Original Article: Simultaneous Optimization of Ultrasound Pretreatment Extraction of Polyphenols Using Response Surface Methodology from *Lamium album* L.

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Abstract

Simultaneous ultrasound pretreatment extraction (UPE) process was optimized by means of response surface methodology (RSM) to maximize the extraction of the two most famous polyphenols, total hydroxycinnamic acids (THAC) derivatives and total flavonoids content (TFC) as hyperoside, from Iranian White Dead Nettle (*Lamium album* L.). A 5-level full factorial central composite design was successfully implemented for UPE optimization, in which temperature, extraction time, ethanol percent, ultrasonic pretreatment time and liquid/solid ratio were relevant independent variables. For maximizing the two responses simultaneously, the optimal processing conditions were as follows: 60% ethanol–water (volume-by-volume); temperature, 55 ºC; extraction time, 24 h; liquid/solid ratio, 12 (mL/g); and time of ultrasound pretreatment, 20 min which the desirability factor was 0.780. Statistical analysis and verification test indicate a satisfactory correlation between the experimental data and predicted values.

Keywords: *Lamium album* L, Response surface methodology (RSM), Hydroxycinnamic acid derivatives, Total flavonoids, Ultrasound.

Introduction

The genus Lamium (Lamiaceae) spread in northern America, Europe, central and northern Asia (Heber,2004; Pereira *et al*.,2012). The genus Lamium is represented in flora of Iran by seven species (Morteza - Semnani *et al*., 2016). *L. album*, commonly is called white dead-nettle, has been widely distributed in Iran in different provinces (Mozaffarian,2015). The genus Lamium can be used in food, tea, medicines and food supplements preparation that all of these applications are attributed to different phytochemical compounds (Paduch *et al*., 2007). According to different references, *L. album* L. is one of the richest sources of phytochemicals that Iridoid monoterpenes, secoiridoid glucosides, triterpene saponins, essential oils, mucilage and polyphenols are the most important (Jaromir Budzianowski and Skrzypczak, 1995).

Polyphenols are one of the biggest groups of secondary metabolites compound in the plant kingdom and more than 8,000 of them have been identified in various plant species (Pinela *et al*.,2016). Polyphenolic compounds can be classified into four main classes: phenolic acids, flavonoids, stilbenes and lignans; flavonoids such as rutin, hypersoid and quercetin. Caffeic acid derivatives including among others rosmarinic acid, chlorogenic acid, vanillic acid that in the last few decades; scientists have favored mostly flavonoids and phenolic acids (Balasundram *et al*., 2006; Chmelová *et al*., 2020; Del Bubba *et al*., 2021; Kashyap *et al*., 2021; Khedher *et al*., 2020).

Numerous studies have been shown that optimizing the extraction of active ingredients in plants is one of the most important determinants for the production of effective herbal products (Tomaz *et al*., 2016). Different factors such as type, percent and volume of the solvent, the particle size, solid-to-liquid ratio, the extraction time and temperature are the most important independent variables that can help us to reach the aim (Kashyap *et al*., 2021; Khedher *et al*., 2020; Lai *et al*., 2014). Furthermore, during the last years, various new extraction techniques, which generally that effectively extract phytochemical compounds, have been introduced including: microwave assisted extraction

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(MAE), pressurized liquid extraction (PLE), and ultrasound-assisted extraction (UAE) *(*Agregán *et al.*,2021*;* Ameer *et al.* ,2017*;* Kashyap *et al.*, 2021; Mohammad Azmin *et al.*, 2016).

Among different alternative methods, ultrasonic pretreatment is widely used for increasing the yield of extraction (Esclapez *et al.*, 2011; Pongmalai *et al*., 2015). Studies on the ultrasonic pretreatment related to improving the quality of bioactive compounds extraction from cabbage outer leaves, extraction of pectin from grapefruit and phenolic compounds from wheat dried distiller's grain (DDG) (Bagherian *et al*., 2011; Izadifar, 2013), show that ultrasonic pretreatment led to higher yield of extraction with financial benefits. During propagation of ultrasound waves, intermolecular distance of solvent molecules varies and acoustic cavitation is generated. Upon the formation of the cavitation, the physical, chemical, and mechanical effect will be created. As a result, the mass transfer from herbs in a liquid medium is enhanced breaking down the cell walls. In the ultrasonic methods, power, time and frequency is important (Chemat *et al.*,2017; Xu *et al.*,2017).

Response surface methodology is a collection of mathematical and statistical techniques that uses data from appropriate experimental designs to determine the multivariate model that can show the individual interaction and combined effects of all independent factors on the response equations with minimum deviation from the exact response (Bezerra *et al*., 2008).

Based on our reviews, no data was reported about the simultaneous optimization of two polyphenols extraction in L. album by ultrasonic pretreatment and RSM. Therefore, optimization extraction conditions of flavonoids and hydroxycinnamic acid derivatives as the two most famous polyphenol of aerial flowering parts of L. album were done by using RSM and central composite rotatable design (CCRD).

Material and methods

Plant Material

Aerial parts of white dead nettle (*L. album*), during the flowering time in spring 2019, were harvested in Nowshahr county, Mazandaran Province in the north of Iran (36°39' 18.7"N, 51°28' 37.1"E), Northern Iran and were dried in shade. Voucher specimen (MPH-2675) of the plant was identified by Dr. Ali Sonboli, and it was deposited in the Medicinal Plants and Drugs Research Institute Herbarium of Shahid Beheshti University, Tehran, Iran. The dried samples were stored in well-closed container desiccators at 4°C.

Chemicals and Reagents

Silica gel 60F254 - precoated TLC plates, methanol, vanillin, sulphuric aicd, ethanol, acetone, hydrochloric acid, sodium molybdate, sodium nitrite, anhydrous sodium sulphate hexamethylenetetramine, ethyl acetate, aluminum chloride hexahydrate, acetic acid, formic acid, rutin, hypersoid, quercetin, chlorogenic acid, caffeic acid, polyethylene glycol 4000 and sodium hydroxide were purchased from Merck (Germany). Diphenylboric acid-p-ethylamino ester (diphenylboryloxyethylamine) was purchased from Roth Company, and chicoric acid purchased from Sigma-Aldrich Company.

TLC Identification

TLC method is the simplest method for initial identification. According to Plant Drug Analysis (A Thin Layer Chromatography Atlas) from Hildebert Wagner and Sabine Bladt (Wagner and Bladt,1996), general mobile phase for polyphenol compounds was used: Ethyl acetate-formic acid-glacial acetic acid-water (100:11:11:26). For sample preparation, the powdered aerial part (1 g leaf and flower) was extracted with l0ml methanol and ethanol separately for 5 min in a water bath at about 60°C, and then filtered; next, 20 µL was used for TLC. Rutin, hypersoid, quercetin, chlorogenic acid, caffeic acid and chicoric acid were used as the standard compounds with 0.1 mg/ml concentration in methanol and 10 µL was used separately in spotting. For detection of each spot, the mobile phase had to be thoroughly removed from the silica gel layer and then the plate was sprayed with 1% methanolic diphenylboric acid-p-ethylamino ester (=diphenylboryloxyethylamine, and then by 5% ethanolic polyethylene glycol-4000(PEG) (10 ml and 8 ml, respectively). Different zones with intense fluorescence were detectable in UV-365 nm.

Experimental Design and Statistical Analysis

A five-factor and central composite rotatable design (CCRD) was employed to evaluate the effects of the independent variables and to optimize the extraction conditions of TF and THAC from *L. album* L. The

design consisted of 50 experiments with 32 factorial points, 10 axial points or α (2 axial points on the axis of each design variable at a distance of 2.38 from the design center) and eight replicates at the center points were used. The parameters used in the experimental design ranging are as follows (Hernández-Carranza *et al*., 2016; Jovanović *et al*., 2017; Liyana-Pathirana and Shahidi, 2005):

- temperature ranging from 21.22 to 68.78ºC(X1)
- extraction time ranging from 18.49 to 37.51 h(X2)
- ethanol concentration ranging from 26.22 to 73.78(v/v) % (X3)
- solvent/solid ratio ranging from 9.24 to 18.76 mL/g (X4)
- ultrasonic pretreatment ranging from 0 to 28.27 min (X5)

Each factor was coded at five levels of -2.38, -1, 0, +1, and 2.38. The following equation was used to convert real values to coded ones (Lai *et al*., 2014):

$$X_i = \frac{x_i - x_0}{\triangle x} \qquad (1)$$

Where X_i is the dimensionless value, x_i is the corresponding actual value, x_0 is the actual value of independent variable i at the central point and $\triangle x$ is the change of x_i corresponding to a unit variation of the dimensionless value. The coded and real values of the independent variables are shown in Table 1. Value of independent variable i at the central point and Δx is the change of x_i corresponding to a unit variation of the dimensionless value. The coded and real values of the independent variables are shown in Table 1.

In order to predict the optimal conditions, experimental data were analyzed using the Design- Expert software and then fitted to an empirical second-order polynomial regression model as follows (Chmelová *et al*.,2020; Kashyap *et al.*,2021; Sedraoui *et al.*,2020):

$$Y = \beta_0 + \sum_{i=1}^{k} \beta_i X_i + \sum_{i=1}^{k} \beta_{ii} X_{ii}^2 + \sum_{i}^{k-1} \sum_{j}^{k} \beta_{ij} X_i X_j \qquad (2)$$

Where Y is the predicted values (TF and THAC yield), β_o is intercept, β_i, β_{ii} and β_{ij} are the regression coefficients for linear, quadratic and interaction terms, respectively, X_i and X_j are independent variables affecting the response. Experimental data were analyzed using Design Expert software (v. 11 Trial, State-Ease, Minneapolis, Minnesota, USA). Analysis of variance (ANOVA) with 95% confidence level and response surface analysis were employed to determine the statistical significance of the model. The relationship between the predicted values and independent variables was studied by a response surface plot.

In order to find the conditions offering the greatest responses for each response simultaneously, the total desirability, using geometric mean must be calculated (Dranca and Oroian,2016) which was carried out with Design-Expert software (v. 11 Trial, State-Ease, Minneapolis, Minnesota, USA). The data reported in all of the tables are the average of triplicate observation.

Ultrasound- Pretreatment Extraction Procedure

The powdered of the herb (1g) was introduced to 100 ml flasks, according to Table 1. Appropriate solvent was added and ultrasonic pre-treatment was done at a specific time for each sample in ultrasonic water bath (Elma, Elmasonic S, S40H, with an effective volume of 3.2 L (internal dimensions: 24 cm× 13.7 cm× 15 cm) that operated at a constant ultrasonic power and frequency of 140 W and 37 kHz, respectively. After pre-treatment, samples extraction was done, according to the condition in Table1, without any shaking. After extraction, they were filtered immediately and the residue was washed with the same solvent to compensate for the lost volume. A rotary evaporating at 40ºC was used to evaporate the supernatants from each extraction condition to dryness. 10 mL methanol 70% (v/v) was applied for dissolving the residue. A cellulose filter (0.45 µm) was applied to filter each solution that was transferred to 25mL volumetric flask; afterwards, methanol 70% (v/v) was applied to wash the residue to 25mL that was stored at 4ºC until analysis.

Quantitative Determination of Total Flavonoid Content (TFC)

The obtained extracts (10 g) in different conditions (Table 1), 1 ml of a 5 g/L solution of hexamethylenetetramine, 20 mL of acetone and 7 mL of hydrochloric acid 25% (w/w) were added to a 100 ml round-bottomed boiled flask under a reflux condition for 30 min. After cooling, the solution filters through a plug of absorbent cotton into a 100 mL volumetric flask. After adding the absorbent cotton to the residue in the round-bottomed flask, extraction was performed twice each time with 20 mL acetone under

a reflux condenser for 10 min and each time was filtered to first volumetric flask. Ultimately, a filter paper was applied to filter the combined acetone solution into the volumetric flask that was then diluted to 100.0 mL with the same solvent by washing the flask and filter. Next, 20.0 mL of this solution was introduced into a separating funnel followed by the addition of 20 mL of water and performing extraction in four steps (15, 10, 10 and 10, respectively) with ethyl acetate. Afterwards, the ethyl acetate extracts were mixed in a separating funnel, and washing was done with two times with 50 mL of water; the organic solvent filter over 10 g of anhydrous sodium sulphate into a 50 mL volumetric flask and dilute to 50 mL with same solvent (mother solution).

In the final step, 10 ml of the mother solution, solution was transferred separately to two 25 ml volumetric flasks and to only one of them, 2% aluminum chloride hexahydrate (w/v) in 5% (v/v) glacial acetic acid solution is added but each both diluted to 25 mL with 5% (v/v) glacial acetic acid solution. The sample with the aluminum chloride hexahydrate reagent, named as test solution and another as blank solution. After 30 min, the absorbance of solution with aluminum chloride was measured by comparison with the blank solution at 425 nm and the percentage content of total flavonoids as hyperoside was calculated as: (Commission, 2016):

$$\mathrm{TFC} = \frac{\mathrm{A}*1.25}{\mathrm{m}} \qquad (3)$$

In other words, the specific absorbance of hyperoside was taken to be 500.

A= absorbance at 425 nm

m= mass of the drug to be examined, in grams

Determination was done by UV/Vis Shimadzu spectrophotometer (model 1700).

Quantitative Determination of Total Hydroxycinnamic Acid (THAC)

To 10 g of the obtained extracts in different condition (Table 1) and 80 mL of ethanol (50% v/v) were mixed. The solution was boiled in a water-bath under a reflux condenser for 30min. After cooling, filtration was done and then 5 mL of ethanol (50% v/v) was applied to wash the filter, which was diluted to 100 ml with same solvent and considered as stock solution. To prepare the test solution, 2 ml of 0.5 M hydrochloric acid was added to 1.0 ml of the stock solution; 10 g of sodium nitrite and 10 g of sodium molybdate were dissolved in 100 ml of water to prepare a 2 ml solution. Afterwards, 2 mL of sodium hydroxide solution (8.5% w/v) was added and dilution was done to 10 mL with water.

Determination was done by UV/Vis Shimadzu spectrophotometer (model 1700); for preparing the blank solution, 1 ml of the stock solution was mixed with 2 mL of 0.5 M hydrochloric acid, 2 ml of sodium hydroxide (8.5% w/v) and dilute to 10.0 mL with water. The absorbance of the test solution was determined at 525 nm immediately. The percentage content of total hydroxycinnamic derivatives, expressed as chlorogenic acid (THAC), was calculated according to equation 4; (Commission, 2016);

$$THAC = \frac{\mathrm{A}*5.3}{m} \qquad (4)$$

A= absorbance of the test solution at 505 nm,

m= mass of the sample to be tested, in grams.

Determination was done by UV/Vis Shimadzu spectrophotometer (model 1700).

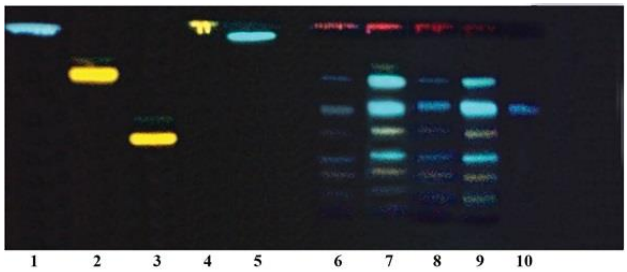

Fig. 1 TLC picture of white dead nettle with different parts, with different standards; caffeic acid (1), hyperoside (2), rutin (3), quercitin (4), chicoric acid (5), ehanolic extract of flower (6), methanolic extract of flower (7), ethanolic extract of leaf (8), Methanolic extract of leaf (9) and chlorogenic acid (10), respectively.

Results and Discussion

Initial Identification with TLC Method

As shown in Figure 1, with different solvent and different part of herb, the results confirmed the presence of some of the flavonoids and phenolic acids, especially rutin and chlorogenic acid, respectively (Fig. 1). Other compounds such as quercetin, chicoric acid, caffeic acid and hyperoside were not detectable. Other important points are:

- More density of different spots in flower related to leaves extract. As a result, in the extraction process, leaves and flowers must be employed together and their combination is economical in industrial extraction.
- More density of spots in methanolic extract relative to ethanolic extract. Based on the latter result, the aqueous methanol is better solvent for extraction of polyphenols (Liyana-Pathirana and Shahidi, 2005). However, since aqueous ethanol is environmentally friendly, economic accessibility and relatively safe for using in health related industries, ethanol has been utilized in this study as the best solvent in optimization processes (Chmelová *et al*., 2020).

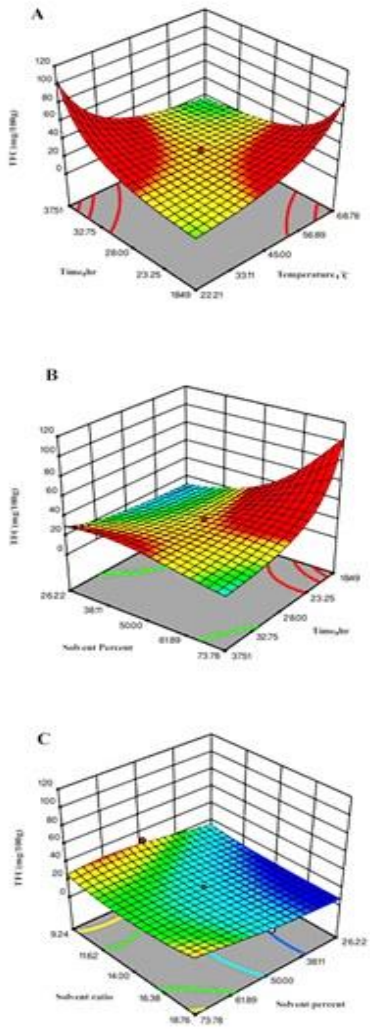

Fig. 2 Response surface plots showing interaction effects of process variables: (A) time and temperature, (B) solvent percent and time and (C) solvent ratio and solvent percent

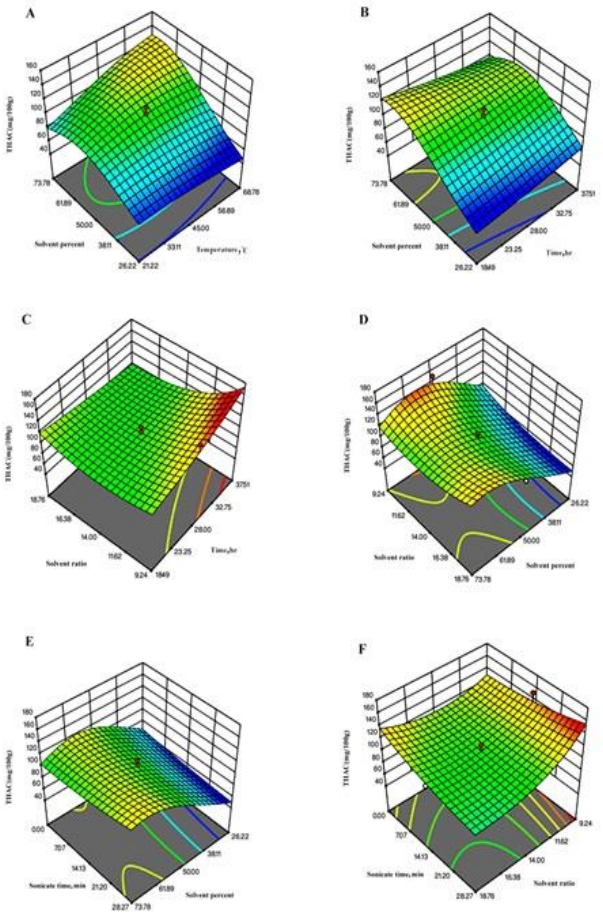

Fig. 3 Response surface plots showing interaction effects of process variables: (A) solvent percent and temperature, (B) solvent percent and time, (C) solvent ratio and time, (D) solvent ratio and solvent percent, (E) sonicate time and solvent percent and (F) sonicate time and solvent ratio.

Optimization of TF and THAC Extraction Process Using RSM

Model Fitting

According to the developed design, 50 experiments were carried out in triplicate and performed in random order to investigate the effect of different variables on the yields of TFC and THAC. Different conditions of 50 experiments (un-coded, coded and their levels) as well as the results for the yield of TFC and THAC are shown in Table 2. The amounts of TFC varied from 8.45 to 30.02 mg/100 g and THAC amounts varied from 55.65 to 150.35 mg/100 g. Least squares regression analysis of variables was used to generate quadratic models and determine the regression coefficients and their suitability to predict the responses (Syahariza *et al.*,2017). Regression coefficients and the summarized results of the analysis

of variance (ANOVA) of the quadratic model for the TFC and THAC are shown in Table 3.

Effects of Extraction Parameters on TFC

The amounts of TFC are presented in Table 2. The highest TFC yield was obtained in run 1 with 50 % (v/v) ethanol, solvent ratio 9.24, temperature 45ºC, extraction time 28h, and sonicate pre-treatment 14 min. For fitting the model equation and experimental data, the analysis of variance (ANOVA) was applied to assess the independent variables; moreover, the regression coefficients were determined and the results are shown in Table 3. According to the ANOVA results, the model was significant at F-value of 14.05 and applicable because of a very low p-value ($p<0.0001$) (Table 3). The fitness and adequacy of the model was evaluated by the determination coefficient (R^2) and the significance of lack-of-fit, respectively (Lai et al., 2014). R^2 value for the TFC equation extraction model was 0.9065, which indicated that 90.65% of the variation could be explained by the equation 6. The ‘‘Lack of Fit of F-value” of 0.8187 implies that the lack-of-fit was not significant relative to pure error ($p = 0.6671$) at 95% confidence verify the validity of the model ($p= 0.6671>0.05$).

The adjusted R^2 ($R^2_{Adj}=0.8419$) was agreement with R^2, which further indicated a high degree of correlation between the observed and predicted values. The value of the coefficient of variation (C.V.=6.42%) indicated a good reproducibility of the model. The influences of five independent variables (temperature, extraction time, ethanol concentration, solvent/solid ratio, and ultrasonic pre-treatment) on of yield of TFC are shown in Table 3. Based on testing the significance of the model at 95% confidence level, coefficient with the p-value lower than 0.05 is significant and retained in the model but the others must be neglected (Equation 5).

$$TFC = 0.2989 - 0.0063\,X_2 - 0.0365\,X_3 + 0.0187\,X_4 + 0.0130X_1\,X_2 + 0.0148X_2\,X_3 - 0.0079X_3\,X_4 + 0.0074\,X_3^2 - 0.008\,X_4^2 \quad (5)$$

Where TFC is the yield of total flavonoids (mg/100g), X1, X2, X3 and X4 are the coded variables for temperature (ºC), extraction time (h), ethanol concentration and solvent ratio, respectively. Among the significant first-order factors, by comparing the relevant coefficients and p-values, the importance of ethanol concentration and solvent ratio is greater than the extraction time. On the other hand, the effect of ethanol concentration is stronger than the solvent ratio, which was derived by comparing the relevant coefficients. According to Table 2, the quadratic effect of ethanol concentration and solvent ratio are also important like the first order as the positive coefficient of solvent percent shows that the effect of ethanol concentration is highly important, and according to the negative quadratic effect of solvent ratio, there was a maximum apparent TFC at a certain ratio. In the (Equation 5), similar reported result by Pandey *et al*. (2018) indicates that the effect of pre-treatment sonication time in each state (linear, interaction and quadratic) is not significant.

According to Table 3, the temperature did not have significant linear and quadratic effects ($p>0.05$), but its interaction with time is significant ($p =0.04<0.05$).

The graph in Figure 2 A shows that higher temperature and longer exposure of extraction time together reduced the yield of TFC. This result is consistent with other reports (Lai *et al*., 2014; Pandey *et al*., 2018) reporting that the temperature and extraction time together could have destructive effect on the extraction of total flavonoids in higher amounts. The higher amount of TFC is obtained in longer time with the lowest temperature or higher temperature with the shortest time (Chmelová *et al*.,2020) also, as shown in Figure 2A, the TFC is more in longer time. Figure 2B shows that time and percent of ethanol have opposite effects on each other. In other words, we can reach the maximum value by increasing the solvent percentage in a shorter time of extraction or by increasing the time with a lower solvent percentage. Nevertheless, via visual inspection, we can see that the with higher ethanol percentage, the TFC is reached its maximum but the TFC in the maximum amounts of solvent percent and extraction time will be at a minimum. However, the best result approximately is obtained in 50% (v/v) ethanol that this value selected as the best solvent in TFC extraction in wild garlic (*Allium ursinum* L.) and Thymus serpyllum L. (Jovanović *et al.*, 2017; Khedher *et al.*, 2020; Tomšik *et al.*, 2016) respectively, within 28 h. According to TFC model equation, solvent ratio has positive effect on the first order term of equation 5 but in interaction with solvent percent and in quadratic state has negative effect on TFC as the interaction can be seen in Figure 2C. The coefficient and the p-value of its interaction with the

solvent percentage indicate that this interaction is less significant than the other interactions.

However, it is worth noting that at higher solvent percentages, without considering the solvent ratio, we can achieve more TFC. This result indicates that the solvent percentage is more significant than the solvent ratio.

Effects of Extraction Parameters on THAC

Experimental results have obtained for total hydroxycinnamic acid derivatives as chlorogenic acid (THAC) on different conditions according to experimental design, regression coefficients and other result of statistical analysis are presented in Table 2 and Table 3 respectively. At 95% confidence level, the ANOVA analysis confirmed that the model was significant (model p-value < 0.0001) and the lack-of-fit was not significant (p = 0.0695>0.05) which confirmed that the fitted model was considered adequate. The predictive equation for describing the effect of studied factors on extraction of THAC compounds from *L. album* L. is as follows (Equation 6):

$$THAC = 0.0973 - 0.001\,X_2 - 0.0078X_3 + 0.0022X_4 + 0.0011X_5 - 0.0018X_1\,X_3 + 0.0014X_2\,X_3 + 0.0012X_2\,X_4 - 0.0013X_3\,X_4 - 0.0012X_3\,X_5 + 0.0011X_4\,X_5 + 0.0008\,X_1^2 + 0.003\,X_3^2 - 0.0011X_4^2 \quad (6)$$

The yields of THAC varied from 55.65 to 150.35 (mg/100g). The determinant coefficient (R^2=0.9429) and the adjusted determination coefficient (R^2_{Adj}=0.9035) indicated a good relation between the studied and predicted values. The coefficient of variation of the model (C.V.=2.95%) showed a good reproducibility. In equation 6, similar to equation 5, the effect of temperature in the first order is not observed but in this state, time of sonicate pre-treatment is significant. Based on the p-values and the coefficients of equation 6, as shown in Table 3, it is concluded that the linear effect of ethanol percentage and solvent ratio in the first order is more significant than time and sonicate pre-treatment. Another notable point in equation 6 is the presence of quadratic term of temperature with high p-value (p=0.0475) in border line; thus, it could be concluded that the temperature factor such as equation 5 is not really significant in linear and quadratic terms but the interaction with solvent percentage is significant. Figure 3 shows the interaction influence of different parameters on the extraction of THAC and help us to understand these interaction effects better. Figure 3A shows that the THAC increased with temperature increasing and it reached a maximum value at about 60–70 ºC and then it decreased; however, the lowest THAC was obtained below 40ºC. Earlier studies have shown that higher temperature may increase the diffusion coefficient; thus, the rate of extraction of polyphenolic compounds is enhanced. However, the temperature exceeding certain values might lead to concurrent decomposition thermo-sensitive compounds which is already mobilized at a lower temperature or even its breakdown is still in the plant cells (Lai *et al*., 2014; Liyana-Pathirana and Shahidi, 2005). Since hydroxycinnamic acid derivatives are subgroups of polyphenolic compounds this behavior seems normal. The small negative coefficient of linear term of time and its positive interaction with ethanol concentration and solvent ratio shows that the slope of THAC variation due to time is slow, but extraction is preferred at shorter times as much as possible (Fig. 3B, C). Various researches have investigated the impact of the ethanol concentration in the extraction of the phenolic compounds. According to these studies, the effect of ethanol percentage is more significant and this impact can be seen in the interaction with other factors. As Figure 3 A, B, D and E shows, the THAC rapidly rises by increasing ethanol concentration and reaches its highest value at about 60% ethanol. This was expected based on the results about polyphenols group and antioxidant features (Kashyap *et al*., 2021; Syahariza *et al*., 2017), as this group is the subgroup of polyphenols compound. According to general principle ‘‘like dissolves like’’ ethanol, due to its effect on the polarity of the extraction medium, has a significant role in the extraction of the phenolic compounds. (Kashyap *et al*., 2021; Lai *et al*., 2014).

According to Table 3, solvent ratio in linear term (p<0.0001), interaction with time and ethanol percent (p=0.0302 and p=0.0146, respectively) and in quadratic terms is significant. In fact, visual analysis of the surface plots shows that the THAC increases with the decrease of solvent ratio in longer time of extraction or in shorter time with the increase of solvent ratio (Fig. 3C). However, according to the negative quadratic effect of solvent ratio, there was a maximum apparent THAC at a certain ratio. Figure 3D shows this difference in the THAC.

Table 1 Independent variables and their coded and actual values used in rotatable central composite design

Independent variables	Symbol	Coded levels				
		-α	-1	0	+1	+α
Temperature (ºC)	X1	21.22	35	45	55	68.78
Extraction time (h)	X2	18.49	24	28	32	37.51
Ethanol concentration (V/V)%	X3	26.22	40	50	60	73.78
Liquid / solid ratio	X4	9.24	12	14	16	18.76
Ultrasonic pretreatment (min.)	X5	0	8	14	20	28.27

Table 2 Rotatable central composite design setting in the coded form (x_1 to x5) and real values of the independent factors (X_1 to X5) with their observed responses

Run order	Coded values (Real values)					Responses	
	x_1	x_2	x_3	x_4	x_5	TF (mg/100g)	THAC (mg/100g)
1	0(45)	0(28)	0(50)	-2.38(9.24)	0(14)	30.0231	150.35
2	1(55)	1(32)	-1(40)	-1(12)	1(20)	14.542	93.7701
3	0(45)	0(28)	0(50)	0(14)	0(14)	11.8972	115.662
4	1(55)	-1(24)	-1(40)	-1(12)	1(20)	13.5436	85.13
5	-1(35)	-1(24)	-1(40)	1(16)	-1(8)	9.0039	94.4982
6	-1(35)	1(32)	-1(40)	-1(12)	-1(8)	16.2969	100.448
7	0(45)	0(28)	0(50)	0(14)	2.38(28.27)	13.7524	108.19
8	-1(35)	-1(24)	-1(40)	-1(12)	-1(8)	10.9944	79.0355
9	-1(35)	-1(24)	-1(40)	-1(12)	1(20)	11.5335	80.705
10	-1(35)	-1(24)	1(60)	-1(12)	1(20)	22.9391	113.301
11	0(45)	2.38(37.51)	0(50)	0(14)	0(14)	14.3905	109.432
12	1(55)	-1(24)	1(60)	-1(12)	1(20)	20.1438	115.048
13	1(55)	1(32)	1(60)	1(16)	-1(8)	15.8937	117.646
14	1(55)	-1(24)	-1(40)	1(16)	1(20)	11.9045	71.2525
15	0(45)	0(28)	0(50)	0(14)	0(14)	15.7101	107.218
16	-1(35)	1(32)	-1(40)	-1(12)	1(20)	15.6863	105.863
17	1(55)	1(32)	1(60)	1(16)	1(20)	16.1308	122.101
18	1(55)	1(32)	1(60)	-1(12)	1(20)	17.9231	127.568
19	0(45)	-2.38(18.49)	0(50)	0(14)	0(14)	16.9781	107.947
20	-1(35)	1(32)	-1(40)	1(16)	1(20)	12.7949	84.8876
21	1(55)	1(32)	-1(40)	-1(12)	-1(8)	14.9612	100.226
22	0(45)	0(28)	0(50)	0(14)	0(14)	13.7168	105.388
23	-1(35)	-1(24)	1(60)	1(16)	-1(8)	16.53	115.888
24	1(55)	1(32)	1(60)	-1(12)	-1(8)	14.0174	126.452
25	0(45)	0(28)	-2.38(26.22)	0(14)	0(14)	8.44928	55.6531
26	-2.38(21.22)	0(28)	0(50)	0(14)	0(14)	15.1832	89.5021
27	0(45)	0(28)	0(50)	0(14)	0(14)	14.6643	103.787
28	1(55)	-1(24)	1(60)	-1(12)	-1(8)	19.2185	125.704
29	-1(35)	1(32)	1(60)	1(16)	-1(8)	19.5117	108.343
30	1(55)	1(32)	-1(40)	1(16)	1(20)	10.8258	73.0507
31	1(55)	-1(24)	-1(40)	1(16)	-1(8)	10.1446	83.3766
32	-1(35)	1(32)	1(60)	-1(12)	-1(8)	18.9358	107.964
33	-1(35)	1(32)	-1(40)	1(16)	-1(8)	15.2245	97.8208
34	1(55)	1(32)	-1(40)	1(16)	-1(8)	11.0169	80.8734
35	0(45)	0(28)	0(50)	2.38(18.76)	0(14)	13.0826	103.834
36	1(55)	-1(24)	1(60)	1(16)	1(20)	17.2048	119.317
37	1(55)	-1(24)	1(60)	1(16)	-1(8)	16.1193	127.574
38	-1(35)	1(32)	1(60)	1(16)	1(20)	16.89	108.155
39	0(45)	0(28)	0(50)	0(14)	0(14)	16.1837	105.074
40	2.38(68.78)	0(28)	0(50)	0(14)	0(14)	14.7465	108.119
41	-1(35)	-1(24)	-1(40)	1(16)	1(20)	8.71771	73.7343

42	0(45)	0(28)	0(50)	0(14)	0(14)	14.7618	102.978
43	-1(35)	-1(24)	1(60)	-1(12)	-1(8)	18.3571	111.376
44	-1(35)	-1(24)	1(60)	1(16)	1(20)	17.7518	109.689
45	0(45)	0(28)	0(50)	0(14)	0(14)	12.6991	102.348
46	0(45)	0(28)	2.38(73.78)	0(14)	0(14)	18.5217	115.933
47	1(55)	-1(24)	-1(40)	-1(12)	-1(8)	12.6252	93.3746
48	0(45)	0(28)	0(50)	0(14)	-2.38(0)	12.7018	114.323
49	0(45)	0(28)	0(50)	0(14)	0(14)	14.9665	105.588
50	-1(35)	1(32)	1(60)	-1(12)	1(20)	22.0155	117.585

X1: Temperature (ºC), X2: Extraction time (h), X3: Ethanol concentration (v/v) %, X4: Solid/liquid ratio(g/mL),X5:Ultrasonic pre-treatment(min.).

Table 3 Estimated coefficients of the fitted second-order polynomial model for TFC, THAC, and analysis of variance ANOVA of the system.

Terms	Regression coefficients			
	TFC	P value	THAC	P value
Intercept				
β_0	0.2989	-	0.0973	-
Linear				
β_1	0.0024^{ns}	0.4145	-0.0008^{ns}	0.0697
β_2	-0.0063^{s}	0.0378	-0.0010^{s}	0.0257
β_3	-0.0365^{s}	< 0.0001	-0.0078^{s}	< 0.0001
β_4	0.0187^{s}	< 0.0001	0.0022^{s}	< 0.0001
β_5	-0.0034^{ns}	0.2480	0.0011^{s}	0.0160
Interaction				
β_{12}	0.0130^{s}	0.0006	0.0007^{ns}	0.1934
β_{13}	0.0059^{ns}	0.0888	-0.0018^{s}	0.0019
β_{14}	-0.0005^{ns}	0.8789	0.0010^{ns}	0.0601
β_{15}	-0.0032^{ns}	0.3442	0.0004^{ns}	0.4427
β_{23}	0.0148^{s}	0.0001	0.0014^{s}	0.0091
β_{24}	-0.0034^{ns}	0.3266	0.0012^{s}	0.0302
β_{25}	0.0038^{ns}	0.2717	-0.0008^{ns}	0.1127
β_{34}	-0.0079^{s}	0.0255	-0.0013^{s}	0.0146
β_{35}	-0.0031^{ns}	0.3647	-0.0012^{s}	0.0313
β_{45}	0.0038^{ns}	0.2664	0.0011^{s}	0.0360
Quadratic				
β_{11}	-0.0018^{ns}	0.4969	0.0008^{s}	0.0475
β_{22}	-0.0030^{ns}	0.2435	-0.0001^{ns}	0.8522
β_{33}	0.0074^{s}	0.0068	0.0030^{s}	< 0.0001
β_{44}	-0.0080^{s}	0.0038	-0.0011^{s}	0.0067
β_{55}	0.0025^{ns}	0.3419	-0.0003	0.5035
ANOVA results				
Model	s	< 0.0001	s	< 0.0001
$R^{2\,a}$	0.9065	-	0.9429	-
Adjusted R^2	0.8419	-	0.9035	-
Predicted R^2	0.7047	-	0.7941	-
CV% [b]	6.42	-	2.95	-
Lack of fit	ns	0.6671	ns	0.0695
Regression degree of freedom	20	-	20	-
Pure error degree of freedom	22	-	22	-
Lack of fit degree of freedom	7	-	7	-
Total degree of freedom	49	-	49	-

s Significant ($p < 0.05$)

ns Not significant ($p > 0.05$)

a Coefficient of multiple determination

b Coefficient of variance

Table 4 Estimated optimum conditions and predicted, and observed values of each individual response.

Response	Predicted mean	95%CI low	Observed value	95%CI high
TF	25.087	20.4859	26.012	31.7962
THAC	130.826	119.563	129.976	143.761

Conclusions

Simultaneous optimization of extraction conditions for recovery of TFC and THAC from white dead nettle (*L. album* L.) with ultrasound pre-treatment were performed by applying different effective factors and using RSM as a mathematical and statistical method for optimization of extraction. Also, the second order polynomial model provided sufficient mathematical description of TFC and THAC responses. Optimization of extraction conditions was successfully performed to provide maximum yields for each observed response. The most dominant effects on this study were time, solvent percent and solvent-to-solid ratio, especially sole interaction, while Sonicate pretreatment did not have any effect on flavonoids extraction except on hydroxycinnamic acid derivatives extraction. Moreover, temperature in flavonoids and hydroxycinnamic acid derivatives extraction, due to the longtime of extraction, is not an effective parameter.

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