Research Article

Characterization of *in vitro* bioactive performance of *Hypericum perforatum* using response surface methodology

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ABSTRACT

Hypericum perforatum is a widely used medicinal plant known that it has high bioactive performance due to its special biochemical compounds in its structure. The current study aimed to determine the optimum processing parameters of extraction conditions of bioactive constituents in *H. perforatum* by ultrasound application and its some bioactive performance. For this purpose, different processing variables namely sonication period (min), liquid/solid ratio and ethanol concentration (%) were selected to characterize the best conditions for extraction of *H. perforatum*. The statistical analysis results revealed that the most effective factor on the studied parameters was ethanol concentration and increment of ethanol level increased the characterized responses until a constant value. The constructed regression models fit very well with quite high determination of coefficients ($R^2 > 0.906$). Total phenolic content was in the range of 39.54-63.55 mg GAE/g sample while total hypericin concentration ranged between 211.5-858.7 mg/kg. Multiple response optimization results revealed that the optimum extraction conditions would be at 47.6% ethanol concentration and 22.2 mL of liquid/solid ratio for 60 min ultrasonic process application. The results revealed that the optimized extraction conditions could be utilized for ultrasound extraction system by industrial application.

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INTRODUCTION

Hypericum perforatum L. (St. John's Wort) is a medicinal herb in Hypericum genus of Hypericaceae family. About 400 species are present in the Hypericaceae family and *H. perforatum* is the most significant species which is used traditionally in medicinal treatments. Many health problems namely burns or wounds on the skin and eczema, and also some psychological disorders could be cured by *H. perforatum* or its different pharmaceutical form [1]. Butterweck [2] also reported that one of the most attracted properties of *H. perforatum* was its antidepressant effect. In addition to this, *H. perforatum* extracts contain many different compounds having strong bioactive performance like phenolic substance and naphthodianthrones like hypericin and these compounds could perform a good activity as anti-inflammatory agent [3].

Extraction by ultrasonic process is a green application having some advantages compared to classical solvent extraction. Ultrasonic extraction is faster, more efficient, easier, inexpensive and solvent saving technique [4, 5]. Mane et al. [6] reported that the enhancement of phenolic yield by ultrasonic extraction is higher compared to classical extraction system. Toma et al. [7] informed that the enhancement of phenolic yield is related to waves occurred due to the cavitations in the solvent because it accelerates the movement of molecules and increases the penetration performance of solvent into the substance. Due to many advantages of ultrasonic extraction system, it has been used to extract the constituents having bioactivity from the cells of plant materials and it is also suitable to reduce solvent usage having some toxic effects for human [8].

Response surface methodology widely used methodology in optimization researches, is a mathematical and statistical approach. It is used to reveal the relationship and interaction effects between selected processing variables, and it is efficiently used to optimize the multifaceted processes [9]. Several studies [10, 11] were conducted to understand the effects of processing parameters on selected responses and to determine the best conditions to perform the extraction for the phenolic compounds from different plants.

In the current study, *H. perforatum* which is a quite popular medicinal plant in traditional medicine was subjected to extraction by using ultrasonic process. Response surface methodology approach was used to determine the optimum conditions for extraction and to observe the linear and interactive effects of processing variables namely sonication time, ethanol concentration and liquid/solid ratio on bioactive feature of the samples.

MATERIALS AND METHODS

Materials
H. perforatum L. harvested in 2018 was dried in a closed room for three days at room temperature and ground as a whole plant.

Extraction of Phytochemical Constituents by Ultrasound Process

For the extraction of *H. perforatum* L. samples, ultrasound assisted extraction (UAE) process was applied. The grounded plant samples were weighed as exactly one gram in glass bottles and extraction solvents were incorporated into the samples and the bottles were covered tightly to prevent the solvent leakage. At the end, the extraction was started in an ultrasonic water bath (Elmasonic S ultrasonic device S10/S10H, Singen, Germany) at constant temperature for different sonication periods at 37 kHz frequency. Table 1 shows the levels of processing variables determined using Box-Behnken design. Three different sonication times (10, 35 and 60 min), ethanol concentrations (15, 45 and 75%) and liquid/solid ratios (20, 30 and 40 mL/g sample) were applied for the samples and totally 15 samples (including three repetitions) were prepared and exposed to extraction by using a ultrasonic process at constant temperature (50 ºC). After extraction process, the bottles were removed from the bath and waited for a time to cool down. At the end, the samples were filtrated using a filter paper and liquid extracts were obtained and kept in glass tubes for further analysis.

Characterization of *H. perforatum* Extracts
Analysis of Total Phenolic Content (TPC)

TPC was determined using the method of Singleton and Rossi [12]. For this purpose, 0.2 mL of diluted extract (1:20) and 1.8 mL of distilled water were mixed in a tube and 1 mL of Folin Cioceltaeu reagent (1:10 diluted) was added. Finally, 2 mL of $NaCO_3$ (2% w/v) was placed and the tubes were vortexed. All samples were incubated in a dark and room conditions for 2 h. Finally, the absorbances of samples were recorded at 765 nm using a spectrophotometer (UV-vis-1800 spectrophotometer, Shimadzu Corp., Japan) and TPC of the samples was calculated as mg gallic acid equivalent (GAE)/g. The analysis was repeated two times with four replications.

Analysis of Total Flavonoid Content (TFC)

For this purpose, 500 μL of diluted samples (1:20) and 2000 μL distilled water were placed in a tube. After that, 150 μL of $NaNO_2$ was added and, 150 μL of $AlCl_3$ (10% w/v)

Table 1. Box-Behnken design for coded and uncoded levels of processing variables for ultrasonic extraction

Factor levels	Processing variables		
	Sonication time (min) (X_1)	Ethanol concentration (%) (X_2)	Liquid/solid ratio (mL/g sample) (X_3)
−1	10	15	20
0	35	45	30
+1	60	75	40

was placed after 5 min later. After 6 min, 1 mL of NaOH (1 M) and 1.2 mL of distilled water were added immediately, and the samples were incubated for 10 min at room conditions. At the end of the incubation period, the absorbance values of the samples were measured at 510 nm using a spectrophotometer and the TFC of samples was calculated as mg catechin equivalent (CE)/g sample by using a calibration curve [13]. The analysis was repeated two times with four replications.

Analysis of Condensed Tannins (CT)

One mL of diluted samples (1:10) was placed in a tube and then 2.5 mL of vanillin solution (1% w/v) and then 2.5 mL of H_2SO_4 (25%) were added. The samples were incubated for 10 min at 30 °C in a water bath, and the absorbance values of the samples were recorded at 500 nm by using a spectrophotometer and CT levels were expressed as mg catechin equivalent (CE)/g sample [14]. The analysis was repeated two times with four replications.

Analysis of Total Hypericin Content (THC)

THC of the extracts was determined by spectrophotometrically. One mL of extract was diluted with methanol in 5 ml volumetric flask. Then the sample was vortexed, and the absorbance value of this solution was recorded at 590 nm using a spectrophotometer. The following equation (Eq.1) was used for the calculation (Eq.1).

$$THC(\text{mg/kg}) = \frac{A \times V}{780 \times m} \qquad (1)$$

where A is the measured absorbance, m is the weight of sample in 5 mL of extract (V). 780 is the specific absorbance of hypericin at 590 nm [15]. The analysis was repeated two times with four replications.

Analysis of antiradical activities of *H. perforatum* extracts

ABTS·+ Radical Scavenging Activity

Firstly, ABTS·+ radical was created by preparation of ABTS·+ stock solution. For this purpose, 7 mmol/L ABTS·+ solution was prepared and 2.45 mmol/L potassium persulfate was added and this mixture was incubated for 16 h for the radical occurrence. After that, the absorbance value of the final radical solution was adjusted as 0.7±0.05 at 734 nm with the phosphate buffer solution (pH 7.4). Then, different concentrations (15, 30, 45 and 60 μL) of diluted extracts (1:20) and 2 mL of ABTS·+ solution were placed into the spectrophotometer cuvettes and incubated for 6 min. Finally, the sample absorbances were recorded at 734 nm using a spectrophotometer. The following equation (Eq.2) was used to calculate the radical scavenging activity as % inhibition.

$$\% \text{ Inhibition} = \frac{A_{\text{control}} - A_{\text{sample}}}{A_{\text{control}}} \times 100 \qquad \text{Eq. (2)}$$

where A_{sample} is the sample absorbance; A_{control} is the stock solution absorbance. The % inhibition values were converted into the Trolox values (μg TEAC/g sample) [16]. The analysis was repeated two times with four replications.

DPPH Radical Scavenging Activity

For this purpose, four different concentrations (20, 37.5, 50 and 75 μL) of diluted extracts (1:20) were mixed with 2 mL of DPPH solution (0.1 mM in methanol) and the mixture was incubated for 30 min. Then, the sample absorbances were measured at 517 nm using a spectrophotometer. The following equation. (Eq.3) was used to calculate the radical scavenging activity of the samples as % inhibition.

$$\% \text{ Inhibition} = \frac{A_{\text{control}} - A_{\text{sample}}}{A_{\text{control}}} \times 100 \qquad \text{Eq. (3)}$$

where A_{sample} is the sample absorbance; A_{control} refers to the absorbance of DPPH solution. The %inhibition values were converted into the Trolox values (μg TEAC/g sample) [17]. The analysis was repeated two times with four replications.

Analysis of Antioxidant Activities of *H. perforatum* Extracts

Ferrous Ions Chelating Activity

A 1 mL of the diluted extract samples (1:30) was placed into the tubes and 3.7 mL of ethanol (96% v/v) was added. Then 100 μL of $FeCl_2$ (2 mM) was incorporated and the final mixture was mixed with 200 μL of ferrozine (Sigma) and after 10 min waiting, the sample absorbances were measured at 562 nm using a spectrophotometer. The results were expressed as chelating ability as shown in Eq.4

$$\text{Chelating ability (\%)} = \frac{A_{\text{control}} - A_{\text{sample}}}{A_{\text{control}}} \times 100 \qquad \text{Eq. (4)}$$

where A_{sample} is the sample absorbance; A_{control} refers to the control absorbance [18]. The analysis was repeated two times with four replications.

Ferric Reducing Antioxidant Activity (FRAA)

For this aim, 2.5 mL of phosphate buffer (pH 6.6) was placed into the tubes having 1 mL of the diluted extract samples. Then, 2.5 mL of potassium ferricyanide (1% w/v) was added and all samples were placed in a water bath for incubation at 50 °C for 20 min. After the incubation, 2.5 mL of trichloroacetic acid (10% w/v) was placed into the tubes to terminate the reaction. Then the samples were exposed to centrifugation at 5000 g for 5 min., 2.5 mL of sample from the upper phase was diluted with 2.5 mL of distilled water. After that, 400 μL of $FeCl_3$ (0.1 w/v) was placed into the tubes and the final mixture was vortexed and immediately the absorbances was recorded at 700 nm using a

spectrophotometer. The recorded absorbances were used to calculate the antioxidant activity of the samples using ascorbic acid calibration curve and the results were expressed as mg ascorbic acid equivalent (mg AAE)/g sample [19]. The analysis was repeated two times with four replications.

Data Modeling, Statistical Analysis and Optimization

In the current study, Box-Behnken experimental design [20] was used for the determination of optimum extraction conditions. For this aim, multiple factors namely sonication times (X_1) (10, 35 and 60 min), ethanol concentration (X_2) (15, 45 and 75%) and liquid/solid ratio (X_3) (20, 30 and 40 mL/g sample) were selected as the processing variables. In this regard, a 3-factor-3-level Box-Behnken experimental design having three replicates at the center point was created using Design Expert software (Design-Expert® Software Version 7.0 (Stat-Ease Inc., Minneapolis, USA). Table 1 shows the all studied factors (processing variables) and their levels in terms of coded and uncoded values. The recorded mean values of the studied responses were fitted to the second order polynomial model as shown in Eq. 5.

$$Y - \varepsilon = \beta_0 + \sum_{i=1}^{N} \beta_i x_i + \sum_{i=1}^{N} \beta_{ii} x_i^2 + \sum_{\substack{i=1 \\ i<j}} \sum_{j=i+1} \beta_{ij} x_i x_j, \qquad \text{Eq. (5)}$$

where Y is the response value, β^0 is the intercept term, β_i is the linear term, β_{ii} is the quadratic term, β_{ij} is the interaction term, and X_i and X_j are the coded levels of the independent variables. The regression coefficients were calculated by using Design Expert package software for each analyzed responses.

All computational works like graphical representations as well as optimization were performed by using Design-Expert® Software Version 7.0 (Stat-Ease Inc., Minneapolis, USA). Analysis of Variance (ANOVA) was also applied to determine the differences among the processing variables. Also, determination coefficients (R^2) were calculated and F values were determined to reveal the significance of the dependent variables ($p<0.05$).

RESULTS AND DISCUSSION

Bioactive Constituent Levels of *H. perforatum* Extracts

Table 2 shows the experimental values of bioactive constituents for *H. perforatum* extracts. As is seen from the table, TPC of all samples was in the range of 39.54–63.55 mg GAE/g sample. The lowest phenolic level was in sample extracted with 15% ethanol at 40:1 liquid/solid ratio for 35 min while the highest phenolic content was determined in the sample subjected to extraction with 45% ethanol at 20:1 liquid solid ratio for 10 min. The linear effects of both ethanol concentration ($p<0.001$) and liquid/solid ratio

($p<0.05$) were found as significant on TPC of the samples while sonication time was not effective. It means that there was no significant difference among the TPC of the samples depending on the sonication time for the samples (Table 3). Table 3 also shows the regression coefficients of the constructed predictive models. It could be said that the second order polynomial model fitted very well with quite high determination coefficient ($R^2=0.962$ and Adj. $R^2=0.894$) and the model was found to be very significant (F value=14.13, $p<0.001$). The constructed mathematical model for TPC was acceptable due to non-significant lack of fit value (F value=11.76, $p>0.05$). Fig. 1 illustrates the change in TPC of the samples. It was seen an increase in TPC by the increase of ethanol concentration until approximately 45-50% and after this level, the phenolic concentration started to decrease with the increase of ethanol level. Also, TPC of the samples increased with the increase of solvent level, which means that the added solvent amount increase provided an increment on the extractable phenolic constituents from the structure of the samples. Chen et al. [21] reported that the extraction yield of total anthocyanin increased with the increase of liquid/solid ratio and stated that the increase of solvent to sample ratio is significant for the enhancement of the extractable phenolics because the increase in solvent level provided to dissolve the solute in solvent easily. At constant conditions (at 50 ℃ and 45% ethanol concentration), TPC of the *H. perforatum* extract was measured as 50.7 mg GAE/g sample for 1:20 liquid/solid ratio while it was 60.5 mg GAE/g sample for 1:40 liquid/solid ratio. And also ultrasonic processing for the extraction increased the TPC because the cell walls were disrupted due to occurred cavitation which promotes the solvent penetration into the sample matrix [22]. So, TPC of conventionally extracted sample (45% ethanol concentration, 50 ℃ and 1:30 liquid/solid ratio) was 45.5 mg GAE/g sample while the TPC was determined as 52.7 mg GAE/g sample for the sample extracted at similar extraction conditions. Sonication time did not affect the TPC values of the samples.

TFC of the samples was given in Table 2 for all runs. As is seen from the table, the highest TFC was calculated for the sample having 40 ml solvent per g and extracted at 60 min by 45% ethanol concentration (run 9) while the lowest TFC was in the sample extracted by 15% ethanol for 10 min at 1/30 solvent ratio. TFC of the samples was affected by all processing variables studied significantly ($p<0.001$). The linear effects of sonication time, ethanol concentration and liquid/solid ratio were found to be very significant ($p<0.001$). The regression coefficients for the constructed model for TFC could be seen also in Table 3. As it is clear, R^2 (0.998) and Adj R^2 (0.997) values were calculated to be quite satisfied and the non-significant lack of fit (F=7.22) indicated that the model fitting ability had a good prediction. The effects of processing variables on TFC were illustrated in Fig.1. As could be seen, increment in ethanol concentration increased the extracted total flavonoids until

Table 2. Experimental data for the studied responses according to Box-Behnken design

Run	Processing variables			Bioactive compound levels				Bioactivity performance parameters			
	ST (min)	EC (%)	L/S (mL/g)	TPC (mg GAE/g)	TFC (mg CE/g)	CT (mg TAE/g)	THC (mg /kg)	ICA (% Inh.)	FRAA (mg AAE/g)	ABTS$^+$ (µg TE/g)	DPPH (µg TE/g)
1	35	45	30	57.33	75.02	14.95	531.7	69.91	82.83	232.7	130.5
2	10	15	30	39.57	44.60	6.22	227.9	74.60	58.15	161.3	70.4
3	35	15	20	40.93	55.08	8.99	225.0	87.22	56.76	155.1	86.0
4	35	15	40	39.54	50.78	8.76	278.2	71.05	63.26	188.3	80.6
5	10	45	20	63.55	85.41	16.44	571.2	81.77	77.89	229.8	111.3
6	60	15	30	42.89	58.89	9.88	211.5	80.09	68.19	211.4	87.6
7	60	75	30	53.39	62.20	14.50	858.7	43.69	88.44	226.7	105.2
8	35	45	30	60.63	75.44	16.34	470.2	69.67	97.36	243.5	147.2
9	60	45	40	60.07	93.40	17.23	519.2	39.55	104.78	275.9	120.7
10	35	45	30	56.93	74.75	14.78	522.1	52.42	90.13	267.1	135.3
11	10	45	40	53.69	75.35	15.99	548.7	71.08	90.86	273.5	153.5
12	35	75	40	53.01	77.11	14.69	841.0	34.93	88.74	261.1	133.9
13	10	75	30	50.34	71.67	13.26	783.7	34.57	75.18	220.6	115.5
14	35	75	20	52.92	90.50	14.97	757.7	39.68	73.70	193.9	127.8
15	60	45	20	50.73	84.66	16.43	569.9	75.64	80.62	222.3	127.4

ST: Sonication time (min), EC: Ethanol concentration (%), L/S: Liquid/solid ratio, TPC: Total phenolic content, TFC: Total flavonoid content, CT: Condensed tannins, THC: Total hypericin content, ICA: Iron chelating activity, FRAA: Ferric reducing antioxidant activity.

approximately 45-50% level and then the TFC was started to decrease. In addition to this, increment of sonication time increased the TFC of the samples due to the cavitation occurred by sonication which provides to promote solvent penetration into the sample matrix [22].

Condensed tannin (CT) levels of the samples were given in Table 2. The highest CT value was calculated 17.23 mg CE/g for the sample extracted with 45% ethanol and 1:40 liquid/solid ratio for 60 min while the lowest value was for the sample extracted by 15% ethanol for 10 min at 1/30 solvent ratio. The linear effect of only ethanol concentration was found as very significant on CT levels of the samples (p<0.001) while the other two processing variables showed no significant effect. The regression coefficients for the constructed model for CT were tabulated in Table 3. Similar to previous responses, R² (0.967) and Adj R² (0.908) values and the non-significant lack of fit (F=1.66) showed that the model fitting ability had a good prediction performance. The change of CT according to the processing variables was shown in Fig.1. As is seen, CT levels tended to increase clearly by the increase of ethanol concentration until 45-50% and after this level; a decrement was detected by the increase of ethanol. Kartnig et al. [23] informed that the condensed tannins present in high concentrations in Hypericum species.

The levels of total hypericin which is the main active substance of the *H. perforatum* plants were also determined

as spectrophotometrically and given in Table 2. The lowest total hypericin content (THC) (211.5 mg/kg) was measured for the sample extracted with 15% ethanol at 1:30 liquid/solid ratio level for 60 min while the highest total hypericin concentration (858.7 mg/kg) was determined for the sample extracted with 75% ethanol for 60 min. It was observed that the ethanol concentration is very effective factor on the hypericin isolation and its isomers' extraction because the highest total hypericin levels were obtained with the highest ethanol concentrations. So, the linear effect of ethanol concentration was found to be very significant (p<0.001). Fig.2 illustrates the contour plots showing the change in total hypericin content of the samples and it could be seen from the figure, only the major increment in total hypericin was determined with the increase of ethanol concentration significantly (p<0.01). The regression coefficients for the constructed model for THC were given in Table 3. R² (0.985) and Adj R² (0.958) values and the non-significant lack of fit (F=2.41) showed that the model fitting ability had a good prediction performance.

Yücel et al. [24] reported that the total hypericin level was 700 mg/kg for dried *H.perforatum* while similar total hypericin results were also reported by Southwell and Bourke [25]. Hypericin which is one the most important bioactive compounds of *H. perforatum* is a naphthodianthrone, an anthraquinone derivative. Jendzelovska et al. [26] reported that hypericin shows good bioactive
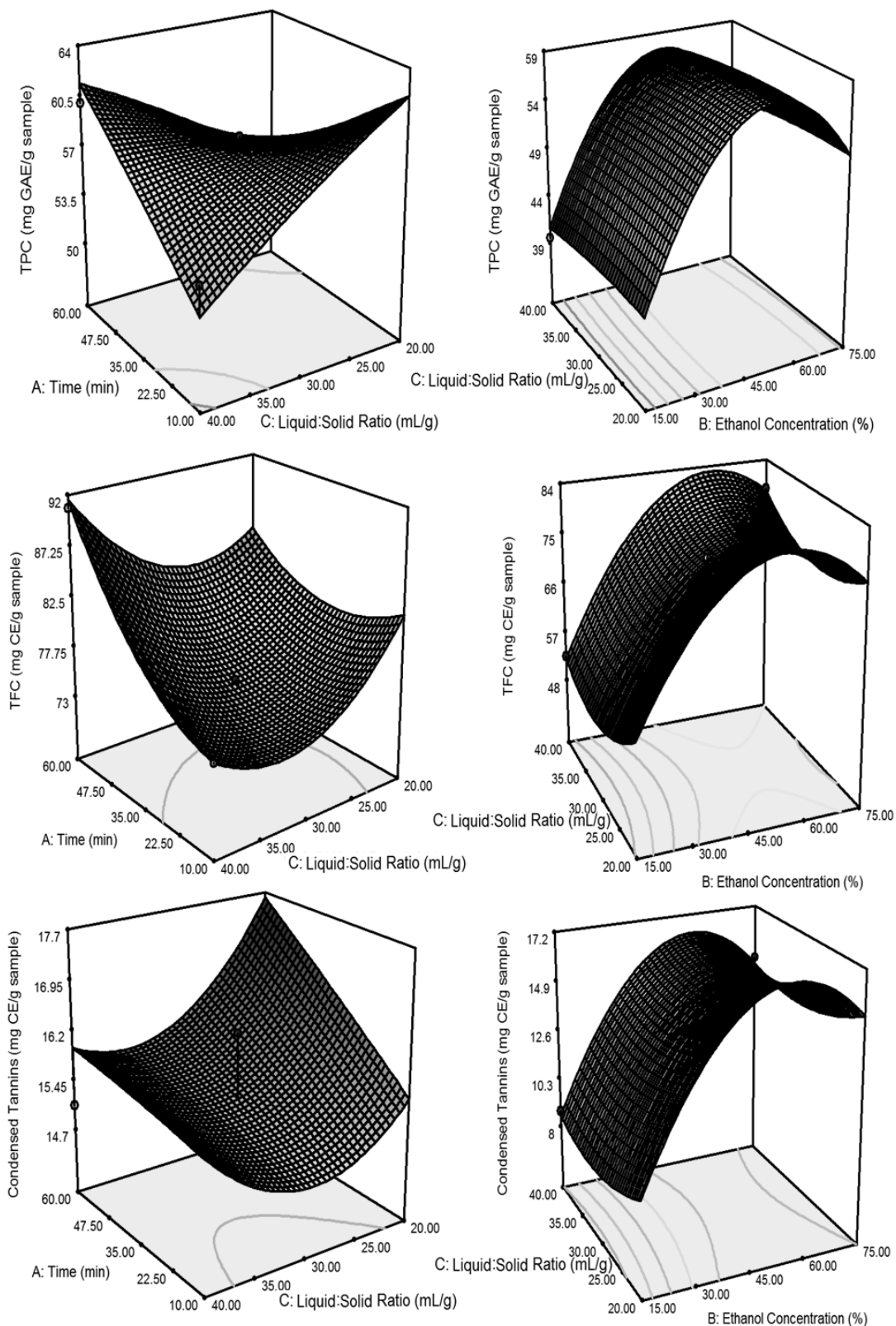
Figure 1. Response surface plots for interactions between the processing variables on total phenolic content, total flavonoid content and condensed tannins.

Table 3. Predicted regression coefficients$^\phi$ of the constructed regression models and ANOVA results for the studied parameters

Factor	Coefficients (β)							
	TPC	**TFC**	**CT**	**THC**	**ICA**	**FRAA**	**ABTS^{+}**	**DPPH**
Intercept	57.77	76.35	15.77	510.2	72.11	86.01	235.1	135.8
X_1	−2.32	2.89***	0.82	7.01	0.29	3.60	5.17	4.90
X_2	5.18***	10.53***	2.95***	283.5***	−21.4**	8.89*	19.02*	18.28**
X_3	−0.05*	−7.66***	−1.88	−25.06	−11.9	13.59*	39.2*	4.53
Cross Product								
X_1X_2	0.89	−1.94***	−0.60	22.84	0.91	0.80	−10.98	−6.89
X_1X_3	7.20	5.22***	−0.65	−10.58	−9.52	4.20	2.16	−18.35
X_2X_3	−0.2	2.52***	−0.02	11.3	4.28	3.20	12.77	4.31
Quadratic								
X_1^2	−0.18	3.29***	−0.03	19.59	−0.49	0.15	4.00	−10.91
X_2^2	−11.1***	−15.1***	−4.36**	−7.17	−9.28	−17.8**	−46.8**	−32.08**
X_3^2	−1.01	11.5***	1.94	55.44	−1.13	−3.88	−3.19	3.35
R^2	0.962	0.998	0.967	0.985	0.906	0.948	0.934	0.943
Adj. R^2	0.894	0.997	0.908	0.958	0.737	0.855	0.814	0.840
F value (model)	14.13***	451.7*	16.37***	36.5***	5.37*	10.18**	7.81*	9.14*
F value (Lack of fit)	11.76	7.22	1.66	2.41	14.93	0.25	0.76	1.60

X_1: Sonication time (min), X_2: Ethanol concentration (%), X_3: Liquid/solid ratio (mL/g), TPC: Total phenolic content, TFC: Total flavonoid content, CT: Condensed tannins, THC: Total hypericin content, ICA: Iron chelating activity, FRAA: Ferric reducing antioxidant activity.

The regression coefficients are presented for the coded levels

Significance levels: *$p<0.05$, **$p<0.01$, ***$p<0.001$

performance, such as antimicrobial, anticancer and anti-inflammatory effects. Similar findings were also reported by different researchers [27].

Antioxidant Capacity of *H. perforatum* Extracts

Antioxidant capacity of *H. perforatum* extracts was evaluated by two different tests namely ferrous ions or metal chelating activity and ferric reducing antioxidant activity (FRAA). Metal chelating activity was determined for the extract diluted as 1:30 to compare the sample and determine the processing effects. Metal chelating activity values of the sample ranged between 34.57-87.22%. The highest chelating activity (87.22%) was determined for extract sample obtained by 15% ethanol at 1:20 liquid/solid ratio for 35 min sonication time while the lowest value (34.57%) was seen in the sample extracted using 75% ethanol. The linear effect of ethanol concentration among the processing variables showed very significant effect on the metal chelating activity of samples ($p<0.01$). Fig. 3 shows the change in the chelating activity, and it is seen clearly that the increment of ethanol concentration increased the chelating activity of the extract samples. The regression coefficients for the constructed model for metal chelating activity were tabulated in Table 3. R^2 (0.906) and Adj R^2

(0.737) values and the non-significant lack of fit (F=14.93) showed that the model fitting ability had a good prediction performance. Iron chelating activity is important because chelating therapy reduces iron-related complications in human body and so improves the quality. It was reported that some iron chelators could be show adverse effect and so, recent researches focused on natural extracts having good iron chelating performance [28]. Zou et al. [29] reported that the iron chelating activity depends on flavonoid concentration of the sample and chelating ability is the main antioxidant action of the flavonoid rich extract of the plants.

Antioxidant performance of the samples characterized by ferric reducing capacity of extracts was given in Table 2. As is it seen from the table, the highest antioxidant performance (104.78 mg AAE/g sample) was determined for the sample extracted with 45% ethanol for 60 min at 1:40 liquid/solid ratio while the lowest value (56.76 mg AAE/g sample) was for the sample extracted with 15% ethanol for 35 min at 1:20 liquid/solid ratio. The linear effects of ethanol concentration and liquid/solid ratio were determined to be significant ($p<0.05$) on FRAA values of the samples (Table 3). The regression coefficients of the constructed polynomial model fitted quite well (R^2=0.948 and Adj R^2=0.855) for the
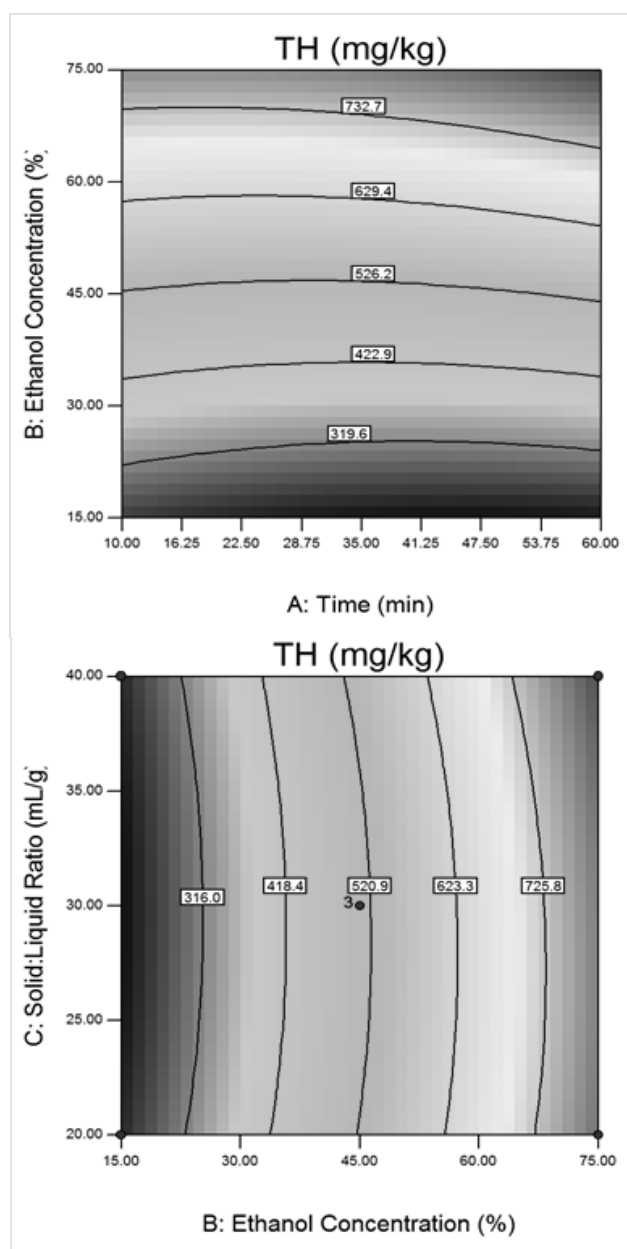
Figure 2. Response contour plots for interactions between the processing variables on total hypericin content (THC).

FRAA values were given in Table 3 and it could be seen that the prediction ability of the model was found to be good due to non-significant lack of fit (F=10.18). Fig. 3 illustrates the change of FRAA values depending on the processing variables and it was observed that the increase in ethanol concentration increased the antioxidant activity until 45-50% ethanol level and after this concentration, antioxidant performance started to decrease. It was also determined a significant positive correlation (r=0.827) between TPC and FRAA values of the samples. So, decrement in

TPC of the extracts due to the increase of the ethanol concentration caused a weakness in the antioxidant capacity of the samples. Iqbal et al. [30] reported that there was a significant correlation between the TPC and FRAA values of bark and leaf extracts of *Goniothalamus velutinus*.

Antiradical Activity of *H.perforatum* Extracts

Antiradical activity of *H.perforatum* extracts was evaluated by ABTS[+] and DPPH radical scavenging performance tests. ABTS[+] radical scavenging activity of the samples was given in Table 2 as μg TE/g sample. The highest ABTS[+] scavenging activity was calculated as 275.9 μg TE/g sample for the sample extracted with 45% ethanol for 60 min at 1:40 liquid/solid ratio while the lowest value (155.1 μg TE/g sample) was for the sample extracted with 15% ethanol for 35 min at 1:20 liquid/solid ratio. The highest and the lowest FRAA and ABTS[+] values were determined in same samples and it was determined a positive significant correlation (r=0.923) between antioxidant activity (FRAA) and antiradical activity (ABTS[+]) of the samples. The linear effects of ethanol concentration and liquid/solid ratio on ABTS[+] radical scavenging activity were determined as statistically significant (p<0.05) while the linear effects of sonication time were insignificant (p>0.05). Table 3 shows the coefficients of regression equation for ABTS[+] values and it is seen that the model fit very well (R²=0.934 and Adj R²=0.814 and non-significant lack of fit as F=7.81). Fig. 4 illustrates the change in ABTS[+] values of the samples depending on the processing variables. Increment in the ethanol concentration and liquid level tended to increase the ABTS[+] radical scavenging activity while there was no a clear change with the increase of sonication time. Chen et al. [21] reported that the ABTS[+] radical scavenging activity of *Lycium ruthenicum* extract was affected by solvent to sample ratio significantly and the sonication time and ultrasound power also caused a decrease in the antiradical activity. Zheleva-Dimitrova et al. [31] investigated the antiradical activity of different Hypericum species and measured that ABTS[+] radical scavenging activity of *H. perforatum* extracts showed quite high scavenging activity compared with other species and BHT as a standard antioxidant substance.

Table 2 shows the DPPH radical scavenging activity of the samples. The highest DPPH radical scavenging activity (153.5 μg TE/g sample) was determined for the samples extracted with 45% ethanol concentration for 10 min at 1:40 liquid/solid ratio while the lowest value (70.4 μg TE/g sample) was measured in the sample extracted with 15% ethanol for 10 min. Only the linear effect of only ethanol concentration was determined as significant (p<0.05). Table 3 shows the coefficients of regression equations for DPPH values and it is seen that the model fit very well (R²=0.943 and Adj R²=0.840 and non-significant lack of fit as F=1.60). Fig. 4 illustrates the change of DPPH scavenging values and it was observed that the increase in ethanol concentration increased the DPPH scavenging activity
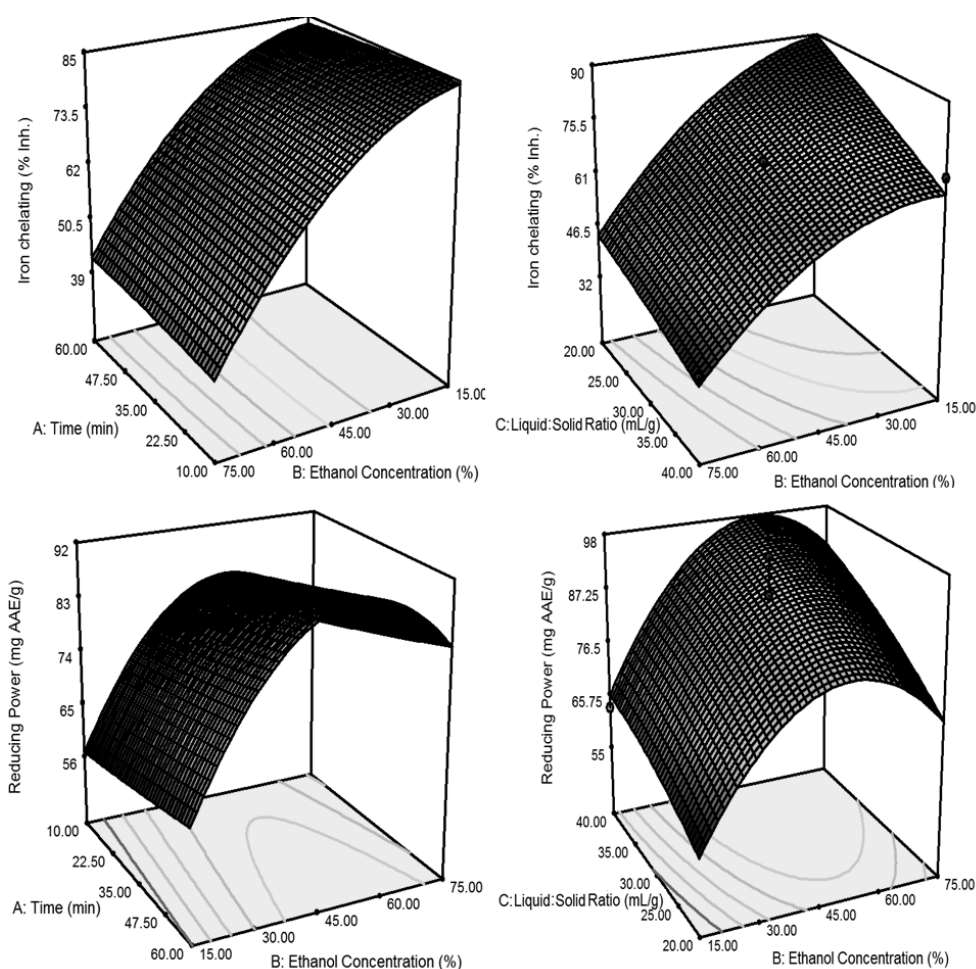
Figure 3. Response surface plots for interactions between the processing variables on iron chelating activity and ferric reducing antioxidant activity.

until 45-50% level and excessive ethanol amount caused a decrease in the activity. DPPH scavenging activity of samples correlated well with TPC (r=0.747) and TFC (r=0.864) and CT (0.908). Fathi and Ebrahimzadeh [32] reported that the DPPH scavenging activity was higher at 100 μg/mL and higher concentrations of *H. perforatum* extracts compared with BHT as a synthetic antioxidant.

Optimization of the Studied Response

Table 4 shows the multiple response optimization results for the studied parameters and the optimum conditions were determined by application of minimization and maximization process. Minimization process showed that minimum values for phytochemical constituent levels and bioactivity parameters would be at 15% ethanol concentration at 1:37.9 liquid/solid ratio for 15.1 min ultrasonic treatment. According to the maximization process, maximum values of the studied parameters would be at 47.6% ethanol concentration at 1:22.2 liquid/solid ratio level for 60 min

ultrasonic treatment. At these conditions, maximum TPC was 62.61 mg GAE/g sample while minimum TPC was 38.27 mg GAE/g sample while maximum THC was 611.5 mg/kg while minimum THC was 241.5 mg/kg sample.

CONCLUSION

In this study, ultrasound assisted extraction process was performed and the processing variables conditions were optimized to increase the yield of the phytochemical constituents from *H. perforatum*. Response surface methodology was used and changes in the selected responses were modeled successfully also the effects of factors were analyzed. It was concluded that ethanol concentration among processing variables was the most effective factor and also sample to solvent ratio affected some studied responses. All responses were affected by ethanol percentage in extraction solvent and until a constant value of ethanol level (approximately 45–50%), after this level, increase in ethanol caused
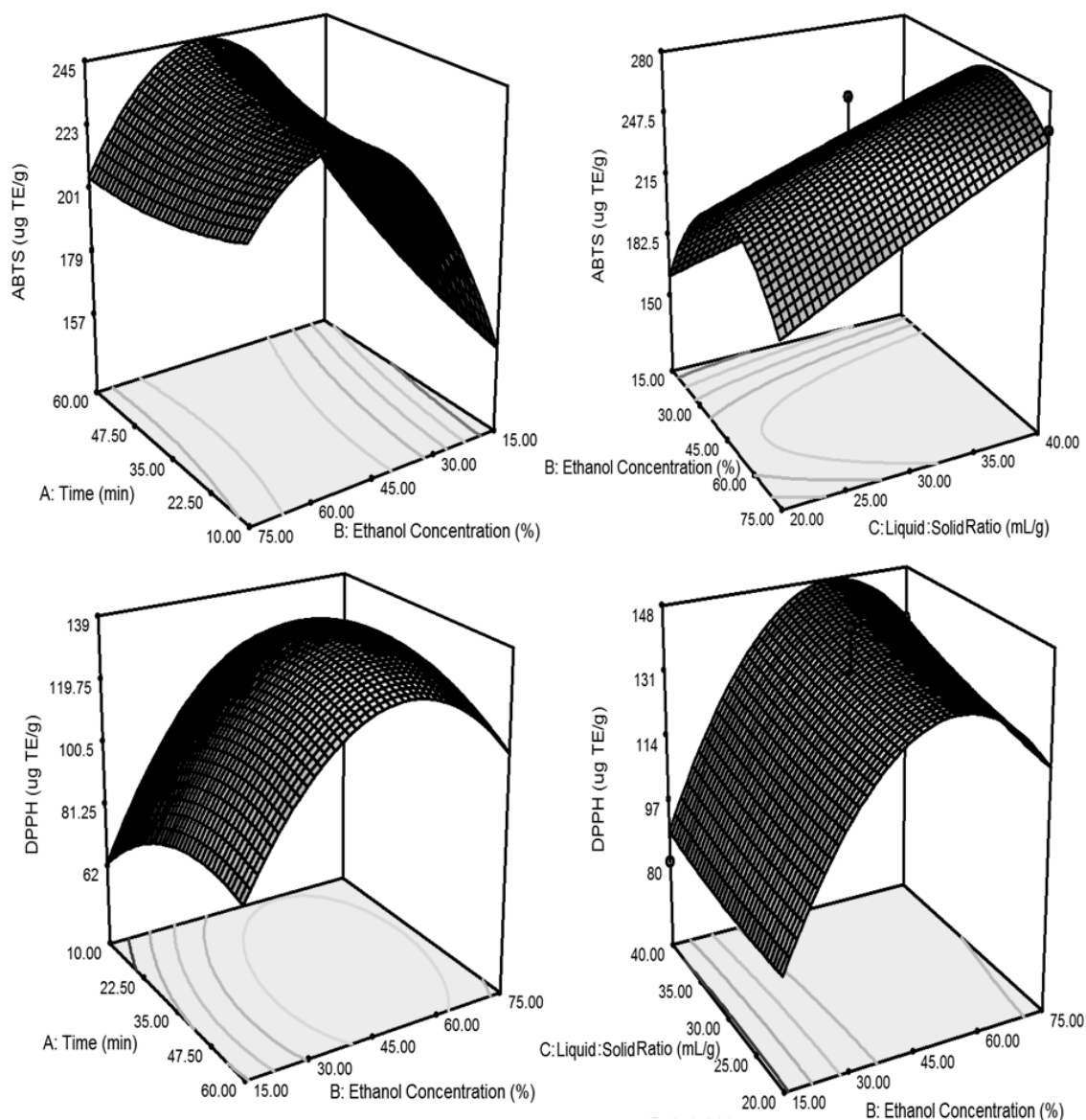
Figure 4. Response surface plots for interactions between the processing variables ABTS$^{.+}$ scavenging activity and DPPH scavenging activity.

Table 4. Multiple response optimization values of *H. perforatum* extracts

	Minimization process				Maximization process			
	X_1 (min)	X_2 (%)	X_3 (mL/g)	*Desirability*	X_1 (min)	X_2 (%)	X_3 (mL/g)	*Desirability*
Response parameters	15.1	15.0	37.9		60.0	47.6	22.2	
TPC (mg GAE/g)		38.27		0.733		62.61		0.790
TFC (mg CE/g)		48.23				83.97		
CT (mg TAE/g)		8.11				17.25		
THC (mg/kg)		241.5				611.4		
ABTS$^+$ (µg TE/g)		184.8				236.7		
DPPH (µg TE/g)		89.37				133.1		
ICA (% Inh.)		71.6				73.82		
FRAA (mg AAE/g)		62.42				87.1		

X_1: Sonication time (min), X_2: Ethanol concentration (%), X_3: Liquid/solid ratio (mL/g), TPC: Total phenolic content, TFC: Total flavonoid content, CT: Condensed tannins, THC: Total hypericin content, ICA: Iron chelating activity, FRAA: Ferric reducing antioxidant activity.

a decrement in the studied parameters values except hypericin. Optimum ethanol concentration was determined as to be 47.6% for all parameters by multiple response optimizations, on the other hand in single response optimization process, only 75% ethanol concentration was calculated for the total hypericin content. Sonication time did not show significant effect on many responses, which means 10 min was enough compared with 60 min ultrasound application. The results of the current study could be applicable for the extraction of *H. perforatum* by different industries.

AUTHORSHIP CONTRIBUTIONS

Authors equally contributed to this work.

DATA AVAILABILITY STATEMENT

The authors confirm that the data support the findings of this study are available within the article. Raw data that support the finding of this study are available from the corresponding author, upon reasonable request.

CONFLICT OF INTEREST

The author declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

ETHICS

There are no ethical issues with the publication of this manuscript.

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