their relative total antioxidant and total phenol contents and also antibacterial activities of extracts. Extraction yields for each species were obtained. The most efficient extraction yield was obtained for *Liquidambar orientalis* as 24.7 %, and followed with *Ziziphus jujuba* and *Olea europaea* with 21.5% and 15.6%, respectively. 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, *Liquidambar orientalis* exhibited the highest phenol content as 0.372 GAEq (mgGA/g sample). Afterward, antioxidant capacities were determined for each plant material. Antioxidant activities were ranged between 8.009 and 0.527 TEAC (mmol/g

sample). According to the results, *Liquidambar orientalis* has the highest antioxidant capacity with 8.009 TEAC (mmol/g sample).

In the next step, antibacterial activities of plant extracts were evaluated by using disc diffusion assay and minimum inhibition concentration assay. Disc diffusion results were obtained for each plant material. Inhibition zones were ranged between 20.5 and 7.8 mm. According to results, *Liquidambar orientalis* has the highest inhibition activity with 20.5 and 19.2 against P. aeruginosa and E. coli respectively. Second powerful plant in terms of antimicrobial activity was *Olea europaea* and it showed the strong activity against E.coli which the diameter of inhibition zone was 13.6 mm. *Ziziphus jujuba* and *Juniperus communis* were showed approximately the same inhibition activity. On the other hand, the microdilution tests (MIC) were performed by 96 well plates for each plant extract. Inhibition concentration values were ranged between 0.4 and 100 mg/ml. According to results, *Liquidambar orientalis* has the impressive minimum inhibition activity with 0.4 and 0.8 mg/ml against S.aureus and S.epidermidis, B.subtilis respectively. Second powerful plant in terms of antimicrobial activity was *Juniperus communis* and it showed the strong activity against S.epidermidis, S.aureus and B.subtilis which the minimum inhibition concentration values were 3.125 mg/ml. *Ziziphus jujuba* and *Olea europaea* were showed approximately the same inhibition concentration value.

The last step was loading olive leaf crude extract onto silk fibroin micro fiber (MF, regenerated silk fibroin (RSF) and silk fibroin nanofiber (NF). These structures were compared in terms of absorb amount of oleuropein. The analysis were performed with HPLC and results were expressed as mg oleuropein/g silk fibroin MF, RSF and NF. The maximum adsorption capacity was calculated as 228.34 mg oleuropein/g silk fibroin nanofiber. On the other hand, the release amount of oleuropein in a medium was found approximately the same. The released amounts of MF, RSF and NF were determined as 86.08, 91.29 and 96.67%, respectively.

Finally, nanofibers can be used in a great deal of industrial area and it seems that nanofibers will became a popular year by year. Especially, medical and textile applications will be more famous in coming years. In conclusion, natural compounds can be loaded on to silk fibroin based nanofibers and nanofibers are good candidate for the development of these kind of biofunctional materials in order to use in medical textile industry.

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APPENDIX A

CALIBRATIONS CURVES OF OLEUROPEIN, GALLIC ACID AND ANTIOXIDANT CAPACITY

Calibration Curve of Oleuropein

Amounts of oleuropein (ole) in the olive leaf extract was calculated using peak area values obtained from HPLC analysis results. On the other hand, while performing adsorption and desorption experiments, oleuropein amount in the medium was calculated by using the same calibration curve.

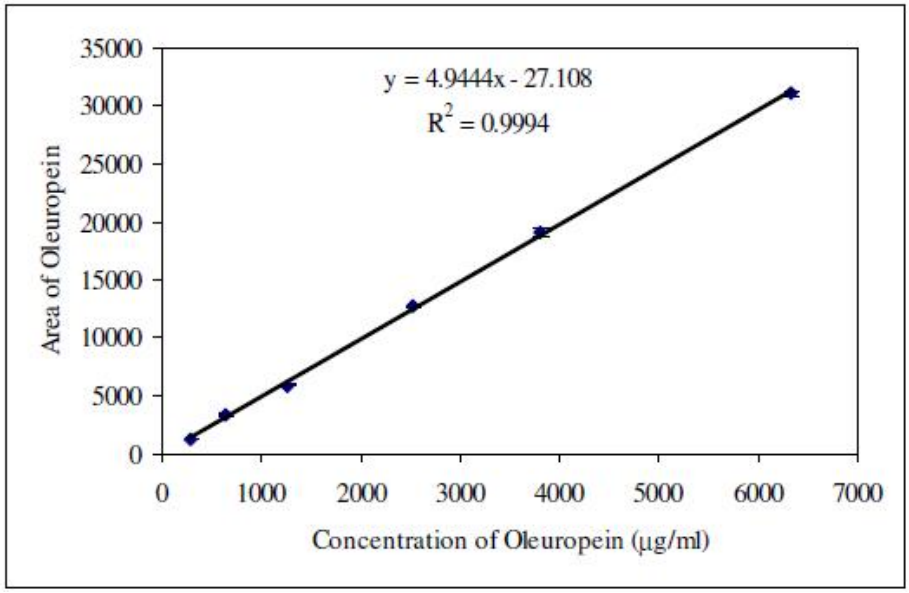

Figure A1. Calibration Curve of Oleuropein

Sample Calculation:

One gram of olive leaf powder is extracted at a solid/liquid ratio of 1/20. According to the results of the HPLC analysis of the crude extract obtained with the extraction process, oleuropein peak area was calculated as 12417. Using the calibration curve equation y=4.9444x-27.108 given in figure A1, x value (concentration, µg/ml) was calculated. According to the equation, x value was found 2517 µg oleuropein/ml. Accordingly, it was calculated that one mililiter olive leaf extract include 2.517mg oleuropein.

Calibration Curve of Gallic acid

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°. The standard concetrations that were prepared for calibration curve are 0.02-0.03-0.04-0.05 and 0.06 mg/ml.

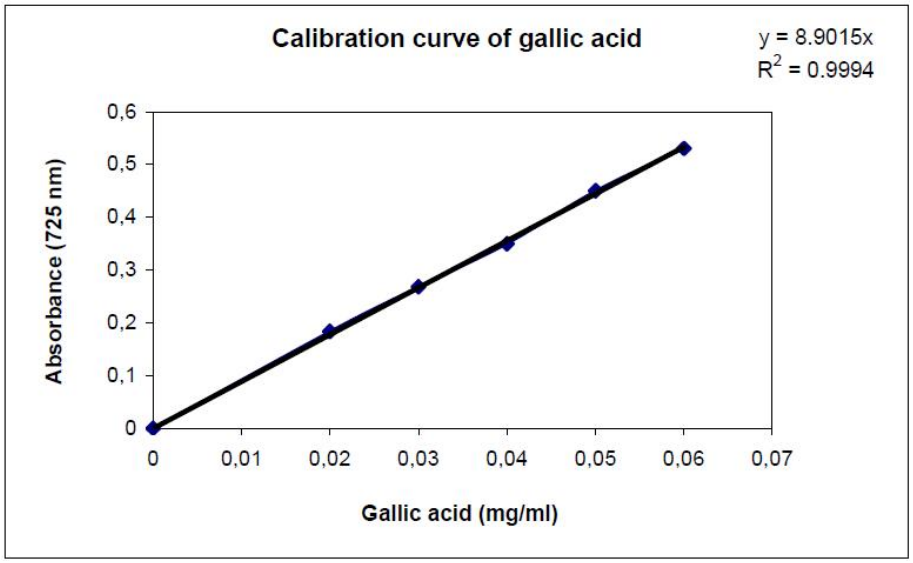

Figure A2. Calibration Curve of Gallic Acid

Calculation of Total Phenol Contents in Gallic acid Equivalent (GAEq)

‘ GAEq(mg GA/g sample) = [A*DF*$V_{solvent}$ (mL)]/[slope of calibration 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

Calibration Curve of Trolox and and Sample Calculation

The polyphenols scavenge the $ABTS^+$ radical cations and they exhibit inhibition. This inhibition can be observed as a decrease in the absorbance values at 734 nm in UV-Visible Spectrophotometer. Firstly, the absorbance of $ABTS^+$ radical cation solutions was measured. It was adjusted to absorbance of 0.7 ± 0.02. Then sample solution was added to $ABTS^+$ radical cation solution and the absorbance values were taken at each 1 minute during 6 minutes. Average of these 6 data was taken and its decrease from the absorbance value of $ABTS^+$ radical cation solution was calculated in order to find out percentage inhibition. After find out the percentage inhibition value, calibration curve for trolox was used so as to find the antioxidant capacity for plant extract (Figure A3).

Example calculation for *Liquidambar orientalis* crude extract;

Initial absorbance value of $ABTS^+$ radical cation was 0.706. The six measured absorbance values after adding the *Liquidambar orientalis* extract solution were, 0.520, 0,442, 0.430, 0.444, 0.442 and 0.439. The average of these values were calculated as 0.452. The inhibition percentage is;

‘ [(Initial absorbance value – Average absorbance value) / Initial absorbance value]*100 = 35.859 %.

The inhibition percentage of samples were measured at least three different Concentrations (10-20-30 µl) and at three replicates.

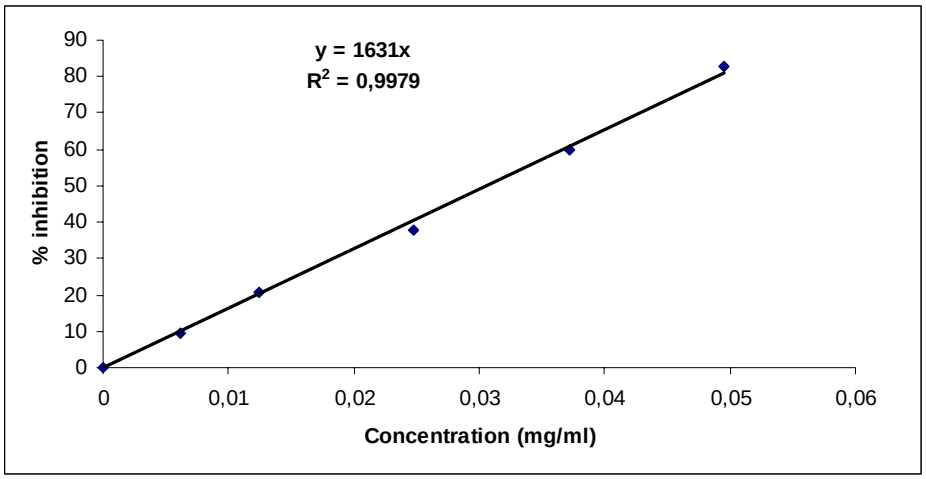

Figure A3. Calibration Curve for Trolox

APPENDIX B

GROWTH CURVES OF MICROORGANISMS

In this study, six different microorganism was used in order to determine the antimicrobial activity of plant extracts. *Escherichia coli* and *Pseudomonas aeruginosa* were used as a gram negative bacteria. *Staphylococcus epidermidis*, *Staphylococcus aureus* and *Bacillus subtilis* were used as a gram positive bacteria. On the other hand, *Candida albicans* was used as a fungus. The growth curves of microorganisms are given below in figures B1, B2, B3, B4, B5 and B6.

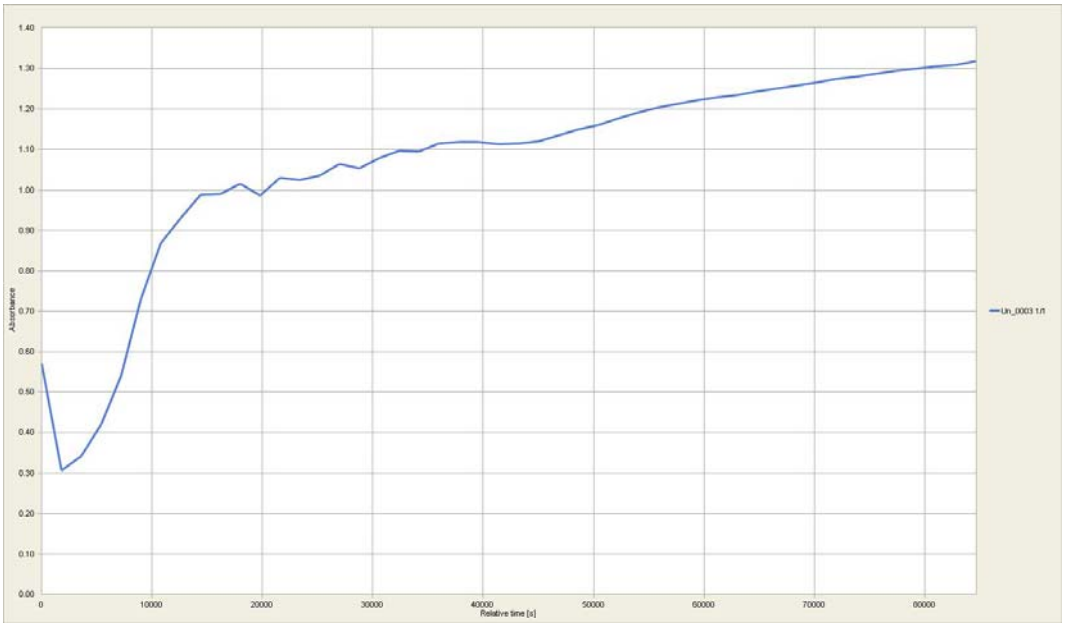

Figure B1. *Escherichia coli* Growth Curve

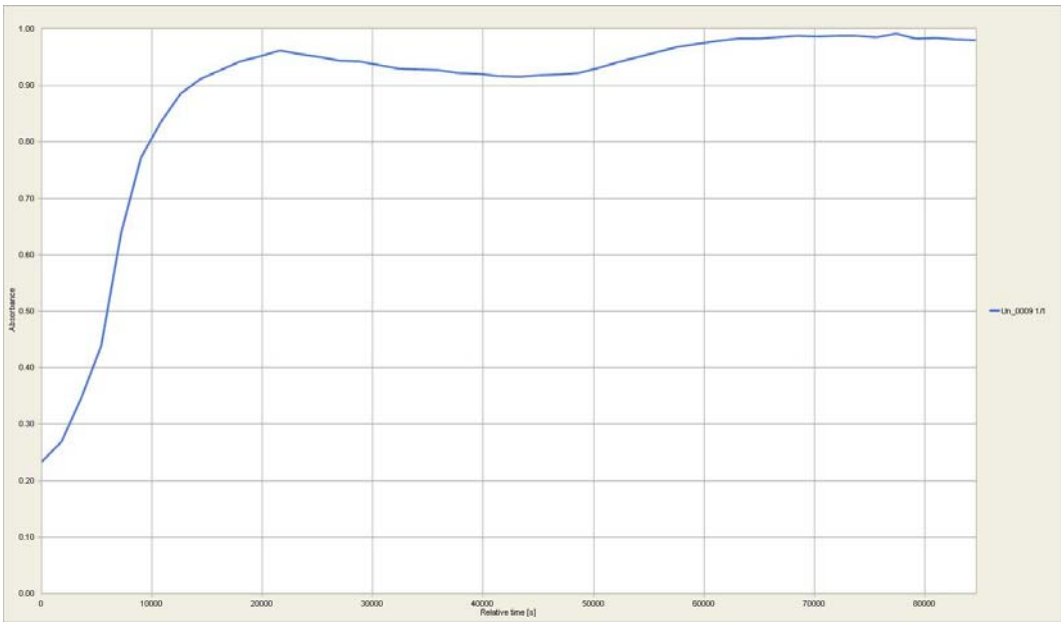

Figure B2. *Staphylococcus epidermidis* Growth Curve

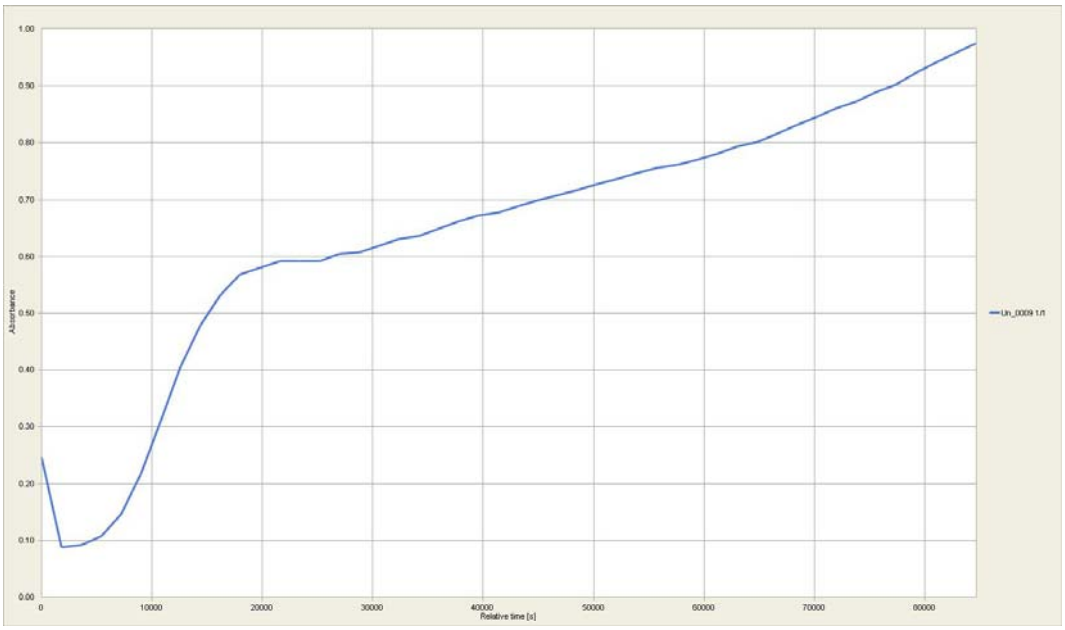

Figure B3. *Staphylococcus aureus* Growth Curve

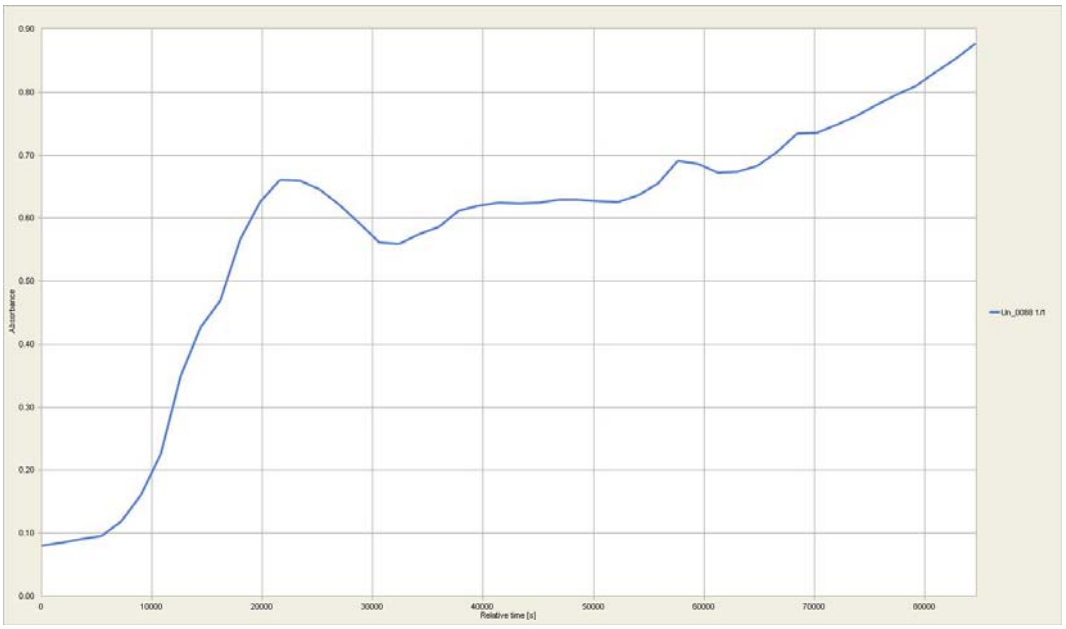

Figure B4. *Bacillus subtilis* Growth Curve

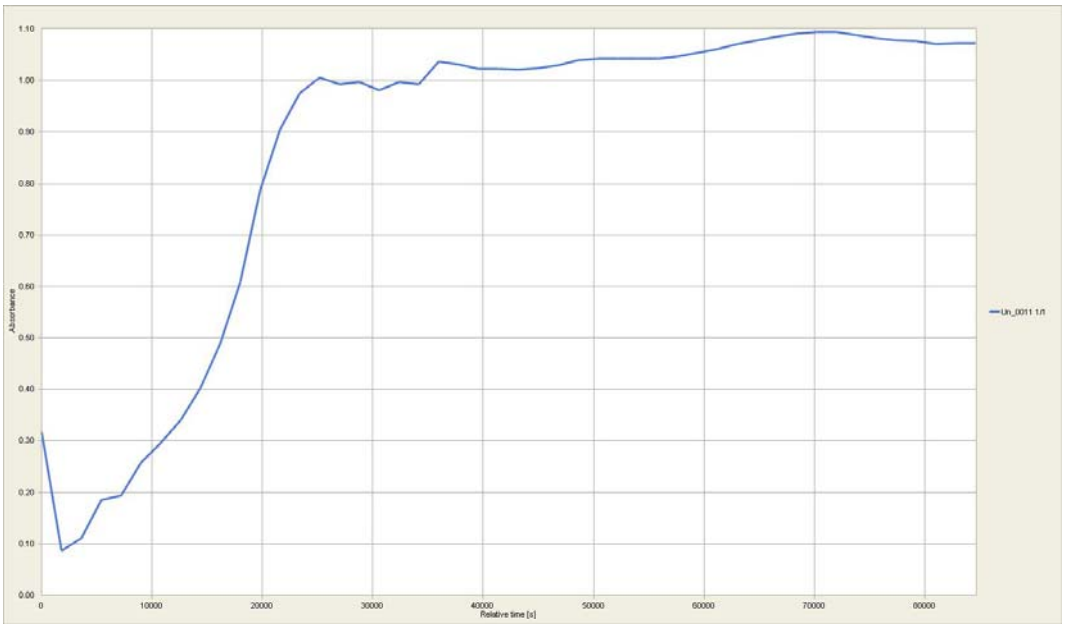

Figure B5. *Pseudomonas aeruginosa* Growth Curve

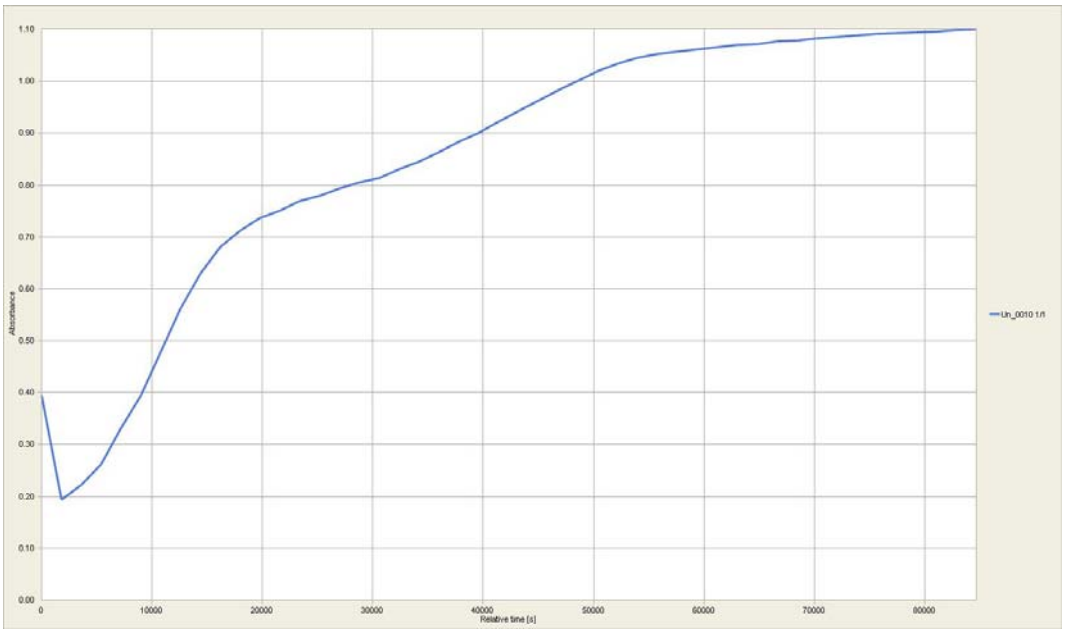

Figure B6. *Candida albicans* Growth Curve

APPENDIX C

DISC DIFFUSION IMAGES OF PLANTS

Escherichia coli, *Staphylococcus epidermidis*, *Staphylococcus aureus*, *Bacillus subtilis*, *Pseudomonas aeruginosa* and *Candida albicans* disc diffusion images are given in figure C1, C2, C3, C4, C5 and C6 respectively for each microorganism.

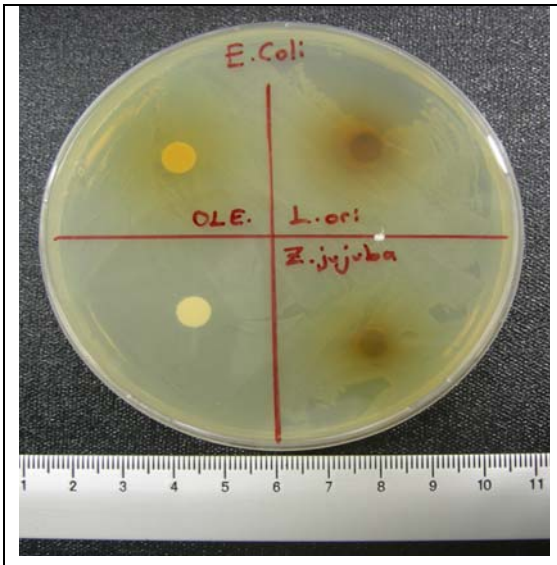

Figure C1. *E. coli* for OLE, L.ori and Z. jujuba extracts

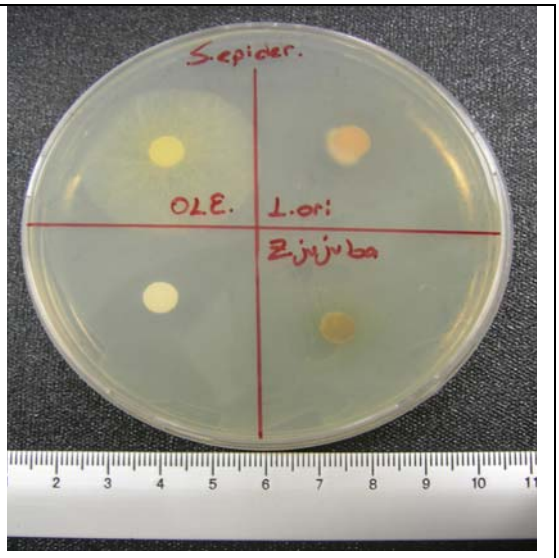

Figure C2. *S. epidermidis* for OLE, L.ori and Z. jujuba extracts

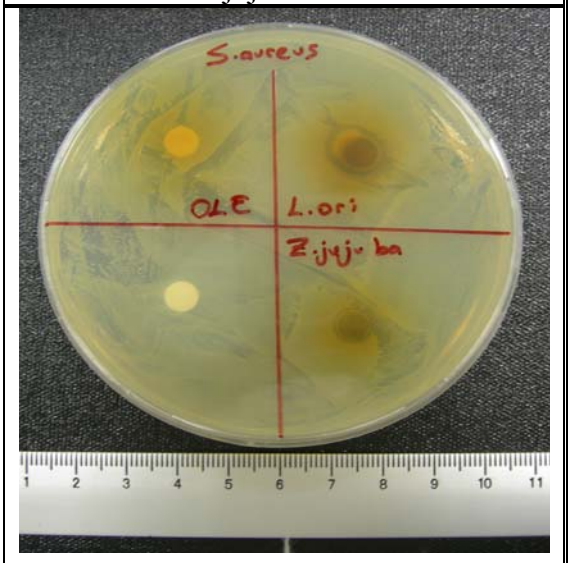

Figure C3. *S. aureus* for OLE, L.ori and Z. jujuba extracts

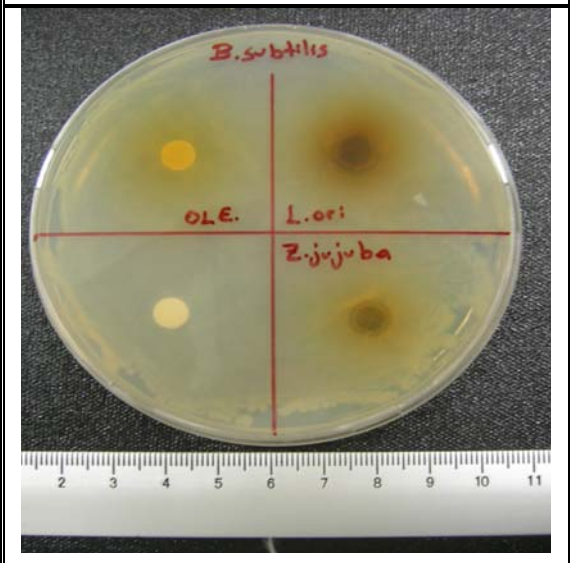

Figure C4. *B. subtilis* for OLE, L.ori and Z. jujuba extracts

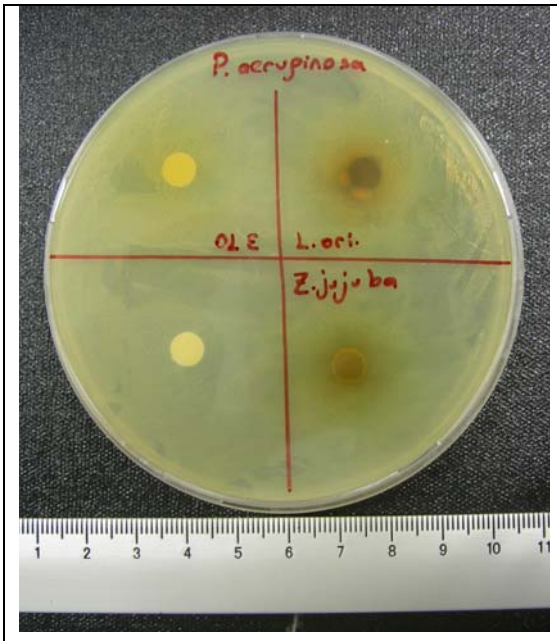

Figure C5. *P. aeruginosa* for OLE, L.ori and Z. jujuba extracts

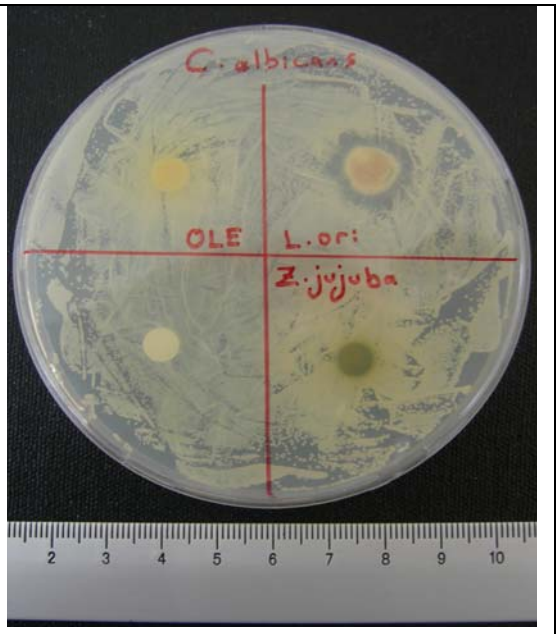

Figure C6. *C. albicans* for OLE, L.ori and Z. jujuba extracts

APPENDIX D

DISC DIFFUSION IMAGES OF ANTIBIOTICS

Penicillin, Gentamycin, Streptomycin and Vancomycin antibiotic disc diffusion images are given below in figures for each microorganism.

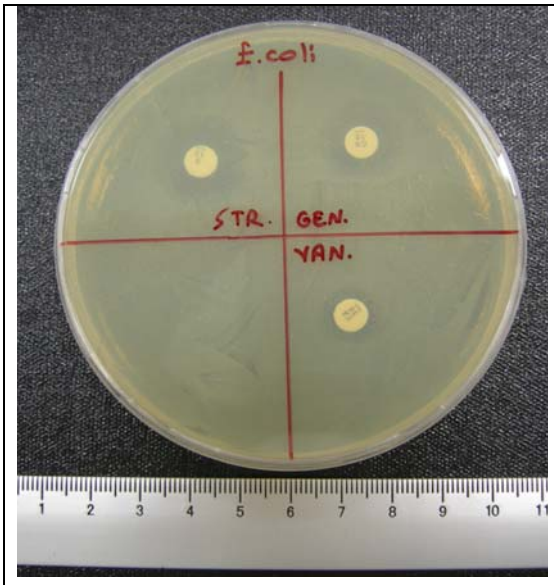

Figure D1. *E. coli* for STR, GEN and VAN antibiotics

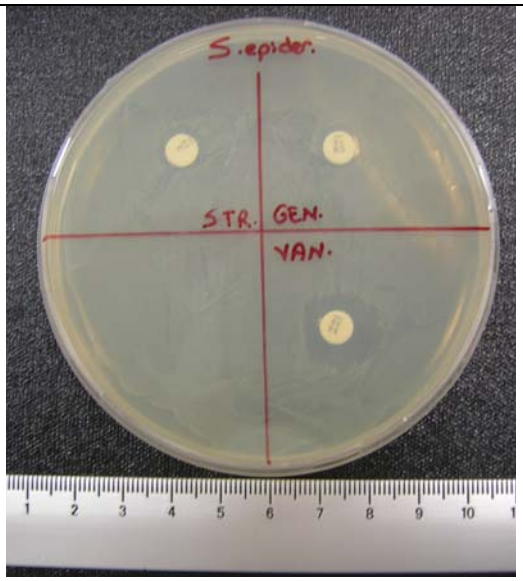

Figure D2. *S. epidermidis* for STR, GEN and VAN antibiotics

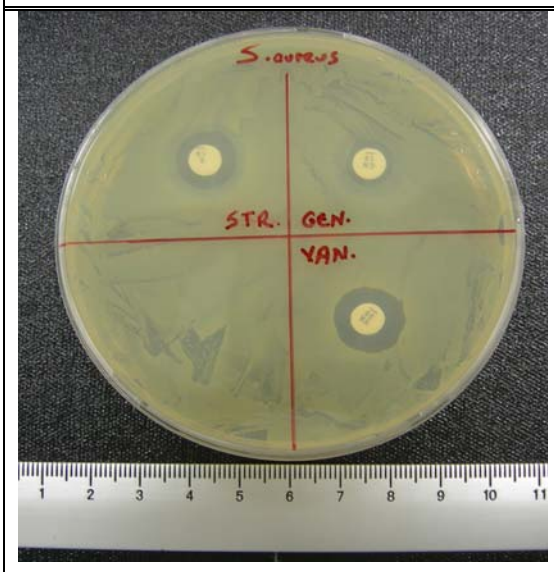

Figure D3. *S. aureus* for STR, GEN and VAN antibiotics

Figure D4. *B. subtilis* for STR, GEN and VAN antibiotics

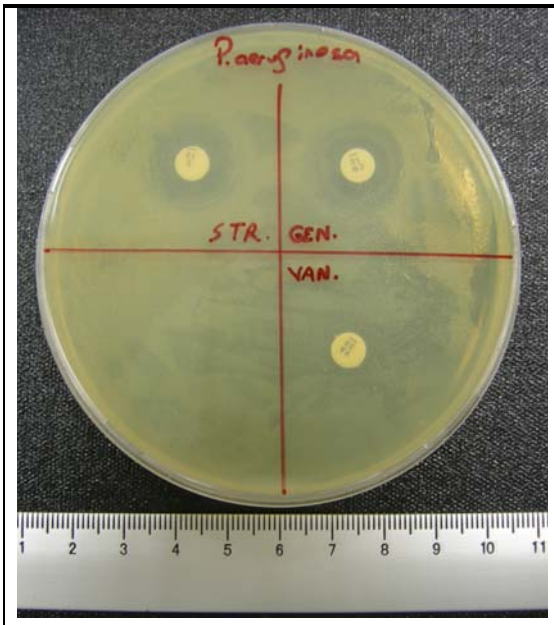

Figure D5. *P. aeruginosa* for STR, GEN and VAN antibiotics

Figure D6. *E. coli* for PEN, VAN and GEN antibiotics

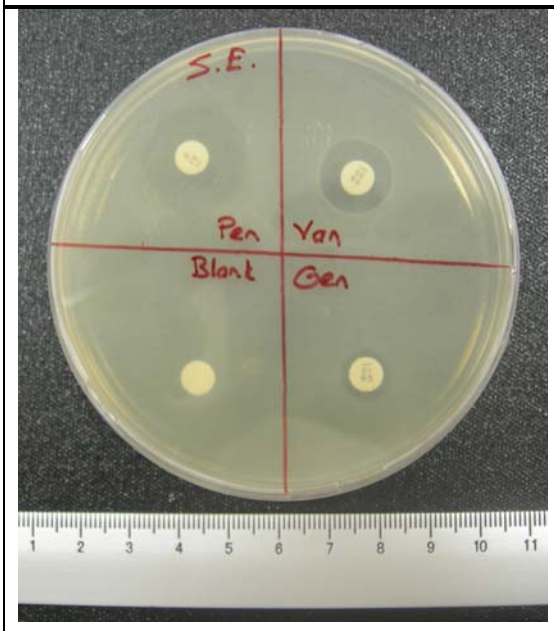

Figure D7. *S. epidermidis* for PEN, VAN and GEN antibiotics

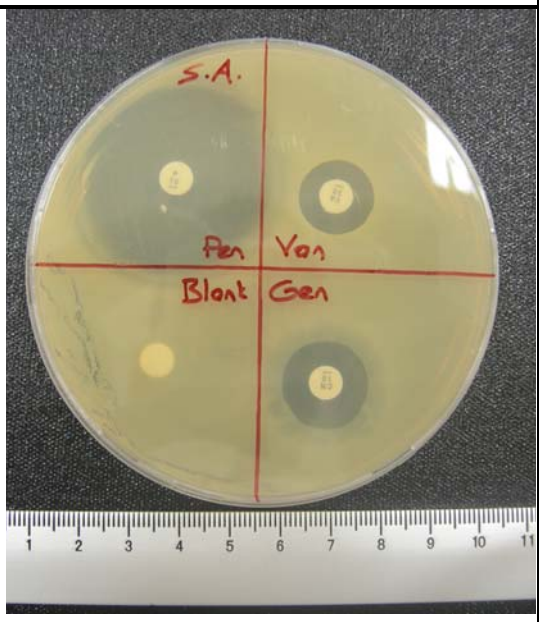

Figure D8. *S. aureus* for PEN, VAN and GEN antibiotics

Figure D9. *B. subtilis* for PEN, VAN and GEN antibiotics

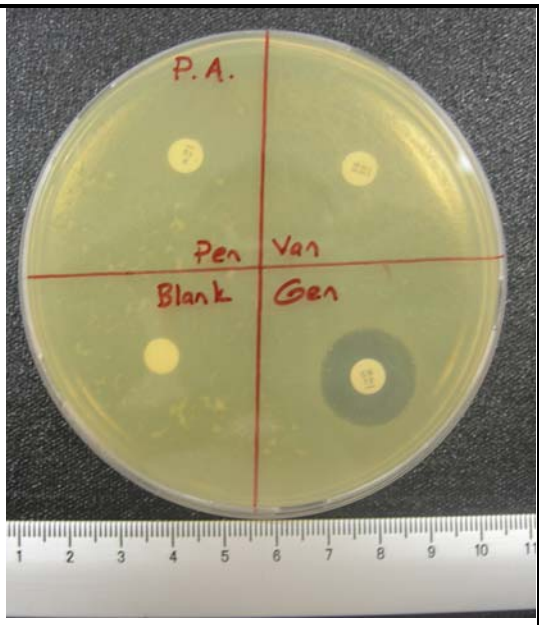

Figure D10. *P. aeruginosa* for PEN, VAN and GEN antibiotics

APPENDIX E

MINIMUM INHIBITION CONCENTRATIONS OF PLANTS

In this study, Minimum inhibition concentrations (MIC) of plants were determined against six microorganism in order to find out their antimicrobial activity. *Escherichia coli* (Gram -), *Staphylococcus epidermidis* (Gram +), *Staphylococcus aureus* (Gram +), *Bacillus subtilis* (Gram +) and *Pseudomonas aeruginosa* (Gram -) were used as a bacteria; *Candida albicans* was used as a fungus. The inhibition graphics were given for each microorganism in figures below.

Minimum Inhibition Concentration of Olive Leaf Extraxt

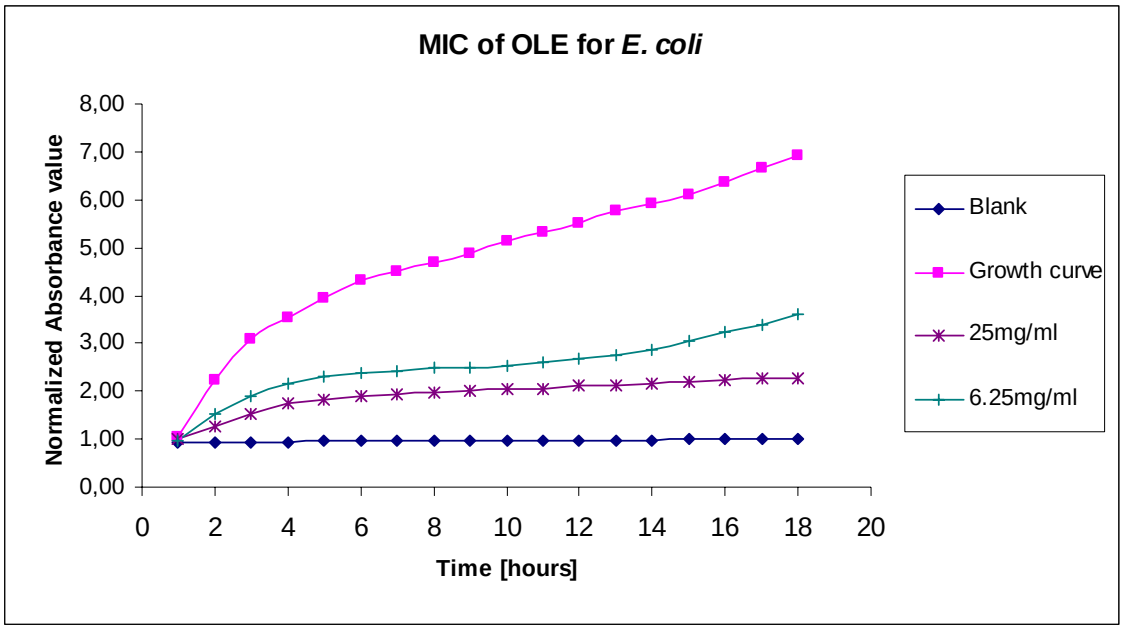

Figure E1. Minimum inhibition concentration of Olive leaf extract for *Escherichia coli*

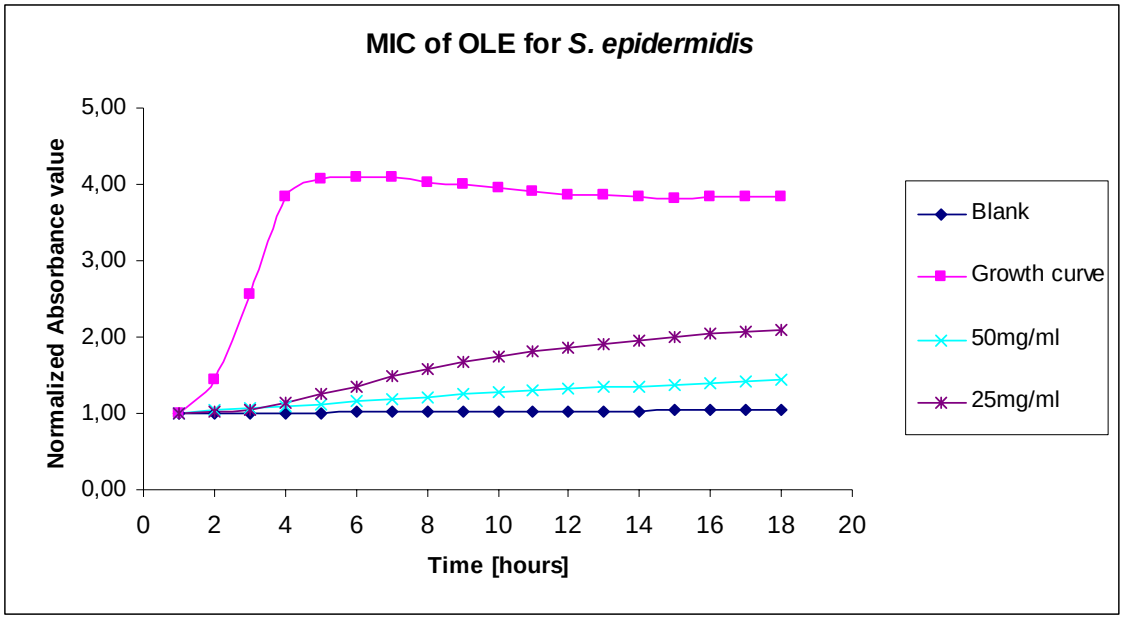

Figure E2. Minimum inhibition concentration of Olive leaf extract for *Staphylococcus epidermidis*

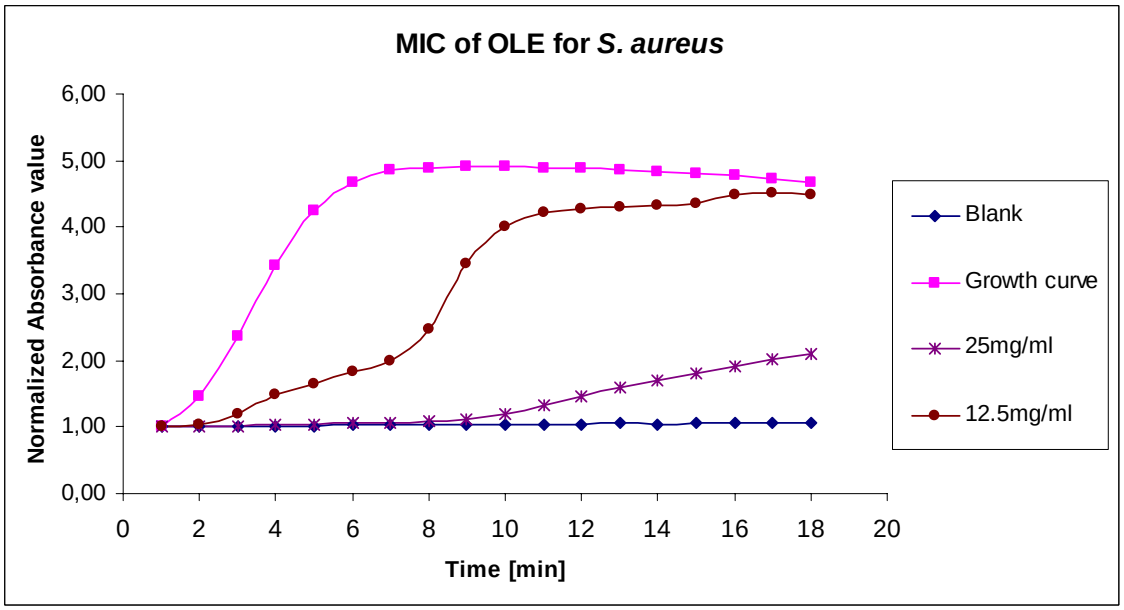

Figure E3. Minimum inhibition concentration of Olive leaf extract for *Staphylococcus aureus*

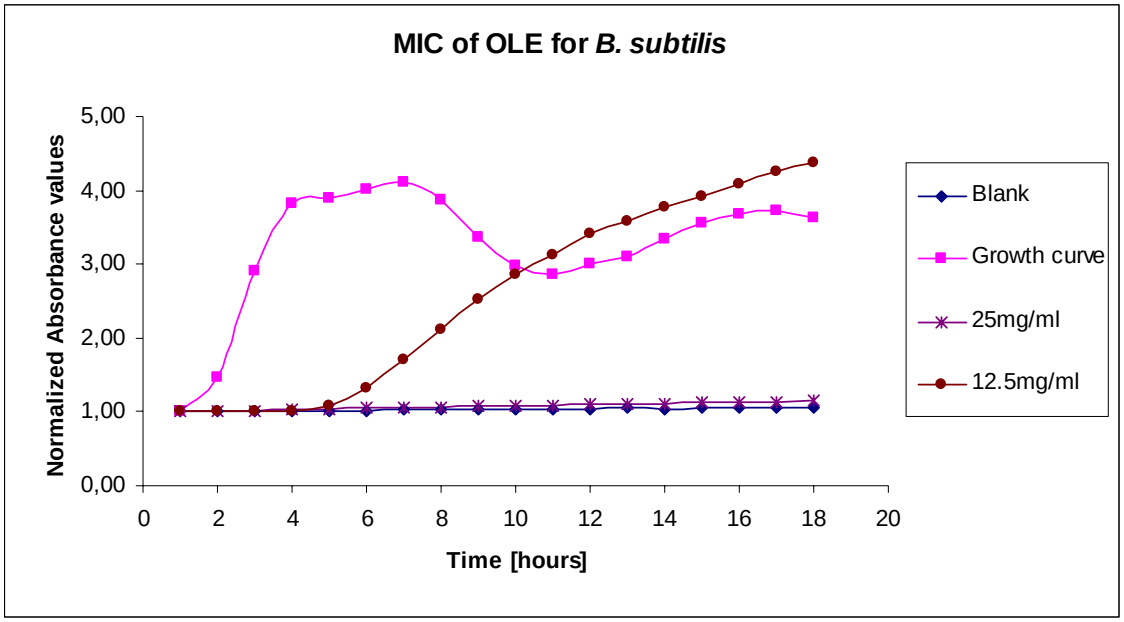

Figure E4. Minimum inhibition concentration of Olive leaf extract for *Bacillus subtilis*

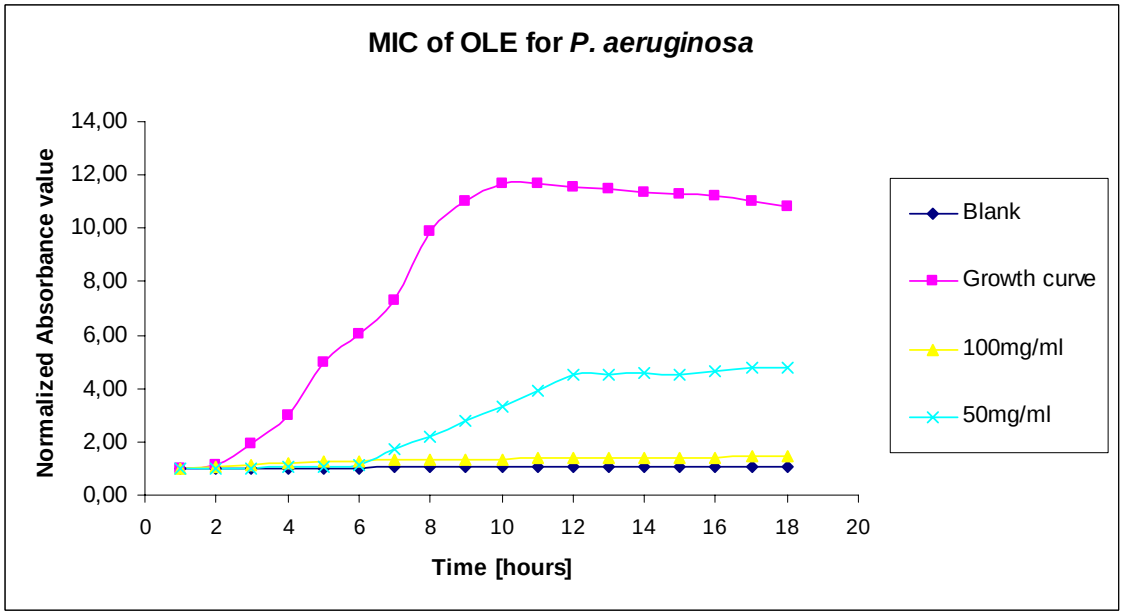

Figure E5. Minimum inhibition concentration of Olive leaf extract for *Pseudomonas aeruginosa*

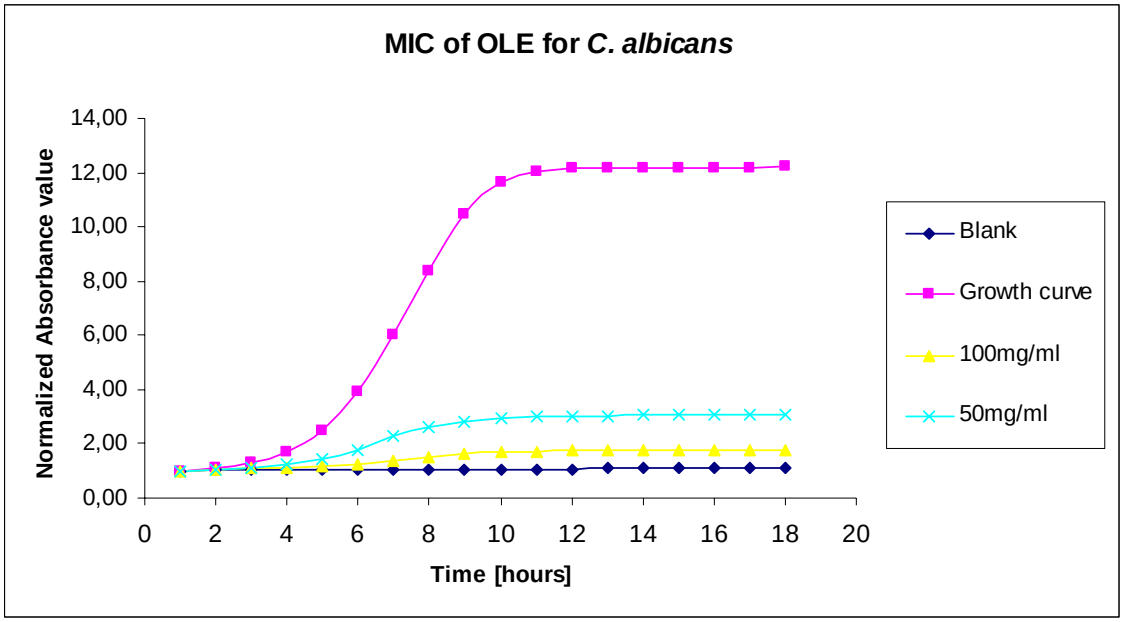

Figure E6. Minimum inhibition concentration of Olive leaf extract for *Candida albicans*

Minimum Inhibition Concentration of *Liquidambar orientalis* Extract

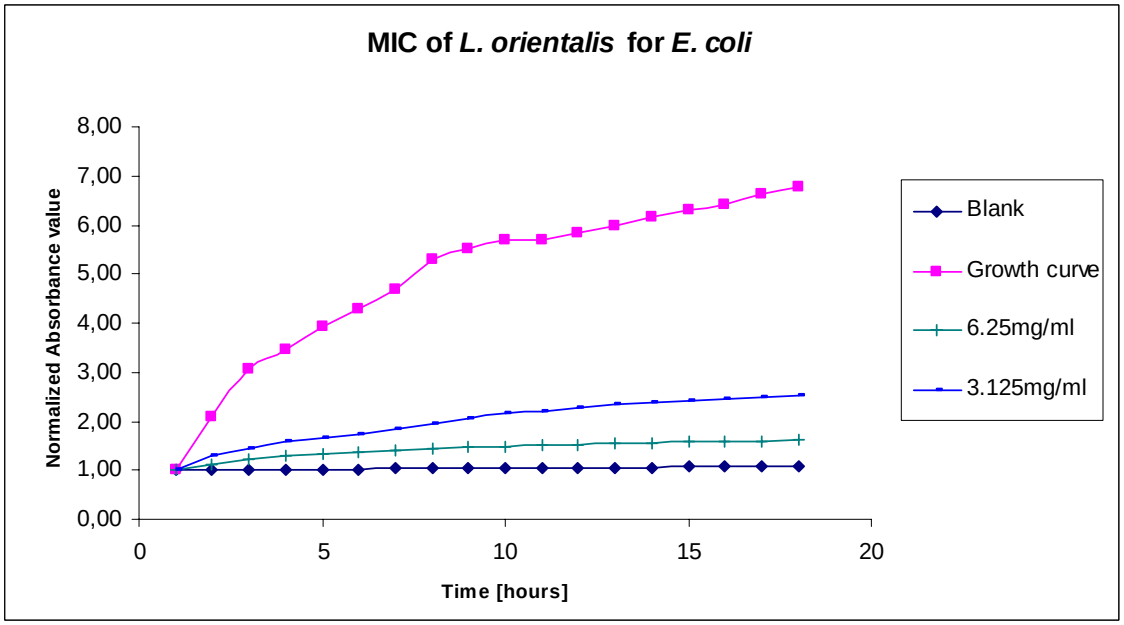

Figure E7. Minimum inhibition concentration of *Liquidambar orientalis* extract for *Escherichia coli*

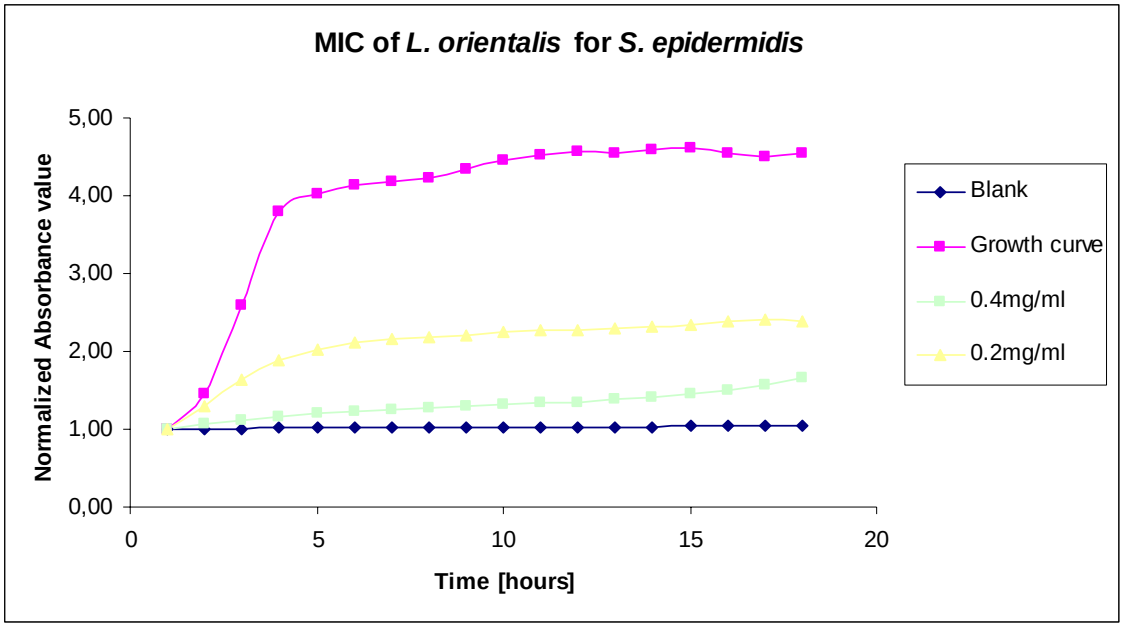

Figure E8. Minimum inhibition concentration of *Liquidambar orientalis* extract for *Staphylococcus epidermidis*

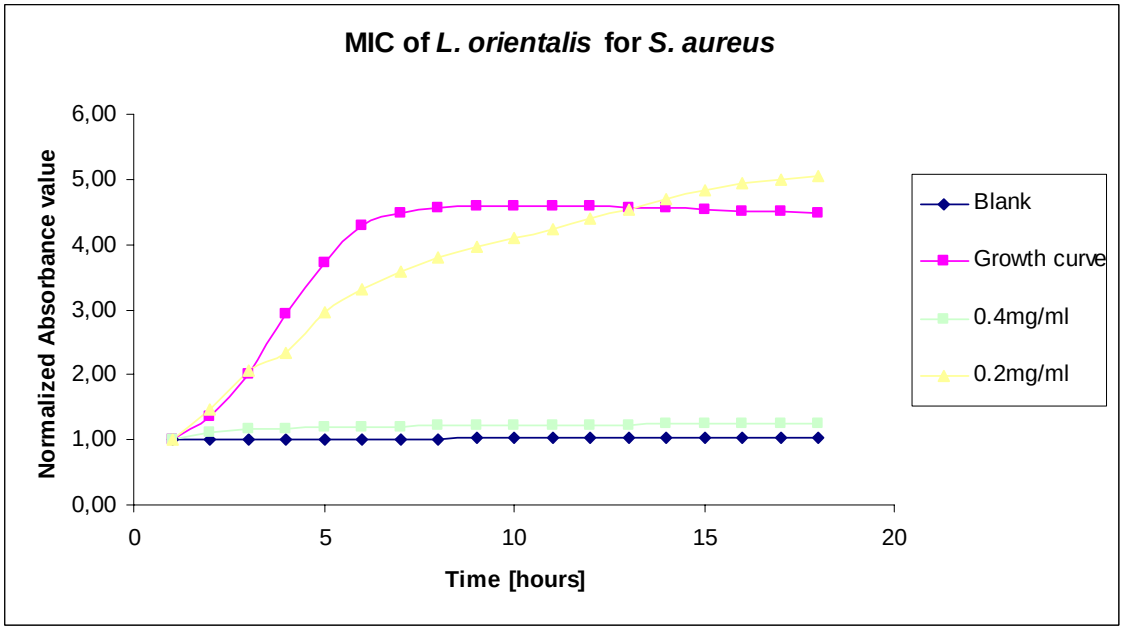

Figure E9. Minimum inhibition concentration of *Liquidambar orientalis* extract for *Staphylococcus aureus*

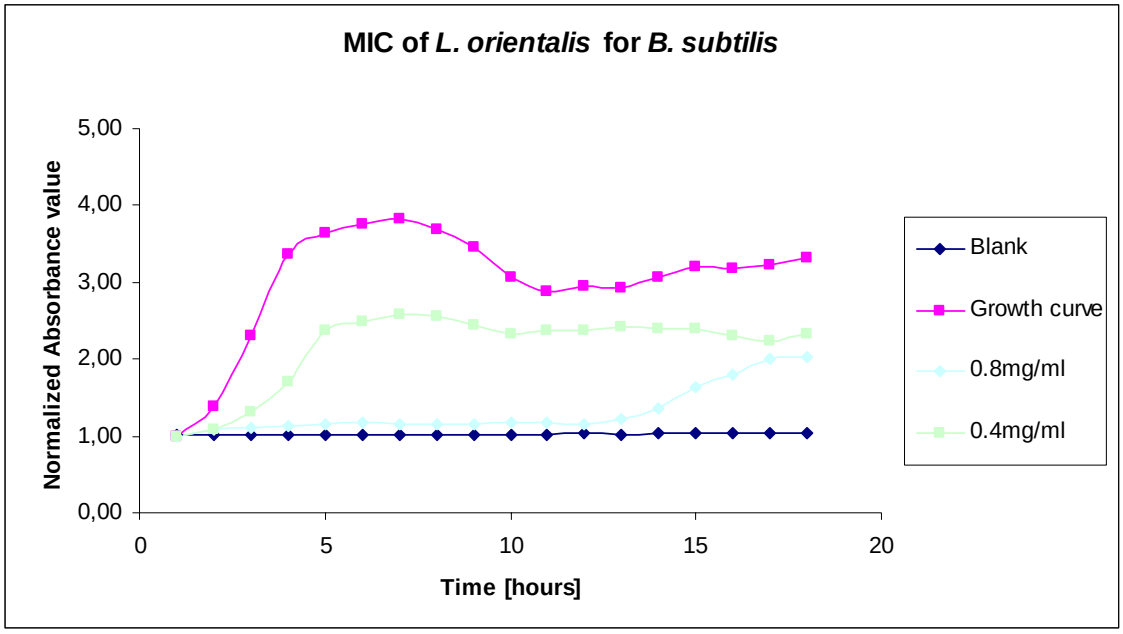

Figure E10. Minimum inhibition concentration of *Liquidambar orientalis* extract for *Bacillus subtilis*

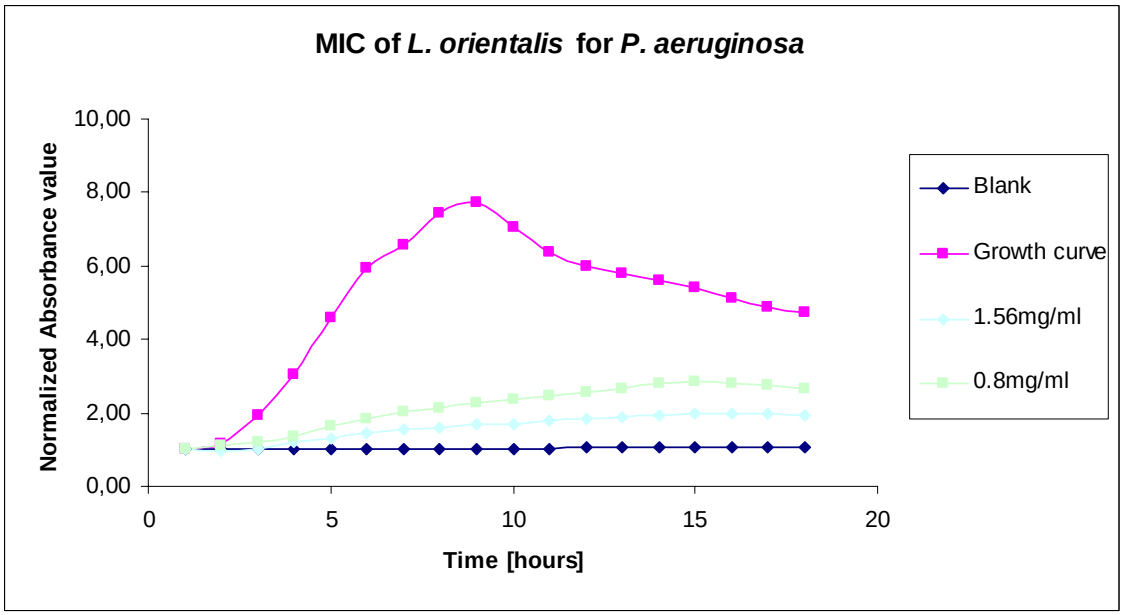

Figure E11. Minimum inhibition concentration of *Liquidambar orientalis* extract for *Pseudomonas aeruginosa*

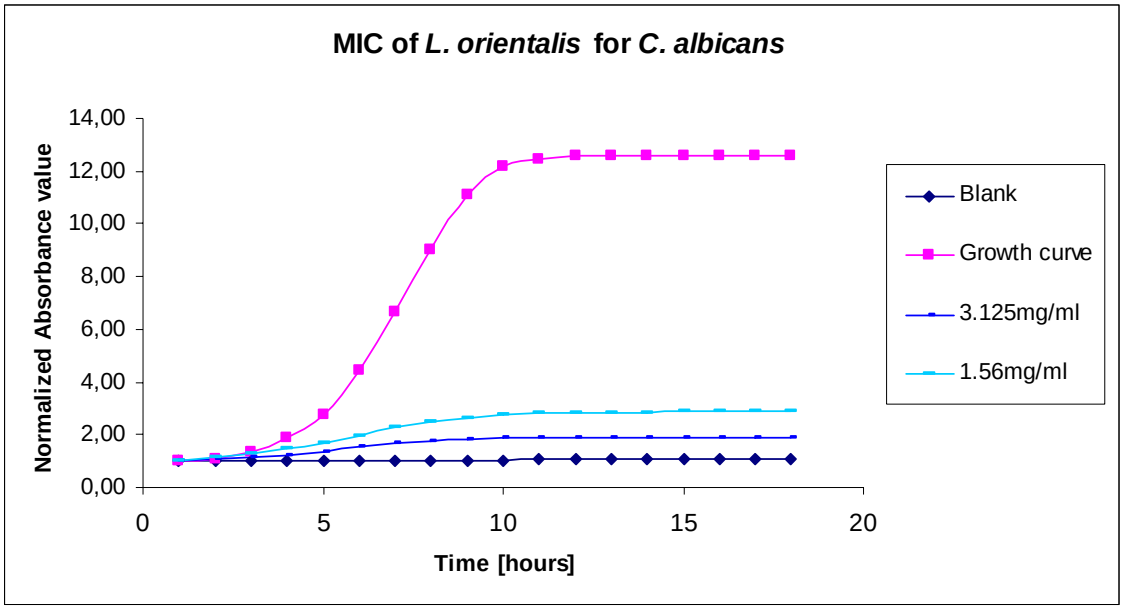

Figure E12. Minimum inhibition concentration of *Liquidambar orientalis* extract for *Candida albicans*

Minimum Inhibition Concentration of *Ziziphus jujuba* Extract

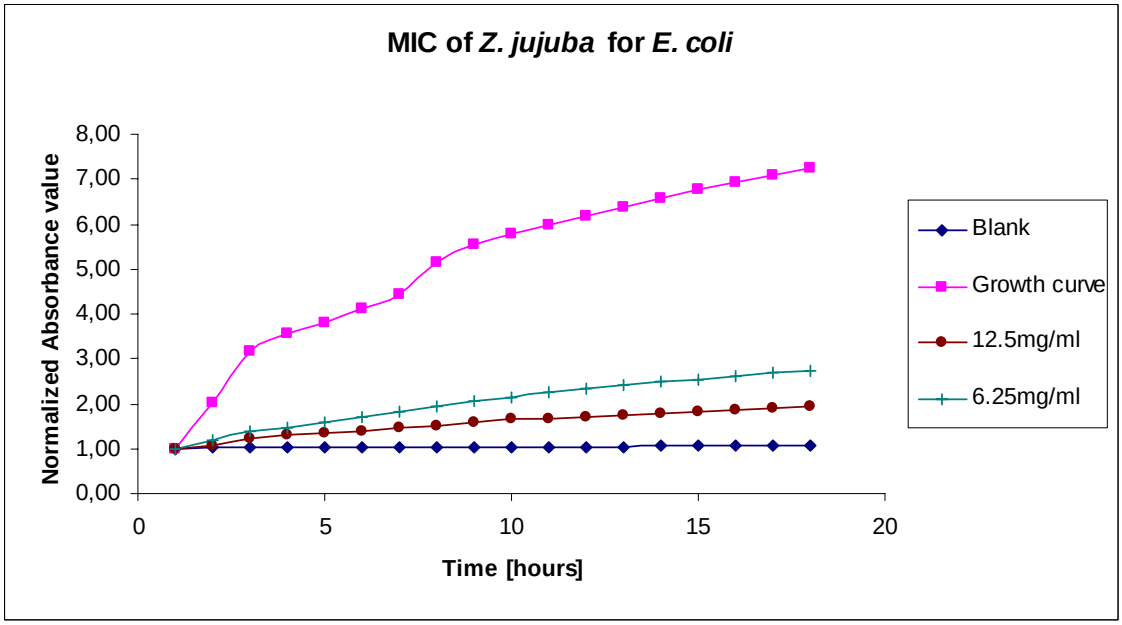

Figure E13. Minimum inhibition concentration of *Ziziphus jujuba* extract for *Escherichia coli*

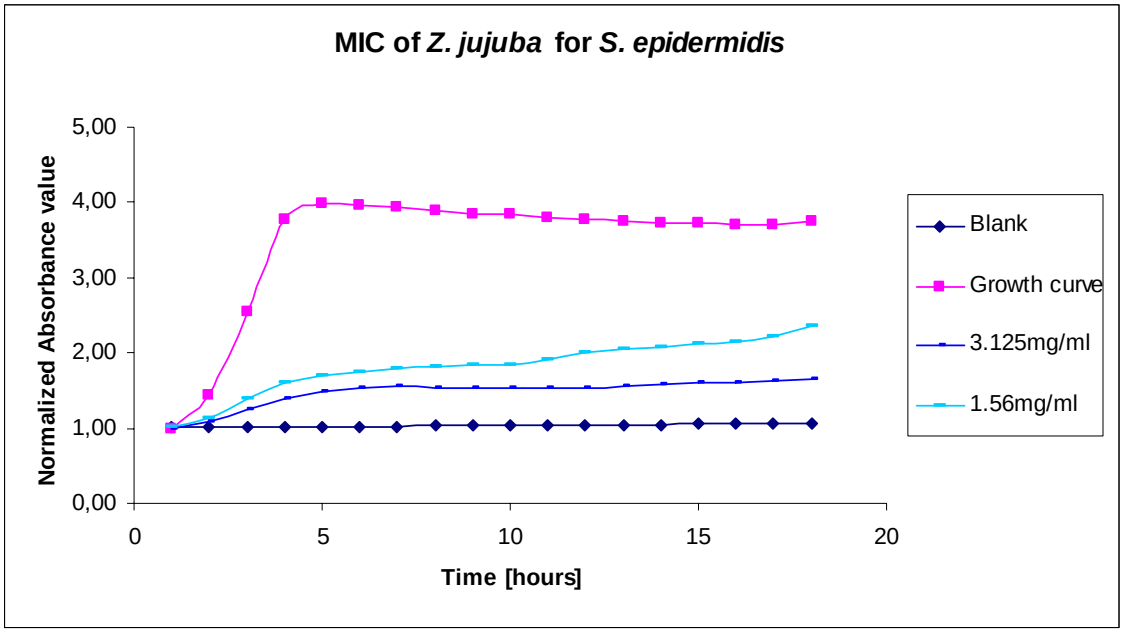

Figure E14. Minimum inhibition concentration of *Ziziphus jujuba* extract for *Staphylococcus epidermidis*

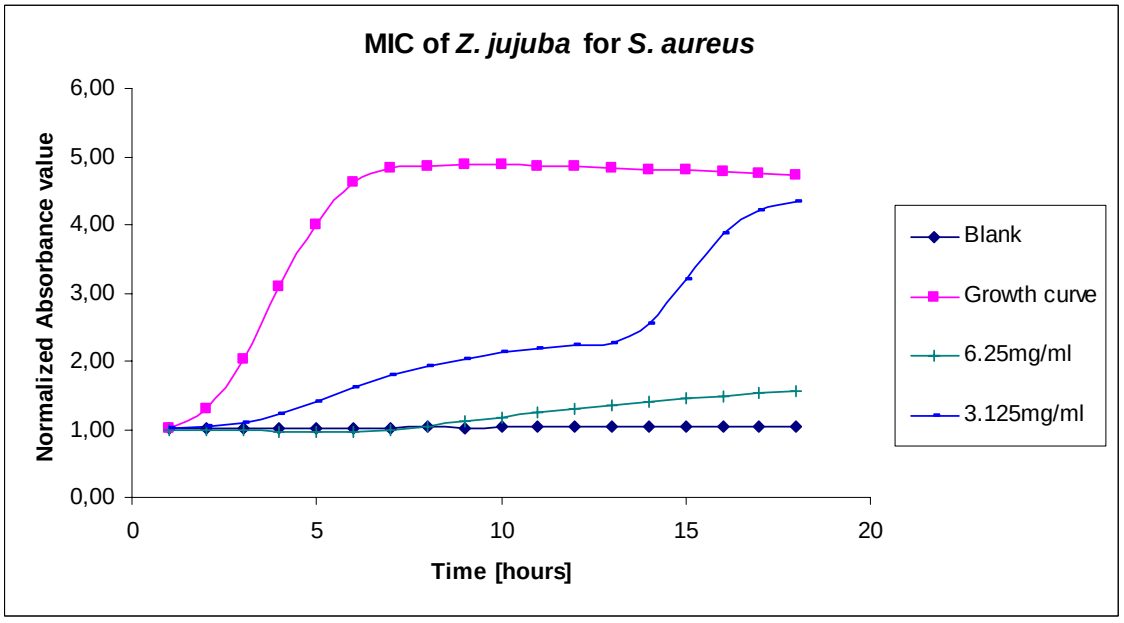

Figure E15. Minimum inhibition concentration of *Ziziphus jujuba* extract for *Staphylococcus aureus*

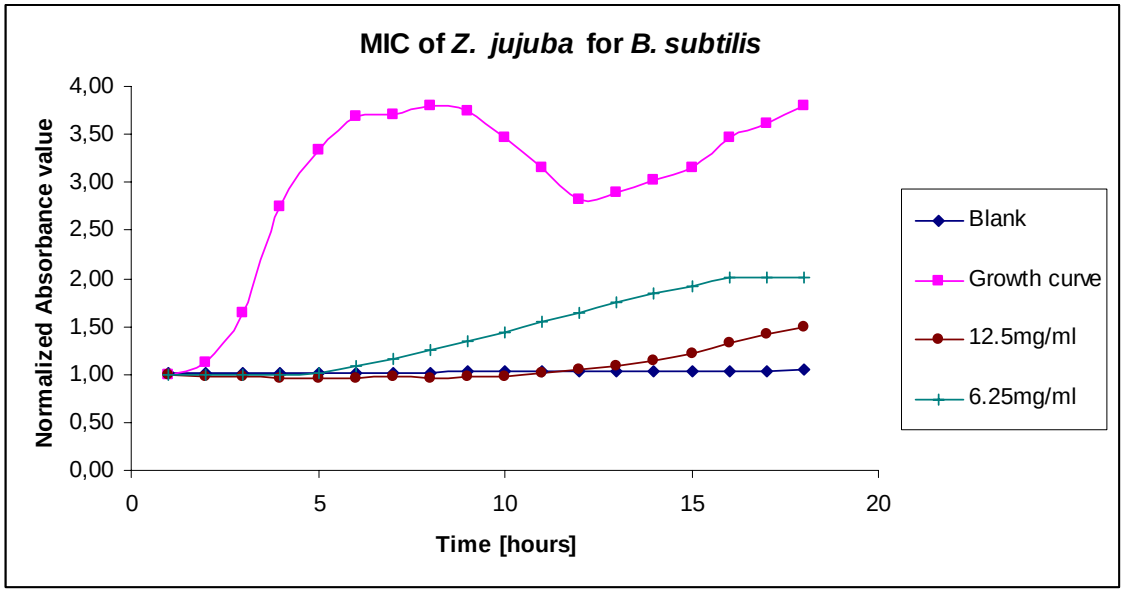

Figure E16. Minimum inhibition concentration of *Ziziphus jujuba* extract for *Bacillus subtilis*

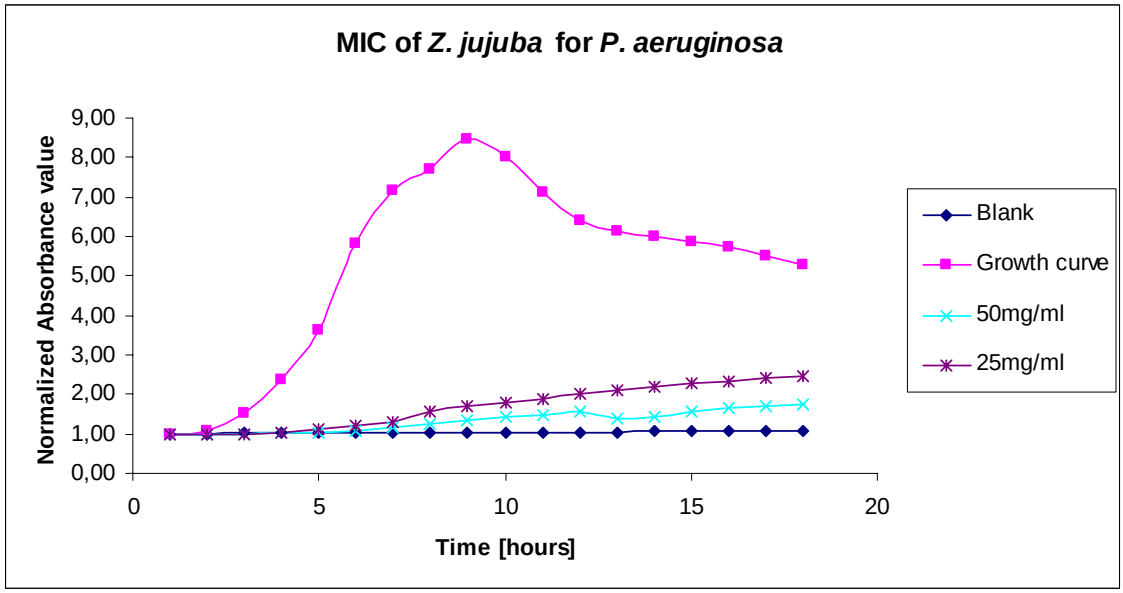

Figure E17. Minimum inhibition concentration of *Ziziphus jujuba* extract for *Pseudomonas aeruginosa*

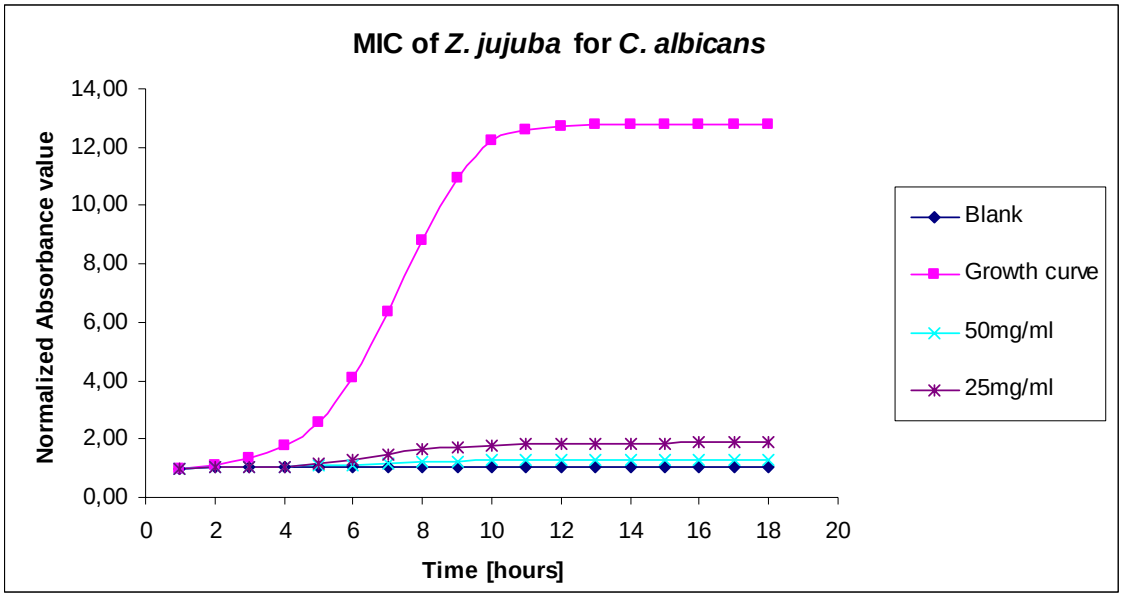

Figure E18. Minimum inhibition concentration of *Ziziphus jujuba* extract for *Candida albicans*

Minimum Inhibition Concentration of *Juniperus communis* (seed) Extract

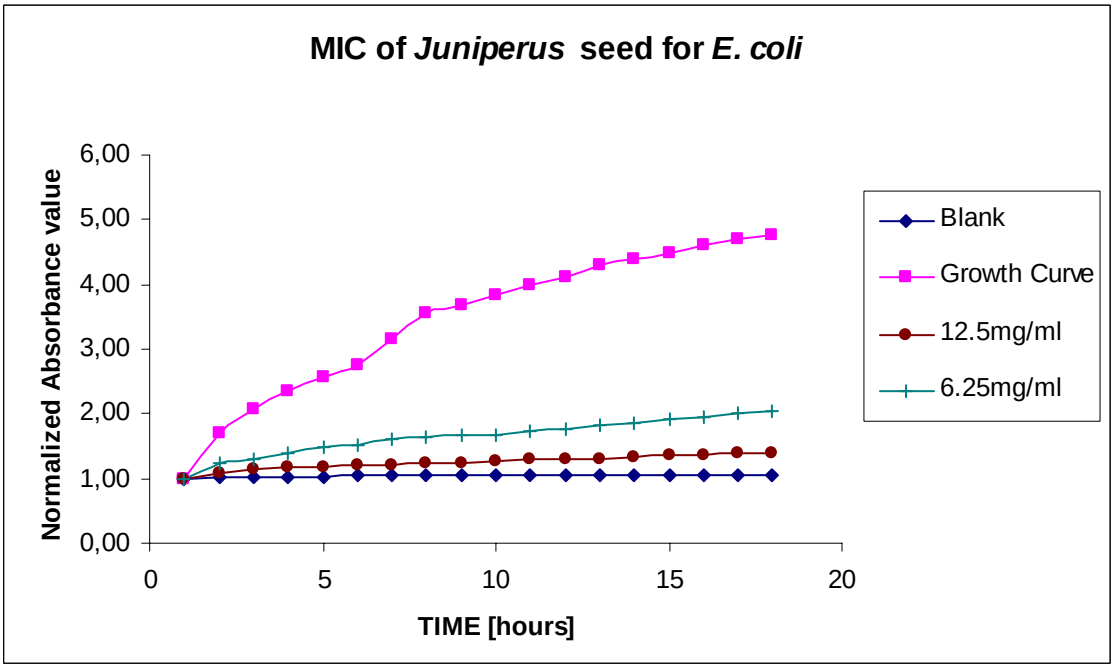

Figure E19. Minimum inhibition concentration of *Juniperus communis* extract for *Escherichia coli*

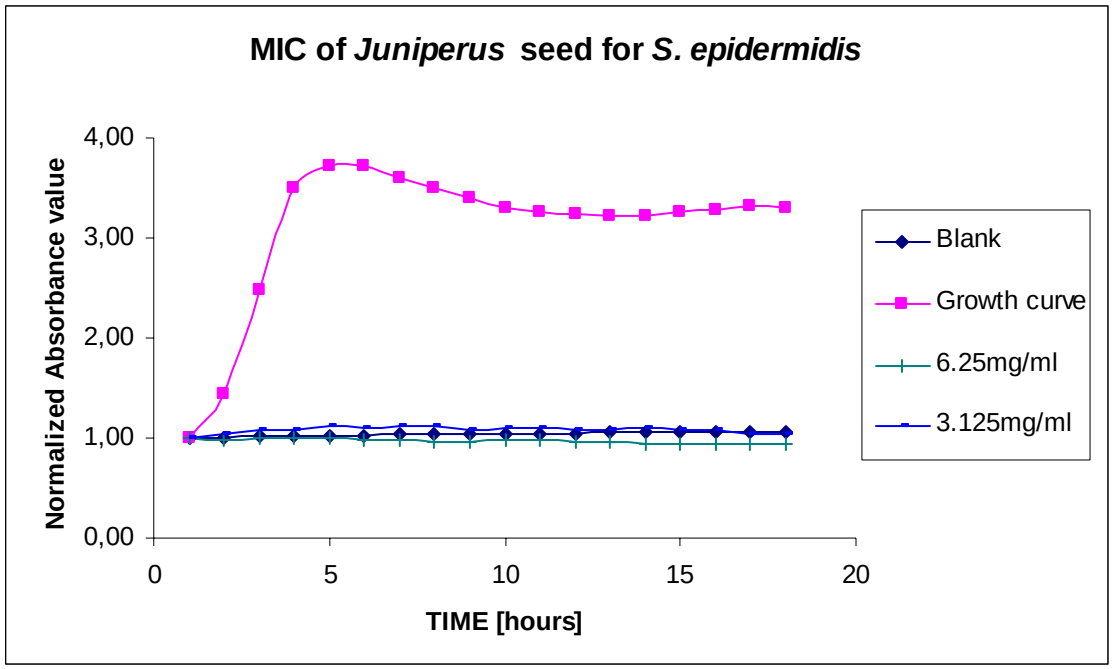

Figure E20. Minimum inhibition concentration of *Juniperus communis* extract for *Staphylococcus epidermidis*

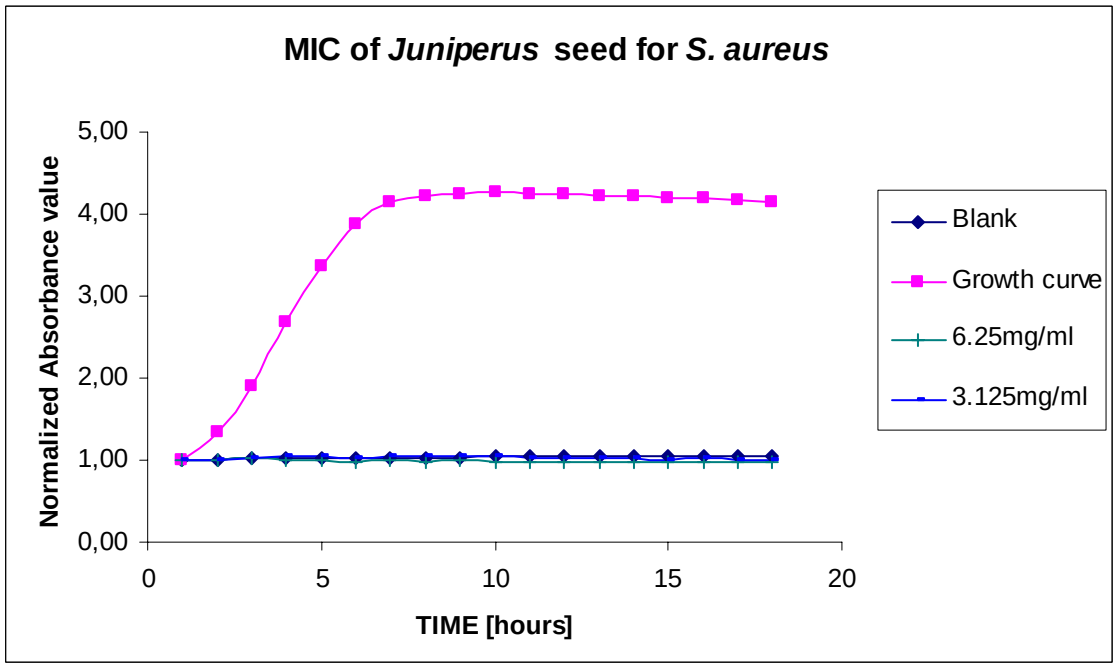

Figure E21. Minimum inhibition concentration of *Juniperus communis* extract for *Staphylococcus aureus*

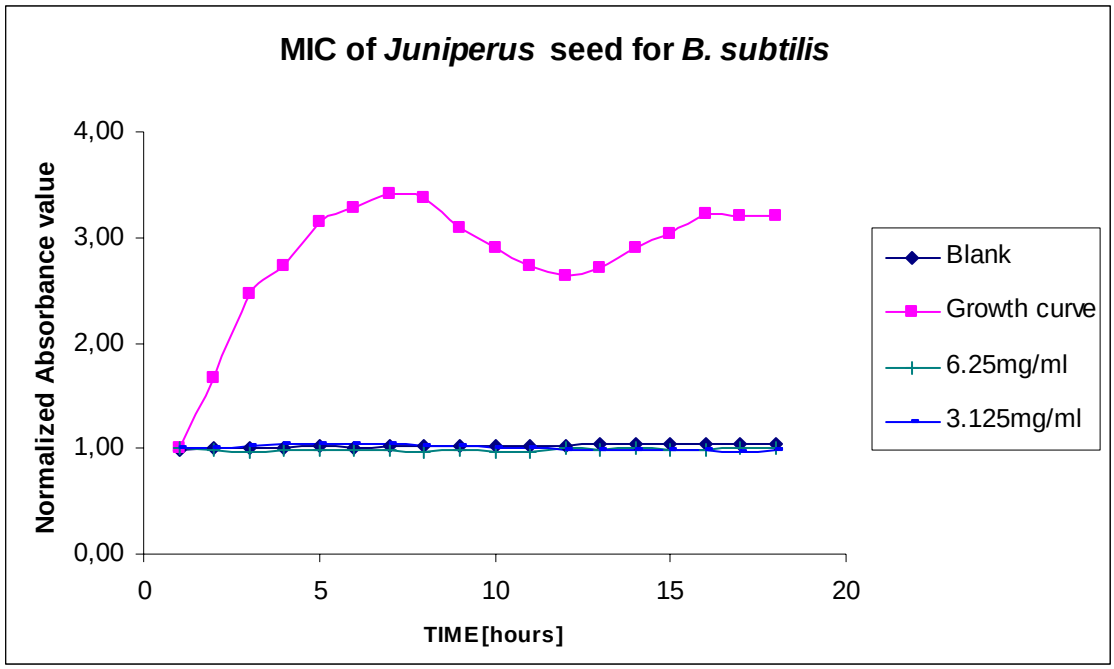

Figure E22. Minimum inhibition concentration of *Juniperus communis* extract for *Bacillus subtilis*

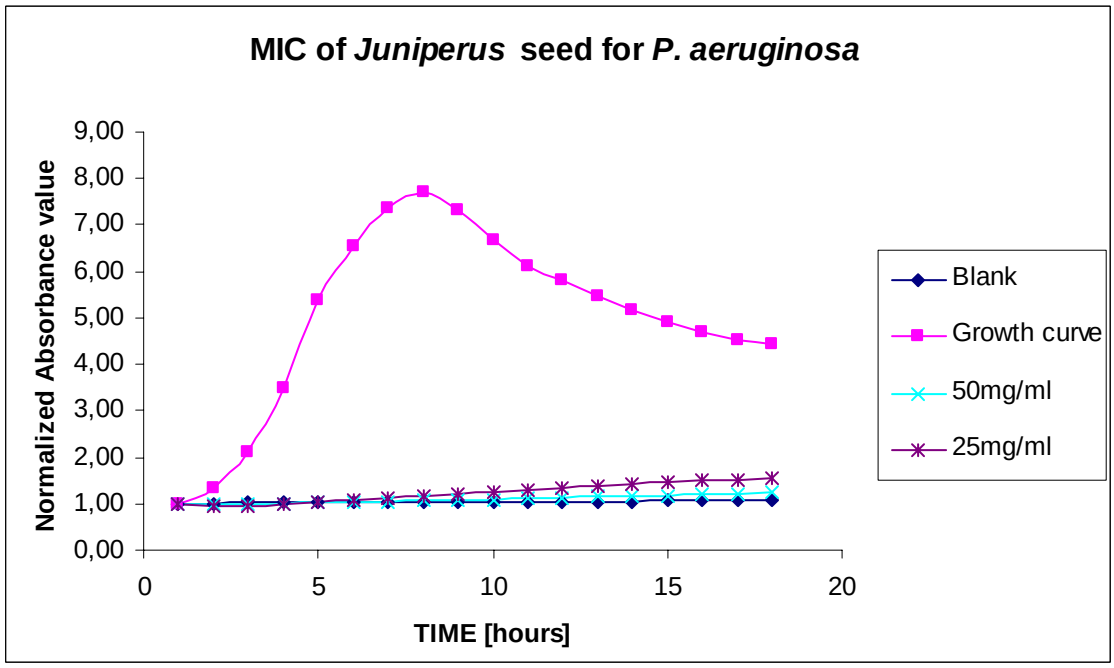

Figure E23. Minimum inhibition concentration of *Juniperus communis* extract for *Pseudomonas aeruginosa*

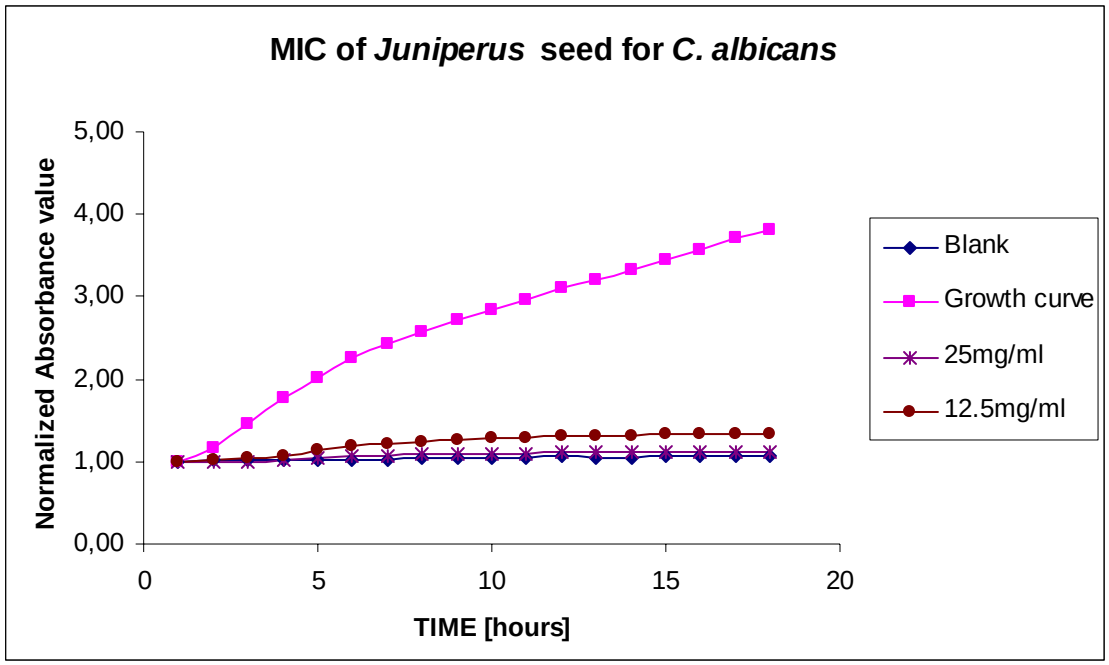

Figure E23. Minimum inhibition concentration of *Juniperus communis* extract for *Candida albicans*

APPENDIX F

MINIMUM INHIBITION CONCENTRATIONS OF ANTIBIOTICS

In this study, Minimum inhibition concentrations (MIC) of antibiotics were determined against five microorganism in order to find out their resistance to antibiotics. *Escherichia coli* (Gram -), *Staphylococcus epidermidis* (Gram +), *Staphylococcus aureus* (Gram +), *Bacillus subtilis* (Gram +) and *Pseudomonas aeruginosa* (Gram -) were used. The inhibition graphics were given for each microorganism in figures below.

Minimum Inhibition Concentration of Ampicillin

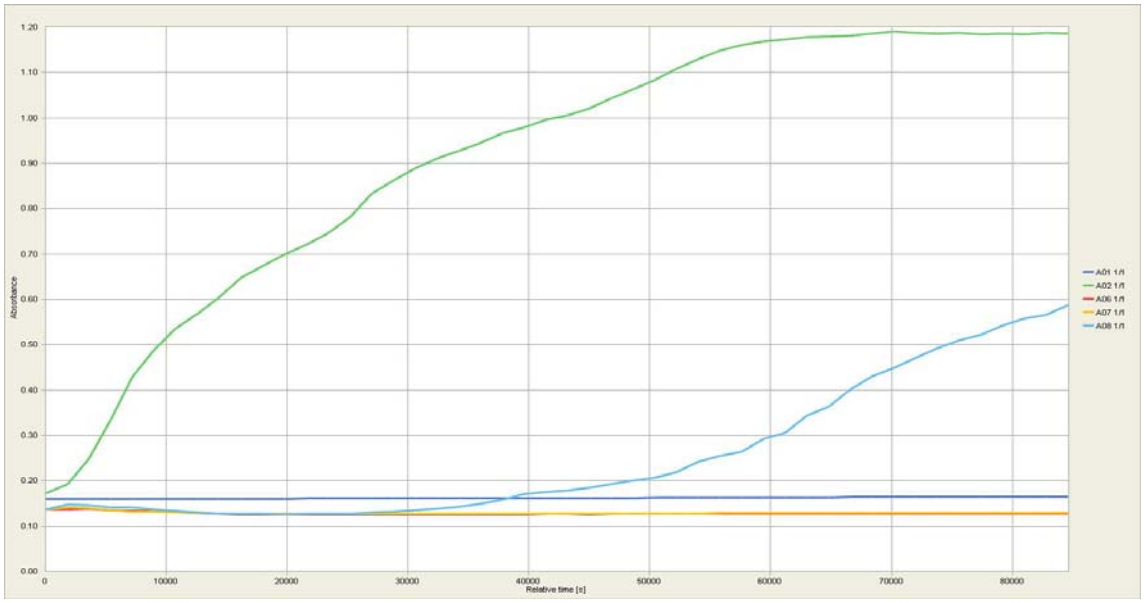

Figure F1. Minimum inhibition concentration of Ampicillin for *Escherichia coli* A6: 12.5 µg/ml, **A7: 6.25 µg/ml**, A8: 3.125 µg/ml

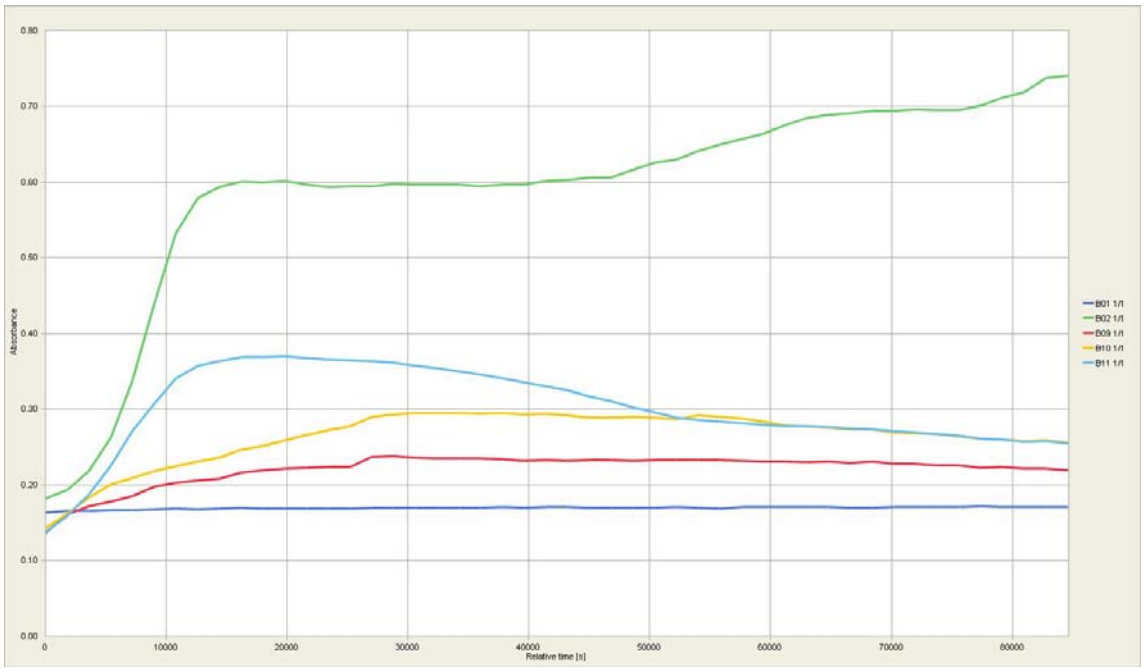

Figure F2. Minimum inhibition concentration of Ampicillin for *Staphylococcus epidermidis*
B9: 1.56 µg/ml, **B10: 0.8 µg/ml**, B11: 0.4 µg/ml

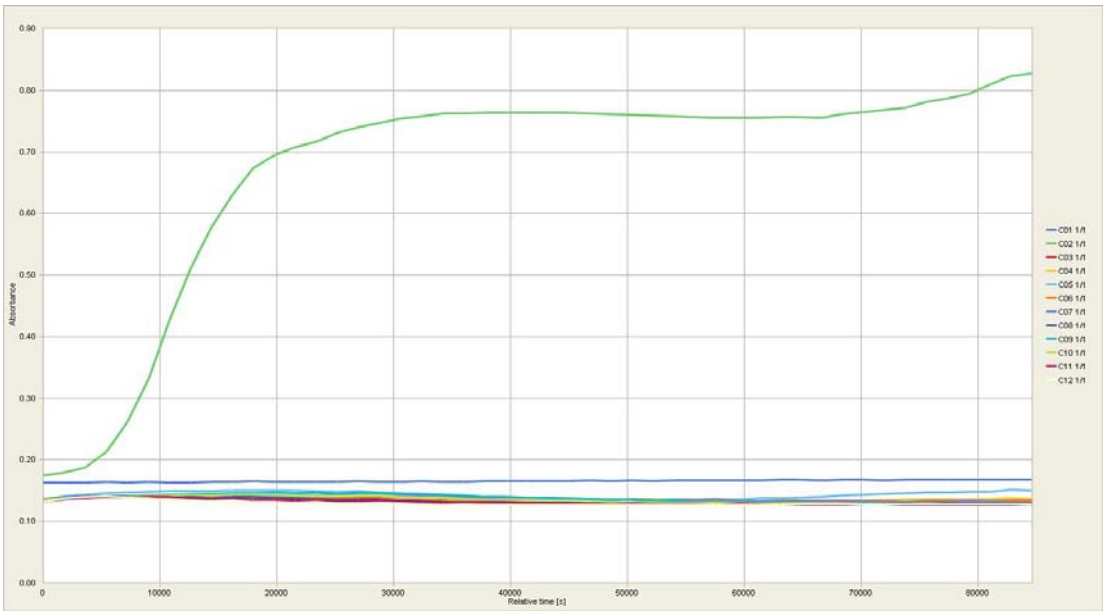

Figure F3. Minimum inhibition concentration of Ampicillin for *Staphylococcus aureus*
C10: 0.8 µg/ml, **C11: 0.4 µg/ml**, C12: 0.2 µg/ml

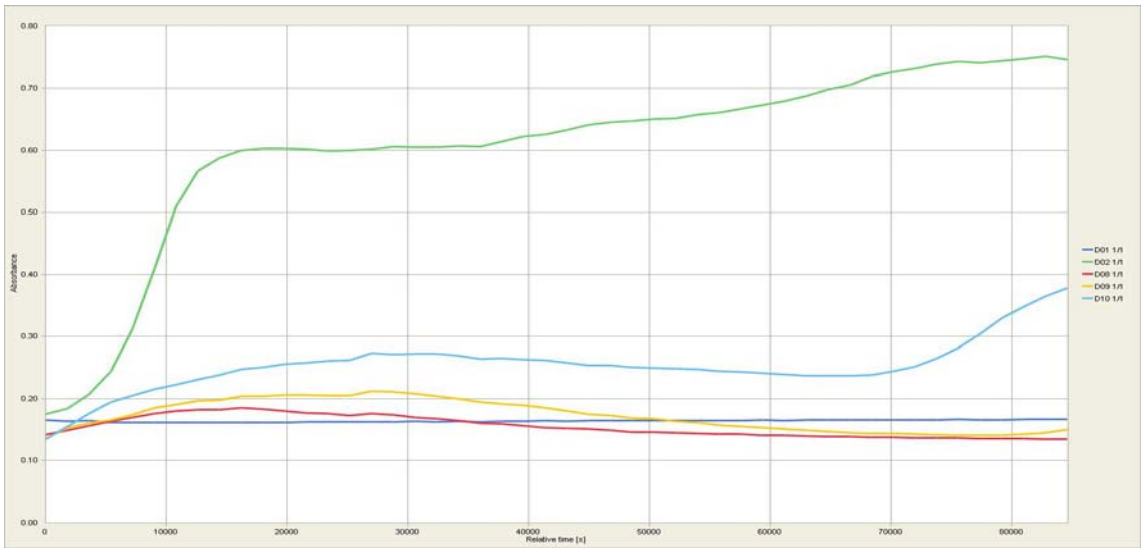

Figure F4. Minimum inhibition concentration of Ampicillin for *Bacillus subtilis*
D8: 3.125 µg/ml, **D9: 1.56 µg/ml**, D10: 0.8 µg/ml

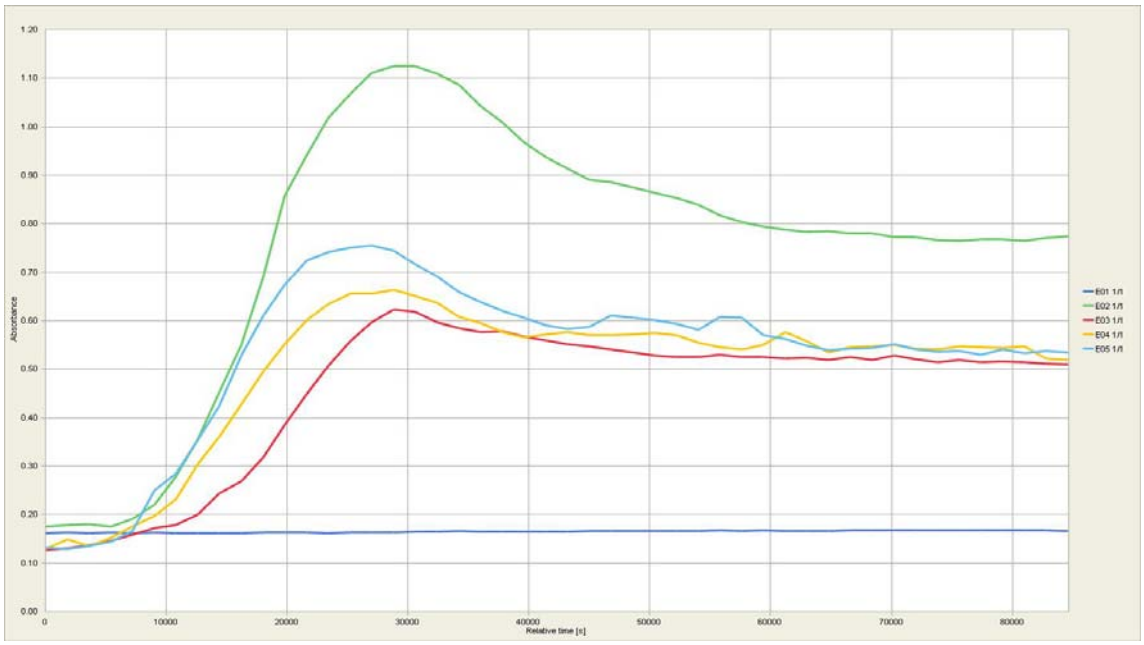

Figure F5. Minimum inhibition concentration of Ampicillin for
Pseudomonas aeruginosa **(No Inhibition)**
E3: 100 µg/ml, E4: 50 µg/ml, E5: 25 µg/ml

Minimum Inhibition Concentration of Gentamycin

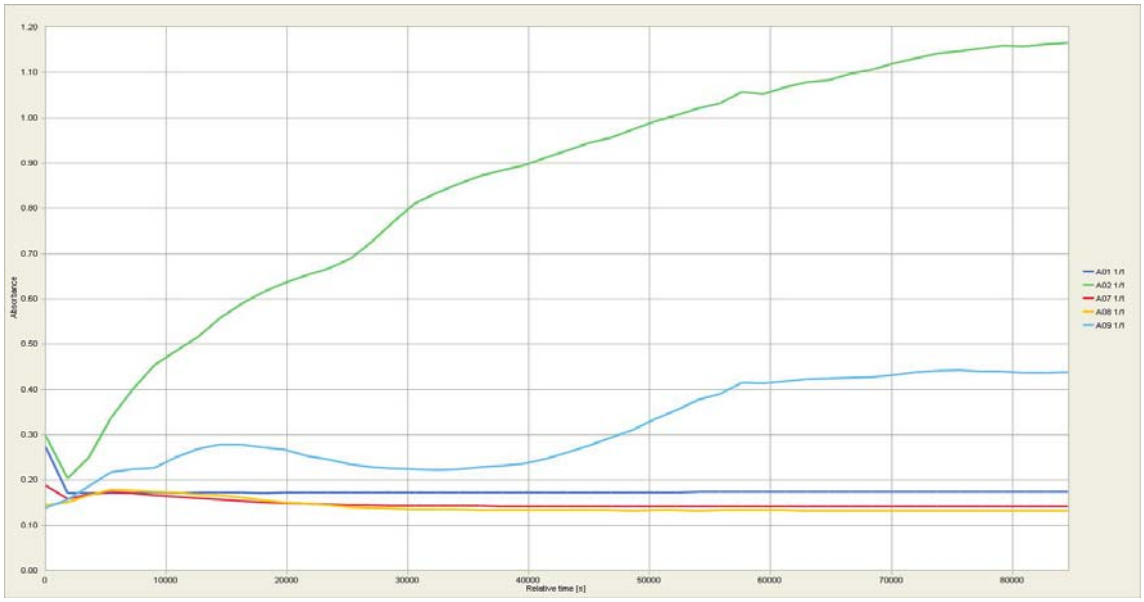

Figure F6. Minimum inhibition concentration of Gentamycin for *Escherichia coli*
A7: 6.25 µg/ml, **A8: 3.125 µg/ml**, A9: 1.56 µg/ml

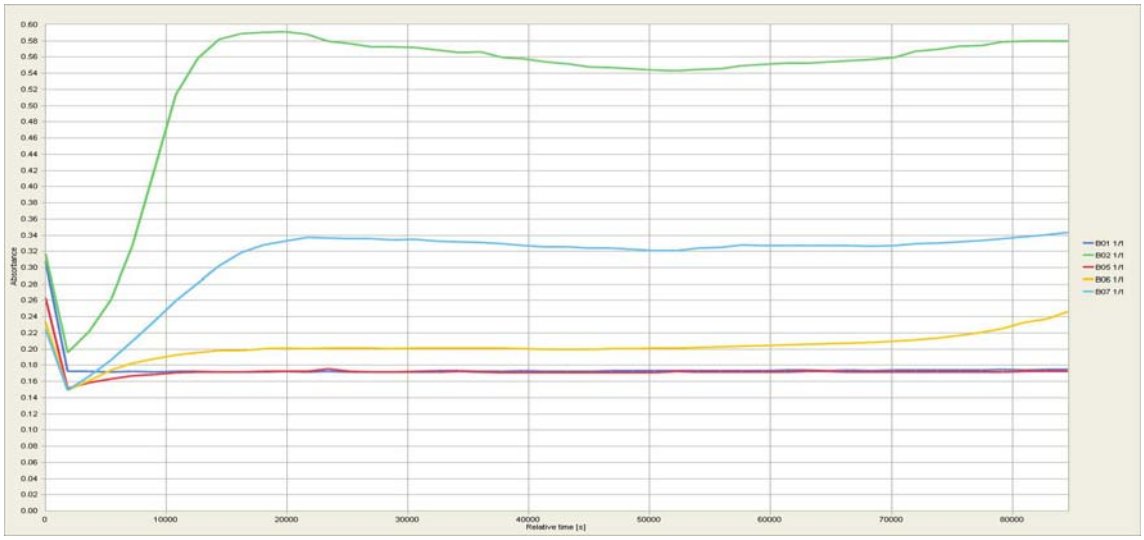

Figure F7. Minimum inhibition concentration of Gentamycin for
Staphylococcus epidermidis
B5: 25 µg/ml, **B6: 12.5 µg/ml**, B7: 6.25 µg/ml

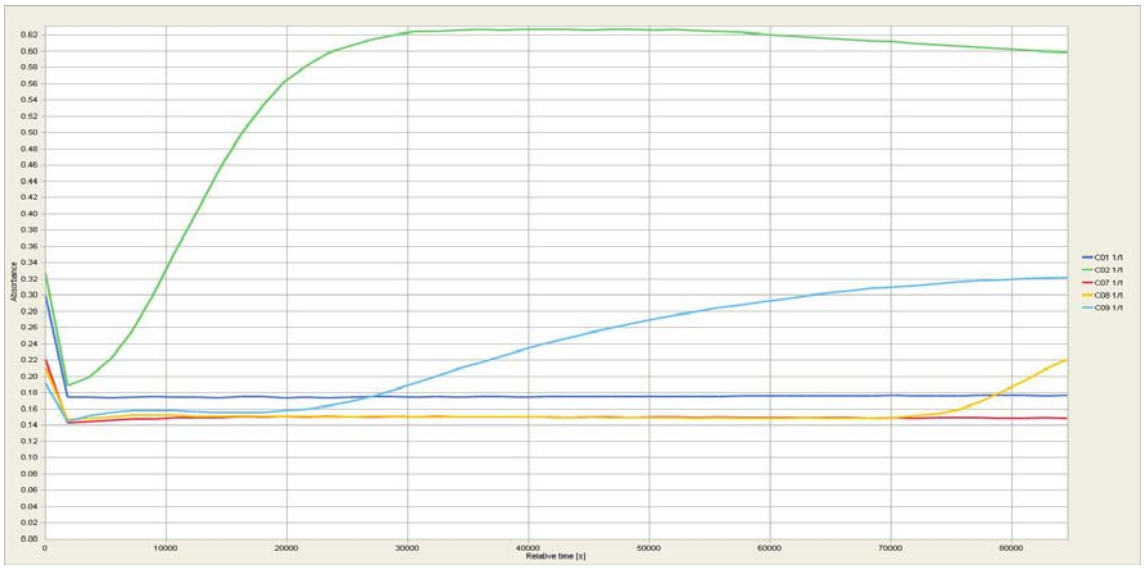

Figure F8. Minimum inhibition concentration of Gentamycin for *Staphylococcus aureus* C7: 6.25 µg/ml, **C8: 3.125 µg/ml**, C9: 1.56 µg/ml

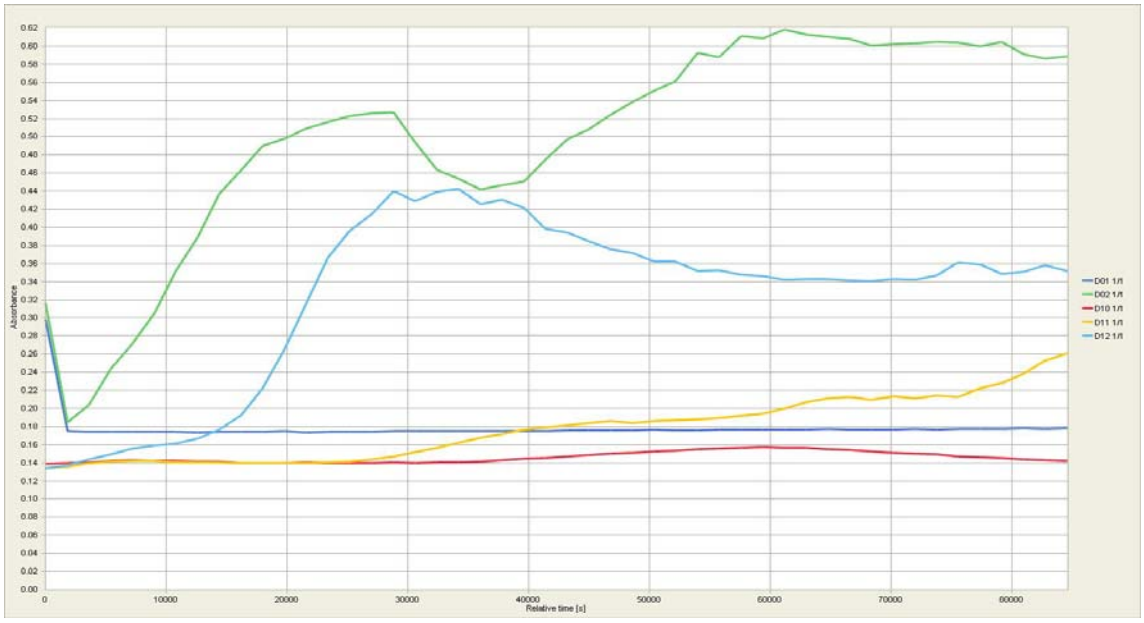

Figure F9. Minimum inhibition concentration of Gentamycin for *Bacillus subtilis* D10: 0.8 µg/ml, **D11: 0.4 µg/ml**, D12: 0.2 µg/ml

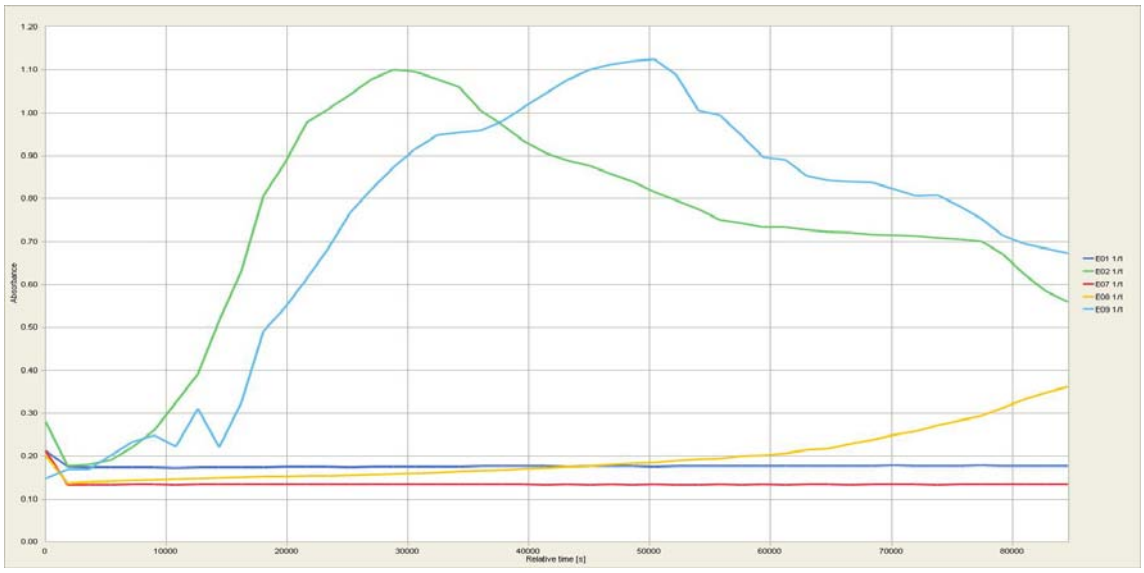

Figure F10. Minimum inhibition concentration of Gentamycin for *Pseudomonas aeruginosa*
E7: 6.25 µg/ml, **E8: 3.125 µg/ml**, E9: 1.56 µg/ml

Minimum Inhibition Concentration of Penicillin

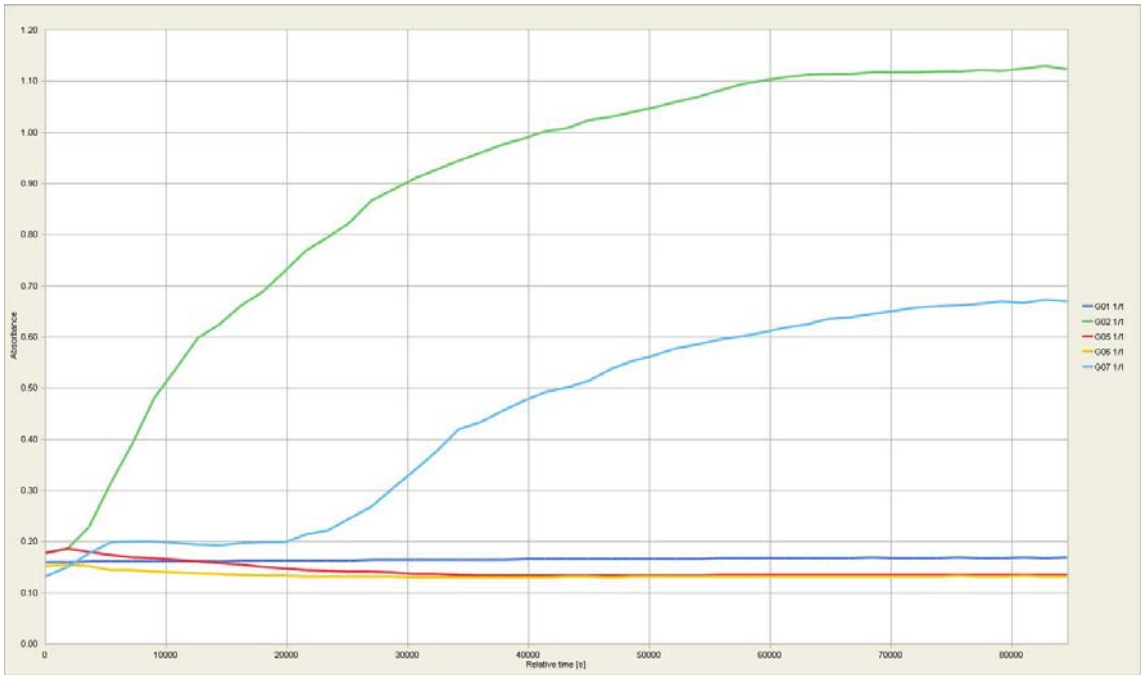

Figure F11. Minimum inhibition concentration of Penicillin for *Escherichia coli*
G5: 2500 µg/ml, **G6: 1250 µg/ml**, G7: 625 µg/ml

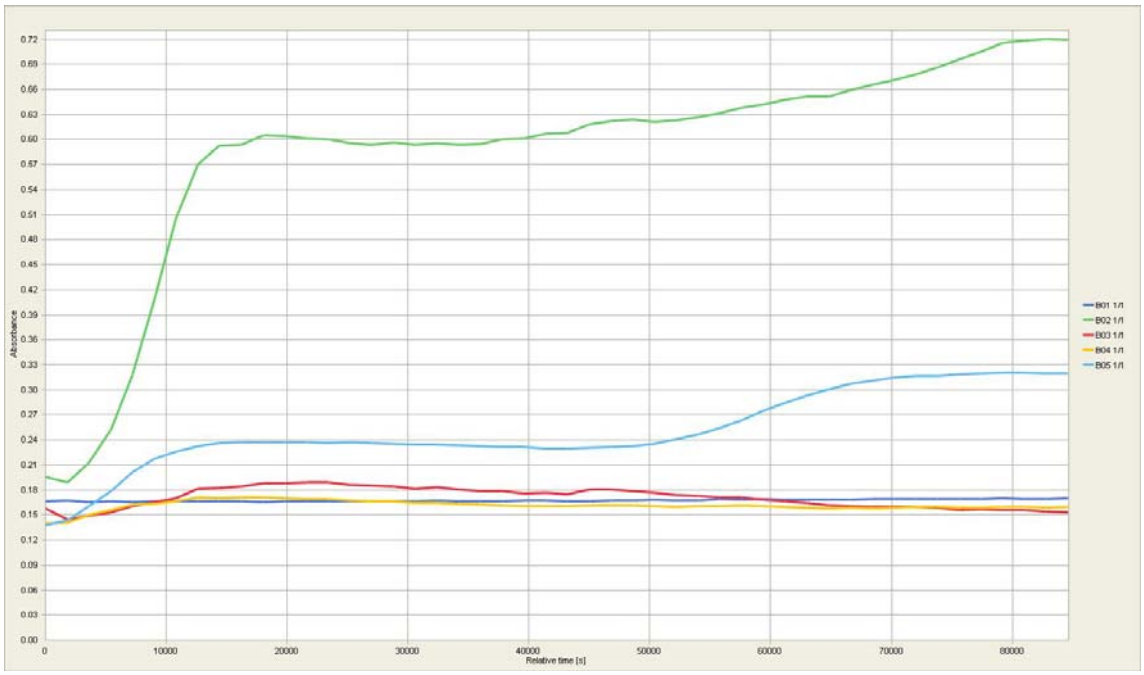

Figure F12. Minimum inhibition concentration of Penicillin for *Staphylococcus epidermidis*
B3: 100 µg/ml, **B4: 50 µg/ml**, B5: 25 µg/ml

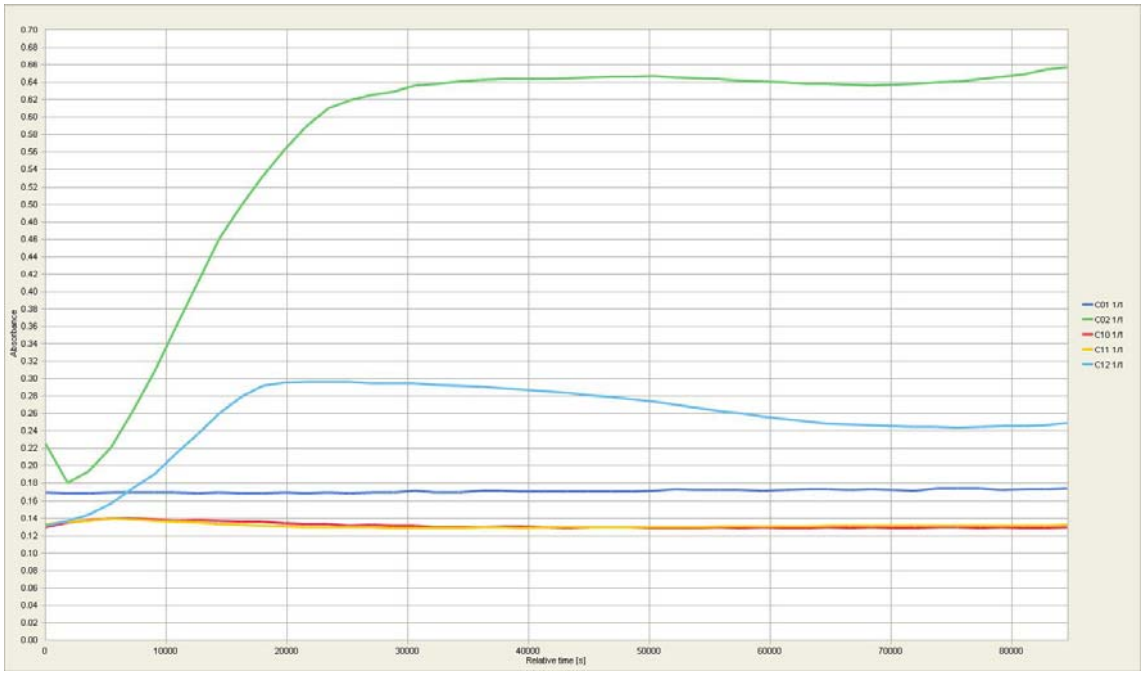

Figure F13. Minimum inhibition concentration of Penicillin for *Staphylococcus aureus*
C10: 0.8 µg/ml, **C11: 0.4 µg/ml**, C12: 0.2 µg/ml

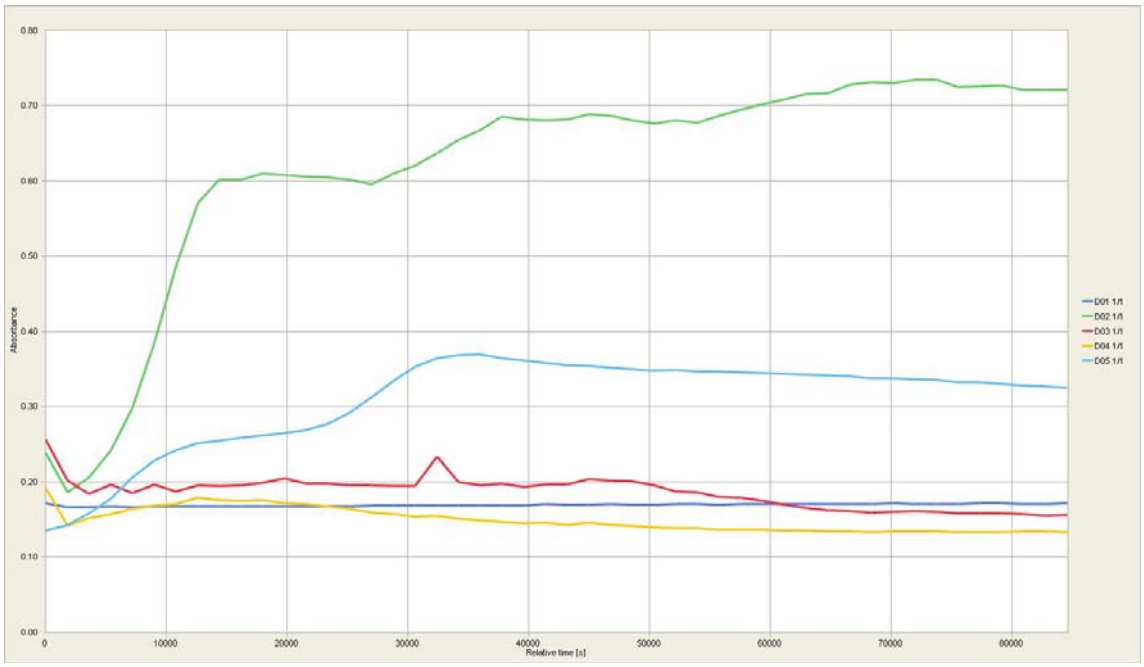

Figure F14. Minimum inhibition concentration of Penicillin for *Bacillus subtilis*
D3: 100 µg/ml, **D4: 50 µg/ml**, D5: 25 µg/ml

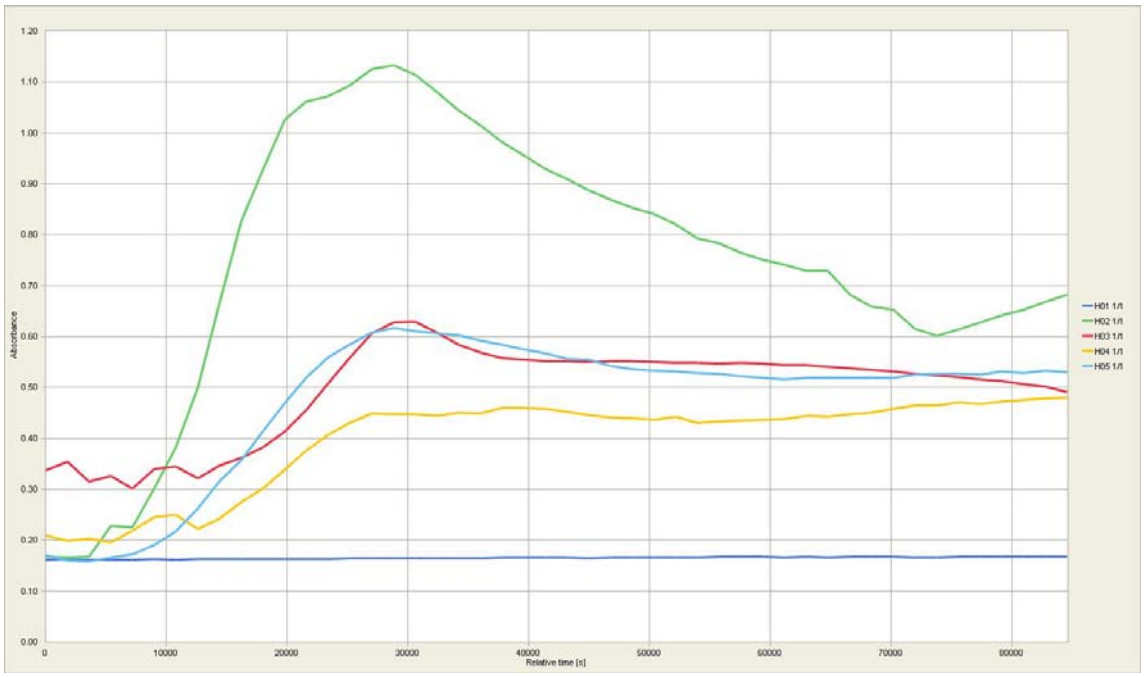

Figure F15. Minimum inhibition concentration of Penicillin for
Pseudomonas aeruginosa **(No Inhibition)**
H3: 10000 µg/ml, H4: 5000 µg/ml, H5: 2500 µg/ml