

Evaluation of the antioxidant activity of *Coreopsis tinctoria* Nuff. and optimisation of isolation by response surface methodology

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The aim of the present study was to determine the antioxidant activity of *Coreopsis tinctoria* flowering tops (CTFT). Studies were conducted to obtain suitable extraction conditions for chlorogenic acid, quercetin, luteolin, apigenin and kaempferol, which were identified and quantified by HPLC. Response surface methodology was employed to optimise the ultrasound-assisted extraction conditions including extraction time, ethanol concentration and liquid-solid ratio. The antioxidant activity of the extracts was analysed using various antioxidant models, such as DPPH, ABTS and hydroxyl radical scavenging assay. CTFT extracted for 15.0 min with ethanol at a concentration of 60.4 % and with liquid-solid ratio 27.5:1 possessed a considerable amounts of total flavonoids and polyphenols (18.9 %). This extract showed higher scavenging activity of ABTS and hydroxyl radical activity than rutin, however not in the DPPH test. We may assume that CTFT possess antioxidant and free radical scavenging potentials.

Keywords: *Coreopsis tinctoria* (Asteraceae), flavonoids, polyphenols, antioxidant activity, response surface methodology, HPLC

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Coreopsis tinctoria Nutt. is a plant native to North America but spread worldwide. North American Indians have used it to treat several disorders. They traditionally used root tea against diarrhea and as an emetic and dried tops for blood, internal pains and bleeding (1). In Portugal, *C. tinctoria* flowering tops infusion has been traditionally used to reduce hyperglycaemia in diabetic patients. Its use is also documented in traditional Chinese medicine where a decoction of the whole plant is used for diarrhoea (2) and diabetes (3). The aim of this study was to elucidate the chemical composition of *Coreopsis tinctoria* flowering tops by HPLC after optimisation of ultrasound-assisted extraction by response surface methodology (RSM). In addition, evaluation of the antioxidant capacity of ethanol extracts by 1,1-diphenyl-2-picrylhydrazyl (DPPH), 2,2-azino-bis (3-ethyl-benzthiazoline-6-sulphonic acid) diammonium salt (ABTS) and hydroxyl radical assay were undertaken.

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EXPERIMENTAL

Plant materials and apparatus

Coreopsis tinctoria Nutt. (*Asteraceae*) flowering tops were acquired from Guangzhou crude medicine companies and identified at the College of Life Science and Biopharmacology, Guangdong Pharmaceutical University, Guangdong (China). To avoid degradation, the air-dried plant material was ground just before extraction.

Chemicals and reagents

DPPH, ABTS and crocus (used in the hydroxyl radical scavenging assay) were obtained from Sigma-Aldrich, Germany. Ascorbic acid was purchased from Sigma Chemical Co. (USA). Water was purified using a Milli-Q water purification system (Milipore, USA). Standards of chlorogenic acid, quercetin, luteolin, apigenin, kaempferol and rutin were purchased from the National Institute for the Control of Pharmaceutical and Biological Products (China). All other chemicals and solvents used for extraction were analytical grade and were obtained from Tianjin Reagent Company (China).

Extraction of Coreopsis tinctoria flowering tops

Coreopsis tinctoria flowering tops were crushed in a 0.7 mm screen. To dried powder (5.0 g), the solvent was added (ethanol of 40, 60 or 80 %) in a defined liquid-solid ratio (20:1, 30:1, 40:1) and extracted with in ultrasonic bath for defined time intervals (15, 30 and 45 min) at 50 kHz. The filtrate was collected and the residue was extracted two times according to the protocol given above. The extract was then concentrated to no ethanol by rotary vaporisation at 60 °C under reduced pressure and residue was constant to a certain volume by methanol. Then, the filtered solution was stored at 4 °C for further analysis.

HPLC analysis

Concentrations of the main flavonoid and polyphenol compounds were determined by calculating the area under the HPLC peak and presented as the mean of two measurements. The extracts were quantified using an Agilent 1100 HPLC system equipped with ChemStation software (Agilent Technologies, USA), a quaternary pump, an online vacuum degasser, an autosampler, a thermostated column compartment and a diode array detector. Separation was achieved on a Hypersil ODS C18 column (4.6 mm × 250 mm, 5 µm) from Dalian Elite Analytical Instruments Co. Ltd. (China). Column temperature was set at 25 °C and the detection wavelength was 360 nm. The mobile phase consisted of methanol and 0.15 % phosphoric acid solution (45:55, V/V). Flow rate was 1.0 mL min⁻¹. The injection volume was 10 µL. All the main flavonoid and polyphenolic compounds were identified by matching their retention times against those of standard compounds.

Box-Behnken design for response surface methodology

To obtain suitable extraction conditions for *Coreopsis tinctoria*, Box-Behnken designs (BBDs, Design Expert software, Trial Version 8.0.4, Stat-Ease Inc., Minneapolis, MN, USA)

were applied to the experimental design, data analysis and model building. Box-Behnken designs for multivariate optimization are a class of rotatable or nearly rotatable second-order designs based on the three-level incomplete factorial design (4). R^2 (adjusted determination coefficient) is the measure for determining the goodness-of-fit of the regression equation (5).

Based on the preliminary tests, a total of 17 runs from BBDs were employed to optimise the main extraction conditions, including the extraction time (X_1), ethanol concentration (X_2) and liquid-solid ratio (X_3), as shown in Table I.

Table I. Levels of extraction in designed experiments

Parameter/level	Extraction time (min, X_1)	Ethanol concentration (% , X_2)	Liquid-to-solid ratio (X_3)
-1	15	40	20
0	30	60	30
1	45	80	40

Antioxidant activity

Scavenging capacity on DPPH radical. – Free radical scavenging activity of each extract and rutin was determined based on the extract's ability to react with the stable DPPH free radical. In brief, 2.5 mL of the extract was added to 2.5 mL of DPPH (250 $\mu\text{g mL}^{-1}$, dissolved in 95 % ethanol). The mixture was vigorously shaken and maintained at room temperature for 30 min in the dark. Absorbance was measured using a spectrophotometer (Lambda 35 UV-visible, Perkin Elmer, USA) at 517 nm against a blank.

ABTS radical scavenging assay. – The antioxidant activity was determined using ABTS radical cation measurements (6–8), with some modifications. ABTS radical cations were produced by reacting 7 mmol L^{-1} ABTS solution with 2.45 mmol L^{-1} potassium persulfate. The mixture was stored in the dark, at room temperature, for 16 h. Upon use, the ABTS solution was diluted with ethanol to an absorbance value of 0.70 ± 0.02 at 734 nm. 0.3 mL of each extract sample of varying concentration was added to 3.0 mL of ABTS solution and mixed vigorously. The reaction mixture was allowed to stand at room temperature for 6 min and the absorbance at 734 nm was immediately measured. The percentual ABTS scavenging effect was calculated.

Hydroxyl radical scavenging assay. – The hydroxyl radical scavenging activity of each extract was measured according to the method described by Wang *et al.* (9–11) with some modifications. Extract samples were incubated with 2.0 mmol L^{-1} EDTA-Fe (0.5 mL), 3 % H_2O_2 (1.0 mL) and 360 $\mu\text{g mL}^{-1}$ crocus in 4.5 mL sodium phosphate buffer (150.0 mmol L^{-1} , pH 7.4) for 30 min at 37 °C. Hydroxyl radical was detected by monitoring absorbance at 520 nm. The percentual hydroxyl radical scavenging effect was calculated. In the control, the sample was substituted by distilled water and sodium phosphate buffer was replaced with H_2O_2 .

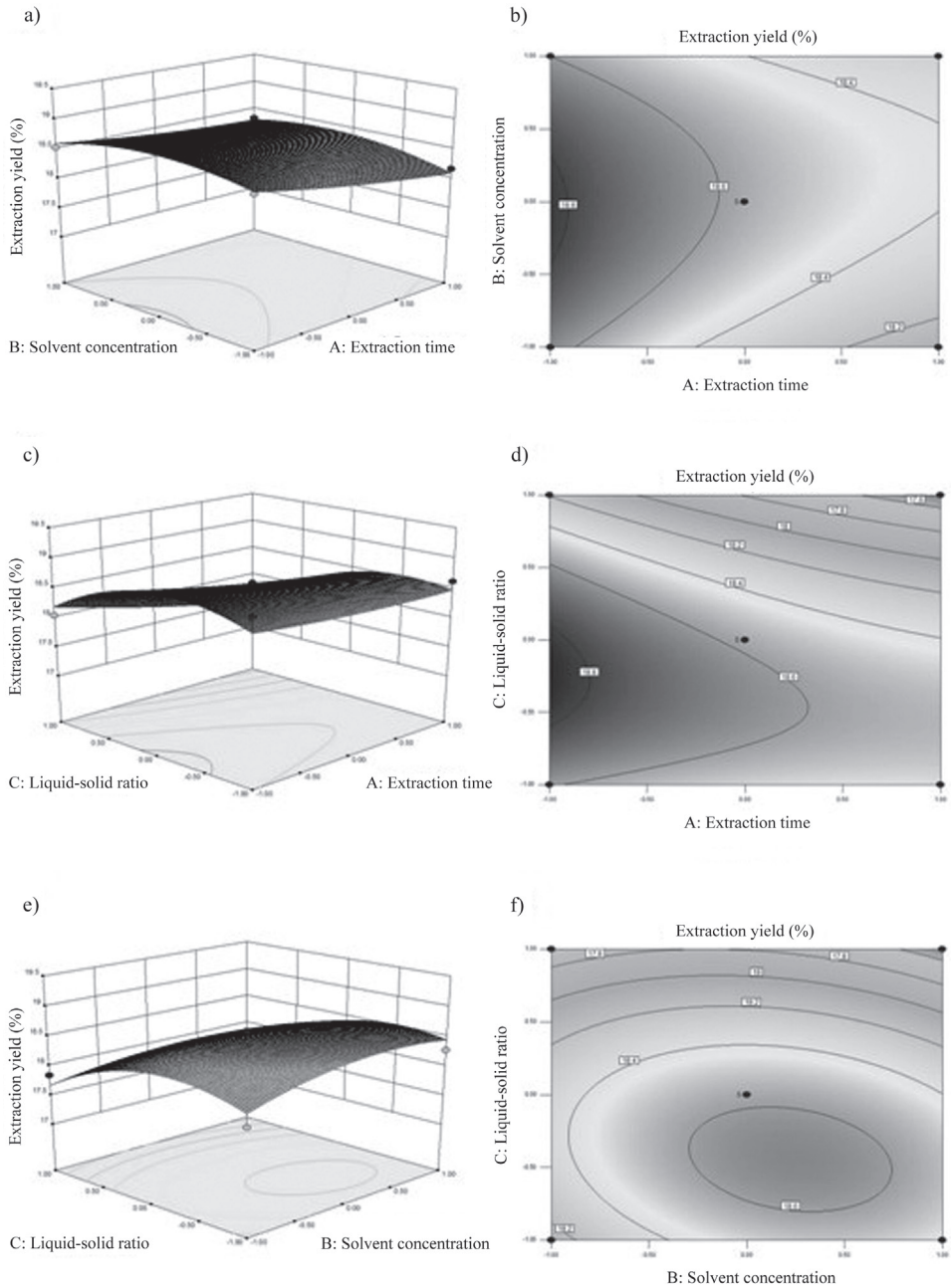


Fig. 1. Response surface plots and contour plots of extraction time (minutes, min), ethanol concentration (% *V/V*) and solid-to-liquid ratio showing their effect on extraction yield.

Statistical analysis

All experimental data (in tables and figures) represent mean values \pm standard deviation ($n = 3$). Statistical significance was set at a value of 0.05. The response obtained from each set within experimental design was subjected to multiple non-linear regressions using the Design Expert software, Trial Version 8.0.4 (Stat-Ease, Minneapolis, USA). The quality of the fit of the polynomial model equation was checked using the *F*-test and *p*-value.

RESULTS AND DISCUSSION

Optimization of response surface methodology (RSM)

The results indicated that the RSM model used to fit the response variable was significant ($p = 0.0135$) and adequate to represent the relationship between the response and the independent variables (12, 13). The model demonstrated a very high *F*-value ($F = 6.0442$). The R^2 value of the RSM model was 0.986, which indicates that only 1.40 % of the total variations were not covered by the model. Meanwhile, relative standard deviation ($RSD = 0.8\%$) demonstrated good precision of the experiments performed (14).

Table II shows that the extraction yield was mostly affected by the liquid-solid ratio (X_3 , $p = 0.0023$), followed by extraction time (X_1 , $p = 0.0275$). Ethanol concentration (X_2) was not found to affect the extraction yield significantly. It was evident that X_3^2 was significant at the level of $p < 0.01$. The predicted response *Y* for the extraction yield could be expressed by the following second-order polynomial equation:

$$Y = 18.57 - 0.21X_1 + 0.037X_2 - 0.35X_3 + 0.060X_1X_2 - 0.14X_1X_3 - 0.12X_2X_3 + 0.047X_1^2 - 0.02X_2^2 - 0.43X_3^2$$

Regression equation was graphically represented by the 3D response surface and 2D contour plots (15). From the 3D response surface curves and contour plots shown in Fig. 2, the effect of the independent variables and their mutual interaction on the extraction yield can be seen. Figs. 2a and b represent the response surface and contour plots showing the effect of the extraction time and ethanol concentration at a fixed liquid-to-solid ratio. As shown in the figure, the extraction yield decreased by increasing the extraction time. Any decrease in the extraction time had only a slight effect on extraction yield. Figs. 2c and d depict the interaction of the extraction time and the liquid-to-solid ratio on the extraction yield at fixed ethanol concentration. By increasing the liquid-solid ratio, the yield reached a maximum, and beyond this point, no increase in the liquid-to-solid ratio improved the yield. Figs. 2e and f describe the effect of the ethanol concentration and liquid-to-solid ratio on the yield at a fixed extraction time. By increasing the ethanol concentration, the yield reached a maximum, and beyond this point, no increase in the ethanol concentration improved the yield.

One aim of this study was to identify the conditions that produced the maximum extraction yield in *Coreopsis tinctoria*. According to the results of designed experiments, the software predicted the optimum extraction time, ethanol concentration and liquid-to-solid ratio to be 15.0 min, 60.4 % and 27.5:1, respectively. Under these conditions, the extraction yield of all five main flavonoid and polyphenol compounds (chlorogenic acid, quercetin,

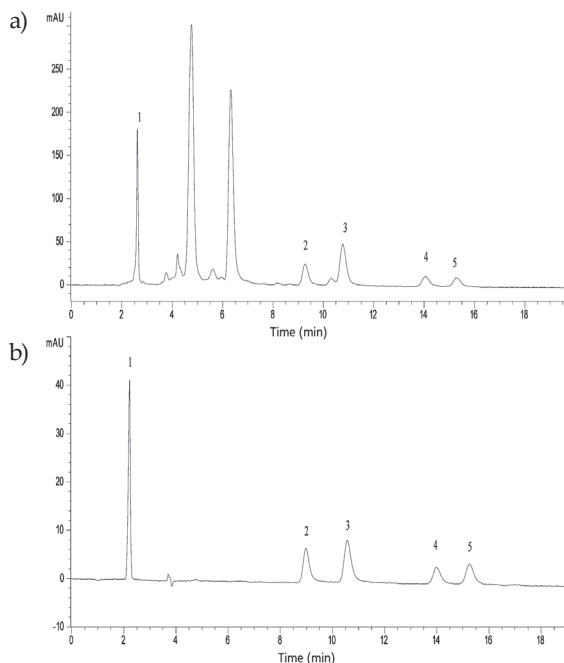


Fig. 2. HPLC chromatogram at 360 nm for: a) standard solution of five flavonoids and polyphenols, b) *Coreopsis tinctoria* flowering top extract. Keys to the peaks: 1 – chlorogenic acid, 2 – quercetin, 3 – luteolin, 4 – apigenin and 5 – kaempferol.

luteolin, apigenin and kaempferol) predicted by the software was 18.85 %. To test the validity of the response surface analysis, the extraction was carried out under the proposed conditions and the extraction yield was determined to be 18.87 ± 0.52 % ($n = 3$). The high correlation between these results confirmed that the response model was adequate to predict optimal operating conditions.

Identification and quantification of extract constituents

Five compounds were identified, namely, chlorogenic acid, quercetin, luteolin, apigenin and kaempferol, on the basis of their retention times compared to the standards. To confirm linearity, standard solutions in the concentration range were prepared and injected into the HPLC system (Table III). The HPLC profiles of flavonoids and phenolic acids extracted from *Coreopsis tinctoria* are shown in Fig. 1.

Antioxidant activity

Scavenging of DPPH radical. – Our results indicate that CTFT extract and rutin were effective in reducing the stable radical DPPH to the yellow-coloured diphenylpicrylhydrazine (Fig. 3a). CTFT active components had significant scavenging effects with the increasing concentration of active components (range of 0.05 – $1.6 \mu\text{mol L}^{-1}$). However, the scaveng-

Table II. Analysis of variance (ANOVA) for experimental results

Factor	Sum of squares for error	Mean square error	df	F-value	p-value
Model	2.4879	0.2764	9	6.0442	0.0135
X ₁	0.3519	0.3519	1	7.6953	0.0275
X ₂	0.0110	0.0110	1	0.2411	0.6384
X ₃	0.9877	0.9877	1	21.5955	0.0023
X ₁ X ₂	0.0143	0.0143	1	0.3122	0.5937
X ₁ X ₃	0.0792	0.0792	1	1.7325	0.2295
X ₂ X ₃	0.0595	0.0595	1	1.3017	0.2914
X ₁ ²	0.0092	0.0092	1	0.2008	0.6676
X ₂ ²	0.1762	0.1762	1	3.8518	0.0905
X ₃ ²	0.7661	0.7660	1	16.7497	0.0046
Lack of fit	0.3184	0.1061	3	236.9869	< 0.0001
Error	0.0018	0.0004	4		
Total	2.8081		16		

df – degrees of freedom

Table III. Linear regression data

Standard solution	Correlation coefficient (R)	Molar concentration (μmol L ⁻¹)
Chlorogenic acid	0.9992	0.189–7.795
Quercetin	0.9991	0.278–10.788
Luteolin	0.9900	0.238–6.407
Apigenin	0.9997	0.241–6.520
Kaempferol	0.9993	0.307–7.602

ing effect of CTFT was significantly lower than that of rutin. At 200 μg mL⁻¹, active components in CTFT and rutin exhibited 68.7 and 89.5 % inhibition, respectively, and the EC₅₀ values were 93.29 and 42.43 μg mL⁻¹ for active components in CTFT and rutin, respectively (Table III). Both CTFT and rutin showed antioxidant activities in a dose dependent manner in DPPH radical scavenging.

Savenging ABTS radical. – Scavenging power correlated well with increasing concentrations of active components. Aliquots of CTFT or rutin (100 μg mL⁻¹) exhibited 73.7 and

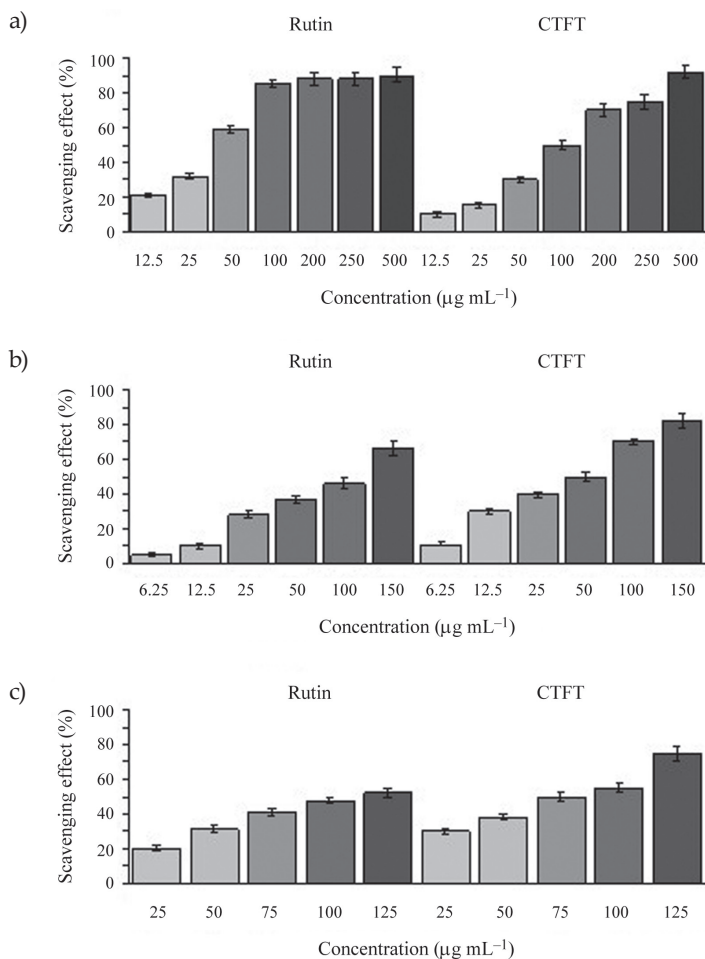


Fig. 3. Radical-scavenging activity of active components from *Coreopsis tinctoria* flowering tops (CTFT) and rutin. Radical-scavenging activities were assessed by measuring: a) DPPH, b) ABTS and c) hydroxyl scavenging activity (mean \pm SD, $n = 3$).

49.1 % inhibition, respectively. The EC_{50} value of CTFT for ABTS radical scavenging activity was 46.29 $\mu\text{g mL}^{-1}$, whereas the EC_{50} value of rutin was found to be 132.47 $\mu\text{g mL}^{-1}$.

Scavenging effects on hydroxyl radicals. – All results showed antioxidant activity in a dose dependent manner. The EC_{50} values of CTFT and rutin were 66.17 and 122.25 $\mu\text{g mL}^{-1}$, respectively (Table III). The ability of CTFT extracts to quench hydroxyl radicals seems to be directly related to the prevention of propagation of lipid peroxidation. Since CTFT seems to be a good scavenger of active oxygen species, it will reduce the rate of hydroxyl radicals' chain reaction.

Table IV. Effect of active components from *Coreopsis tinctoria* on different radical scavenging activities

Samples	EC_{50} ($\mu\text{g mL}^{-1}$) ^a		
	DPPH	ABTS	Hydroxyl
CTFT	93.29 \pm 1.56	46.29 \pm 1.31	66.17 \pm 1.15
Rutin	42.43 \pm 2.13	132.47 \pm 1.85	122.25 \pm 1.39

EC_{50} – measure of radical scavenging activity being the concentration required to inhibit 50 % free radical activity.

^a Mean \pm SD values ($n = 3$).

CONCLUSIONS

In the present study, we demonstrated that CTFT possesses considerable amounts of flavonoids and polyphenols (18.9 %). The data obtained clearly indicate that the extract possesses potent ABTS and hydroxyl radical scavenging activity. CTFT was also able to scavenge DPPH. These antioxidant activities could have contributed, at least partly, to the therapeutic benefits of certain traditional claims for *Coreopsis tinctoria* flowering tops. In view of the potential use of *Coreopsis tinctoria* flowering tops in the functional food industry, its therapeutic benefits and bioactive compounds warrant further investigation.

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