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Full Length Research Paper

Optimization of extraction efficiency of tannins from *Cichorium intybus* L.: Application of response surface methodology

Muhammad Aslam Shad*, Haq Nawaz, Tanzila Rehman, Hafiz Badaruddin Ahmad and Mazhar Hussain

Department of Chemistry, Bahauddin Zakariya University, Multan-60800, Pakistan.

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The effect of solvent concentration and time on the extraction efficiency of tannins from root, stem, leaves, and seeds of *Cichorium intybus* L. was studied. A 2^2-factorial central composite design was employed by using response surface methodology to optimize the extraction conditions. The analysis of variance showed the significant effect of both variables on the extraction yield of tannins. The lower P-values ($P < 0.05$) indicated the positive significant effect of both variables on the extraction of tannins from different parts of *C. intybus*. The values of correlation coefficient (R^2) indicated that more than 96% of the variability in tannins content of *C. intybus* could be explained by response surface model. The 3D surface plots showed a quadratic effect of methanol concentration and linear effect of time on the extraction efficiency of tannins from *C. intybus*. The optimum tannins yield of 0.607, 0.529, 0.26, and 0.431 g/100 g dry weight from root, stem, leaves, and seeds of *C. intybus*, respectively was observed at 60% methanol concentration and extraction time of 80 min. Moreover, the data suggests that *C. intybus* is a rich source of tannins and can be preferably used to fulfil the requirements of tannins in medicinal field.

Key words: *Cichorium intybus* L., chicory, medicinal plants, tannins extraction, central composite design (CCD), response surface methodology (RSM).

INTRODUCTION

Cichorium intybus L. commonly known as chicory, belongs to family Asteracea and is a non native herbaceous plant. The genus *Cichorium* comprises six species mainly distributed in Asia and Europe (Baise and Ravishankar, 2001). *C. intybus* has been found to possess a great medicinal importance because of its marvellous biochemical, phytochemical, and antioxidant composition. All parts of *C. intybus* are used for the treatment of jaundice, because of its antihepatotoxic activity (Abbasi et al., 2009). It can be used to protect against hepatocellular damage, lipid peroxidation, and diabetes. It also possesses antiulcerogenic, anti-inflammatory, diuretic, and anticancer activities (Reddy et al., 1997; Ahmed et al., 2003). Dysmenorrhoea, insomnia, splentitis, obesity, hyperlipidemia, AIDS, and tachycardia are some other clinical conditions which can be treated with the use of *C. intybus* L. (Duke, 1983; Rastogi and Mehrorta, 1994; Afzal et al., 2009). Leaves and roots of this plant also have strong nematicidal and antibacterial activities (Aqil and Ahmad, 2003). The methanolic extract of *C. intybus* has been found to enhance glucose uptake, inhibit adipogenesis, and possesses hepatoprotective effect (Muthusamy et al., 2008; Atta et al., 2010). All parts of chicory contain a number of biochemical compounds such as carbohydrates, lipids, and proteins and some medicinally important compounds such as alkaloids, inulin, sesquiterpene lactones, coumarins, vitamins, chlorophyll pigments, unsaturated sterols, flavonoids, saponins, and tannins (Molan et al., 2003; Muthusamy et al., 2008; Nandagopal and Ranjitha Kumari, 2007; Atta et al., 2010). Tannins are high molecular weight polyphenolic compounds present in plants, foods, and beverages,

*Corresponding author. E-mail: shadaslam@yahoo.com. Tel: 092-061-9210391.

Table 1. Coded and actual levels of independent variables as per chosen by CCD.

Experimental runs	Coded levels of variables		Actual levels of variables	
	x_1	x_2	Solvent concentration (%)	Extraction time (min)
1	-1	1	20	80
2	0	-2	40	20
3	1	-1	60	40
4*	0	0	40	60
5	-1	-1	20	40
6	-2	0	0	60
7	2	0	80	60
8	0	2	40	100
9*	0	0	40	60
10	1	1	60	80
11*	0	0	40	60

Coded level: -2, -1, 0, 1, and 2; x_1 (Solvent Concentration (%)): 0, 20, 40, 60, 80; x_2 (Extraction time (min)): 20, 40, 60, 80, 100. *Center points.

soluble in water and polar organic solvents, and these tannins are classified as hydrolysable and condensed tannins based on their chemical structure and biological activity (Haslam, 1996; Makkar and Becker, 1998). Both types of tannins are capable of forming strong complexes with certain type of proteins depressing the rate of their digestion (Feeny, 1970). Tannins may also bind to bacterial enzymes or form indigestible complexes with cell wall carbohydrates reducing the cell wall digestibility (Barry and Manley, 1984; Barry et al., 1986; Reed et al., 1990). In recent years, tannins have been investigated to possess high antioxidant (Amarowicz et al., 2004), free radical scavenging (Koleckar et al., 2008), antimicrobial (Ho et al., 2001), gastroprotective, and anti-ulcerogenic activities (Ramirez and Roa, 2003). Moreover, tannins have been investigated as potent inhibitors of lipid peroxidation in heart mitochondria (Hong et al., 1995) and possess antifibrotic effects (Chuang et al., 2011). Due to these therapeutic properties tannins can be used in the treatment of various diseases to improve human health.

The extraction yield of tannins from plant material depends on the method of extraction, nature of the extracting solvent, and extraction conditions. It has been investigated that the particle size, temperature, solvent concentration, and time have a significant effect on the extraction of tannins from plant material (Makkar and Becker, 1993; Chavan et al., 2001; Lokeswari et al., 2010; Chew et al., 2011). Various solvents such as water, acetone and methanol are used for the extraction of tannins from plant material. The varying concentrations of tannins have been reported in *C. intybus* (Muthusami et al., 2008). The variation in tannin content of *C. intybus* may be attributed to various factors, including climatic, environmental, and extraction conditions.

In present study, we planned to evaluate the effect of solvent concentration and time on the extraction of tannins from different parts of *C. intybus*. We developed a 2^2-factorial central composite design (CCD) to optimize the solvent concentration and time for extraction of tannins and analyzed the data by regression analysis using response-surface methodology (RSM).

MATERIALS AND METHODS

Roots, leaves and stem of *C. intybus* were collected from the agriculture fields of Bait Hazara, Dera Ghazi Khan Division, Punjab, Pakistan. These parts of *C. intybus* were transported to the laboratory, washed with distilled water and dried under shade. The seeds of *C. intybus* were purchased from local market. All parts of the plant were ground using electric grinder and stored in air tight jars for further analysis.

Experimental design for the extraction of tannins

Response-surface analysis is used to create response-surface models for the prediction of changes in response variables as a function of change in independent variables. It is also used for the determination of optimum levels of the independent variables leading to the desired response goals (Khuri and Cornell, 1987). Being an effective statistical technique for optimization of process conditions, RSM has also been used previously for the optimization of extraction procedures (Quanhong and Caili, 2005; Pierozan et al., 2009; Liu et al., 2009; Li et al., 2010; Sun et al., 2011). A 2^2-factorial CCD was employed to study the effect of extraction parameters (x_1: concentration of extracting solvent and x_2: time) on the extraction of tannin contents from different parts (root, stem, leaves, and seeds) of *C. intybus*. CCD was arranged in such a way that it allowed the development of appropriate empirical equations. Based on the developed CCD, a total of 11 experimental runs were assigned with two independent variables at five levels of each of the solvent concentration (0, 20, 40, 60, and 80% methanol) and extraction time (20, 40, 60, 80, and 100 min), and a value of $\alpha = 2$. The centre point was repeated three times to calculate the repeatability of the method and the effects of unexplained variables in the actual responses were minimised by randomizing the experiments (Table 1). The codes for variables were calculated by the following equation.

$$x_i = (X_i - X_0) / \Delta X_i \quad (1)$$

where x_i is the coded value of an independent variable, X_i is the actual value of an independent, X_0 is the actual value of independent variable at center point, and ΔX_i is the interval between two levels of an independent variable.

The specific codes for the two factors are:

$$x_1 = [\text{Solvent concentration (\%)} - 40] / 20 \quad (2)$$

and

$$x_2 = [\textit{Time (min)} - 60] / 20 \quad (3)$$

Estimation of tannins

Tannins content of methanolic extracts of each part of *C. intybus* was estimated by following the method as described by Fagbemi et al. (2005). The methanolic extract (1 ml) was mixed with Folin-Ciacolate's reagent (0.5 ml), followed by the addition of saturated Na_2CO_3 solution (1 ml) and distilled water (8 ml). The reaction mixture was allowed to stand for 30 min at room temperature. The supernatant was obtained by centrifugation and absorbance was recorded at 725 nm using UV-Visible Spectrophotometer (Janeway, 6405). Tannins content was calculated as mg tannic acid equivalent from a linear regression equation obtained from a calibration curve (R^2 = 0.9944):

$$Abs_{725nm} = 7.061 \times [TA]_{(mg)} \quad (4)$$

where $[TA]_{(mg)}$ is the concentration of tannic acid taken as standard.

Statistical analysis

Response-surface analysis was conducted to study the effect of solvent concentration and time on the extraction of tannins content in different parts of *C. intybus*. Regression analysis and analysis of variance (ANOVA) were performed to analyse the statistical significance of the model terms. Regression coefficients were determined by employing a least-squares technique to predict the quadratic polynomial models for the response variables (Myers and Montgomery, 2002).

The generalized polynomial model equation for prediction of the changes in response variable is given as follows:

$$Yi = \beta_o + \beta_1 x_1 + \beta_2 x_2 + \beta_{11} x_{11} + \beta_{22} x_{22} + \beta_{12} x_1 x_2 \quad (5)$$

where Yi is the predicted response, β_o is a constant, β_1 and β_2 are the regression coefficients for the main variable effects, β_{11} and β_{22} are quadratic effects and β_{12} is the interaction effect of independent variables. The coefficient of determination (R^2) was analysed for the determination of adequacy of the response surface models (Weng et al., 2001). Lack of fit test (F-ratio) at a probability (P) of 0.05 was used for the assessment of the significance of the estimated regression coefficient for each response variable. Lack of fit measures the degree of failure of a model to fit the data in experimental domain, particularly for reduced points in a randomized experiment. An insignificant value of lack of fit indicates the adequacy of the model in describing the response (Montgomery, 2001). Corresponding variables with larger F-values and smaller P-values were considered more significant (Amin and Anggoro, 2004). |Reduced model contained only those terms which were found statistically significant ($P < 0.05$). Construction of experimental design, data analysis, and optimization procedure were performed using the statistical software Design Expert 8.0.4.1 (Stat-Ease, Inc).

Optimization and validation of the procedures

Individual and multiple-optimization procedures were carried out to predict the optimum levels of two independent variables (x_1 and x_2) leading to the overall desired response goals. For graphical optimization, three-dimensional plots were constructed between response and the two independent variables. Adequacy of the response-surface models was verified by comparing the experimental data with the predicted ones by the final reduced models.

RESULTS AND DISCUSSION

Tannins are the natural polyphenolic compounds which can influence the nutritive value of different food stuffs utilized by human and other animals. Tannins also have large influence on the phytochemical and phytotherapeutical value of medicinal plants. Various methods have been used to increase the extraction efficiency of tannins from different medicinal plants for their use in pharmaceutical field (Cobzac et al., 2005). Methanol has been found to be the most commonly used solvent for the extraction of tannins rather than other organic solvents (Harborne, 1973). However, no investigation has been reported regarding the optimization of extraction efficiency of tannins from *C. intybus* which has been traditionally used as medicinal plant. In this study, RSM was used for the optimization of the extraction conditions to increase the yield of tannins from *C. intybus*.

The concentration of tannins (g/100 g dry weight) in different parts of *C. intybus* at random levels of extraction parameters as per chosen by CCD is shown in Table 2. Tannins content in different parts of *C. intybus* were found to be comparatively lower than those reported earlier (Muthusamy et al., 2008). The results indicate that the increase in concentration of extracting solvent as well as in the time of extraction led to an increase in the tannins content in each part of *C. intybus*.

RSM optimization results and validation of the procedure

Optimization process predicted an optimum level for each of the independent variables that resulted in the desirable goals. The non-significant terms were dropped in the initial model and the experimental data were fitted again only to the significant parameters to obtain the final reduced model. However, the non-significant linear terms were included in the final reduced model if the quadratic or interaction terms containing these variables were found to be significant (Mirhosseini and Tan, 2009).

The application of RSM yielded the regression equations showing an empirical relationship between

Table 2. Tannins content (g/100 g dry weight) in different parts of *C. intybus* at random levels of extraction parameters as per chosen by CCD.

Experimental runs	Solvent concentration (%)	Time (min)	Root	Stem	Leaves	Seeds
1	20	80	1.177	1.261	0.634	0.919
2	40	20	0.494	0.431	0.177	0.443
3	60	40	0.754	1.011	0.454	0.853
4	40	60	1.475	1.278	0.642	0.875
5	20	40	0.691	0.958	0.398	0.819
6	0	60	0.899	0.887	0.589	0.233
7	80	60	1.286	1.286	0.737	0.91
8	40	100	1.487	1.328	0.684	0.946
9	40	60	1.475	1.278	0.642	0.875
10	60	80	1.53	1.334	0.766	0.955
11	40	60	1.475	1.278	0.642	0.875

Table 3. Final polynomial regression equation for coded and actual levels of variables.

Part of *C. intybus*	Yi	Tannin concentration (g/100 g dry weight)
Root	$1.38 + 0.099x_1 + 0.27x_2 + 0.072x_1x_2 - 0.090x_1^2 - 0.12x_2^2$	$- 0.59035 + 0.012049x_1 + 0.040882x_2 + 0.00018125\ x_1x_2 - 0.000224572\ x_1^2 - 0.000288322X_2^2$
Stem	$1.28 + 0.077x_1 + 0.20x_2 + 0.005x_1x_2 - 0.048x_1^2 - 0.10x_2^2$	$-0.53830 + 0.012730\ x_1 + 0.039554x_2 + 0.0000125x_1x_2 - 0.000120378x_1^2 - 0.000249753x_2^2$
Leaves	$0.63 + 0.040x_1 + 0.13x_2 + 0.019x_1x_2 + 0.00607x_1^2 - 0.052x_2^2$	$-0.17252 - 0.00204846\ x_1 + 0.020223x_2 + 0.0000475x_1x_2 + 0.000015189x_1^2 - 0.000130123x_2^2$
Seed	$0.88 + 0.10x_1 + 0.042\ x_1^2 - 0.00525\ x_1x_2 - 0.044x_2^2 - 0.005293x_2$	$0.29313 + 0.014709x_1 + 0.00420033x_2 - 0.000013125x_1x_2 - 0.000110419x_1^2 - 0.00001323x_2^2$

tannins content and the extraction variables in coded and actual units. Polynomial equation includes the coefficient for intercept, main effects, interaction terms, and quadratic effects (Table 3). The influence of each factor on the response is shown by the sign and magnitude of the main effect. RSM indicated that the relationship between extraction conditions and the tannins content could be explained by significant second order polynomial regression models.

The linear, quadratic, and interaction effects of the concentration of extracting solvent and time on tannins content of root, stem, leaves, and seeds of *C. intybus* obtained by the analysis of variance (ANOVA) are shown in Tables 4, 5, 6, and 7, respectively. The significance and adequacy of the model were measured in terms of F-value and P-value at 5% significance level ($P < 0.05$). The measurement of F-value and P-values indicated that methanol concentration has positive significant effect on the extraction of tannins from stem, leaves, and seeds of *C. intybus*. The effect of methanol concentration on extraction of tannins from root of *C. intybus* was found to be non-significant. The extraction time was also found to possess a positive significant effect on tannins extraction from root, stem, and leaves of *C. intybus*. The interaction effects were found to be non-significant in each case. The quadratic effects of both independent variables on tannin extraction from root and stem of *C. intybus* were found to be significant. The quadratic effect of time on tannin extraction from leaves and seed was found to be non-significant.

Correlation coefficient (R^2) measures the variability of the model in the observed response values. A value of R^2 closer to unity gives the better prediction of the response and high significance of the model. The values of R^2 for tannins content of stem, leaves, and seeds of *C. intybus* indicated that more than 96% of the variability in the tannins content could be explained by the model. The values of adjusted R^2 for these responses also advocate the significance of the model.

Three dimensional (3D) response surface plots were drawn to show the main and interaction effects of methanol concentration and time on the extract yield of tannins from root, stem, leaves, and seeds of *C. intybus* as presented in Figures 1A, 2A, 3A, and 4A, respectively. The 3D surface plots show that methanol concentration has both quadratic and linear effects on the extraction

Table 4. Analysis of variance (ANOVA) for extraction of tannins from root of *C. intybus*.

Source	Regression coefficients		Sum of squares	df	Mean square	F-value	P-value
	Coded factors	Actual factor					
Model	1.38	-0.59035	1.32	5	0.26	9.79	0.0128
x_1	0.099	0.012049	0.12	1	0.12	4.36	0.0906
x_2	0.27	0.040882	0.88	1	0.88	32.62	0.0023
x_1x_2	0.072	0.00018125	0.021	1	0.021	0.78	0.4175
x_1^2	-0.09	-0.00022457	0.16	1	0.16	5.78	0.0613
x_2^2	-0.12	-0.00028832	0.26	1	0.26	9.53	0.0273
Residuals	-	-	0.13	5	0.027	-	-
Lack of fit	-	-	0.13	3	0.045	-	-
Pure error	-	-	0.000	2	0.000	-	-

Mean = 1.16; SD = 0.16; C.V (%) = 14.17; R^2 = 0.9073; R^2 (adjusted) = 0.8147; R^2 (predicted) = 0.3446; df: Degree of freedom; CV: Coefficient of variation; SD: Standard deviation.

Table 5. Analysis of variance (ANOVA) for extraction of tannins from stem of *C. intybus.*

Source	Regression coefficients		Sum of squares	df	Mean square	F-value	P-value
	Coded factors	Actual factor					
Model	1.28	-0.5383	0.75	5	0.15	30.22	0.001
x_1	0.077	0.01273	0.071	1	0.071	134.26	0.0129
x_2	0.2	0.03955	0.49	1	0.49	97.84	<0.0002
x_1x_2	0.005	0.0000125	0.0001	1	0.0001	0.020	0.8929
X_1^2	-0.048	-0.00012037	0.045	1	0.045	8.97	0.0303
x_2^2	-0.1	-0.00024975	0.19	1	0.19	38.62	0.0016
Residuals	-	-	0.025	5	0.004988	-	-
Lack of fit	-	-	0.025	3	0.008313	-	-
Pure error	-	-	0.000	2	0.000	-	-

Mean = 1.12; SD = 0.071; CV (%) = 6.30; R^2 = 0.9680; R^2 (adjusted) = 0.9359; R^2 (predicted) = 0.6616; df: Degree of freedom; CV: Coefficient of variation; SD: Standard deviation.

Table 6. Analysis of variance (ANOVA) for extraction of tannins from leaves of *C. intybus*.

Source	Regression coefficients		Sum of squares	df	Mean square	F-value	P-value
	Coded factors	Actual factor					
Model	0.63	-0.17252	0.29	5	0.059	96.56	<0.0001
x_1	0.04	-0.00204846	0.02	1	0.02	32.17	0.0024
x_2	0.13	0.020223	0.2	1	0.2	335.1	<0.0001
x_1x_2	0.019	0.0000475	0.001444	1	0.001444	2.38	0.1835
x_1^2	0.006076	0.000015189	0.0007125	1	0.0007125	1.17	0.3280
x_2^2	-0.052	-0.000130123	0.052	1	0.052	86.18	0.0002
Residuals	-	-	0.003034	5	0.0006067	-	-
Lack of fit	-	-	0.003034	3	0.001011	-	-
Pure error	-	-	0.000	2	0.000	-	-

Mean = 0.58; SD = 0.025; CV (%) = 4.26; R^2 = 0.9898; R^2 (adjusted) = 0.9795; R^2 (predicted) = 0.9239; df: Degree of freedom; CV: Coefficient of variation; SD: Standard deviation.

efficiency of tannins from root, stem, and leaves of *C. intybus.* The effect of methanol concentration on extraction of tannins from seeds of *C. intybus* was found to be non-significant. The duration of time has both the quadratic and linear effects on the extraction efficiency of tannins from stem, leaves, and seeds of *C. intybus*, but

Table 7. Analysis of variance (ANOVA) for extraction of tannins from seeds of *C. intybus*.

Source	Regression coefficients		Sum of squares	df	Mean square	F-value	P-value
	Coded factors	Actual factor					
Model	0.88	0.29313	0.19	5	0.037	26.49	0.0013
x_1	0.10	0.014709	0.12	1	0.12	88.31	0.0002
x_2	0.042	0.00420033	0.021	1	0.021	14.87	0.0119
x_1x_2	-0.00525	0.000013125	0.0001103	1	0.0001103	0.078	0.7907
x_1^2	-0.044	-0.000110419	0.038	1	0.038	26.76	0.0035
x_2^2	-0.005293	-0.000013231	0.0005407	1	0.0005407	0.38	0.5624
Residuals	-	-	0.007034	5	0.007034	-	-
Lack of fit	-	-	0.007034	3	0.002345	-	-
Pure error	-	-	0.000	2	0.00264	-	-

Mean = 0.82; SD = 0.038; CV (%) = 4.55; R^2 = 0.9636; R^2 (adjusted) = 0.9273; R^2 (predicted) = 0.6162; df: Degree of freedom; CV: Coefficient of variation; SD: Standard deviation.

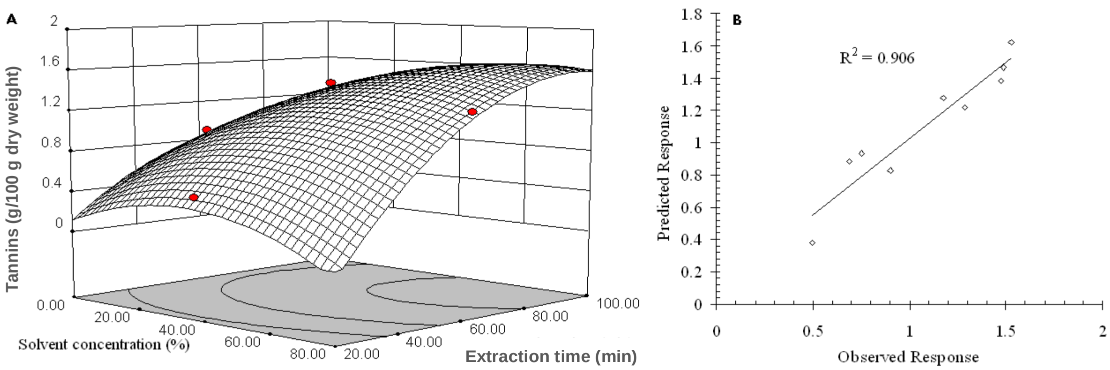

Figure 1. (A) 3D response surface plot and (B) agreement between observed and predicted values of tannins in root of *C. intybus.*

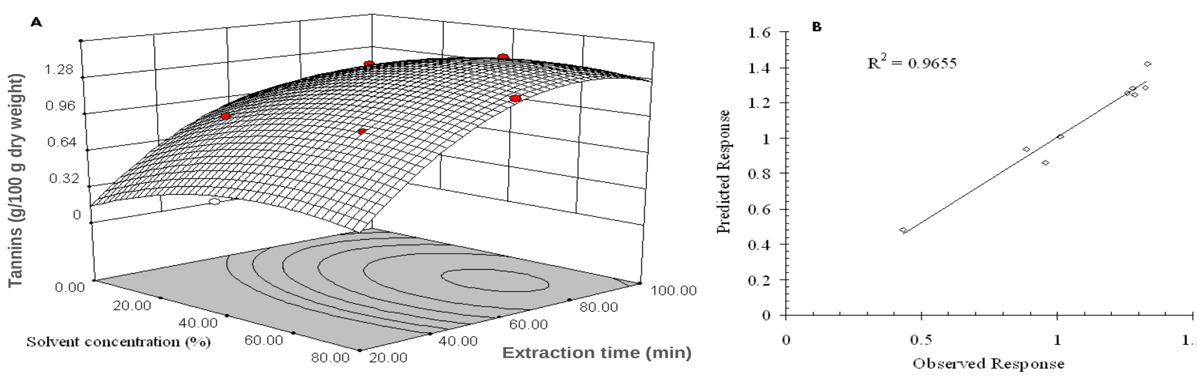

Figure 2. (A) 3D response surface plot and (B) agreement between observed and predicted values of tannins in stem of *C. intybus.*

only quadratic effect of time was observed on the extraction of tannins from roots of *C. intybus.* The optimum yield of tannins was found to be 0.607, 0.529, 0.26, and 0.431 g/100 g dry weight from root, stem, leaves, and seeds of *C. intybus*, respectively at 60% methanol concentration and extraction time of 80 min. To test the applicability of the model, the predicted values of tannins content calculated from the polynomial regression equations were drawn against the observed values of tannins from root, stem, leaves, and seeds of *C. intybus* as shown in Figures 1B, 2B, 3B, and 4B, respectively. A good agreement between the observed and predicted

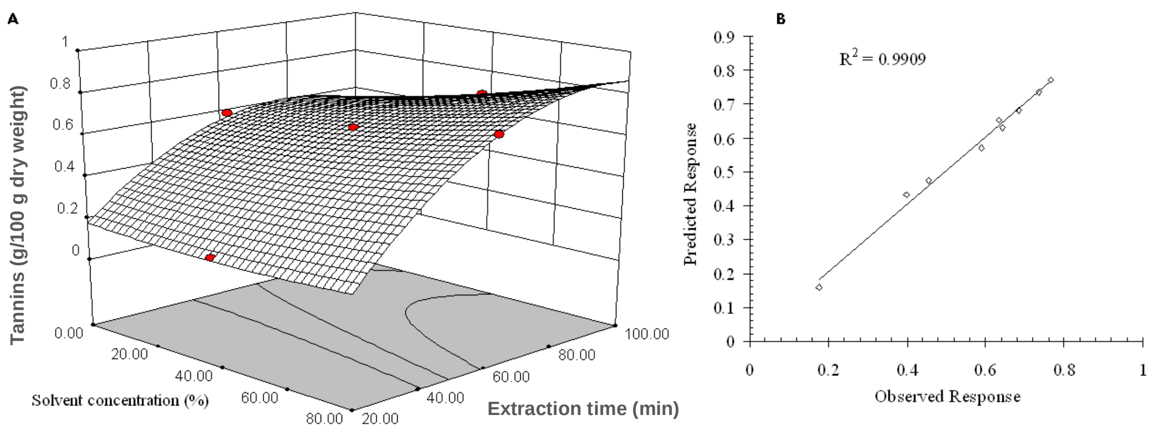

Figure 3. (A) 3D response surface plot and (B) agreement between observed and predicted values of tannins in leaves of *C. intybus.*

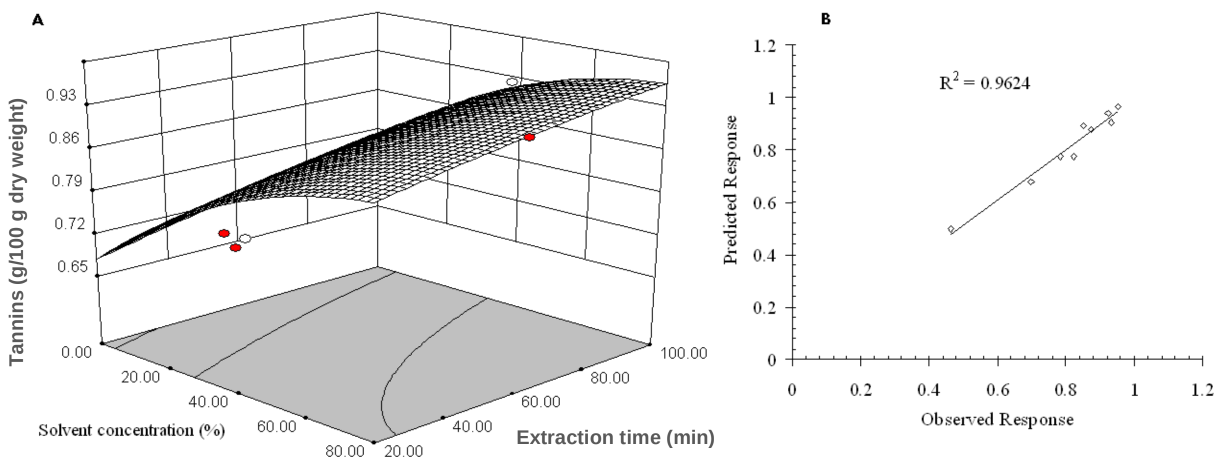

Figure 4. (A) 3D response surface plot and (B) agreement between observed and predicted values of tannins in seeds of *C. intybus.*

values of tannins content was observed with high values of coefficient of determination (R^2) such as 0.906, 0.9655, 0.9909, and 0.9624 for root, stem, leaves, and seeds, respectively. Higher values of R^2 suggest that the applicability of purposed model with greater accuracy for the extraction system of tannins from the plant materials, particularly, *C. intybus*.

Conclusion

The effect of two independent variables: solvent concentration (x_1) and extraction time (x_2) on the extraction efficiency of tannins from various parts of *C. intybus* was optimized by CCD using RSM. The analysis of variance showed the significant effect of both variables (solvent concentration and extraction time) on the yield of tannins. The optimum yield of tannins in methanolic extracts of studied parts of *C. intybus* could be obtained at 60% methanol concentration and an extraction time of 80 min. Moreover, the data suggest that root and leaves of *C. intybus* are rich source of tannins and can be preferably used to fulfil the requirements of tannins in medicinal field.

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