INVESTIGATION OF HEMOSTATIC BIOMATERIALS CONTAINING PLANT EXTRACTS

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ABSTRACT

INVESTIGATION OF HEMOSTATIC BIOMATERIALS CONTAINING PLANT EXTRACTS

Haemostasis in other words a process which causes bleeding to stop is very important in injury. Recent researches were focused on discovery of haemostatic agents and developing biomaterials which transfer them to the injury side. Therefore plant extracts and three dimensional biomaterials were widely investigated. In this research the extract of *Equisetum arvense* was investigated as a coagulatory agent. Silk fibroin-hyaluronic acid mixture was used for the preparation of three dimensional sponge like biomaterials. The prepared sponge forms could also be used as scaffold for wound healing. In this research effect of extraction parameters on extract composition and bioactivity was investigated. Effect of extraction parameters were analyzed on 6 factors at 3 levels. It was seen that extraction parameters had high influence on both composition and bioactivity of the prepared extract. Although it was reported that the extract of *equisetum arvense* had anticoagulant activity, in this research it was shown that changing parameters caused variation of extract bioactivity from anticoagulant to coagulant as a result of changing extract composition. Addition of extract into silk fibroin-hyaluronic acid mixture caused proteins to precipitate as a result of interaction between protein and phenolic compounds. As a result of this precipitation significant decrease in the mechanical strength of biomaterial was observed. In order to minimize this interaction, plant extract was added into the silk fibroin after mixing with hyaluronic acid solution. As a result, mechanical strength and pore size of the biomaterial were increased and pore distribution became more regular. Also biomaterial gained tubular network on both vertical and horizontal dimensions. This would help the proliferation and migration of the fibroblast cells and moreover prevent the formation of scar tissue.

ÖZET

BİTKİ ÖZÜTÜ İÇEREN HEMOSTATİK BİYOMALZEMELERİN ARAŞTIRILMASI

Hemostaz olarak adlandırılan kanama durdurma yaralanmalarda en öncelikli konudur. Günümüzde kanama durdurucu ajanlar ve bunların yara bölgesine uygulanmasına yönelik sistemlerin geliştirilmesi öncelikli çalışma alanlarındandır Bu ajanlar arasında bitki ekstraktları ve uygulama yöntemi olarak da üç boyutlu biyomalzemeler önem kazanmaktadır. Bu çalışma içerisinde antioksidan ve antimikrobiyal özellikleri üzerinde sıkça çalışılan *Equisetum arvense* bitki ekstraktı kanama durdurucu özellikleri bakımından incelenmiş, ipek fibroin-hyaluronik asit karışımından elde edilen üç boyutlu yapıda ajanın ilgili bölgeye taşınması ve yara iyileşmesini destekleyici yapı iskelesi olarak tasarlanmıştır. Bitki ekstraktlarının içeriğinde ve biyoaktivitedeki değişim ekstraksiyon parametlerinin 6 değişken ve 3 seviye üzerinden gösterilmiştir. Literatürde antikoagulant olarak bilinen *Equisetum arvense* özütünün farklı ekstraksiyon koşullarına bağlı olarak değişen içeriği sayesinde koagulant etkisinin de olduğu gösterilmiştir. İpek fibroin ve hyaluronik asit karşımına sıvı halde eklenen bitki ektraktının içeriğindeki fenolik bileşiklerin ipek fibroin proteinleri ile etkileşime girmesi sonucu malzemenin mekanik kuvvetini bozacak şekilde çökeltilere sebep olduğu gözlemlenmiştir. Bu bağlamda daha kullanışlı malzemelerin tasarlanması için farklı metotlar denenmiştir. İpek fibroin içerisine bitki ekstraktının, hyaluronik asit ile karıştırıldıktan sonra eklenmesi sonucu protein-fenolik bileşik etkileşiminin engellendiği ve malzemenin yapısını düzenlendiği gözlemlenmiştir. Artan hyaluronik asit sayesinde malzemenin hem dikey hem yatay eksende daha düzgün tübüler bir yapıya sahip olduğu, içeriğindeki bitki ekstraktı artışının ise daha düzenli ve geniş boyutlu gözenekleri oluşturduğu gözlemlenmiştir. Kanama durdurucu etkileri göz önüne alındığında hyaluronik asitin antikoagulant etkiye sahip olduğu, malzemeye katılan bitki ekstratının ise bu etkiyi azalttığı gözlemlenmiştir.

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INTRODUCTION

1.1 General Information

Blood loss is one of the main death reasons around world. One does not have to lose literally all of his blood to die. Depending upon the many factors like fitness level, sex, age, health, genetic backup, people can die from losing half or two-thirds of their blood. Losing one-third of the blood volume considered as a very serious event. A single deep cut can need a suturing and hospitalization, and without these processes even a single bleeding can cause death. In technical speech blood loss is known as hemorrhaging and defined as blood loss or blood escape from the circulatory system via leaking from the blood vessels (i.e. vagina, mouth, nose, ear) or through a break in the skin. It can be said that there are two types of blood loss. Desanguination and exsanguination were defined as blood loss in massive amounts and complete blood loss respectively. Exsanguination can be seen if injured part is not sutured. Exsanguination is the most common cause of death on the battlefield. Non-battlefield causes can include injuries by shooting or stabbing; motor vehicle accidents; suicides, and partial or complete cuts of limbs with keen objects. Also trauma to the spleen, kidneys, lungs and liver can cause severe internal bleeding which cause death. More over to these, serious trauma can cause tearing of major blood vessels and cause bleeding which results in death. It can be said that there are four stages of hemorrhage:

In stage 1, 15% (≈750 mL) of the blood loss can be observed. The blood pressure can be maintained, the respiratory rate is normal. There is no change in vital signs and fluid support is not necessary.

In stage 2, there can be up to 15–30% blood volume loss (≈750–1500 mL) occur. Arterial constrictions cannot maintain the cardiac output, rapid heartbeats (tachycardia) starts, blood pressure need to be maintained and diastolic pressure increased slightly. In this stage blood transfusion is strictly necessary.

The blood loss of 30 to 40 % (≈1500–2000 mL) is seen stage 3. In this stage systolic blood pressure drops to 100 mmHg or less. Hypovolemic shock was seen, heart beat rate increased (more than 120 bp). Blood transfusion will be necessary.

In step 4, more than 40% (>2000 mL) blood loss occurs. There is an extreme tachycardia seen with more than 140 bpm however the pulse is weak, systolic pressure drops to 70 mmHg, unconsciousness or even coma can be seen. Finally the limit of the body's compensation is reached and aggressive resuscitation is required in order to prevent death.

With its different types and different stages hemorrhage is a complicated event and handled seriously. As mentioned before it is the main reason of human deaths around the world, so bleeding should be stopped at the early stages. Many pharmaceutical and natural products were used and investigated in order to overcome the bleeding. In this project one of the alternative medicinal plant *equisetum arvense* which would describe in the further parts was going to be investigated for its homeostatic effects. After optimization of extraction parameters according to different responses by response surface methodology, the products were tested for their homeostatic activity. The most active extract was than embedded into a biomaterial and its release profile was studied.

Before going into the experimental part, one should clearly understood some topics like human skin, wound, bleeding and the plant that was investigated for its homeostatic effect. Our aim is to stop bleeding, most of external bleeding occurs as a result of wounds and every wound is a damage that occurs on the skin. So human skin will be the next chapter that should be mentioned.

1.2Human Skin

Basically human skin is the outer covering of human body. It is the first defense mechanism that body forms in order to have a protection against pathogens. The skin is formed of three layers of tissue. The outermost layer, epidermis which forms the protective structure, the dermis or stratum corneum which is a fibrous layer that supports and strengthens epidermis and lastly subcutaneous layer, the subcutis which is developed from fatty tissues that provide nutrients to the above two layers and insulates body. As seen in Figure 1, the epidermis is contains no blood vessels, it has a protective function. It consist of five types of cells which are: melanocytes, Langerhans cells, keratinocytes and merkels cells. The second layer under the epidermis is dermis, which is made up of connective tissues. It connected to epidermis strictly and contains nerve endings that provides senses. Dermis contains sweet glands, apocrine glands, hair follicles sebaceous glands, lymphatic vessels and blood vessels.

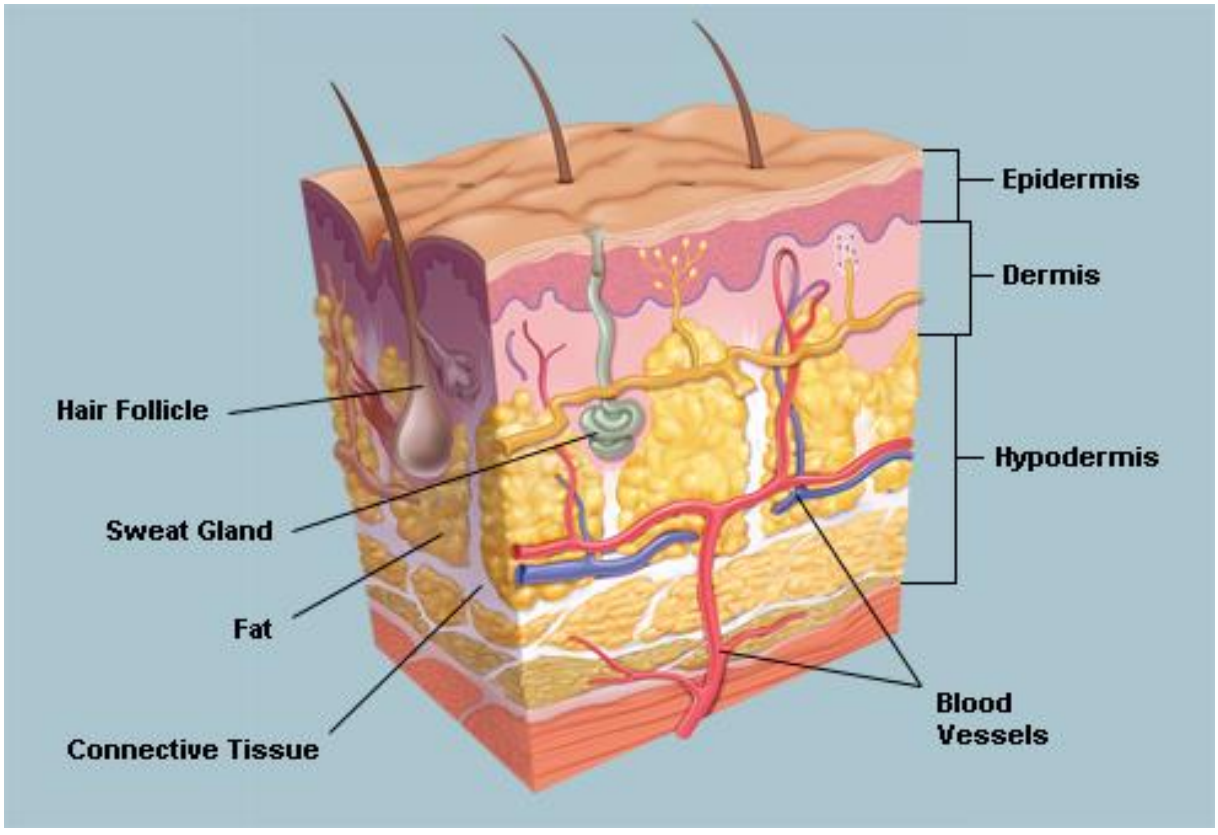

Figure1. Structure of the skin (1).

The last and the most important (for the research that was carried out) section of the skin is beneath the two layer, hypodermis. Actually the hypodermis is not part of the skin. Its function is only to attach the skin to the underlying bone and muscle by loose connective tissues and elastin. Fibroblasts, macrophages and adipocytes are the main cell types that seen on this layer. The adipocytes help the body to isolate; fibroblast synthesizes the extracellular matrix and collagen, the structural framework for animal tissues, and plays a critical role in wound healing. Macrophages function in both non-specific defense (innate immunity) as well as help initiate specific defense. In this layer, just below the surface of human skin, there is enormously well established network of blood vessels can be seen. Skin is covered by mass of arteries, veins, and capillaries. There are two reasons for this massive network system. Firstly this network supply maximum biologic needs of the skin, in exchange the skin is at the service of the blood vascular system, by functioning as a cooling device. The evaporation of sweat over the skin absorbs heat and cools down blood temperature. If the environment is cold, the body heat can be conserved, by contraction of vessel in quick, successive rhythm, which allows only a small amount of blood to flow through them. Secondly in addition to body temperature control, skin also plays a role in the regulation of blood pressure. By opening and closing of certain sphincter like vessels in the skin, flow of blood can be controlled. By this way, the circulation of the blood through the peripheral capillary beds could be allowed or it can be bypassed by parallel connections directly from small arteries to veins (2 - 4).

A trauma can disrupt the skin integrity and cause damage on the blood network. The total of these events are resulted in wound formation. In the next chapter wound will be explained briefly.

1.3 Wound

If the continuity of bodily tissue is disrupted this situation is called a wound. This continuity can be broken by an injury in which skin was damaged and open wound formed or a blunt force causes a contusion. In open wounds skin or mucous membrane which are the protective body surfaces were broken and by this way bleeding occurs and pathogens can easily enter into the tissue. As a result of bleeding these pathogens can easily exposed themselves to the other regions of the body. However in closed wounds no exposure to the outer environment can be seen and by this way healing occurs without any contamination. In both closed and open wounds hemorrhage occurs. In closed wounds the level of the injury depends mostly on the force and the direction of the force. Small forces may only cause damage on the skin and underlying soft tissues in the case of closed wounds. In these bruises infiltration of blood into the neighboring tissues can be seen as a result of rupturing of small vessels. Bleeding stops suddenly and the injury restored to normal. A stronger force may cause bigger damage in which larger vessels rupture and more blood leak to the extracellular matrix and causes hematoma. In open wounds the skin gets injured. The area exposed to the foreign materials and as a result infection can be occur after injury. If the area is big enough the tissue can be easily dry and the cooling effect of the air can give more harm to the tissue. Normally the healing process of the open wound in the skin is rapid, however if the injured area is deep and wide, death will be occur as a result of high blood vessel numbers under the skin.

The healing of a wound is a complex biological event which finalized in a long period of time. When the tissue continuity disrupted the edges of the tissue separated as a result of the elastic property of the skin. Blood start to fill the void as a result of bleeding, it overflow the cavity and coagulation of the blood occurs. The clots starts to dry and harden. Incrustation occurs. This crust starts to shrink and move the edges of the wound to each other. After a week time the scab removed from the injury part, newly formed tissue covers the area. Skin formation occurs and it covers the whole area. Figure-2 a-b shows the wound area after 3 days and 5 days respectively (5 - 7).

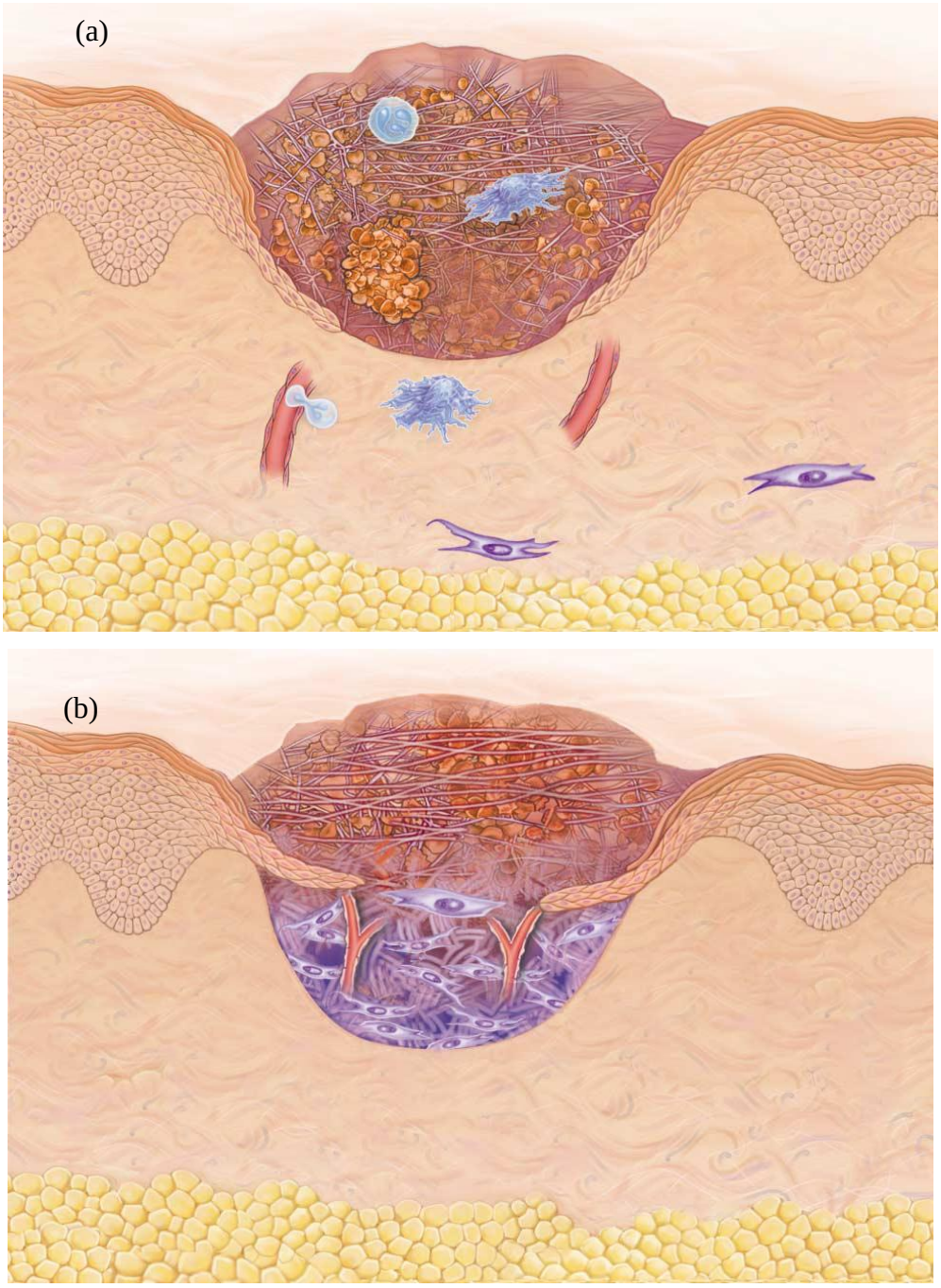

Figure 2. (a) Cutaneous Wound Three Days after Injury. (b) Cutaneous Wound Five Days after Injury.Blood vessels are seen sprouting into the fibrin clot as epidermal cells resurface the wound (8).

It can be said that in order to heal the wounds the first thing that should be done is to stop bleeding and stimulate the formation of fibrin clot rapidly. Blood coagulation will be the next chapter for understanding the whole process completely.

1.4 Blood Coagulation

Blood is a mobile tissue that transports both nutrients and oxygen to the cells and carries away the waste products and carbon dioxide from the cells. It can be said that blood is both tissue and fluid. Since it consists of similar cell types which have specific functions, it can be accepted as tissue. These cells are suspended in plasma and this plasma gives fluidity to the blood. If any interruption occurs in this liquid flow death will occur within few minutes because of the unsuitable environment for the tissues.

As mentioned above blood can be thought as a tissue because of the similar specialized cell types. The majority of blood volume is occupied by erythrocytes (red blood cells) with 45% and the rest of the cells like white blood cells and platelets only fill 1% of the total volume. Erythrocytes, platelets, lymphocytes and phagocytic cells are the four major types of blood cells. Both lymphocytes and phagocytic cells constitute white blood cells. White blood cells generate the immune system. They both protect body from invaders and clean up the system from pathogens. Erythrocytes transfer oxygen from lungs to cells by their hemoglobin molecule and transfer some of the carbon dioxide backwards. Platelets involve in the blood clotting. Although all of these cells are essential for life, from now on focusing on platelets can be more important because of key role in blood coagulation.

Platelets have the average size of two to four micrometers in diameter and they are the smallest cells in the blood. However their number in the circulating blood is 150000 to 400000 per mm^3, which is much more than white blood cells. They formed in the bone marrow and lack nucleus as so they have no proliferation ability. They have a high tendency to attach each other via hair like filaments on their membranes. The granules in the platelets contain factors important for clot promoting activity. Platelets take function in the hemostasis which is the control and prevention of bleeding. Normally the inner endothelial lining of the blood vessel inhibits the platelet activation by producing nitric oxide, endothelial-ADPase and PGI_2 (prostacyclin). ADP is the platelet activator and ADPase clears this factor from the lining. A cell adhesion ligand, von Willabrand factor (vWF), was produced by endothelial cells to adhere the collagens in the basement membrane layer. Without any injury these collagen does not exposed to the blood stream. Collagen, vWF and tissue factors were exposed to the stream when

the endothelial cells injured. Platelets contact with these substances and get activated. In the existence of calcium ions coagulation factors can bind to other platelets and accumulation of platelet plug occurs. This aggregation results in stop of bleeding and form a suitable site for blood clot. Without platelets this defense mechanism in which starts with stoppage of bleeding and ends with blood clot formation never occurs. Resistance of the capillary walls will be decreased in a platelet deficiency case and abnormal bleeding occurs. By the factors they secreted, platelet also become essential for the coagulation of the blood. After blood clot forms, the factors provide clot shrinkage and retraction of the clot.

After the formation of platelet plug coagulation of the blood occurs. Basically coagulation will define as removal of unstable plug and formation of stronger, more resilient blood clot by enzyme mediated, mutually dependent series of reactions that forms the thrombin and fibrin from fibrinogen. The formation of a clot is often referred to as secondary hemostasis. The first stage, primary hemostasis, is characterized by blood vessel constriction and platelet aggregation at the site of vessel injury. Numerous blood components that called coagulation factors involved in the coagulation process. It was discovered that there are thirteen factors that are take a role in coagulation. Coagulation can be triggered by two independent and separate pathways, extrinsic and intrinsic pathways. Both pathways intersect at the formation of factor X. After that point common pathway of coagulation begins which resulted in formation of the clot. The pathways and factors can be seen in Figure 3.

Intrinsic pathway involves a series of proteins, protein cofactors and enzymes, which interacts in reactions that take place on membrane surface. Tissue injury starts these reactions and at the end fibrin clot forms. As a result of negatively charged surface contact factor XII were activated and form the enzyme form of factor XII (factor XIIa). This enzyme catalyzes formational change of the factor XI to its enzyme form factor XIa. In the presence of Ca^{++} ions factor Xia convert factor IX to factor IXa. Factor IXa makes a complex with factor VIII on the surface of platelets membranes. This complex requires Ca^{++} ions in order to be stabilized. After stabilization factor X binds to that complex and activated into factor Xa. Factor Xa also forms another complex with factor V in the presence of calcium ions on the membrane surface. Prothrombin, a glycoprotein essential for clotting, binds to that complex and converted into thrombin which is an enzyme cleaves fibrinogen monomer to fibrin polymer. Thrombin also

transform factor XIII into factor XIIIa. This enzyme stabilizes fibrin polymers by cross-linkages.

To generate a thrombin burst is the main role of the extrinsic pathway. After the damage occurs in the blood vessel factor VII leaves the circulation and get in touch with tissue factor which is expressed on leukocytes and fibroblasts. So it can be said that extrinsic pathway activated by the tissue factor, which also known as tissue thromboplastin. Then tissue factor- factor VIIa complex forms. This complex can activate both factor IX and factor X. Factor VII is also activated by thrombin, factor VIIa, factor XII and factor Xa. The activation of factor X to factor Xa initiates the activation thrombin as same as on intrinsic pathway and resulted in fibrin clot formation (9-11).

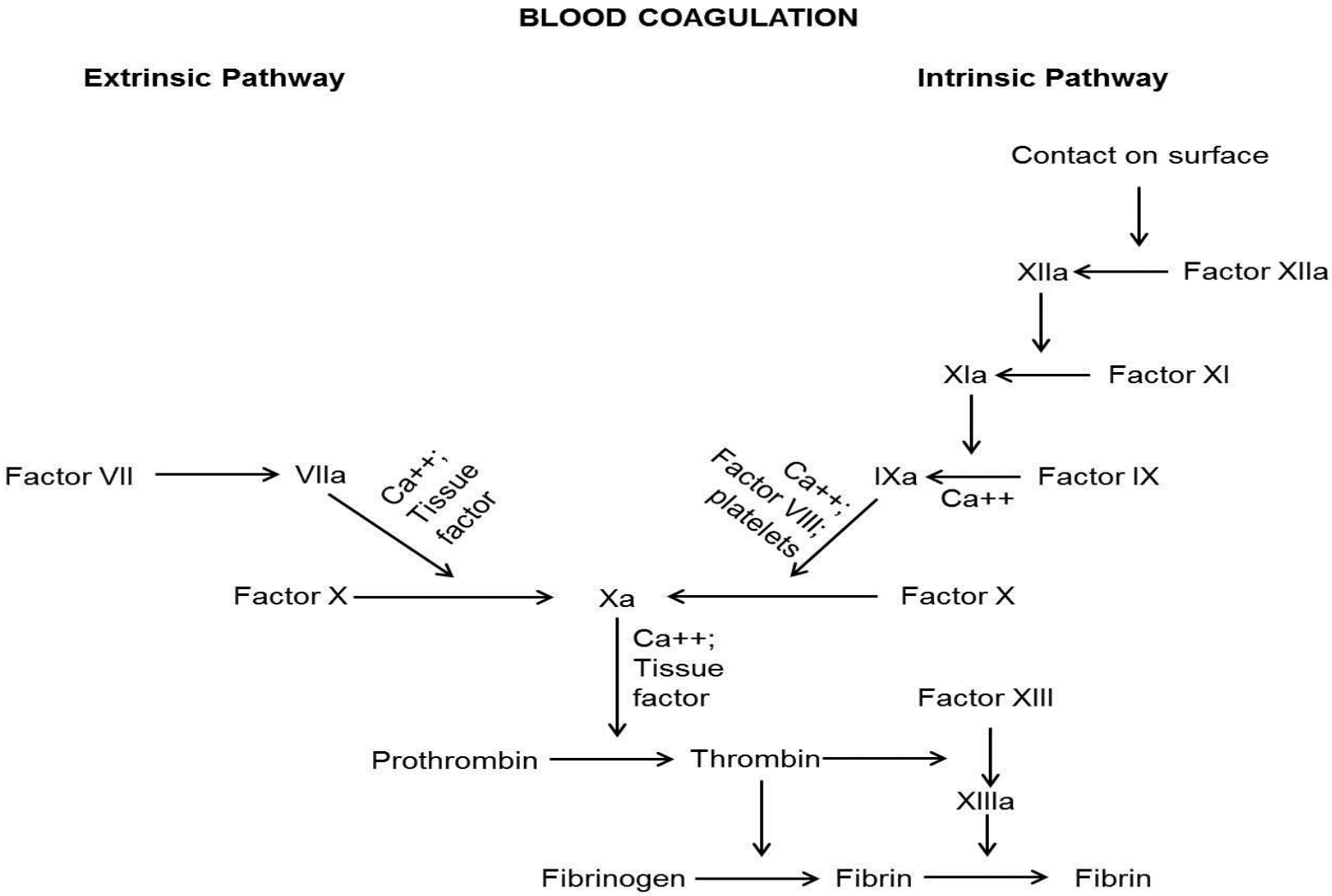

Figure-3. The extrinsic and intrinsic pathway of blood coagulation.

To prevent loss of excess amount of blood in which may resulted in death or coma, the blood coagulation cascade should act immediately and forms the clot with high success. However as a result of many factors like patients physiological condition, genetic backup, size, number and place of the injury or wound can be determinative for the time period for clot formation. As a result there is need for usage of natural or synthesized compounds to promote the coagulation occurs. Pharmaceuticals, biomaterials and natural compounds are the ones that can be used for this purpose. In this research, extract of *equisetum arvense* was investigated for its blood coagulation activity. Therefore, detailed information about *equisetum arvense* will be introduced in the next chapter.

1.5 *Equisetum Arvense*

Equisetum arvense (Horsetail) is an herbal remedy dating back to ancient Roman and Greek medicine. The plant contains several substances which can be used medicinally. It is rich in the minerals silicon (10%), potassium, and calcium. Horsetail contains silicon, which plays a role in strengthening bone. For that reason, it is sometimes suggested as a treatment for osteoporosis. It is also used as a diuretic, and as an ingredient in some cosmetics. *Equisetum arvense* is a widespread species along stream banks and in meadows in North America and Eurasia. In Turkey this plant grows in agricultural fields and in river banks with its seven different subspecies, as a result of this high number of subspecies population and big growth area *Equisetum arvense* becomes the most abandoned and used sanatory plant. As it contains saponins, silicic acid, potassium salt, tannins and alkaloids; it was used traditionally by healers to cure different kinds of illnesses. Kidney and urinary bladder illnesses, eczema, rheumatism, chronic cough, lung diseases, edema, stop bleeding, heal ulcers and wounds are major diseases. It was thought that the therapeutic effect of the plant maintained as a result of high silicic acid content. Silicic acid can be seen in this plant because mostly this plant grows in the silicated soils. In 1967 Jones et al showed and described the silicification in plants (12). Although silica does not involve in plant growth, it has an important role in plant defense mechanism against pathogens as a mineral barrier and also take role in replacement of water and mineral salts (13). Plant takes this silica from the soil in its basic form, ortosilicic acid . This monomer stored in leafs as thin layer of a film (13). In traditional medicine, leafs of was dipped into hot water and then filtered, the water will be used as beverage or it applied externally to the unhealthy region. However as a result of contained substance, over doses or toxic effects as a result of high steaming time or poison can be seen. So before use characterization of the extract by different extraction parameter should be done. In 2002 Science and Technology agency published a report and stated that *equisetum arvense* contains high amount of tannins, folic acid, vitamins, nicotinic acid, and pantothenic acid. In 2005 Takashi Nagai and his colleagues investigate the antioxidant effect of horsetail extracts solved in alcohol and water. The antioxidant effects were showed in this investigation (14). In literature it was seen that the hypoglycemic activities, diuretic activities and anti-inflammatory activities of *Equisetum arvense* were investigated (15-19). Do Monte and

his colleagues work on mice at 2004 and they investigate the antigenic and anti-inflammatory effects of different doses of *equisetum arvense* body part extracts by using heat and chemical tests. It was seen that the pain caused by chemicals diminished after plant extract used, however no effect was seen on pains produced by heat. Also the inflammation decreased after extract usage. They showed that *equisetum arvense* extract has an analgesic effect on central nervous system for chemical stimuli but not on heat stimuli, The mechanism cannot be found. On the same paper the anti-inflammatory effect of the extract was also mentioned. (20) At USA Patent Institute there is patent application which has an id number of 12/231,798 and a heading of transdermal therapy devices and methods wants the *Equisetum arvense*'s patent rights for cure in bleeding stoppage, cancer healing, cramp healing, ulcer healing. In this institute, more than seven thousand applications can be found on the topics of silicic acid and horsetail. Cosmetics and healthcare applications are the main subjects for this plant. Silicic acid takes parts in the biomaterials subject for patent applications. In 2005 Dos Santos and co-worker showed that in mice *equisetum arvense* extract has a sedative and pain killing effect (21). In 2007 Milovanovic et al showed the antioxidant properties of *equisetum arvense* as a result of high phenolic content (22). In 2005 Nagai et al showed the high flavonoid and vitamin content of horsetail and also showed the high content of zinc and copper in the plant, can take part in removal of superoxide anion radicals and as a result plant extract can be used as antioxidant (14). At 2005 on old mice Dos Santos and colleagues investigate the role of *equisetum arvense* extract on removal of oxidative damages on brain. They showed that an addition of equisetum addition to the nutrition's of mice can increase the mental conditions (23). Also at 2009 Stajner et al and at 2008 Milica-Dukic et al showed the antioxidant capacity of horsetail (24,25). The antimicrobial effect of horsetail was investigated by three different scientists. At 2006 Raduvolic et al showed the antimicrobial effect of *equisetum arvense* (26). At 2007 Milovanovic et al investigated and showed that horsetail was a strong antimicrobial agent on fungus and gram negative bacterial (22). Lastly at 2009 Canadanovic-Brunet et al showed the n-butanol and ethyl acetate extract of *equisetum arvense* has antimicrobial effects (27). In 2007 two scientists worked on the anticancer effect of horsetail. Tepkeeva et al showed that extract can be used as tumor development inhibitor on breast cancer cells (28) and Alexandru et al showed that horsetail extract has a cytotoxic effect on leukemia (29).

With all the benefits *equisetum arvense* is one of the most investigated herbal remedy in literature. However, although it was used as a blood coagulator in folk medicine, there was no investigation found on that subject in literature. *Equisetum arvense* contains many substances and without proper dose it can become toxic and poisonous. Also extracts can diminished their effects as time passes as a result of natural compounds properties. So to stabilize the effect for a long period of time extract should be embedded into a biomaterial. On the next chapter information on biomaterials will be given briefly.

1.6 Biomaterials

Biomaterial is a natural or artificial material that circles entire or part of a living structure or device which can perform, fulfill and replace a natural process. Biomaterials were defined as inorganic or organic materials which have high biocompatibility. They can be structured from metal, ceramic or polymer. In this research the biomaterials that was designed to carry the plant extract was going to arranged from polymers. Because as the topic of the research is investigation of homeostatic biomaterials containing plant extracts, interaction between blood components and biomaterial should not give any damage to blood cells or healthy cells on wound area and should not change the protein activity. With their rigid properties metals and ceramic can easily give more damage to the wounded area. Also they have to be biocompatible, biodegradable, and bioresorbable. As working space is fluidic and viscous proper handling should be required. After getting wet by blood the biomaterial should not become slippery. Also a hemostat should absorb liquids from the injury site properly and effectively. As a result of these properties polymers would be the best choice for biomaterial formation.

From the beginning of polymer sciences, the materials were used in medicine, and medical application. Every newly discovered synthetic polymer finds a way of usage in surgeries and clinical practices. Nylon was the first synthetic polymer that was used in surgeries as a suturing material. Then poly methylmetacrylate, polyvinyl chloride were the next polymers published in journals for their medical applications, like catheters, vascular crafts, lenses, and hip implants. Later on they started to used not only as permanent prosthetic device but also for controlled drug delivery and gene therapy. The last step in their evolution was to used in tissue engineering as three

dimensional scaffolds for newly generated tissues. Some polymers currently used in medicine and their applications were shown in Table 1.

Table 1. Polymers and their applications in medicine

Poly(methylmethacrylate)	Rigid contact lenses, intra-ocular lens
Nylon-type polyamides	Sutures
Poly(ethylen terephtalate)	Vascular protheses, cardiac valves
Polyurethanes	Catheters, cardiac pumps
Polymeric compoundsbased on methylmethacrylate	Acrylic cements for orthopedy and odontology, facial prostheses, joint surgeries, filling of bone cavities and porous bony tissues
Silicones	Plastic surgery, tubes, oxygenators
Polytetrafluoroethylene	Orthopedy, vascular clips
Nylon-type polyamides	Sutures
Poly(vinyl chloride)	Blood pushes, catheters
Poly(2-hydroxyethyl methacrylate)	Flexible contact lenses, plastic surgery, hemocompatibility of surfaces

There are three properties to be taken into consideration during polymer generation. Biocompatibility, biodegradability and bioresorbability are the properties. First of all the polymer should be biodegradable. After degradation of polymers, there shouldn't be any toxic leftovers formed in the surrounding tissue, organelle or living being. More to this the remnants should stimuli the immune system. On the other hand mutagenesis shouldn't be observed in short or long time periods after degradation. Lastly and more importantly after degradation the particles shouldn't contact with blood components to form clots. However in this research the aim was to form a blood clot as soon as possible with the help of natural product released from the biomaterial. The further clotting after degradation will cause strokes and heart attacks. These were the unwanted and most dangerous side effects. Secondly the material should be biocompatible, which means that "the ability of a material to perform with an appropriate host response in a specific application". This topic includes all the aspects

like no toxic effect, no immunological response, high or low interaction with host. Thirdly bioresorbability is re-adsorption of the material by the host. The polymeric biomaterial should have also some functional desired aspects. It has to be sterilizable, biological and chemical usage, and should be handled easily. All of these aspects are shown in Table 2.

Table 2. Functional and biological properties of polymeric biomaterials

Biocompatibility, Bioresorbability, Biodegradibility	**Biofunctionality**
None toxicity	Chemical and biological use
No immunogenicity	Adequate material property
No mutagenicity	Strerilizability
No thrombogenicity	Stability on storage
Re-Adsorbtion	Easy to use

When the literature searched it was seen that there were many hemostatic biomaterials investigated and marketed. In common they use tend to increase the adsorption capacity of the biomaterial in order to get rid of the excess amount of aqueous part of the blood and increase the blood content in that injured area in a relative mean. By adding chemicals they also provide antibacterial ability to that side (30 - 35). According to their growth area every population uses different kind of plant for different illnesses. The most needed cure is to stop bleeding because bleeding is an instantly developing incident. *Musa paradisiac* (banana) in Ghana, *Jatropha Gossypifolia* in Mexico and *Ageratum conyzoides* in Brazil are the some examples of plants that were used for blood coagulation. In Turkey *Equisetum arvense* and nicotiana species were also used as blood coagulator agents for centuries. However *equisetum arvense* or commonly known field horsetail is a toxic and poisonous substance if over dose occurs. So before working on the homeostatic effects of the plant the characterization and standardization of the plant extract should be handled. This characterization and standardization of extract will be the first section of this study (36 - 38).

CHAPTER 2

AIM OF THE STUDY

The main aim of this study was to investigate a homeostatic biomaterial which contains plant extracts. As the study was carried out the aim was changed to, "Investigation of a hemostatic sponge biomaterial, formed with silk fibroin and hyaluronic acid including *Equisetum arvense* extract as coagulating agent". For each segment of the study sub-aims were targeted to achieve the final goal. Those are:

1. For plant extract part, the aim was to investigate the effect of changing extraction parameter on the composition of *Equisetum arvense* extract along with its biological activities.

2. For blood coagulation part, the aim was to investigate the effect of different *Equisetum arvense* extracts on both extrinsic (fibrinogen time) and intrinsic pathways (prothrombin time) of coagulation cascade. The correlation between coagulation cascade and the content of plant extract was aimed as well.

3. For biomaterial part, the aim was to prepare a biomaterial in the form of sponge using a hyaluronic acid and silk fibroin and to integrate plant extract into prepared biomaterial for blood coagulation activity.

CHAPTER 3

MATERIALS & METHODS

3.1 Materials

Previously collected and dried *equisetum arvense* (horsetail) were purchased from the local herbalist. Analytical grade ethanol (C_2H_5OH) was used during extraction and purchased from Fluka. In ordered to determine the total phenolic components Folin-ciocalteu reagent and sodium carbonate were used. Gallic acid was used to obtain calibration curve for the phenolic compound determination assay. Trolox (6-hydroxy-2,5,7,8,-tetramethylchroman-2-carboxylic acid), ABTS (2,2'-azinobis(3-ethylbenzothiazoline-6-sulphonic acid) reagents and potassium persulfate ($K_2O_8H_8$) were used for antioxidant analysis.

For antibacterial property determination assays, disk diffusion method was used. Both nutrient broth and nutrient agar was used as food supply. For positive control penicillin, vancomycin and amphotericin, antibiotics and antifungals were used

To prepare the silk fibroin solution dialysis was used. Sodium sulfide hydrate and sulfuric acid were used for the treatment of dialysis tubing. Calcium chloride-2-hydrate ($CaCl_2.2H_2O$) was used for the preparation of aqueous silk fibroin solution. HPLC grade acetonitrile and HPLC grade acetic acid were used as mobile phases in HPLC analyses.

For cytotoxicity tests mouse fibroblast cell line, DMEM medium, fetal bovine serum, antibiotic solution (streptomycin and penicillin) and L-glutamine were used. Tetrazolium salt (3-[4, 5-dimethylthiazolyl-2-y]-2,5-diphenyltetrazolium bromide) was used to determine cell viability.

The blood samples collected from investigator himself were used for blood coagulation assays.

3.2 Methods

3.2.1 Experimental Design

Response surface methodology (RSM) was used to analyze the effects of selected extraction parameters on the selected responses. The responses were yield percentage, phenolic content, total antioxidant capacity and silicic acid content. Half face centered surface composite design was used to investigate the responses (39, 40). Each independent variable was coded at three levels between -1 to +1 where the factors were determined as temperature (x_1), stirring speed (x_2), Ethanol percent (x_3), extraction time (x_4) and solid-liquid ratio (x_5). Factors and levels are summarized in Table 3.

Table 3. The coded levels and corresponding actual levels of factors used in MINITAB software to generate experimental design

		Coded Levels		
FACTORS	Symbols	-1	0	1
Temperature (°C)	x_1	4	24.5	45
Stirring speed (rpm)	x_2	50	150	250
Ethanol (%)	x_3	10	50	90
Extraction time (hr)	x_4	2	7	12
Solid-Liquid Ratio (gr:ml)	x_5	1:10	1:20	1:30

3.2.2 Extraction of Plant Materials

Pre-dried *equisetum arvense* leaves were grinded in a bench top mill. Ten grams of grinded particles were weighed and extracts were prepared based on the experimental conditions listed in Table 4. At the end of the required extraction time, the liquid extract was vacuum filtered, and then ethanol content was removed by using rotary evaporator under vacuum at 40°C. The supernatant of aqueous extract was frozen at -20°C for overnight and frozen extracts were subjected to lyophilization.

Table 4. Experimental design for response surface analysis of *equisetum arvense* in terms of uncoded levels

Sample	**X_1** **Temperature (°C)**	**X_2** **Stirring speed (rpm)**	**X_3** Ethanol %	**X_4** **Extraction time (hr)**	**X_5** **Solid-liquid ratio (gr:ml)**
1	4	50	10	2	01:30
2	45	50	10	2	01:10
3	4	250	10	2	01:10
4	45	250	10	2	01:30
5	4	50	90	2	01:10
6	45	50	90	2	01:30
7	4	250	90	2	01:30
8	45	250	90	2	01:10
9	4	50	10	12	01:10
10	45	50	10	12	01:30
11	4	250	10	12	01:30
12	45	250	10	12	01:10
13	4	50	90	12	01:30
14	45	50	90	12	01:10
15	4	250	90	12	01:10
16	45	250	90	12	01:30
17	4	150	50	7	01:20
18	45	150	50	7	01:20
19	24.5	50	50	7	01:20
20	24.5	250	50	7	01:20
21	24.5	150	10	7	01:20
22	24.5	150	90	7	01:20
23	24.5	150	50	2	01:20
24	24.5	150	50	12	01:20
25	24.5	150	50	7	01:10
26	24.5	150	50	7	01:30
27	24.5	150	50	7	01:20
28	24.5	150	50	7	01:20
29	24.5	150	50	7	01:20
30	24.5	150	50	7	01:20
31	24.5	150	50	7	01:20
32	24.5	150	50	7	01:20

3.2.3 Determination of Phenolic Content

Total phenolic content of *equisetum arvense* extracts was determined by Folin-ciocalteu method. Folin-ciocalteu reagent was prepared by 1:10 dilution of stock solution. Sodium carbonate solution of 7% was prepared in distilled water. Gallic acid was used as standard in the calibration curve. *Equisetum arvense* extract was dissolved in distilled water. 20 µl of each sample was mixed with 100 µl Folin-ciocalteu reagent and incubated for 2.5 minutes. Then 80 µl of sodium carbonate solution was added. The mixture was kept in dark for 1 hour. Samples were subjected to photometric measurement at 725 nm. Results were expressed as mg of gallic acid equivalents (GAE)/gr dry weight extract.

3.2.4 Determination of Antioxidant Activity

Antioxidant capacity of *equisetum arvense* extract was determined by Trolox-Equivalent Antioxidant Capacity (TEAC) method. ABTS (14 mM) and potassium persulphate (4.9 mM) were mixed in a 1:1 ratio and kept in dark for 16 hours. Trolox was used as standard in the calibration curve. Plant extract was dissolved in water. ABTS solution was dispensed in each well at a volume of 200 µl. Samples were subjected to kinetic measurement for 30 minutes at 734 nm. Percent inhibition of ABTS cation as a result of antioxidant activity of *equisetum arvense* extract was calculated by the formula showed below:

$$\text{ABTS Inhibition \%} = (1- (A_f / A_0)) \times 100$$

A_f refers to final absorbance value measured on the last measurement and A_0 refers to absorbance value measured directly after dispensing ABTS on the sample.

3.2.5 Determination of Silicic Acid Content

Calorimetric determination of silicic acid method was modified from Metrohm group's application bulletin. Silicic acid was dissolved in distilled water at 70 °C for 5 hours at 200 rpm for standard preparation. 5 ml of *Equisetum arvense* extracts,

dissolved in distilled water, was mixed with 0.1 ml of 10% oxalic acid (w/v), 0.25 ml of 10% Hepta molybdate tetrahydrate (w/v) and 0.1 ml of 22.8% hydrochloric acid (v/v) solutions. Spectrophotometric measurements were performed at 400 nm.

3.2.6 Cell Culture and Determination of Cytotoxic Activity

NIH3T3 mouse fibroblast cell line was maintained in DMEM supplemented with 10%FBS and 100 µg ml^{-1} streptomycin, 100 U/ml penicillin. Cytotoxic activity of *Equisetum arvense* extract was evaluated by the MTT assay in two stages. *Equisetum arvense* was extracted in 70% aqueous ethanol, at 180 rpm at room temperature for 2 hours. After lyophilization, extracts were dissolved in DMEM supplemented with 10% FBS. Aqueous phase of the extract was separated by centrifugation at 4000 rpm for 5 minutes and subjected to serial dilution in DMEM with FBS (10%) to determine IC50 of *Equisetum arvense*. Cells were later exposed to IC50 of extracts obtained in varying conditions for 24, 48 and 72 hours. Cells subjected to MTT assay were incubated in dark at 37 ^{o}C for 4 h and spectrophotometric measurement was performed at 545 nm. Cell viability was calculated by using the formula:

Cell viability: 100% × (Average absorbance value of treated cells/Average absorbance value of control cells)

3.2.7 HPLC Analysis

High pressure liquid chromatography (HPLC) analysis was modified from the studies of Canadanovic-Brunet et al (25). HPLC was performed with an Agilent 1100 series device equipped with diode array detector. A reversed-phase column, Lichrospher 100- RP 18 with a 5-µm particle size (Agilent Technologies, USA), was used at the flow rate of 0.8 mL min^{-1}. Mobile phase gradient was performed by varying the proportion of solvent A (%2.5 acetic acid) to solvent B (%100 acetonitrile) as follows: initial 1% B; linear gradient to 40% B in 40 minute. The samples were prepared at concentration of 10 mg/ml in water and the injected sample volume was 20 µl. All solutions were filtered prior to injection through 0.20 µm membrane filters (Millipore,

Bedford, MA, USA). The column temperature was at 35 °C. The measurements were held at 254 nm and 280 nm.

3.2.8 Blood Sample

Blood was taken voluntarily from healthy donor (investigator) at the medical center located in IYTE. From donor, 7 ml of blood was collected into heparinize tubes (vacutest kima, Italy). Tubes were centrifuged at 1500 rpm for 7 min. at 4 °C. Serum was collected from each tube and aliquoted into eppendorf tubes and freezed at -80 °C for further analyses.

3.2.9 Blood Coagulation Time

Both Prothrombin time (PT) and fibrinogen time (FT) were measured in order to see the effect of plant extract on intrinsic and extrinsic pathways. Coalyzer 410 (Farmasina, Istanbul, Turkey) was used in order to determine the coagulation time by electromagnetic mechanical analysis.

For PT test, tiny metal marble was put into the test tube and 50 µl of serum was added. Sample was incubated at 37 °C for 60 seconds. 100 µl of PT reagent (Diagon, Hungary) was mixed with 10 µl of 20 mg/ml plant extract in an eppendorf tube. Mixture was added into the test tube and coagulation was monitored. For blank test sample without extract was used.

For FT test, tiny metal marble was put into the test tube and 90 µl of immidazol (Diagon, Hungary) was added. Sample was incubated at 37 °C for 60 seconds. 50µl of FT reagent (Diagon, Hungary) was mixed with 10 µl of 20 mg/ml plant extract in an eppendorf tube. Mixture was added into the test tube and coagulation was monitored. For blank test, sample without extract was used.

3.2.10 Determination of Antimicrobial Activity with Disc Diffusion

Extract was subjected to antimicrobial activity test by disc diffusion method. Discs were sterilized by ethylene oxide. Sterile blank discs were soaked into *Equisetum arvense* extract for 24 hours. Antibiotic discs of penicillin, vancomycin and amphotericin B were used as positive control. Bacterial culture of *Escherichia coli, Staphylococcus epidermidis,* fungal culture of *Candida albicans* were used for the tests. By cotton swab they were spread onto agar surface. Extract-soaked blank discs and antibiotic discs were then placed on the plates with different microorganisms. After incubation for 24 hours at 37 °C, inhibition zone diameters were measured by using a compass.

3.2.11 Cell Culture

NIH/3T3 mouse fibroblast cell line were maintained in DMEM supplemented with 2 mM L-glutamine, 10% fetal bovine serum, 100 µg/ml streptomycin and 100 U/ml penicillin; in 5% CO_2 at 37 $^{\circ}$C. Cells were subcultured every 48 hours.

3.2.12 Cytotoxic Activity Determination by MTT

Cytotoxicity of *equisetum arvense* extract was evaluated by the MTT (tetrazolium (3-{4,5-dimethylthiazol-2-yl}-2,5-diphenyl tetrazolium bromid**e)** assay. Extract was dissolved and diluted serially in DMEM with FBS(10%) and filter-sterilized. NIH/3T3 cells were grown in the absence or presence of various concentrations of *equisetum arvense* for 1, 3 and 5 days. Then the cells were exposed to MTT for 4 h in dark at 37^{o}C. Mitochondrial hydrogenases in the viable cells reduce MTT into formazan crystals which can be dissolved in DMSO. Absorbance was measured at 545 nm. Cell viability was calculated by using below formula:

Cell viability: 100% × (Average absorbance value of treated cells/ Average absorbance value of control cells)

3.2.13 Preparation of Silk Fibroin

Sericin which is sticky protein was removed from the raw silk. During the process, raw silk was kept in 1:50 (v/w) concentration of boiling aqueous 0.05% Na_2CO_3 for 30 minutes. Repeating this procedure with three time followed by washing, the sericin was removed and raw silk was stored at room temperature.

3.2.14 Preparation of Dialysis Tubes

Glycerin was removed from tubes by washing in running water for 2-3 hours. By putting tubes in a 0.3 % (w/v) sodium sulfide solution at 80°C for one minute and washing with water at 60°C for 2 minutes sulfur components were removed. For the tubes were incubated with 0.2% (v/v) sulfuric acid for one minute. Process was completed by rinsing with water at 60°C for 2 minutes, then tubes were left to dry at ambient conditions.

3.2.15 Preparation of Silk Fibroin in Aqueous Solution

1.2 g sericin free silk was dissolved in 1:28 (v/w) of a ternary solvent system of $CaCl_2/CH_3CH_2OH/H_2O$ (1:2:8 in molar ratio). Incubation for 2 hours in a shaking water bath at 78 °C and 150 rpm to form a clear solution. Then the solution was dialyzed against deionized water for at least 3 days to remove the neutral salts using a cellulose tubing (MWCO 10000 kDa). Dialysis water was changed every 30 minutes for the first 2 hours, and every 12 hour for the rest of the time.

3.2.16 Preparation of Silk Fibroin Sponge with the addition of Hyaluronic Acid and *E. Arvense* Extract Separately

Aqueous silk fibroin solution was mixed with hyaluronic acid at the ratios of 3 % and 15 % (w/w). *Equisetum arvense* extract was put into the silk fibroin hyaluronic acid mixture at concentrations of 10, 50 and 100 mg/ml. First *equisetum arvense* extract was added into silk fibroin solution then hyaluronic acid powder was added into this

mixture. The mixtures were put into the lyophilization bottles and freezed at -24 °C and -80 °C. After freezing overnight, the samples were lyophilized.

Table 5.The preparation conditions of sponge matrices and their codes

	% 3 Hyaluronic acid		**% 15 Hyaluronic Acid**	
Extract Concentration (mg/ml)	**-24°C**	**-80°C**	**-24°C**	**-80°C**
0	A_1	A_2	A_3	A_4
10	B_1	B_2	B_3	B_4
50	C_1	C_2	C_3	C_4
100	D_1	D_2	D_3	D_4

3.2.17 Preparation of Silk Fibroin Sponge with Dissolved Hyaluronic Acid

Aqueous silk fibroin solution was mixed with aqueous hyaluronic acid (HA) solution in the ratios of 0, 5, 7.5 and 10% (w/w). Hyaluronic acid was added as dissolved form. Sufficient amount of HA was dissolved in 5 ml of dH_2O and added to 35 ml silk fibroin (1.2 gr). The mixtures were put into 24 well plates. After freezing overnight, the samples were lyophilized.

3.2.18 Addition of *Equisetum Arvense* Extract to Sponges

Equisetum arvense extract was dissolved in dH_2O to prepare aqueous solution at concentrations of 0, 20 and 50 mg/ml. After filtration, 1 ml of extract solution was added into mixture of silk fibroin and hyaluronic acid. Sponges were dried at 40 °C.

3.2.19 Dipping Sponges into *Equisetum Arvense* Extract

Equisetum arvense extract was dissolved in dH_2O, aqueous ethanol solution (50%) to prepare liquid extract solution at concentrations of 0, 20 and 50 mg/ml. After filtration, the prepared sponges were dipped into extract solution for 3 seconds.

3.2.20 Coating Sponges with Aqueous Hyaluronic Acid Solution Including *Equisetum arvense* Extract

Equisetum arvense extract solution was prepared at a concentration of 5 mg/ml in dH_2O. After filtration, hyaluronic acid (%0.05, v/v) was added into the extract solution. Then sponges were dipped into aqueous hyaluronic acid solution including extract for 10 seconds. Then samples were dried in the oven at 35°C for 3 hours.

3.2.21 Preparation Of Silk Fibroin Sponge With Addition Of Aqueous Hyaluronic Acid And *Equisetum Arvense* Mixture

Aqueous silk fibroin solution was mixed with hyaluronic acid (HA) in the ratios of 0, 5, 7.5 and 10% (w/w) and *equisetum arvense* with concentrations of 0, 10 and 20 mg/ml. Hyaluronic acid was dissolved in 2 ml of *equisetum arvense* extract. Hydrogels were than mixed with silk fibroin. The mixtures were put into 24 well plates. After freezing overnight, the samples were lyophilized.

3.2.22 Fluid Uptake and Degradation

Cylinders with a dimensions of 0.5 cm height and 1 cm radius were cut from each sponge sample materials. These cylinders were put into 9 deep well plates and mixed with 2.5 ml 1X sterile PBS for time periods of 24, 48, 72 and 96 hours. Weight loss of in each sample was measured to observe their degradation.

3.2.23 Release Studies

Silk fibroin sponge with plant extract were subjected to batch release studies. For batch release, pre-cut foams were soaked in PBS solution up to 3 days by sacrificing material for each day. Medium was removed in order to determine phenolic compounds by HPLC, total phenol content by Folin-ciocalteu method and antioxidant capacity by Trolox Equivalent Antioxidant Capacity.

CHAPTER 4

RESULTS AND DISCUSSION

4.1. Extraction

4.1.1 Effect of Extraction Factors on Yield

After lyophilization of each sample the extracts were weighted and the percentages of the yield were calculated. The percent yields are presented in Table 6.

Table 6. Percentage of the extraction yield
(10 grams of dry leafs of *equisetum arvense* was used for extraction)

Sample	% Yield	Sample	% Yield
1	11.46	17	12.77
2	21.71	18	9.07
3	13.09	19	13.79
4	5.71	20	12.83
5	12.21	21	13.83
6	4.86	22	5.496
7	3.45	23	14.81
8	3.78	24	19.01
9	14.40	25	10.05
10	17.63	26	14.66
11	18.52	27	13.52
12	20.88	28	15.16
13	10.35	29	13.88
14	5.20	30	13.42
15	11.90	31	12.76
16	8.95	32	11.47

As seen in the Table 6, the yield percentages were varied from 3.45 % to 21%. The minimum yield was obtained for the sample number 7 as the highest yield was observed for the sample number 2. The extraction parameters for the preparation of sample number 2 and 7 are presented in Table 2. The main differences between the extraction parameters of those were temperature, stirring speed, ethanol percent and solid-liquid ratio. It was a known and well accepted fact that as the temperature

increases the mass transfer would also increase (41). Also it was found that as the ethanol percent decreased the yield increased. The polarity of the solvent was an important issue in extraction (42). The polar ingredients of the plant could dissolve much better in high water ratio. The differences between the yields of samples were also support this fact.

The results of the yield percentage were analyzed in Minitab Software. After eliminating four outliers the analysis variance of total extract yield percentage (y_1) showed that the regression model had low dispersion with a R^2 value of 99.87. The mathematical model could be obtained by using significant ($p<0.05$) coefficients of the extraction parameters. The predicted model for yield percentage was as follows:

$$y_1 = 31.3087 + 0.6086\,x_1 - 0.0255\,x_2 + 0.1577x_3 - 2.8456\,x_4 - 1.4168\,x_5 - 0.0095\,{x_1}^2 - 0.0024\,{x_3}^2 + 0.1380\,{x_4}^2 + 0.0257\,{x_5}^2 - 0.0003\,x_1x_2 - 0.0020\,x_1x_5 + 0.0046\,x_2x_4 - 0.0002\,x_2x_5 - 0.0023x_3x_4 + 0.0018\,x_3x_5 + 0.0355\,x_4x_5$$

The surface and contour plot of the yield percentage vs. ethanol concentration and temperature can be seen in Figure 4. The other parameters were set constant at the center points. It was seen from the plots that the maximum yield would be obtained in the conditions where ethanol percent was kept constant at 10% to 50% and temperature at 20°C to 30°C.

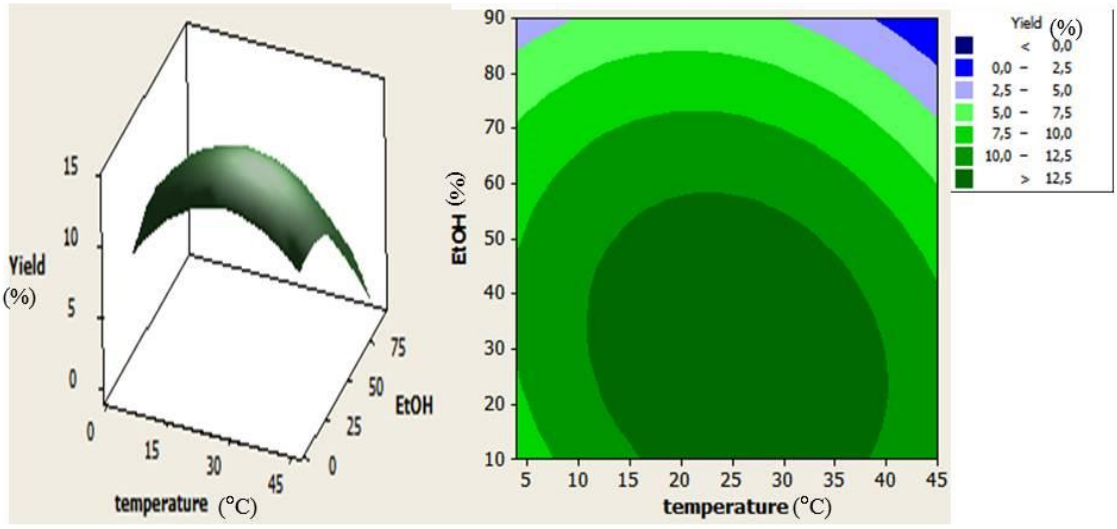

Figure 4. Surface and contour plot of the yield percentage vs. EtOH percentage and temperature. Stirring speed was 150 rpm, solid-liquid ratio was 1:20 and extraction time was 7 hour.

4.1.2 Effect of Extraction Factors on Total Phenol Content (TPC)

The extracts were analyzed for their total phenolic content by Folin-ciocalteu method. The results can be seen in Table 7 as milligram Gallic acid equivalent per gram dry weight of extract.

Table 7. Total phenol content of *equisetum arvense* extract prepared at different extraction conditions

Sample	**TPC** (mg Gallic Acid equivalent/ gr DW)	**Sample**	**TPC** (mg Gallic Acid equivalent/ gr DW)
1	82.94	**17**	86.38
2	35.32	**18**	52.08
3	20.47	**19**	86.15
4	35.41	**20**	79.57
5	25.39	**21**	51.85
6	41.90	**22**	63.46
7	34.50	**23**	53.27
8	41.57	**24**	80.58
9	59.25	**25**	93.25
10	106.46	**26**	103.63
11	53.56	**27**	85.47
12	57.13	**28**	79.82
13	150.04	**29**	94.85
14	182.80	**30**	100.92
15	115.40	**31**	75.77
16	149.14	**32**	115.41

The highest total phenol content was observed for samples 14, 13, 16 as the lowest total phenol content were obtained for samples 3 and 4 in terms of gallic acid equivalent. It can be concluded that samples extracted in high percentage of ethanol and for longer time had more phenolic content than the lower ones as seen in Table 7. This could be understandable because the polarity of the solvent had a high influence on the solubility of the phenolic compounds. The phenolic content of *equisetum arvense* consists of substances with polar properties (27). In 2005 the phenolic content of *equisetum arvense* was published as 23.9 gram gallic acid gram^{-1} dry weight for ethanol extract and 7.98 gram gallic acid / gram dry weight. In that research it was also said that as the alcohol concentration increases the phenolic content becomes richer (14). In literature it was said that the total phenolic content of *equisetum arvense* leaves was

0.212 ±0.044 gram gallic acid equivalent / L for dry leaves (43). All of our results were found in that range and according to the alcohol concentration, the amount of phenolic compounds were changed. Caffeic acid, (-)-epicatechin, p-coumaric acid, vanilic acid and rutin are some of the polyphenolics with polar nature. Although some components dissolve in moderately polar solvents, they have better solubility in weak polar or non-polar solvents. (42, 44)

Results of total phenol content of the *equisetum arvense* extracts were then analyzed in Minitab Software for surface plot analysis. The analysis variance of total phenolic content (y_2) showed that the regression model had low dispersion with a R^2 value of 95.46 after eliminating two outliers. The mathematical model could be obtained by using significant ($p<0.05$) coefficients of the extraction parameters. The predicted model for total phenolic content was as follows

$$y_2 = 59.0996 + 1.1801\, x_3 - 3.3278\, x_4 - 0.015\, {x_3}^2 + 0.1099 x_3 x_4$$

The surface and contour plot of the total phenolic content vs ethanol concentration and extraction time are shown in Figure 5. The other parameters were set constant at the center points. It was seen from the plots that the maximum total phenolic content would be obtained for the conditions where EtOH percent was held between 50 % to 90 % and extraction time was held longer than 12 hours.

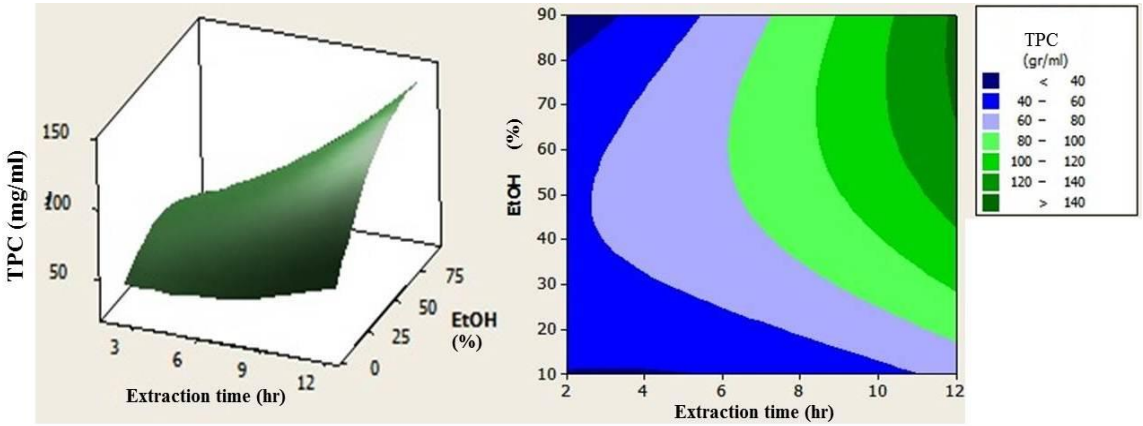

Figure 5. Surface and contour plot of the total phenol content (TPC) vs. EtOH percentage and extraction time. Stirring speed was 150 rpm, solid liquid ratio was 1:20 and temperature was 24.5 °C.

4.1.3 Effect Of Extraction Factors On Total Antioxidant Capacity (TAOC)

Antioxidant capacity of *equisetum arvense* extracts was determined by ABTS method. The results obtained can be seen in Table 8. Samples of 13, 14, 15 and 16 had the highest total antioxidant capacity while samples of 3 and 4 had the lowest total antioxidant capacity.

Table 8. Total antioxidant capacity of extracts prepared at different conditions (Micro Molar TEAC of milligram dry weight of extract)

Sample	µM TEAC / mg DW	Sample	µM TEAC / mg DW
1	830.89	**17**	838.07
2	395.40	**18**	523.70
3	**292.75**	**19**	651.98
4	**338.80**	**20**	572.58
5	412.14	**21**	357.18
6	550.20	**22**	532.75
7	585.21	**23**	817.09
8	820.69	**24**	1116.61
9	939.18	**25**	885.20
10	725.69	**26**	1099.02
11	424.02	**27**	814.92
12	412.35	**28**	866.76
13	1214.94	**29**	954.65
14	1246.82	**30**	915.48
15	1268.09	**31**	1203.40
16	1270.79	**32**	1026.65

When the extraction parameters of the highest and lowest values of trolox equivalent antioxidant capacity (TEAC) results were analyzed in Table 8, it can be concluded that ethanol percentage and extraction time were the most important factors on total antioxidant capacities of extracts. In the literature, the total antioxidant capacity of horsetail was reaported as 39±4 µM TEAC g^{-1} dry weight of plant (45). In this method one gram of dry plant was added to 100 ml boiling water and infusions were made. After the unit conversions it was seen that our results were much higher than the

literature as they were within the range of 293 to 1270 µM TEAC mg^{-1} dry weight of plant extract. This huge difference occurred as a result of the technique that was used. The alcohol content of extraction medium and other parameters that were played an important role for total phenol content and total antioxidant capacity of the extracts, were generated these differences. The correlation could be seen in Figure 6. Antioxidant capacity increases with increasing amount of phenolic substances in the extracts. Similar correlation between TPC and TAOC of plant extracts were reported in the literature. (46). Polarity of the extract medium is one of the major components of solubility of the phenolic compounds. Caffeic acid, (-) epicatechin, p-coumaric acid, vanilic acid and rutin were some of the phenolics with relatively polar nature according to the HPLC chromatograms. The amount of these substances could be changed by changing polarity and may increase antioxidant capacity along with moderately apolar compounds having antioxidant capacity.

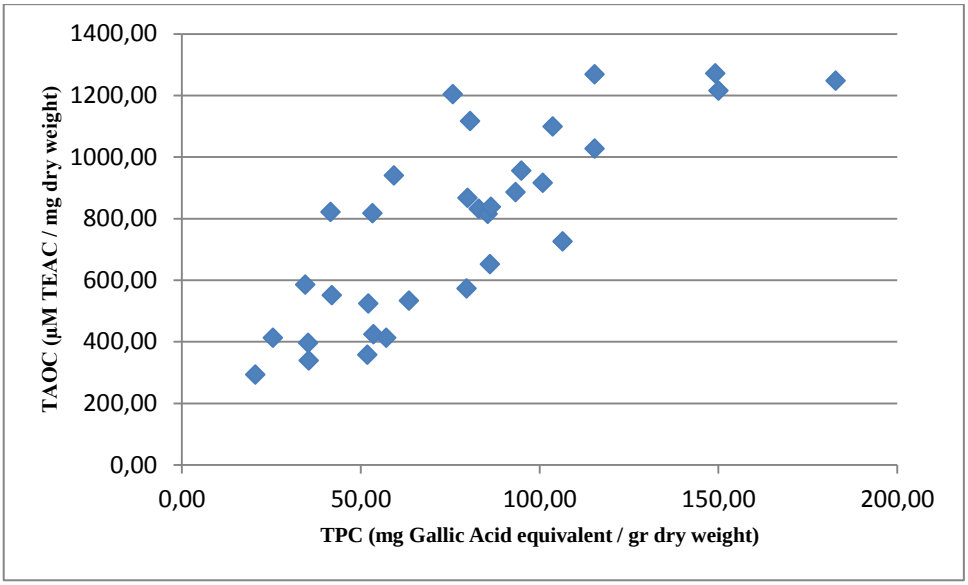

Figure 6. Correlation between total phenol content (TPC) and total antioxidant capacity (TAOC) of extracts prepared at different conditions.

TAOC results were evaluated on Minitab program for surface plot analysis. The analysis variance of total phenolic content (y_3) showed that the regression model had low dispersion with a R^2 value of 96.91 after eliminating three outliers. The mathematical model could be obtained by using significant ($p<0.05$) coefficients of the extraction parameters. The predicted model for total antioxidant capacity was

$$y_3 = 886.78 - 13.966\,x_1 + 3.998\,x_2 + 16.729x_3 - 58.573x_4 - 62.701x_5 - 0.018\,{x_2}^2 - 0.219\,{x_3}^2 + 6.89\,{x_4}^2 + 1.975\,{x_5}^2 + 0.078\,x_1x_3 + 0.03\,x_2x_3 + 0.622\,x_3x_4$$

The surface and contour plots of the TEAC vs. ethanol concentration and extraction time were shown in Figure 7. The other parameters were set constant at the center points. It was seen from the plots that the maximum total antioxidant content would be obtained for the conditions where ethanol percent was between 70% and 90% and extraction time was chosen more than 12 hours.

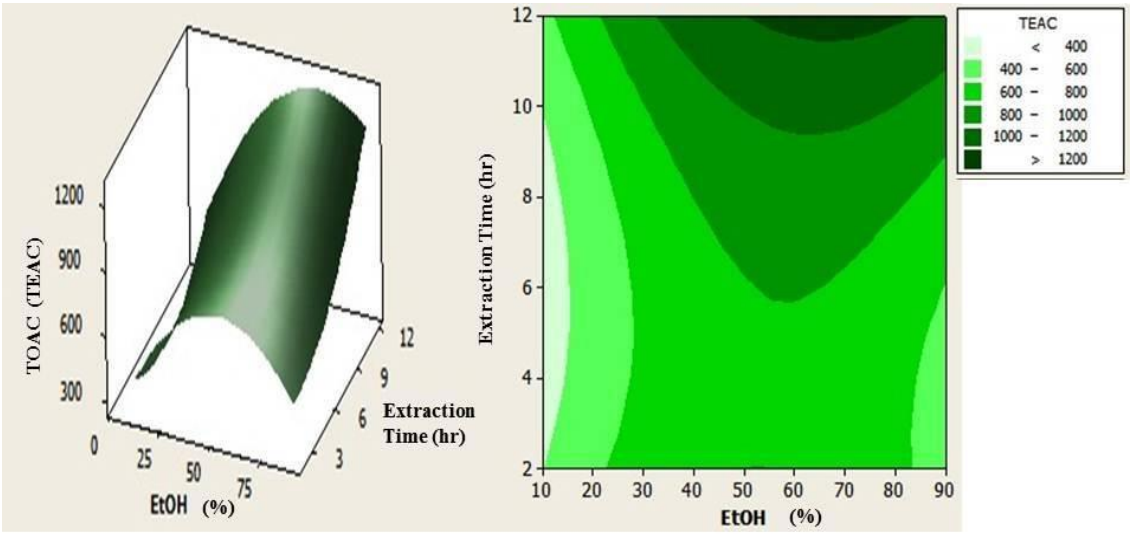

Figure 7. Surface and contour plot of the total phenol content vs. Ethanol percentage and extraction time. Stirring speed was 150 rpm, solid liquid ratio was 1:20 and temperature was 24.5 °C.

4.1.4 Effect of Extraction Parameters on Silicic Acid Amount

Silicic acid amount of *equisetum arvense* extracts were analyzed with silicic acid determination assay. The results that were obtained from the assay could be examined in Table 9. The highest silicic acid amount was detected for the sample 32 and the lowest silicic acid amount was obtained for sample 13. When the results compared to literature of which gave the silicic acid amount up to 6%, it was seen that our finding were in that range (47).

Table 9. Milligram silicic acid amount per mg dry weight of extract

Sample	mg Silicic Acid / mg DW	Sample	mg Silicic Acid / mg DW
1	0.023	**17**	0.023
2	0.014	**18**	0.022
3	0.013	**19**	0.034
4	0.015	**20**	0.035
5	0.010	**21**	0.014
6	0.021	**22**	0.014
7	0.016	**23**	0.021
8	0.020	**24**	0.025
9	0.016	**25**	0.022
10	0.015	**26**	0.026
11	0.013	**27**	0.024
12	0.017	**28**	0.028
13	0.008	**29**	0.022
14	0.021	**30**	0.013
15	0.015	**31**	0.032
16	0.018	**32**	0.046

When the extraction parameters of highest and lowest values were analyzed in Table 4, it was seen that the center point values of each selected extraction parameters should be used to obtain the highest silicic acid amount in extracts, the extreme levels for each factors gave the lowest value.

The results for silicic acid amount were analyzed by using Minitab software for response surface methodology. The analysis variance of silicic acid (y_4) showed that the regression model had low dispersion with a R^2 value of 95.14 after eliminating three outliers. The mathematical model could be obtained by using significant ($p<0.05$) coefficients of the extraction parameters. The predicted model for silicic acid amount was

$$y_4 = 0.007734 + 0.000292\,x_1 - 0.000275x_2 + 0.000647x_3 + 0.001786x_4 - 0.001118x_5 + 0.000001{x_2}^2 - 0.000007\,{x_3}^2 + 0.000003\,x_1x_3 - 0.000039\,x_4x_5$$

The surface and contour plot of the silicic acid for solid liquid ratio vs. extraction time; extraction time vs. temperature and solid liquid ratio vs. temperature were shown in Figure 8. The other parameters were set constant at the center points in each plot. It was seen from the plots that the maximum silicic acid amount would be obtained for the conditions if solid-liquid ratio fixed at 1:20; temperature was set to 25 °C and extraction time was set to 7 hours.

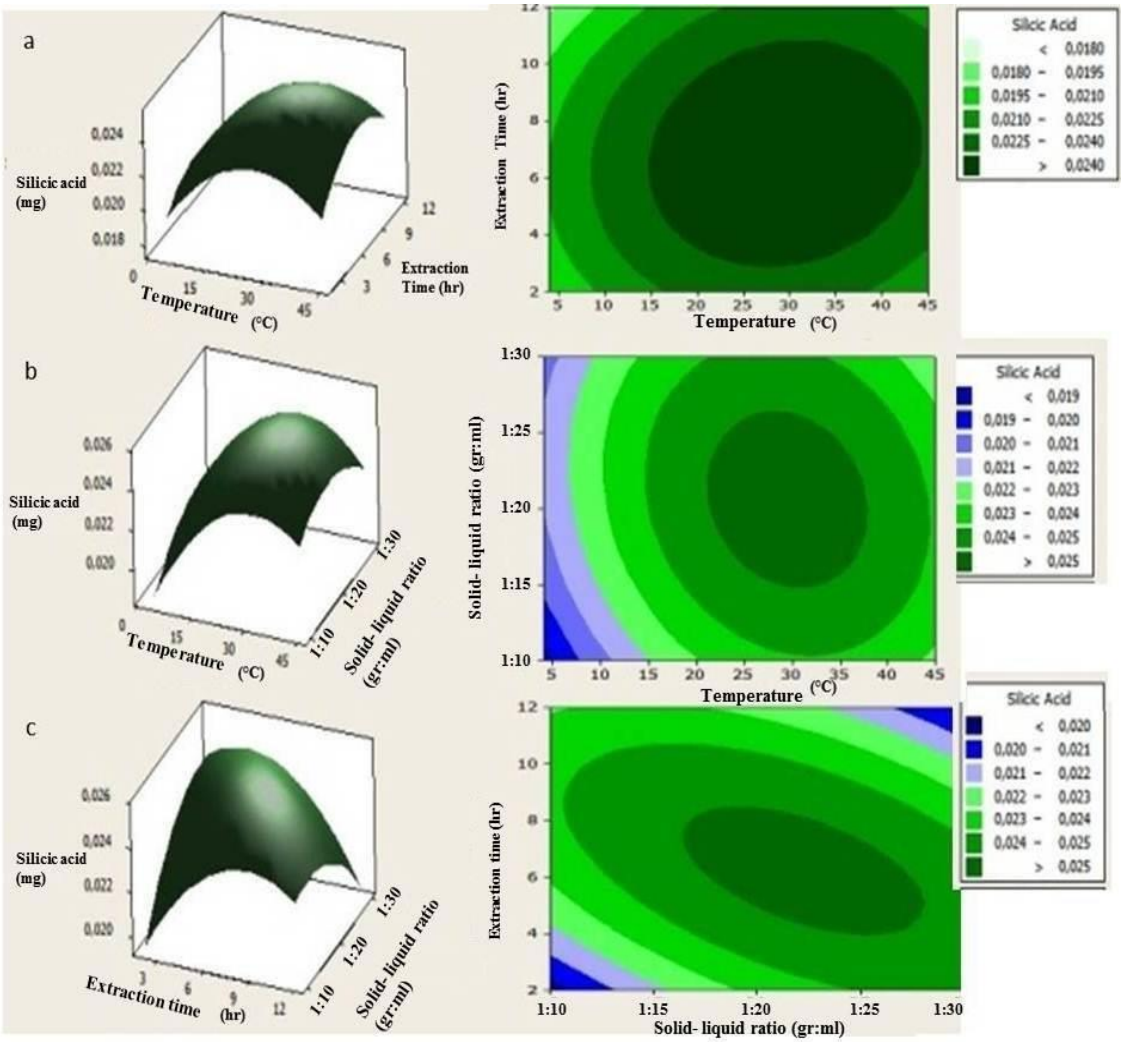

Figure 8. Contour and surface plots of silicic acid amount for temperature vs. extraction time (a); temperature vs. solid-liquid ratio (b); extraction time vs. solid liquid ratio (c).

4.1.5 Effect of Extraction Parameters on Cytotoxicity

Cytotoxic evaluation was performed in two stages to observe the cytotoxic effect gradually. Concentration range was between 100-3000 µg/ml for the first trial to determine IC*50* value, which was 500 µg ml^{-1} in 48 hour. For this concentration, viability was determined as 49.61% as seen in Figure 9. IC*50* value of the extract constituted the limitation for further cytotoxicity evaluation by using products of parametric extraction of *Equisetum arvense*. Cytotoxic evaluation of IC*50* for 24, 48 and 72 hour was compared with total phenol content and antioxidant activity of the extracts (Table 10). Cytotoxic activity was higher in extracts 13, 14, 15 and 16, which also have higher antioxidant activity and total phenol content. Extracts coded as 2, 6 and

8, having lowest antioxidant activity and total phenol content, also show the lowest cytotoxic activity for all time periods. These two extract sample groups differ in extraction medium composition and extraction time, extracts resulting in significantly higher cytotoxic activity was processed with extraction medium containing in 90% ethanol for 12 hour, while extracts obtaining with 10% ethanol for 2 hour did not decrease viability upon exposure to fibroblast cells. Extracts two, six and eight had AOC and TPC with tolerable cytotoxicity.

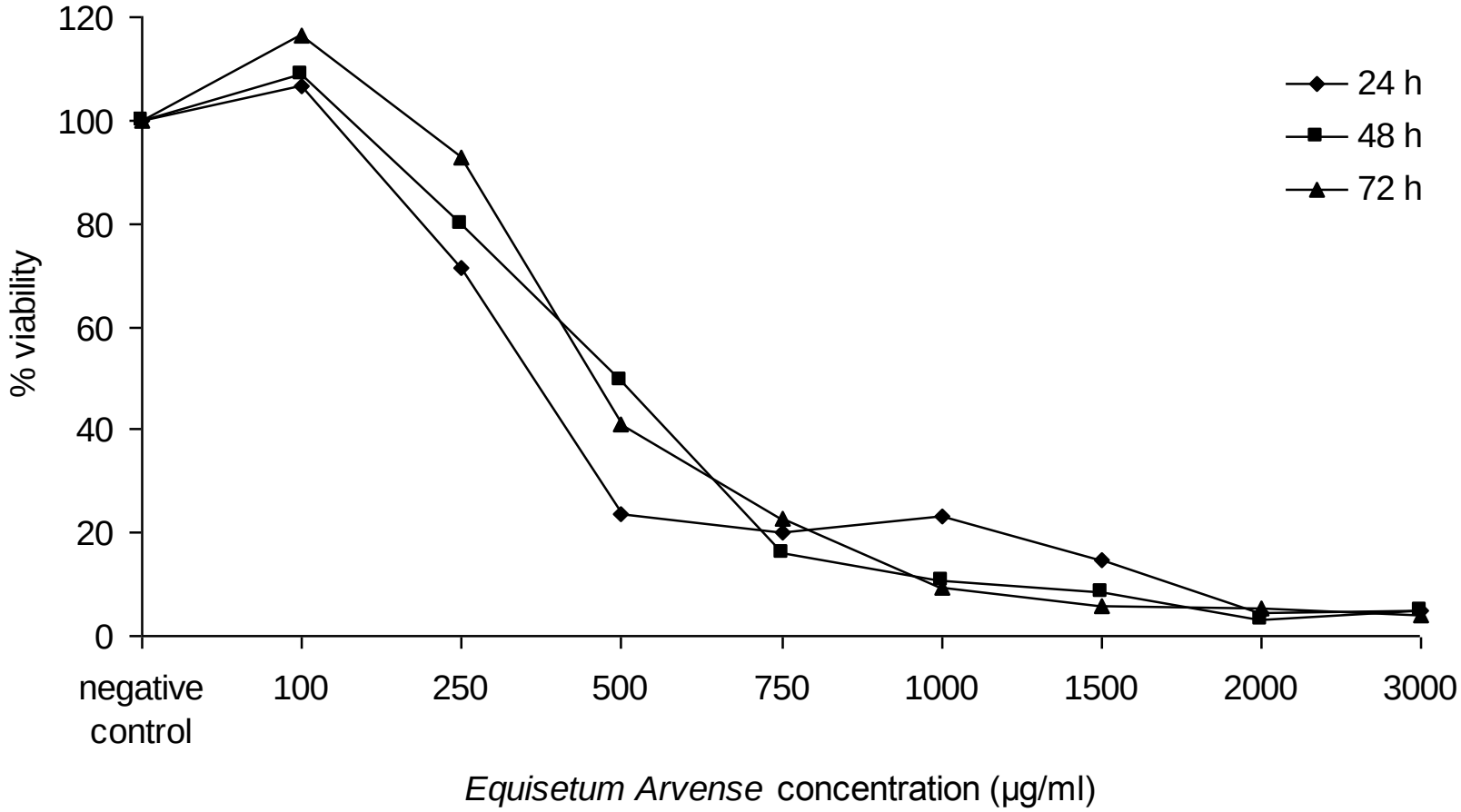

Figure 9. Graph of cytotoxic activity of *Equisetum arvense*. Plant *was* extracted in 70% aqueous ethanol at 180 rpm at room temperature for 2 hours. IC50 indicates the inhibitory effect of the extract on the half of the population.

Table 10. Cytotoxic activity of *Equisetum arvense* extracts obtained from parametric extraction study. Negative control cell viability was assumed as 100%. (L) indicates lowest total antioxidant activity (TAOC) and total phenol content (TPC) as (H) indicating the highest values

	% Viability				
Extract code	24 hour	48 hour	72 hour	Antioxidant activity (μM TEAC/mg extract dry weight)	Total phenol content (mg GAE/g extract dry weight)
1	117.90	85.27	77.95	830.89	82.94
2 (L)	142.65	104.57	104.11	395.40	35.32
3	152.04	86.29	98.75	292.75	20.47
4	119.23	86.49	102.03	338.80	35.41
5	119.34	80.67	99.68	412.14	25.39
6 (L)	117.24	93.25	114.98	550.20	41.90
7	121.55	89.97	105.46	585.21	34.50
8 (L)	126.63	101.13	109.94	820.69	41.57
9	132.38	82.53	81.88	939.18	59.25
10	83.65	67.21	60.38	725.69	106.46
11	155.36	108.27	88.72	424.02	53.56
12	125.52	68.58	77.35	412.35	57.13
13 (H)	18.90	12.09	8.97	1214.94	150.04
14 (H)	41.99	6.56	_	1246.82	182.80
15 (H)	34.36	23.69	28.99	1268.09	115.40
16 (H)	18.12	12.68	1.99	1270.79	149.14
17	62.43	61.58	66.57	838.07	86.38
18	137.13	91.34	77.30	523.70	52.08
19	83.43	61.04	57.79	651.98	86.15
20	73.81	55.36	53.40	572.58	79.57
21	125.52	91.04	61.72	357.18	51.85
22	132.82	104.85	105.78	532.75	63.46
23	120.66	89.43	77.99	817.09	53.27
24	116.13	66.81	53.54	1116.61	80.58
25	61.22	74.30	64.59	885.20	93.25
26	108.07	67.55	53.35	1099.02	103.63
27	61.99	59.03	44.34	814.92	85.47
28	60.44	72.93	52.33	866.76	79.82
29	64.64	59.47	46.46	954.65	94.85
30	164.31	83.90	63.80	915.48	100.92
31	98.23	76.06	52.43	1203.40	75.77
32	74.81	57.66	35.78	1026.65	115.41

High antioxidant capacity and total phenol content represents scavenging activity of a compound against free radical formation in the cell. Free radicals, having importance in energy generation and metabolic activities, shares electrons of other atoms to meet the absence of electron in the outermost orbit. Otherwise cellular lipids, proteins, DNA, enzymes and cellular respiration are affected negatively due to oxidative stress and it leads to cell death (48). Oxidant-producing enzymes specific for bacterial killing cannot work efficiently due to low amount of oxygen in the wound area (49). Wound area becomes rich in microbial flora and wound healing process is negatively affected. Wound area is also exposed to repeated perfusion, resulting in increase in oxygen amount in wound area and free radical release. Oxidative stress is increased due to high concentration of free radicals and cells playing role in regeneration phase of wound healing are degraded (50). This repeated anemia/perfusion activity leads to prolonged wound healing process.

Compounds having antioxidant activity inhibits free radicals, intermediate products of free radicals and oxidation reactions which cause oxidative stress (51). Amount of these antioxidant compounds is critical for their benefit. Excessive amount of antioxidants decrease activity of oxidant enzymes which has inhibitory role against microorganisms in the wound area. It has been observed in this study that extracts having higher antioxidant activity and total phenol content leads to cytotoxic activity on fibroblast cells, in accordance with the theory. Antioxidant activity and total phenol content is found as closely related with the extraction parameters such as extraction time, extraction medium system and temperature. It can be concluded that optimization of extraction plays a key role in properties of the extract, that is also relevant with its use.

4.1.6 HPLC Analysis

The compositional differences between the extracts which caused the diversity in the total antioxidant capacity and total phenol contents were investigated and confirmed by HPLC analyses. *Equisetum arvense* extracts of samples 3, 4, 13 and 16 were chosen to be analyzed. Sample 13 and 16 had higher total antioxidant capacity and total phenol content whereas sample 3 and 4 had lower antioxidant capacity and total phenol content. The reason to choose two samples for high and low values was to determine the effects of extraction parameters on antioxidant properties of extracts due to changes of extract composition. Sample 3 was extracted at lower temperature and solid-liquid ratio than sample 4, sample 13 was extracted at lower temperature and stirring speed than sample 14. HPLC analysis was done at 254 and 280 nm. The results were shown in Figure 10.

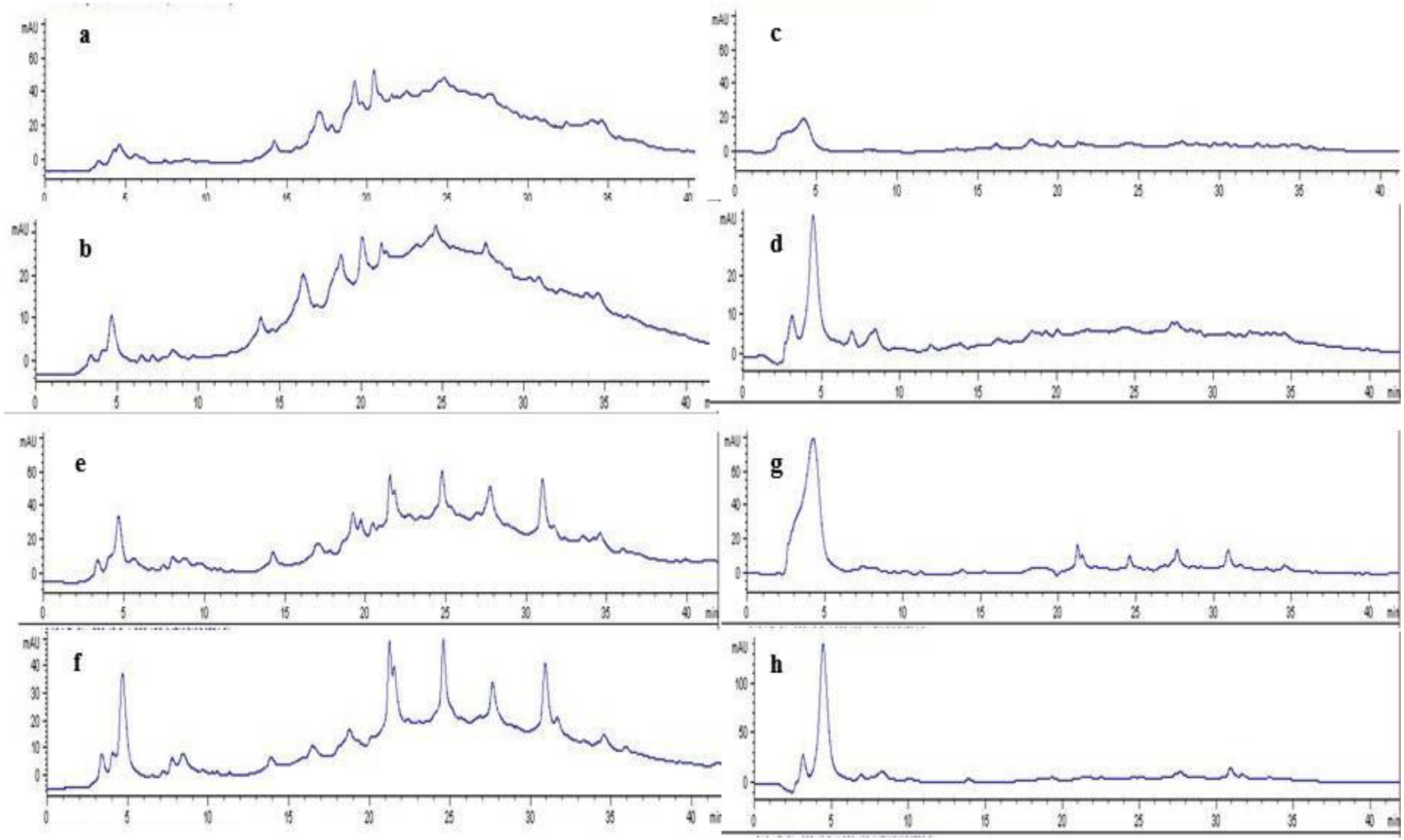

Figure 10. HPLC chromatograms of 280 nm (a to d) and 254 nm (e to h). Samples were 16 (a, e); 13 (b, f); 3 (c, g) and 4 (d, h).

HPLC chromatograms showed consistency with total phenolic content results. The areas of peaks for sample 16 and 13 were at least 4 times higher than those areas of samples 3 and 4. These differences were occurred because of the non-polar substances in the plant material as expected. As the polarity of the mobile phase decreased the non-polar substances were washed out of the column. Differences between the total phenol contents of the *equisetum arvense* extract resulted in the difference of total antioxidant capacities of extracts.

Samples have differences in HPLC analysis regarding amount of phenolic content. When sample 13 and 16 are compared, it can be seen that sample 16 has little more non-polar compounds than sample 13 according to areas of peaks observed in chromatogram areas. Same result can be seen between sample 4 and 3. The only differences for sample 13 and 16 were extraction temperature and stirring speed. As the temperature and stirring speed increased during extraction, the amount of soluble non-polar substances in the extract also increased.

In 2009 phenolic contents of *equisetum arvense* were investigated using HPLC analysis (27). Phenolic contents that were determined were caffeic acid, (-)-epicatechin, p-coumaric acid, vanilic acid and rutin. Those phenolic standards were also used in this research. The retention times of those standards were 19.58 min.; 17.725 min.; 20.085 min.; 27.869 min.; 17.048 min. for (-)-epicatechin, caffeic acid, p-coumaric acid, rutin and vanilic acid respectively. Chromatogram areas of the major compounds present in the *equisetum arvense* extract are given in Figure 11.

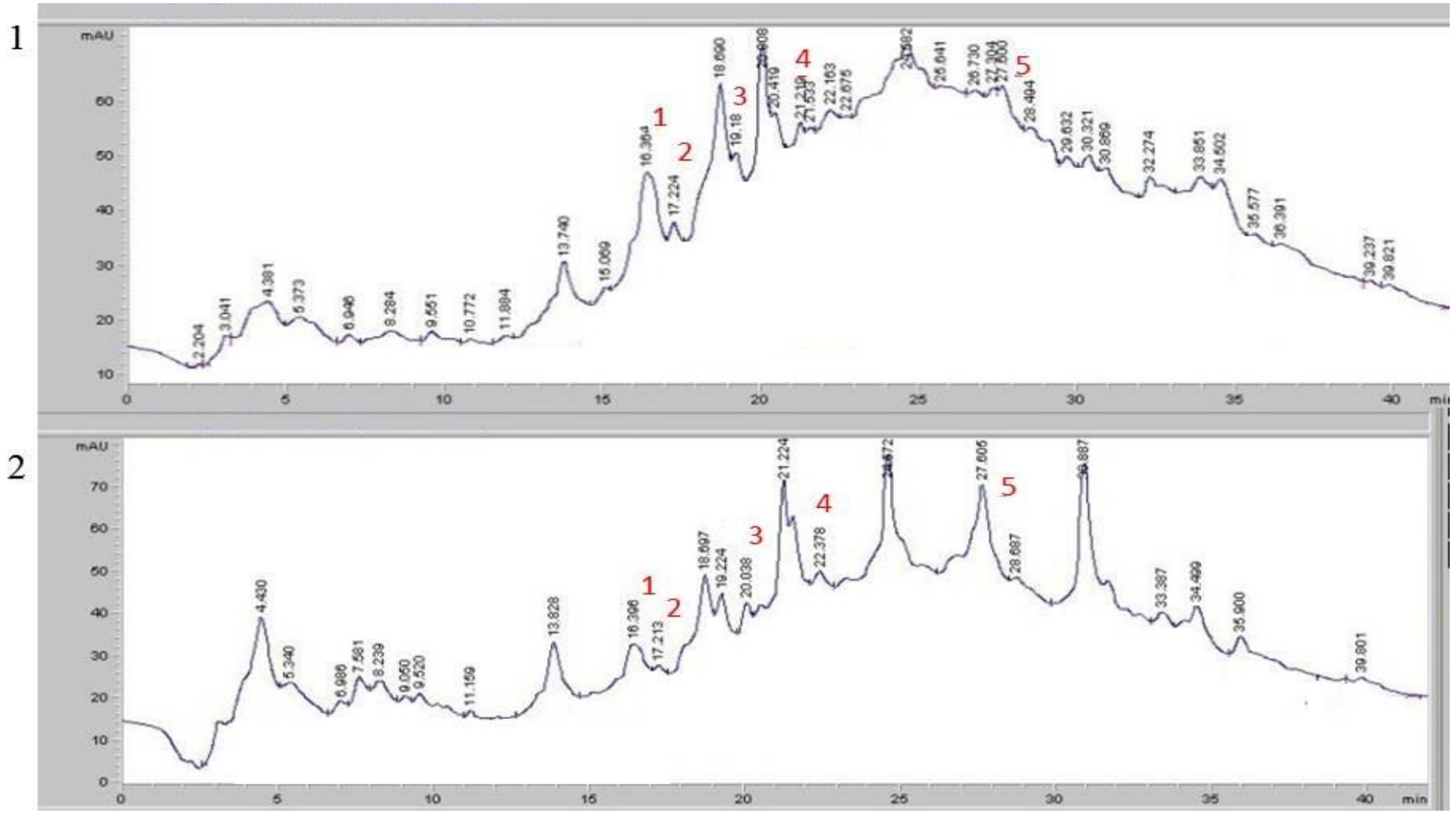

Figure 11. HPLC chromatogram of phenolic compounds in the *equisetum arvense* extract of sample 16, based on the retention times were given above. The phenolic compounds were caffeic acid (1); vanilic acid (2) ;(-)-epicatechin (3); p-coumaric acid (4); rutin (5). The chromatograms were analyzed at 280 nm (1) and 254 nm (2).

4.2. Blood Coagulation

4.2.1 Prothrombin Time

For each sample the prothrombin time were measured. The prothrombin time is the time it takes plasma to clot after addition of tissue factor. To determine the prothrombin time, the quality of extrinsic pathway could be measured. The rate could be affected by the levels of factor VII. In literature the *equisetum arvense* extract was mentioned as inhibitor for platelet aggregation (19), anti-aggregant for thrombin (52) and also had a antithrombin activity (53) although in folkloric medicine the plant was known for its haemostatic effect. Our study was confirming both of the claims. Parallel to the change in extraction parameters, the coagulation effect of the extract was shifted from anticoagulant to coagulant. The results can be seen in Table 11. In the table the time difference between coagulation of negative control and plant added sample was shown as ΔPT. Negative ΔPT means coagulation rate was higher than negative control. Positive ΔPT refers to blood slower coagulation rate than negative control. For prothrombin time blood coagulates approximately in 20 seconds.

Table 11. Change in the prothrombin time due to addition of plant extract (ΔPT).

Sample	ΔPT (sec)
1	2.5
2	3.5
3	10.9
4	20
5	-2.9
6	1.6
7	-5.1
8	2.4
9	-3
10	-0.2
11	-0.6
12	-1.4
13	5.3
14	-0.3
15	-0.8
16	3.9

Sample	ΔPT (sec)
17	1.5
18	3.7
19	2.9
20	0.8
21	-1.5
22	5.4
23	-1
24	0.5
25	2
26	0.5
27	2.1
28	3.2
29	0.6
30	-0.2
31	3.7
32	1.6

Also the relations between prothrombin time and antioxidant capacity and phenolic content were analyzed. In Figure 12 and Figure 13 the relations were shown. It was seen that as the phenolic content and antioxidant capacity decreases the extract became more coagulant. The low antioxidant capacity and phenolic content showed us that the major content of the extract were hydrophilic substances and were seen on the polar region of the HPLC chromatogram in Figure 10. By the help of prothrombin time we could determine the extrinsic pathway of coagulation. So it can be said that the apolar substances trigger the extrinsic pathway of coagulation.

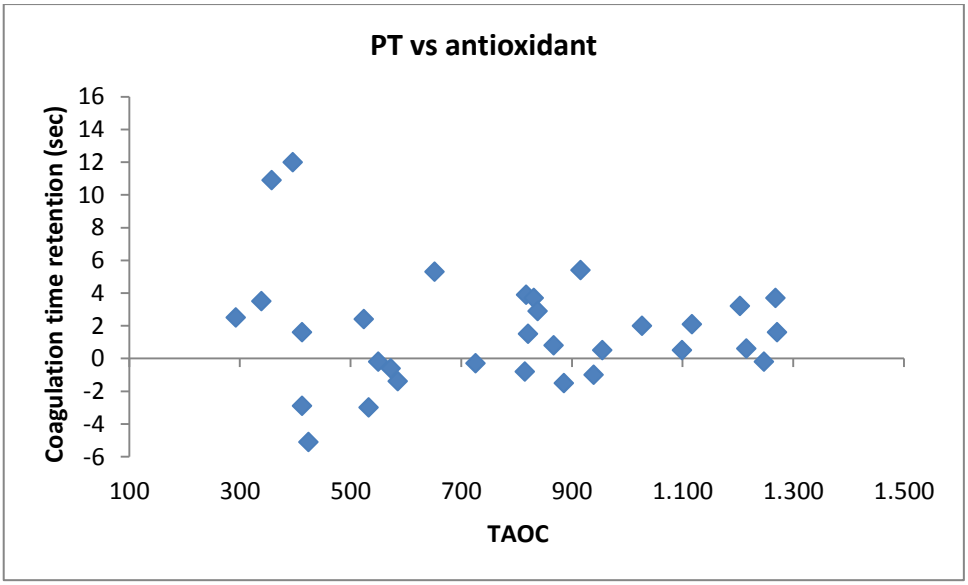

Figure 12. The relation between ΔPT time (sec) and total antioxidant capacity (TAOC).

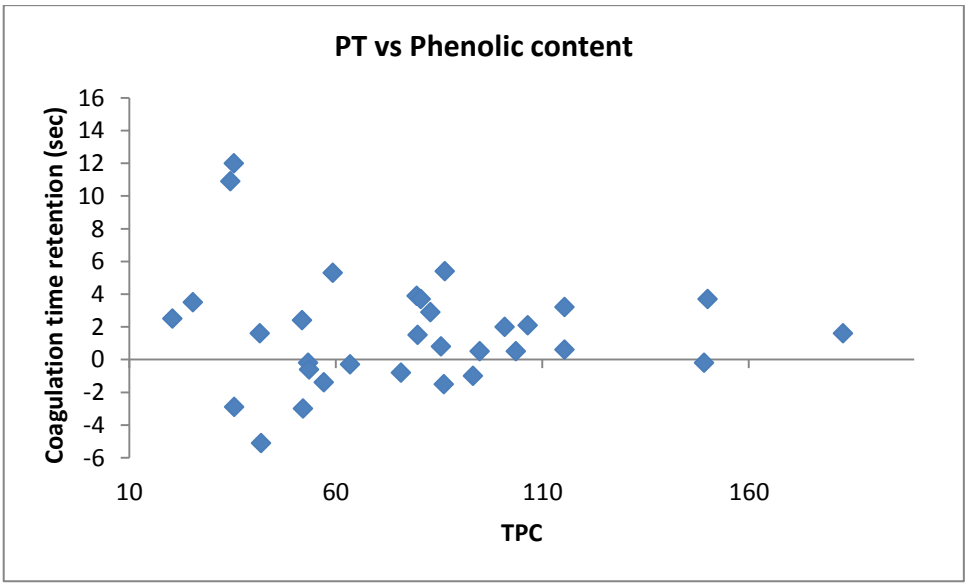

Figure 13. The relation between ΔPT time (sec) and total phenolic content (TPC).

4.2.2 Fibrinogen Time

The study on fibrinogen time (FT) gave similar results to PT results. But in fibrinogen time the results were much more significant. Specialized proteins were needed in the intrinsic pathway of blood coagulation. This pathway involved a series of proteins, protein cofactors, and enzymes, which interacted with the membrane surface of thrombin. As a result of this interaction at final stage fibrinogen was converted into fibrin. Parallel to the change in extraction parameters, the coagulation effect of the extract was shifted from anticoagulant to coagulant. The results can be seen on Table 12. In the table the time difference between coagulation of negative control and plant added sample was shown as ΔFT. Negative ΔFT means blood was coagulating faster than negative control. Positive ΔFT means blood was coagulating slower than negative control. For prothrombin time blood coagulates approximately in 10 seconds.

Table 12. Change in the fibrinogen time due to addition of plant extract (ΔFT).

Sample	ΔFT (sec)
1	-0.2
2	2.1
3	4.4
4	1
5	4
6	1.2
7	-0.1
8	0.3
9	2.2
10	27.4
11	0.8
12	0.9
13	-0.3
14	0.4
15	1.2
16	1

Sample	ΔFT (sec)
17	2.5
18	-0.8
19	-1.5
20	-1.2
21	0.4
22	-1.7
23	0.3
24	-1.5
25	-0.9
26	-0.5
27	-1
28	0.2
29	-0.3
30	-1.4
31	-0.8
32	-0.5

However the relations between fibrinogen time and antioxidant capacity and phenolic content were different than those of prothrombin time results. In Figure 14 and Figure 15 these relations can be seen. It was seen that as the phenolic content and antioxidant capacity of extract increased the coagulation activity of extract increased as

well. The high antioxidant capacity and phenolic content showed us that the major content of the extract were hydrophobic substances and were seen in the relatively apolar region of the HPLC chromatogram. By the help of fibrinogen time we could determine the intrinsic pathway for coagulation. So it can be said that the apolar substances trigger the intrinsic pathway of coagulation cascade severely. The proteins in the intrinsic pathway were affected by the phenolics explicitly.

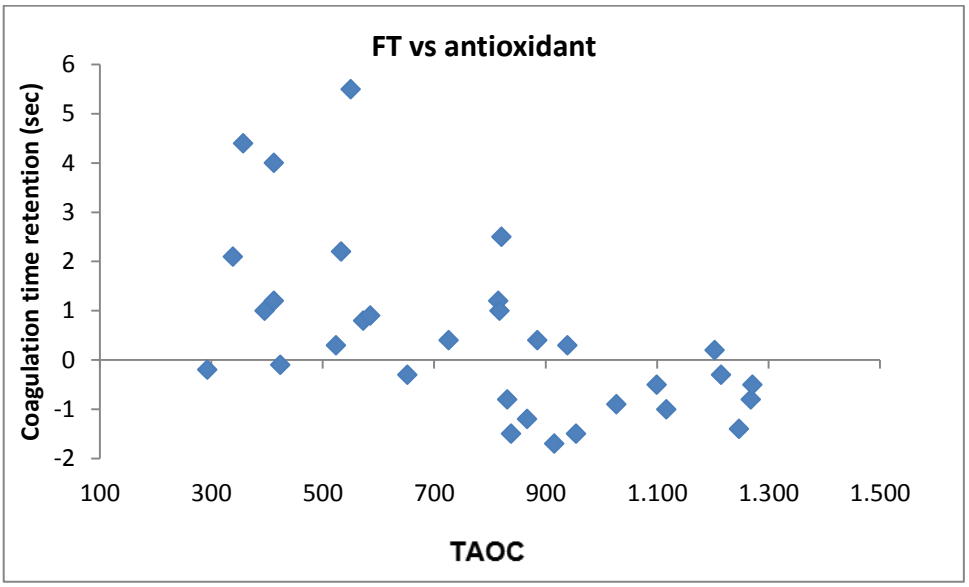

Figure 14. The relation between ΔFT time (sec) and total antioxidant capacity (TAOC).

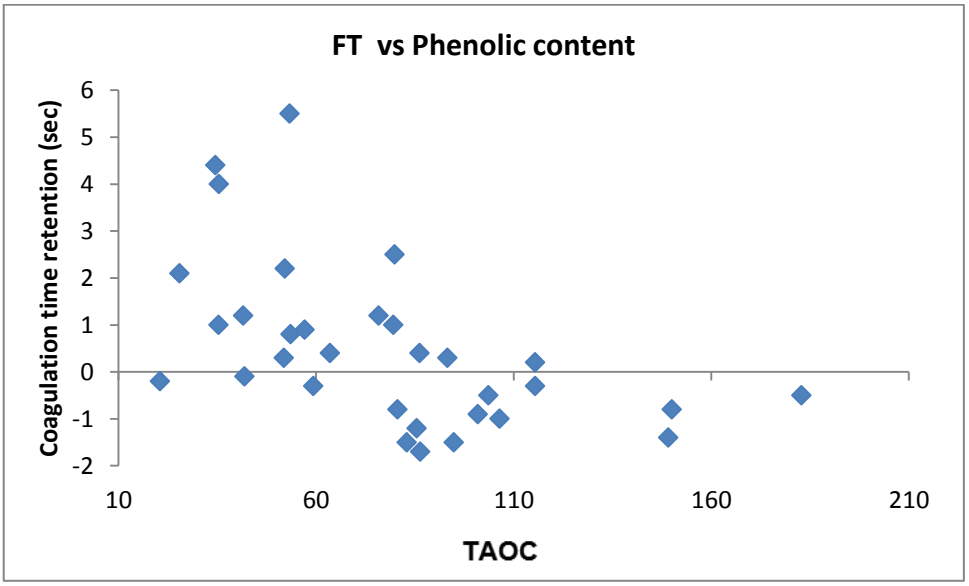

Figure 15. The relation between ΔFT time (sec) and phenolic content (TPC).

4.2.3 HPLC Analyses

In order to detect the active phenolic compound on blood coagulation, HPLC analyses were performed. Sample 9, 21, 30 and 19 were chosen for their inhibitory and accelerating effect on prothrombin time. Their ΔFT were 2.6, 1.2, - 1.6 and -1.2 respectively. However no reasonable changes could be observed on their ΔPT results. They were 4, -1.7, -1.37 and 0.53 respectively. HPLC chromatograms of those samples can be seen in Figure 16.

Phenolic acid which has a retention time of 13.6 minute may have potential on decreasing ΔFT. It was the only phenolics with a decreasing concentration and supported the change in ΔFT. According to the retention times between 12 minutes and 16 minutes caffeic acid and its derivatives were seen on the HPLC chromatogram. Chao *et al.* 2009 showed caffeic acid could provide anti-coagulatory protection in diabetic mice (54). The unknown peak on the chromatogram with a retention time of 13.6 was thought to be a caffeic acid derivative. The results were confirmed with Chao's findings. The extract acted as an anti-coagulant substance while the concentration of this derivative was high and other relatively polar phenolics concentration low. However when the caffeic acid derivative concentration decreased and the polar phenolics concentration increased, extracts started to act like haemostatic agent.

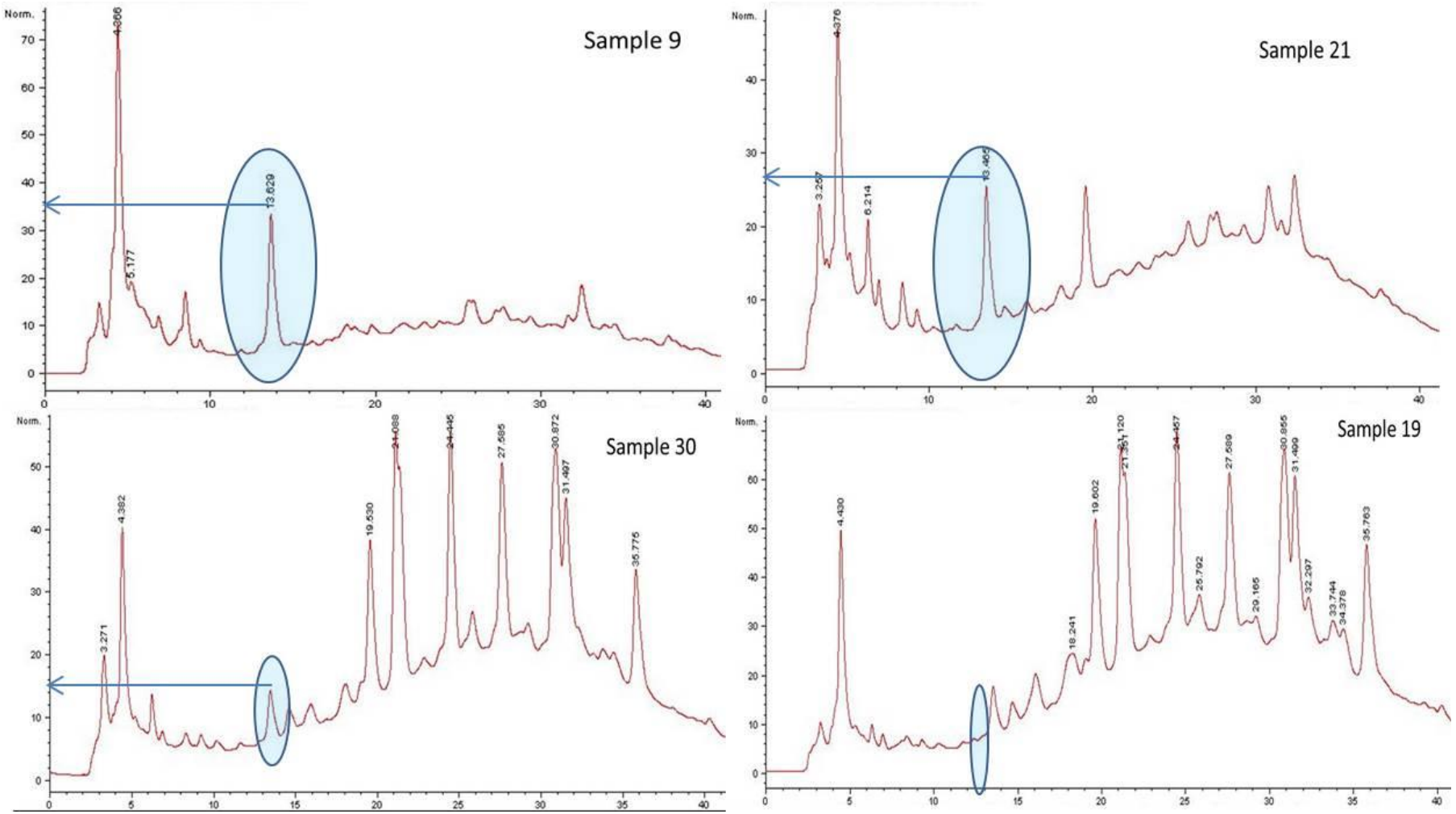

Figure 16. HPLC chromatograms of samples 9, 19, 21, 30. ΔPT time (sec) were -3, 2.9, -1.5 and -0.2 respectively. The phenolic acid which was thought to be responsible for lower the PT was circled.

4.3. Biomaterial

4.3.1 Morphology of Prepared Biomaterials

To prepare a sponge form biomaterial which has a plant extract was a difficult task to overcome. Because phenolic compounds had a high tendency to interact with proteins (53, 54). As a result of these interactions, morphology of proteins were changed and precipitations occurred. For preliminary research; effect of freezing temperature, hyaluronic acid concentration and plant extract concentration was investigated.

The morphology of sixteen different sponge samples was analyzed using SEM micrographs. The preparation methods and results of the SEM analyzes of the sample were given in Figure 17. First two columns of pictures show the sponge made with adding 3% hyaluronic acid and the last two columns show the sponges made with adding 15% hyaluronic acid. Also the freezing temperatures (-24 $^{\circ}$C, -80 $^{\circ}$C) of each sponge material were given on top of the columns. On the other hand rows show the concentration of plant extract which was added to the sponges before freezing.

It was seen that hyaluronic acid concentration was an important factor for the layer formation in the sponges. As the concentration increased, more rigid layers formed. Also increasing concentration of hyaluronic acid in the sponge effected the pore formation. More regular and spherical pores were observed. The freezing temperature was also an important factor for the pore formation. As freezing temperature decreased the number of the pores increased. On the other hand the sponge matrices changed their structure. No pore formation or no layer formation was seen as the *equisetum arvense* extract concentration increased. Disruption in the pore and layer formation as a result of plant extract addition was negatively affected the mechanical strength of the sponge material.

In this method aqueous plant extract was added after hyaluronic acid-silk fibroin mixture was formed. In order to obtain homogenous mixture materials were mixed for at least 15 minutes. In this period of time phenolics in the plant extract interacted with silk fibroin proteins and white precipitate was formed. Since silk fibroin concentration was decreased due to precipitation and the mechanical strength of the biomaterial was affected negatively.

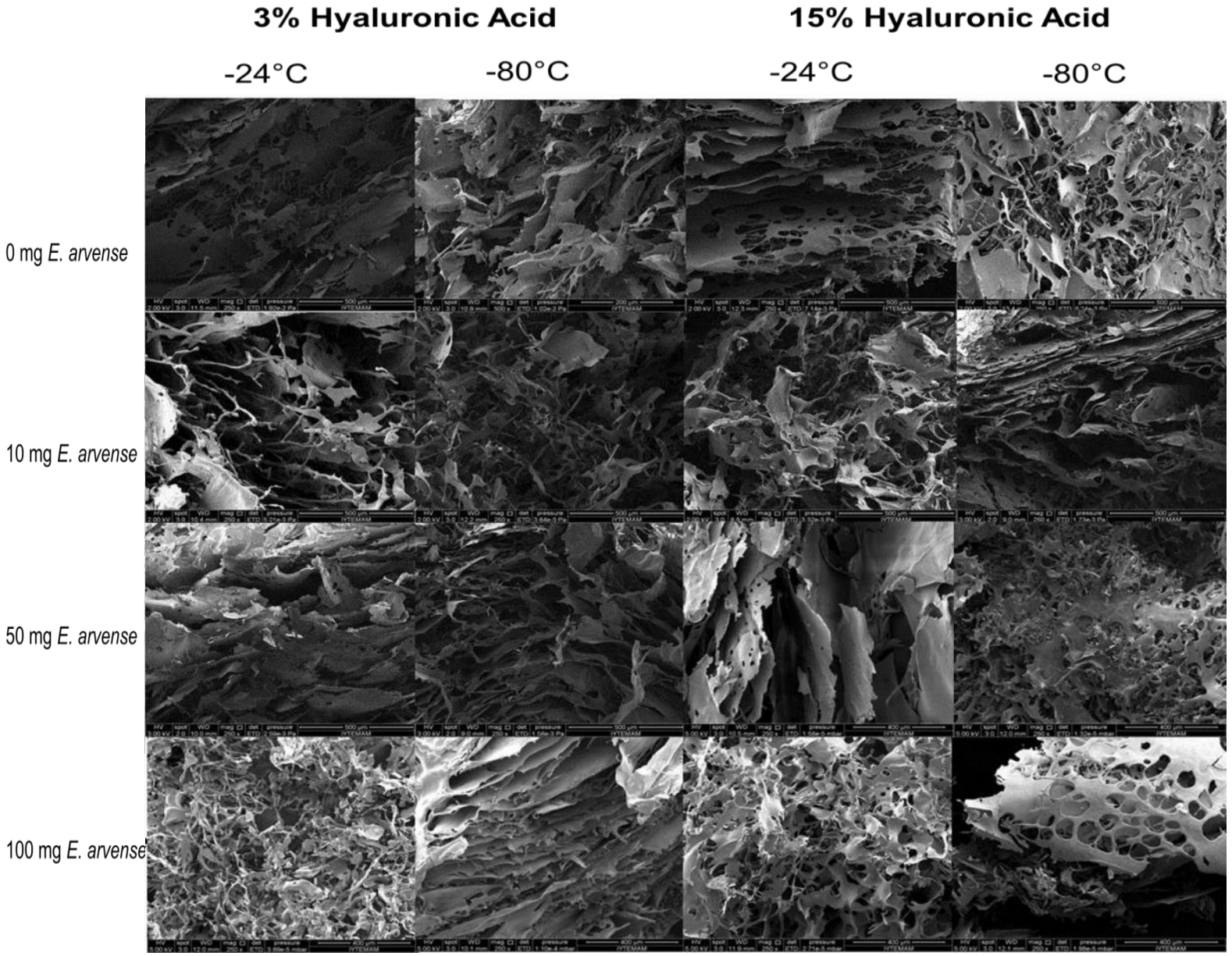

Figure 17. SEM micrographs of sponge matrices with varying extract amounts.

4.3.2 Fluid Uptake and Degradation Biomaterial

The sponges prepared by addition of hyaluronic acid and *equisetum arvense* extract into silk fibroin separately were subjected to fluid uptake and degradation tests. The dry weights of sponge matrices with varying extract amounts after 96 hours PBS treatment were shown in Figure 18. As it was seen from Figure 18 as the amount of extract increased the weight loss increased. The matrices with no extract lost 50% of their initial weight after 96 hours. The sponge matrices which had a 100 mg plant extract dissolved completely in PBS after 2 hours.

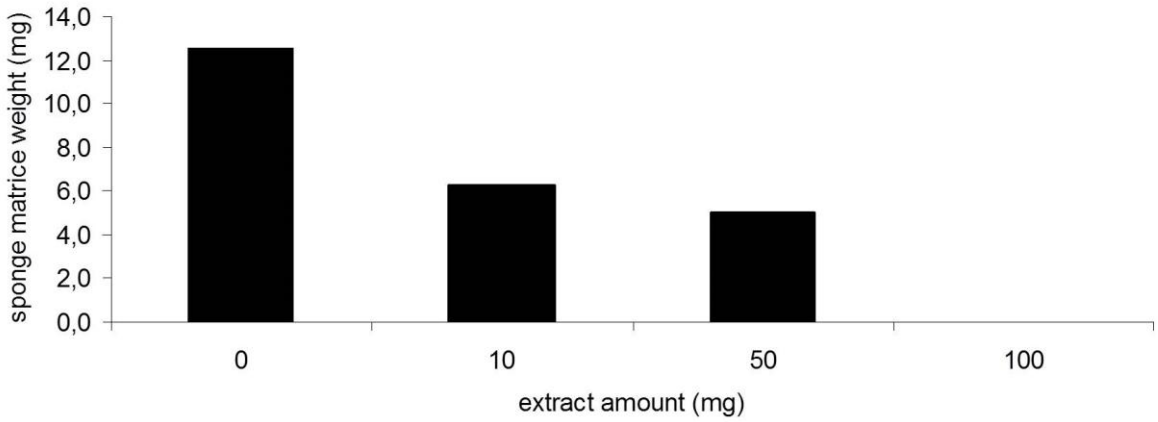

Figure 18. Weight of matrices with varying extract amounts after 96 hours' PBS release

Also it was seen that increases in the extract amount, increased the fragility of the matrices. Sponge materials' mechanical strength and their release properties changed as the extract amount increased. The matrices which had higher extract amount disintegrated immediately. This disintegration and fragility of the extracted added sponge matrices would be as a result of protein-phenolic compound interactions by weak bonds (55). As a result of this interactions precipitation of complexes occur and solubility decreases (56). Also it was seen that if hyaluronic acid amount increased the mechanical strength and degradation time. Also increasing freezing temperature had a negative effect on mechanical strength and degredation time. The reason for this phenomenon was described in the previous section. As a result of protein-phenolic interaction, silk fibroin was precipitated. Due to the decrease in silk fibroin concentration in the solution, the structure of the sponge was non-uniform. This non uniformity affected mechanical strength of the sponge material negatively.

4.3.3 Phenolic Content and Total Antioxidant Capacity Analysis of Sponges with Extract

The sponges prepared by addition of hyaluronic acid and *equisetum arvense* extract into silk fibroin separately were subjected to phenolic content and total antioxidant capacity analyses. In Table 13 and 14, the phenolic content and antioxidant capacities of release medium of each sponge matrices were tabulated. As treatment time increased the viscosity of release medium increased due to dissolution of hyaluronic acid. Because of dissolved hyaluronic acid there were deviations in the phenolic content and total antioxidant capacity results. However most of the plant extract in the matrices were released during first 24 hours.

Table 13. Total phenolic content in the release medium of sponge matrices

	TPC (mg Gallic Acid equivalent/ml)			
	24 hr	**48 hr**	**72 hr**	**96 hr**
A1	6,5	7,5	13,7	2,2
A2	6,9	12,3	7,2	1,2
A3	11,0	6,2	4,8	0,8
A4	11,2	0,2	10,0	1,6
B1	5,7	7,2	12,3	2,0
B2	5,0	4,9	11,7	1,9
B3	5,7	1,8	13,4	2,2
B4	4,0	16,0	38,6	6,3
C1	11,6	25,9	32,8	5,3
C2	13,9	17,1	23,9	3,9
C3	10,1	16,0	16,6	2,7
C4	14,7	39,1	41,5	6,8
D1	10,4	10,7	15,1	2,5
D2	5,9	14,8	17,9	2,9
D3	8,3	17,4	12,5	2,0
D4	13,4	25,3	31,2	5,1

Table 14. Total antioxidant capacities in the release medium of sponge matrices

TAOC (µM TEAC / µl)			
24 hr	**48 hr**	**72 hr**	**96 hr**
103,08	70,53	79,55	-3,95
133,32	39,29	76,15	-0,94
78,89	64,73	81,85	-37,89
134,47	-31,61	167,01	105,56
145,81	70,10	142,13	111,40
83,20	67,09	84,13	232,58
175,87	27,49	101,91	167,37
84,59	173,41	220,45	148,90
125,30	252,02	229,01	215,34
157,11	227,46	217,71	239,91
87,53	198,78	201,55	236,86
159,42	295,01	256,73	264,17
126,85	113,87	187,99	127,77
85,88	150,91	188,03	125,00
151,08	238,18	206,35	140,93
145,82	255,85	249,74	291,57

When the results were analyzed in detailed, it was observed that as the release of extract from the sponge occurred in a burst manner when the concentration of plant extract increased in the sponge. The sudden burst release can be attributed to disintegration of sponge structure caused by its relatively low mechanical strength. This result could be used for developing double layer sponge materials. Inside layer could be arranged with high hyaluronic acid and low extract concentration in order to give

structural support to wound area with its relatively high tensile strength whereas the outer layer which had high extract concentration could make sudden release of the extract into the wound area in order to stop bleeding.

New ways of constituting sponge materials were analyzed after the failure of previous trials. In the next three chapters the results of addition of aqueous *Equisetum Arvense* extract to sponges, dipping sponges into the aqueous plant extract and, coating sponges with extract were discussed.

4.3.4 Addition of Aqueous *Equisetum Arvense* Extract to Sponges

In this method blank discs with different hyaluronic acid content were prepared. Than plant extract which was dissolved in dH_2O, was added on top of the blank sponges. The sponges were disintegrated immediately right after addition of aqueous plant extract. No reformation of the sponges was seen after drying. The result can be seen in Figure 19.

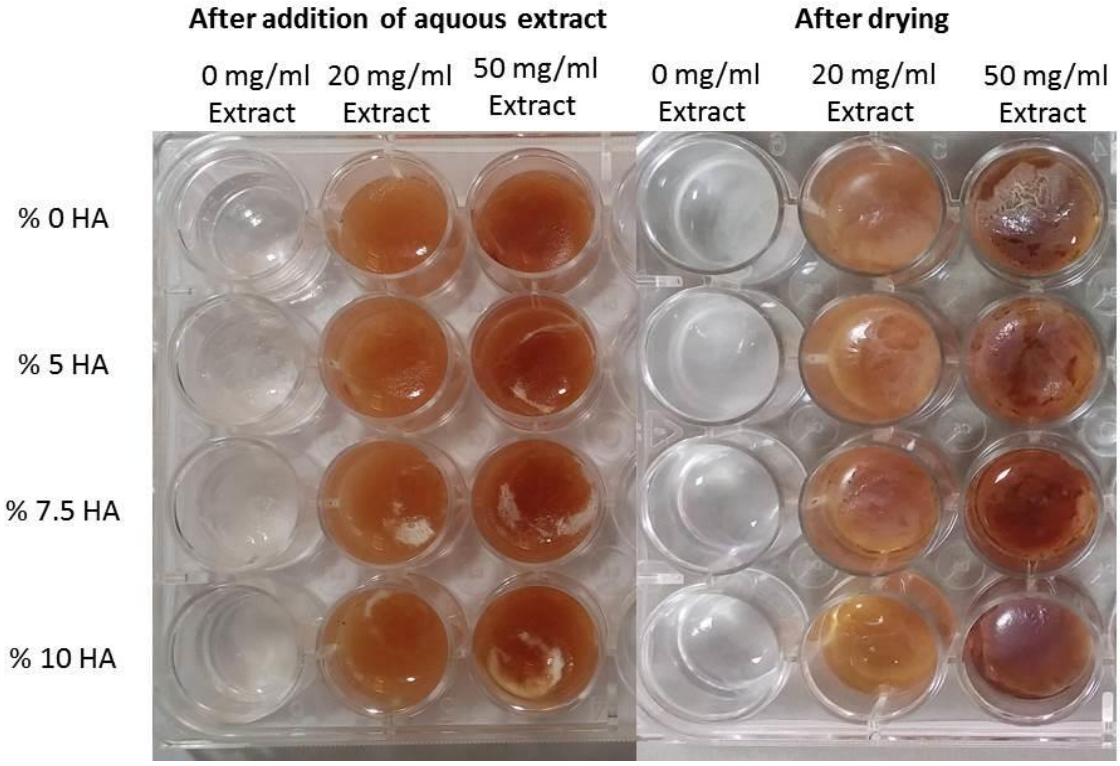

Figure 19. The picture of prepared sponges with addition of aqueous solution of *equisetum arvense* extract. Image on the left hand side showed samples after addition of aqueous plant extract, on the right hand side after drying was performed.

This method was not effective because the concentration of hyaluronic acid was

not high enough to prevent disintegration of the material in aqueous solutions. However 10% of hyaluronic acid could be the highest amount for formation of 3D material in this case. Higher than 10% hyaluronic acid resulted in dense and rough material.

4.3.5 Dipping Sponges into Solution of *Equisetum Arvense* Extract

After failure of addition method, dipping blank sponge discs into the aqueous plant extract for 2 seconds was tested. By this method interaction of sponges with aqueous solution for longer time period was prevented. The idea behind this method was absorption of extract by the pores of sponge to prevent disintegration. For this method plant extract was dissolved both in dH_2O and 50% ethanol. Absolute ethanol converted silk fibroin into β form which had low solubility. Sponges which had 5 and 7.5 % hyaluronic acid were dissolved immediately after dipping into both aqueous plant extract prepared by dH2O and 50% EtOH. The method was successful for the sponges containing 10% hyaluronic acid. However after drying, those sponges lost their shapes and became more rigid. That would be unsuitable for wound healing applications. The result can be seen in Figure 20. Both blank discs, discs after dipping and after drying were seen in the figure. On the outer circle negative controls and in the inner circle sponges which were dipped into aqueous solutions of plant extracts were shown.

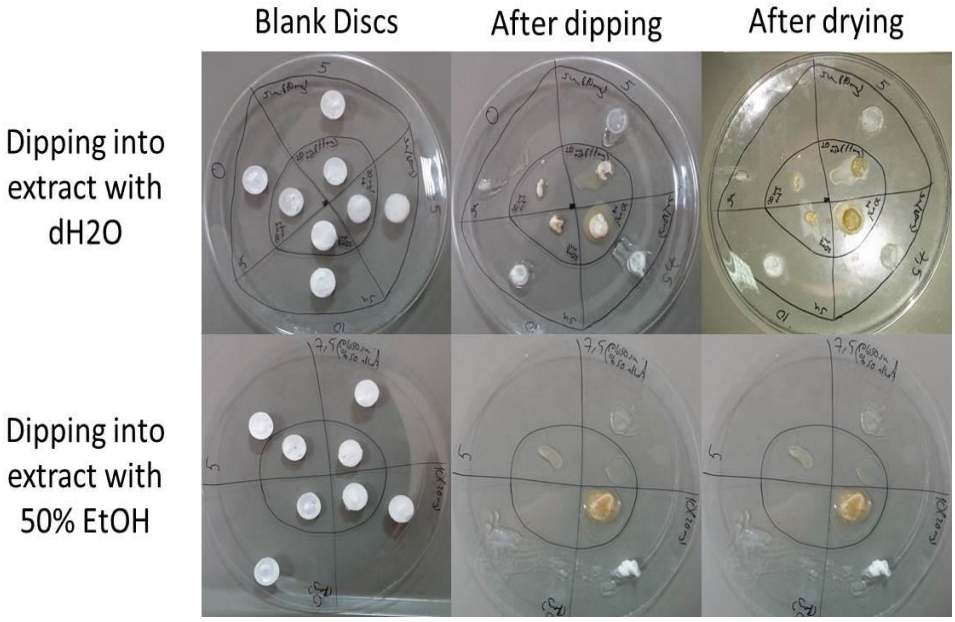

Figure 20. Images of sponges prepared by dipping method.

4.3.6 Coating Sponges with Mixture of Hyaluronic Acid and *Equisetum Arvense* Extract

In order to prevent disintegration of the sponges in the dipping and addition methods coating of blank sponges were investigated. Extracts were dissolved in the dH_2O which had hyaluronic acid. By this way hydrogelated extracts were obtained. Those hydrogels would cover the outer layer of blank sponges and could not penetrate into the sponges. So disintegration of the material would be prevented. As expected sponges were not dissolved after covering with hydrogelated aqueous plant extract. However they were harder after drying. Due to formation of a shell on the outer surface this hard shell could not be appropriate for the wound healing applications. The result can be seen in Figure 21.

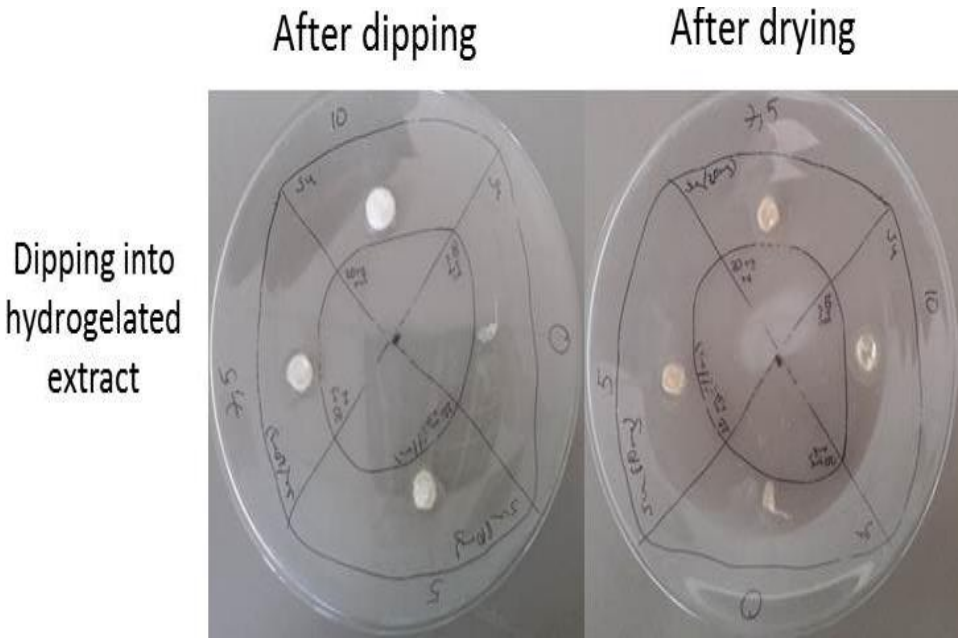

Figure 21. The image of sponges prepared by dipping method.

4.3.7 Preparation of Silk Fibroin Sponge with Addition of Aqueous Hyaluronic Acid Solution Including *Equisetum Arvense* Extract

The negative effect of plant extract on silk fibroin was observed because of significant interaction of its phenolic content with silk fibroin protein. In order to alter this interaction between phenolics and protein the phenolics in the extract should be encapsulated. The easiest way was to encapsulate the phenolics with hyaluronic acid. Plant extract was dissolved to obtain hyaluronic acid solutions with different extract concentrations. After encapsulation of plant extract these solutions were added into the aqueous silk fibroin. After adding plant extract - hyaluronic acid solution into the silk fibroin there were no precipitation observed. After lyophilization the prepared sponges were in intact form and no phase separation was seen. Results can be seen in Figure 22. As hyaluronic acid (HA) ratio increased the density of sponge increased as well. Also the mechanical strength of the sponge samples in the solution increased in parallel with increasing HA concentration.

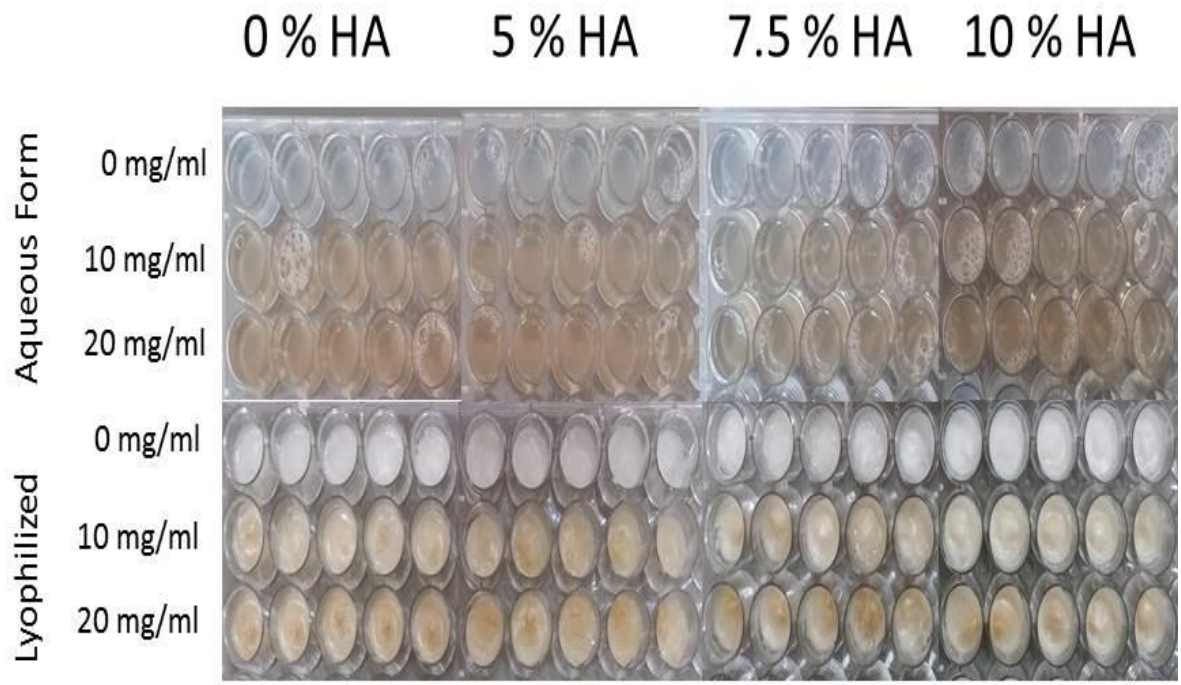

Figure 22. Silk Fibroin sponges prepared with liquid Hyaluronic acid and *E. Arvense* mixture. On top aqueous form of sponge material was seen. On the bottom sponges were seen after lyophilization. The changing ratios of both hyaluronic acid and plant extract can be seen in the figure.

4.3.8 Morphology of Silk Fibroin Sponge with Addition of Aqueous Hyaluronic Acid Solution Including *Equisetum Arvense* Extract

In order to determine the structural properties of the sponges prepared by addition of *Equisetum arvense* hyaluronic acid mixture, SEM images of both vertical and horizontal sections were investigated. The results could be seen in Figure 23 and 24.

When both of the sections were analyzed it was seen that hyaluronic acid increased the regularity of the matrices. Sponges consisted of multiple layers as the hyaluronic acid concentration inside the material increased. On vertical section tubular formations could be seen. More distinct layer formations were seen on horizontal section of sponges as seen in SEM images. Both of the structural forms were important for both cell proliferation and vein formations. Also this structure might support the new growing tissue. The sizes of both vertical tubes and horizontal layer were between the ranges of 2 to 50 micrometers. These ranges of sizes were sufficient enough for the movement of fibroblast cells and white blood cells. As the hyaluronic acid concentration increased the mechanical strength and the rigidity of the sponges were also increased.

Concentration of the plant extract effected pore size and the distribution of the pores of sponge materials. In low concentrations distributions of the pores were nonuniform. As the concentration of plant extract increased they became more uniform. These results revealed that preparation technique with hyaluronic acid encapsulated plant extract was successful. Also plant extract hyaluronic acid mixture might act as crosslinking agent and increased the performance of sponge material. Uniform pores and structure of sponge material were important for enhanced cell migration. As a result of uniform structure, the formation of scar tissue could be prevented. Growing of healthy tissue would support the healing process further. Also the concentration of the plant extract determined the size of the pores on both sections. As the concentration of extract increased the pore sizes were also increased. This was important because by this way size-selective sponges could be arranged for cell migration. With this preparation technique sponges having pore size structure could be designed for to different tasks in wound healing and haemostatisis. The sizes of the cells in the body are different from each other. By controlling the size of the pores it could be possible to control cells that enter the scaffold. That would be resulted in quicker and smoother healing.

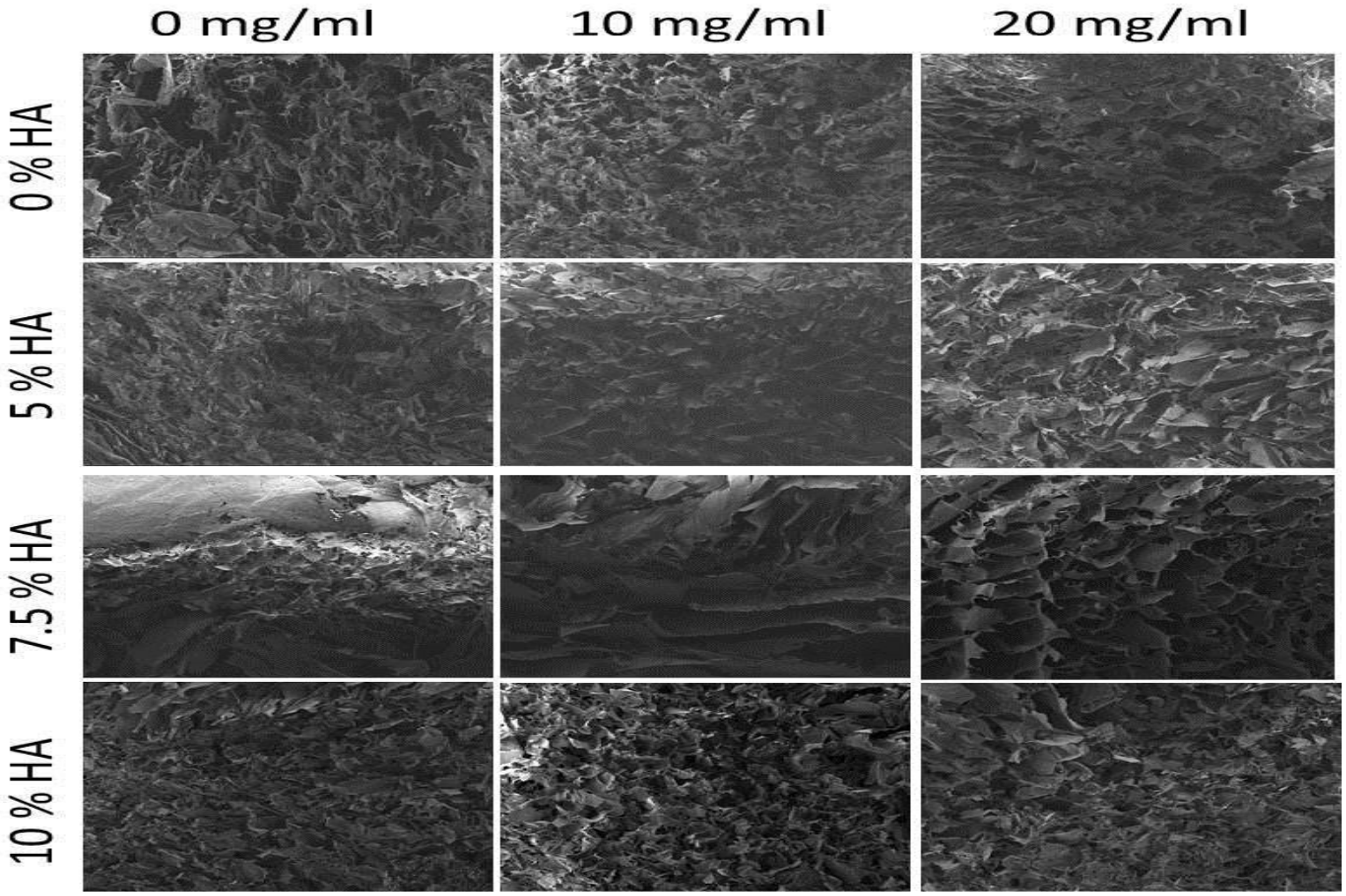

Figure 23. SEM pictures of vertical sections of silk fibroin sponge prepared with *equisetum arvense* hyaluronic acid mixture. Top row shows the concentrations of plant extract added into the sponges. On the left column the percent amount of hyaluronic acid added into the sponges were shown.

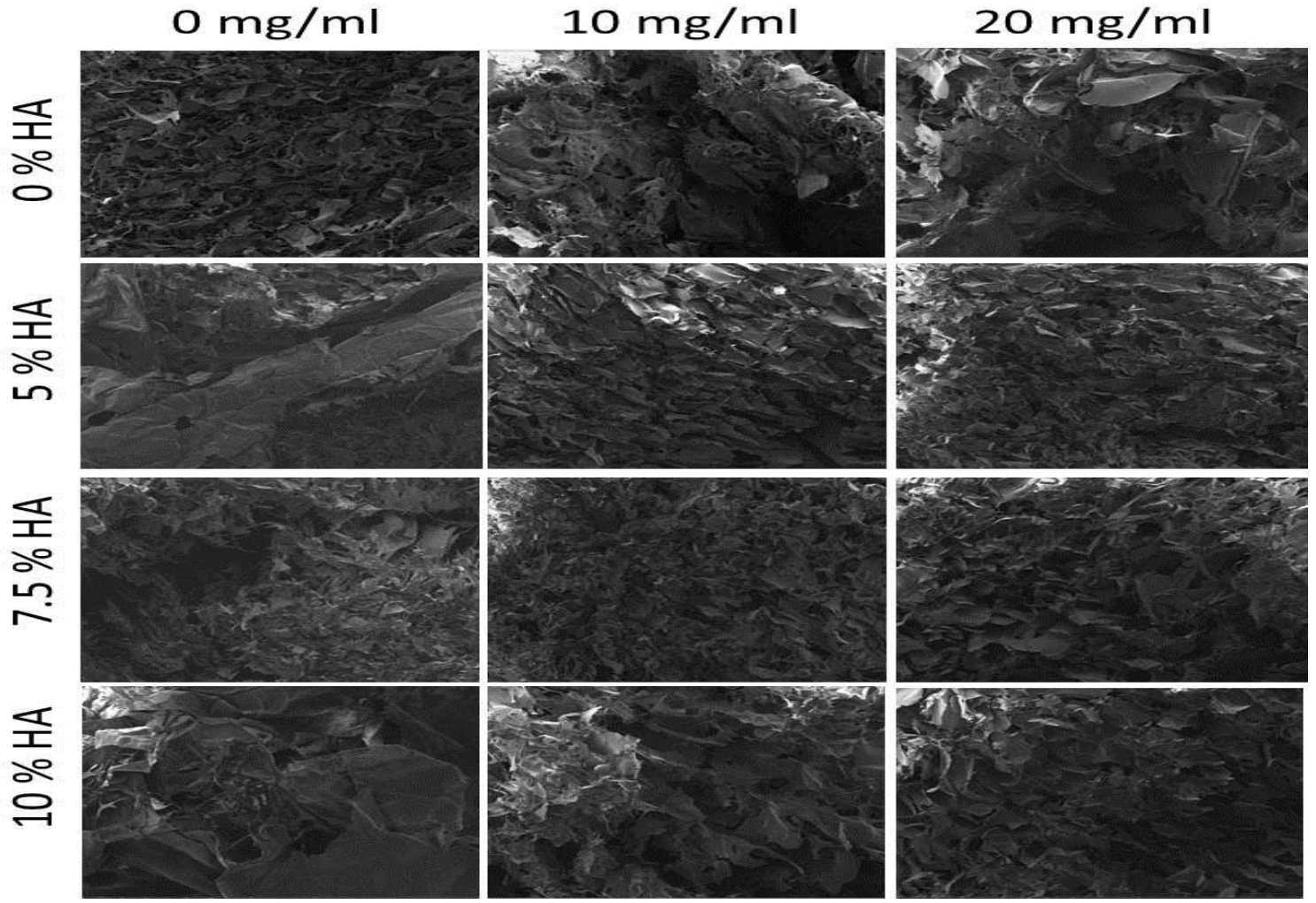

Figure 24. SEM pictures of horizantal sections of silk fibroin sponge prepared with *equisetum arvense* hyaluronic acid mixture. Top row shows the concentrations of plant extract added into the sponges. On the left column the percent amount of hyaluronic acid added into the sponges were shown.

4.3.9 Cytotoxicity of Silk Fibroin Sponge with Addition of Aqueous Hyaluronic Acid Solution Including *Equisetum Arvense* Extract

One of the most important properties of the biomaterial was its cytotoxic behavior. So the cytotoxic activities of the sponge samples were investigated. Results could be seen in Figure 25. According to results it could be observed that first 24 hour the release medium had low cytotoxic activity on fibroblast cells. In 48 hour release medium of the sponge samples caused decreasing viability. The average was 30% after 48 hours. However when 72 hour MTT results were analyzed, rapid cell proliferation can be seen. This phenomenon occurred as result of rapid burst release of plant extract into the medium from sponge biomaterial. High level of antioxidants acted as cytotoxic agent. Fibroblast cells consumed those materials in the first 24 hour time and metabolized them within 48 hour. The hyaluronic acid left in the medium started to effect on fibroblast cells and help their proliferation. This result was in accordance with the findings reported in the literature. In 1998 Greco et al. showed that hyaluronic acid stimulates human fibroblast proliferation (57).

According to this cytotoxicity results it could be claimed that sponge matrices could be used in both wound healing and haemostasis. Sudden burst release of the plant extract could stop bleeding immediately and remaining hyaluronic acid could stimulate fibroblast cells for proliferation while silk fibroin matrices give structural support.

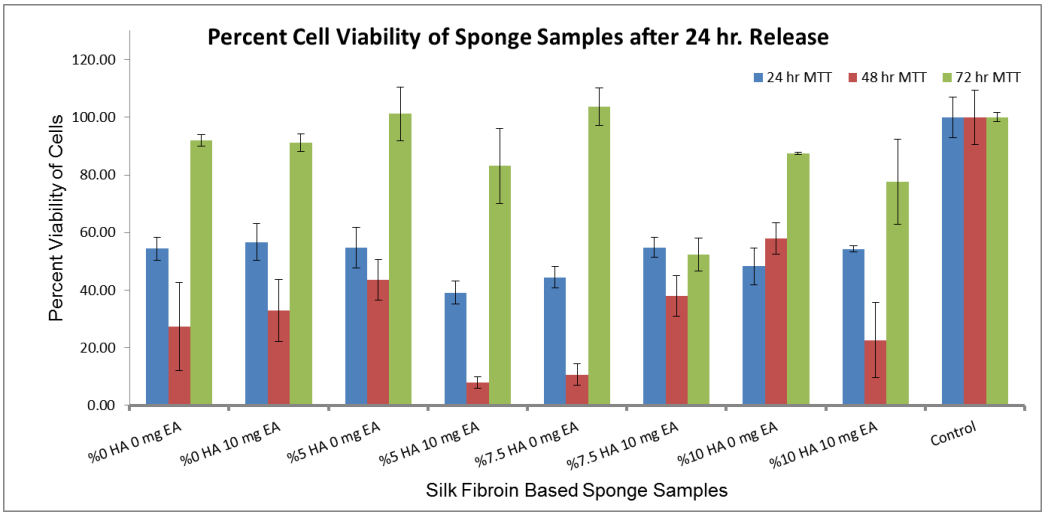

Figure 25. Percent cell viability of 24 hour release medium of sponge samples. MTT tests were done for 24, 48 and 72 hours.

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4.3.10 Haemostatic Effect of Silk Fibroin Sponge with Addition of Aqueous Hyaluronic Acid Solution Including *Equisetum Arvense* Extract

The haemostatic effects of release medium of sponge material were analyzed. Both fibrinogen and prothrombin time were investigated. The results can be seen in Figure 26. When the released medium results compared with the positive control it can be seen that as a result of hyaluronic acid in the release medium tended to act as anticoagulant substance. The effect of plant extract was more distinctive in fibrinogen time. In fibrinogen time, sponge prepared without hyaluronic acid gave similar results as plant extract alone. These results indicated that silk fibroin had no effect on coagulation cascade as well. The sponges prepared without plant extract showed high anticoagulant activity. According to Verheye *et al.* hyaluronic acid inhibited platelet adhesion and aggregation and prolonged bleeding (58). Therefore, hyaluronic acid in the sponge material had anticoagulant activity. For fibrinogen time, plant extract reduced this effect.

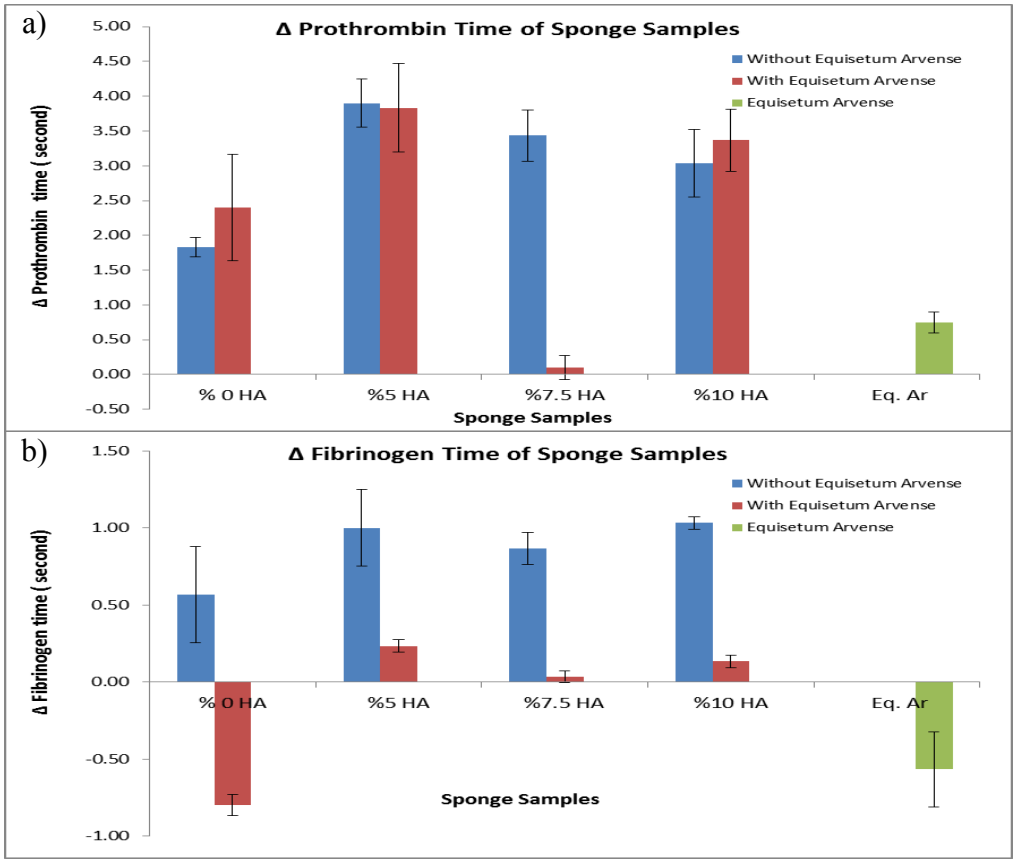

Figure 26. Haemostatic effect of 15 minute release medium of sponge samples. Both ΔFT and ΔPT results were shown.

4.3.11 HPLC Analyses of Silk Fibroin Sponge with Addition of Aqueous Hyaluronic Acid Solution Including *Equisetum Arvense* Extract

Release medium of sponges were analyzed with HPLC to detect its content. It was seen that the phenolic content of the extract could not be detected in the HPLC chromatograms. Results can be seen in Figure 25. Those results were in parallel with haemostatic tests of release medium. Sponge sample prepared without hyaluronic acid had contained little amount of phenolics. In haemostatic test only this sample gave same result with plant extract. Rest of the release medium of samples had lower amount of phenolics according to HPLC chromatograms. Those amounts were not enough to extinguish the anticoagulant effect of hyaluronic acid.

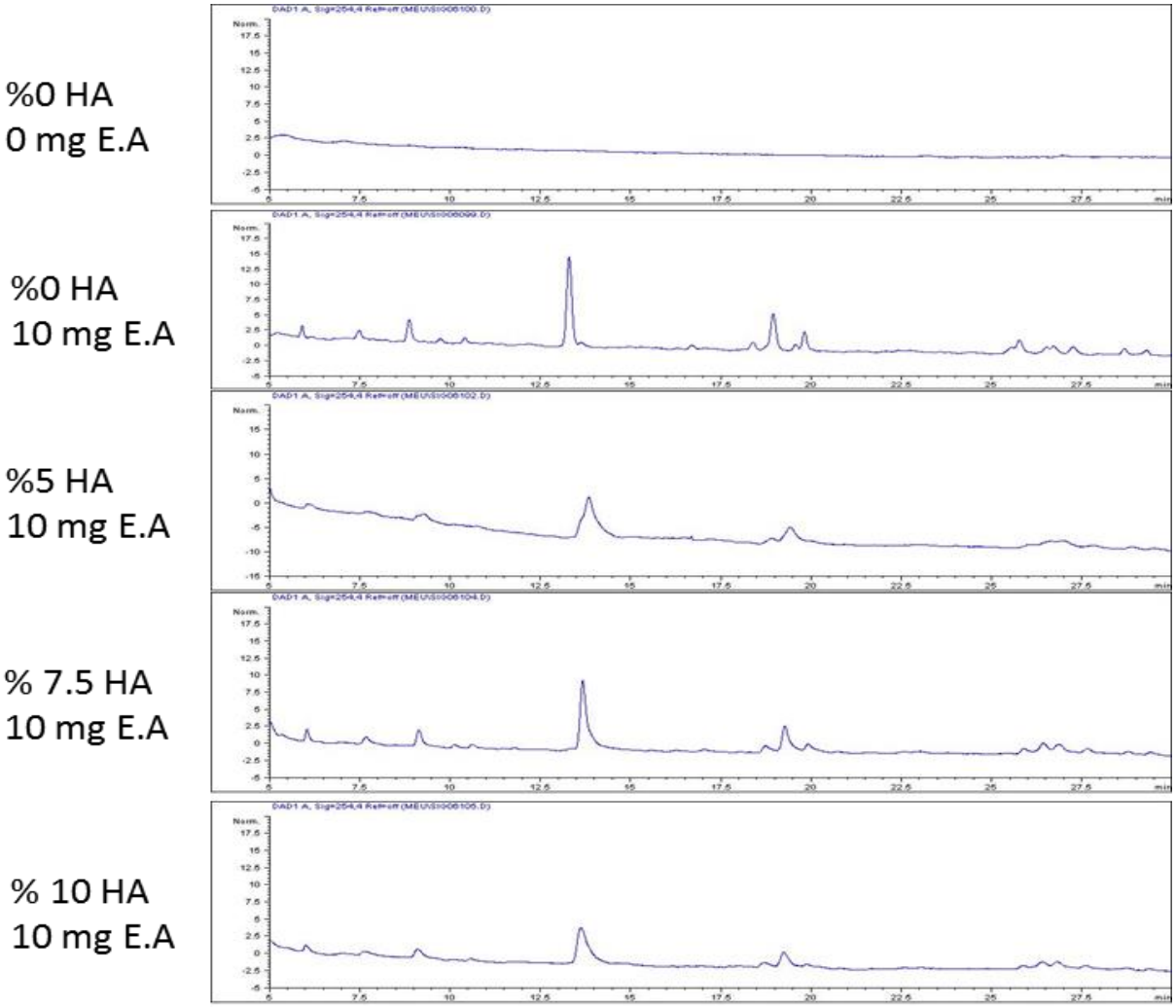

Figure 27. HPLC chromotograms of 15minute release medium of sponge samples.

CHAPTER 5

CONCLUSION

In this research a hemostatic biomaterial containing plant extract was investigated. For plant material *equisetum arvense* was used for its hemostatic properties. Firstly standardization of extraction was done. It was seen that low alcohol content and long extraction time increased the extraction yield. However high alcohol content and long extraction time increased both antioxidant capacity and total phenolic content of the plant extract. The highest silicic acid amount was obtained in the mid-ranges of all parameters. Also correlation between antioxidant capacity and phenolic content was found. The plant extract showed cytotoxic activity on fibroblast cells as their antioxidant capacities increased. HPLC analyses showed the differences in the contents of extracts obtained with changing extraction conditions.

Secondly the hemostatic properties of the plant extracts were investigated. It was seen that as the phenolic content and antioxidant capacity decreased, the extract became more coagulant. The low antioxidant capacity and phenolic content showed us that the major content of the extract were hydrophilic substances. By the help of prothrombin time we could determine the extrinsic pathway of coagulation. Therefore the apolar substances triggered the extrinsic pathway of coagulation. The high antioxidant capacity and phenolic content showed us that the major content of the extract were hydrophobic substances and were seen in the relatively apolar region of the HPLC chromatogram. The proteins that plays important role in the intrinsic pathway (fibrinogen time related) affected by the phenolics present in the extract.

According to the retention times between 12 minutes and 16 minutes caffeic acid and its derivatives were seen on the HPLC chromatogram. The extract acted as an anti-coagulant agent while the concentration of caffeic acid derivative was high and other relatively polar phenolic content was low. However when the caffeic acid derivative concentration decreased and the polar phenolics content increased, extracts started to act like haemostatic agent.

The development of silk fibroin based sponge biomaterials were also carried out. It was seen that high hyaluronic acid content enhanced the mechanical strength and

extended the time, on the other hand high freezing temperature decreased mechanical strength and degradation time. Also adding plant extract decreased mechanical strength and shortened degradation time as a result of the interaction between protein and phenolic compounds. Precipitation occurred and this changed the morphology of the sponge material. As the hyaluronic acid concentration increased the more rigid layers form and pore formation increased. The freezing temperature was also an important factor for the pore formation. As freezing temperature decreased the number of the pores increased. On the other hand the sponge matrices changed their structure. No pore formation or no layer formation was seen as the *equisetum arvense* extract amount increased. Increase in pore formation and disruption of layers negatively affected mechanical strength of the sponge material. Lastly as a result of dissolved hyaluronic acid, gel formation occurred in the release medium which caused interference in the total phenolic content and total antioxidant capacity analyses. During the first 24 hour time most of the plant extract was released from sponge material into the release medium. Different methods of sponge preparation were tested. Those were addition of aqueous *Equisetum arvense* extract to sponges, dipping sponges into the aqueous plant extract and coating sponges with extract were discussed. None of the techniques gave efficient results. Blank sponges were disintegrated or became harden. The negative effect of plant extract on silk fibroin was occurred because of high interaction of its phenolic content with silk fibroin proteins. In order to alter this interaction between phenolics and protein, plant extract should be encapsulated. Increased hyaluronic acid concentration increased regularity of the matrice structure. As the hyaluronic acid concentration increased the mechanical strength and the rigidity of the sponges were also increased. Concentration of the plant extract affected pore size and the distribution of the pores of sponge materials. In high extract concentrations distributions of the pores were uniform.

These results revealed that sponge matrices could be used in both wound healing and haemostasis. Sudden burst release of the plant extract could stop bleeding immediately and remaining hyaluronic acid could stimulate fibroblast cells for proliferation while silk fibroin matrices give structural support.

REFERENCES

1. http://www.webmd.com/skin-problems-and-treatments/picture-of-the-skin

2. Breathnach, A.S. An Atlas of the Ultrastructure of Human Skin. *J Anat*. 1971, 110 (3), 484–485.

3. Kanitakis, J. Anatomy, Histology And İmmunohistochemistry Of Normal Human Skin. *European Journal of Dermatology*. 2002 (12), 4, 390-401.

4. Gross, J.; Francis, O.; Gross, S. The Structureof Human Skin Collagen As Studied With The Electron Microscobe. *Jour. Exp Med*. 1948, 88(5),555-568.

5. Laurens, N.; Koolwijk, P.; De Maat, M.P.M. Fibrin Structure And Wound Healing. *Journal Of Thrombosis And Haemostasis*. 2006 4: 932–939.

6. Singer, A.J.; Clark, R.A.F. Cutaneous Wound Healing. *The New England journal of Medicine*. 1999, 738-746.

7. Martin, P. Wound Healing-Aiming For Perfect Skin Regenaration, *Science* 1997 276, 75-81.

8. http://bme240.eng.uci.edu/students/07s/ngunn/wound_healing.html

9. Butenas,S.; Mann, K. G. Blood Coagulation, *Biochemistry (Moscow)*, 2002, 67(1),3-12.

10. Gentry, P.A. Comparative Aspects Of Blood Coagulation, *The Veterinary Journal*. 2004, 168, 238–251

11. Recinos, G.; Inaba, K.; Dubose, J.; Demetriades, F.; Rhee, P. Local And Systemic Hemostatics İn Trauma: A Review. *Turkish Journal of Trauma & Emergency Surgery* , 2008, 14(3),175-181.

12. Jones, L.H.P.; Handreck, K.A. Silica in Soils, Plants, and Animals. *Advances in Agronomy*. 1967, 19, 107-149.

13. Holzhüter, G.; Narayanan, K.; Gerber, T. Structure of Silica in *Equisetum arvense*. *Anal. Bioanal. Chem.* 2003, 376 , 512–517.

14. Nagai, T.; Myoda, T.; Nagashima, T. Antioxidative Activities of Water and Ethanol Extract from Field Horsetail (Tsukushi) *Equisetum arvense* L. *Food Chemistry*. 2005, 91, 389 - 394.

15. Andrade-Cetto, A.; Heinrich, M. Mexican Plants with Hypoglycaemic Effect Used in The Treatment of Diabetes. *Journal of Ethnopharmacology*. 2005, 99, 325–348.

16. Gutiérrez, R.M.F.; Laguna, G.Y.; Walkowski, A. Diuretic Activity of Mexican Equisetum. *Journal of Ethnopharmacology*. 1985, 14(2-3), 269–272.
17. Schmeda-Hirschmann, G.; Loyola, J.I.; Sierra, J.; Retamal, R.; Rodriguez, J. Hypotensive Effect and Enzyme Inhibition Activity of Mapuche Medicinal Plant Extracts. *Phytotherapy Research*. 1992, 6(4), 184–188.

18. Lemusa, I.; Garcíaa, R.; Erazoa, S.; Peñaa, R.; Paradab, M.; Fuenzalidab, M. Diuretic Activity of an *Equisetum Bogotense* Tea (Platero Herb): Evaluation in Healthy Volunteers. *Journal of Ethnopharmacology*. 1996, 54 (1), 1996, 55–58.

19. Shimizu, M.; Matsuzawa, T.; Hase, K.; Tsurumi, Y.; Seki, T.; Morohashi, M.; Toriizuka, K.; Terasawa, K.; Honda, T.; Morita, N. Studies On Bathing Agent 1. Anti-Infiammatory Effect of Bathing Agent Used for Skin Disease. *The Japanese Society of Pharmacognosy*. 1993, 47(1), 1-4.

20. Do Monte, F. H. M.; Dos Santos, Jr, J. G.; Russi, M.; Lanziotti, V. M. N. B.; Leal, L. K. A. M.; Cunhab G. M. de A. Antinociceptive and Anti-Inflammatory Properties of the Hydroalcoholic Extract of Stems from *Equisetum arvense* l. in mice. *Pharmacological Research*. 2004, 49, 239 - 243.

21. Dos Santos Jr., J.G.; Blanco, M.M.; Do Monte, F.H.M.; Russi, M.; Lanziotti, V.M.N.B., Leal, L.K.A.M.; Cunha, G.M. Sedative and Anticonvulsant Effects of Hydroalcoholic Extract of *Equisetum arvense*. *Fitoterapia.*2005,76, 508–513.

22. Milovanovic, V.; Radulovic, N.; Todorovic, Z.; Stakovic, M.; Stojanovic, G.; Antioxidant, Antimicrobial ad Genotoxicity Screening of Hydroalcoholic Extracts of five Serbian Equisetum Species. *Plant Foods Hum Nutr.* 2007, 62,113–119.

23. Dos Santos Jr., J.G.; Do Monte, F.H.M.; Blanco, M.M.; Lanziotti, V.M.N.B.L.; Maia, F.D.; Leal, L.K.A. Cognitive Enhancement in Aged Rats After Chronic Administration of *Equisetum arvense* l. with Demonstrated Antioxidant Properties in Vitro.; *Pharmacology, Biochemistry and Behavior.* 2005, 81, 593 – 600.

24. Stajner, D.; Popovic, B.M.; Canadanovic-Brunet, J.; Anackov, G. Exploring *Equisetum arvense* L., *Equisetum ramosissimum* L. and *Equisetum telmateia* L. as Sources of Natural Antioxidants. *Phytother.* 2009, 23, 546–550.

25. Mimica-Dukic, N.; Simin, N.; Cvejic, J.; Jovin, E.; Orcic, D.; Bozin, B. Phenolic Compounds in Field Horsetail (*Equisetum arvense* L.) as Natural Antioxidants. *Molecules.* 2008, *13*, 1455-1464.

26. Radulovic, N.; Stojanovic, G.; Palic, R. Composition and Antimicrobial Activity of *Equisetum arvense* L. Essential Oil. *Phytother. Res*. 2006,20, 85–88.

27. Canadanovic´-Brunet, J. M.; Cetkovic´, G. S.; Djilas, S. M.; Tumbas, V. T.; Savatovic´, S. S.; Mandic´, A. I.; Markov, S. L.; Cvetkovic, D. D. Radical Scavenging and Antimicrobial Activity of Horsetail (*Equisetum arvense* L.) Extracts. *International Journal of Food Science and Technology*. 2009, 44, 269 - 278.

28. Tepkeeva, I. I.; Moiseeva, E. V.; Chaadaeva, A. V.; Zhavoronkova, E. V.; Kessler, Yu. V.; Semushina, S. G.; Demushkin, V. P. Evaluation of Antitumor Activity of Peptide Extracts from Medicinal Plants on the Model of Transplanted Breast Cancer in CBRB-Rb(8.17)1Iem Mice. *Bulletin of Experimental Biology and Medicine*. 2008, 145 (4), 464 - 466.

29. Alexandru, V.; Petrusca, D.; Gılle, E. Investigation of Pro-apoptotic Activity of *Equisetum arvense* L. Water Extract on Human Leukemia U 937 Cells. *Romanian Biotechnological Letters.* 2007, 12(2)

30. Kabiri, M.; Emami, S.H.; Rafinia, M.; Tahriri, M. Preparation and Characterization of Absorbable Hemostat Crosslinked Gelatin Sponges for Surgical Applications. *Current Applied Physics*. 2011,11, 457-461.

31. Basmadjian, D.; Sefton, M.V.; Baldwin, S.A. Coagulation on Biomaterials in Flowing Blood: Some Theoretical Considerations. *Biomaterials.* 1997, 18, 1511-1522.

32. Griffith, L. G. Polymeric Biomaterials. *Acta Mater.* 2000, 48, 263-277.

33. Seal, B.L.; Otero, T.C.; Panitch, A. Polymeric Biomaterials for Tissue and Organ Regenaration. *Material Science and Engineering.R* 2001, 34, 147-230.

34. Vert, M. Polymeric Biomaterials: Strategies of the Past vs. Strategies of the Future; *Prog. Polym. Sci.* 2007, 32, 755–761.

35. Gouzy, M.F.; Sperling, C.; Salchert, K.; Pompe, T.; Streller, U.; Uhlmann, P.; Rauwolf, C.; Simon, F.; Ohme, F.B.; Voit, B.; Wernera, C. In Vitro Blood Compatibility Of Polymeric Biomaterials Through Covalent Immobilization Of An Amidine Derivative. *Biomaterials* 2004, 25, 3493–3501.

36. Weremfo, A.; Adinortey, M.B.; Pappoe, A.N.M. Haemostatic Effect of the Stem Juice of *Musa paradisiaca* L.(Musaceae) in Guinea Pigs. *Advances in Biological Research*. 2001,5 (4), 190-192.

37. Oduola, T.; Adeosun, G.O.; Oduola, T.A.; Avwioro, G.O.; Oyeniyi, M.A. Mechanism of Action of Jatropha Gossypifolia Stem Latex as a Haemostatic Agent;. *Eur J Gen Med* 2005, 2(4), 140-143.

38. Bamidele, O.; Akinnuga, A.M.; Anyakudo, M.M.C.; Ojo, O.A.; Ojo, G.B.; Olorunfemi, J.O.; Johnson, O.P. Haemostatic Effect of Methanolic Leaf Extract of *Ageratum Conyzoides* in Albino Rats. *Journal of Medicinal Plants Research.* 2010, 4(20), 2075-2079.

39. Myers, R. H. Response Surface Methodology. Allyn and Bacon: Boston, MA, 1971.

40. Kwon, J.H.; Beälanger, J.M.R.; Pareä, J.R.J. Optimization of Microwave-Assisted Extraction (MAP)† for Ginseng Components by Response Surface Methodology. *J. Agric. Food Chem.* 2003, *51,* 1807 - 1810.

41. Chimuka, L.; Michel, M.; Cukrowska, E.; Buszewsk,i B. Influence Of Temperature On Mass Transfer In An Incomplete Trapping Supported Liquid Membrane Extraction Of Triazole Fungicides. *J. Sep. Sci*. 2009, 32, 1043 - 1050.
42. Lin, F.; Giust, M.M. Effects of Solvent Polarity and Acidity on the Extraction Efficiency of Isoflavones from Soybeans (Glycine max). *J. Agric. Food Chem*. 2005, 53, 3795 - 3800.

43. Giao, M.S.; Gonzalez-Sanjose, M.L,; Rivero-Perez, M.D.; Pereira, C.L.; Pintado, M.E.; Malcata, F.X. Infusions of Portuguese Medicinal Plants: Dependence of Final antioxidant Capacity and Phenol Content on Extraction Features. *J. Sci. Food Agric*. 2007, 87, 2638 - 2647.

44. Liu, F. F.; Ang, C.Y.W.; Springer, D. Optimization of Extraction Conditions for Active Components in Hypericum perforatum Using Response Surface Methodology. *J. Agric. Food Chem*. 2000, 48, 3364 - 3371.

45. Gil, D.M.A.; Fale, P.L.V.; Serralheiro, M.L.M.; Rebelo, M.J.F. Herbal Infusions Bioelectrochemicals Polyphenolic Index: Green Tea – The Gallic Acid Interference. *Food Chem*. 2011, 129, 1537 - 1543.

46. Duda-Chodak, A.; Tarko, T.; Rus, M. Antioxidant Activity and Total Polyphenol Content of Selected Herbal Medicinal Products Used in Poland. *Herba Polonica*. 2011, *57* (1), 48 - 60.

47. European Medicines Agency; Community Herbal Monograph on *Equisetum arvense* L.. HERBA. 2008, Doc. Ref. EMEA/HMPC/394895/2007.

48. Antolovich, M.; Prenzler, P. D.; Patsalides, E.; Mcdonald, S.; Robards, K. Methods for Testing Antioxidant Activity. *The Analyst*. 2002, 127, 183 - 198.

49. Mustoe, T. Understanding Chronic Wounds: A Unifying Hypothesis On Their Pathogenesis And İmplications For Therapy. *The American Journal of Surgery*. 2004, 187, 65 - 70.

50. Schäfer, M.; Werner, S. Oxidative Stress İn Normal And İmpaired Wound Repair. *Pharmacological Research*. 2008, 58, 165 - 171.

51. Aehle, E., Grandic, S.R.; Ralainirina, R., Baltora-Rosset, S.; Mesnard, F.; Prouillet, C.; Maziere, J.; Fliniaux, M. Development and Evaluation of an Enriched Natural Antioxidant Preparation Obtained from Aqueous Spinach (Spinacia oleracea) Extracts by an Adsorption Procedure. *Food Chemistry*. 2004, 86, 579 - 585.

52. Mekhfia, H; El Haouari, M; Legssyer, A; Bnouhama, M; Aziz, M; Atmani, F; Remmalb, A; Ziyyat, A. Platelet Anti-Aggregant Property Of Some Moroccan Medicinal Plants. *Journal of Ethnopharmacology*. 2004, 94, 317–322.

53. Goun, E. A.; Petrichenko, V.M.; Solodnikov, S.U.; Suhinina, T.V.; Kline, M. A.; Cunningham, G; Nguyen, C; Miles, H. Anticancer and Antithrombin Activity Of Russian Plants. *Journal of Ethnopharmacology*. 2002, 81, 337-342.

54. Chao, P.C.; Hsul, C.C; Yin, M.C. Anti-Inflammatory and Anti-Coagulatory Activities of Caffeic Acid And Ellagic Acid iİn Cardiac Tissue of Diabetic Mice. *Nutrition & Metabolism*. 2009, 6(33), 1-8.
55. Karl, J. S.; Troukhanova, N. V.; Lynn, P. Y. Nature of Polyphenol-Protein Interactions. *J. Agric. Food Chem*. 1996, 44, 80 - 85.

56. Bartolomé, B.; Estrella, I.; Hernández; M.T. Interaction of Low Molecular Weight Phenolics With Proteins (BSA). *Journal of Food Science*. 2000, 65 (4), 617 - 621.

57. Greco, R.M.; Iocono, J.A.; Ehrlich, H.P. Hyaluronic Acid Stimulates Human Fibroblast Proliferation Within A Collagen Matrix. *J Cell Physiol.* 1998 ,177(3), 465-473.

58. Verheye S.; Markou, C.P.; Salame, M.Y.; Wan, B.; King III, S.P.; Robinson, K.A.; Chronos, N.A.F.; Hanson, S. R. Reduced Thrombus Formation By Hyaluronic Acid Coating Of Endovascular Devices. *Arterioscler Thromb Vasc Biol.* 2000, 20, 1168-1172.

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Publications:

- **Uslu ME**, Ceylan C, Bayraktar O. 2014. Kinetic and structural characterization of interaction between trypsin and *Equisetum arvense* extract. *Turkish Journal of Biochemistry* 39(4):463–471
- **Uslu ME**, Erdogan I, Bayraktar O, Ates M. 2013. Optimization Of Extraction Conditions for Active Components in *Equisetum arvense* Extract. *Romanian Biotechnological Letters* 18(2): 8115-8131.

Proceedings

- Erdogan I, Sunguc C, **Uslu ME**, Bayraktar O. 2013. Investigation of anti-tumoral activity of Cistus creticus extract against PC-3 cell line. *European Journal of Pharmaceutical Sciences* 50, 29.
- Sunguc C, Erdogan I, **Uslu ME**, Bayraktar O. 2013. In vitro antitumor activity of Sarcopoterium spinosum leaf extract with bioactive natural compounds. *European Journal of Pharmaceutical Sciences* 50 (supplement 1), 4.
- **Uslu ME**, Erdogan I, Bayraktar O. 2013. Incorporation of *Equisetum arvense* extract into silk fibroin–hyaluronic acid sponge matrices for wound dressing applications. *New Biotechnology* 29: 117

Oral presentations:

- **Uslu ME**, Gonzales Gil A, Erdogan I, Bayraktar O. 2-5 September 2014. Fenolik Bileşiklerin İpek Fibroin Kullanılarak Köpük Fraksiyonlama Yöntemi ile Ayrılması. 11. Ulusal Kimya Müh. Kongresi
- **Uslu ME**, Erdogan I, Bayraktar O. 25-26 September 2014. Preparation and Characterization of Silk Fibroin-Chitosan- Clinoptilolite Composite Films with Olive Leaf Extract for Wound Dressing Applications. 2nd International Congress On Healthcare And Medical Textiles.
- Erdogan I, **Uslu ME**, Bayraktar O. 27-31 August 2014. "Effects of Some Selected Plant Extracts on 3T3 Fibroblast Cell Line Wound Model. 6th International Conference on Oxidative Stress in Skin Biology and Medicine.
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- Erdogan I, Balcı B, Sunguc C, **Uslu ME**, Bayraktar O. 9-11 May 2013. Olive Leaf Extract and Clinoptilolite Loaded Silk Fibroin Chitosan Blend Films as a Potential Coating on Packaging Materials. 7th International Packaging Congress-Izmir.
- **Uslu ME**, Erdogan I, Bayraktar O. 23-26 September 2012. Yara Örtüsü Uygulamaları İçin *Equisetum arvense* Özütü Eklenmiş İpek Fibroin-Hyaluronik Asit Karışımlarından Köpük Matrislerin Oluşturulması. 17. Ulusal Biyoteknoloji Kongresi-Istanbul.