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Bioactivity of Hawthorn (*Crataegus monogyna*) and Heather (*Calluna vulgaris*) Leaf Tinctures: An Optimization Study Using Response Surface Methodology

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Highlights:

- Bioactivity of tinctures is affected by the plant genus.
- Total phenolic content changed significantly by the ethanol concentration.

Keywords:

- Tincture
- Bioactivity
- Optimization

ABSTRACT:

In the current research, two medicinal important plants namely hawthorn (*Crataegus monogyna*) and heather (*Calluna vulgaris*) leaf were used for the tincture production according to central composite rotatable design and the produced tinctures were subjected to bioactive analysis to reveal the optimum manufacturing conditions. As response, total phenolic content and radical scavenging performance of the produced tinctures were analyzed. The levels of total phenolics and radical scavenging performance activity of the hawthorn and heather tinctures ranged between 2996.9-5415.8 mg GAE/L and 5514.9-13923.6 mg GAE/L and 26.9-71.9 and 51.0-94.1%, respectively. Effect of both liquid/solid ratio and ethanol concentration affected the bioactivity of the samples. Optimization process revealed that the optimum levels for the highest total phenolic content and antiradical activity were 4.03 mL/g for liquid/solid ratio and 67.07% for ethanol level for the hawthorn tincture and 4.03 mL/g for liquid/solid ratio and 53.77% for ethanol level for the heather tincture. The results of the current work revealed that the processing variables had important effect on the bioactivity of the tinctures.

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INTRODUCTION

Tincture is a hydroalcoholic extract used in the pharmaceutical applications. It is widely used in the medicinal treatments especially in phytotherapy. Bone (2003) informed that the tincture which is a plant extract manufactured by especially hydroalcoholic extraction is quite efficient for the medicinal cares compared to whole plant because tincture is in liquid form and shows good bioactive performance due to all bioactive compounds present in solution and easily they can start the reaction. To provide high effectiveness of the plant to treat the disease, tinctures are used widely and efficiently in the phytotherapy.

Medicinal plants are widely used for the treatment of many diseases in the worldwide because they are rich in many phytochemical substances showing bioactive performance. Seifried et al. (2007) informed the bioactive properties of phytochemicals in medicinal plants having an important role in the protecting the body against free radicals because these substances can easily reduce the oxidative stress due to their radical scavenging properties. The hawthorn plant is an effective herbal remedy used to treat chronic heart failure in the worldwide (Koch and Malek, 2011). It was reported that the pharmacological researches revealed that hawthorn extracts performed cardio- and vasoprotective properties in addition to prophylactic and therapeutic treatment of such as coronary heart disease, endothelial dysfunction and, atherosclerosis, (Koch and Malek, 2011). Heather is also another important medicinal plant and it has long been used as a medicinal herb against some disorders such as rheumatism, arthritis, as well as an antiseptic, vulnerary, choleretic and expectorant. Additionally, its decoction is also effective on some problems of urinary tract because of its diuretic, anti-inflammatory and antimicrobial properties (Orhan et al., 2007; Vučić et al., 2014; Wadekar et al., 2015; Moreira et al., 2015; Chepel et al., 2020).

The response surface methodology which is an important statistical modeling technique makes complex operations easier and widely used to optimize processing parameters (Sheng et al., 2013; Wang et al., 2013). It is used effectively due to need fewer attempts to evaluate multiple parameters and their interactions (Ma et al., 2010). It determines optimum working conditions depending on the variables by introducing functions that can predict responses conditions (Myers and Montgomery, 2002; Koç and Kaymak Ertekin, 2010). There are some studies about response surface methodology applications for the optimization of tincture production from origanum (Kaplan et al., 2019), clove (Kaplan et al., 2020) and lemon balm (Akçura, 2020).

In the current study, effect of two important parameters namely liquid/solid ratio and ethanol concentration on total phenolic content and antiradical activity of hawthorn (*Crataegus monogyna*) and heather (*Calluna vulgaris*) leaf tincture were deterrmined. To reveal the best processing variable levels to manufacture the most bioactive tincture, response surface methodology-central composite rotatable design was used.

MATERIALS AND METHODS

Materials

Hawthorn and heather leaves harvested in 2019 were provided from Karakas Food Co (Kayseri, Turkiye). The moisture content of the hawthorn and heather leaves were 9.1 and 6.2%, respectively. The samples were ground and stored in a plastic covered bag until analysis.

Experimental procedure

Preparation of tincture samples

To produce the tincture samples, two different processing parameters were used to determine the effect of these parameters on the bioactive performance of the final products. For this purpose, ethanol concentration and liquid/solid level were selected as parameters and the levels of these factors were tabulated in Table 1. Central composite rotatable design was applied to optimize the production parameters for the tincture samples. Liquid/solid ratio was in the range of 3-10 g/mL while the ethanol concentration ranged between 25-75% (Table 1). To manufacture the tincture sample, the relevant parameters according to the Table 1 were applied and the final mixture was kept for 14 days at dark place in room conditions. At the end, the samples were centrifuged at 7500 g and +4 ºC for 5 min and the supernatant was used as tincture sample for the bioactivity tests.

Table 1. Experimental Design for the Production of Hawthorn and Heather Leaf Tinctures

	Coded Values		Actual Values	
Runs	**A**	**B**	**A**	**B**
1	0	0	6.5	50
2	-1	1	4.03	67.68
3	1	-1	8.97	32.32
4	0	0	6.5	50
5	0	1.414	6.5	75
6	1	1	8.97	67.68
7	0	0	6.5	50
8	-1	-1	4.03	32.32
9	0	-1.414	6.5	25
10	1.414	0	10	50
11	-1.414	0	3	50

A: Liquid/Solid Ratio (mL/g), B: EtOH Concentration (%)

Determination of total phenolic content (TPC)

The method reported by Köprü et al. (2021) was used to determine the total phenolic content of the tincture samples. For this aim, 0.2 mL of tincture sample was mixed with 1.800 mL of distilled water and then 1 mL of Folin reagent (diluted as 10^{-1}) was added. Afterthat, 2 mL of Na_2CO_3 (2% w/v) was placed into the mixture and mixed well using vortex. The final mixtures were kept for 2 h in a dark place at 25 ºC. At the end, the sample absorbance was recorded using a spectrophotometer (Shimadzu, UV-vis 1800, Japan) at 765 nm. A calibration curve prepared by gallic acid standard was used to calculate as mg gallic acid equivalent (mg GAE/L). The measurement was replicated with four repetitions.

Determination of antiradical activity

To determine the radical scavenging performance of the tincture samples, DPPH (2,2-diphenyl-1-picrylhydrazyl) radical substance was used. A 100 µL of tincture sample (40^{-1} v v^{-1}) was mixed with 3.9 mL of DPPH methanolic solution (0.1 mM) and mixed well by vortex. Then the samples were kept for 30 min in a dark place at 25 ºC and then, the sample absorbances were recorded at 550 nm using a spectrophotometer (Shimadzu UV-vis 1800, Japan). The antiradical activity (ARA) of the samples was calculated by Equation 1:

$$ARA\ (\%\ Inh.) = [(Abs_{control}) - (Abs_{sample})/(Abs_{control})] * 100 \quad (1)$$

where Abs is the absorbance. The measurement was replicated with four repetitions (Köprü et al., 2021).

Data optimization

In this study, response surface methodology-central composite rotatable design was used. Table 1 shows the levels of processing variables by coded and uncoded terms. The regression models were fitted to the following polynomial equation (Equation 2) and the model constants were calculated.

$$Y - \varepsilon = \beta_0 + \sum_{i=1}^{N} \beta_i x_i + \sum_{i=1}^{N} \beta_{ii} x_i^2 + \sum_{\substack{i=1 \\ i<j}} \sum_{j=i+1} \beta_{ij} x_i x_j \quad (2)$$

Design-Expert® Software Version 7.0 (Stat-Ease Inc., Minneapolis, USA) was used to calculate the regression coefficients of linear, quadratic and interaction terms for each output parameter. Also, the same software was used to perform variance analysis and optimization process.

RESULTS AND DISCUSSION

Total phenolic content of the tincture samples

Total phenolic content (TPC) of the both hawthorn and heather leaf tinctures were tabulated in Table 2. Total phenolics of the hawthorn tinctures ranged between 2996.9-5415.8 mg GAE/L and the tincture sample produced with 50% EtOH and 3 mL/g liquid/solid level (run 11) had the highest total phenolics while the lowest was for the sample prepared with 50% EtOH and 10 mL/g liquid/solid level (run 10). Similarly, total phenolic contents of the heather samples ranged between 5514.9-13923.6 mg GAE/L and the highest TPC level was measured for the run 11 (3 mL/g). Total phenolics of the samples also changed significantly based on the tincture type and the highest total phenolic content was monitored for the heather leaf tincture. For the tincture sample prepared according to the run 1 and 6, total phenolic content of the hawthorn and heather samples were determined as 4540.1 and 8653.4 and 4050.3 and 5717.6 mg GAE/L, respectively. Figure 1 illustrates the change in total phenolics of the hawthorn leaf tincture and it is seen clearly from the figure that the increase in the liquid/solid ratio decreased the total phenolic content of the tincture sample ($p<0.05$) while the ethanol concentration increment provided an increase in TPC ($p<0.05$) of the samples. Linear effect of liquid/solid ratio was determined to be very significant ($p<0.01$, Table 3) for both tincture samples while ethanol concentration was also effective on the total phenolic content of the samples ($p<0.1$).

Table 2. Total Phenolic Content and Antiradical Activity of Hawthorn and Heather Leaf Tinctures

Runs	Liquid/Solid Ratio (mL/g)	EtOH Concentration (%)	TPC (mg GAE/L)		ARA (%)	
			Hawthorn	Heather	Hawthorn	Heather
1	6.5	50	4540.1	8653.4	48.1	79.7
2	4.03	67.68	5325.3	11785.1	71.9	90.8
3	8.97	32.32	3294.2	5514.9	32.1	51.0
4	6.5	50	4406.4	8261.5	47.4	71.3
5	6.5	75	4771.2	7548.6	57.5	66.3
6	8.97	67.68	4050.3	5717.6	45.9	58.2
7	6.5	50	4666.5	9815.5	51.2	86.0
8	4.03	32.32	4513.8	10396.6	50.9	87.9
9	6.5	25	2996.9	6440.5	26.9	59.0
10	10	50	3575.0	5849.3	35.3	55.7
11	3	50	5415.8	13923.6	71.3	94.1

TPC: Total phenolic content, ARA: Antiradical activity

Table 4 shows the predictive regression models for the total phenolics of hawthorn and heather tinctures. As is seen, the models could be effectively used to estimate the total phenolic content of the sample due the high coefficient of determination ($R^2 > 0.935$). Chepel et al. (2020) investigated the variation of phenolic compounds and bioactive performance of various parts of the heather and they reported that the highest level of bioactive constituents was detected in leaves and roots at all growth

stages of heather. They reported that the total phenolic contents were 14.52, 26.88 and 32.67 mL/g for the stages of vegetative, flowering and seed ripening, respectively. Various parts (roots, flowers, shoots) of the heather plant contain important bioactive compounds and chemical composition of the heather plant is depended on some factors such as the climate, season, altitude, soil characteristics or growth stages (Cucu et al., 2022).

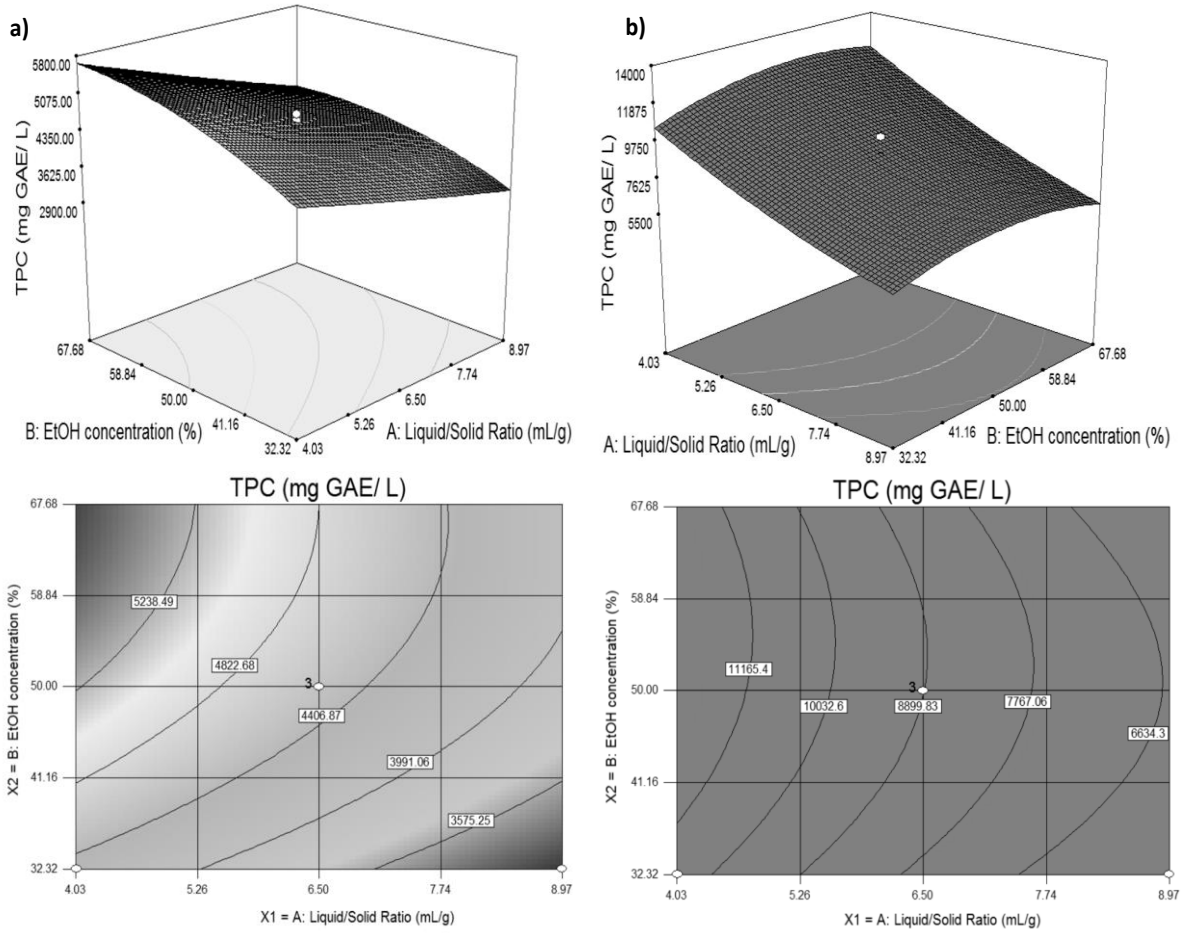

Figure 1. Change in total phenolic content of hawthorn (a) and heather (b) leaf tincture

Antiradical activity of tincture samples

Table 2 shows the antiradical activity of both hawthorn and heather leaf tinctures. As is seen, antiradical activity values of hawthorn tinctures ranged between 29.9-71.3% while the antiradical activity of heather tincture was in the range of 51.0-94.1%. The highest antiradical performance was monitored for both tincture sample produced with 3 mL/g and 50% ethanol (run 11). As is clearly seen from the values given in Table 2, heather tincture showed better radical scavenging performance compared to hawthorn tincture. Figure 2 illustrates the variation in the antiradical activity of the both samples. Increase in the ethanol level provided an increase in the antiradical activity of the samples while there was a significant decrease with the increase in the liquid level per g of leaf sample ($p<0.01$, Table 3). Effects of both liquid/solid ratio and ethanol concentration were determined as significant ($p<0.01$) and a significant effect was not observed on the interaction the processing variables ($p>0.05$). Table 4 shows the predictive regression models for also antiradical activity of hawthorn and heather tincture samples. As is seen, the models could be effectively used to estimate the antiradical activity of the sample due the high coefficient of determination ($R^2> 0.935$). High correlation was determined between total phenolic content and antiradical activity of both hawthorn and heather leaves as 0.977 and 0.956, respectively. It was reported that the heather showed good antiradical activity and its radical scavenging

performance showed a significant change depending on the growth stage of the plant. The highest DPPH radical scavenging performance was recorded for the flower and leaf samples collected in the stage of flowering (Chepel et al., 2020).

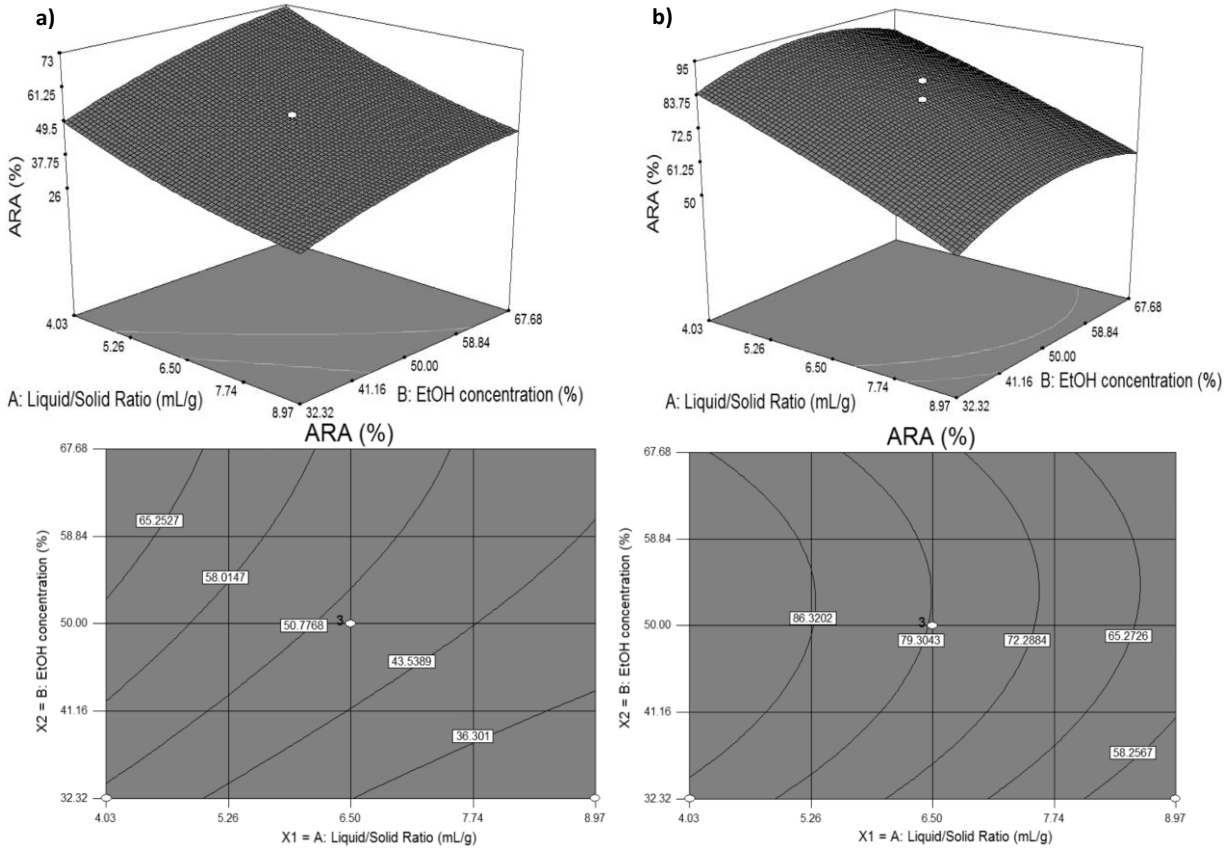

Figure 2. Change in antiradical activity of hawthorn (a) and heather (b) leaf tincture

Table 3. ANOVA Table for the Effects of Studied Parameters on Total Phenolic Content and Antiradical Activity

		Total Phenolic Content				Antiradical Activity			
		Hawthorn		Heather		Hawthorn		Heather	
		Mean	F	Mean	F	Mean	F	Mean	F
Source	**df**	**Square**	**Value**	**Square**	**Value**	**Square**	**Value**	**Square**	**Value**
Model	5	1367872	41.02[a]	14616163	54.30[a]	408.02	59.94[b]	457.70	14.45[a]
A-Liquid/Solid Ratio (mL/g)	1	3777869	113.30[a]	62544256	232.34[a]	1141.63	167.71[a]	1918.97	60.60[a]
B-EtOH concentration (%)	1	2505223	75.13[a]	1246873	4.63[c]	761.56	111.88	51.45	1.62
AB	1	51848.52	1.55	351536.8	1.31	13.03	1.91	4.79	0.15
A^2	1	5135.407	0.15	1229549	4.57[c]	44.51	6.54	8.86	0.28[b]
B^2	1	427752.8	12.83b	5409593	20.10[b]	42.83	6.29	307.98	9.73
Residual	5	33343.63		269188.2		6.81		31.67	
Lack of Fit	3	44291.26	2.62	13170.95	0.02	8.70	2.20	16.63	0.31
Pure Error	2	16922.18		653214		3.96		54.23	
Cor Total	10	1367872	41.02	14616163	54.30	408.02			

[a] $p<0.01$, [b]$p<0.05$, [c] $p<0.1$

Table 4. Regression Equations for the Studied Parameters of Hawthorn and Heather Leaf Tinctures

Parameters	Regression model	R^2	Adj R^2
Hawthorn TPC	$Y_{TPC}=4537.9-687.9A+559.6B-114.1AB+30.2A^2-275.3B^2$	0.976	0.952
Hawthorn ARA	$Y_{ARA}=48.91-11.96A+9.76B-1.81AB+2.81A^2-2.75B^2$	0.984	0.967
Heather TPC	$Y_{TPC}=8910-2798.8A+394.8B-297AB+467.1A^2-979B^2$	0.982	0.964
Heather ARA	$Y_{ARA}=79.02-15.50A+2.54B-1.10AB-1.25A^2-7.39B^2$	0.935	0.870

TPC: Total phenolic content, ARA: Antiradical activity

Optimization of the processing variables

According to the experimental design in the work, total phenolic content and antiradical activity of the both heather and hawthorn tinctures were determined and the results were subjected to multiple optimization process using desirability function to see the processing points giving the maximum and minimum bioactive parameter values. According to the optimization, the highest total phenolic content and antiradical activity of the hawthorn tincture samples were determined as 5654 mg GAE/L and 72.49% at 4.03 mL/g solid/liquid ratio and 67.07% ethanol concentration. For the heather tincture sample, liquid/solid and ethanol levels were determined to be 4.03 mL/g and 53.77% for the highest total phenolic content (12279 mg GAE/L) and antiradical activity (93.24%), respectively. Figure 3 shows that the change in desirability levels calculated based on multiple optimization process and it was determined that the desirability levels were quite acceptable for the optimized conditions. For the heather tincture sample, it was calculated as 0.987 and 0.888 for the total phenolic content and antiradical activity, respectively.

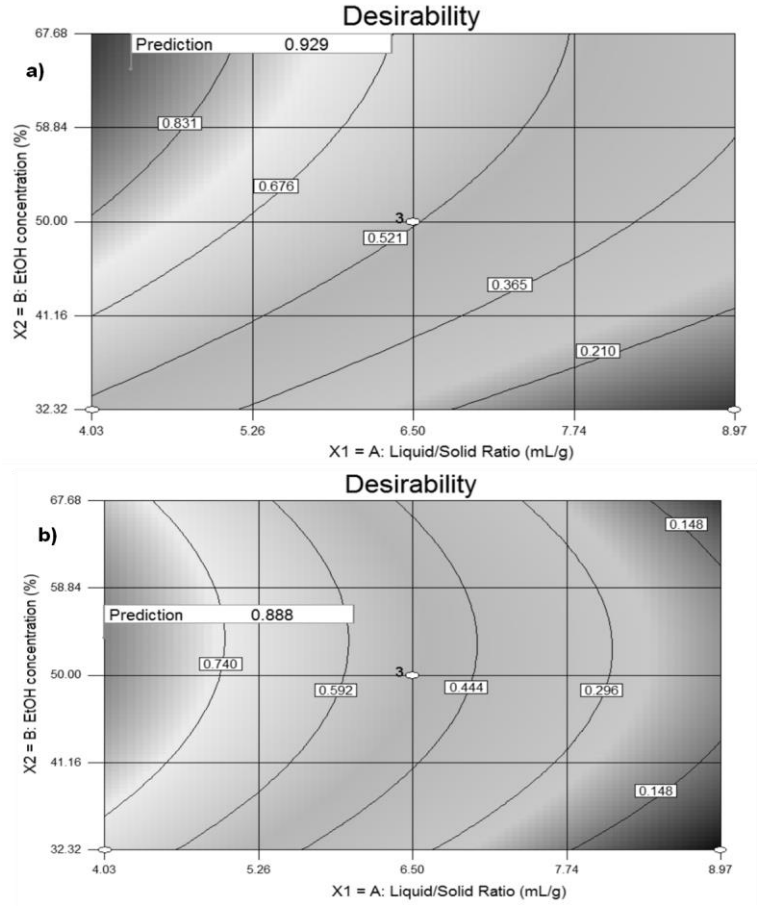

Figure 3. Change in desirability values of hawthorn (a) and heather (b) leaf tincture depending on processing variables

CONCLUSION

Tinctures of both hawthorn and heather leaves showed high bioactivity and two important processing variables namely liquid/solid ratio and ethanol concentration showed significant effect on the bioactive performance of the tincture samples. Optimization results revealed that the highest total phenolic content and antiradical activity of the samples could be obtained with the lowest liquid/solid ratio for the tincture production. Also ethanol concentration level changed depending on the leaf type and heather tincture performed better bioactivity compared to hawthorn tinctures

Conflict of Interest

The article author declares that there is no conflict of interest

Author's Contributions

The author declare that he prepared all parts of the article by himself.

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