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Evaluation of the hemostatic activity of *Equisetum arvense* extract: the role of varying phenolic composition and antioxidant activity due to different extraction conditions

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ABSTRACT

Hemostasis is a procedure that causes bleeding to stop and is very important in injuries. Recent researches were focused on the discovery of plant hemostatic agents. Therefore, plant extracts were investigated as a major source as a hemostatic agent. In this research, *Equisetum arvense* extract was investigated as a candidate coagulatory agent. Effect of extraction parameters on coagulation was investigated. Although it has been stated in the literature that the extract of *Equisetum arvense* has anticoagulatory activity, it has been shown in this study that alteration of the extraction parameters changes the extract bioactivity from the anticoagulant to the coagulant.

Keywords: *Equisetum arvense, Hemostasis, Blood coagulation, Plant extracts.*

1. INTRODUCTION

One of the main death reasons worldwide is blood loss. It is considered as a very serious event when one-third of the blood volume is lost. A single cut can need a stitch and hospitalization, and without these processes, a single bleeding can cause death easily. Bleeding with different types and different stages is a complex event and is handled seriously. Many natural and pharmaceutical products were used commercially, and new products investigated to stop the bleeding. When we look at the layers of skin the outermost layer is epidermis and has the protective structure; the second layer is the dermis. This layer is a fibrous tissuethat strengthens and supports epidermis and beneath that layer the subcutis is seen which is developed from fatty tissues that insulates body and provide nutrients to other two layers. The last and the most important section of the skin are beneath the epidermis and dermis is hypodermis. In fact, the hypodermis is not part of the skin. Its role is only to connect the skin to the underlying muscle and bone by elastin and loose connective tissues. Skin is covered by mass of veins, capillaries and arteries [1- 5].

Blood is a tissue that transports oxygen and nutrients to the cells. Also, it carries away the carbon dioxide and waste products from the cells. Most of the blood volume is occupied by erythrocytes (45%) and the rest is white blood cells (44%) and platelets (1%). Platelets is important component of the blood because they involve in the blood clotting. The average size of platelets ranges from two to four micrometers in diameter. They are the smallest cells found in the blood. By the help of hair like filaments on their membranes, platelets have a high tendency to attach to each other. The platelet granules contain important factors for clot promoting activity. When the endothelial cells are injured, Von Willebrand factor, collagen and tissue factors becomes exposed to the blood stream. Platelets get activated whenever they contact with these substances. Coagulation factors can bind to other platelets in the presence of calcium ions and by this way platelet plug accumulation occurs. This aggregation stops bleeding and form a favorable site for clot. Coagulation of the blood occurs just after the platelet plug formation. Coagulation can be initiated by two separate and independent pathways, intrinsic and extrinsic pathways. A series of proteins cofactors, enzymes and proteins involves in intrinsic pathway, which interacts in reactions that take place on membrane surface. These reactions are initiated by tissue injury and results in fibrin clot. The main role of the extrinsic pathway is to generate a thrombin burst. Factor VII leaves the circulation after the damage occurs in the blood vessel. It gets in touch with tissue factor which is expressed in fibroblasts and leukocytes. So, it can be said that the tissue factor activated the extrinsic pathway [6-10]. To prevent loss of excess amount of blood, clot formation should be initiated by the blood coagulation cascade with high success. Despite of, place of the injury or wound, genetic backup, patients' physiological condition can be determinative for the time period of the clot formation. In order to promote the coagulation, biomaterials, pharmaceuticals and natural compounds are used.

Equisetum arvense (Horsetail) is an herbal drug found in ancient Greek and Roman medicine. Several substances that the plant contains can be used medicinally. It was used traditionally by healers to cure different kinds of illnesses as it contains saponins, tannins, alkaloids, silicic acid and potassium salt. *Equisetum arvense* can be found in stream banks of meadows in North America and Eurasia. Two species, *E. arvense* and *E. ramosissimum* are also common in South Africa. *Equisetum hyemale*, known as rough horsetail, scouring rush, scouring rush horsetail and South Africa snake grass, is an invasive species of moist natural habitats in South Africa and Australia [11].

Science and Technology agency published a report in 2002 and stated that *Equisetum arvense* contains a high concentration of

vitamins, folic acid, tannins, pantothenic acid, and nicotinic acid. Nagai and his colleagues investigated the antioxidant activities of horsetail extracts in water and alcohol in 2005 and were reported the antioxidant activities [12]. *Equisetum arvense* diuretic, hypoglycemic antioxidant, anticancer, antimicrobial, smooth muscle relaxant effects of the vessels and ileum, anticonvulsant, sedative, anti-anxiety, and anti-inflammatory activities were also reported in the literature [13-18]. In 2004, Do Monte and his colleagues worked on mice to investigate the anti-inflammatory and antigenic effects of different doses of *Equisetum arvense* body part extracts by using chemical and heat tests. They reported that after plant extract was used, the pain caused by chemicals diminished. However, no results were seen on heat triggered pains. Also, it was reported that after extract usage the inflammation was decreased. They reported that on central nervous system *Equisetum arvense* extract has an analgesic effect for chemical stimuli but no evidence found on heat stimuli and the mechanisms for those results cannot be found. Same investigation also mentioned the anti-inflammatory effect of the extract [19].

In this research, extracts of *Equisetum arvense* were evaluated for their hemostatic activities. The role of varying phenolic composition and antioxidant activity of extracts as a result of different applied extraction conditions was investigated.

2. MATERIALS AND METHODS

2.1. Experimental design.

The experimental design was performed according to methods described in the literature [20, 21]. To examine the responses, half face centered surface composite design was used for analyses. Every independent variable was numbered at three levels between (-1) to (+1). The factors were determined as ethanol percent, stirring speed, temperature, solid-liquid ratio and extraction time for the extraction.

2.2. Preparation of Extracts.

Extracts of *Equisetum arvense* were prepared by following the method described in the literature [20]. Briefly, dried leaves were weighed and subjected to extraction process in aqueous ethanol solution with changing temperature, extraction time, solid-liquid ratio, stirring speed and ethanol percent based on the experimental design. Thirty-two extract samples were prepared at the conditions predetermined in experimental design.

2.3. Determination of Phenolic Content and Antioxidant Activity.

Folin- Ciocalteu method was picked to determine total phenolic content of *equisetum arvense* extracts. 1:10 dilution of stock solution from Folin-Ciocalteu reagent was prepared. 7% sodium carbonate solution in distilled water was prepared. Gallic acid was used in the calibration curve as standard. *Equisetum arvense* extract was dissolved in distilled water. 100 µl Folin-Ciocalteu reagent was mixed with 20 µl of each sample and waited for two and a half minutes. Then 80 µl of Na_2CO_3 solution was added above that mixture. For 1 hour the mixture was kept in dark. 725 nm wavelength was used in spectrophotometer to detect phenolic contents in the samples. Results were expressed as mg of gallic acid equivalents (GAE)/g-1 dry weight extract.

Trolox- Equivalent Antioxidant Capacity (TEAC) method was used in order to determine the total antioxidant capacity of *equisetum arvense* extract. Potassium persulphate (4.9 mM) and ABTS (14 mM) were mixed in a 1:1 ratio. Mixture kept in dark for 16 hours. In the calibration curve Trolox was used as standard. *Equisetum arvense* extract was dissolved in distilled water. 200 µl of ABTS solution was added in each microplate wells.. Kinetic measurement was held at 734 nm for thirty minutes. Percent inhibition of ABTS cation as a result of *equisetum arvense* extract's antioxidant activity was calculated as:

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

A_f refers to final absorbance detected on the last reading and A_0 refers to absorbance measured just after dispensing ABTS on the sample

2.4. Blood Sample.

Blood was taken voluntarily from healthy donor (investigator) at the medical center located in Izmir Institute of Technology. 7 ml of blood was collected from donor into heparinize tubes (vacutest kima, Italy). At 1500 rpm for 7 minutes tubes were centrifuged at 4 ºC. Plasma was collected from each tube then aliquoted into eppendorf tubes and freeze at -80 ºC.

2.5. Determination of Blood Coagulation Time.

Both fibrinogen time (FT) and prothrombin time (PT) were measured to investigate the effect of plant extract on extrinsic and intrinsic pathways. To determine the coagulation time, Coalyzer 410 (Farmasina, Istanbul, Turkey) was used.

For PT test, 50 µl of plasma was added into the test tube and tiny metal marble was added for electromagnetic mechanical analysis. For 60 seconds sample was incubated at 37 ºC. 10 µl of 20 mg/ml plant extract was mixed with 100 µl of PT reagent (Diagon, Hungary) in an eppendorf tube. Coagulation was monitored after mixture was added into the test tube. Sample without extract was used for blank test.

For FT test, 90 µl of immidazol (Diagon, Hungary) was added into the test tube and tiny metal marble was added for electromagnetic mechanical analysis. For 60 seconds sample was incubated at 37 ºC. 10 µl of 20 mg/ml plant extract was mixed with 50µl of FT reagent (Diagon, Hungary) in an eppendorf tube. Coagulation was monitored after the mixture was added into the test tube. For blank test, sample without extract was used.

2.6. High Performance Liquid Chromatography (HPLC) Analysis.

Agilent 1100 series device with DAD detector was used to perform HPLC analyses. LiChrospher 100-RP18 (Agilent Technologies, USA), a reversed-phase column with a 5-µm particle size was used and the flow rate was 0.8 mL min^{-1}. Mobile phase was performed as gradient. The proportion of solvent A (%2.5 acetic acid) to solvent B (%100 acetonitrile) as: initial 1% B; linear gradient to 40% B in 40 minutes. 10 mg/ml of samples was prepared in water. 20 µl sample volume was then injected to device. Through 0.20 µm membrane filters (Millipore, Bedford, MA, USA), before injection all solutions were filtered. The

column temperature was at 35 ºC. The measurements were carried out at 280 nm and 254 nm.

2.7. Liquid Chromatography Quadrupole Time-Of-Flight Mass Spectrometry (LC-QToF-MS) Analysis.

The mobile phases were used with the gradient washing (elution) steps given in the following table 1. Mobile phase A was the mixture of water and 0.1% formic acid and mobile phase B was the acetonitrile. The column was maintained at 35 ºC, the injected sample volume was 2 µL and the flow rate was determined as 0.4 mL/min.

MS analysis was carried out using an Agilent 6550 iFunnel high resolution Accurate Mass QTOF-MS equipped with Agilent Dual Jet Stream operating in positive ion electrospray ionization (Dual AJS ESI) interface and desiccant gas flow 14.0 L/min; nebulizer gas pressure 35 psi; drying gas temperature 290 ºC; sheath gas temperature 400 ºC; the sheath nitrogen gas flow at 12 L/min. Mass spectra were recorded in the negative ionization mode in a mass range of 50-1800 m/z. Integration and data detailing were performed using the station MassHunter Workstation "software. Agilent METLIN Metabolomics database, library and full mass personal composite database and library (METLIN_AM_PCDL) and Metlin Metabolomics have been used to identify analytes. Positive and negative modes are conducted in the same conditions.

Table 1. Percentage of mobile phase in elution steps according to time interphase.

Elution Steps	
Time (min)	Mobile phase
0	% 5 B
5	% 62 B
10	% 68 B
19	% 80 B
34	% 95 B
37	% 5 B
3	Conditioning cycle

3. RESULTS

3.1. Total Phenolic Content and Antioxidant Capacity.

The antioxidant capacities and total phenolic contents of extracts prepared at different extraction conditions were already reported in our earlier publication [20]. A new set of 32 plant extracts was prepared in order to study the haemostatic activities of these extracts reported in this present paper. The total phenolic contents and total antioxidant capacities results of the plant extracts are given in Figure 1 and 2.

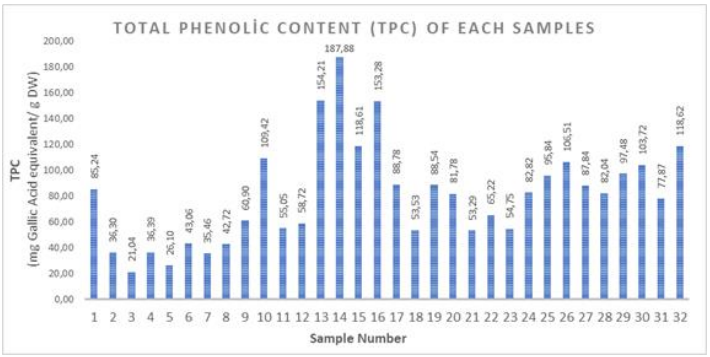

Figure 1. Total phenol contents of *equisetum arvense* extracts prepared at different extraction conditions.

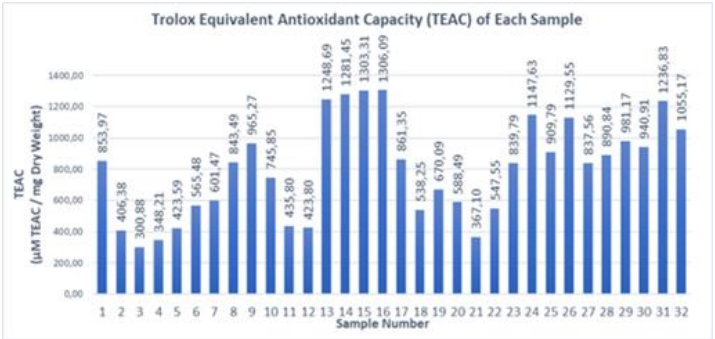

Figure 2. Trolox equivalent antioxidant capacities of extracts prepared at different extraction conditions (Micro Molar(µM) TEAC of milligram dry weight (DW) of extract).

These results clearly indicated that antioxidant capacities and phenolic contents changed meaningfully as a result of the changing extraction conditions. These changes were mainly due to the different compositions of the extracts prepared at different extraction conditions [20].

3.2. Effect of Plant Extract on Blood Coagulation.

3.2.1. Effect on Prothrombin Time. Prothrombin time was measured for each sample. Prothrombin time is the time that passed for plasma coagulation after tissue factor added. In order to determine the prothrombin time, one should measure the quality of extrinsic pathway. The levels of factor VII affected the rate of coagulation. The *Equisetum arvense* extract was mentioned as both inhibitors for platelet aggregation [18] and anti-aggregate for thrombin [22, 23] in literature. Although in folkloric medicine the plant was known for its hemostatic effect, it also had an antithrombin activity according to studies reported in literature [24]. Both claims were confirmed in our present study. The coagulation effect of the extract was shifted from coagulant to anticoagulant by changing the extraction parameters. The results can be seen in Table 2. The time difference between plant extract added sample and coagulation of negative control was shown as ΔPT in the table. If coagulation rate was higher than negative control, this resulted in a negative value for ΔPT. If the rate of blood coagulation was slower than negative control, a positive value of ΔPT was observed. Normally blood coagulates approximately in 20 seconds for prothrombin time.

In this study also, the relations between prothrombin time and total phenolic content and antioxidant capacity of the extracts were evaluated and given in Figure 3 and 4. As seen from these figures some of the extracts became more coagulant as antioxidant capacity and phenolic content of these extracts decreased. The relatively low phenolic content and antioxidant capacity were due to a few polar compounds constituting the major content of the extracts. The presence of these polar compounds was confirmed by observing them in the polar region of the HPLC chromatogram in Figure 7. The extrinsic pathway of coagulation could simply be determined with the help of prothrombin time measurements. Therefore, it can be said that the extrinsic pathway of coagulation

was triggered by the presence of polar compounds present in the extracts.

Table 2. Change in the prothrombin time (ΔPT) due to addition of plant extract.

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
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

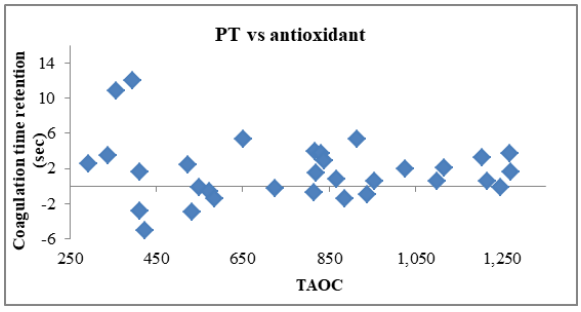

Figure 3. The relation between ΔPT time (sec) and total antioxidant capacity (TAOC).

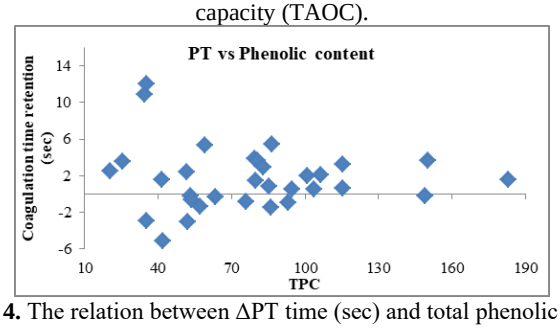

Figure 4. The relation between ΔPT time (sec) and total phenolic content (TPC).

3.2.2. Effect on Fibrinogen Time. The results from the study of fibrinogen time (FT) determination were in accordance with those of prothrombin time determination experiments. But the results for fibrinogen time were much more significant compared with those for prothrombin time. In the intrinsic pathway of blood coagulation, specific proteins are needed. This pathway involves a series of proteins, enzymes, and protein cofactors. Those substances interact with the thrombin membrane surface. At final stage fibrinogen is converted into fibrin as a result of this interaction. In our case, the activity of some extracts shifted from coagulant to anticoagulant based on the changing extraction parameters at which these extracts prepared. The results for fibrinogen time can be seen in Table 3. In this table, the values for the time difference between coagulation of plant extract added sample and negative control are tabulated as ΔFT. If blood was coagulating faster than negative control, this resulted in a negative value for ΔFT. If blood was coagulating slower than negative control, a positive value of ΔFT was observed. Normally, blood coagulates approximately in 10 seconds for fibrinogen time.

Table 3. Change in the fibrinogen time due to the 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
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 phenolic content and antioxidant capacity were different than those for prothrombin time results. As seen from Figure 5 and 6 the increased antioxidant capacities and phenolic contents of extracts increased the coagulation activities of the extracts. The relatively nonpolar compounds constituting the major content of the extracts resulted in relatively high total phenol content and antioxidant capacity. These compounds can be observed in the relatively nonpolar region of the HPLC chromatogram for samples 19 and

30 (Figure 7). The intrinsic pathway of coagulation could be determined with the help of measurements for fibrinogen time. So, it can be said that the nonpolar compounds trigger the intrinsic pathway of coagulation cascade severely. The proteins in the intrinsic pathway were affected by the phenol content of the extracts explicitly.

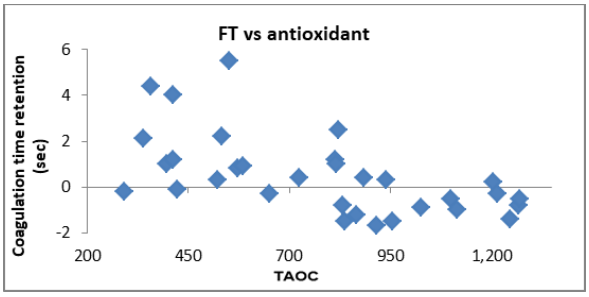

Figure 5. The relation between ΔFT time (sec) and total antioxidant capacity (TAOC).

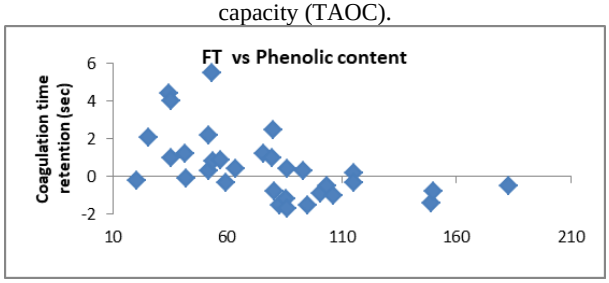

Figure 6. The relation between ΔFT time (sec) and phenolic content (TPC).

3.3. HPLC and LC-QToF-MS Analyses.

HPLC analyses were performed to detect the active phenolic compounds on blood coagulation. For their accelerating and inhibitory effect on prothrombin time, sample 9, 21, 30 and 19 were chosen. Their ΔFT were 2.2, 0.4, -1.4 and -1.5 respectively. However, no reasonable changes could be observed on their ΔPT results. They were -3, -1.5, -0.2 and 2.9 respectively. HPLC chromatograms of those samples can be seen in Figure 7.

The peak corresponding to the possibly a phenolic acid observed in HPLC chromatogram with retention time of 13.6 minute was thought to be one of the key components for the decreases in ΔFT values. The changes in ΔFT values could mainly be explained with the presence of phenolic acid content of the extracts. This phenolic acid was identified as caffeic acid with HPLC using caffeic acid standard. The ΔFT values were affected significantly with the decreases in the content of relatively polar compounds including caffeic acid and other phenolic acids present in the extracts. In diabetic mice, anti-coagulatory protection of caffeic acid has already been reported by Chao et al. 2009 [25]. In 2017 Luo et al. worked on seven different types of caffeic acid derivatives to investigate their effects on blood coagulation. In their research they concluded that all the caffeic acid derivatives they investigated had certain procoagulant and anticoagulant activities [26]. In 2016 Pallag et al. identified the predominant flavonoids present in *Equisetum arvense* L. species. The most dominant flavonoid in the *Equisetum arvense* L. was reported as caffeic acid [31]. In accordance with those findings reported in literature caffeic acid or its derivatives can be strong candidates for their observed effects on blood coagulation. Our findings were also in accordance with those reported in the literature [25-30]. The extracts with relatively high caffeic acid and other phenolic acids contents acted as an anti-coagulant substance. However, the extracts with relatively lower caffeic acid and other phenolic acids contents started to act like hemostatic agent. As seen in HPLC chromatograms for samples 9 and 21, the % of peak area fractions for the relatively polar compounds corresponding to the peaks appearing at retention times between 0 and 15 minutes was found to be higher than that for relatively nonpolar compounds corresponding to the peaks appearing at retention times between 15 and 40 minutes. However, In HPLC chromatograms for samples 19 and 30, the % of peak area fractions for the relatively nonpolar compounds corresponding to the peaks appearing at retention times between 15 and 40 minutes was found to be high.

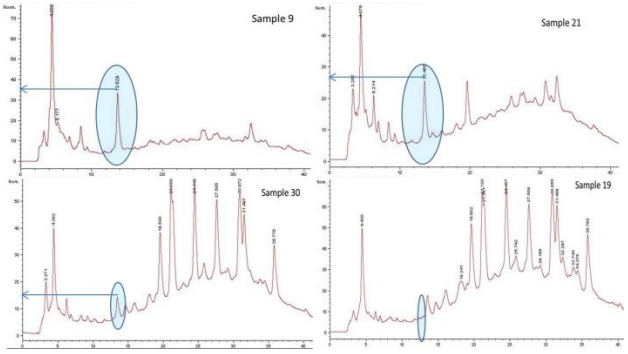

Figure 7. HPLC chromatograms of samples 9, 19, 21, 30. ΔPT time (sec) were -3, 2.9, -1.5 and -0.2 respectively. The phenolic acid which was thought to be responsible for decreasing the PT was circled.

Table 4. Retention time, related MS data of compounds present in extracts analyzed with LC-QToF MS.

		Formula	m/z	Polarity	RT
Sample 19	Rutin	$C_{27}H_{30}O_{16}$	609.1464	Negative	3.854
	Kaempferol-7-o-glucoside	$C_{21}H_{20}O_{11}$	447.0991	Negative	4.814
	Luteolin	$C_{15}H_{10}O_6$	287.0557	Positive	5.164
	Quercetin	$C_{15}H_{10}O_7$	301.0356	Negative	5.627
	Kaempferol	$C_{15}H_{10}O_6$	285.0403	Negative	6.068
	(-)-Naringenin	$C_{15}H_{12}O_5$	271.0612	Negative	6.079
Sample 9	Vanillic acid	$C_7H_6O_4$	153.0186	Negative	3.368
	Rutin	$C_{27}H_{30}O_{16}$	609.1468	Negative	3.865
	Caffeic Acid	$C_9H_8O_4$	179.0344	Negative	4.272
	Ferulic acid	$C_{10}H_{10}O_4$	193.051	Negative	4.758
	Kaempferol-7-o-glucoside	$C_{21}H_{20}O_{11}$	447.0962	Negative	4.814
	Luteolin	$C_{15}H_{10}O6$	287.0554	Positive	5,166
	(-)-Naringenin	$C_{15}H_{12}O_5$	271.0611	Negative	6.079

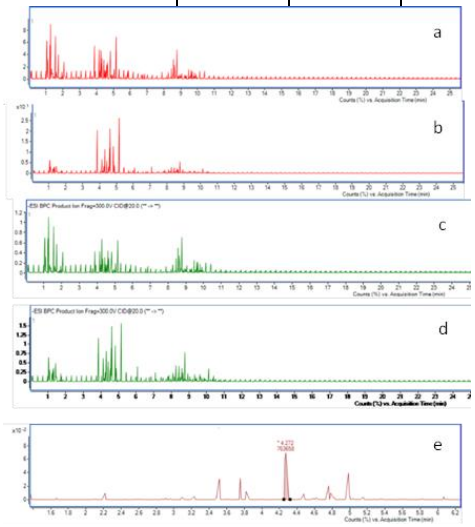

Figure 8. LC-QToF-MS (ESI TIC) profiles for samples 9 (a) and 19 (b); LC-QToF-MS (ESI BPC) profiles for samples 9 (c) and 19 (d) in negative modes; peak area for caffeic acid (e).

LC-QToF-MS method was also used for the identification of phenolic contents of the extracts (prepared at different extraction conditions resulting in different effects on blood coagulation) labeled as sample 9 and 19. Figures 8 a, b show the LC-MS (ESI) profiles in negative modes for samples 9 and 19, respectively. The molecules were identified by their m/z values released as protonated ions [M+H]+. The identification was performed based on comparisons with similar data found in Agilent METLIN Metabolomics database, library and full mass personal composite database and library (METLIN_AM_PCDL) and Metlin Metabolomics. The presence of relatively nonpolar compounds such as rutin, kaempferol 7-o-glucoside, luteolin, quercetin, naringenin was detected with LC-ESI+ QToF-MS analysis for both extracts (Table 4).

Figures 8c, d show the base peak chromatograms obtained by LC-ESI+ QToF-MS analysis for samples 9 and 19, respectively. The major peaks in extract (sample 9) corresponded to phenolic acids (vanillic acid, caffeic acid and ferulic acid), corresponding to more than 26% of peak area fractions, while caffeic acid represented 11.7% from area fraction. The LC-QToF-MS analyses revealed that caffeic acid was present in extract samples having relatively high anti-coagulant activity and that extracts showing coagulant activity contained a very small amount of polar constituents (mainly caffeic acid), but high amount of nonpolar constituents.

4. CONCLUSIONS

In this paper *Equisetum arvense* plant extracts' hemostatic properties were explained. It was seen that the extract became more coagulant as the antioxidant capacity and phenolic content decreased. According to the low phenolic content and antioxidant capacity it can be said that the major content of the extract was hydrophilic compounds. The extrinsic pathway of coagulation helped us to determine the prothrombin time. Therefore, the extrinsic pathway of coagulation was triggered by nonpolar compounds.

The high phenolic content and antioxidant capacity showed us that the major content of the extract was hydrophobic compounds.

Those compounds were seen in the relatively nonpolar region of the HPLC chromatograms. The proteins that play an important role in the intrinsic pathway (fibrinogen time related) affected by the phenolics present in the extract.

Caffeic acid and its derivatives were observed at the retention times between 12 minutes and 16 minutes in the HPLC chromatograms. The extract tent to act as an anti-coagulant agent while the concentration of caffeic acid was high. However, when the concentration of caffeic acid decreased extracts started to act like hemostatic agent.

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