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Optimization of Bioactive Extraction Conditions and Radical Scavenging Activity of *Cassia fistula* Leaves using Response Surface Method

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Abstract: Composite design of three factors, leaves concentration, time and temperature were studied as an independent variables factors influencing in the total phenolics and flavonoids extraction from *Cassia fistula* leaves. Predicting phenolics and flavonoids extraction yield (y) were assumed by cubic polynomial regression model for the independent variables. The optimum conditions produced the highest phenolics and flavonoids yields were 6% leaves concentration at 54°C for 10 min; 6% leaves concentration at 72°C for 7.4 min, respectively. Multiple regressions of three independent variables and their effect on the radical scavenging activity of *Cassia fistula* leaves extracts were also studied. The predicted conditions which lead to the highest radical scavenging activity (97.55%) were 6 % leaves concentration at 66°C for 10 min.

Key words: *Cassia fistula* leaves · Phenolics · Flavonoids · Antiradical activity

INTRODUCTION

Cassia fistula Linn. (Fabaceae) is widely grown in tropical and subtropical areas as an ornamental plant in homesteads and along the roadside due to its beautiful, bright yellow flowers [1]. Leaves fall during cold weather, at the early part of hot season and are quickly replaced by new leaves [2]. As mentioned by Indian literature, *Cassia fistula* plant has been described to be useful against skin diseases, liver troubles, tuberculoses glands and its use into the treatment of hematemesis, leucoderma, pruritus and diabetes [3, 4]. Leaves were found to be effective against cough and ring- worm infections [5, 6]. The leaves extract is indicated for its anti-tussive and wound healing properties [7, 8]. The leaves are also one of the most important ingredients in preparing Ayurvedic medicine [9]. The plant organs are known to be an important source of secondary metabolites notably phenolic compounds; the leaves are laxative, antiperiodic, depurative, anti-inflammatory, carbuncles, ulcers, intermittent fever, gouty arthritis and rheumatalgia [10]. Also, the plant extracts using different solvents were shown as potent antibacterial, antifungal, anti-inflammatory and antioxidant properties [11, 12]. Leave extracts of *Cassia fistula* using by different solvents have shown promising antifungal activity [13, 14, 15]. *Cassia fistula* is an important source of naturally occurring bioactive compounds [16]. Polyphenolics abundantly present in both *in vivo* and *in vitro* extracts may prove to be very important, non-toxic chemo-preventive agents against various oxidative stresses [17]. The aqueous alcoholic extracts of stem bark and leaves showed significant antioxidant activity, which may be accounted for their high phenolic content [18]. The different parts of *C. fistula* contain flavonoids, phenolics compounds and proanthocyanidins [16, 19]. Leaves of *Cassia fistula* contain flavonoids, tannins and anthraquinones as a main phytochemicals [17, 20 and 21]. It is an established fact that polyphenolic compounds, such as flavonoids, anthraquinones, anthocyanidins and xanthones, possess remarkable antioxidant activities which are present quite commonly in the plant family leguminosae [18]. Duh *et al.* [22] mentioned that antioxidant activity of plants might be due to their phenolics compounds. The recent studies have investigated that the antioxidant effect of plant products is mainly attributed to phenolic compounds such as flavonoids, phenolic acids, tannins [23, 24]. Currently there is much interest to protect low density lipoprotein and important cells and organs, as well as food systems, against oxidative damage. There has been an increasing demand to evaluate the antioxidant properties of direct plant extracts or isolated products from plant origin rather than looking for synthetic ones [25]. Response surface methodology is considered to be the best statistical

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approach to assess the influence of different experimental factors and their linear and quadratic interactions on analytical responses and to model and optimize the extraction conditions of phenolics [26].

This study aimed to prepare an aqueous extraction from *Cassia fistula* leaves with a high potential antiradical effect. The three dimension response surface method was used to optimize the extraction conditions. Leaves concentration, temperature and extraction time were the independent variables. At the same time, the total extracted phenolics and flavonoids were dependent variables.

MATERIALS AND METHODS

Cassia fistula leaves were collected from Faculty of Agriculture, Ain Shams University, Cairo, Egypt.

Preparation of Leaf Extracts: Shaded-dried leaves were extracted by water at different concentrations (3, 5, 7 and 9 g/150 ml water) and extraction temperatures (50, 65, 80 and 100°C) for different times (4, 6, 8 and 10 min). The filtered crude extracts were subjected to different antioxidants analysis as follows:

Total Phenolics Content: Leaves extract (0.5 ml) was mixed with 2.5 ml of Folin- Ciocalteu's phenol reagent and kept for 5 min at room temperature (30±4°C). Two ml of Na_2CO_3 (7.5%) was added to the mixture and adjusted to 10 ml with de-ionized-distilled water. The mixture was maintained in dark place at room temperature for 60 min. Absorbance of the mixture was measured at 765 nm against a reagent as a blank using a UV–Vis spectrophotometer (HITACI, ü-1900) [27]. Gallic acid equivalent (GAE) was used as a standard ($y = 0.006x + 0.039$, $R^2 = 0.9971$) and total phenolics content was calculated as mg of GA equivalent per gram of extract on dry basis.

Total Flavonoids Content: $NaNO_2$ solution, 5% (75µl) was added to 1.5 ml of extract and incubated at room temperature (30±4°C) for 6 min. $AlCl_3.6H_2O$ solution, 10% (150µl) was added to the mixture and allowed to stand for 5 min. NaOH, 1mol (0.5ml) and distilled water (2.5ml) were added and the mixture was mixed well then the absorbance was measured at 510 nm by UV–Vis spectrophotometer (HITACI, ü-1900) [28]. Quercetin (QE) was used as a standard ($y= 0.0106 x + 0.0052$, $R^2 = 0.9927$) and total flavonoids content was calculated as mg QE/g dry matter.

Antiradical Activity: *Cassia fistula* leaf extracts were tested for its ability to capture DPPH radicals according to the method of Miliauskas *et al.* [29]. Radical scavenging activity was calculated as percent of inhibition by the difference between absorption of the control and the sample.

Statistical Analysis: The optimum extracted phenolics or flavonoids yield (EPY or EFY) obtained data were analyzed by non-linear regression models. The model proposed for response of EPY or EFY presented as follows:

$$EPY \text{ or } EFY = y_0 + aC + bT + ct + dC^2 + eT^2 + ft^2 + gC^3 + hT^3 + it^3 + JCT + Kct + lTt + mC^2T^2 + nC^2t^2 + oT^2t^2 + pC^3T^3 + qC^3t^3 + rT^3t^3 \quad (1)$$

where y_o, a, b, c, d, e, f, g, h, I, j, k, l, m, n, o, p, q, r and s are intercept, linear, quadratic and cubic regression coefficient terms, respectively. C (leaves concentration), T (temperature) and t (time) are independent variables. Regression analysis using PROC REG procedures was carried out by Statistical Analysis System [30]. Three-dimension (response surface and contour plots) were used as a methods to study the response of EPY and EFY extraction and their radical scavenging ability as dependent variables with leaves concentration, temperature and time as independent variables. The response surface methodology applied using SigmaPlot [31] to locate the optimum conditions.

RESULTS AND DISCUSSION

Effect of Leaves Concentration, Temperature and Time on the Total Phenolics Yield: The polynomial trends of leaves concentration (LC), temperature (T) and extraction time (t) as independent variables versus the extracted phenolics yield (EPY) as dependent variable are presented in Fig. 1. It introduced the predictive values those generated from the quadratic regression on the original data. The concentration of *Cassia* leaves had a significant effect on the extraction yield of phenolics according to the Eq. 1 (Fig. 1A). Extracted phenolics were increased from 109.6 to 188.8 mg/g as gallic acid equivalent with increasing the leaf concentrations from 2 to 6 % (w/v). The high temperature showed a negative effect on extraction yield according to the Eq. 2 (Fig. 1B). As the output data refers, increasing the temperature of water lead to destroy the phenolic compounds.

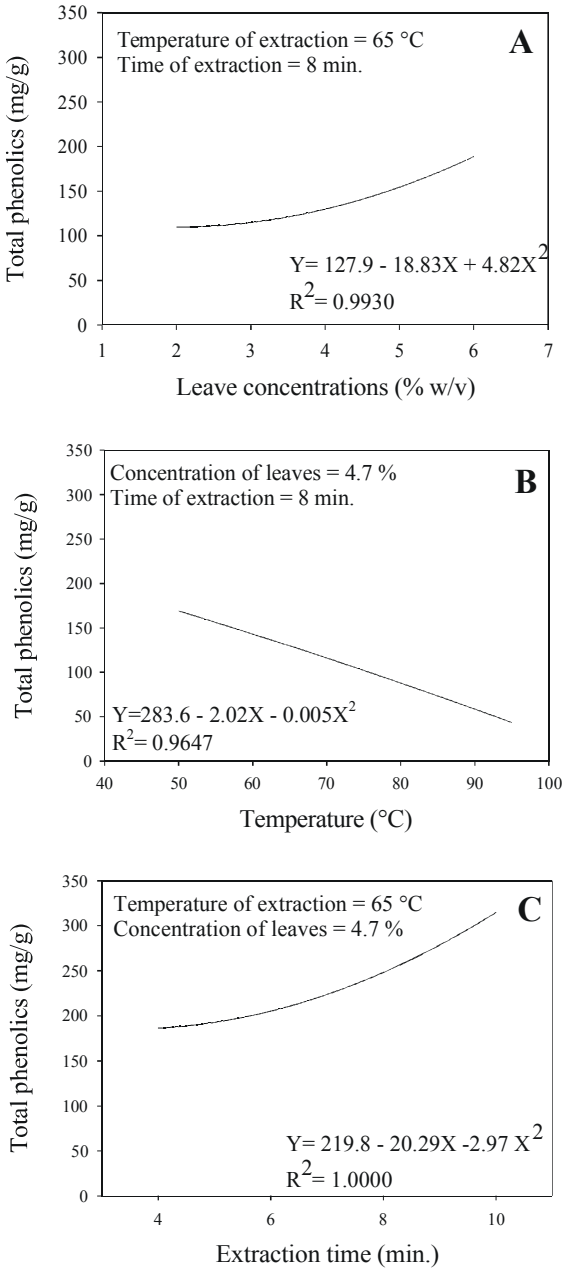

Fig. 1: Polynomial quadratic trend of different leaves concentrations (A), temperatures (B) and extraction times (C) on the extraction yield of total phenolics (mg/g)

The extracted phenolics compound was 169.3 mg/g at 50°C. It could be noticed, the extraction efficiency decreased by 74.3% with increasing the extraction temperature till 90°C (43.4 mg/g). The heat treatment may simultaneously degrade phenolic compounds as mentioned by Lim and Murtijaya [32]. The temperature affected in the stability of phenolic compounds in herbal infusions [33]. Extracted phenolics gradually increased from 186.3 to 214.8 mg/g at the extraction times of 4 and 10 min, respectively according to the Eq. 3 (Fig. 1C). El Hajj *et al*. [34] noticed an increasing in the extraction time lead to increasing in the extraction of total phenolic compounds.

Effect of Leaves Concentration, Temperature and Time on the Total Flavonoids Yield: The effect of leaves concentration, temperature and extraction time on the total flavonoids yield (mg/g) are presented as a polynomial trend of different variables in Fig. 2. The leaves concentration had a significant effect on the yield of extracted flavonoids according to the Eq. 4 (Fig. 2A). Extracted flavonoids were increased from 52.2 to 104.2 mg/g with increasing the leaves concentration from 2 to 6 % (w/v), with increasing percentage of 49.9 %. Sathishkumar *et al*. [35] found that the content of extracted flavonoids were achieved the maximum value at 16.6 % leaves in solvent. The effect of temperature on flavonoids extraction yield is shown in Fig. 2B. Extracted flavonoids were gradually increased from 4.0 to 108.3 mg/g with increasing the temperature from 50 to 74.7°C, respectively according to the Eq. 5 at Fig. 2B. Then, the yield was dramatically decreased to the minimal value (39.5 mg/g) with continuously increasing the temperature till 95°C. The highest extraction yield of raw flavonoids was observed at temperature ranged between 50 to 80°C. The flavonoids could be oxidized at temperature of surpassing 80°C so that the extracted flavonoids decreased gradually [36]. The effect of time on flavonoids extraction yield according to Eq.6 is shown in Fig. 2C. The highest predicted flavonoids yield was 98.6 mg/g at 4.3 min then it was dramatically decreased to 24.5 mg/g up to 10 min. Thereafter, increased the extraction time showed a negative effect on the extraction yield. This decrease may be due to the effect of long extracted periods on the phenolic compounds which threatened by oxidation or degradation. The duration time of extraction had a significant effect on the obtained polyphenols yield [37].

Independent Variables Interaction Effect on the Extracted Phenolics Yield: According to the previous polynomial quadratic trend, the relationships between those different variables were not clear. Therefore, three-dimension response surface cubic plot between each two independent variables and the extracted phenolics yield was established. The predictive output data are presented

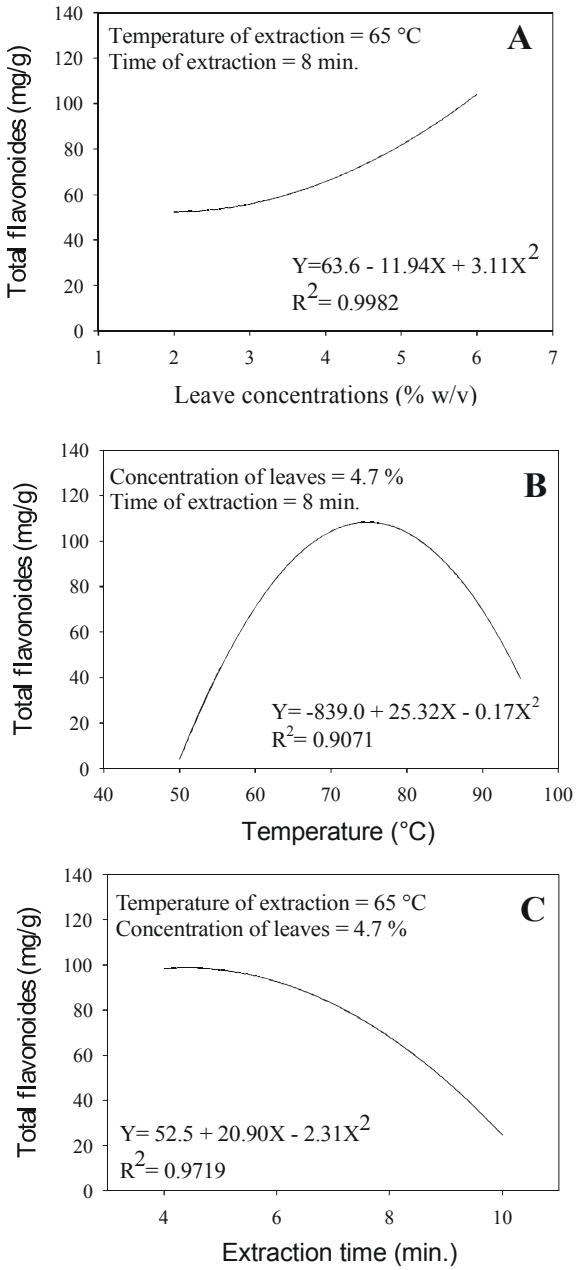

Fig. 2: Polynomial quadratic trend of different leave concentrations (A), temperatures (B) and extraction times (C) on the extraction yield of total flavonoids (mg/g).

in Fig. 3. It explains the relationships between the extracted phenolics yield as dependent variable and leaves o f concentration, temperature and extraction time as independent variables. Phenolics yield was slightly increased with increasing the leaves concentration from 2 to 6% under the same extraction temperature. On contrary, the yield was decreased with increasing the extraction temperature from 50 to 95°C as in Fig. 3A. The significantly ($p<0.05$) relationships was observed between both of leaf concentrations and temperatures as independent variables and extracted phenolics yield as response variable. The predicted model (Eq. 7) had a high correlation coefficient (R^2=0.9548). From output data, it could be noticed that the best predicted extracted phenolics yield for that equation was 208.6 mg/g at 6 % leaves concentration and 55.6°C.

$$EPY = -1719.2 + 35.0LC + 84.0T - 9.85LC^2 - 1.24T^2 + 1.13LC^3 + 0.0057T^3 + 7.63LC^2T^2 \quad (2)$$

Fig. 3B shows the effect of leaves concentration and extraction time at 65 °C on extracted phenolics yield. Predicted model (Eq. 8) cleared the effect of leaves concentration and extraction time, on EPY as follows:

$$EPY = -1126.2 + 78.1LC + 540.0T - 4.68LC^2 - 84.36T^2 - 0.494LC^3 + 4.10T^3 + 0.052LC^2T^2 \quad (3)$$

El Hajj *et al*. [34] and Rajha *et al.* [38] showed a negative effect of time on phenolics content yield which decreased after 97 hours and 75 min., respectively. In contrary, the obtained data appeared a positive effect of time on the EPY. It may be due to that the time was not enough to start the degradation kinetics. The increase of extraction temperature was reported to reduce times of extraction of total phenolics [39]. The time had a quadratic linear effect on the EPY. On the other hand, the leaves concentration had a negative effect on the EPY. According to the predicted equation increased the leaves concentration more than 5.75% lead to a decrease the EPY. This decrease might be due to the fact that when the material ratio reached a certain level, the extract has well dissolved in the solution that may lead the contents of the extract become saturated and prevent further increase [36]. The highest EPY was 317.7 mg/g at 5.75 % leaves concentration after 10 min with R^2= 0.8619. The response surface of extracted phenolics yield that affected by temperature of extraction and extraction time at 4.7 % leaves concentration is presented in Fig. 3C. The extracted phenolics yield was increased with increasing both of temperature (up to 58.43°C) and time of extraction. The temperature had not linear effect on EPY since it decreased with increasing the temperature more than 58.43°C. The temperature limit differed from study to other. It was 50°C according to Cacace and Mazza [39] or

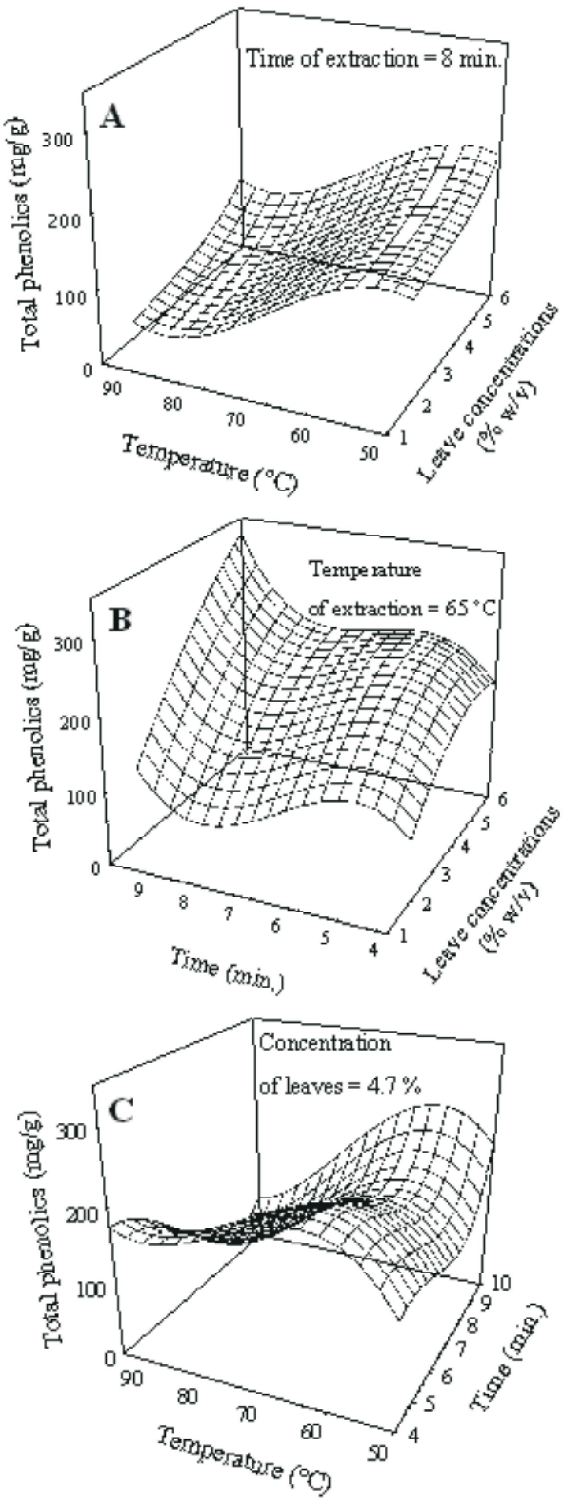

Fig. 3: Three dimension response surface plot of total phenolics yield at different leaf concentrations, temperatures and extraction times as independent variables

60°C as mentioned by Spigno *et al*. [40]. Regarding the duration of the extraction process, short [41, 42, 43] and long extraction periods can be found in the literatures [42, 44]. Many authors mentioned to the enhancing capacity of the temperature parameter on the extraction efficiency of phenolic compounds [38, 40]. It enhances the mass transfer, improves the solubilization of the solutes in the solvent and reduces the surface tension and viscosity [45]. Nevertheless and beyond a certain value the denaturation of the phenolic compounds can occur. Output data of response surface study showed significant ($p<0.05$) relationships between both of temperature and extraction time as independent variables and EPY as response variable. The predictive equation (Eq. 9) shows the effect of extraction temperature (T) and extraction time (t) on extracted phenolics yield.

$$EPY = -3515.0 + 274.3LC + 131.9T - 46.5LC^2 + 1.75T^2 + 2.56LC^3 + 0.007T^3 - 0.0003LC^2T^2 \quad (4)$$

The highest observed predictive EPY was 257.6 mg/g at 58.43°C and 10 min with R2= 0.7150. Bassani *et al*. [26] studied the function of time and temperature on the extracted phenolics yield from yerba mate tea leaves. The results showed that polynomial equations were significant with 10 min and 90°C as an optimum extraction conditions.

Independent Variables Interaction Effect on the Extracted Flavonoids Yield: Three dimension Gaussian model used to study the effect of leaves concentration, temperature and time on the extracted flavonoids yield are presented in Fig. 4. Response surface study could use to explaining the interaction effect between two independent variables and their effects on the dependent variable. The effect of interaction between leaves concentration and temperature on extracted flavonoids yield at 8 min is presented in Fig. 4A. Flavonoids yield was affected by each of the leaves concentration and temperature. Temperature effect on extraction was dual. On one hand, higher temperature can accelerate the solvent flow and thus increase the flavonoids content and on the other hand, higher temperature can decrease the fluid density that may reduce the extraction efficiency [46]. The predicted values of studied variables gave the highest flavonoids yield (160.4 mg/g) at 6% leaves concentration (w/v) and 75.3°C. The predicted model (Eq. 10) reflects the relation between the variables with $R^2 = 0.9267$.

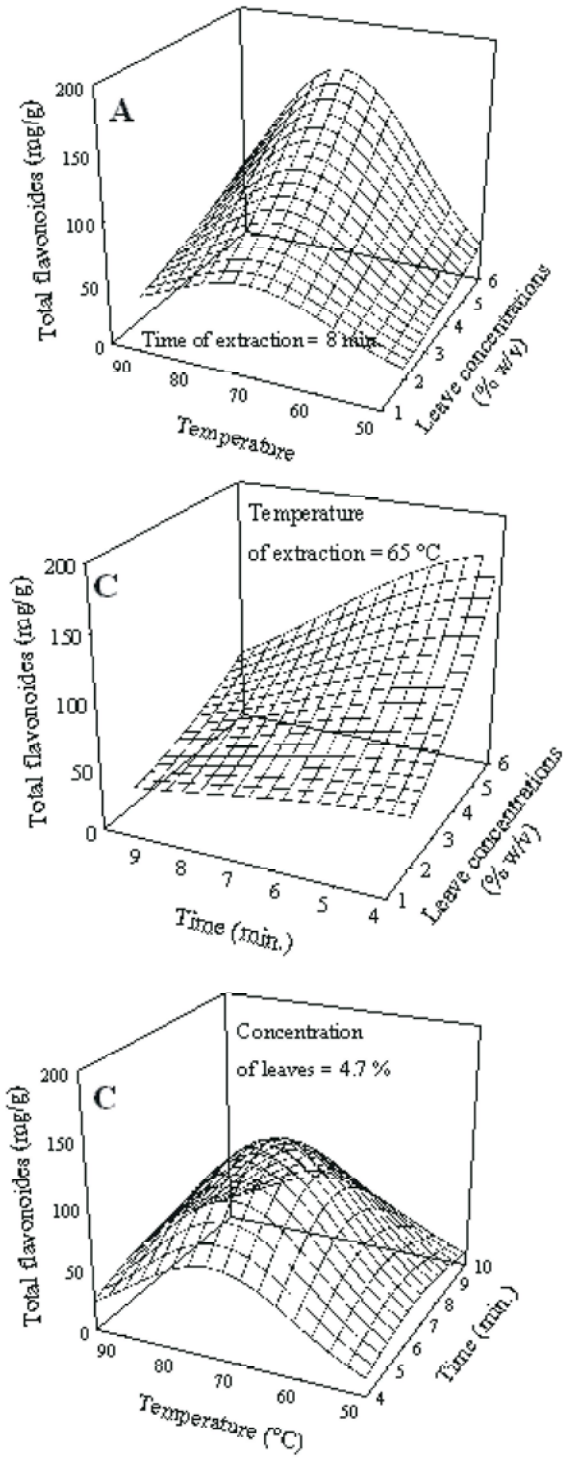

Fig. 4: Three dimension response surface plot of total flavonoids yield at different leaf concentrations, temperatures and times as independent variables.

$$EPY = 304.0e^{-0.5\left[\left(\frac{LC-11.6}{5.04}\right)^2+\left(\frac{T-75.7}{14.0}\right)^2\right]} \quad (5)$$

Each leaves concentration and extraction time had a significant (P<0.05) effect on the extracted flavonoids yield at 65°C (Fig. 4B). Flavonoids yield was increased with increasing the leave concentrations from 2 to 6% (w/v). In contrary, increased the extraction time had a negative effect on the extracted yield. The Gaussian predicted equation of different independent variables was presented in (Eq. 11) with $R^2 = 0.9267$.

$$EPY = 2922.1e^{-0.5\left[\left(\frac{LC-23.2}{7.2}\right)^2+\left(\frac{T-3.4}{4.3}\right)^2\right]} \quad (6)$$

According to the Eq. 11, the highest predictive flavonoid yield was 170.4 mg/g at 6% (w/v) leaves concentration for 4 min. Three dimension regression of the flavonoids yield at different temperatures and extraction times are presented in Fig. 4C. Upon the basis of noticeable changes in flavonoids extraction yield which affected by temperatures and extraction times at 4.7% (w/v) concentration, a higher predictive flavonoids yield (128.5 mg/g) was observed at 75.3°C for 6.6 min. Bassani *et al*. [26] reported the quadratic effects of the interaction between extraction time and temperature were significant (p<0.05). The model which linked between temperatures and extraction times is shown in equation 12.

$$EPY = 128.9e^{-0.5\left[\left(\frac{LC-6.7}{2.5}\right)^2+\left(\frac{T-74.5}{13.4}\right)^2\right]} \quad (7)$$

Multiple Regression of Independent Variables to Phenolic and Flavonoid Compound Yields: Multiple regression coefficients are presented in Table 1 to predict cubic models for extracted phenolics and flavonoids yield. The models were tested for adequacy by analysis of variance. The regression models for extracted phenolics and flavonoids yield data were highly significant (P<0.01) with $R^2 = 0.7219$ and 0.6412, respectively. Consequently, the obtained predicted models are recommended for identify the optimum conditions which required to produce the highest yield of extracted phenolics or flavonoids. It could be concluded that, the extraction of 6% (w/v) leaves concentration at 54°C for 10 min, give the highest predictive phenolics yield. Whereas, the highest predictive flavonoids yield can be obtained with the same leaves concentration at 72°C for 7.4 min.

Radical Scavenging Activity: The contour plot in Fig. 5 shows the response surface of antiradical activity as observed in the presence of different extracts stable

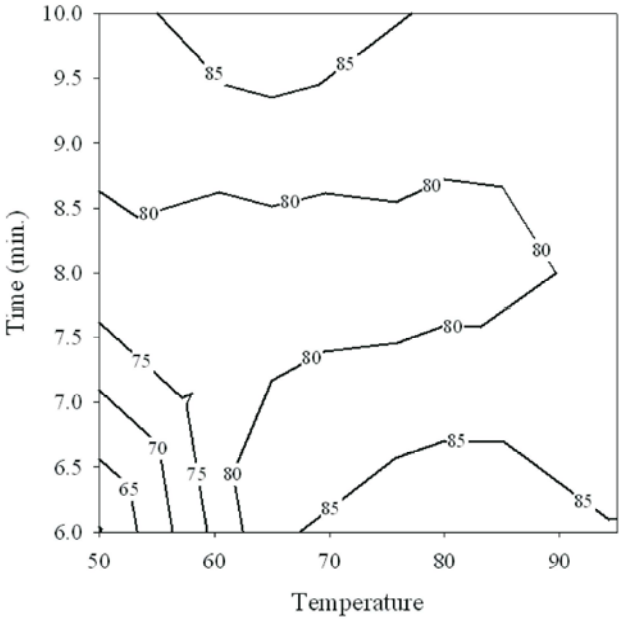

Fig. 5: Contour plot of antioxidant activity (%) of cassia leaf extracts at different temperature and times and concentration 6%

Table 1: Regression coefficients of predicted cubic model for response of the extracted phenolics and flavonoids yield

Extracted phenolics yield model		Extracted flavonoids yield model	
Variables	Parameter estimate	Variables	Parameter estimate
Linear	--	Linear	--
Intercept	-2655.7	Intercept	1711.65
C	-355.07	C	5.7740
T	66.778	T	-37.909
T	750.22	t	-529.2001
Quadratic	--	Quadratic	--
C^2	36.411	C^2	-11.427
T^2	-0.6465	T^2	0.31523
t^2	-74.392	t^2	43.5984
Cubic	--	Cubic	--
C^3	-1.6360	C^3	1.57418
T^3	0.0022	T^3	-0.00136
t^3	3.0541	t^3	-1.56563
Interaction	--	Interaction	--
CT	6.1175	CT	-0.24395
Ct	11.140	Ct	9.76259
Tt	-7.6065	Tt	6.53759
C^2T^2	-0.0094	C^2T^2	0.00123
C^2t^2	-0.1516	C^2t^2	-0.18884
T^2t^2	0.0072	T^2t^2	-0.00654
C^3T^3	0.0000068	C^3T^3	-0.00000152
C^3t^3	0.0017	C^3t^3	0.00136
T^3t^3	-0.0000028	T^3t^3	0.00000268
CTt	-0.1086	CTt	0.00396
R^2	0.7219	R^2	0.6412
Probability value	0.0001	Probability value	0.0001

R^2 = correlation coefficient

Table 2: Regression coefficients of predicted cubic model for response of the antioxidant activity

Linear		Quadratic		Cubic		Interaction	
Intercept	-859.51	C^2	-3.3400	C^3	0.15554	CT	0.0465
C	11.968	T^2	0.4539	T^3	0.00193	Ct	0.9705
T	35.396	t^2	5.6484	t^3	0.21734	Tt	-0.9474
T	58.322	--	--	--	--	C^2T^2	0.00052
--	--	--	--	--	--	C^2t^2	0.0174
--	--	--	--	--	--	T^2t^2	0.0013
--	--	--	--	--	--	C^3T^3	-3.68×10^{-7}
--	--	--	--	--	--	C^3t^3	-0.000097
--	--	--	--	--	--	T^3t^3	-6.48×10^{-7}
--	--	--	--	--	--	CTt	-0.0285

DPPH• radical at 6% leaves concentration, different extraction temperatures (50, 65, 80 and 95°C) and extraction times (6, 8 and 10 min.). From the obtained data, 6% leaves concentration gave the highest phenolics and flavonoids yield which be expected to give the highest antiradical values. This expectation is in accordance with the results of Irshad *et al.* [47], who referred the radical scavenging effects of *C. fistula* pulp and seed extracts were directly proportional to the phenolic content present in extracts. Similar results were obtained by Sahu and Saxena [48] with the extract of *C. longa* which contained the highest amount of phenolic and flavonoid compounds and exhibited the highest antiradical activity. The antiradical activity was increased with increasing the temperature and extraction time. On contrary, at temperature more than 65°C the antiradical activity was decreased. The strongest antiradical activity effect was observed at optimum conditions (65°C for 9.3 min) with 85% antiradical activity. The same high antiradical activity was observed at high extraction temperature degrees (from 68 up to 90°C) for short extraction times (from 6 to 6.6 min). This behavior of antiradical activity of extracts may be due to the inverse relation between the concentration of total phynolics content and flavonoids content in different extracts with different extraction temperatures and times. Whereas, total phynolics content concentration increases with low extraction temperature and long extraction time, the flavonoid content concentration increases with the adverse conditions. Raupp *et al.* [49] found that the antioxidant capacity increased significantly in the Cafona fruits as a consequence of drying temperature from 55 to 75°C as polyphenol contents increased.

According to the multiple regression of leaves concentration, temperature and extraction time versus the antiradical activity, the output data are presented in Table 2. The obtained model had a high significant ($P<0.01$) with $R^2 = 0.6870$. The predicted model can be used to identify the optimum conditions which required obtaining the highest antiradical effect. The highest predicted antiradical activity was 97.55%. The predicted conditions which can be used to prepare the extract of cassia leaves with the highest antiradical potential effect were 6 % leaves concentration at 66°C for 10 min. Bassani *et al.* [26] referred to the total flavonoids content correlated closely to the antioxidant capacity, corroborating the fact that this phenolic class is responsible for the beneficial health effects of yerba-mate tea consumption. The best combination of extraction time and temperature were found to be 10 minutes at 90°C, which rendered a mean phenolic content of 427.74 mg GAE/L and 80.02% of inhibition of the DPPH radical. They also demonstrated that the quality of the extracts was not affected by the heightened temperature if short periods of time were adopted.

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