ENCAPSULATION OF *SARCOPOTERIM SPINOSUM* EXTRACT IN ZEIN PARTICLE BY USING ELECTROSPRAY METHOD

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

MASTER OF SCIENCE

in Biotechnology

by
Ceren SÜNGÜÇ

July, 2013
İZMİR

We approve the thesis of **Ceren SÜNGÜÇ**

Examining Committee Members:

_____

Prof. Dr. Oğuz BAYRAKTAR
Department of Chemical Engineering, İzmir Institute of Technology

_____

Assist. Prof. Dr. Ayşegül BATIGÜN
Department of Chemical Engineering, İzmir Institute of Technology

_____

Assist. Prof. Dr. Çağatay CEYLAN
Department of Food Engineering, İzmir Institute of Technology

5 July 2013

_____	_____
Prof. Dr. Oğuz BAYRAKTAR Supervisor, Department of Chemical Engineering İzmir Institute of Technology	**Assist. Prof. Dr. Mehmet ATEŞ** Co-Supervisor, Department of Laboratory Animal Science Dokuz Eylül University
_____	_____
Prof. Dr. Volga BULMUŞ Head of the Department of Biotechnology and Bioengineering	**Prof. Dr. R. Tuğrul SENGER** Dean of the Graduate School of Engineering and Sciences

ACKNOWLEDGMENTS

Firstly, I would like to express my deep and sincere gratitude to my advisor Prof. Dr. Oğuz BAYRAKTAR for his suggestions, guidance, encouragement and support throughout my M.Sc. study. I also would like to thank him for his generosity providing materials and laboratory facilities. I thank to my co-advisor Assist. Prof. Dr. Mehmet ATEŞ for his critical suggestion on my study.

I warmly express my special thanks to my co-workers, İpek ERDOĞAN, Mehmet Emin USLU, Sedef TAMBURACI, Esra AYDINLIOĞLU, Sezen Duygu ALICI, Derya KÖSE and Damla TAYKOZ for their supports, helps, patience and friendships.

I would like to extend my thanks to Semiha and Reşit ÇAĞLIYANGİL, especially, I want to express special thanks to my sweety mother, Gül and my father, İlhan SÜNGÜÇ for their love, encouragement and supports during my education.

I strongly thank Mehmet ÇAĞLIYANGİL for his endless support, patience, helps, friendship and his kind love.

ABSTRACT

ENCAPSULATION OF *SARCOPOTERIUM SPINOSUM* EXTRACT IN ZEIN PARTICLE BY USING ELECTROSPRAY METHOD

Sarcopoterium spinosum species has valuable and common medicinal plant in the Mediterranean region. The optimum conditions for the extraction of *S. spinosum* leaves to obtain bioactive extract were investigated using response surface methodology (RSM). Total phenol contents, total antioxidant and antibacterial activities, phenolic composition of *S. spinosum* extract were studied. The prepared *S. spinosum* extract showed high antioxidant activity when compared with many other medicinal plants in the literature. It was determined as 3143.5 mmole Trolox per gr dry weight. The phenolic content of *S. spinosum* extract was examined with High Performance Liquid Chromatography (HPLC). Hyperoside and isoquercetin were detected in *S. spinosum* extract. Especially, isoquercetin was the major compound in the extract. In addition, the antimicrobial activity of *S. spinosum* extract was investigated. The extract showed fungicide activity against *Candida albicans*. *S. spinosum* extract were encapsulated within zein particle via electrospray method in order to enhance its stability. The effects of process parameters for electrospraying method on the particle morphology and size distribution were extensively investigated. The best process conditions were determined as zein concentration of 5% (w/v) in 70% aqueous ethanol solution, flow rate of 0.3 ml/h and applied voltage of 14 kV depending on narrow size distribution, spherical and smooth particle morphology. The best *S. spinosum* extract loading was achieved at extract to zein weight ratio of 1:5. The prepared extract loaded zein microparticles showed significant antioxidant activity.

ÖZET

ELEKTROSPREY YÖNTEMİ KULLANILARAK *SARCOPOTERİUM SPİNOSUM* ÖZÜTÜNÜN ZEİN PARTİKÜLÜ İLE ENKAPSÜLASYONU

Sarcopoterium spinosum türü Akdeniz Bölgesi'ndeki kıymetli ve tıbbi amaçlı olarak yaygın kullanılan bir bitkidir. Tepki yüzeyi metodolojisi kullanılarak, *S. spinosum* yapraklarından ideal şartlar altında biyoaktif özüt elde edilmesi araştırıldı. *S. spinosum* özütünün fenolik komposizyonlarından kaynaklanan toplam fenol içeriği, toplam antiokisidan ve antimikribiyal aktivitesi çalışılmıştır. Elde edilen *S. spinosum* özütü literatürdeki diğer tıbbi amaçlı kullanılan bitiklerle karşılaştırıldığında yüksek antioksidan aktivitesi göstermiştir. Bu değer gram kuru ağırlık başına 3143,5 mmol Trolox olarak ölçülmüştür. *S. spinosum* özütünün fenolik içeriği yüksek performanslı sıvı kromatografisiyle belirlenmiştir. *S. spinosum* özütünde Hyperoside ve isoquercetin varlığı tespit edilmiştir. Özellikle, özütün ana bileşiği isoquercetin olduğu gösterilmiştir. Ayrıca, *S. spinosum* özütünün antimikrobiyal aktivitesi araştırılmştır. Özüt, *C. albicans* gibi zorlu bir patojene karşı antifungal aktivite göstererek bertaraf etmiştir. *S. spinosum* özütü zein partikülü ile elektrosprey yöntemi kullanılarak enkapsüle edilmiştir ve bu yöntem özütü stabilizesini geliştirmesi amaçlanmıştır. Elektrosprey yönteminin partikül morfolojisi ve boyut dağılımı üzerindeki proses parametrelerinin etkileri detaylı bir şekilde incelenmiştir. Bu bağlamda, en iyi üretim koşuları %5 (hacimde ağırlıkça yüzde) zein konsantrasyonu, %70 su ethanol solüsyonu içinde hazırlanarak, 0,3 ml/sa akış hızı ve 14kV voltaj uygulanarak elde edilmiştir. Ayrıca, elektrospreyle elde edilen zein partikül morfolojisini etkilemeksizin en iyi *S. spinosum* yükleme oranı 1:5 (ağırlık oranı) olarak tespit edilmiştir. Hazırlanan özüt yüklü zein mikropartiküller anlamlı antioksidan aktivite göstermiştir.

TABLE OF CONTENTS

LIST OF FIGURES

Figure **Page**

LIST OF TABLES

Table **Page**

CHAPTER 1

INTRODUCTION

Medicinal plants have been used for the treatment of many diseases since ancient times. Nowadays, the importance of medicinal plants is gaining increasing attention to solve health care problems all the over world. Since medicinal plants are the source of natural compounds, they are widely used in medical, cosmetic, food and pharmaceutical industries and they are required to be examined by scientific approach.

Medicinal plants include phytochemicals that have both antioxidant and antimicrobial activities. Their antioxidant activities contribute to protect against oxidative damage of biologically important cellular components such as, proteins, membrane lipids and also DNA. In addition, phytochemicals act as antimicrobial agent because phytochemicals, secondary metabolites of medicinal plants, also is a kind of defense mechanism of the plant. Polyphenol structures are among the phytochemicals which are found in large amounts in most of the plants. However, the extraction of bioactive phenolic compounds from plant materials is the first and significant step for identification of bioactive phytochemicals in medicinal plants. In the literature, the effect of extraction parameters on extract content and its observed bioactivity has rarely taken into consideration.

Another important issue is the preservation of their bioactivity during their processing conditions and storage. The main problem of using plant derived natural compound is their degradation in gastrointestinal system before reaching the circulation system which limits the area of usage of these compounds. Therefore, it is necessary to apply encapsulation systems, in order to maximize the potential therapeutic benefits of natural compounds. Encapsulation provides good protection for sensitive compounds present in plant extracts against oxidation and dehydration reactions which reduce the bioactivity of natural compounds. Recently, many biopolymers are widely studied as an alternative encapsulating material since synthetic polymers have many undesired properties. Still, there is need for new biopolymers that are biocompatible and biodegradable to be used encapsulating material. Zein, a corn protein, is one of the most commonly used natural encapsulating materials in food and pharmaceutical industry

(Neo et al., 2013; Parris, Cooke, & Hicks, 2005). In the light of this information, plant extracts are preferred as bioactive components with their antioxidant and antimicrobial activities caused mainly from their high phenolic contents. The reported to have, *Sarcopoterium spinosum,* endemic specie for the Mediterranean Region, has high antioxidant and phenolic content (Al-Mustafa & Al-Thunibat, 2008). To the best of our knowledge, no study was reported on the composition of the extract of *S. spinosum* in the literature.

The main objective of this study is to investigate the changes of chemical composition with changing extraction parameters. Therefore, detailed extracts based on antioxidant and antimicrobial activities were performed. Since *Sarcopoterium spinosum* extract is proposed as a highly valuable, the aim of the thesis is to further investigate the potential use of *S. spinosum* extract using by electrospray encapsulation method with in zein.

CHAPTER 2

LITERATURE REVIEW

2.1. Medicinal Plants

Medicinal plants have traditionally been used in folk medicine for their natural healing and therapeutic effects. They can, for example, be used to regulate blood glucose level, to decrease depression or stress in molecular level of cell, or to reduce the risk of cancer (Nostro, Germano, D'angelo, Marino, & Cannatelli, 2001; Patel, Prasad, Kumar, & Hemalatha, 2012; Su et al., 2007; Zheng, Viswanathan, Kesarwani, & Mehrotra, 2012). In addition, pharmacological industry utilizes medicinal plants because of the presence of active chemical substances as agents for drug development (Cragg & Newman, 2005). Plants are also valuable for food and cosmetic industry as additives, due to their preservative effects because of the presence of antioxidants and antimicrobial constituents (Gómez-Estaca, Balaguer, Gavara, & Hernández-Muñoz, 2010; Kosaraju, Labbett, Emin, Konczak, & Lundin, 2008).

2.1.1. *Sarcopoterium spinosum* as a Medicinal Plant

The *Sarcopoterium spinosum* species is a common medicinal plant in the Mediterranean region. The ethnobotanical survey reported that *S. spinosum* is used in traditional medicine for the management of diabetes, pain relief, digestive problems or cancer (Rao, Sreenivasulu, Chengaiah, Reddy, & Chetty, 2010). It is also known as thorny burnet and also synonym of *Poterium spinosum.* The plant *Sarcopoterium spinosum* belongs to the Rosaceae plant family. It is an abundant and characteristic species of the semi-steppe shrublands in Mediterranean region (Rao et al., 2010). While in the Middle East the species dominates large areas, westwards it is less common and many populations have become extinct since the late 19th century (Gargano, Fenu, Medagli, Sciandrello, & Bernardo, 2007).

In the late 1960s and 1980s, several studies were performed to show the extract of *Sarcopoterium spinosum* exhibits a hypoglycemic effect in rats. Although the detail identification of phenolic composition of *S. spinosum* is not found, the wide scanning of medicinal plants is encountered with the name of it (Al-Mustafa & Al-Thunibat, 2008; Barbosa-Filho et al., 2008; Hamdan & Afifi, 2008; Kasabri, Afifi, & Hamdan, 2011; Sarıkaya & Kayalar, 2010). In particular, it is widely used as an anti-diabetic drug. A few studies confirmed this information and measured its anti-diabetic activity (Reher, Slijepcevic, & Kraus, 1991; Smirin et al., 2010). According to these studies, the *Sarcopoterium spinosum* extract exhibited an insulin-like effect on glucose uptake in hepatocytes by inducing about 150 % increase in glucose uptake, respectively, compared to 160 % increase in glucose uptake obtained by insulin (Patel et al., 2012; Rao et al., 2010). Another study reported the insulin like action of the extract of *Sarcopoterium spinosum* in targets tissues, as it increases insulin secretion *in vitro*, and has an improved glucose tolerance *in vivo (*Kasabri et al., 2011). In addition, the *S.spinosum* extract appeared to have high effect on the control of blood glucose level by inhibitory activity of α-amylase (Hamdan & Afifi, 2008). The recent studies revealed the potential of the extract of *Sarcopoterium spinosum* for the treatment of type II diabetes (Kasabri et al., 2011; Smirin et al., 2010). The *Sarcopoterium spinosum* extract also caused GSK-3 Phosphorylation in myotubes to increase (Rao et al., 2010). Phosphorylation of a protein by GSK-3 usually inhibits the activity of its downstream target. The GSK-3 protein play crucial role in a number of central intracellular signaling pathways, including cellular proliferation, migration, inflammation and immune responses, glucose regulation, and apoptosis.

The identification of chemical composition of the *Sarcopoterium spinosum* plant extract could help for the explanation of its bioactivity. As a result this extract can be good source of natural compounds with potential antioxidant activity for medical, cosmetic, food and pharmaceutical industries.

2.2. Antioxidant Activity and Role of Polyphenols

Antioxidants contribute to protect against oxidative damage of biologically important cellular components such as, proteins, membrane lipids and also DNA, from reactive oxygen species attacks. Free radicals and reactive oxygen species (ROS) are

released continuously during the essential aerobic metabolism as unwanted metabolic by-products. The role of antioxidants may directly react with and inactive free oxygen radical. Antioxidants show functions as terminators of free radicals chain, or chelators of redox active transition metal ions that are capable of catalyzing lipid peroxidation (Al-Mustafa & Al-Thunibat, 2008; Wellwood & Cole, 2004). There are many pathways of antioxidant to intercept free radical oxygen species in the biological systems, such as, act as reducing agents, induce the preparation of anti-oxidative enzymes, or suppress the production of oxidative enzymes, i.e. cyclooxygenase, telomerase, lipoxygenase (Naasani et al., 2003; Su et al., 2007). The activity of these enzymes are responsible for inhibiting free radical oxygen species under normal circumstance, but the enzymes can deform or the gene of the enzymes cannot make transcription during stress conditions. The antioxidant activity can prevent stress response.

Natural antioxidants are recently in high demand because of their potential in health improvement and disease prevention, and their developed safety and consumer acceptability (Bellik et al., 2012). The properties of antioxidant in medicinal plants depend on the plant which phytochemical contains secondary metabolites. In addition, concentration and composition of present phytochemical are related to antioxidant activity. Plants, the main sources of antioxidants, comprise a great diversity of compounds. These compounds, phytochemicals, vary in structure, the number of phenolic hydroxyl groups and their position, leading to variation in their anti-oxidative capacity (Buchanan, Gruissem, & Jones, 2000). Phytochemicals are classified as carotenoids, alkaloids, nitrogen-containing compounds, organ sulfur and phenolic compounds, based on their biosynthetic origins. The most studied of the phytochemicals are the phenolics and carotenoids. The basic classification of phytochemicals has been adopted by Liu (Liu, 2004) coming together most of phytochemical classes and the structures of their main chemically relevant components. These groups have also several subgroups and these are demonstrated in Figure 2.1.

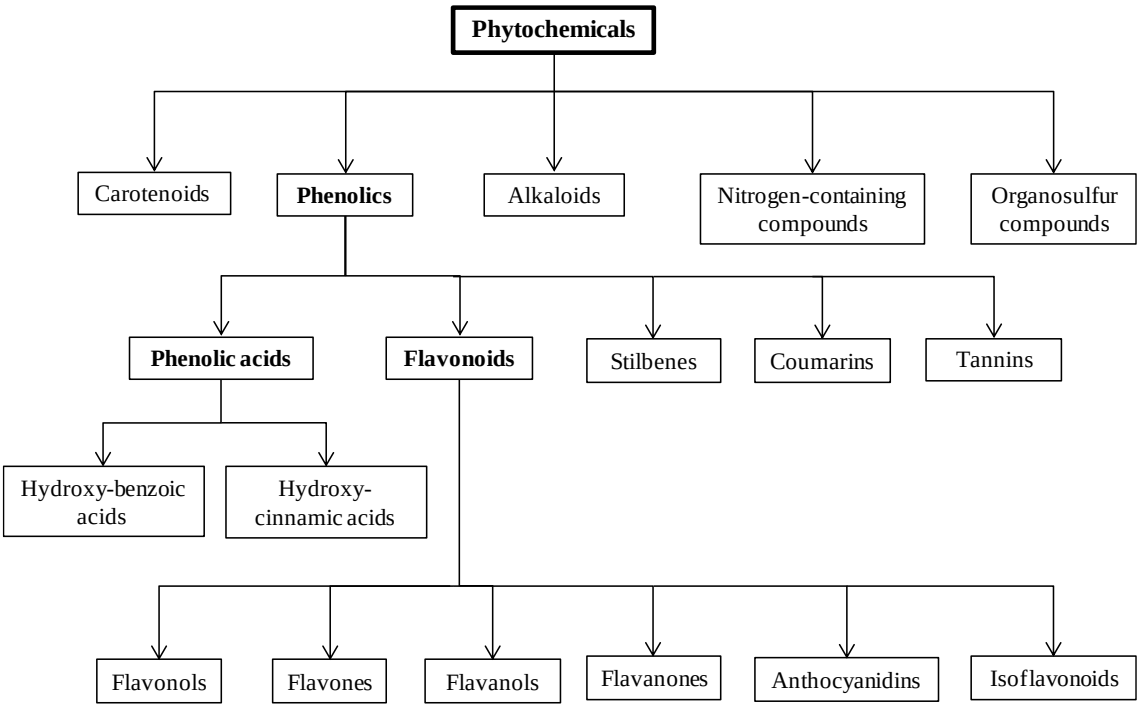


Figure 2.1. Classification of phytochemicals.
(Source; Liu, 2004)

Phenolic compounds (POH) are bioactive substances widely distributed in plants. Phenolic compounds prevent oxidative damage with a number of different mechanisms. Basically, the action of phenolic compounds as antioxidants respectively acts as free radical acceptors. Thus, they inhibit or delay the oxidation of lipids and other molecules by rapid donation of a hydrogen atom to radicals (R) and suppressing the formation of reactive oxygen species (ROS) (Dai & Mumper, 2010).

$$R^{\cdot} + POH \rightarrow RH + PO^{\cdot} \quad (2.1)$$

The phenoxy radical intermediates ($PO^{\cdot}$) are less stable due to forming of resonance structure. Thus, the phenoxy radical intermediates also continue to interfere with chain-propagation reactions by reacting with other free radicals.

$$PO^{\cdot} + R^{\cdot} \rightarrow POR \quad (2.2)$$

Phenolic compounds are dominant and ideal structure chemistry for free radical scavenging activities. The one of reasons of crucial role of phenolic compounds in

antioxidant activity are that they have many phenolic hydroxyl groups that are prone to donate a hydrogen atom or an electron to a free radical. The other of it is that phenolic compounds have lengthened conjugated aromatic system to delocalize an unpaired electron (Denev, Kratchanov, Ciz, Lojek, & Kratchanova, 2012). The mechanism of phenolic compounds as antioxidant activity is defined with major chemical expression in equation 2.1 and 2.2. The detail mechanisms of antioxidant, such as transition metal chelation free radical scavenging, and interactions with lipid membranes, proteins and nucleic acids (Al-Mustafa & Al-Thunibat, 2008; Wellwood & Cole, 2004), are version of the equations. Therefore, phenolic compounds have high antioxidant potential. According to classification depicted by Liu and Bravo (Bravo, 1998; Liu, 2004), plant phenolics consist of flavonoids, phenolic acids, tannins, which are illustrated in Figure 2.2 and Figure 2.3, and less common lignans and stilbenes in Figure 2.4. Flavonoids are the most plentiful polyphenols in human diets. The basic structure of flavonoid is flavan nucleus, including fifteen carbon atoms arranged in three rings. The rings showed in Figure 2.2 and as A, B and C (Dai & Mumper, 2010). Flavonoids are divided into subgroups in terms of the oxidation state of the central C ring (Bellik et al., 2012; Dai & Mumper, 2010).

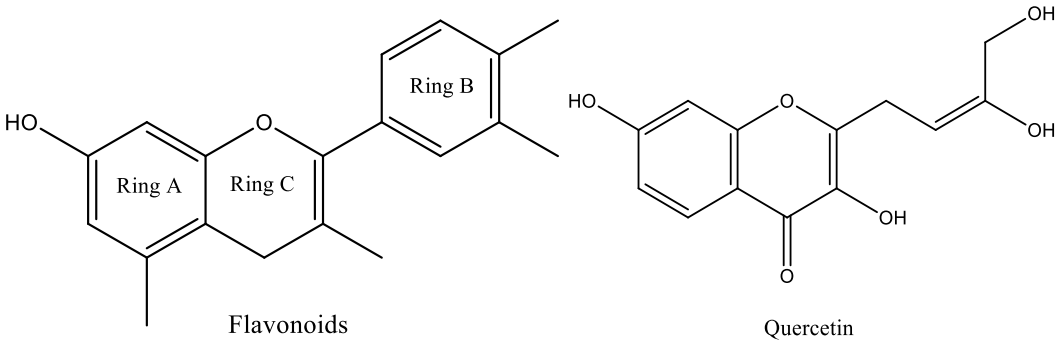
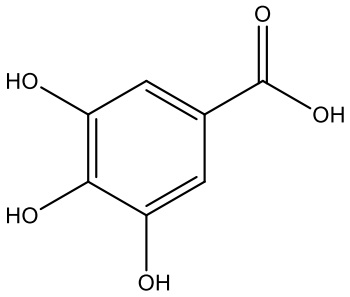
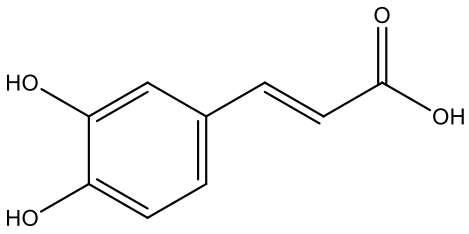


Figure 2.2. Structure of flavonoids

Flavonols have a double bond between second carbon and third carbon in C rings (Figure 2.2), with a hydroxyl group in third carbon of the C ring. It represents one of the most costly flavonoids with quercetin. Colorimetric methods and HPLC combined with UV detector or mass spectrometry have been used to determine the total content of phenolics.

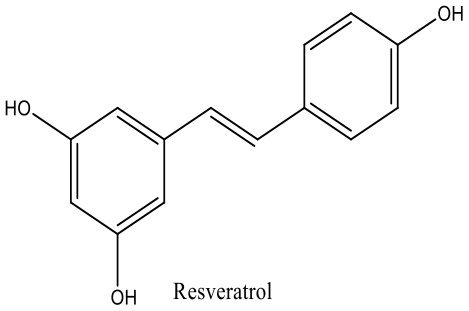


Gallic Acid



Caffeic Acid

Figure 2.3. Structure of phenolic acids



Resveratrol

Figure 2.4. Structure of stilbenes

Phenolic acids are divided into two subgroups which are derivatives of benzoic acids, i.e. gallic acid, and derivatives of cinnamic acids, i.e. coumaric acid and caffeic acid. In addition, the major phenolic compound found in coffee is chlorogenic acid, which is formed when caffeic acid is esterified (D'Archivio et al., 2007; Dai & Mumper, 2010). They are illustrated in Figure 2.3. Tannins are another major group of polyphenols and classified into two groups that are hydrolysable tannins and condensed tannins. Hydrolysable tannins are compounds including a central core of polyhydric alcohol such as glucose or hydroxyl groups. When these are esterified with hexahydroxydiphenic acid, it was called as ellagitannins, or esterified with gallic acid, it was called as gallotannins (D'Archivio et al., 2007). On the other hand, condensed tannins have more complex structure than hydrolysable tannins. Condensed tannins consist of oligomers or polymers of flavan-3-ol, such as catechins (D'Archivio et al., 2007).

2.2.1. Applications of Polyphenols

Polyphenols presents a wide range of pharmacological attribution. Phenolic compounds are known for their antioxidant activity that is useful for diabetes mellitus or preservation against cancer. Interestingly, several studies showed that some polyphenols, such as tannins or flavonoids, cause oxidative strand breakage in DNA in the presence or absence of metal ion such as cupper (Nobili et al., 2009; Wamtinga Richard Sawadogo & Mario Dicato, 2012; Ziech et al., 2012). The reason of this is that cancer cell lines are known to include high amount of cupper ion. When they are exposed to redox reactions with polyphenols, these cancer cell lines cause to generate reactive oxygen species and then phenoxyl radicals lead to breakdown of the structure of DNA, lipid or protein (Fukumoto & Mazza, 2000; Wamtinga Richard Sawadogo & Mario Dicato, 2012). It means that polyphenols, sometimes, can act as pro-oxidants by degrading DNA in the presence of transition metal ions. In addition, the ability of polyphenols to scavenge free radicals is used empirically for many other diseases, especially anti-diabetic. Polyphenols exhibit anti-diabetic activity by renewing the function of pancreatic activity or regulating insulin and metabolites in insulin dependent process, or preventing the intestinal absorption of glucose (Wamtinga Richard Sawadogo & Mario Dicato, 2012). Phenolic compounds, such as coumarins, □avonoids and terpenoids, show reduction in blood glucose levels. About 800 plant species having potential strategy to control diabetic activity have been available in literature (Alarcon-Aguilara et al., 1998; Patel et al., 2012); nevertheless, searching for new anti-diabetic drugs from natural plants is an attractive subject because the plants have potential to contain many phytochemicals which are implicated as having alternative and safe effects on diabetes mellitus, also called as anti-diabetic effect. Diabetes Mellitus is a complex metabolic disorder and it is characterized by high blood glucose level due to the inability of the body cells to utilize glucose level properly (Rao et al., 2010). Although chemical therapies and insulin treatment can restrain the diabetes, numerous complications are common case of the diabetic treatment. There are many recent researches that were focused on effects of plant secondary metabolites to use as a treatment of different type diabetic. It was investigated that polyphenols protect pancreatic β-cells from degeneration and diminish lipid peroxidation of cells (Singab, El-Beshbishy, Yonekawa, Nomura, & Fukai, 2005) and the effect of rutin and o-

coumaric acid against the obesity in rats fed a high-fat diet was clarified (Hsu, Wu, Huang, & Yen, 2009).

2.2.2. Antimicrobial Activity of Phytochemicals

Plants have limitless ability to synthesize phytochemicals, as secondary metabolites, which also show antimicrobial effects and serve as defense mechanisms of the plants against pathogenic microorganisms. Phytochemicals with antioxidant activity may show pro-oxidant behavior under pathogenic microorganisms' circumstances like acting against highly mitotic cells i.e. cancer. It is thought that the toxicity of bioactive polyphenols to microorganisms is associated with the sites and number of hydroxyl groups they have (Cowan, 1999; Das, Tiwari, & Shrivastava, 2010). In addition, some researchers have observed that more highly oxidized phenols are more inhibitory against pathogenic microorganisms (Das et al., 2010; Paiva et al., 2010). According to the researches, there are many mechanisms of antimicrobial action of phytochemicals, yet they are also not fully understood. It is thought that flavonoids act as inhibiting cytoplasmic membrane function while they are able to change cell morphology with damage formation of filamentous cells (Cushnie & Lamb, 2005). Moreover, they may inhibit DNA gyrase and β-hydroxyacyl-acyl carrier protein dehydrates activities, thus the synthesis of DNA and RNA is inhibited (Cushnie & Lamb, 2005; Paiva et al., 2010). Some compounds have been reported that, for example, tannins show antimicrobial activity as blocking microorganism membranes by the help of binding to polysaccharides or enzymes on the surface of cells, terpenes directly cause membrane disruption and coumarins can reduce in cell respiration of microorganisms (Cowan, 1999). It is important that not only a single compound is responsible for observed microbiological activity but also the combination of compounds may show bioactivity when they interact in synergistic manner. In addition to molecular antimicrobial activity of antioxidants, antimicrobial compounds can be classified as bacteriocidal, bacteriostatic and bacteriolytic in terms of observing their effects of bacterial culture (Madigan, Martinko, Dunlap, & Clark, 2008). Bacteriostatic compounds are inhibitors of protein synthesis and affect by binding to ribosomes. If the concentration of compound is lowered, the compounds are released from the ribosome and growth is resumed. Bacteriocidal compounds attach to their cellular targets and are not removed

by dilution and kill the cells. However, dead cells are not destroyed and, total cell numbers remain constant. Finally, bacteriolytic compounds include antibiotics that prevent the cell wall synthesis. Because the cell wall and its synthesis mechanisms are highly unique to the bacteria, the antibiotics have very selectivity. Antimicrobial activity is measured by deciding the smallest amount of agent needed to inhibit the growth of test organism, called as minimum inhibition concentration (MIC) (Madigan et al., 2008). The growth of the test organism in the broth is indicated by turbidity or cloudiness of the broth. The lowest concentration of the extract which inhibited the growth of the test organism was taken as MIC value. The MIC is not a constant for a given agent; it varies with the test organism, the inoculum size, the composition of the culture medium, the incubation time, and the conditions of incubation, i.e. aeration and temperature. When the culture conditions are standardized, different antimicrobial sample can be compared to determine which most effective against given organism.

2.3. Extraction of Polyphenols

The extraction of bioactive compounds from plant materials is the first step to the recovery of the phytochemicals which are commonly used as pharmaceutical, food ingredients and cosmetic products. The extraction involves the separation of bioactive portions of plant tissue from the inactive components by using selective solvents (Handa, Khanuja, Longo, & Rakesh, 2008) .

Solvent extraction is the most commonly used method to prepare extracts from plant materials for diverse applications. The conventional extraction techniques such as maceration and soxhlet extraction have shown low efficiency and potential environmental pollution due to large volumes of organic solvent used and long extraction time required in those methods (Handa et al., 2008). The extraction technique that solvent is used and its methodology are significant for the utilization of phenolic and their antioxidant efficiency. It is generally known that the yield of phenolics extraction depends on the type and polarity of solvents, extraction time and temperature, sample-to-solvent ratio as well as on their chemical composition and physical characteristics (Dai & Mumper, 2010). Moreover, the chemical composition of phenolics may also be associated with other plant components. For this reason, it may also contain some non-phenolic substances i.e. carbohydrates, protein, organic acids and

lipids. As a result, additional steps may be required to remove those unwanted components. Water, ethanol, methanol, acetone and solvent mixtures with different proportions of water are frequently used to extract phenolic compounds from plants. Before solvent application step, plant samples should be treated by milling, grinding and homogenization. Methanol has been generally found to be more efficient in extraction of lower molecular weight polyphenols while the higher molecular weight flavanols are better extracted with aqueous acetone. In particular, ethanol is widely used solvent for polyphenol extraction because of the safe for human health (Dai & Mumper, 2010; Handa et al., 2008). The extraction of phenolic compound from plant materials is also influenced by the time and temperature of exposing plant sample to solvent. The extraction time and temperature cause the conflicting actions of solubility and phenolic degradation by oxidation (Dai & Mumper, 2010). It means that an increase in the extraction temperature and time can promote higher yield of phenolic by increasing both solubility and mass transfer rate. However, many phenolic compounds are easily hydrolyzed and oxidized during long extraction time and high temperature. As a result, long extraction times and high temperature increase the chance of oxidation of phenolics which decrease the yield of phenolics in the extracts. The solid-liquid ratio is another important parameter in extraction of plant materials and generally, studies indicate that mostly ratios between 1:10 and 1:50 are used. Therefore, there is no universal extraction procedure suitable for extraction of all plant phenolics. Extraction parameters need to be varied to optimize the biological activity of interest.

2.4. Encapsulation of Polyphenols

In recent years, polyphenols have attracted great interest due to their potential health benefits and antioxidant properties. Antioxidant activities of polyphenols are effective only when active compounds preserve their bioactivity and stability. To be used as cosmetic, nutritional or pharmaceutical active ingredients, polyphenols increase in the application fields of biotechnological interest (Munin & Edwards-Lévy, 2011). However, polyphenols can be easily loss of effective bioactivity due to stability problems.

The fundamental use of natural polyphenols is also delicate because of their sensitivity to environmental factors, such as chemical, physical and biological

conditions. The unpleasant taste of phenolic compounds even limits their application. Processing conditions, for instance, also cause a loss of bioactivity due to low solubility, permeability, loss of stability or while storing, degradation in gastrointestinal system before reaching the circulation system which limits the area of usage of these compounds. It is crucial that the activity of plant bioactive chemicals depends on conserving their stability (Li, Lim, & Kakuda, 2009; Y. Luo et al., 2013). Therefore, the usage of polyphenols requires that the formulation of a protecting natural product can maintain structural integrity of the phenolic compounds until proper time. It is well known that encapsulation is a proper process to preserve active agents (Nedovic, Kalusevic, Manojlovic, Levic, & Bugarski, 2011). In addition, the usage of encapsulation technology on natural compounds has gained great interest. Instead of direct implementation, it is necessary to apply delivery or carrier systems, like encapsulation, in order to maximize the potential therapeutic benefits of antioxidants (Munin & Edwards-Lévy, 2011). Basically, encapsulation is described as a process to cover active agents within another substance for a specific period of time (Nedovic et al., 2011). Main reasons for encapsulation are to protect plant extracts from devastating environment effects such as undesirable effects of light, moisture and oxygen, to prohibit reactions such as oxidation and dehydration which reduce the shelf life of compounds and to improve processing step.

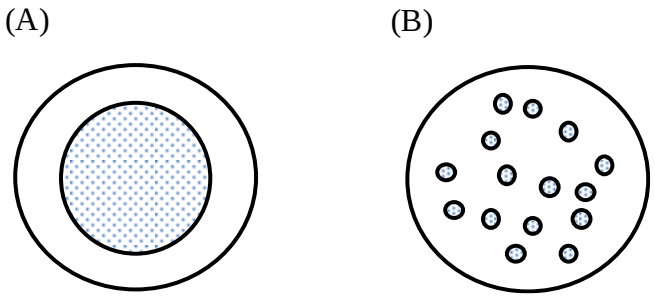


Figure 2.5. Major forms of encapsulation: mononuclear capsule (A) and aggregate (B).

In addition to protect environmental stress on the polyphenols, encapsulated systems can be useful tool to improve delivery of bioactive molecules. There are numerous works for the use of encapsulated polyphenols instead of free compounds. Beside the

point of protective mission of encapsulation, morphology of encapsulated polyphenols is crucial. Most common morphologies for encapsulation of polyphenols are divided in terms of internal structure which is mononuclear capsule and aggregates type as seen in Figure 2.5 (Fang & Bhandari, 2010). They are also called core-shell like and matrix (Munin & Edwards-Lévy, 2011). These coating materials may include polymers of natural or synthetic origin, or lipids instability. Based on this information, there are many techniques for encapsulation of polyphenols.

2.4.1. Encapsulation Techniques; Electrospray

Nowadays, various encapsulation techniques are available. The current encapsulation techniques for polyphenols consist of spray drying, emulsion, coacervation, liposomes, freeze drying and finally electrospray.

Spray Drying

Spray drying has been used in the industry since 1950s (Fang & Bhandari, 2010), so it is one of the oldest and the most widely used technology for encapsulation. Spray drying technique is performed by forming particles from dispersion of the active agent in the solution that is used as coating agent (Munin & Edwards-Lévy, 2011). Since spray drying is low cost, flexible and continuous operation, it is preferred in industrial technology. The basic principle of spray drying is based on the homogenization of the core material with the wall material and atomization of this dispersion with a nozzle or spinning wheel in the spray-drying chamber. The atomization enables to promote fast removal of water. Then, the particles are separated from the drying air and fallen to the bottom of the drier (Desai & Jin Park, 2005; Fang & Bhandari, 2010; Nedovic et al., 2011). The size of the particle is produced with range of 10-100 μm (Fang & Bhandari, 2010). Although spray-dryers are widespread in the food industry, there are several limitations and disadvantages of this technique, such as immobilization of the process, harsh and non-uniform conditions in the drying chamber. Besides, it is not always easy to control particle size. Another advantages is also that there are only a few coating material found.

Emulsions

Emulsion technology is generally utilized in the case of water soluble food active agents. Since the emulsification is by dispersing one liquid in a second immiscible liquid as small spherical droplets, an emulsion consists of at least two immiscible liquids which are usually oil and water. If oil droplets are dispersed in an aqueous phase, it is called as oil-in-water (O/W) emulsion, whereas if water droplets are dispersed in an oil phase, it is called as water-in-oil (W/O) emulsion(Fang & Bhandari, 2010). The size of the droplets range from 0.1 to 100 μm. Emulsions can be used when polyphenols have low solubility in water and oil. Thus, high concentration polyphenols have advantages for this technology. However, some research was reported that polyphenols showed different characteristic when different polyphenols were used in emulsion systems (Fang & Bhandari, 2010).

Coacervation

Coacervation is a modified emulsification technology. The methodology is simple and based on the phase separation of one or many hydrocolloids from the initial solution and deposition of the newly formed coacervate phase around the active ingredient emulsified in the same reaction media (Fang & Bhandari, 2010). The driving force for the phase separation is mainly due to the electrostatic interactions through that process. Coacervation is an expensive method for encapsulation. This technique is an immobilization rather than an encapsulation technique and most of the core material is essential oils rather than polyphenols. At the end of that process, shape of sample is not associated with a definite form.

Liposomes

The mechanism of forming liposomes is basically the hydrophilic–hydrophobic interactions between polar lipids and water molecules. Liposomes are defined as the colloidal particles comprised from the spherical bilayers which enclose bioactive molecules (Fang & Bhandari, 2010). The size of the particles varies from 30 nm to a several microns (Nedovic et al., 2011). Although the ability to control the release rate of encapsulated material through the bilayer and delivering to desired site at the right time are the major advantages of usage of liposomes, it is one of the less current methods due to its high cost.

Freeze Drying

Freeze drying, also called as lyophilization, is a simple technique for water soluble compounds. The principle of freeze drying is based on the dehydration of heat-sensitive sample. It means that freeze-drying performs by freezing the material and declining the surrounding pressure and then, adding enough heat (Fang & Bhandari, 2010). By this procedure, the frozen water in the material converts solid phase to gas phase. The results of freeze drying encapsulation procedure are usually the uncertain form. However, the long dehydration procedure is required for freeze drying encapsulation and the high energy input through that process of freeze drying (Nedovic et al., 2011). Some researchers think that the barrier of an open porous structure forms between the active ingredient and its surrounding and changes bioactivity of sample (Nedovic et al., 2011).

2.4.1.1. Electrospray (Electrohydrodynamic Atomization)

Electrohydrodynamic atomization is a process that uses an electric field to control the formation of micro/nano polymeric material (Jaworek, 2007). There are two main electrohydrodynamic atomization techniques: electrospray and electrospinning. They are cost effective application to produce fibers and particles through proper selection of the processing parameters. When it is chosen proper studied parameters, which will be mention in section 2.4.1.2., electrospray that organizes the center of the encapsulation studied is able to form the fine sphere particle. Basically, the principle of operation in electrospray depends on that liquids can readily interact with electric fields. The interaction between pumping liquids with syringe and electric field that is maintained by high voltage causes liquids to disperse into fine droplets, seen in Figure 2.6 (Y. Wu & Clark, 2008). Then, the forming charged droplets with high electric potential can be controlled by grounding collector (Jaworek & Sobczyk, 2008; Salata, 2005; Xie & Wang, 2007).

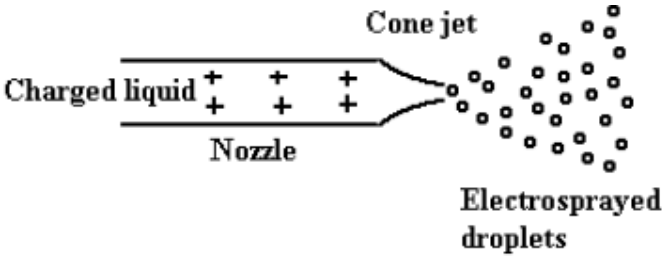


Figure 2.6. Basic schematic principle of electrospray.
(Source; Wu & Clark2008)

Lord Rayleigh was first to describe instabilities of a charged liquid droplet in 1882 (Salata, 2005). He wrote that an excessive charged droplet is unstable and the high charge potential forces the liquid droplet into smaller droplets. It is defined as Coulombic fission of the droplets cause that the original droplet disperses forming many smaller, more stable droplets (Jaworek & Sobczyk, 2008). By this way, he described relationship between the surface charge density and surface tension forces of the droplets. According to him (Jaworek, 2007; Salata, 2005), the limit on the surface charge density, also called as Rayleigh limit, where maximum point is, the electrohydrodynamic forces overcome the surface tension forces of the droplets. Thus, the fission of the droplets into smaller ones is occurred. The first scientific observation was reported by the physicist John Zeleny in 1914 (Salata, 2005). The theoretical explanation of conical droplet shape at the capillary exit by investigating the hydrostatic balance between electrical and surface tension forces was established by Taylor in 1964 (Y. Wu & Clark, 2008). In 1994, Cloupeau and Fernandez separately studied and clarified about the different electrospray patterns in terms of the liquid physical properties, the liquid flow rate and the setup geometry of system, the electric potentials and current. In addition, the multi-jet electrospray mode was observed by Shtern *et al.* in 1994, Jaworek *et al*. in 1996 (Y. Wu & Clark, 2008).

The technology behind the electrospray process is simple. The summary of the physics governing electrospray, which was developed by Jaworek and Sobczyk, is that the bulk forces include electrodynamic forces (proportional to the electric fields induced by the charged nozzle and emitted droplets), inertia, gravity and drag force (proportional to jet velocity and the viscosity of the gas surrounding the jet). When the induced droplet flows and deforms, called as Taylor cone-jet, surface stresses acting

against surface tension include electrodynamic stress (proportional to the charge density on the surface of the jet, and on the local electric field), pressure differential across the jet-air interface, and stresses due to liquid dynamic viscosity and inertia. It is illustrated as the following equation (Jaworek & Sobczyk, 2008);

$$d = a\frac{Q^{aQ}\ \varepsilon_0^{a\varepsilon}\ \rho_l^{a\rho}}{\sigma_l^{a\sigma}\ \gamma_l^{a\gamma}} \tag{2.3}$$

The constant α in equation 2.3 depends on the liquid permittivity and d: particle diameter, Q: volume flow rate, ε: permittivity of free space, ρ: liquid density, σ: liquid surface tension, γ: liquid bulk conductivity. The derivation of equation has been improving as long as the field of electrospray continues to expand significantly. The advantage of electrospray is that droplets can be formed very fine particles, in special cases down to nanometers. Moreover, adjusting applied voltage and the flow rate to the nozzle can be altered the charge and size of the droplets. Due to its properties, electrospray constructs monodisperse particles with high loading capacity and minimum active material lost. Electrospray technique is great flexibility in the choice of starting material because it can carry out a variety of organic, inorganic, or polymeric materials. In addition, the ease of operation and cost-effectiveness makes this process attractive. The processing parameters are the flow rate of the liquid, the working distance, the applied voltage the nozzle diameter and the concentration of coating material and solvent properties.

2.4.1.2. Electrospray Parameters

Concentration of Polymer and Solvent Properties

Concentration of polymer is crucial factor that control to form droplets. The viscosity of used polymer can directly change and originate particles. The variying polymer concentration and molecular weight also affects the surface tension of the solution and thus, influence the characteristics of encapsulated product (Chakraborty, Liao, Adler, & Leong, 2009). By the help of equation described by Jaworek and his friend, a decrease

in surface tension of the liquid is related with an increase in particle diameter. Therefore, it can be said that the diameter of the particles directly depends on the polymer concentration and molecular weight. According to the hydrophilicity and hyrophobicity of the used polymer, the solvent change in order to dissolve the polymer. The typed of used solvents also play a crucial role in the particle formation because it is responsible parameter for viscosity of the polymer solution. Based on the viscosity of it, the most significant properties of solvents are emphasized as miscibility, surface tension and volatility. According to the article (Chakraborty et al., 2009), if the surface tension of the used solvent is high, the result of electrospray of the polymer solution would be broad distribution in particle size. The other crucial solvent property is volatility. The solvent should evaporate during the flight of the particle between the needle and collector. If not, it may lead to the formation of large diameter particles (Chakraborty et al., 2009; Jaworek & Sobczyk, 2008). Miscibility of solvent is another critical property of solvent. Unless miscibility of solvent polymer solution enhance properly, particle defects can form. In our study, ethanol is used with water as a solvent because aqueous ethanol and solution are good solvent choice to dissolve the used bioactive extract along with zein as a carrier biopolymer. In addition, ethanol is an excellent intermediate because of its good miscibility in water, low surface tension (22 mN/m) and low boiling point at 78,3 ºC at 1 atm with respect to water.

Voltage

The applied voltage is critical parameters as a driving force for the electrospray process. If the applied electrical field is not sufficient, it would not overcome the surface tension of the polymer solution and particles would not be formed. In order to deal with the surface tension stress, the high voltage is applied. It is expected to see that increasing field strength is significantly reducing the size of particles (Chakraborty et al., 2009; Salata, 2005). On the other hand, if increase in field strength is so high, it may stop the size-reducing effect and introduce particle size variability since it cause to instability of jet mode. According to the article (Gomez-Estaca, Balaguer, Gavara, & Hernandez-Munoz, 2012), it should be arranged to find optimum level for applied voltage in terms of used polymer, solvent and experimental conditions.

Flow Rate

Flow rate is another parameter that affects the particle size as can be seen from the equation 2.3. According to this equation, decreased flow rate results in particles smaller diameter. In addition, decreased flow rate allows generating particles with spherical smooth morphology (Chakraborty et al., 2009). However, it causes that it takes longer time for the production of particle.

Other Parameters

The additional parameters that affect the size and morphology of the electrospray particles are working distance (that is distance between the needle and collector), temperature and humidity. An increase in working distance results in the smaller particles in size. However, if the distance is not within the optimum range, it will result in significant particle loss to the surroundings. If the case is vice versa, that is, the working distance is inadequate; the particles are prone to forming aggregates due to presence of organic solvent rather than evaporation of it during the deposition (Chakraborty et al., 2009; Jaworek & Sobczyk, 2008). Temperature and humidity also affect the product morphology that is produced by electrospray method. If the temperature is high, the molecular mobility and in relation the solution conductivity will increase and this results in decreased solution viscosity and surface tension (Chakraborty et al., 2009). Furthermore, it causes to increase the surface roughness of the product. The evaporation rate of polymer solvents may be increased by increasing relative humidity in the electrospray process and resulted in larger particle diameters.

2.4.2. Zein

Zein, the prolamin fraction of corn protein, has been examined as a possible raw material for its coating ability for encapsulation of bioactive compounds. It has been used in industry since the early part of the 20th Century (Lawton, 2002). Gorham, in 1821, first described zein after isolating the protein from maize (Lawton, 2002) and, in 1897, zein was first identified based on its solubility in aqueous alcohol solutions (Fu, Wang, Zhou, & Wang, 2009). Zein is 40 kDa in molecular weight and it exhibits amphiphilic properties due to containing nearly an equal amount of hydrophilic and lipophilic amino acid residues, illustrated in Figure 2.7. Since zein is a brick like shape,

it has a high potential for encapsulation or film application to carry other molecules inside them. Moreover, it is a natural polymer because of the generated form corn. In contrast to synthetic materials, it has the advantages for its absorbability and for the low toxicity of the degradation end products.

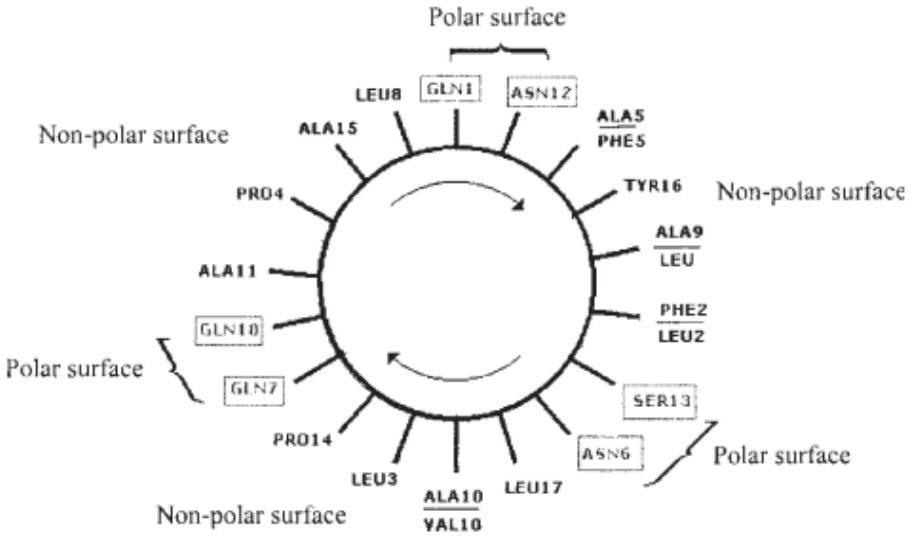


Figure 2.7. Molecular structure of zein.
(Source; Corradini et al., 2004)

Zein offers several potential advantages as a raw natural material for its coating ability, film and plastics application. Thus, it already has long been applied in pharmaceutical and food industries because its material has film forming ability, improving stability, potential biodegradability and biocompatibility (Yunpeng Wu, Luo, & Wang, 2012). For instance, Zein microspheres have been used to deliver insulin (Fu et al., 2009). Flu and his co-workers have studied about zein microspheres, loaded both ivermectin and heparin along with biodegradable ciprofloxacin–zein microsphere film (Fu et al., 2009). Encapsulated hydrophilic nutrient with high bioactivities and the release profile of hydrophilic nutrient can be greatly improved after the particles are coated by zein protein (Y. Luo, Zhang, Whent, Yu, & Wang, 2011). Thus, zein can also overcome the drawback of hydrophilic polymeric system in order to achieve sustained drug release (Y. Luo et al., 2013). It has long been recognized for the applications of natural bioactive compounds (Yangchao Luo, Zhang, Cheng, & Wang, 2010; Parris et

al., 2005; Quispe-Condori, Saldaña, & Temelli, 2011; Yunpeng Wu et al., 2012). It can provide extended shelf life by avoiding contact between the bioactive compound and prooxidant factors such as oxygen barrier property, its low water uptake values, high resistance to temperature or UV light (Gomez-Estaca et al., 2012; Neo et al., 2013). Zein particulate structures were generally recognized-as-safe (GRAS) biopolymers for incorporation into food matrices (Xiao, Davidson, & Zhong, 2011) and also approved by FDA to be commercialized in pharmaceutical industry (Yunpeng Wu et al., 2012).

CHAPTER 3

OBJECTIVES

The primarily aim of this study was to investigate *S. spinosum* extract that have important biological activities including antioxidant and antimicrobial activities. The goals in this study can be summarized as follows:

- Evaluation of extraction parameters for *S. spinosum* to maximize the total phenol content, antioxidant and antimicrobial activities of the prepared extract.

- Investigation of electrospraying parameters to encapsulate *S. spinosum* extract efficiently.

- Examination of the antioxidant activity of *S. spinosum* extract loaded zein microparticles.

CHAPTER 4

MATERIALS AND METHODS

4.1. Materials

4.1.1. Chemicals

Ethanol, methanol, phosphoric acid, ethylacetate, formic acid, acetic acid, tetrahydrafuran were obtained from Merk (Dermstadt, Germany). Acetonitrile, HPLC gradient, was purchased from Sigma Aldrich (Steinheim, Germany). Gallic acid was purchased from Merck (Dermstadt, Germany). Dimethyl sulfoxide (DMSO) was obtained from Riedel (Seelze, Germany). ABTS (2,2'-azinobis(3-ethylbenzothiazoline-6-sulphonic acid) from Sigma Aldrich (Steinheim, Germany), zein and all standards were obtained from Sigma Aldrich (Steinheim, Germany). Folin-ciocalteu reagent was obtained from Merck (Dermstadt, Germany), sodium carbonate anhydrus (99.5%) was obtained from Riedel (Seelze, Germany), Dulbecco's Modified Eagle Medium (DMEM), Fetal Bovine Serum (FBS), Phosphate-Buffered Saline (PBS), Penicillin–Streptomycin were purchased from Gibco (New York, USA). 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl) tetrazolium bromide, MTT, purchased from Sigma Aldrich (Steinheim, Germany). Bacteria growth media (broth and agar) were used to ensure the cultivation of the microorganisms. Nutrient Broth was purchased from Sigma Aldrcih (Steinheim, Germany). Potato broth was purchased from BD biosciences. Agar and peptone were used to dilute the microbial cultures and obtained from Oxoid. A thin layer chromatography (TLC) Aluminum sheet (20x20 cm) with silica gel 60 F_{254} and INT (Iodonitrotetrazolium chloride) were purchased from Merck (Dermstadt, Germany). 2-Aminoethyl diphenylborinate (NP) and Polyethylenglycol (PEG) were used as a dye and obtained from Fluka (Steinheim, Germany) and Merck (Dermstadt, Germany), respectively.

4.1.2. Equipment and Experimental Set Up

Extraction was performed with a thermo-shaker. Extraction solvent was evaporated in the rotary evaporator (Aldrich, Heidolph Laborota 4001). In order to remove water phase of extraction, freeze dryer (Telstar Cryodos A50) was used to dry aqueous extract. In order to carry out micro dilution test for MIC, phenol and antioxidant measurement from sample, Varioskan (Thermo) and Multiskan Spectrum (Thermo) were used. The HPLC equipment used was Agilent 1100 equipped with UV-DAD for the identification of chemical composition. The stationary phase was a LiChrospher® 100RP- C_{18} column (4× 250 mm) 35°C. For the electrospray process, syringe pump (Programmable Single Syringe Pump Model NE-1000, U.S.A) was used and for the voltage adjustment, GAMMA High Voltage Supplier was used. In order to analyze the surface morphology of the prepared particles, Philips XL30 SFEG (FEI Company, Oregon, USA) and Quanta FL ESEM scanning electron microscopies were used. In order to determine cytotoxicity of extract and extract loaded sphere, while cell culture studies were performed using sterile laminar hood (Jouan MSC12, Thermo) and for cell incubation in Stericycle Incubator, Thermo. Cells were observed under light microscope (Olympus CKX31). The samples were centrifuged with Beckman Coulter Allegra 25R Centrifuge. The cytotoxicity tests were carried out with Elisa Plate Reader (Medispec ESR200).

4.2. Methods

4.2.1. Preparation of Plant Extracts

Sarcopoterium spinosum sample used in this study was freshly collected from the region located in Gülbahçe, İzmir, Turkey. They were collected during spring season of 2012 before flowering period. Collected plant material was dried at room temperature and kept under clean dark place. All leaves were removed from thorns and stems. The dried *S. spinosum* leaves were ground in a blender to obtain the powdered form of material. 10 g of plant powder was used for each extraction test set, which was determined with design of experiment software (Minitab Inc., State College, PA, USA).

Extraction time, solvent concentration and liquid-solid ratio were the parameters of interest for extraction experiments. At the same time, all test sets were fixed at a shaking speed of 180 rpm in a thermo-shaker. Then, the samples were filtered through Whatman filter paper and centrifuged at 5000 rpm for 10 min. The supernatant parts of the samples were evaporated with a rotary evaporator for removal of ethanol under reduced pressure at 35 ºC. The remaining aqueous extract solutions were lyophilized to obtain solid extract form. The percent (w/w) extraction mass yields of plant materials were calculated.

4.2.2. Determination of Total Phenol Contents (TPC)

Total phenol contents of extracts obtained from *Sarcopoterium spinosum* leaves were determined by using Folin-ciocalteu method (Ainsworth & Gillespie, 2007). *S. spinosum* extracts were dissolved in deionized water (dH_2O) in 1 mg extract in 10 ml dH_2O. Prepared plant extracts or standard (gallic acid) solutions were mixed with Folin-ciocalteu reagent. While taking 20 µl from each sample, 100 µl Folin- ciocalteu reagent (1:10 dilution with deionized water) was added to each sample. After 2.5 min, sodium carbonate solution (7% in deionized water) was added to this mixture. Then, it was left to incubate for 1 hour at room temperature in a dark place. After that, the absorbance was measured at 725 nm with a UV spectrophotometer. Results were expressed as miligrams of gallic acid equivalents (GAE) per gram dried weight. Calibration curve of gallic acid can be seen in Appendix A. All samples were analyzed at least three times.

4.2.3. Determination of Total Antioxidant Capacity (TAOC)

The Trolox equivalent antioxidant capacity (TEAC) assessment was performed in terms of radical scavenging ability according to the ABTS/$K_2S_8O_2$ method. The $ABTS^+$ radical was generated by a reaction between 14 mM ABTS and activated with 4.9 mM $K_2S_8O_2$. The $ABTS^+$ solution was diluted with absolute ethanol to an absorbance of 0.70 (±0.03) at 734 nm and placed in multi-plate reader (Varioskan, Thermo). 10µl of each sample and their replicates were prepared in 96 well-plates. Then, 200µl $ABTS^+$ solution was added into each sample well and absorbance decrease was recorded during

1h. First absorbance value was used as initial time reading and the absorbance value recorded at the end of analysis was called as final reading. After calibration curve for Trolox was obtained by determining the slope of the plot of the percentage inhibition of absorbance of Trolox versus concentration as given in Appendix B. In this assay, the antioxidant activity was expressed as milimole TEAC per gram dried extract.

$$\% \text{ inhibition} = 1 - (\text{Absorbance}_{final} / \text{Absorbance}_{initial}) \times 100$$

4.2.4. High Performance Liquid Chromatography (HPLC) Analysis

Dired *S. spinosum* extract was dissolved in 50% ethanol in water (v/v) and extract solution was made vortex until completely dissolved. Before given HPLC analysis, prepared *S. spinosum* extract solutions were filtered wtih 0.22μm syringe filter. In addition to this, all standards were prepared at a concentration of 1 mg/ml. The HPLC equipment used was an Agilent 1100 equipped with UV-DAD. The stationary phase was a LiChrospher® 100RP- C_{18} column (4× 250 mm) thermostated at 30 °C. The flow rate was 1 ml/min and the absorbance changes were monitored at 280, 254 and 360 nm. In addition, the mobile phases for chromatographic analysis were (A) phosphoric acid/water (1: 99, v/v) and (B) tetrahydrofuran: acetornitrile (5:95, v/v). A linear gradient was run from 87% (A) and 13% (B) to 82% (A) and 18% (B) during 10 min; it changed to 81%(A) and 19% (B) in further 25min (35 min, total time); in last 10 min it changed to 78% (A) and 22% (B) (35 min, total time). The aid of the HPLC research done by Ying and friends, this method was arranged and improved (Ying X. et al., 2009).

4.2.5. Thin Layer Chromatography (TLC) Analysis

Solvent mixture consists of ethyl acetate: formic acid: acetic acid: water (100: 11: 11: 26). Sample was applied to TLC plate as a line. The plate loaded with sample was put in TLC chamber including desired solvent system. At the end of solvent running, the plate was put in oven at 100 ºC for 3 minutes to prepare coloring step. It was sprayed while still hot with 1% NP which was prepared in methanol. Then, 5%

PEG dissolved in absolute ethanol was sprayed on plate, instantly. The plate was observed under UV light at 366 nm.

4.2.6. Antimicrobial Activity Tests

4.2.6.1. Preparation of Cultures for Antimicrobial Activity Tests

The strains were purchased as lyophilized powders from suppliers, America Type Culture Collections (ATCC). Inoculation medium was potato broth for *Candida albicans* and nutrient broth for *Escherichia coli* and *Staphylococcus epidermidis,* which were incubated overnight at 37^{o}C. In order to prolong usage time, stock cultures and their reserves were prepared in 40% glycerol broths by inoculating the fresh culture (1:1). Stock cultures were kept at -80^{o}C for further studies after they were labeled.

4.2.6.2. Disc Diffusion Assays

Agar disk diffusion method for antimicrobial tests was developed in 1940, commonly known as Kirby-Bauer test (Das et al., 2010; Madigan et al., 2008). Disc diffusion Susceptibility Testing was performed to observe the initial antibacterial susceptibility of *S. spinosum* extracts obtained using different extraction parameters which along with their results in extracts with the highest antioxidant capacity (# 9), phenolic content (# 6), the lowest ones (# 3), respectively. Each of the plant extracts, 125 mg, was added to 2.5 ml of appropriate broth in order to obtain sample extract at a concentration of 50 mg/ml. In the disc diffusion assays, three microorganisms, *E. coli, S. epidermidis* and *C. albicans,* were chosen to determine the antimicrobial activities of plant extracts. *E. coli* is a gram negative bacteria, *S. epidermidis* is a gram positive bacteria and *C. albicans* is a fungus. Fresh cultures were prepared daily in 10 ml broth by transferring one loop of stock bacteria which were kept at –80 ºC. These cultures were incubated overnight. The inoculated culture was transferred to the surface of appropriate agar plates by a sterile loop and incubated overnight. A sterile cotton swab which was touched to four-five isolated colonies of the test bacterial strains grown on the agar plate was used to inoculate a tube of peptone water (1: 1000, w/v). The

inoculated tube was standardized to match with 0.5 McFarland turbidity standards. A sterile cotton swab was dipped into the standardized bacterial test suspension and used to evenly inoculate the entire surface of agar plates. Inoculated culture was dispersed by streaking the sterile swab over the entire sterile agar surface by rotating the plate 60^{o} each time to ensure the inoculum uniformly spread. Blank discs were placed on the surface of inoculated plates with sterile forceps. The 15µl of prepared sample (50 mg/ml) was added into the blank discs. All plates were incubated at 37 °C for 24 hours. The diameters of the zones of inhibition appearing around the discs were measured in millimeter (mm) and recorded. To obtain more visible images, the plates were colored with p-Iodonitrotetrazolium violet, INT, except gram positive type after incubation. INT reacts with the metabolites produced by the microorganisms and the region of plate with the microorganism turn to pink color.

4.2.6.3. Minimum Inhibition Concentration Analysis

The Minimum Inhibition Concentration (MIC) measured for antimicrobial agent is the lowest concentration that inhibits growth. The growth of the test organism in the broth is indicated by turbidity or cloudiness of the broth. The lowest concentration of the extract which inhibited the growth of the test organism was taken as MIC value. Each of the plant extract, 125 mg, was added to 2, 5 ml of appropriate broth in order to obtain sample extract (50 mg/ml). These sample extract solutions were prepared with serial dilution; to a final concentration 20, 10, 5, 2 mg/ml to be used for minimum concentration analysis. Each well was filled with 100 µl sample and 95 µl appropriate broth. Finally, 5 µl of bacterial specie in the inoculated tube which was standardized to match with 0.5 McFarland turbidity standards was added into each well of 96-well plate. The assay plates were incubated at 37 ^{0}C for 24 h and shake with 60 rpm. The growth kinetic assays for each strain were performed in triplicate. Growth curves were observed with turbidity measurement with a micro plate reader (Varioskan) at 600 nm. These results for each extract sample were reported as MIC values (mg/ml). These spectrophotometric measurements for MIC values were carried out with a standardized protocol of Varioskan multi plate reader.

4.2.7. Cell Viability Tests

NIH 3T3 mouse fibroblast cell line was grown in DMEM medium supplemented with 2 mM L-glutamine, 10% fetal bovine serum (FBS), 1% Penicillin–Streptomycin as antibiotic in flasks. The cells were maintained fibroblast morphology between 80-90% of cells for 7 days after initiation of differentiation in a dark cell incubator having 5% carbondioxide (CO_2) and 95% humidity at 37 ºC.

Cell viability tests were carried out for the extracts obtained at different extraction parameters and encapsulated *S. spinosum* extract using 3- (4,5-dimethylthiazol-2-yl) -2,5-diphenyl tetrazolium bromide (MTT) Cell Viability assay. After seeded cells were exposed to test samples for a appropriate time, the living cells reduce a tetrazolium dye, MTT, into its insoluble formazan giving a purple color (Yanez et al., 2004). Briefly, fibroblast cells were seeded a day prior to sample exposure at 10 000 cells per well in 96 well-plate filled with culture medium containing DMEM with 10% FBS. Stock samples of *S. spinosum* extract were prepared in DMEM containing 10% FBS. *S. spinosum* extract-loaded zein particle samples were subjected to pretreatment. Indirect cytotoxicity method was used to measure the toxicity of extract loaded zein particles. Samples were treated with culture medium for 24 hours (h). After that, extraction of encapsulated extract in zein particle was performed in culture medium. This extract was assayed using MTT procedure. Briefly, at the end of the exposure time, the cultures in 96-well plates were washed with PBS and replaced with 100 µl MTT (0.5 mg/ml) in the incubator for 4 h at 37 °C. After centrifugation (1800 rpm, 10 min) to carefully remove non-metabolized MTT, 100 µl of DMSO were added to each well to solubilize the MTT formazan produced by the cultured cells. After shaking for 5 min at room temperature, absorbance values in each plate were measured by using ELISA reader at 575 nm.

$$\text{Cell viability (\%)} = (\text{Absorbance value}_{\text{sample}} / \text{Absorbance value}_{\text{control}}) \times 100$$

4.2.8. Preparation of Zein Particles via Electrospray

Zein was dissolved in 70% ethanol in water (v/v) and stirred for 60 min at room temperature until completely dissolved. Several concentrations of zein in aqueous ethanol were prepared, ranging from 1 % to 20% (w/v). Zein solutions were subjected to electrospray process using injection syringe with a blunt end steel needle which was attached to the positive electrode of a direct current (DC) power supply in Figure 4.1 (Torres-Giner, Martinez-Abad, Ocio, & Lagaron, 2010).

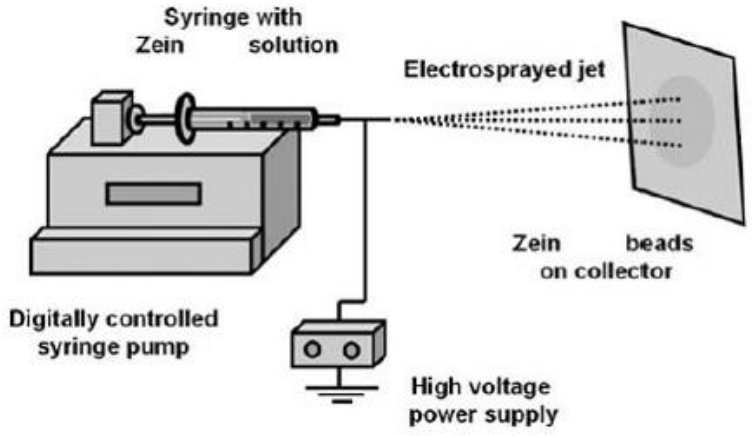


Figure 4.1. The basic experimental set up of the electrospray.
(Source; Torres et al. 2010)

The solution, prepared different concentration, was fed to the spinneret by means of an injection pump (Model NE-1000, Programmable Single Syringe Pump, U.S.A.). The collector plate covered with aluminum foil was fixed at a working distance of 10 cm away from the needle tip and connected to the grounded counter electrode of the power supply. 5 mL plastic syringe was filled with the solution and a syringe pump was used to control the flow rate at which the solution was dispensed. While electrospray, the droplets were dried during the flight time for zein particles on to the surface of the collector plate. For different set of experiments, voltage, zein concentration and flow rate were changed. The selected voltages were 10, 12, 14 and 18 kV; the flow rate were 0.15, 0.3, 0.6, 1 and 1,5 ml/h; solvent concentrations were 80, 70 and 60% (v/v); the zein concentrations were 1, 2.5, 5, 10 and 20% (w/v). The effect of

the main electrospray variables on the shape and size of the zein particles were investigated to determine the optimal conditions for obtaining homogeneously distributed particles. After that, extract solutions with several composition (extract: zein) in %70 aqueous ethanol were prepared, ranging from 2:1 , 1:1 , 1:5 , 1:10 , 1:20, 1:50 and 1:100 (w/w). The morphology of zein particles alone and loaded with extract of *S. spinosum* was investigated using scanning electron microscopy (SEM). Effects of parameters in electrospray experiments on the shapes and sizes of zein particles were studied.

4.2.9. Analyses for *S. spinosum* extract-loaded Zein Microparticles

The morphology of sphere was examined using a scanning electron microscopy, Philips XL30 SFEG (FEI Company, Oregon, USA) and Quanta FL ESEM unit. The samples were collected on an aluminum sample holder, which was placed on the surface of the collector plate. The samples were treated with gold-epalladium immediately prior to analysis. All SEM evaluations were conducted at an accelerating voltage of 5-7 kV.
After the particle diameter was measured with image j software, particle size distribution was evaluated with the aid of minitab 15 software. In order to measure the amounts of encapsulated *S. spinosum* extract, encapsulation efficiency was studied. After the production of *S. spinosum* extract loaded zein microparticles, it was dissolved in 70% ethanol again. The absorbance at 350 nm for before and after electrospray of the mixture was read. The wavelength at 350 nm was used because there was no absorption for zein at 350 nm. Loading efficiency was measure by the aid of antioxidant activity of broken micro sphere with beat beaker. *S. spinosum* extract encapsulation efficiency was determined by the equation;

$$\text{Encapsulation efficiency (\%)} = \frac{\text{Absorbance of the extract in microsphere}}{\text{Absorbance of the extract initially added}} \times 100$$

CHAPTER 5

RESULTS AND DISCUSSION

5.1. Extraction of Sarcopoterium spinosum

The principle of extraction has been reviewed in many comprehensive studies (Mustafa & Turner, 2011). The studies show that the efficiency of extraction in medicinal plants depends on the nature of samples. It is one of the best extraction methods to collect bioanalyte that water-alcohol extraction at room temperature of plant material particles while floating in the liquid (Handa et al., 2008). Ethanol-water mixture was used in this study because it is harmless solvent and phenolic content of *S. spinosum* can be soluble in aqueous ethanol. However, there are some extraction conditions which are especially effective solution for the preparation of extracts from medicinal plants. For example, the particle size of the plant material floating in the liquid must be small due to mass transfer resistances. It is expected to see that the effectiveness of extraction will be higher because of the larger surface area. So, it can be a common sense for efficient extraction of plant material. On the other hand, there are some other extraction parameters such as solvent types, its concentration, extraction time etc. which should be clarified in order to determine the best extraction condition of the plant of interest. In this study, the best parameters for the extraction of *S. spinosum* were investigated to observe their effects on extract yield, total phenol content (TPC), total antioxidant capacity (TAOC). The experimental results were analyzed using response surface methodology.

5.1.1. Determination of Extraction with Design of Experiment

The coded and uncoded independent variables used in the design are listed in Table 5.1. Ranges of EtOH (X_1, %, v/v, water : ethanol), Extraction time (X_2, hour) and liquid solid ratio (X_3, ml/mg, v/w, aqueous-ethanol : plant material) were selected as

preliminary experimental results. In addition, independent variables and their lower, middle and upper design points for response surface analysis are shown in Table 5.1.

Table 5.1. Independent variables and the coded and uncoded levels used for the optimization of extraction of *Sarcopoterium spinosum* leaves extracts.

Independent variable	Units	Coded levels		
		-1	0	+1
		Uncoded levels		
Ethanol concentration, x_1	% (v/v)	10	50	90
Extraction time, x_2	hour	2	8	14
Liquid-solid ratio, x_3	ml/mg	10	20	30

The experimental data were fitted with second order surface response method with following equation (Montgomery, 2008):

$$y=\beta_0 + \sum_{i=1}^{3} \beta_i \, . \, x_i + \sum_{j=1}^{3} \beta_{ii} . \, x_i^2 + \sum \sum_{i<j}^{3} \beta_{ij} . \, x_i . \, x_j \qquad (5.1)$$

Where y represents the measured response variables, β_0 is a constant, β_i , β_{ii} and β_{ij} are the linear, quadratic and interactive coefficients of the model. x_i , x_j and x_{ij} are the levels of the independent variables. The surface response graphs were generated by varying two variables with the experimental range and holding the other constant at the central point. The test of statistical significance was based on the total error criteria with a confidence interval level of 95.0%.

5.1.2. Evaluation of Analysis of *S. spinosum* Extracts Parameters

The initial part includes the determination of design variables employed in response surface analysis. The ability of different conditions in extracting antioxidant phenolic compounds from *S. spinosum* was compared using both Folic-Ciocalteu and ABTS assays. Table 5.2 shows actual experimental both results and predicted results calculated with the equations obtained from response surface analysis.

Table 5.2. Experimental design of three-level, three variable central composite design, and the predicted and experimental results of total phenolic content and total antioxidant capacity.

Test set	x_1, Ethanol Concen-tration	x_2, Extraction Time	x_3, Liquid-solid ratio	TEAC (mmole /g DW)		TPC (mg GAE /g DW)	
				Experimental*	Y_1,Predicted**	Experimental*	Y_2,Predicted***
1	10	2	10	1995.7	1927.1	255.5	238.3
2	90	2	10	3033.0	3592.7	334.2	329.4
3	10	14	10	1790.0	1515.3	205.4	238.3
4	90	14	10	2473.9	3180.9	332.5	329.4
5	10	2	30	2125.4	397.7	301.4	346.6
6	90	2	30	2174.5	1503.3	386.3	437.7
7	10	14	30	2420.6	909.9	266.6	346.6
8	90	14	30	2960.4	2015.5	312.6	437.7
9	10	8	20	3143.5	915.7	280.1	292.4
10	90	8	20	2096.5	2301.3	385.1	383.6
11	50	2	20	2239.5	1855.2	337.5	338.0
12	50	14	20	2379.1	1905.4	276.6	338.0
13	50	8	10	2182.4	2282.2	286.8	283.8
14	50	8	30	2253.9	934.8	360.0	392.2
15	50	8	20	2148.3	1608.5	259.6	338.0
16	50	8	20	1972.0	1608.5	286.1	338.0
17	50	8	20	1981.7	1608.5	305.0	338.0
18	50	8	20	2599.3	1608.5	297.2	338.0
19	50	8	20	2127.5	1608.5	262.1	338.0
20	50	8	20	2361.4	1608.5	346.4	338.0

*Experimental values were average of triplicate **$r^2 = 0.88$ ***$r^2 = 0.87$

Response surface analysis of the data in Table 5.2 demonstrates the relationship between total phenolic content and extraction parameters, and also between total antioxidant activity and extraction parameters is with regression coefficient (r^2= 0.88 and 0.87). Equation 5.2 shows the relationship between total antioxidant activity of extract and extraction parameters. Equation 5.3 also shows the relationship between total phenolic content of extract and extraction parameters.

$$Y_1 = 2805.7 + 24.3x_1 - 193.6x_2 - 80.7x_3 + 7.55\,x_2^2 - 0.35\,x_1x_3 + 3.85\,x_2x_3 \quad (5.2)$$

$$Y_2 = 172.7 + 1.139x_1 + 5.417x_3 \quad (5.3)$$

Table 5.3. Experimental design of three-level, three variable central composite designs, and the predicted and experimental results of mass yield percentage.

Test set	x_1, Ethanol Concen-tration	x_2, Extraction Time	x_3, Liquid-solid ratio	Mass yield (%)	
				Experimental*	Y_3, Predicted**
1	10	2	10	8.6	10.3
2	90	2	10	8.2	14.9
3	10	14	10	17.5	19.0
4	90	14	10	17.5	10.3
5	10	2	30	7.2	10.3
6	90	2	30	11.9	19.0
7	10	14	30	19.7	19.0
8	90	14	30	18.4	19.0
9	10	8	20	21.0	19.0
10	90	8	20	20.4	14.9
11	50	2	20	15.0	14.9
12	50	14	20	14.6	19.0
13	50	8	10	18.1	10.3
14	50	8	30	11.0	19.0
15	50	8	20	19.3	19.0
16	50	8	20	11.2	14.9
17	50	8	20	8.8	19.0
18	50	8	20	12.0	19.0
19	50	8	20	19.0	14.9
20	50	8	20	13.8	11.8

*Experimental values were average of triplicate **r^2=0.91

Response surface analysis of the data in Table 5.3 demonstrates that the relationship between mass yield and extraction parameters, and also between total antioxidant activity and extraction parameters is with good regression coefficient (r^2=0.91). Equation 5.4 shows the relationship between mass yield and extraction parameters.

$$Y_3 = 11.827 + 0.343x_1 - 0.0037x_1^2 \quad (5.4)$$

Correlation between phenolic content and antioxidant activity has been established; Table 5.2 shows that increase of extracted phenolic contents causes increase of antioxidant capacity for *S. spinosum* extract. However, increase of antioxidant capacity may also depend on types of phenolic compounds extracted. Therefore, the analyzed

using response surface methodology to observe the optimum extraction parameters for an extract with desired properties.

5.1.3. Response Surface Analysis

Response surface analysis was applied for determining optimal extraction parameters such as solvent concentration, extraction time and liquid-solid ratio which affect on total antioxidant capacity, total phenolic content and mass yield of the *S. spinosum* extract.

5.1.3.1. Response Surface Analysis for Total Antioxidant Capacity

Total antioxidant activity was determined using the $ABTS^{+}$ method. Trolox equivalent antioxidant capacity (TEAC) was described in terms of radical scavenging ability. As shown in Figure 5.1, the extraction time and liquid-solid ratio had negative quadratic effect on the TAOC of the extract.

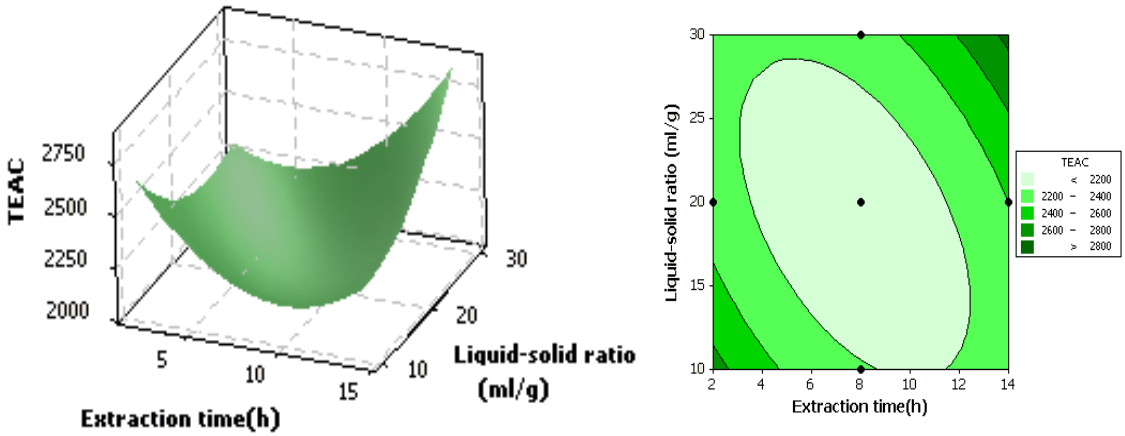


Figure 5.1. Response surface and contour plots of total antioxidant capability (Y_1) for the effects of extraction time and liquid-solid ratio at ethanol concentration of 50%.

The interaction of ethanol concentration with the other variables had similar effect. In addition, the ethanol concentration has positive effects on total antioxidant capacity shown in Figure 5.2.

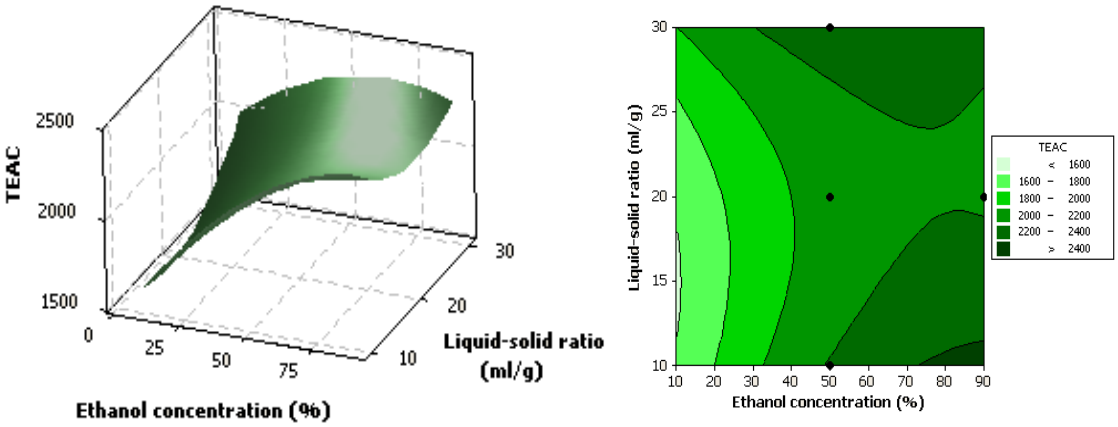


Figure 5.2. Response surface and contour plots of total antioxidant capability (Y_1) for the effects of ethanol concentration and liquid-solid ratio at extraction time of 8h.

5.1.3.2. Response Surface Analysis for Total Phenolic Content

The total phenolic content of *S. spinosum* extract was determined with a spectrophotometric method using Folin-ciocalteu's reagent (Lester, Lewers, Medina, & Saftner, 2012). Figure 5.3 is a response surface plot showing the effect of liquid-solid ratio and extraction time on the total phenol content at the fixed ethanol concentration of 50%. The extraction time has shown a negative effect on the total phenol content.

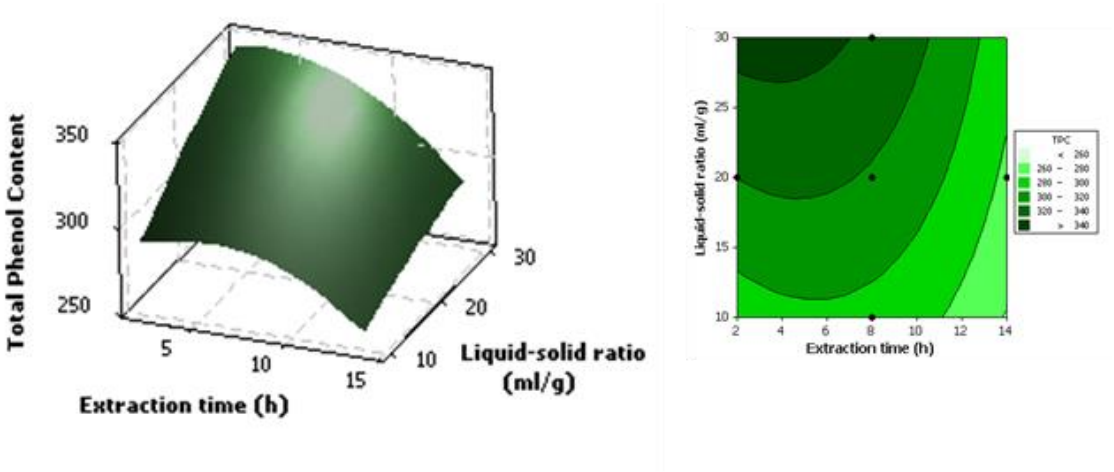


Figure 5.3. Response surface and contour plots of total phenol content (Y_2) for the effects of extraction time and liquid-solid ratio at ethanol concentration of 50%.

In Figure 5.4 and Figure 5.5, the total phenol content drastically increased when ethanol concentration increased. According to the results, the amount of phenolic compound improved when the ethanol increased in solvent composition.

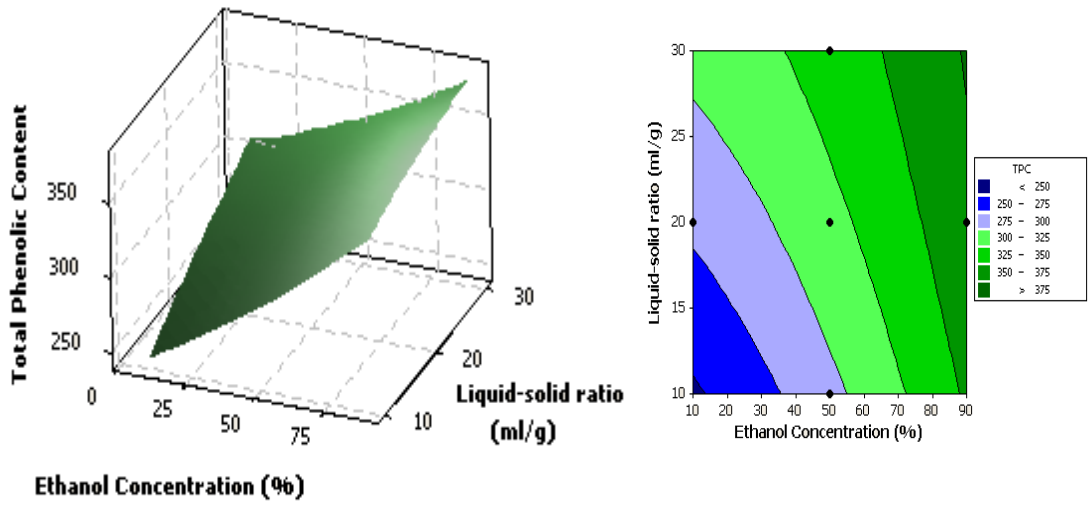


Figure 5.4. Response surface and contour plots of total phenol content (Y_2) for the effects of ethanol concentration and liquid-solid ratio at extraction time of 8h.

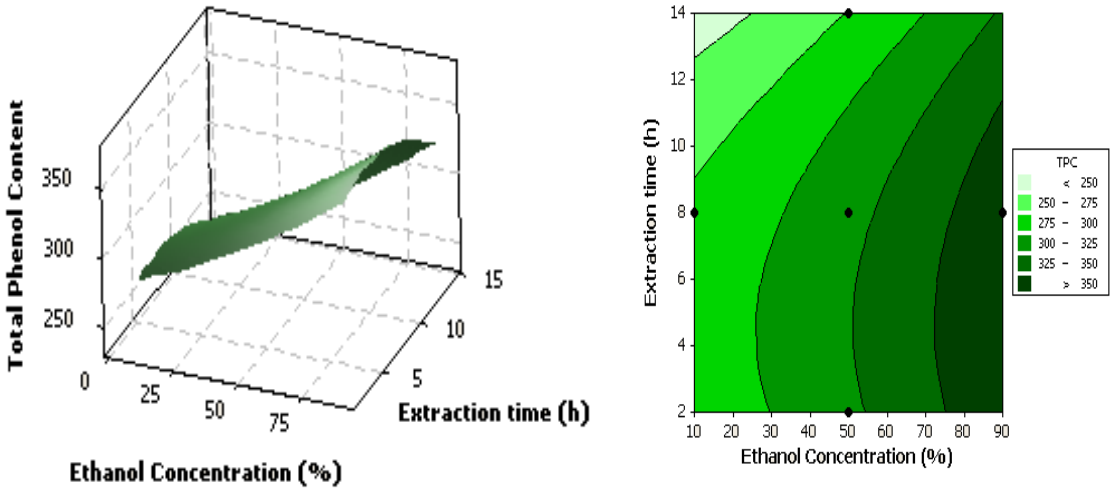


Figure 5.5. Response surface and contour plots of total phenol content (Y_2) for the effects of extraction time and ethanol concentration at constant liquid-solid ratio (20:1).

5.1.3.3. Response Surface Analysis for Total Mass Yield

In general, mass yield of the extraction process is influenced by many parameters such as temperature, time and solvent polarity (Montgomery, 2008). However, in this study, the main focus was the extraction parameters having influence on antioxidant capacity of extract due to its phenolic content. In Figure 5.6, the results of response surface and contour plots revealed that the ethanol concentration in extraction had very significant effect on mass yield of extract.

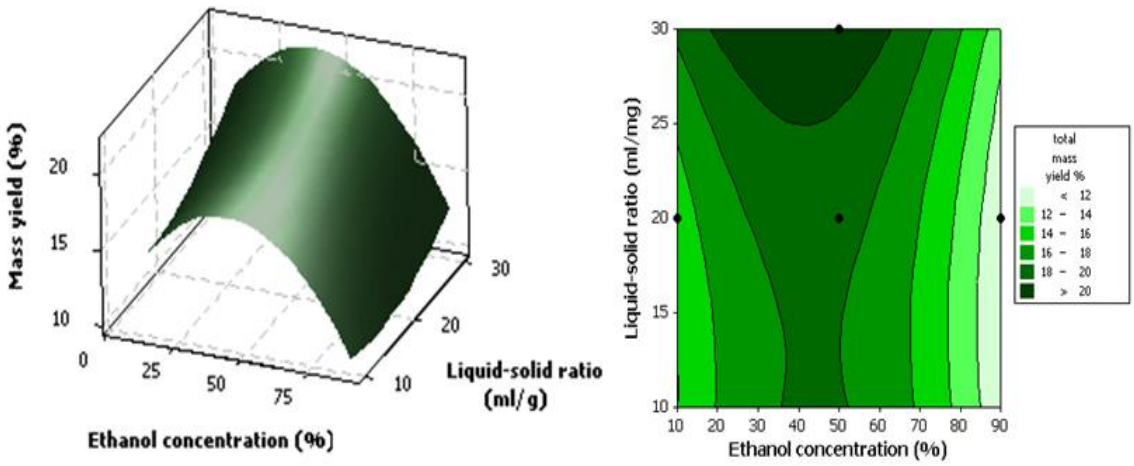


Figure 5.6. Response surface and contour plots of mass yield (Y_3) for the effects of ethanol concentration and liquid-solid ratio at extraction time of 8h.

Also, in Figure 5.7, the results indicated that the effect of liquid-solid ratio was linear regardless of the proportion of the extraction time. This was also confirmed with the expression in Equation 5.3. The relationship between solvent composition and extraction time is properly formed in middle design point. The mass yield increased steadily with increasing liquid-solid ratio.

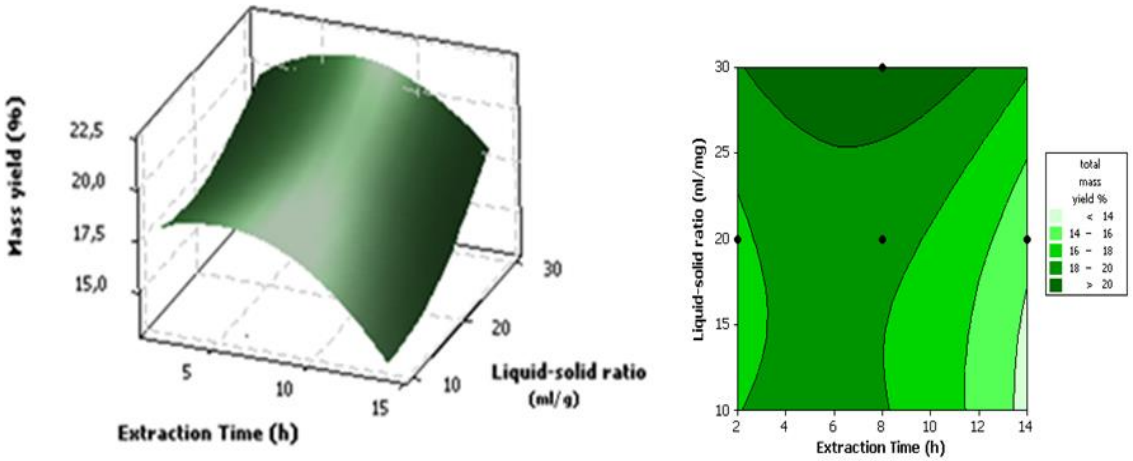


Figure 5.7. Response surface and contour plots of mass yield (Y_3) for the effects of extraction time and liquid-solid ratio at ethanol concentration of 50%.

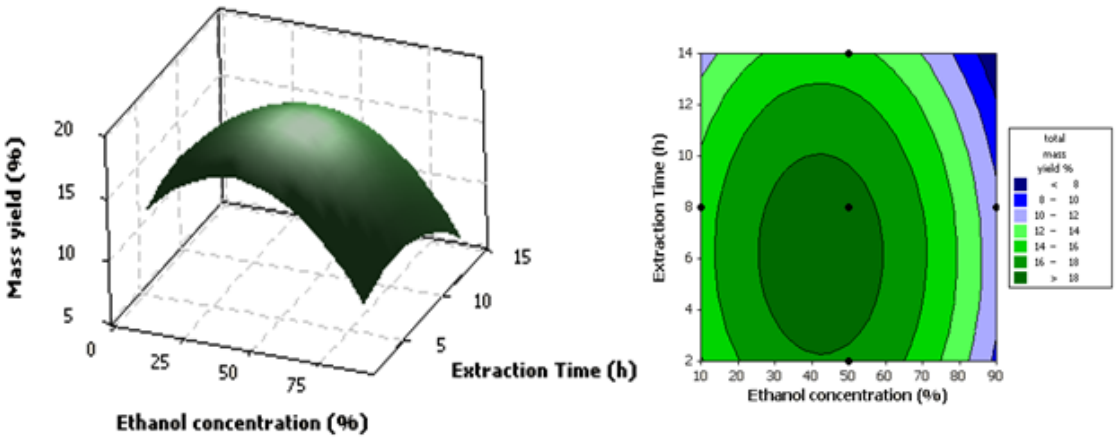


Figure 5.8. Response surface and contour plots of mass yield (Y_3) for the effects of extraction time and ethanol concentration at constant liquid-solid ratio (20:1).

5.1.4. HPLC Analysis and Identification of Phenolic Compounds

Identification of the phenolic compounds in the extract was attempted using high performance liquid chromatography (HPLC-DAD). The retention time of major phenolic substances and their corresponding UV absorbance spectra were determined by HPLC analysis. 3-D HPLC Chromatogram is shown in Figure 5.9 with their UV spectrum. To the best of our knowledge few studies in the literature were reported about the content of *S. spinosum* extract as a medicinal plant. After Reher, in 1991, deduced the presence of catechin, α-tocopherol content of *S. spinosum* was studied by Sarıkaya in 2010 (Reher et al., 1991; Sarıkaya & Kayalar, 2010). However, no detailed study exists in the literature regarding the content of *S. spinosum* extract. In this study, it was tried to analyze the whole extract of *S. spinosum* which was extracted with aqueous ethanol solution under proper extraction conditions. After developing HPLC method, Figure 5.10 shows the representative HPLC chromatogram with specific wavelengths which are characteristics wavelength to identify phenolics. The major compounds were identified with the help of their retention time, their UV spectra in comparison to corresponding reference standards. From the HPLC chromatogram of the extract recorded at 270 nm, two peaks were detected at the retention times 16.99 (peak 4) and 17.8 (peak 5), as shown in Figure 5.10, Peak 4 and Peak 5 were identified as hyperoside and isoquercetin, respectively. The major compound in the extract was isoquercetin.

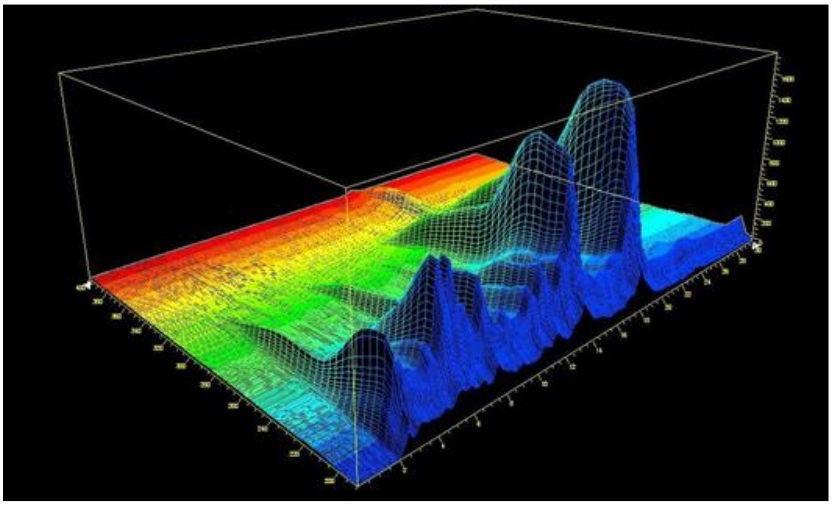


Figure 5.9. 3D HPLC chromatography structure of *S. spinosum* extract

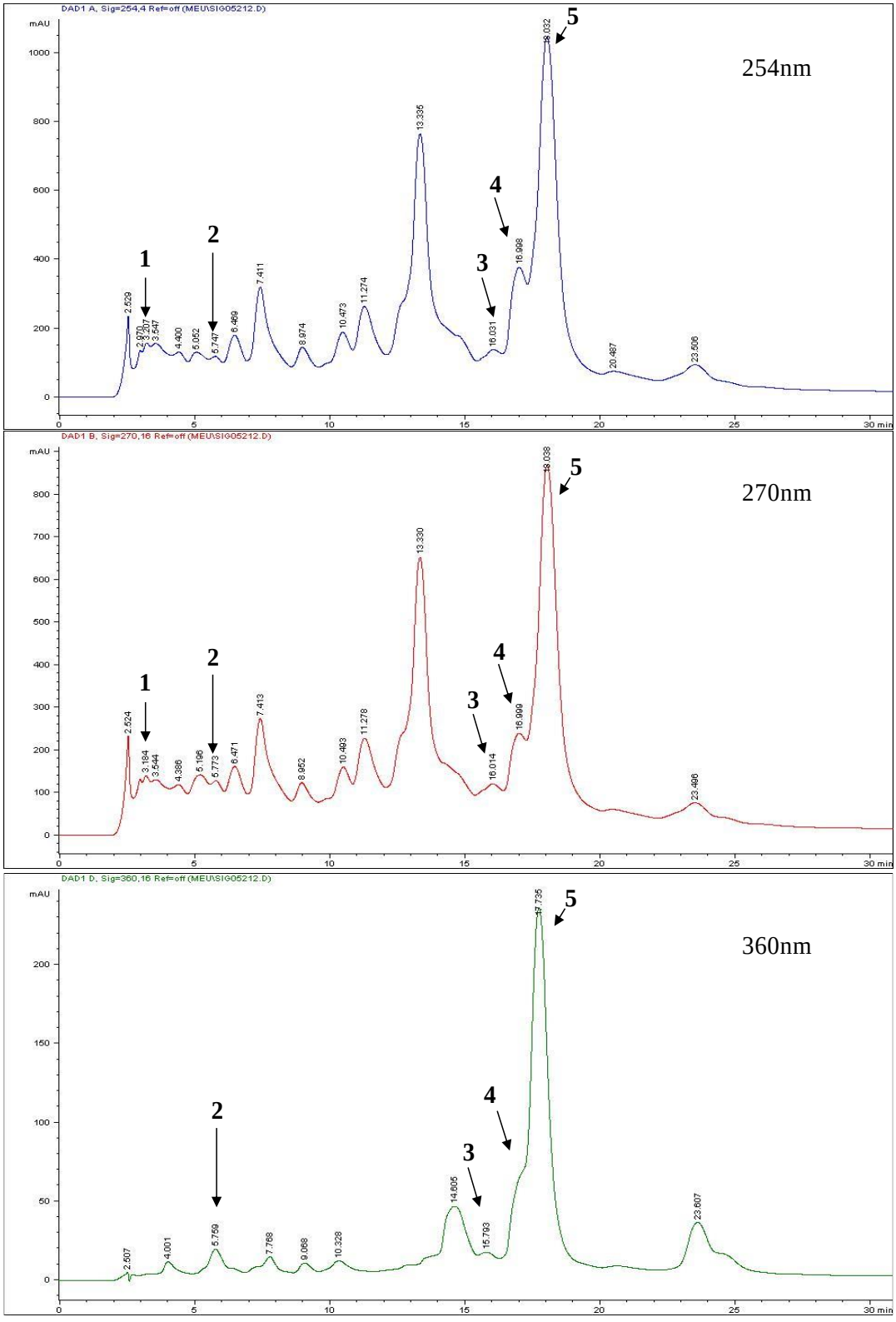
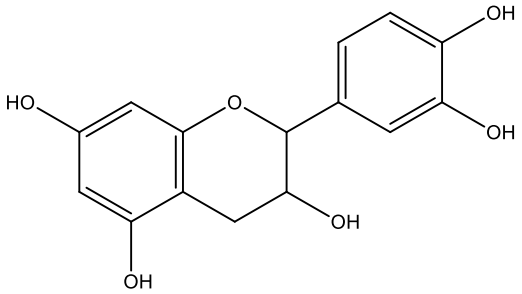


Figure 5.10. HPLC chromatograms of *S. spinosum* extract at the wavelength of 254, 270 and 360 nm. Compounds are denoted with numbers: 1- gallic acid, 2- catechin, 3-rutin, 4-hyperoside and 5-isoquercetin.

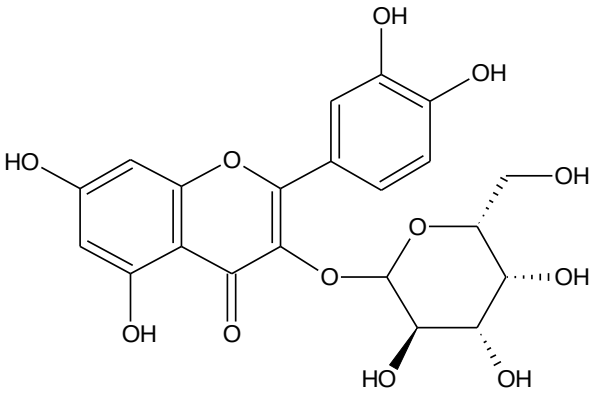
Compoud 1 had similar UV spectra and retention time, characteristic of gallic acid. Due to presence of dominant peaks, it can be observed as a small peak. The utilization of gallic acid commercial standard confirmed the identification of compound 1. Compound 2 had similar UV spectra and retention time with catechin. The amount of catechin was calculated as 17.8 mg per gr dried weight in extract. Especially, as seen in Figure 5.15, it had very characteristic UV_{max} at 220 nm and 280 nm.



catechin

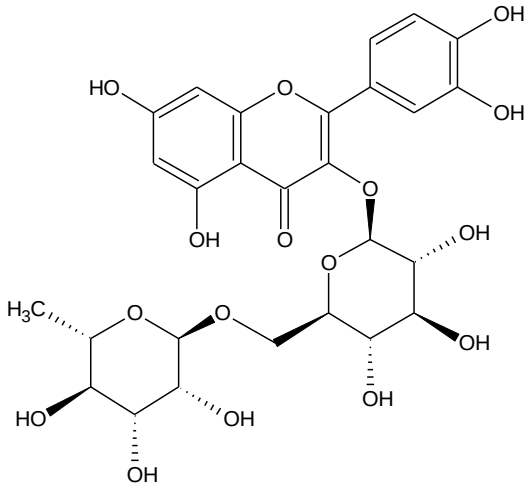
Figure 5.11. Chemical structure of catechin.

The retention time of compound 3 in the chromatogram was determined to be the same with rutin. It had similar UV profile and been confirmed with the help of commercial reference standard. The UV spectra and retention time of compound 4 and 5 were consistent with those of hyperoside (Rt, 16.9 min) and isoquercetin (Rt, 17.8 min). Both compounds showed their UV_{max} at 360 nm, specifically. In addition to UV spectral characterization, the identification of hyperoside and isoquercetin was further confirmed by spiking their commercial standards into extract solution before analysis. The amount of hyperoside was calculated as 12.9 mg per gr dried weight in extract, according to calibration curves in Appendix D. Isoquercetin, the major compound of *S. spinosum* extract, was 36.6 mg per gr dried weight in extract.



Isoquercetin

Figure 5.12. Chemical structure of isoquercetin



rutin

Figure 5.13. Chemical structure of rutin

In addition to HPLC analysis, thin layer chromatographic (TLC) method was also used to analyze and confirm the presence of these compounds. After coloring step, phenolic compounds were distinctively separated on the TLC plate in the extract. Black and white image of the TLC plate is shown in Figure 5.14. The bands can be seen as colorful bands under UV light at 365 nm. The bands on the TLC plate for standards were in good match especially, indicating the presence of hyperoside and isoquercetin in the extract. These results were in accordance with the HPLC results.

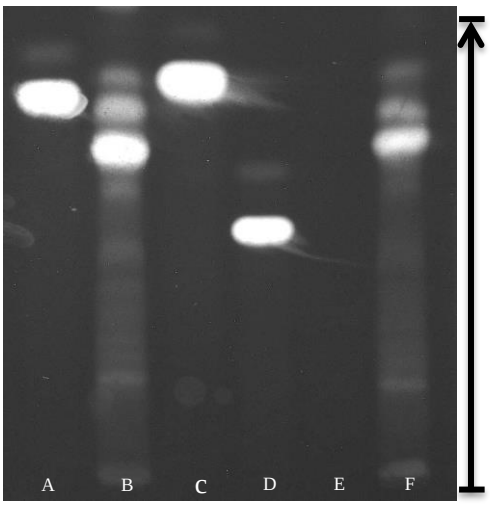


Figure 5.14. The image of TLC plate *S. spinosum* extract (B and F lines) and standards (hyperoside; A, isoquercetin; C, rutin; D and gallic acid; E line).

The novelty of our research was the identification of the content of *S. spinosum* extract. The presence of rutin, isoquercetin and hyperoside were confirmed with the results of TLC and HPLC. There is no supportive published data which can identify the content of *S. spinosum* in the extract with these chemicals. For this reason, all compound identification was carefully studied and confirmed by many different tried and repetition. In the literature, there is no report on the presence of these natural compounds in *S. spinosum*. In this study, especially two main compounds (hyperoisde and isoquercetin) were successfully identified in the extract of *S. spinosum*.

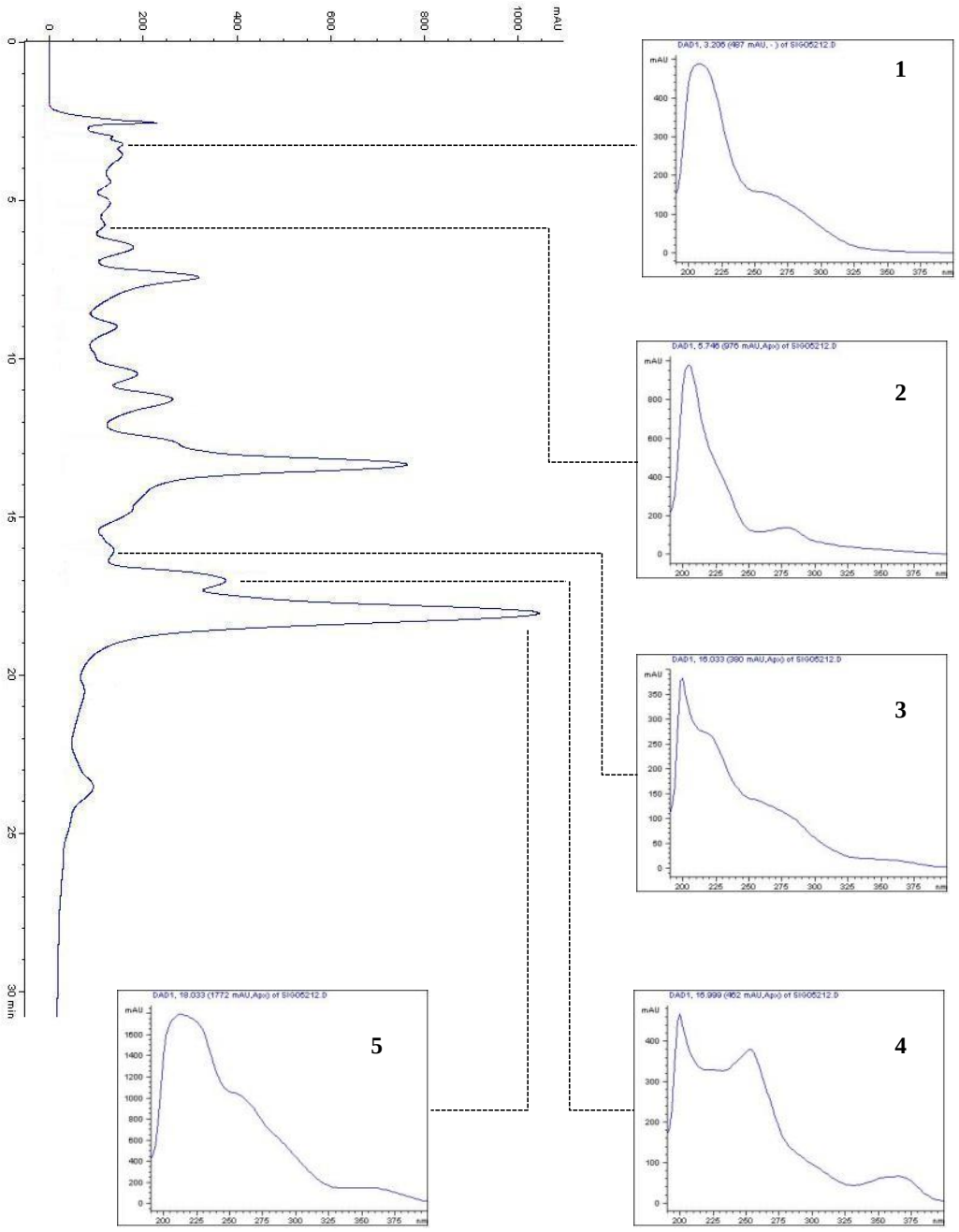


Figure 5.15. HPLC profile with the UV spectra of *S. spinosum* extract for peak 1(gallic acid), peak 2 (catechin), peak 3(rutin), peak 4 (hyperoside) and peak 5 (isoquercetin).

5.1.5. Antimicrobial Activity Analysis

The determination of antimicrobial activity described in the material method section includes the measurement of (i) diameter of the zone of inhibition of bacterial growth around disk that contains antimicrobial compound on agar media and (ii) the

minimum inhibition concentration (MIC) of the antimicrobial compound in liquid media. In antimicrobial assays, three microorganisms were chosen to determine the antimicrobial activities of *S. spinosum* extracts. These were *E. coli*, *C. albicans* and *S. epidermidis*. *E. coli* is a gram negative bacteria and *S. epidermidis* is a gram positive while *C. albicans* is a pathogenic fungi as a microorganism.

5.1.5.1. Disc Diffusion Test

Disc diffusion method enables to determine the antimicrobial activity of the samples. It was performed for *S. spinosum* extract that was prepared with chosen optimum conditions which are 70% ethanol solvent, 20:1 liquid-solid ratio and 4 hour extraction time. Antimicrobial effects of the extract against each bacteria species are shown in Figure 5.16. The antimicrobial effect against *S. epidermidis* is not seen clearly because the gram positive bacteria can not be dyed with INT. *S. spinosum* extract formed an inhibition zone at moderate level against *S. epidermidis.* In addition, *C. albicans* and *E. coli* were more sensitive to the plant extract than *S. epidermidis*.

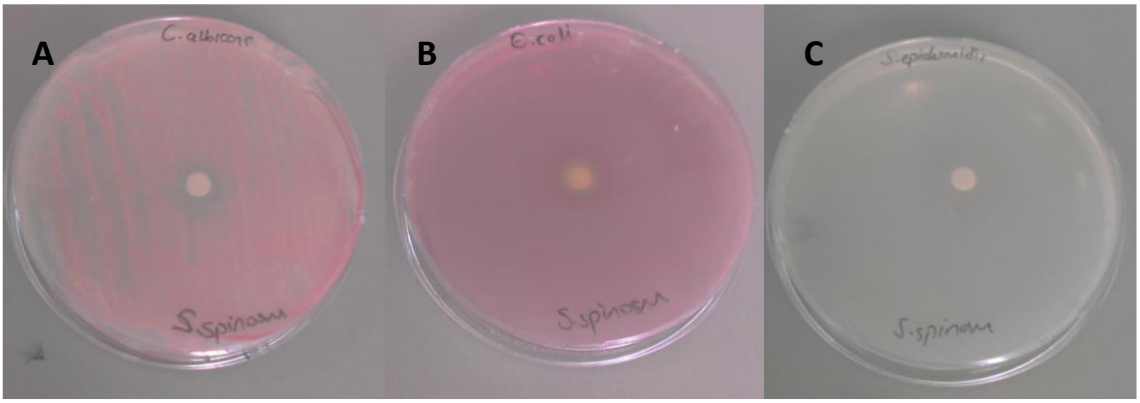


Figure 5.16. Inhibition zone of *S. spinosum* extract for *C. albicans* (A), *E. coli* (B), *S. epidermidis* (C).

Especially, *C. albicans* exhibited weak resistances against plant extract in disc diffusion assays. According to the results of disc diffusion assays, the inhibition zone for *E. coli* revealed the bacteriostatic effect of *S. spinosum* extract. On the other hand, it

was bacteriocidal effect against *C. albicans*. The plant extract was found to be more effective for inhibition of *C. albicans* growth compared with other species. Antimicrobial activities of *S. spinosum* extracts obtained at different extraction parameters were tested. The extract with the highest antioxidant activity coded as test #9, the highest phenolic content coded as test #6 and the lowest value for both coded as test #3 were taken into antimicrobial tests. The aim of this was to observe the effects of different extraction parameters on antimicrobial activities due to changing contents of the extracts. As seen in Figure 5.17, there was no significant difference between the inhibition zone values for extracts obtained at different extraction parameters.

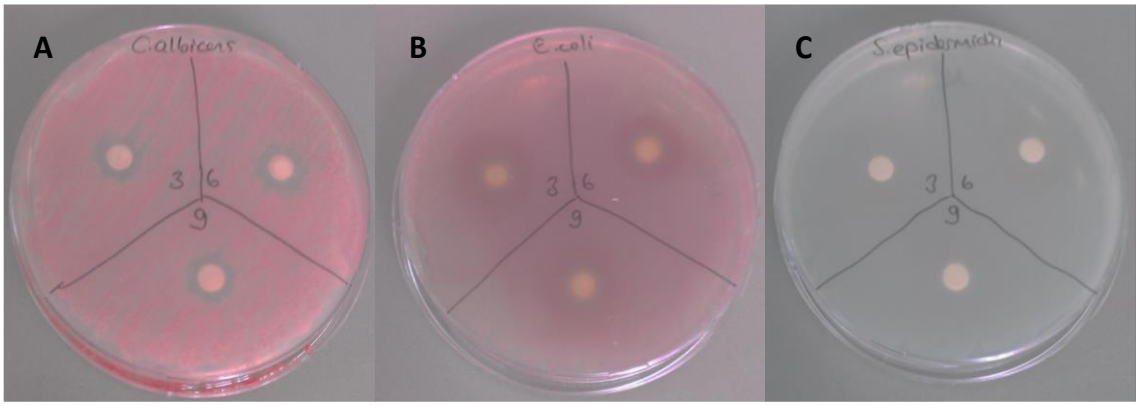


Figure 5.17. Inhibition zones of plant extracts (#3, #6, #9) of *S. spinosum* for *C. albicans* (A), *E. coli* (B), *S. epidermidis* (C). Extracts were obtained at different extraction parameters.

5.1.5.2. Minimum Inhibition Concentration (MIC)

The minimum inhibition concentration (MIC) method provides potentially useful technique for indicating the lowest concentration of antimicrobial test sample at which growth of specific microorganism is inhibited. In order to determine the MIC, the plant extract solutions as subject to serial dilutions were performed to the plant extract in 96 well micro plates with inoculated microorganisms. This method was evaluated by using absorbance results that were examined for changes in turbidity as an indicator of growth. The antimicrobial activity of *S. spinosum* extract against *E. coli* was evaluated by following growth curve, seen in Figure 5.18. The lowest concentration, applied in

test, exhibited antimicrobial activity interestingly. As a result, the low amount of *S. spinosum* extract can be highly effective against *E. coli*. On the other hand, the antimicrobial activity *in vitro* of *S. spinosum* extract against *S. epidermidis*, illustrated in Figure 5.19, showed bacteriostatic effect because of preventing growth at a certain level.

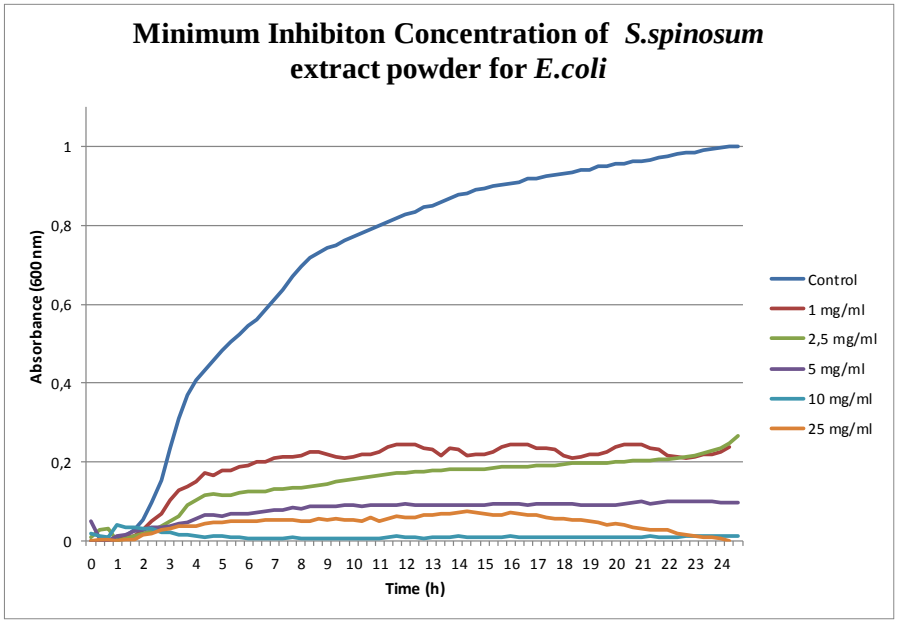


Figure 5.18. The growth curve of *E. coli* in liquid media with different concentration *S. spinosum* extract.

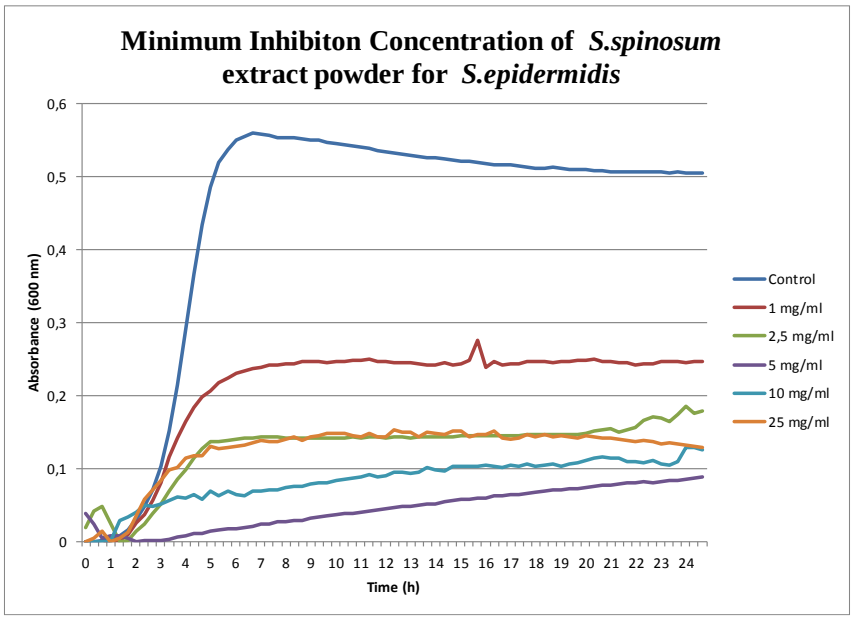


Figure 5.19. The growth curve of *S. epidermidis* in liquid media with different concentration *S. spinosum* extract.

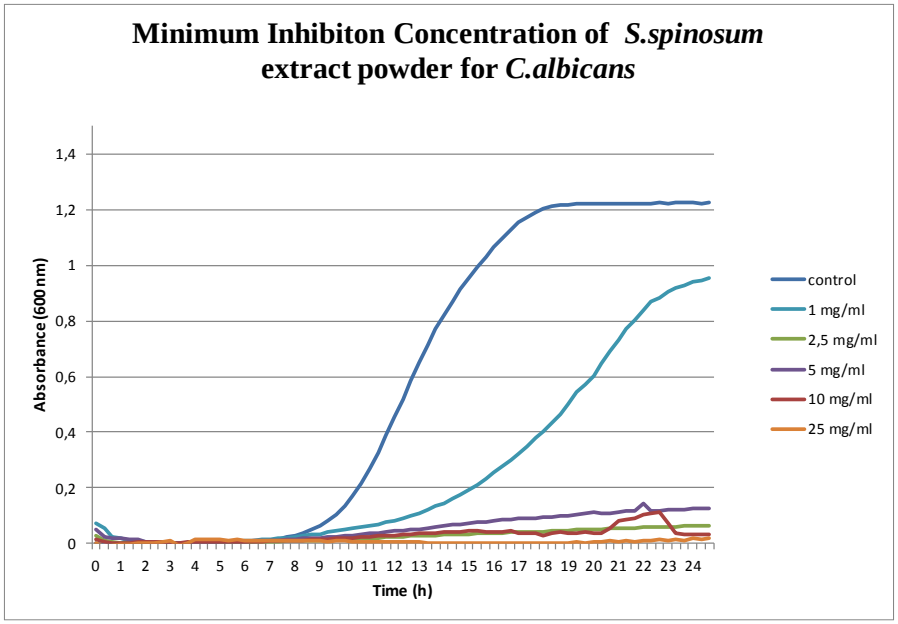


Figure 5.20. The growth curve of *C. albicans* in liquid media with different concentration *S. spinosum* extract.

In addition, the MIC result of *S.spinosum* extract against *C.albicans* supported the result in disc diffusion test. As demonstrated in Figure 5.20, bacteriocidal effect was observed when *C.albicans* was exposed to *S.spinosum* extract (>1mg/ml). According to the results of antimicrobial tests, *S.spinosum* extract showed impressive antimicrobial activity. The results of microbial tests were summarized in Table 5.4.

Table 5.4. The results of antimicrobial activity.

S.spinosum extract parameter	*E. coli*		*S. epidermidis*		*C. albicans*	
	MIC (mg/ml)	DISC (Diameter mm)	MIC (mg/ml)	DISC (Diameter mm)	MIC (mg/ml)	DISC (Diameter mm)
Optimum	>1	16	>2,5	19	>2,5	17
Test #3	->1	16	>2,5	19	>2,5	17
Test #6	>1	16	>2,5	19	>2,5	17
Test #9	>1	16	>2,5	19	>2,5	17

5.1.6. Cytotoxicity Analysis

The NIH 3T3 mouse fibroblast cell line was used as a model for studying the effect of *S. spinosum* extract on cell viability test. In order to evaluate the cytotoxicity of the plant extract, fibroblast cell line viability was measured using MTT method. Figure 5.21 shows the viability of cell detected by MTT assay as a function of the dose applied for 24, 48 and 72h. For all time intervals, similar pattern was observed. It means that concentrations of *S. spinosum* extract, exposed to healthy cell line, were no contradictory for different time exposures. The value of inhibition concentration for 50% cell viability (IC_{50}) is 250µg/ml. On the other hand, the research, realized by Simirin and his coworkers, revealed that *S. spinosum* extract at a dose of 1000 µg/ml increased the pancreatic β- cell proliferation (Smirin et al., 2010).

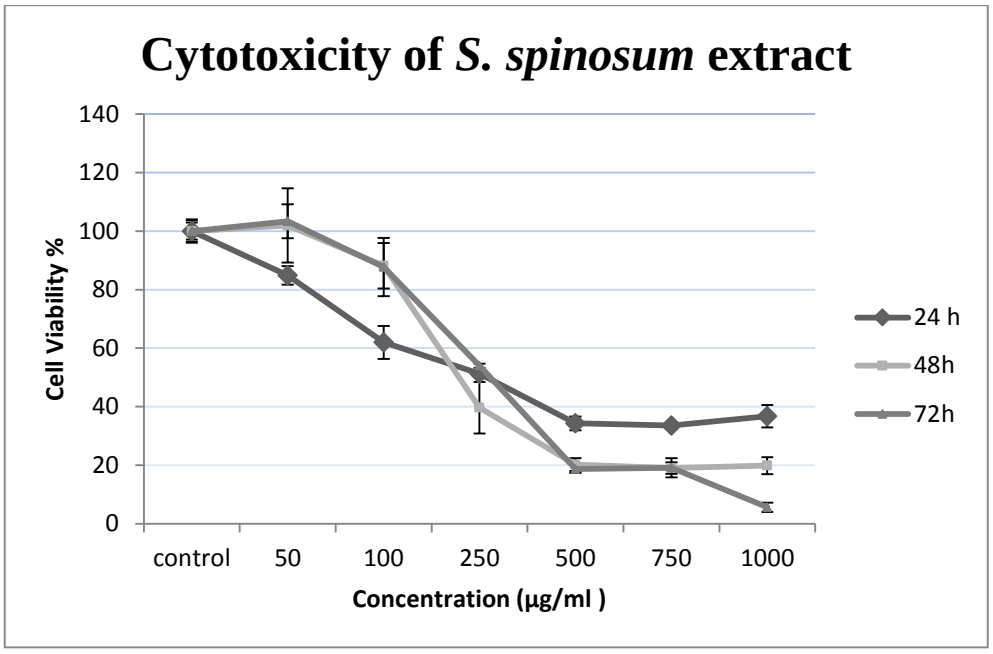


Figure 5.21. Percentage of cell viability of NIH 3T3 mouse fibroblast cells treated with different concentration *S. spinosum* extract.

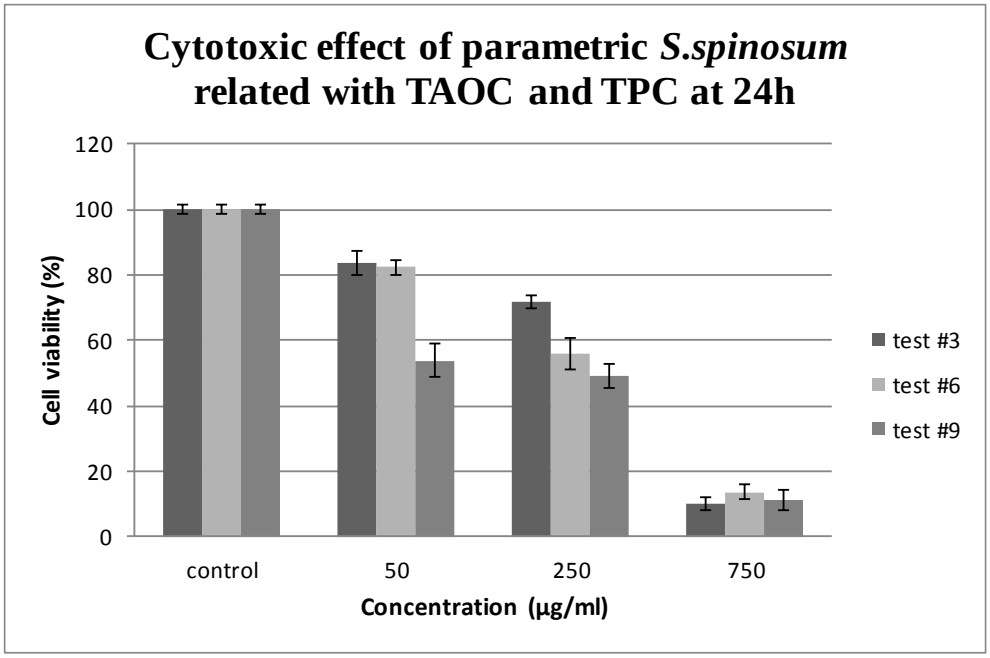


Figure 5.22. Percentage of cell viability of fibroblast cells treated with different concentration of *S. spinosum* extracts having the highest antioxidant activity (test #9), the highest amount phenolic content (test #6) and the lowest both (test #3) at **24h**.

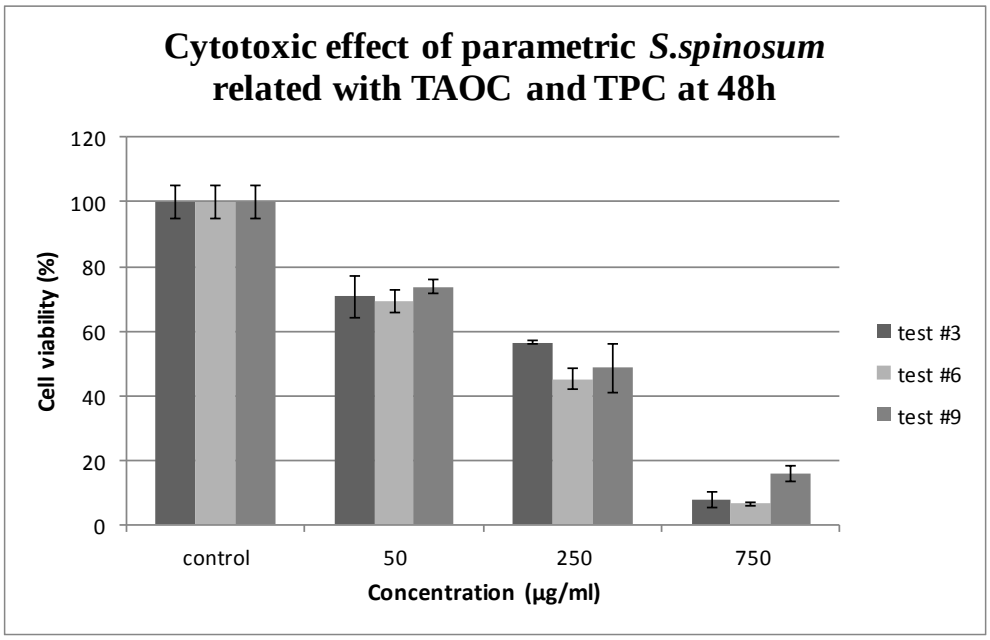


Figure 5.23**.** Percentage of cell viability of fibroblast cells treated with different concentration of *S. spinosum* extracts having the highest antioxidant activity (test #9), the highest amount phenolic content (test #6) and the lowest both (test #3) at **48h**.

After cell viability test was studied for optimum *S. spinosum* extract parameter, three concentration was observed as toxic, nontoxic and IC_{50} for parametric *S. spinosum* extracts in terms of the highest antioxidant activity (test #9), the highest amount phenolic content (test #6) and the lowest both (test #3). At the end of the first 24h interval in Figure 5.22, it showed that IC_{50} was slightly changed in terms of level of antioxidant capacity. However, this impact disappeared at continued 48h. In Figure 5.23, there was a slight different at 750 µg/ml concentration *S. spinosum* extract for test number 9 at 48h. It can be also beginning point for the impressive result of cell viability at 72h. Because test # 9, shown the highest antioxidant activity in parametric studied, dramatically different effect to cell line, it was less toxic effect to the cells than others at 72h.

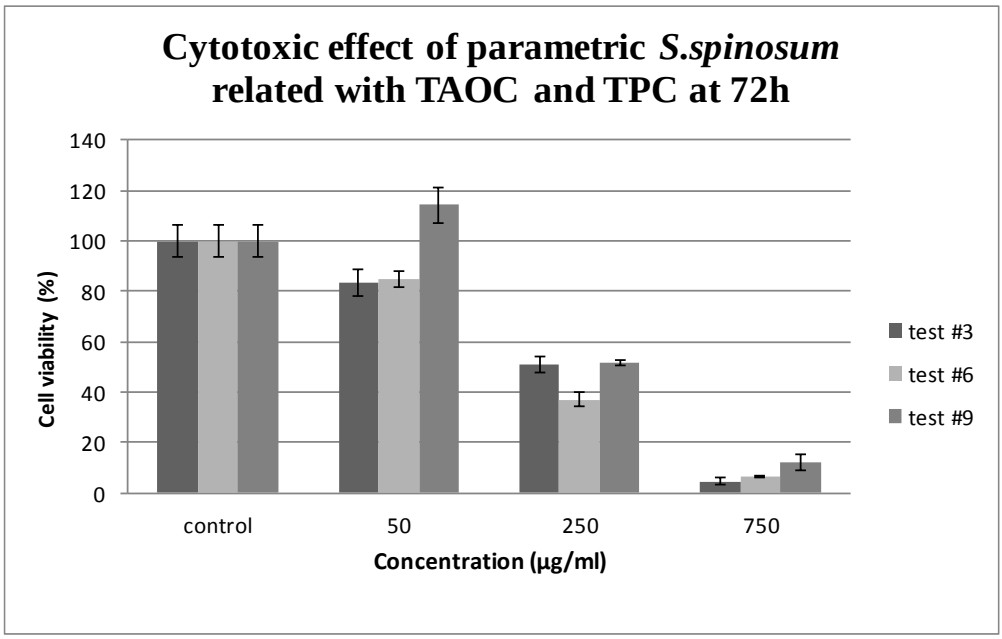


Figure 5.24**.** Percentage of cell viability of fibroblast cells treated by different concentration of parametric *S. spinosum* extracts in terms of the highest antioxidant activity (test #9), the highest amount phenolic content (test #6) and the lowest both (test #3) at **72 h.**

5.2. Encapsulation of *Sarcopoterium spinosum* Extract

5.2.1. Microparticles Preparation and Analysis

S. spinosum extract loaded zein microparticles were obtained using electrospray method. Before the loaded sample was produced, the best condition for encapsulation with zein using by electrospray was determined. The main variables that affect the morphology of the particles include the concentration of zein polymer in solution in terms of viscosity, surface tension, conductivity of the solvent and the processing conditions i.e. flow rate, applied voltage and distance between the tip of the needle and the collector plate. For this reasons, the effect of zein concentration, solvent concentration and applied voltage were investigated on the size and morphology of the zein structures. In order to optimize the process parameters, the studied zein polymer concentrations were chosen as 1, 2.5, 5, 10 and 20%. The most crucial parameter in electrospray principle is polymer concentration because of the viscosity and surface tension linked to viscosity.

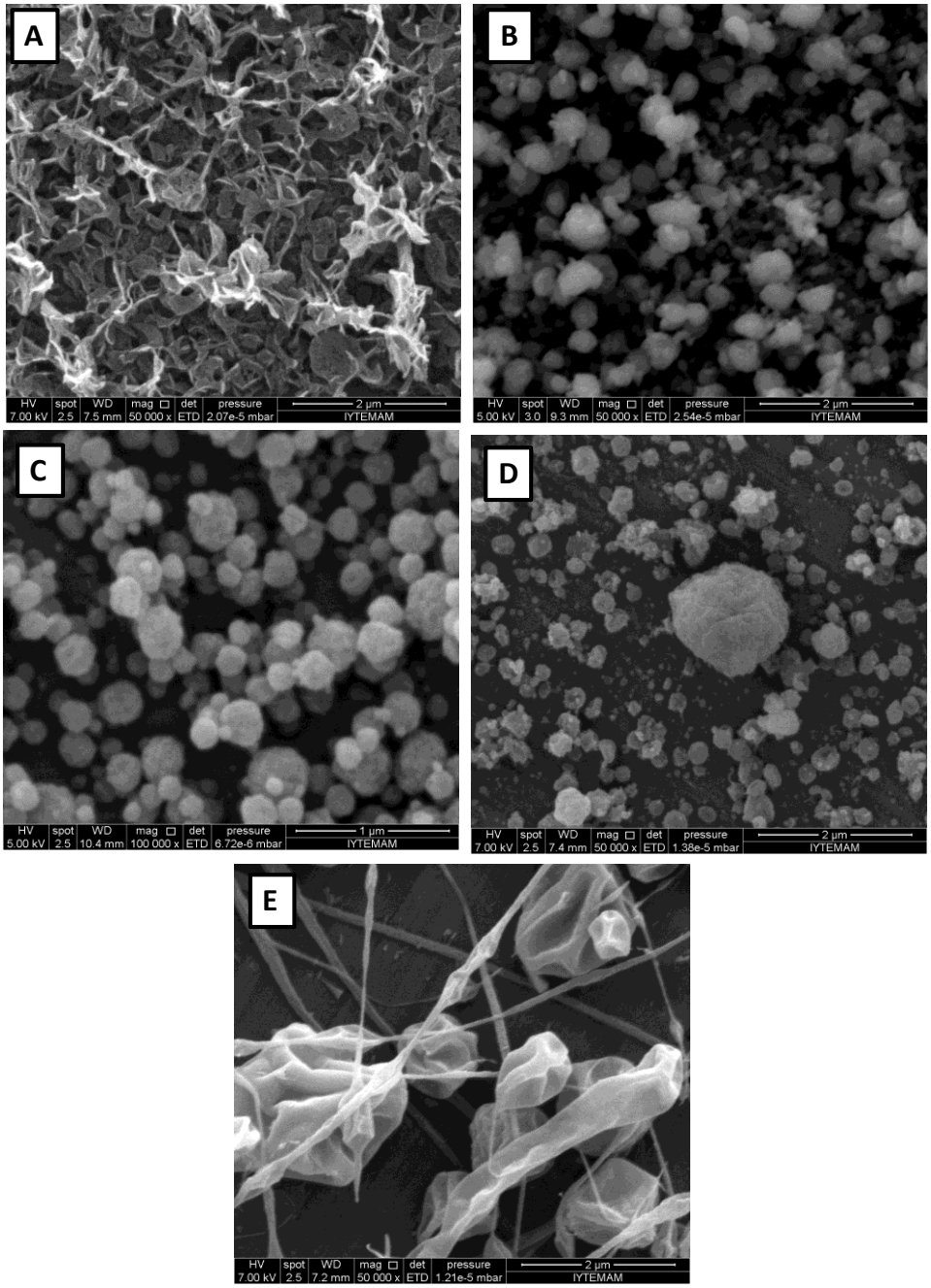


Figure 5.25. SEM images showing the effect of zein concentration on the size and shape of microostructures obtained at a constant flow rate (0.3 ml/h), needle to collector distance (10 cm), and voltage (14 kV). Zein concentration (w/v) ; A: 1%; B: 2.5%; C: 5%; D: 10%; E: 20%.

Figure 5.25 shows the morphology of the zein structures obtained with increasing concentrations of zein. In addition, higher surface tension with high zein concentration causes more instability and resulted in uneven distribution of particle

diameters. Finally, it can be clearly seen that fiber like structure was generated rather than sphere particle structure with a the highest concentration of polymer in Figure 5.25-E. According to the SEM image, the use of 5% zein concentration resulted in particles with smooth surface and narrow size distribution. For example, the polymer concentration in the 70% aqueous ethanol solution was too low for particle formation for 1% (w/v) zein concentration as seen in Figure 5.26-A. Due to low intermolecular entanglements among polypeptide chains (Gomez-Estaca et al., 2012), zein polymer was not able to form spheres after solvent evaporation.

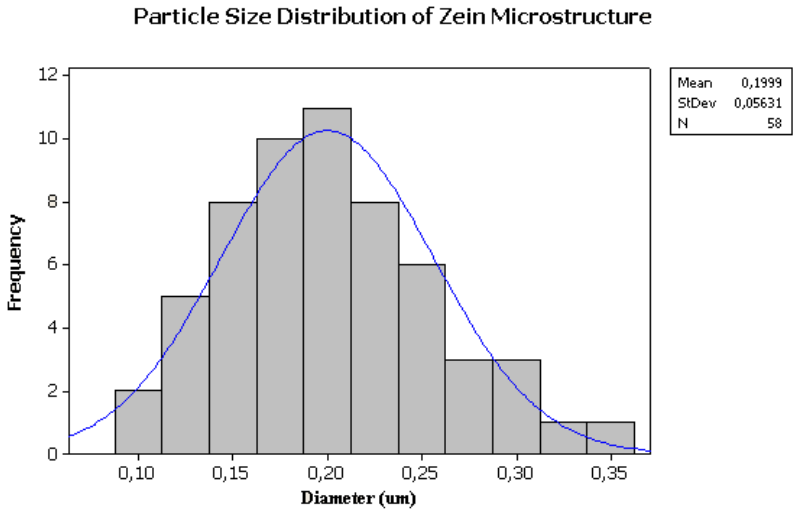


Figure 5.26. Particle size distribution of zein concentration (5%) under flow rate (0.3 ml/h), needle to collector distance (10 cm), voltage (14 kV) and zein concentration 5% (w/v).

The particle size distribution is shown in Figure 5.26. When electrospraying was performed with 5% zein concentration at an apply voltage of 14kV and flow rate 0.3 ml/h. The solvent used was 70% aqueous ethanol solution.The average particle diameter was determined as 200 nm ± 56 nm diameter.

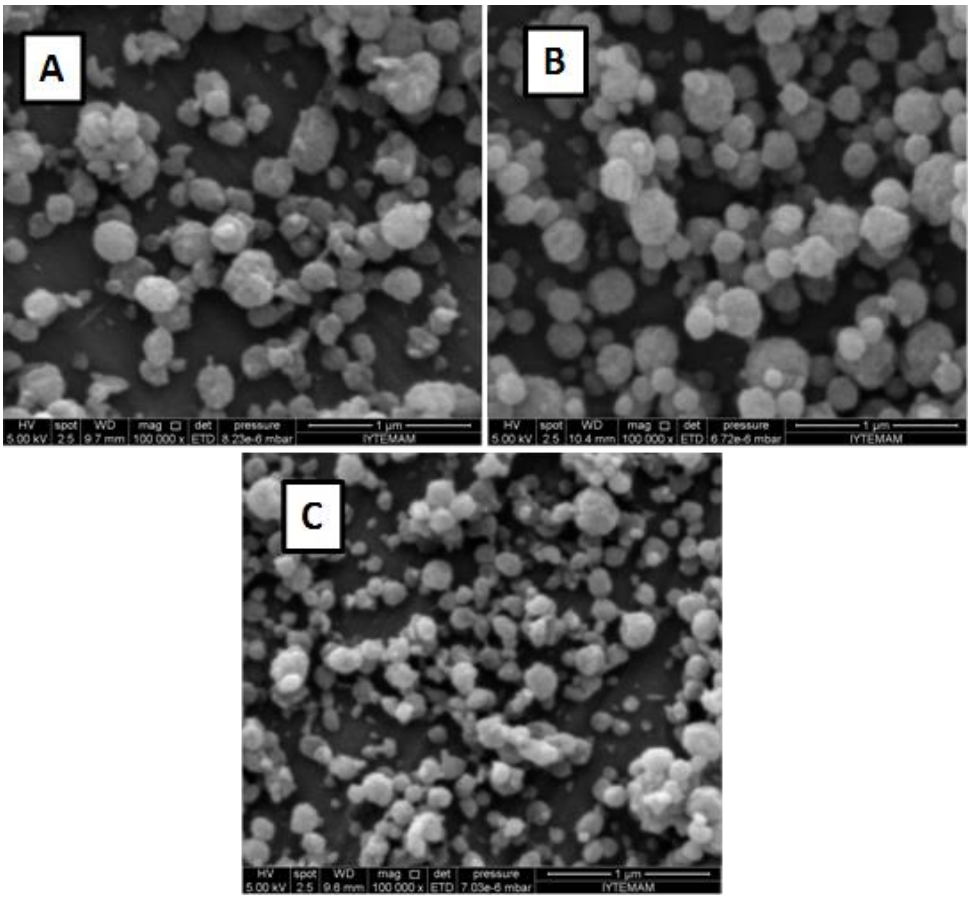


Figure 5.27. SEM images showing the effect of solvent concentration for zein on the size and shape of microparticles obtained at a constant flow rate (0.3 m/h), needle to collector distance (10 cm), and voltage (14 kV), zein concentration (5% , w/v). Solvent concentration (ethanol in dH_2O; v/v), A: 60% ; B: 70% ; C: 80%.

The solvent concentrations were studied at 60, 70 and 80% ethanol. The effect of solvent concentration on the particle morphology was shown in Figure 5.27. It was observed that the particle sizes decreased and perfect spherical shapes were deformed with respect to changing ethanol concentration of 60 and 80%. Increase in ethanol concentration had a slight destructive effect on the shape of spherical particles. The SEM results revealed that 70% aqueous ethanol solution was described as ideal solvent system. It caused desired particle size and morphology. 70% aqueous ethanol solution was a good choice, it can be used as an extraction solvent as well as electrospraying solvent. Gomez and his friends underlined that the applied voltage was another crucial parameter in achieving a stable cone-jet mode for obtaining monodisperse particle (Gomez-Estaca et al., 2012). The SEM results, in Figure 5.28, supported this claim

proposed by Gomez. According to SEM images, low voltage was not sufficient to form perfect spherical shape and narrow size distribution. On the other hand, the particles tended to form aggregates when the applied voltage was high. Compared to the previous results reported in the literature, the range of applied voltage between 12 and 16kV did not cause the shape of the particles to change significantly. Therefore, the results were in agreement with previously the results reported in the literature (Gomez-Estaca et al., 2012).

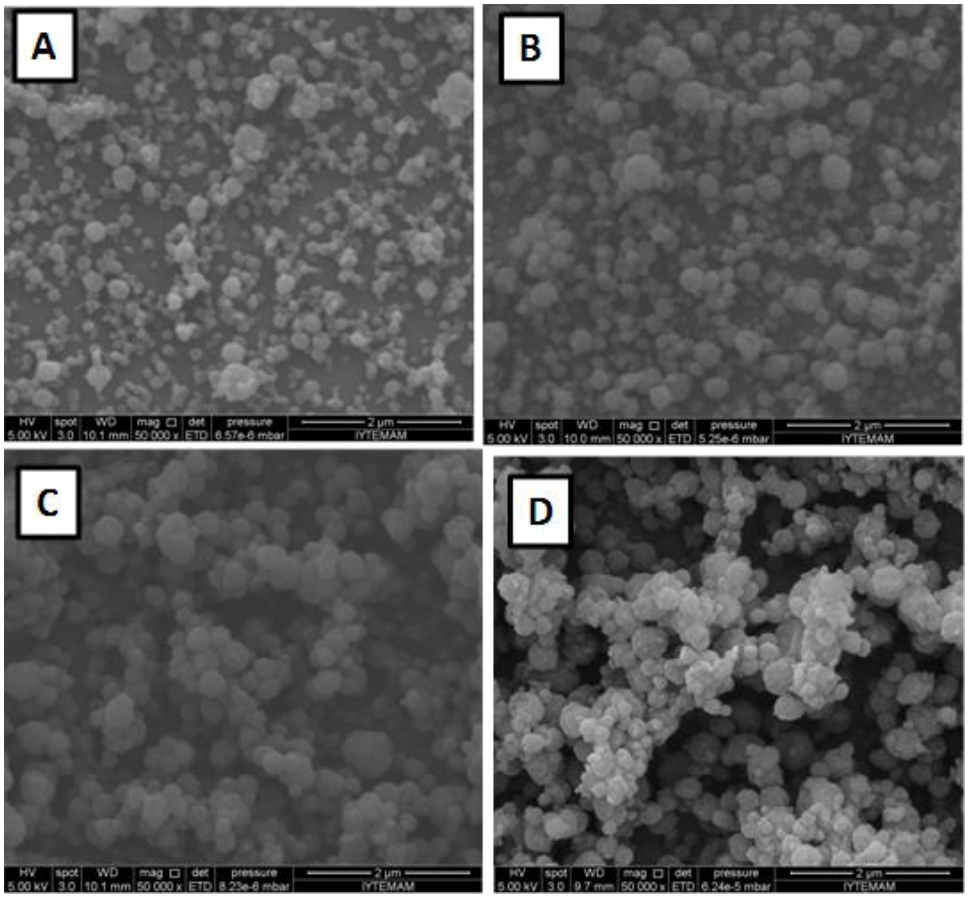


Figure 5.28. SEM images showing the effect of voltage on the size and shape of microparticle obtained at a constant zein concentration (5 % (w/v)), needle to collector distance (10 cm), and flow rate (0.3 ml/h). Voltage; A: 10kV; B: 12kV; C:14 kV ; D:18 kV.

The process of electrohydrodynamic atomization especially depends on the flow rate. According to equation 2.3 that implies the principle of the electrospray, the diameter of particles increases with the flow rate. The effect of the flow rate on the particles can be seen in Figure 5.29. In order to observe the effects of the flow rates between 0.15 and

1.5ml/h, voltage was fixed at 14kV, 5% zein concentration in 70% aqueous ethanol solvent was used in the experiment. As seen in Figure 5.29, the monodisperse particle size distribution with lowest mean particle diameter was obtained for 0.3ml/h.

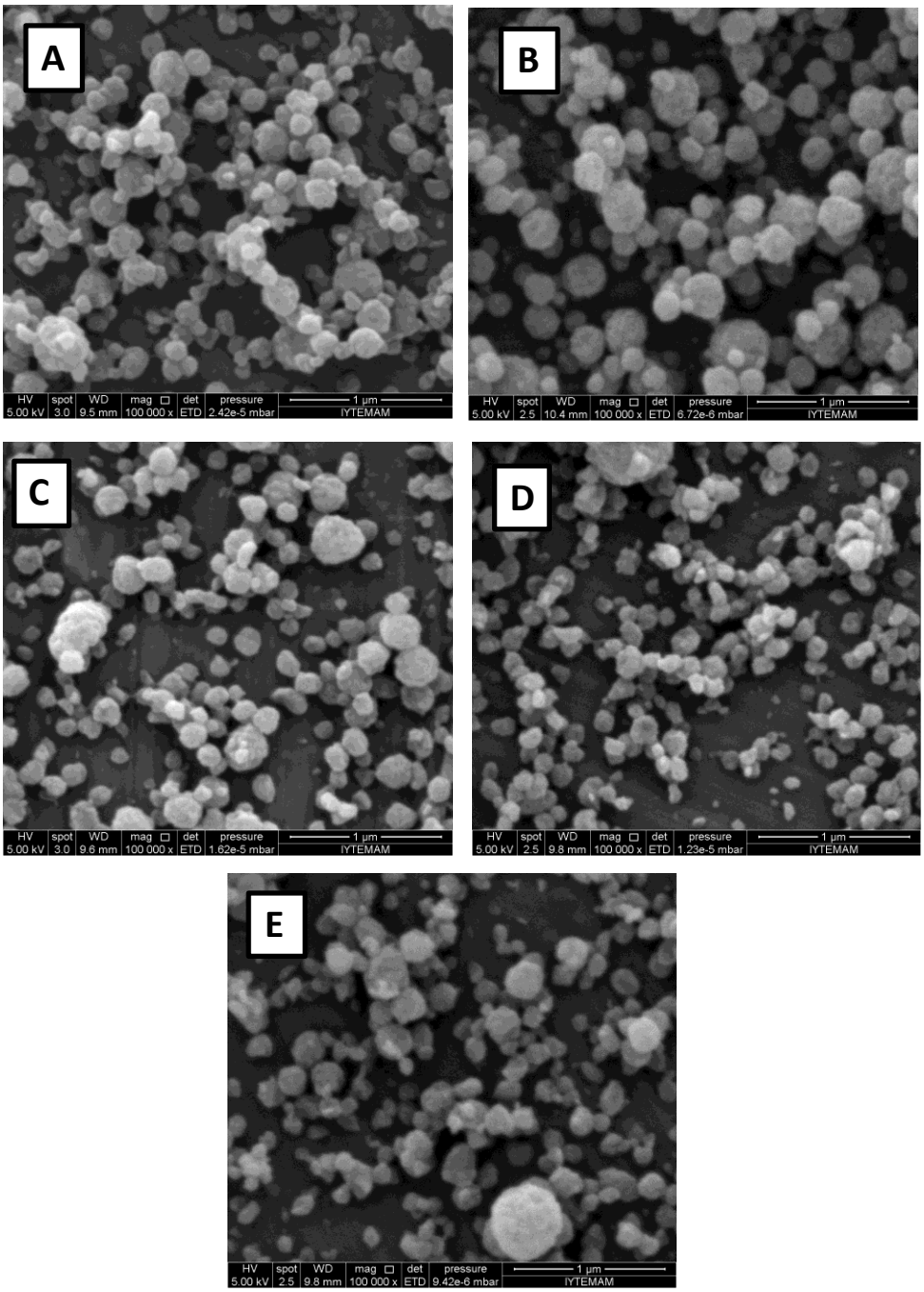


Figure 5.29. SEM images showing the effect of flow rate on the size and shape of microstructures obtained at a constant zein concentration (5 % (w/v)), needle to collector distance (10 cm), and voltage (14 kV). Flow rate; A: 0.15; B: 0.3; C: 0.6; D:1; E:1.5 (ml/h).

5.2.2. Preparation of Extract-loaded Zein Microparticles

After the best processing conditions were determined with respect to the morphology and narrow size distribution of the prepared zein particles, *S.spinosum* extract loaded zein microparticles were prepared. The effects of the plant extract concentration on particle size and morphology were explored. Then, the bioactivities of *S.spinosum* extract loaded zein microparticle were studied.

5.2.2.1. Morphology of Plant Extract -loaded Zein Microparticles

SEM images directly can provide information on the particle morphology and particle size. Based on the results discussed in section 5.2.1., the processing parameters for obtaining *S.spinosum* extract loaded microstructures with a narrow size distribution were zein concentration of 5% (w/v), ethanol concentration of 70% (v/v), flow rate of 0.3 mL/h, applied voltage of 14 kV and a needle to collector distance of 10 cm. For the production of *S.spinosum* extract loaded zein microstructures, *S.spinosum* extract was dissolved in 70% aqueous ethanol solution with a zein concentration of 5% (w/v) at several extract to zein weight ratio of 2:1, 1:1, 1:5 1:10, 1:20 and 1:50. For all of these weight ratios, SEM images of extract loaded micro particles are given in Figure 5.30.

As seen in Figure 5.30, the morphology of the prepared microstructures with (the extract powder: zein) weight ratio of 2:1 and 1:1 were partially spherical but the image of the particles were rough. The reason of this could be insufficient encapsulation capability of zein due to insufficient surface tension caused by high amount of *S.spinosum* extract. The desired morphology with narrow size distribution at high extract loading amount was achieved at a weight ratio of 1:5, as seen in Figure 5.30-C.

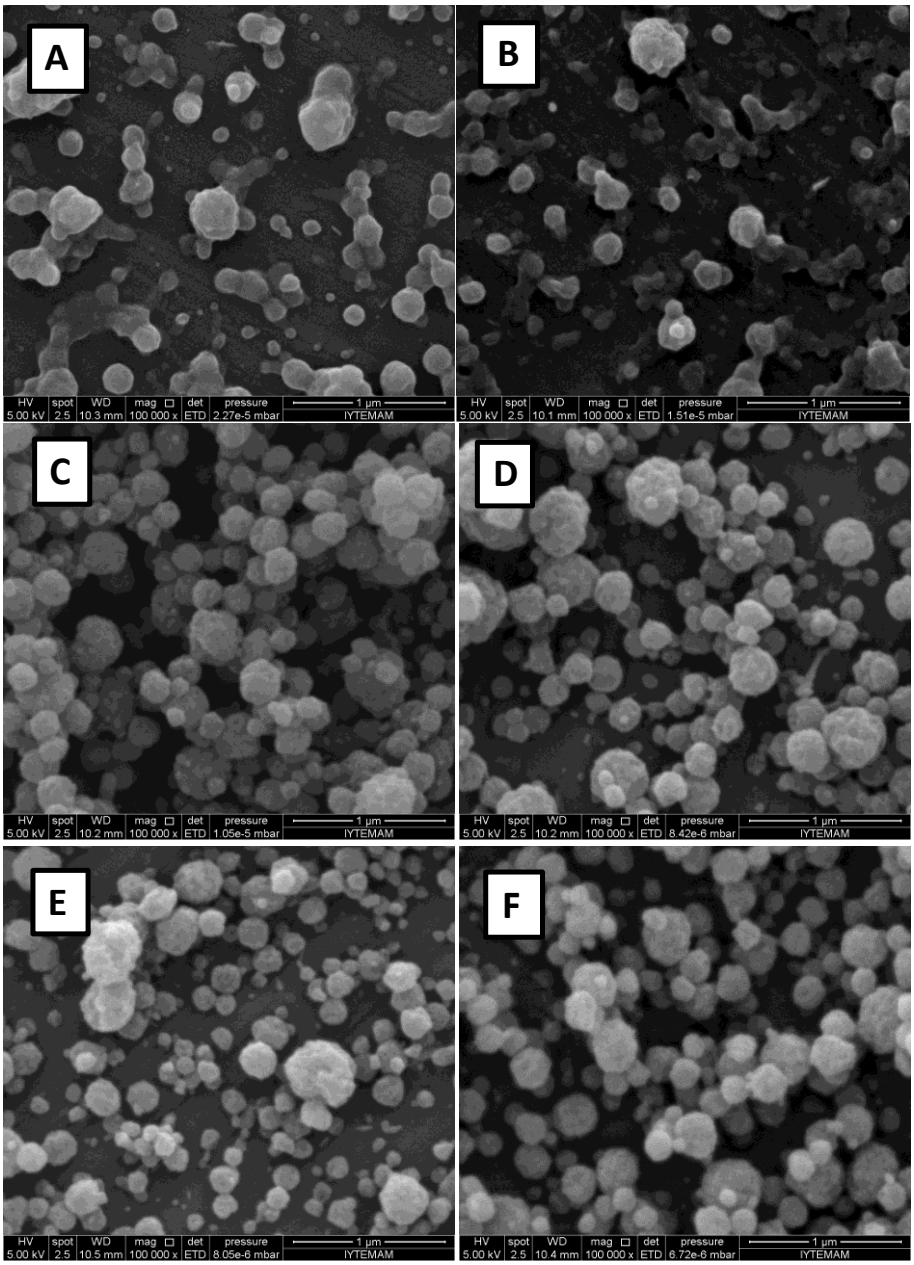


Figure 5.30. SEM images showing the effect of *S.spinosum* extract loading amount on the size and shape of microparticles obtained at a constant flow rate (0.3 m/h), needle to collector distance (10 cm), and voltage (14 kV), zein concentration (5% ,w/v). *S.spinosum* : zein (w/w), A: 2:1 ; B: 1:1 ; C: 1:5 ; D: 1:10 ; E: 1:20 ; F:1:50.

Particle size distribution obtained at a (the extract powder: zein) weight ratio of 1:5 was illustrated in Figure 5.31. The average diameter of *S.spinosum* loaded zein microparticles was found as 193± 57 nm. In addition to particle size, the extract loading efficiency microparticles at the extract to zein ratio of 1:5 was calculated. Encapsulation efficiency was measured as 96 % for 1:5 (*S.spinosum* extract: zein) weight ratio.

Therefore, 1:5 (*S.spinosum* extract:zein) weight ratio was determined as optimum weight ratio to prepare *S.spinosum* loaded zein microstructures for rest of this study.

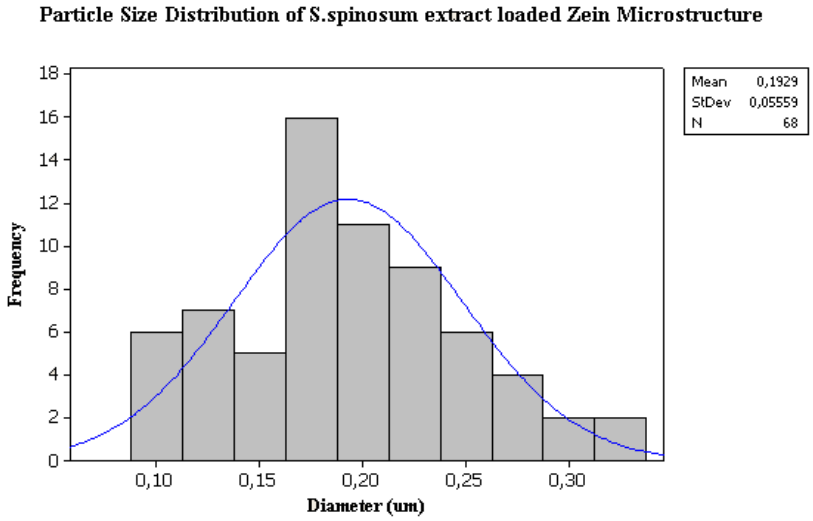


Figure 5.31. Particle size distribution of *S.spinosum* extract loaded zein microparticles prepared 1:5 extract zein weight ratio.

5.2.2.2. The Activity of Extract-loaded Zein Microparticles

The *S.spinosum* extract loaded in zein microparticles that was prepared at (*S.spinosum* extract: zein) weight ratio of 1:5 was analyzed for their total antioxidant activity and cytotoxicity. The antioxidant activity of the extract loaded zein microparticles was measured as 377.8 mmole/g dried weight. The 73.7% of initially loaded *S.spinosum* extract was regained using beat beaker for 15 min. The extract loaded zein microparticles were expected to show antimicrobial activity due to antimicrobial activity of the extract used. Previous studies; done by the researchers have shown that zein had good preservation capability to protect the activity of agent integrated within zein (Li et al., 2009; Y. Luo et al., 2013; Neo et al., 2013; Parris et al., 2005; Yunpeng Wu et al., 2012).

5.2.2.3. Cytotoxicity Analysis

Cell viability was investigated for *S. spinosum* extract loaded zein particles. In order to measure it, cells were exposed to *S. spinosum* extract at a concentration of 50µg/ml because this concentration level was not significantly toxic for fibroblast cell line. In addition to control group, cells were exposed to zein particle containing no extract. As seen in Figure 5.32, *S. spinosum* extract loaded zein microparticles showed similar effect with same concentration of *S. spinosum* extract alone.

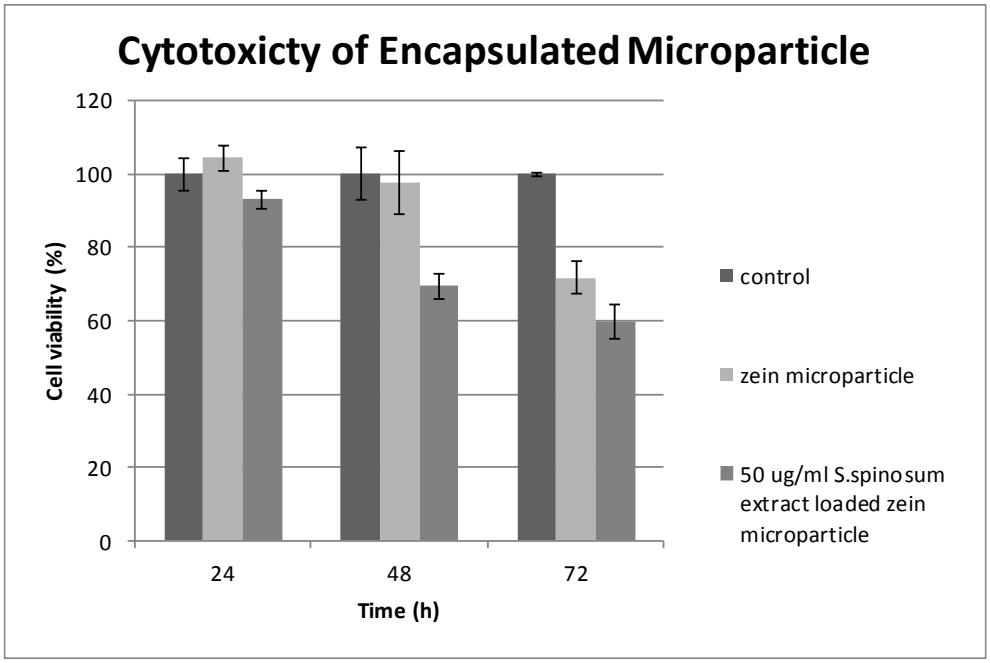


Figure 5.32. Percentage of cell viability of NIH 3T3 mouse fibroblast cells treated with *S. spinosum* extract loaded zein microparticles.

CHAPTER 6

CONCLUSION

Medicinal plants include phytochemicals that can have both antioxidant and antimicrobial activities. *Sarcopoterium spinosum* as a medicinal plant used in this study was mostly found in Mediterranean region. Although *S. spinosum* was used as a medicinal plant, there is only little research about *S. spinosum* in literature. The extraction of bioactive compounds from plant materials is the first step to the recovery of the polyphenols.

The optimum conditions for the extraction of *S. spinosum* leaves were determined using response surface methodology (RSM). The total phenol content, total antioxidant and antimicrobial activities and phenolic composition of *S. spinosum* extract were investigated. *S. spinosum* extract showed relatively high antioxidant activity when compared with many other medicinal plants. The identification of phenolics in *S. spinosum* extract using by HPLC has shown the presence of hyperoside and isoquercetin. Isoquercetin was found to be the major compound in the extract. To the best of our knowledge, this is the first report demonstrating the presence of isoquercetin and hyperoside within *S. spinosum* extract.

In addition to antioxidant activity, *S. spinosum* extract has shown significant antimicrobial activity. *S. spinosum* extract were successfully encapsulated within zein particles via electrospray method in order to contribute to the stability of the extract. The effects of process parameters of electrospray method on particle size distribution and morphology were extensively investigated. By changing process parameters, zein particles were successfully prepared to encapsulate *S. spinosum* extract with high encapsulation efficiency. The extract loaded zein particles had antioxidant capacity. The study demonstrated that electrospray process could be a potential new approach for enhancing the extract stability by encapsulation.

REFERENCES

Ainsworth, Elizabeth A, & Gillespie, Kelly M. (2007). Estimation of total phenolic content and other oxidation substrates in plant tissues using Folin–Ciocalteu reagent. *Nature Protocols, 2*(4), 875-877.

Al-Mustafa, A.H., & Al-Thunibat, O.Y. (2008). Antioxidant activity of some Jordanian medicinal plants used traditionally for treatment of diabetes. *Pak J Biol Sci, 11*(3), 351-358.

Alarcon-Aguilara, FJ, Roman-Ramos, R., Perez-Gutierrez, S., Aguilar-Contreras, A., Contreras-Weber, CC, & Flores-Saenz, JL. (1998). Study of the anti-hyperglycemic effect of plants used as antidiabetics. *Journal of Ethnopharmacology, 61*(2), 101-110.

Barbosa-Filho, J.M., Alencar, A.A., Nunes, X.P., Tomaz, A.C., Sena-Filho, J.G., Athayde-Filho, P.F., . . . Da-Cunha, E.V.L. (2008). Sources of alpha-, beta-, gamma-, delta-and epsilon-carotenes: A twentieth century review. *Revista Brasileira de Farmacognosia, 18*(1), 135-154.

Bellik, Yuva, Boukraâ, Laïd, Alzahrani, Hasan A, Bakhotmah, Balkees A, Abdellah, Fatiha, Hammoudi, Si M, & Iguer-Ouada, Mokrane. (2012). Molecular Mechanism Underlying Anti-Inflammatory and Anti-Allergic Activities of Phytochemicals: An Update. *Molecules, 18*(1), 322-353.

Bravo, Laura. (1998). Polyphenols: chemistry, dietary sources, metabolism, and nutritional significance. *Nutrition reviews, 56*(11), 317-333.

Buchanan, Bob B, Gruissem, Wilhelm, & Jones, Russell L. (2000). *Biochemistry & molecular biology of plants*: American Society of Plant Physiologists Rockville.

Chakraborty, S., Liao, I., Adler, A., & Leong, K.W. (2009). Electrohydrodynamics: A facile technique to fabricate drug delivery systems. *Advanced drug delivery reviews, 61*(12), 1043-1054.

Corradini, E, Mattoso, LHC, Guedes, CGF, & Rosa, DS. (2004). Mechanical, thermal and morphological properties of poly (ε caprolactone)/zein blends. *Polymers for advanced technologies, 15*(6), 340-345.

Cowan, Marjorie Murphy. (1999). Plant products as antimicrobial agents. *Clinical microbiology reviews, 12*(4), 564-582.

Cragg, G.M., & Newman, D.J. (2005). Plants as a source of anti-cancer agents. *Journal of ethnopharmacology, 100*(1), 72-79.

Cushnie, TP, & Lamb, Andrew J. (2005). Antimicrobial activity of flavonoids. *International journal of antimicrobial agents, 26*(5), 343-356.

D'Archivio, Massimo, Filesi, Carmela, Di Benedetto, Roberta, Gargiulo, Raffaella, Giovannini, Claudio, & Masella, Roberta. (2007). Polyphenols, dietary sources and bioavailability. *ANNALI-ISTITUTO SUPERIORE DI SANITA, 43*(4), 348.

Dai, Jin, & Mumper, Russell J. (2010). Plant phenolics: extraction, analysis and their antioxidant and anticancer properties. *Molecules, 15*(10), 7313-7352.

Das, K., Tiwari, RKS, & Shrivastava, DK. (2010). Techniques for evaluation of medicinal plant products as antimicrobial agent: Current methods and future trends. *Journal of Medicinal Plants Research, 4*(2), 104-111.

Denev, Petko N, Kratchanov, Christo G, Ciz, Milan, Lojek, Antonin, & Kratchanova, Maria G. (2012). Bioavailability and Antioxidant Activity of Black Chokeberry (Aronia melanocarpa) Polyphenols: in vitro and in vivo Evidences and Possible Mechanisms of Action: A Review. *Comprehensive Reviews in Food Science and Food Safety, 11*(5), 471-489.

Desai, Kashappa Goud H, & Jin Park, Hyun. (2005). Recent developments in microencapsulation of food ingredients. *Drying Technology, 23*(7), 1361-1394.

Fang, Z., & Bhandari, B. (2010). Encapsulation of polyphenols–a review. *Trends in Food Science & Technology, 21*(10), 510-523.

Fu, Jian-Xi, Wang, Hua-Jie, Zhou, Yan-Qing, & Wang, Jin-Ye. (2009). Antibacterial activity of ciprofloxacin-loaded zein microsphere films. *Materials Science and Engineering: C, 29*(4), 1161-1166. doi: 10.1016/j.msec.2008.09.031

Fukumoto, LR, & Mazza, G. (2000). Assessing antioxidant and prooxidant activities of phenolic compounds. *Journal of Agricultural and Food Chemistry, 48*(8), 3597-3604.

Gargano, Domenico, Fenu, Giuseppe, Medagli, Piero, Sciandrello, Saverio, & Bernardo, Liliana. (2007). The status of Sarcopoterium spinosum (Rosaceae) at the western periphery of its range: Ecological constraints lead to conservation concerns. *Israel Journal of Plant Sciences, 55*(1), 1-13.

Gomez-Estaca, J., Balaguer, MP, Gavara, R., & Hernandez-Munoz, P. (2012). Formation of zein nanoparticles by electrohydrodynamic atomization: Effect of the main processing variables and suitability for encapsulating the food coloring and active ingredient curcumin. *Food Hydrocolloids, 28*(1), 82-91.

Gómez-Estaca, J., Balaguer, MP, Gavara, R., & Hernández-Muñoz, P. (2010). Nanoencapsulation of the functional food ingredient curcumin by electrohydrodynamic atomization.

Hamdan, I.I., & Afifi, F.U. (2008). Screening of Jordanian flora for α-amylase inhibitory activity. *Pharmaceutical Biology, 46*(10-11), 746-750.

Handa, S.S., Khanuja, S.P.S., Longo, G., & Rakesh, D.D. (2008). *Extraction technologies for medicinal and aromatic plants*: Earth, Environmental and Marine Sciences and Technologies.

Hsu, C.L., Wu, C.H., Huang, S.L., & Yen, G.C. (2009). Phenolic compounds rutin and o-coumaric acid ameliorate obesity induced by high-fat diet in rats. *Journal of Agricultural and Food Chemistry, 57*(2), 425-431.

Jaworek, A. (2007). Micro-and nanoparticle production by electrospraying. *Powder technology, 176*(1), 18-35.

Jaworek, A., & Sobczyk, AT. (2008). Electrospraying route to nanotechnology: An overview. *Journal of Electrostatics, 66*(3), 197-219.

Kasabri, V., Afifi, F.U., & Hamdan, I. (2011). In vitro and in vivo acute antihyperglycemic effects of five selected indigenous plants from Jordan used in traditional medicine. *Journal of Ethnopharmacology, 133*(2), 888-896.

Kosaraju, S.L., Labbett, D., Emin, M., Konczak, I., & Lundin, L. (2008). Delivering polyphenols for healthy ageing. *Nutrition & Dietetics, 65*, S48-S52.

Lawton, John W. (2002). Zein: A History of Processing and Use. *Cereal Chem., 79*(1), 1-18.

Lester, G.E., Lewers, K.S., Medina, M.B., & Saftner, R.A. (2012). Comparative analysis of strawberry total phenolics via Fast Blue BB vs. Folin-Ciocalteu: assay interference by ascorbic acid. *Journal of Food Composition and Analysis*.

Li, Y., Lim, L.T., & Kakuda, Y. (2009). Electrospun Zein Fibers as Carriers to Stabilize (−)Epigallocatechin Gallate. *Journal of food science, 74*(3), C233-C240.

Liu, Rui Hai. (2004). Potential synergy of phytochemicals in cancer prevention: mechanism of action. *The Journal of Nutrition, 134*(12), 3479S-3485S.

Luo, Y., Wang, T. T., Teng, Z., Chen, P., Sun, J., & Wang, Q. (2013). Encapsulation of indole-3-carbinol and 3,3'-diindolylmethane in zein/carboxymethyl chitosan nanoparticles with controlled release property and improved stability. *Food Chem, 139*(1-4), 224-230. doi: 10.1016/j.foodchem.2013.01.113

Luo, Y., Zhang, B., Whent, M., Yu, L. L., & Wang, Q. (2011). Preparation and characterization of zein/chitosan complex for encapsulation of alpha-tocopherol, and its in vitro controlled release study. *Colloids Surf B Biointerfaces, 85*(2), 145-152. doi: 10.1016/j.colsurfb.2011.02.020

Luo, Yangchao, Zhang, Boce, Cheng, Wen-Hsing, & Wang, Qin. (2010). Preparation, characterization and evaluation of selenite-loaded chitosan/TPP nanoparticles with or without zein coating. *Carbohydrate Polymers, 82*(3), 942-951. doi: 10.1016/j.carbpol.2010.06.029

Madigan, Michael T, Martinko, John M, Dunlap, Paul V, & Clark, David P. (2008). *Brock Biology of microorganisms 12th edn* (Vol. 11).

Montgomery, Douglas C. (2008). *Design and analysis of experiments*: Wiley.

Munin, A., & Edwards-Lévy, F. (2011). Encapsulation of Natural Polyphenolic Compounds; a Review. *Pharmaceutics, 3*(4), 793-829.

Mustafa, A., & Turner, C. (2011). Pressurized liquid extraction as a green approach in food and herbal plants extraction: A review. *Analytica chimica acta, 703*(1), 8-18.

Naasani, Imad, Oh-hashi, Fujiko, Oh-hara, Tomoko, Feng, Wan Yong, Johnston, Jeffrey, Chan, Kenneth, & Tsuruo, Takashi. (2003). Blocking telomerase by dietary polyphenols is a major mechanism for limiting the growth of human cancer cells in vitro and in vivo. *Cancer research, 63*(4), 824-830.

Nedovic, Viktor, Kalusevic, Ana, Manojlovic, Verica, Levic, Steva, & Bugarski, Branko. (2011). An overview of encapsulation technologies for food applications. *Procedia Food Science, 1*, 1806-1815.

Neo, Y. P., Ray, S., Jin, J., Gizdavic-Nikolaidis, M., Nieuwoudt, M. K., Liu, D., & Quek, S. Y. (2013). Encapsulation of food grade antioxidant in natural biopolymer by electrospinning technique: a physicochemical study based on zein-gallic acid system. *Food Chem, 136*(2), 1013-1021. doi: 10.1016/j.foodchem.2012.09.010

Nobili, S., Lippi, D., Witort, E., Donnini, M., Bausi, L., Mini, E., & Capaccioli, S. (2009). Natural compounds for cancer treatment and prevention. *Pharmacological Research, 59*(6), 365-378.

Nostro, A., Germano, MP, D'angelo, V., Marino, A., & Cannatelli, MA. (2001). Extraction methods and bioautography for evaluation of medicinal plant antimicrobial activity. *Letters in Applied Microbiology, 30*(5), 379-384.

Paiva, PMG, Gomes, FS, Napoleão, TH, Sá, RA, Correia, MTS, & Coelho, L. (2010). Antimicrobial activity of secondary metabolites and lectins from plants. *Current Research, Technology and Education Topics in Applied Microbiology and Microbial Biotechnology, Badajoz: Formatex*, 396-406.

Parris, N., Cooke, P.H., & Hicks, K.B. (2005). Encapsulation of essential oils in zein nanospherical particles. *Journal of agricultural and food chemistry, 53*(12), 4788-4792.

Patel, DK, Prasad, SK, Kumar, R., & Hemalatha, S. (2012). An overview on antidiabetic medicinal plants having insulin mimetics property. *Asian Pac J Trop Biomed, 2*(4), 320-330.

Quispe-Condori, S., Saldaña, M.D.A., & Temelli, F. (2011). Microencapsulation of flax oil with zein using spray and freeze drying. *LWT-Food Science and Technology, 44*(9), 1880-1887.

Rao, M.U., Sreenivasulu, M., Chengaiah, B., Reddy, K.J., & Chetty, C.M. (2010). Herbal medicines for diabetes mellitus: a review. *Int J PharmTech Res, 2*(3), 1883-1892.

Reher, G., Slijepcevic, M., & Kraus, L. (1991). Hypoglycemic activity of triterpenes and tannins from Sarcopoterium spinosum and two Sanguisorba species Pl. *Med, 57*, A57-A58.

Salata, O.V. (2005). Tools of nanotechnology: Electrospray. *Current nanoscience, 1*(1), 25-33.

Sarıkaya, B.B., & Kayalar, H. (2010). Quantitative determination of α-tocopherol and quality control studies in *Sarcopoterium spinosum* L.

Singab, A.N.B., El-Beshbishy, H.A., Yonekawa, M., Nomura, T., & Fukai, T. (2005). Hypoglycemic effect of Egyptian Morus alba root bark extract: Effect on diabetes and lipid peroxidation of streptozotocin-induced diabetic rats. *Journal of ethnopharmacology, 100*(3), 333-338.

Smirin, P., Taler, D., Abitbol, G., Brutman-Barazani, T., Kerem, Z., Sampson, S.R., & Rosenzweig, T. (2010). *Sarcopoterium spinosum* extract as an antidiabetic agent: In vitro and in vivo study. *Journal of ethnopharmacology, 129*(1), 10-17.

Su, L., Yin, J.J., Charles, D., Zhou, K., Moore, J., & Yu, L.L. (2007). Total phenolic contents, chelating capacities, and radical-scavenging properties of black peppercorn, nutmeg, rosehip, cinnamon and oregano leaf. *Food Chemistry, 100*(3), 990-997.

Torres-Giner, S., Martinez-Abad, A., Ocio, M.J., & Lagaron, J.M. (2010). Stabilization of a Nutraceutical Omega-3 Fatty Acid by Encapsulation in Ultrathin Electrosprayed Zein Prolamine. *Journal of food science, 75*(6), N69-N79.

Wamtinga Richard Sawadogo, Marc Schumacher, Marie-He´ le`ne Teiten „ & Mario Dicato, Marc Diederich. (2012). Traditional West African pharmacopeia, plants and derived compounds for cancer therapy. *Biochemical Pharmacology, 84*, 1225-1240.

Wellwood, Carol RL, & Cole, Rosemary A. (2004). Relevance of carnosic acid concentrations to the selection of rosemary, Rosmarinus officinalis (L.), accessions for optimization of antioxidant yield. *Journal of agricultural and food chemistry, 52*(20), 6101-6107.

Wu, Y., & Clark, R.L. (2008). Electrohydrodynamic atomization: a versatile process for preparing materials for biomedical applications. *Journal of Biomaterials Science, Polymer Edition, 19*(5), 573-601.

Wu, Yunpeng, Luo, Yaguang, & Wang, Qin. (2012). Antioxidant and antimicrobial properties of essential oils encapsulated in zein nanoparticles prepared by liquid–liquid dispersion method. *LWT - Food Science and Technology, 48*(2), 283-290. doi: 10.1016/j.lwt.2012.03.027

Xiao, D., Davidson, P.M., & Zhong, Q. (2011). Spray-dried zein capsules with coencapsulated nisin and thymol as antimicrobial delivery system for enhanced antilisterial properties. *Journal of agricultural and food chemistry, 59*(13), 7393-7404.

Xie, J., & Wang, C.H. (2007). Encapsulation of proteins in biodegradable polymeric microparticles using electrospray in the Taylor cone-jet mode. *Biotechnology and bioengineering, 97*(5), 1278-1290.

Yanez, J., Vicente, V., Alcaraz, M., Castillo, J., Benavente-Garcia, O., Canteras, M., & Teruel, J.A.L. (2004). Cytotoxicity and antiproliferative activities of several phenolic compounds against three melanocytes cell lines: relationship between structure and activity. *Nutrition and cancer, 49*(2), 191-199.

Ying X., Wang Y., Jing X., Wenjie Z., Haibo L., & Zhang C., and Li F. (2009). HPLC Determination of Eight Polyphenols in the Leaves of Crataegus pinnatifida Bge. var.major. *Journal of Chromatographic Science, 47*, 201-205.

Zheng, Y.Y., Viswanathan, B., Kesarwani, P., & Mehrotra, S. (2012). Dietary Agents in Cancer Prevention: An Immunological Perspective†. *Photochemistry and Photobiology*.

Ziech, D., Anestopoulos, I., Hanafi, R., Voulgaridou, G.P., Franco, R., Georgakilas, A.G., . . . Panayiotidis, M.I. (2012). Pleiotrophic effects of natural products in ROS-induced carcinogenesis: The role of plant-derived natural products in oral cancer chemoprevention. *Cancer Letters*.

APPENDIX A

CALIBRATION CURVE OF GALLIC ACID

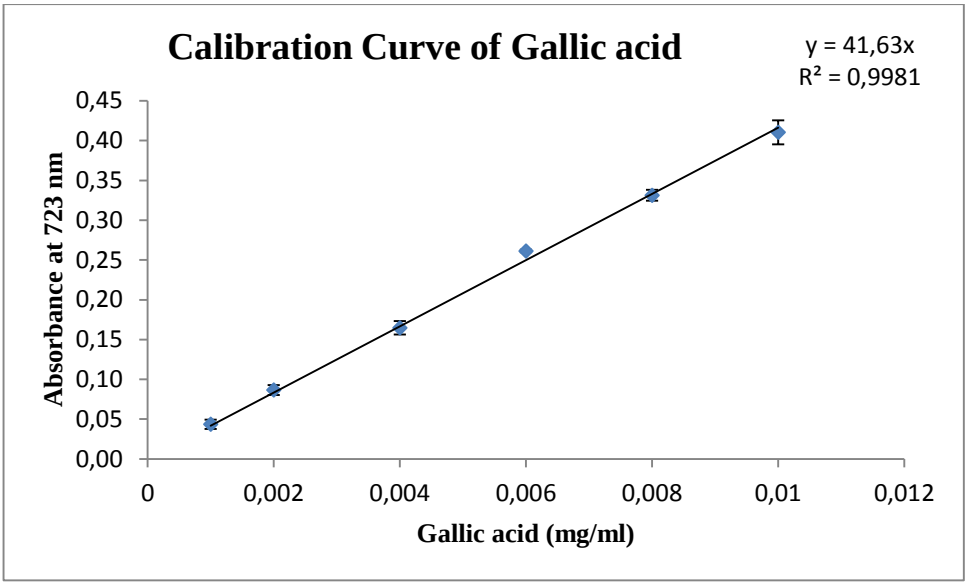


Figure A.1. Calibration curve of gallic acid standard which is used for expression of total phenol contents as gallic acid equivalents.

APPENDIX B

CALIBRATION CURVE OF TROLOX

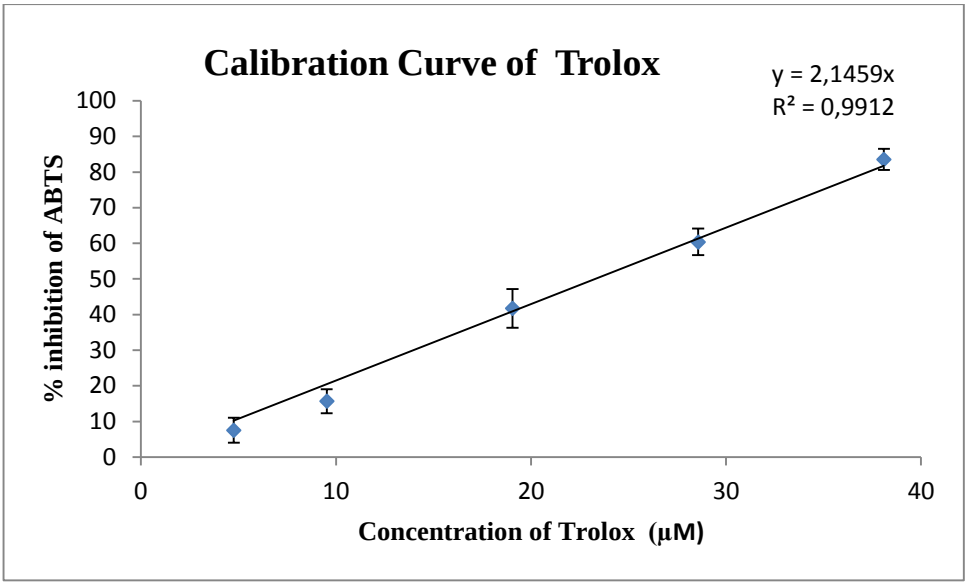


Figure B.1. Calibration curve of gallic acid standard which is used for expression of total phenol contents as gallic acid equivalents.

APPENDIX C

HPLC CHROMATOGRAMS OF USED STANDARDS

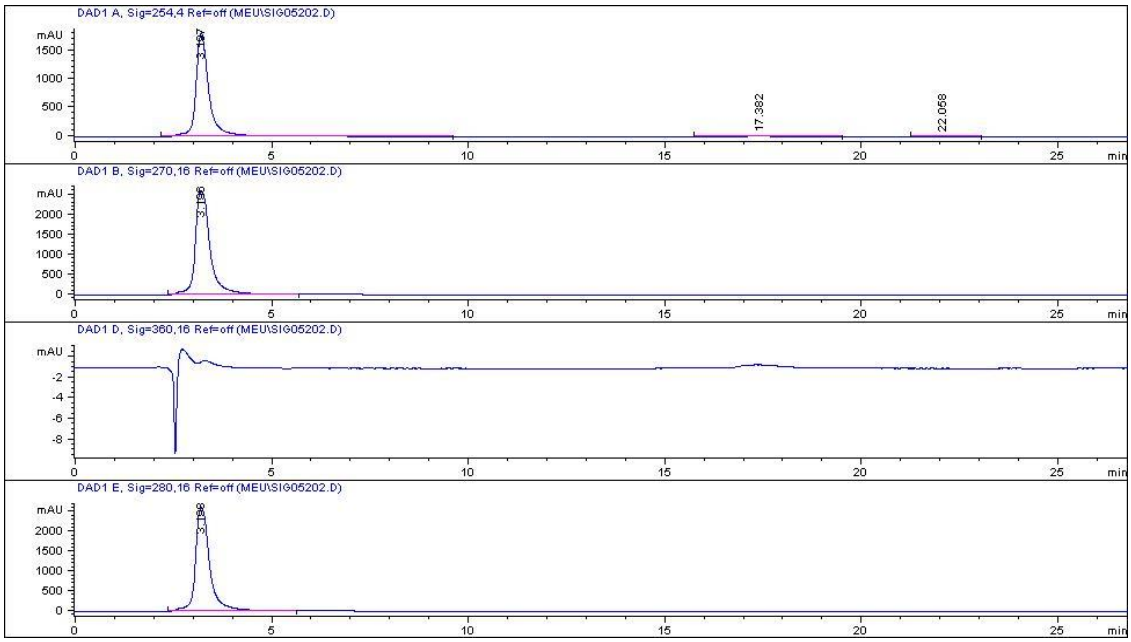


Figure C.1. HPLC Chromatogram of gallic acid as a reference standard.

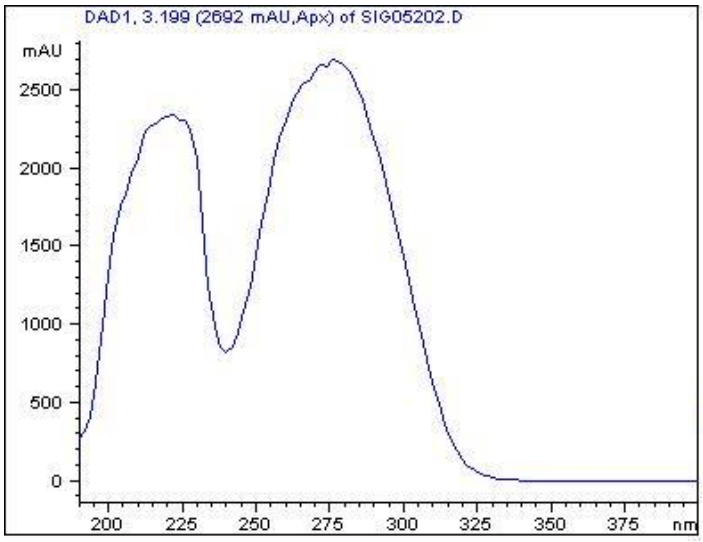


Figure C.2. UV spectra of gallic acid as a reference standard.

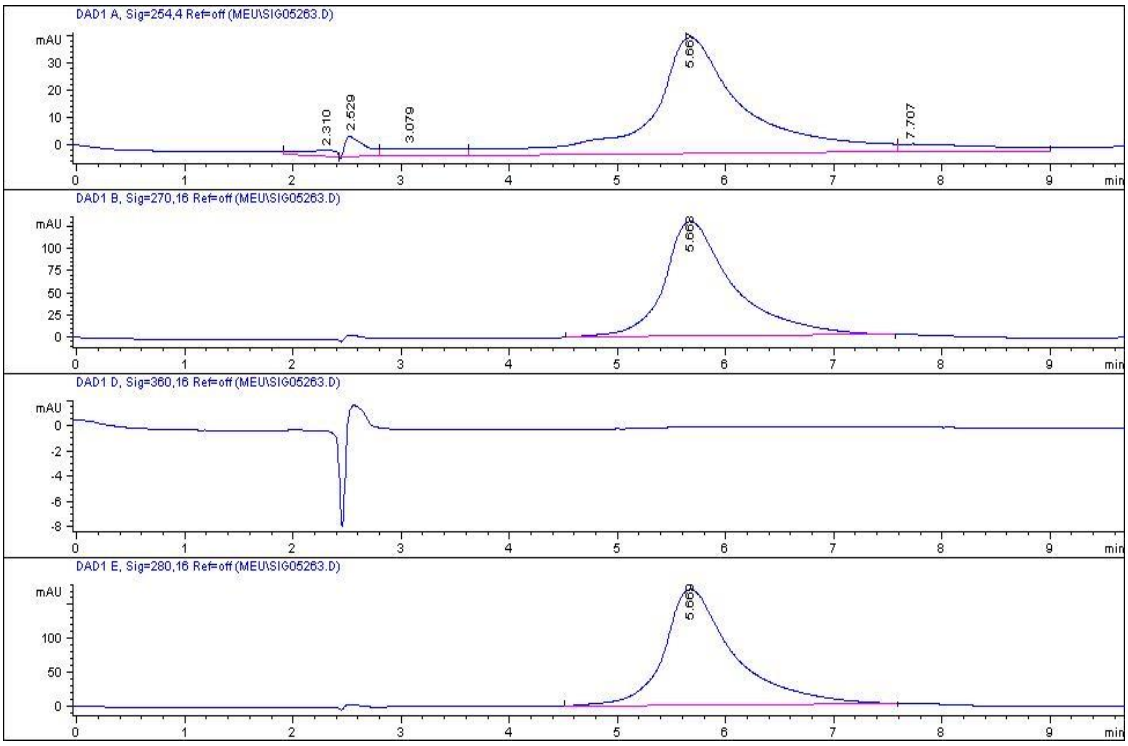


Figure C.3. HPLC Chromatogram of catechin as a reference standard.

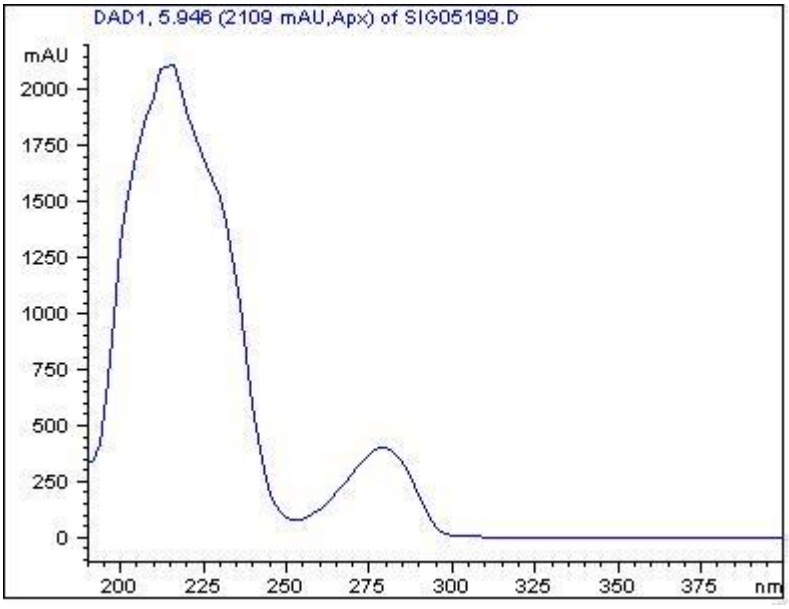


Figure C.4. UV spectra of catechin as a reference standard

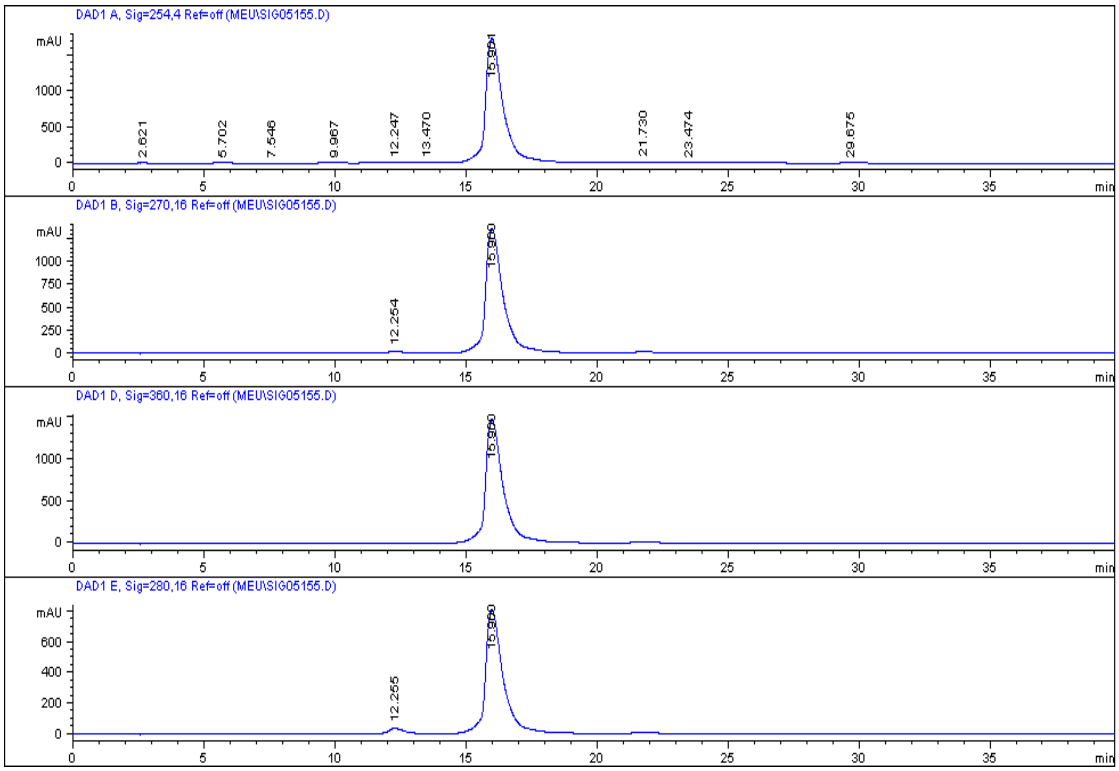


Figure C.5. HPLC Chromatogram of rutin as a reference standard.

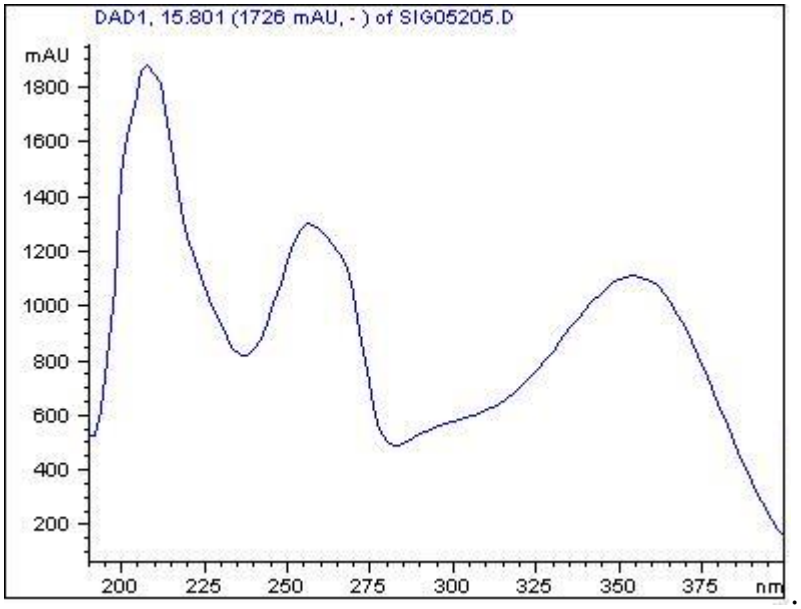


Figure C.6. UV spectra of rutin as a reference standard

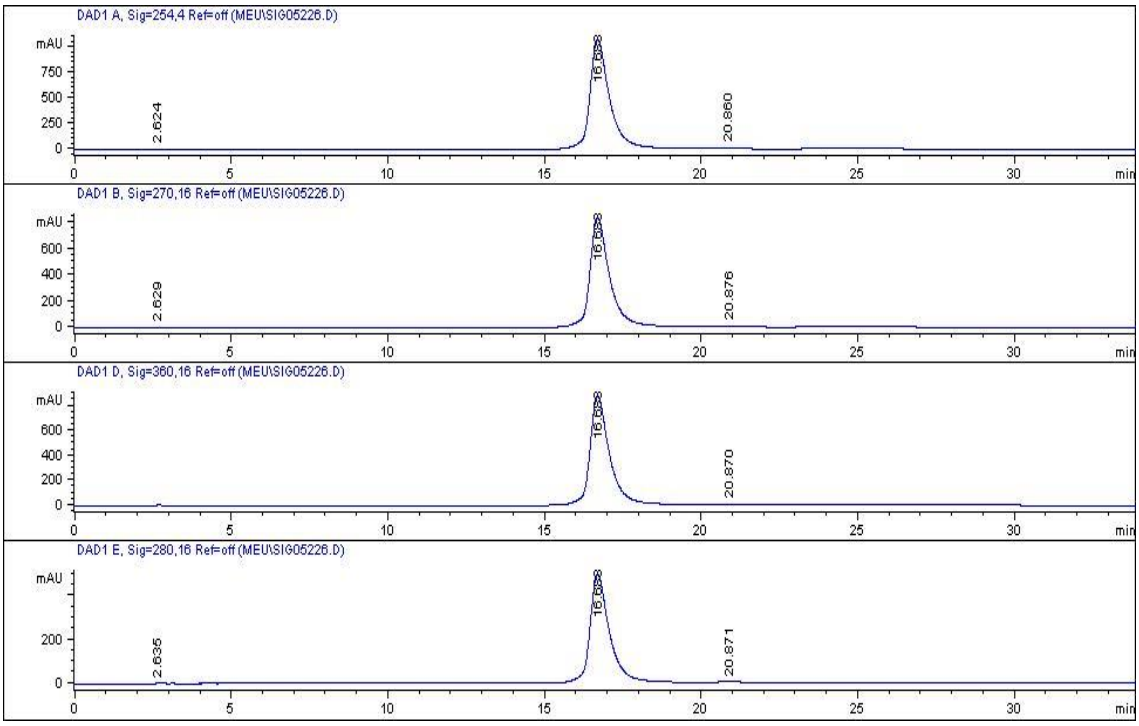


Figure C.7. HPLC Chromatogram of hyperoside as a reference standard.

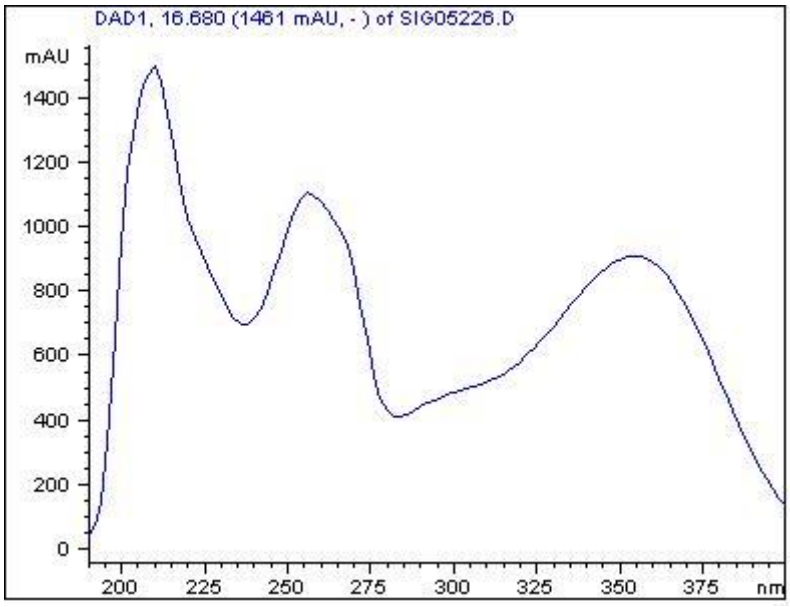


Figure C.8. UV spectra of hyperoside as a reference standard.

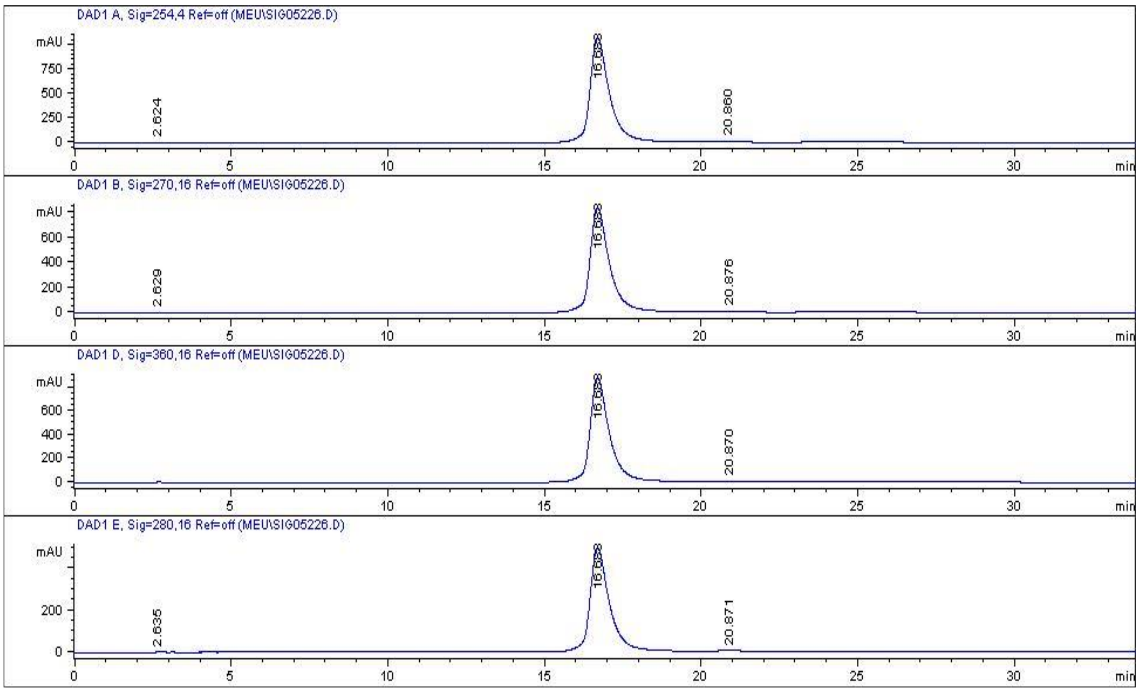


Figure C.9. HPLC Chromatogram of isoquercetin as a reference standard.

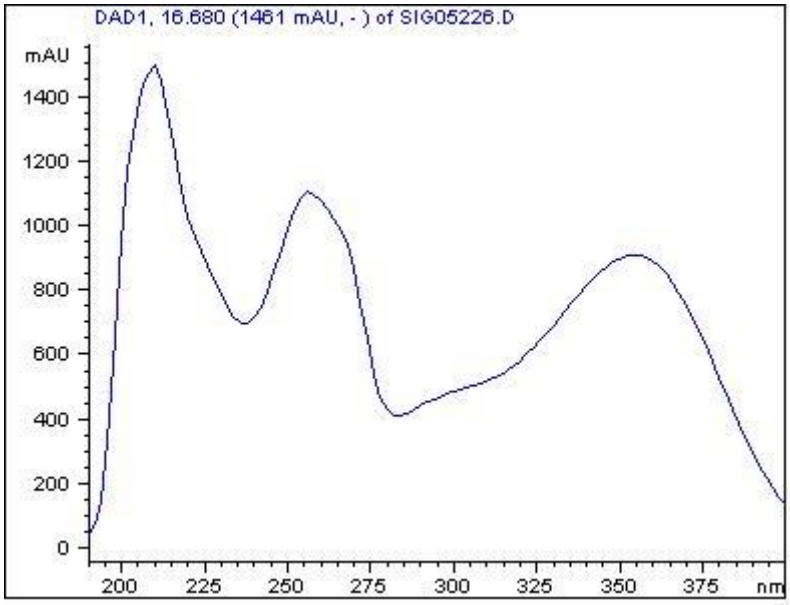


Figure C.10. UV spectra of isoquercetin as a reference standard.

APPENDIX D

CALIBRATION CURVE OF USED STANDARDS IN HPLC

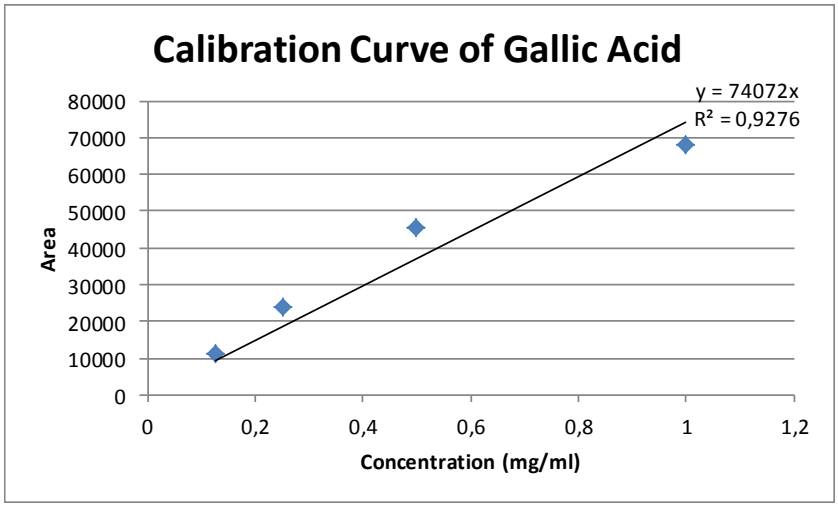


Figure D.1. Calibration curve of gallic acid standard in HPLC which is used for expression of the amount found in extract.

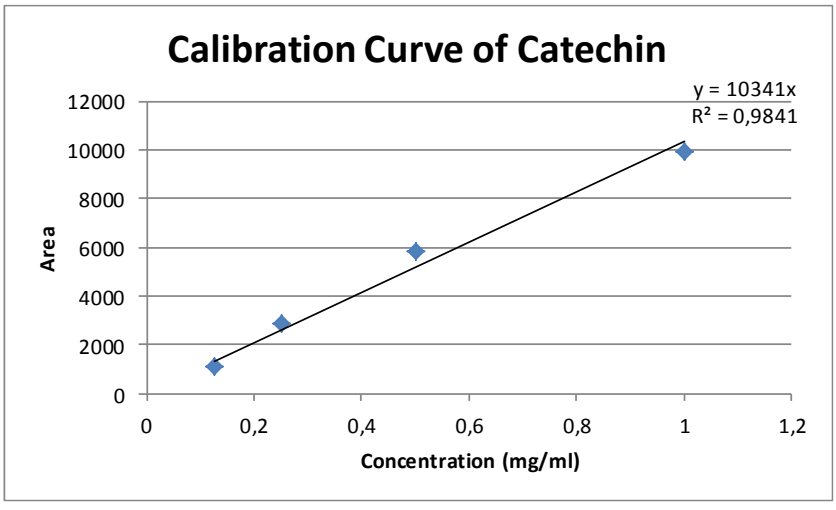


Figure D.2. Calibration curve of catechin standard in HPLC which is used for expression of the amount found in extract.

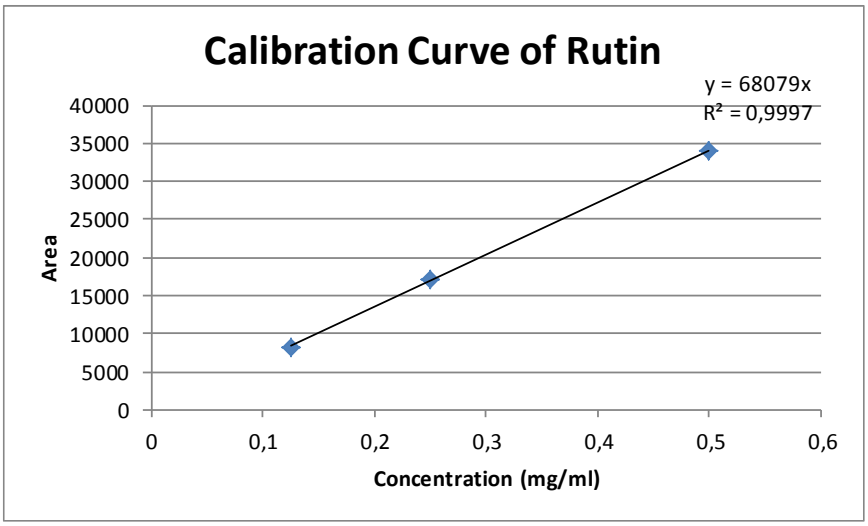


Figure D.3. Calibration curve of rutin standard in HPLC which is used for expression of the amount found in extract.

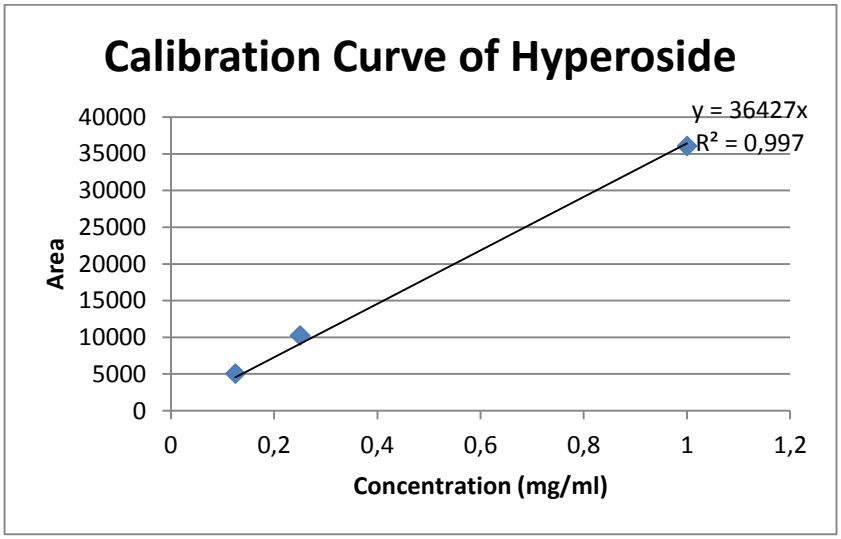


Figure D.4. Calibration curve of hyperoside standard in HPLC which is used for expression of the amount found in extract.

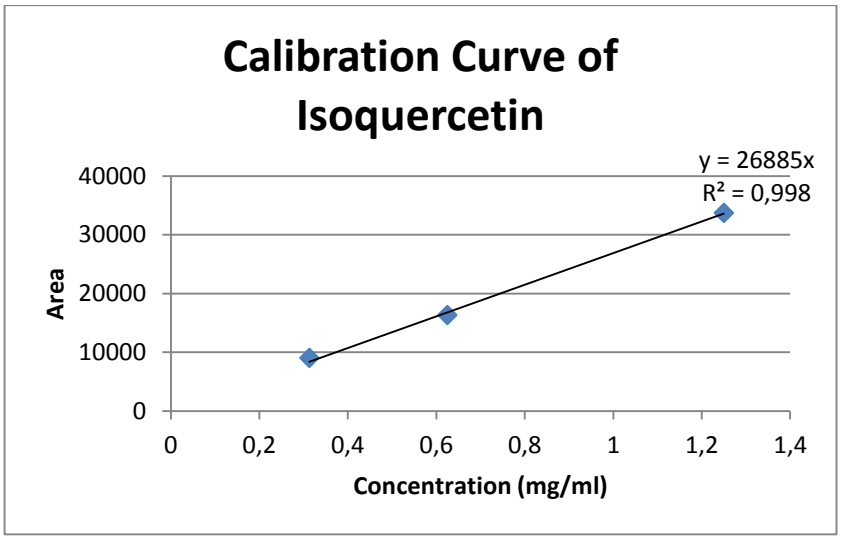


Figure D.5. Calibration curve of isoquercetin standard in HPLC which is used for expression of the amount found in extract.