

## Optimization of extraction conditions for active components in *Equisetum arvense* extract

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### Abstract

Response surface methodology was applied to predict optimum conditions for *Equisetum arvense* extraction. Central composite design was used to monitor the effects of temperature, stirring speed, ethanol percent, extraction time, solid-liquid ratio on dependent variables such as, extract yield percent, total phenol content, total antioxidant capacity, silicic acid amount. According to the mathematical models obtained from the analysis, the highest values for yield percent, total phenol content, total antioxidant capacity and silicic acid amount were found to be 18.67 %, 123 mg gallic acid gr<sup>-1</sup> dry weight extract, 1608 µM TEAC mg<sup>-1</sup> dry weight extract and 0.0049 mg silicic acid mg<sup>-1</sup> dry weight extract, respectively. The plant extracts were analyzed with HPLC to determine the phenolic content and compositional differences of extracts obtained at different extraction conditions. Plant extracts were also analyzed for their cytotoxic and antimicrobial activities. The high total antioxidant capacity and total phenolic content resulted in an increased cytotoxic effect on fibroblast cells. *Equisetum arvense* extracts showed antimicrobial activity against *Staphylococcus epidermidis* and *Escherichia coli* bacteria, however showed no effect against *Candida albicans*.

**Keywords:** *Equisetum arvense*, response surface methodology, extraction, antioxidant, total phenol content, antimicrobial activity, cytotoxicity, silicic acid.

### Introduction

In recent years, the usage of plant materials as food supplement and as alternative medicine has increased due to their phytochemical contents. Among these phytochemicals, alkaloids, carotenoids and phenolics have been widely studied. The most popular area in research is the antioxidant capacities of these substances. Phenolic compounds present in plants exhibit strong antioxidant activities [1, 2]. Cellular activities result in free radical accumulation which causes oxidative stress in the cell. The cells are damaged due to this oxidative stress and harmful side effects. In order to solve this problem, researchers focus on the reduction or elimination of the oxidative stress, by means of plant phenolics. While some researchers working in the area of pharmaceutical research focus on biological activities, some researchers in the area of food manufacturing focus on the phenolic-protein interaction. Phenolics can interact with proteins easily by the weak bonds like hydrogen bonds [3]. Protein-phenolic soluble complexes have been formed and it has been observed that as the particle grows the solubility decreases [4]. Tannins, caffeic acid, chlorogenic acid and catechol are some examples of commonly studied phenolics in this area [5, 6]. As the awareness to the benefits of the phytochemicals increase in the society, researchers have started to focus on the plants which are used in the folk medicine.

One of the widely used and studied plants is *Equisetum arvense*; (field horsetail) in northern hemisphere of wetlands. Silicic acid, tartaric acid, protocatechuic acid and caffeic acid are present in plant [7-9]. Recent investigations also showed that concentrations of isoquercitrin, apigenin and kaempferol are also present in high concentration in plant supplement [10, 11]. *Equisetum arvense* is used in the treatment of kidney and bladder disturbances as folk medicine [12, 13]. Recent investigations not only show that plant have high antioxidant capacity [14] but also it has the anti-inflammatory, anti-microbial, anti-cancer, sedative and anticonvulsant effects [15-18]. In 2006 researchers investigated the composition and antimicrobial properties of essential oils from *equisetum arvense*. The twenty five compounds with antimicrobial activities were identified in the essential oil obtained from the aerial parts of the plant [15]. In 2009 a report was published about the antimicrobial and hydroxyl radical scavenging activities of methanol extract of the aerial parts of the plant [16]. Antitumoral activities of the *equisetum arvense* peptides were also investigated [17]. Pro-apoptotic and cytotoxic activities of aqueous part of *equisetum arvense* extract were also reported in 2007 [18]. Not only *equisetum arvense* but also other species of equisetum family (*equisetum telmateia*) were investigated for their active substance content and biological effects [19]. In the literature, the influence of extraction parameters on extract composition have been well understood but biological activities based on composition changes have rarely taken into consideration in extraction studies. Active phenolic components and effect of these parameters on biological activities can be altered by changing extraction variables. Extraction is mainly based on solubility which also depends on more than one level of many other factors. Solvent polarity, temperature, pressure, particle size, stirring speed and extraction time are well known extraction parameters affecting the content of extract.

Experimental design is an effective method to determine the effects of extraction parameters on extract content and related biological activities. One of the most powerful experimental design procedure is response surface methodology (RSM) for optimizing multiple and interrelated parameters. This method can be used to investigate the relationships between several explanatory input variables and response variables. RSM is useful for the analysis of the results of designed experiments in order to obtain an optimum experimental variable corresponding to desired response behavior. Optimal value of the variables can be determined from surface structure of the plot. The hills and valleys formed on the surface structure can also help to determine if more experiments or new parameters are needed [20-24]. In the literature this technique was used in various areas. Some of them were related to optimize conditions for the extraction of phenolic antioxidants, plant derived compounds, plant pigments and proteins [25-29]. To the best of our knowledge no systematic studies have been reported for the optimization of extraction parameters for active components of *equisetum arvense* extracts.

Aim of this study was to determine the effects of extraction parameters on the content and predetermined biological activities of *equisetum arvense* extract. Response surface methodology with half central composite design for five factors at three levels was used in this research.

## Materials and Methods

### Chemicals and reagents

Dried *equisetum arvense* leaves were purchased from local herbalist (Izmir, Turkey). Ethanol, Folin-Ciocalteu reagent, gallic acid, silicic acid, hydrochloric acid, hepta-molybdate tetrahydrate, acetic acid and rutin were purchased from Merck (Darmstadt, Germany). Sodium carbonate, oxalic acid, acetonitrile, (-)-epicatechin and p-coumaric acid were obtained from Sigma (Steinheim, Germany). Vanilic acid, caffeic acid, Trolox (6-hydroxy-2,5,7,8-

tetramethylchroman-2-carboxylic acid) and ABTS [2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid)] were purchased from Fluka (Steinheim, Germany). Dulbecco's Modified Eagle Medium (DMEM) and fetal bovine serum (FBS) were supplied from Gibco (Paisley, UK). Penicillin and vancomycin were obtained from Oxoid Limited (Hampshire, UK). Amphotericin B was purchased from Applichem. *S. epidermidis* (ATCC 12228) and *C. albicans* (ATCC 64548) were supplied from ATCC (Wesel, Germany). *E. coli* (NRRL B-3008) was obtained from NRRL (Washington D.C, USA).

### 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. 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 1.

**Table 1.** The coded levels and corresponding actual levels of factors used in MINITAB software to generate experimental design

FACTORS	Symbols	Coded Levels		
		-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

### 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 on Table 2. 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 2.** Experimental design for response surface analysis of *equisetum arvense* extraction process 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	1:30
2	45	50	10	2	1:10
3	4	250	10	2	1:10
4	45	250	10	2	1:30
5	4	50	90	2	1:10
6	45	50	90	2	1:30
7	4	250	90	2	1:30
8	45	250	90	2	1:10
9	4	50	10	12	1:10
10	45	50	10	12	1:30
11	4	250	10	12	1:30
12	45	250	10	12	1:10
13	4	50	90	12	1:30
14	45	50	90	12	1:10
15	4	250	90	12	1:10
16	45	250	90	12	1:30

17	4	150	50	7	1:20
18	45	150	50	7	1:20
19	24.5	50	50	7	1:20
20	24.5	250	50	7	1:20
21	24.5	150	10	7	1:20
22	24.5	150	90	7	1:20
23	24.5	150	50	2	1:20
24	24.5	150	50	12	1:20
25	24.5	150	50	7	1:10
26	24.5	150	50	7	1:30
27	24.5	150	50	7	1:20
28	24.5	150	50	7	1:20
29	24.5	150	50	7	1:20
30	24.5	150	50	7	1:20
31	24.5	150	50	7	1:20
32	24.5	150	50	7	1:20

### 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<sup>-1</sup> dry weight extract.

### Determination of Antioxidant Activity

Total 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<sub>f</sub> refers to final absorbance value measured on the last measurement and A<sub>0</sub> refers to absorbance value measured directly after dispensing ABTS on the sample.

### 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. Five milliliters 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.

### Cell culture and determination of cytotoxic activity

NIH3T3 mouse fibroblast cell line was maintained in DMEM supplemented with 10% FBS and 100 µg ml<sup>-1</sup> streptomycin, 100 U ml<sup>-1</sup> 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 4,000 rpm for 5 minutes and subjected to serial dilution in DMEM with FBS (10%) to determine IC<sub>50</sub> of *Equisetum arvense* extract.

Cells were later exposed to IC<sub>50</sub> of extracts obtained in varying conditions for 24, 48 and 72 hours. Cells subjected to MTT assay were incubated in dark at 37 °C for 4 hours and spectrophotometric measurement was performed at 545 nm. Cell viability was calculated using the following formula:

Cell viability:  $100\% \times (\text{Average absorbance value of treated cells} / \text{Average absorbance value of control cells})$

#### Determination of minimum inhibition concentrations (MIC)

Antimicrobial activity of *equisetum arvense* extracts was determined in means of minimum inhibition concentration (MIC). Assays were performed in 96-well plate. Bacterial culture of *Escherichia coli*, *Staphylococcus epidermidis*, and fungal culture of *Candida albicans* were used in order to observe inhibitory effects of *equisetum arvense* extract on microorganisms. The numbers of microorganisms were set to 0.5 McFarland for the assay. The concentrations of *equisetum arvense* extracts used to determine the MIC values were 9.4; 18.75; 37.5 and 75 mg ml<sup>-1</sup>. Penicillin, vancomycin and amphotericin B were used as positive controls. In each well 95 µl growth medium, 100 µl plant extract and 5 µl of microorganism were added respectively. Plates were incubated at 37°C for 24 hours and growth of each strain was determined by spectrophotometric measurements at 600 nm.

#### Determination of antimicrobial activity with disc diffusion

Extracts were subjected to disc diffusion method to determine the antimicrobial activity against bacterial culture of *Escherichia coli*, *Staphylococcus epidermidis*, and fungal culture of *Candida albicans*. Antibiotic discs of penicillin, vancomycin and amphotericin B were used as positive controls. Each sterile blank disc was saturated with 20 µl of extracts with a concentration of 150 mg ml<sup>-1</sup>. Both sample discs and antibiotic discs were placed on plates of each microorganism. Inhibition zone diameters were measured by using a compass after incubation for 24 hours at 37 °C.

#### HPLC analysis

High pressure liquid chromatography (HPLC) analysis was modified from the studies of Canadanovic-Brunet et al [16]. 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<sup>-1</sup>. 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 a 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 35 °C. The measurements were held at 254 nm and 280 nm.

## Results and Discussion

#### 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 3.

**Table 3.** Percentage of the extraction yield (Ten grams of dry leafs of *equisetum arvense* was used for each 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

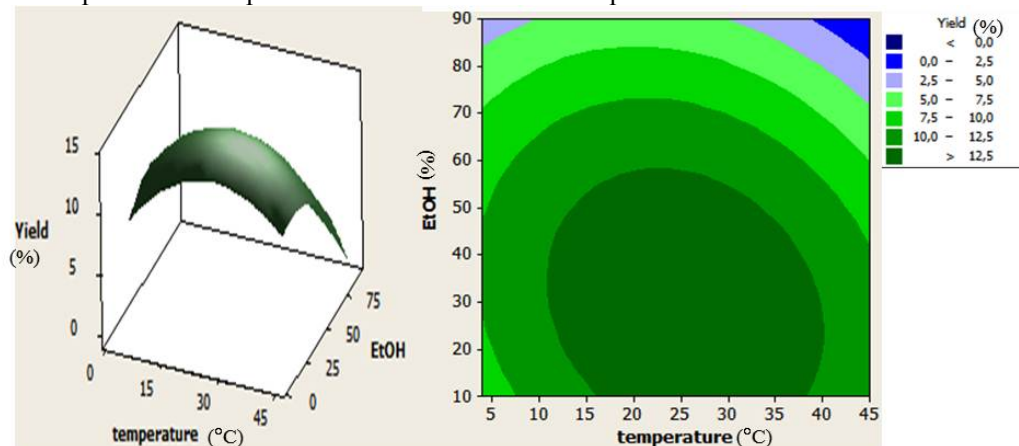
7	3.45	23	14.81
8	3.78	24	19.01
9	14.40	25	10.05
10	17.63	26	14.66
11	18.52	27	13.52
12	20.88	28	15.16
13	10.35	29	13.88
14	5.20	30	13.42
15	11.90	31	12.76
16	8.95	32	11.47

As seen in the Table 3, the yield percentages were varied from 3.45 % to 21.71%. 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 [30]. Also it was found that as the ethanol percent decreased the yield increased. The polarity of the solvent was an important issue in extraction process [31]. The polar ingredients of the plant could dissolve much better in high water ratio. The differences between the yields of samples also supported this fact.

The results of the yield percentage were analyzed using 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:

$$y_1 = 31.3087 + 0.6086 x_1 - 0.0255 x_2 + 0.1577 x_3 - 2.8456 x_4 - 1.4168 x_5 - 0.0095 x_1^2 - 0.0021 x_2^2 + 0.1380 x_4^2 + 0.0257 x_5^2 - 0.0003 x_1 x_2 - 0.0020 x_1 x_5 + 0.0046 x_2 x_4 - 0.0002 x_2 x_5 - 0.0023 x_3 x_4 + 0.0018 x_3 x_5 + 0.0355 x_4 x_5$$

The surface and contour plot of the yield percentage vs. ethanol concentration and temperature can be seen in Figure 1. The other parameters were set constant at the center points. It was seen from the plots that the maximum yield would be obtained for the conditions where ethanol percent was kept constant at 10 to 50% and temperature at 20 to 30°C.



**Figure 1.** 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.

**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 4 as milligram Gallic Acid Equivalent (GAE) per gram dry weight of extract.

**Table 4.** Total phenol content of *equisetum arvense* extract prepared at different extraction conditions

Sample	TPC (mg GAE gr <sup>-1</sup> extract)	Sample	TPC (mg GAE gr <sup>-1</sup> extract)
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

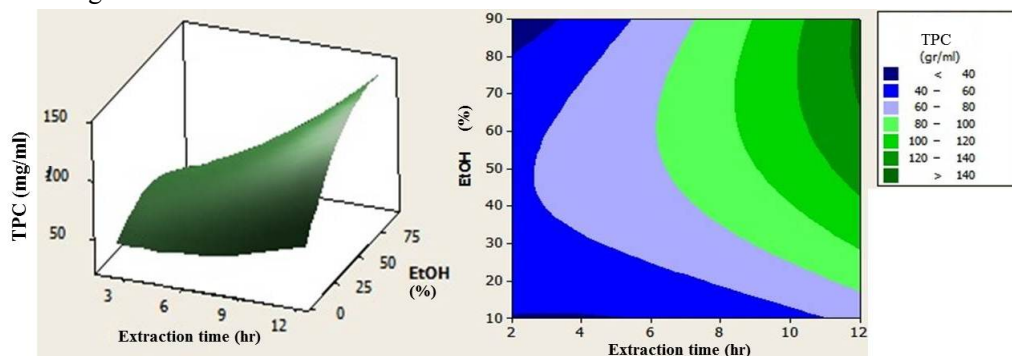
The highest total phenol content was observed for samples 14, 13, 16 as the lowest total phenol content were obtained for samples 3 and 4 in terms of gallic acid equivalent. It can be concluded that extracts obtained with high percentage of ethanol and for longer time had more phenolic content than the ones obtained with lower ethanol content as seen in Table 2. 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 [16]. In 2005 the phenolic content of *equisetum arvense* was published as 23.9 and 7.98 gram GAE gram<sup>-1</sup> for ethanol and water extract, respectively. It was also reported that an increase in the alcohol concentration of the extraction medium resulted in an extract rich in phenolic content [32]. In literature the total phenolic content of *equisetum arvense* leaves was reported as 0.212 ± 0.044 gram GAE L<sup>-1</sup> for dry leaves [34]. All of our results were in close agreement with those reported in the literature. Depending upon the alcohol concentration of extraction medium, the changes for the amount of phenolic compounds in the extracts were observed. 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 [23, 31]

Results of total phenol content of the *equisetum arvense* extracts were then analyzed using 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 versus ethanol concentration and extraction time are shown in Figure 2. 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 2.** 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.

### Effect of extraction factors on total antioxidant capacity (TAOC)

Total antioxidant capacities of *equisetum arvense* extracts were determined with ABTS method. The results obtained can be seen in Table 5. The samples 13, 14, 15 and 16 had the highest total antioxidant capacity while the samples 3 and 4 had the lowest total antioxidant capacity.

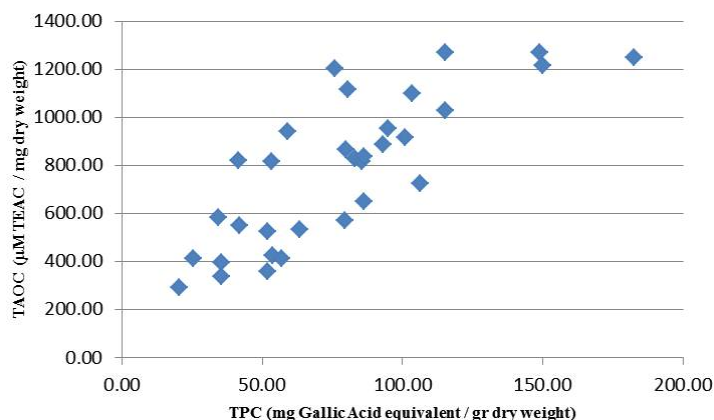
**Table 5.** Total antioxidant capacity of extracts prepared at different conditions

Sample	$\mu\text{M TEAC mg}^{-1}$ extract	Sample	$\mu\text{M TEAC mg}^{-1}$ extract
1	830.89	17	838.07
2	395.40	18	523.70
<b>3</b>	<b>292.75</b>	19	651.98
<b>4</b>	<b>338.80</b>	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
<b>13</b>	<b>1214.94</b>	29	954.65
<b>14</b>	<b>1246.82</b>	30	915.48
<b>15</b>	<b>1268.09</b>	31	1203.40
<b>16</b>	<b>1270.79</b>	32	1026.65

When the extraction parameters of the highest and lowest values of Trolox equivalent antioxidant capacity (TEAC) results were analyzed in Table 2, 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 reported as  $39 \pm 4 \mu\text{M TEAC g}^{-1}$  dry weight of plant [34]. 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  $1270 \mu\text{M TEAC mg}^{-1}$  dry weight of plant extract. This significant difference occurred as a result of the technique that was used. The alcohol content of extraction medium and other parameters that play an important role for total phenol content and total antioxidant capacity of the extracts can be considered as the main causes of these differences. The correlation could be seen in Figure 3. Total 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 [35]. The 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 varied with changing polarity and may increase antioxidant capacity along with moderately apolar compounds having antioxidant capacity.

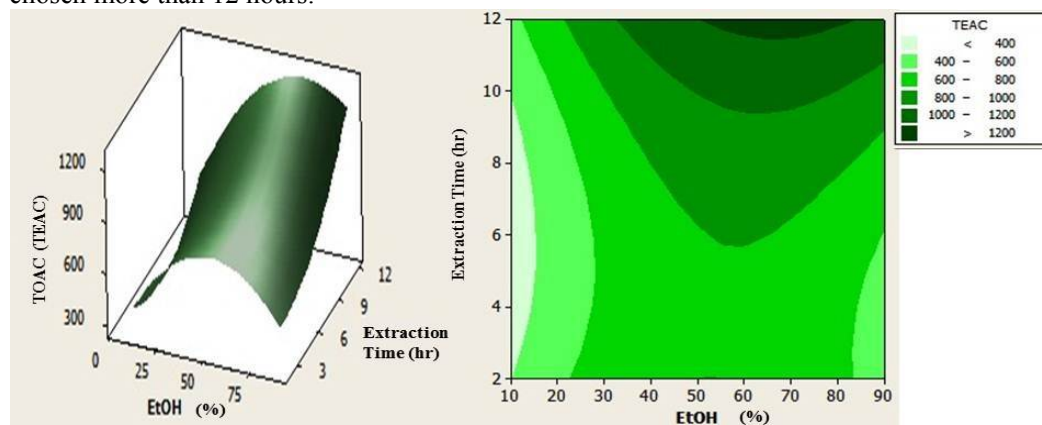


**Figure 3.** Correlation between total phenol content (TPC) and total antioxidant capacity (TAOC) of extracts prepared at different conditions.

TAOC results were evaluated with 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 3 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

$$y_3 = 886.78 - 13.966 x_1 + 3.998 x_2 + 16.729 x_3 - 58.573 x_4 - 62.701 x_5 - 0.018 x_2^2 - 0.219 x_3^2 + 6.89 x_4^2 + 1.975 x_5^2 + 0.078 x_1 x_2 + 0.03 x_1 x_3 + 0.622 x_2 x_4$$

The surface and contour plots of the TEAC versus ethanol concentration and extraction time are shown in Figure 4. The other parameters were set constant at the center points. It was seen from the plots that the maximum total antioxidant content was obtained for the conditions where ethanol percent was between 70% and 90% and extraction time was chosen more than 12 hours.



**Figure 4.** Surface and contour plot of the total antioxidant capacity versus Ethanol percentage and extraction time. Stirring speed was 150 rpm, solid liquid ratio was 1:20 and temperature was 24.5 °C.

**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 are presented in Table 6. The highest silicic acid amount was detected for the sample 32 and the lowest silicic acid amount was obtained for sample 13. When the 6 % silicic acid content of *equisetum arvense* was considered in the literature, it can be concluded that our finding was in accordance with the result reported in the literature [8].

**Table 6.** The silicic acid amount (milligram silicic acid amount per mg dry weight of extract) of extracts prepared at different conditions

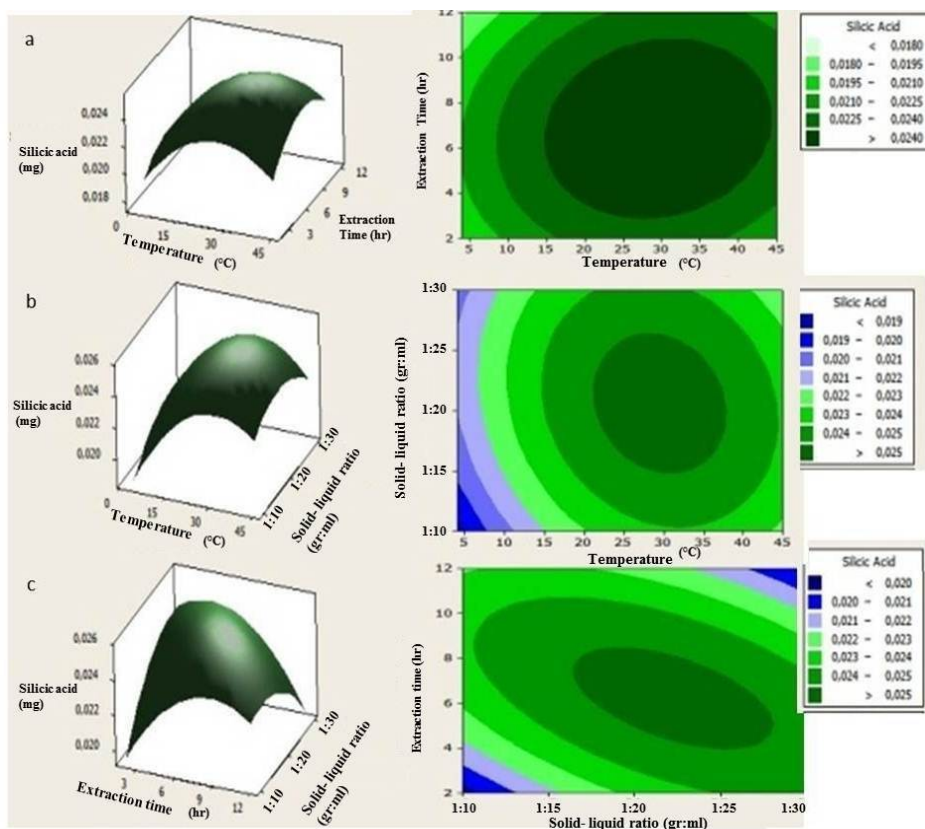
Sample	mg Silicic Acid mg <sup>-1</sup> extract	Sample	mg Silicic Acid mg <sup>-1</sup> extract
1	0.023	17	0.023
2	0.014	18	0.022
3	0.013	19	0.034
4	0.015	20	0.035
5	0.010	21	0.014
6	0.021	22	0.014
7	0.016	23	0.021
8	0.020	24	0.025
9	0.016	25	0.022
10	0.015	26	0.026
11	0.013	27	0.024
12	0.017	28	0.028
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 2, it was seen that the center point values of each selected extraction parameters should be used to obtain the highest silicic acid amount in the extracts, the extreme levels for each factors gave the lowest value.

The results for silicic acid amount were analyzed 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 using significant ( $p < 0.05$ ) coefficients of the extraction parameters. The predicted model for silicic acid amount was

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

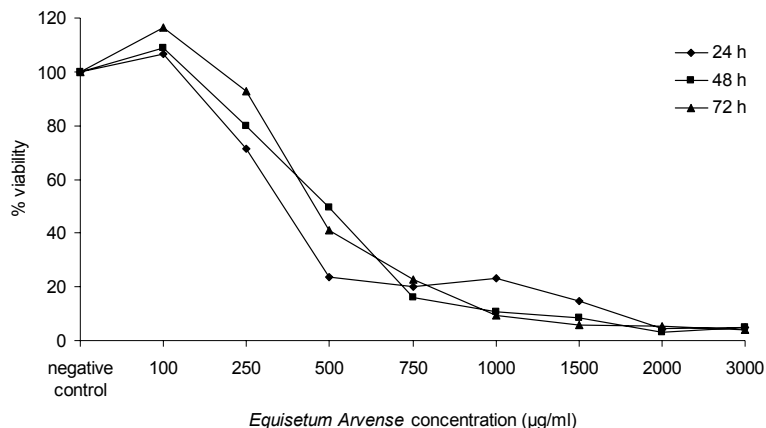
The surface and contour plot of the silicic acid amount for solid-liquid ratio versus extraction time; extraction time versus temperature and solid liquid ratio versus temperature are shown in Figure 5. The other parameters were set constant at the center points in each plot. It was seen from the plots 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 5.** 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).

### Effect of extraction parameters on cytotoxicity

Cytotoxic evaluation was performed at two stages to observe the cytotoxic effect gradually. Concentration range was between 100–3000  $\mu\text{g ml}^{-1}$  for the first trial to determine  $\text{IC}_{50}$  value, which was 500  $\mu\text{g ml}^{-1}$  in 48 hour. For this concentration, viability was determined as 49.61% as seen in Figure 6.  $\text{IC}_{50}$  value of the extract constituted the limitation for further cytotoxicity evaluation by using products of parametric extraction of *Equisetum arvense*. Cytotoxic evaluation of  $\text{IC}_{50}$  for 24, 48 and 72 hour was compared with total phenol content and antioxidant activity of the extracts (Table 7). 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 showed 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 90% ethanol for 12 hour, while extracts obtained with 10% ethanol for 2 hour did not decrease the viability upon exposure to fibroblast cells. The extracts 2, 6 and 8 have possessed good AOC and TPC with tolerable cytotoxicity.



**Figure 6.** Cytotoxic activity of *Equisetum arvense* extracted in 70% aqueous ethanol at 180 rpm at room temperature for 2 hours. IC<sub>50</sub> indicates the inhibitory effect of the extract on the half of the population.

**Table 7.** 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

Extract code	% Viability			Antioxidant activity (µM TEAC mg <sup>-1</sup> extract )	Total phenol content (mg GAE g <sup>-1</sup> extract)
	24 hour	48 hour	72 hour		
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 [36]. Oxidant-producing enzymes specific for bacterial killing cannot work efficiently due to low amount of oxygen in the wound area [37]. 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 [38]. 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 [39]. 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 are found to be 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 for the properties of the extract, that is also relevant with its use.

#### Effects of extraction parameters on antimicrobial activity

*Equisetum arvense* extracts were subjected to disc diffusion assay to determine their antimicrobial activities. Extracts did not form inhibitory zones in the presence of *C. albicans*, while they had effective antimicrobial activity against both gram positive and gram negative bacteria as given in Table 8. These differences can be attributed to the varying extraction parameters which affect the composition of extracts.

**Table 8.** The zone diameters that were formed by 150 mg ml<sup>-1</sup> *equisetum arvense* extracts for both gram positive and gram negative pathogens

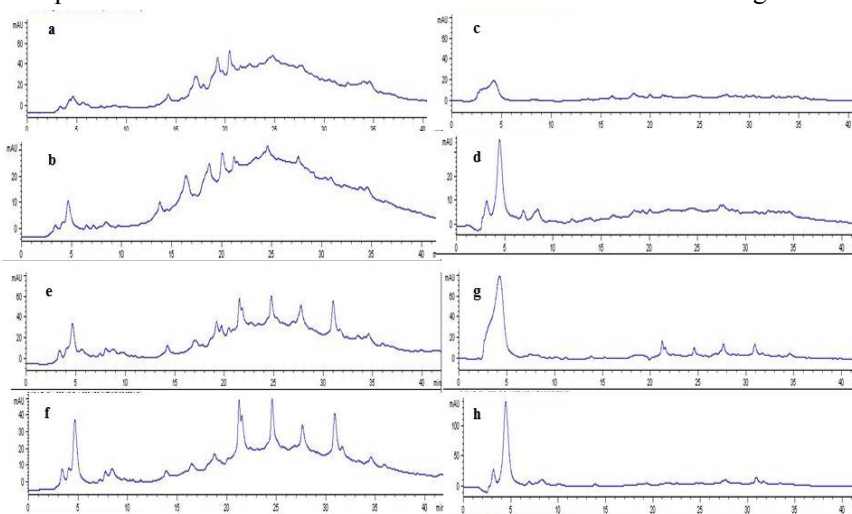
Sample	Zone Diameter (mm)	
	<i>S. Epidermidis</i>	<i>E. Coli</i>
Vancomycin	24	9
Streptomycin	18	21
1	8	20
2	0	21
3	0	18
4	0	19
5	0	16
6	8	0
7	0	8
8	15	0
9	0	0
10	0	21
11	0	20
12	0	18
13	9	8
14	7	10
15	8	17
16	0	0
17	0	0
18	12	0
19	0	8
20	0	8
21	0	19
22	0	0
23	0	0
24	9	0

25	0	8
26	9	0
27	9	0
28	0	0
29	0	0
30	8	8
31	0	8
32	8	8

*Equisetum arvense* extracts prepared by using different extraction parameters had affected the growth of microorganisms differently. In literature the inhibitory zone diameters were reported as 12 mm for *E. coli* and 10 mm for *S. epidermidis* [14, 40]. During assay, the start of growth inhibition was observed for both *E. coli* and *S. epidermidis*; however concentrations of extracts that were used could not be sufficient enough to observe the minimum inhibition concentrations. Although it was reported in the literature, *E. arvense* had an inhibitory effect on *C. albicans* [14], in our study no zone of inhibition was observed for *C. albicans*. Nineteen of the extracts were found to be effective inhibitors against *E. coli* growth, on the other hand only twelve of the extracts effectively inhibited the *S. epidermidis* growth. These results also showed the importance of extractions parameters on antimicrobial activity of extracts as a consequence of their changing chemical composition.

### HPLC analyses

The compositional differences between the extracts which caused the variations in the total antioxidant capacity and total phenol content were investigated and confirmed with HPLC analyses. *Equisetum arvense* extracts of samples 3, 4, 13 and 16 were selected to perform their HPLC analyses. 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 performed at both 254 and 280 nm. The results are shown in Figure 7.



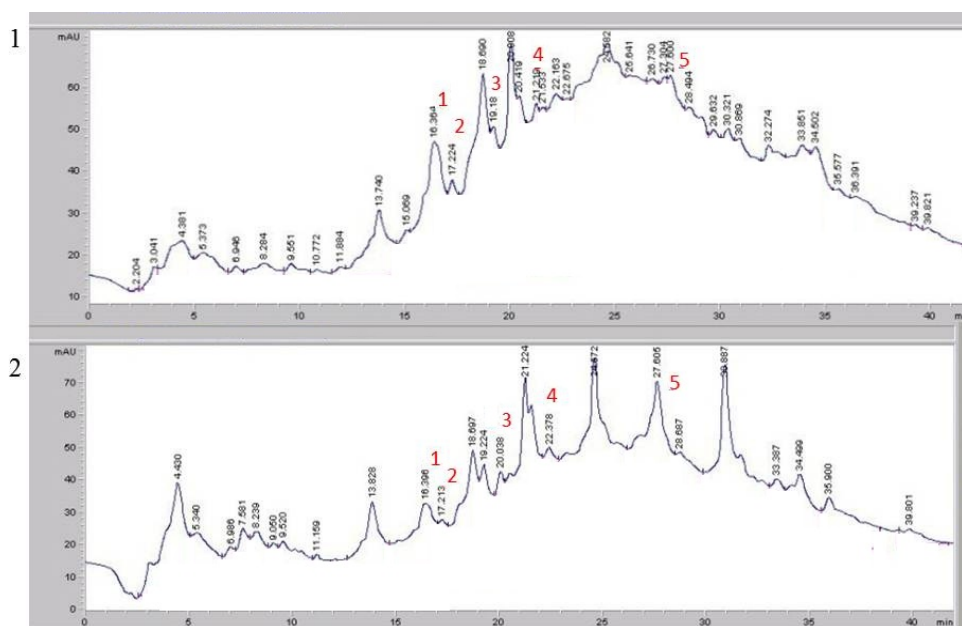
**Figure 7.** HPLC chromatograms of 280 nm (a - d) and 254 nm (e - h). Samples were 16 (a, e); 13 (b, f); 3 (c, g) and 4 (d, h).

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 for samples 3 and 4.

3 and 4. These differences occurred because of the relatively non-polar substances in the plant material as expected. Towards the end of HPLC chromatogram the relatively 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 relatively non-polar substances in the extract also increased.

In the literature, phenolic contents of *equisetum arvense* were investigated using HPLC analysis [16]. Phenolic contents that were determined were caffeic acid, (-)-epicatechin, p-coumaric acid, vanilic acid and rutin. These phenolic standards were also used in this research. The retention times of these standards were determined as 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. The chromatogram areas of the major compounds present in the *equisetum arvense* extract are given in Figure 8.



**Figure 8.** HPLC chromatogram of phenolic compounds in the *equisetum arvense* extract of sample 16. The phenolic compounds: caffeic acid (1); vanilic acid (2); (-)-epicatechin (3); p-coumaric acid (4); rutin (5). The chromatograms were recorded at 280 nm (1) and 254 nm (2).

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