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Phenolics in *Citrus hystrix* leaves obtained using supercritical carbon dioxide extraction

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Abstract: The extraction of phenolics from *Citrus hystrix* leaf was carried out using supercritical fluid extraction and was optimized using response surface methodology (RSM). The effects of CO_2 flow rate, extraction pressure and extraction temperature on yield, total phenolic content and diphenyl-picrylhydrazyl-IC_{50} were evaluated and compared with ethanol extraction. The extraction pressure was the most significant factor affecting the yield, TPC and DPPH-IC_{50} of the extracts, followed by CO_2 flow rate and the extraction temperature. The optimum conditions of pressure, CO_2 flow rate and temperature were at 267 bars, 18 g/min and 50°C, respectively. The yield, TPC and DPPH-IC_{50} obtained were 5.06%, 116.53 mg GAE/g extract and IC_{50} of 0.063 mg/ml, respectively. These values were not significantly different (p<0.05) to their predicted values. Better inhibition and TPC were obtained using SFE method whereas higher yield and phenolic acids were obtained in the ethanol extracts.

Keywords: *Citrus hystrix*, supercritical fluid extraction, antioxidant activity, optimization, phenolic compounds

Introduction

Due to high concentrations of free lipid radicals, both in food *in vitro* and *in vivo* after food digestion, the need to look at antioxidants as functional ingredients in foods has become a trend. Synthetic antioxidants such as, butylated hydroxytoluene (BHT), butylated hydroxyanisole (BHA), tertiarybutyl hydroquanone (TBHQ) and propyl gallate (PG) are conventional food antioxidants. Due to safety issues, consumer concerns and increasing regulatory scrutiny (Shahidi, 2000; Jamilah *et al*., 2009) concerning synthetic antioxidants, the possibility of natural antioxidants as alternatives is aggressively researched. The leaves of *Citrus hystrix*, known locally as *Limau purut*, is used in many Malaysian and South-East Asian region local dishes and medicinal preparations. *C. hystrix* as a potential source of natural antioxidant had been reported (Jamilah *et al*., 1998; Ching and Mohamed 2001; Jaswir *et al*., 2004; Idris *et al*., 2008; Chan *et al*., 2009; Butryee *et al*., 2009; Azlim Almey *et al*., 2010). Reports were based on extracts obtained through the conventional solvents such as ethanol, methanol, acetone and water. To produce extracts of high phenolic content and rich in antioxidants from *C. hystrix* leaves, requires high extraction efficiency which were influenced by factors such as particle size, extraction methods, solvent type, solvent concentration, solvent-to-solid ratio, extraction temperature, pressure and time (Lang and Wai, 2001; Pinelo *et al*., 2005; Silva *et al*., 2007; Wang *et al*., 2008; Banik and Pandey, 2008).

Steam distillation and organic solvent extraction using percolation, maceration and Soxhlet techniques are conventionally used for the extraction of bioactive compounds from plant sources. They are not efficient and economical and this can be overcome by using the supercritical carbon dioxide (SC-CO_2) process (Bimakr *et al*., 2009). Carbon dioxide (critical temperature, pressure and density ~ 31.18°C, 72.0 bar; 0.47 gcm^{-3}, respectively) is safe, residue free, non-flammable, inexpensive and environmentally-friendly (Pyo and Oo, 2007). The optimization of supercritical fluids for the extraction of natural antioxidants and phenolic compounds from the leaves of *C. hystrix* has not been reported. Hence, this study was carried out with the objective of optimizing the extraction of the antioxidant and phenolic acids from the leaves of *C. hystrix* using supercritical carbon dioxide (SC-CO_2) fluid extraction by varying and/or fixing known variables associated with the extraction techniques.

Materials and Methods

Reagents used

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Folin-Ciocalteu Reagent (FCR) and 1,1-diphenyl-2-picryl-hydrazyl (DPPH) were purchased from Sigma (St Louis MO USA). Carbone dioxide, (purity 99.99%) was purchased from Malaysian Oxygen (MOX), Malaysia. Absolute ethanol (99.4%, analytical grade), the modifier for SC-CO_2 process, acetonitrile and methanol (HPLC grade) as the mobile phase for HPLC and phenolic acids standards (vanillic, syringic, *p*-coumaric, *m*-coumaric, trans cinnamic, benzoic, gallic and sinapic acid) were purchased from Fisher Scientific Chemical (Loughborough, England). All other chemicals used were either analytical or HPLC grade.

Sample Preparation

The leaves of *C. hystrix* were obtained from a wholesale market at Puchong, Selangor, Malaysia. Upon arrival at the laboratory, leaves were sorted, washed under running tap water, oven dried at 40°C for 24 h and stored at ambient temperature away from the light. The dried leaves were ground just before extraction in a blender (MX-335, Panasonic, Malaysia) for 10 s to produce a powder with an approximate particle size of 0.5 mm (Bimakr *et al*., 2009).

Solvent extraction

The phenolic compounds in the *C. hystrix* leaves powder were extracted according to Jamilah *et al*. (1998) with slight modifications. The first step involved soaking the powder in 95% ethanol for 24 h at 50°C at an ethanol to leaf ratio of 10:1 (v/w). The crude extract was then filtered and concentrated by evaporating at 40°C in the rotary evaporator (Eyela, A-1000S, Japan). When the ethanol was evaporated off the concentrated extract was transferred into brown glass bottles, flushed with nitrogen and kept at – 25°C until use. The extraction was carried out in triplicate.

Supercritical Carbon Dioxide (SC-CO_2) extraction

Supercritical carbon dioxide (SC-CO_2) fluid extraction using the supercritical fluid extractor (ABRP200, Pittsburgh, PA, USA), with a 500 mL extractor vessel attached, was carried out according to Bimakr *et al*. (2009) with slight modifications. The flow rate of CO_2 and modifier (ethanol), extraction temperature, pressure and time were adjusted using ICE software coupled with the supercritical fluid extractor. The liquid CO_2 was pressurized and heated to the desired pressure and temperature with the aid of the pressure pump (P-50, Pittsburg, PA, USA) to reach the supercritical state prior to passing it into the extraction vessel. The flow rate of absolute ethanol (EtOH), the modifier to improve the extraction of phenolics from *C. hystrix* leaves was fixed at a flow rate of 3 mL/min for all experimental procedures. The duration of the static extraction time was fixed at 30 min, while the dynamic extraction time was kept constant at 90 min.

Fifty grams of *C. hystrix* leaves powder was mixed with 150 g glass beads (2.0 mm in diameters) to systemize the flow rate and the mixture were placed in the extractor vessel. The extraction was then performed under various experimental conditions as generated by the response surface methodology (RSM) design. EtOH was removed from the extracts by vacuum evaporation using a rotary evaporator (Eyela, A-1000S, Japan) at 40°C. The extracts collected in the round bottle flasks wrapped with aluminum foil to minimize light exposure and oxidation were then placed in the oven at 40°C for 30 min before being transferred into desiccators for final constant weight. After which the extracts were transferred into brown glass bottles, flashed with nitrogen and stored in a freezer (-25°C) until further analysis. The extractions were carried out in triplicates.

Determination of total phenolic content (TPC)

The total phenolic content of *C. hystrix* leaf extracts was determined using the Folin-Ciocalteu reagent according to the method described by Singleton *et al*. (1999). An aliquot of the ethanolic extract (0.5 mL) at 1000 ppm was added to 0.5 mL Folin reagent, under dim light before 10 mL (7%) of sodium carbonate was added. The mixture was then left in the dark for 60 min. A UV–Visible spectrophotometer (UV-1650PC, Shimadzu, Kyoto, Japan) was used to measure the absorbance of the mixture at 725 nm and EtOH was used as the blank. The calibration equation for Gallic acid, expressed as Gallic acid equivalent (GAE) in mg/g extract, was $y = 0.0064x + 0.0093$ ($R^2 = 0.9972$).

Determination of free radical scavenging activity

Free radical scavenging activity of *C. hystrix* leaf extracts was measured according to the procedure described by Ramadan and Moersel (2006) with slight modifications. A 0.1 mL aliquot of toluenic (both methanol and ethanol were initially tried but poorer solubility was obtained) sample solution at different concentrations was added with 0.39 mL of fresh toluenic 1,1-diphenyl-2-picryl-hydrazyl (DPPH) solution (0.1 mM). Triplicates were carried out for each concentration. The mixtures were vortexed and left in the dark for 60 min and absorbance was read against pure toluene (blank) at 515 nm using a UV–Visible spectrophotometer (UV-1650PC, Shimadzu,

Kyoto, Japan). The free radical scavenging activities of extracts were calculated as follows:

$$\% \text{ Inhibition} = ([A_{control}-A_{sample}]/A_{control})^{*}100$$

Where $A_{control}$ = absorbance of the control reaction (containing all reagents except samples); A_{sample} = absorbance of the test compound.

Determination of IC_{50} in this test was defined as the concentration of the extract that was able to inhibit 50% of the total DPPH radicals. IC_{50} of the sample was expressed in mg/mL and calculated by the interpolation of linear regression analysis (Brand-Williams *et al*., 1995). The IC_{50} of BHA and α-tocopherol were used a positive controls.

Determination of phenolic acids

The phenolic acids of the *C. hystrix* leaf extracts that were obtained from the optimum SC-CO_2 conditions for yield, TPC and DPPH-IC50 were analyzed using high-performance liquid chromatography (HPLC) (Agilent Technologies 1200 series model, 76337 Waldbronn, Germany) equipped with Diode Array Detector (DAD), and detection at 254 nm. The HPLC parameters were modified from Andersen and Pedersen (1983). The column temperature used was 30°C at a maximum temperature 35°C and the column used was Crespak RP C18S RP C18 (150 mm L* 4.6 mm ID, JASCO). The flow rate of mobile phases used was 1.5 mL/min for 25% acetonitrile in formic acid-water (0.5:99.5), which were run isocratically. The injection volume used was 20 µL in duplicates for each of the SC-CO_2 optimum conditions and ethanol extracts.

The standards used were vanillic, syringic, *p*-coumaric, *m*-coumaric, trans-cinnamic, benzoic and sinapic acids (Fisher Scientific Chemical Loughborough, England). Identification and quantification of phenolic acids in the extracts were based on the standard curves of the standards as well as their peaks retention times.

Experimental Design and Statistical Analysis

Response surface methodology (RSM) was used to determine the optimum conditions for the yield, TPC and DPPH-IC_{50} in *C. hystrix* leaf extracts. The experimental design and statistical analysis were carried out using Minitab V. 14 statistical package (Minitab Inc., PA, USA). Central composite design (CCD) was chosen to evaluate the joint effect of three independent variables i.e. CO_2 rate, extraction temperature and pressure, coded as X_1, X_2 and X_3, respectively. The minimum and maximum values for CO_2 rate were set at 15 and 25 g/min, extraction temperature between 40 and 60°C and pressure between 100 and 300 bars. The dependent values were yield, TPC and DPPH-IC_{50}. For optimization, yield and TPC were maximized to achieve highest values and lowest value for DPPH-IC_{50}.

The whole design consisted of 20 combinations including six replicates of the center point. The ANOVA tables were generated and the effect and regression coefficients of individual linear, quadratic and interaction terms were determined. The significances of all terms in the polynomial were analyzed statistically by computing the F-value at a probability (p) of 0.001, 0.01 or 0.05. The statistically found non-significant (p>0.05) terms were removed from the initial models and only significant (p<0.05) factors were involved in the final reduced model. The non-significant linear terms were also kept in the reduced model in cases where their quadratic or interaction terms were significant (p<0.05). Experimental data were fitted to the following second order polynomial model and regression coefficients were obtained according to the generalized second-order polynomial model proposed for the response surface analysis as below according to Myers *et al*. (2009).

$$y = \beta_0 + \sum_{i=1}^{k} \beta i x i \sum_{i=1}^{k} \beta i i x i^2 + \sum_{i}^{k-1} \sum_{j}^{k} \beta i j x i x j \quad \text{Eq (1)}$$

Where β_0, β_i, β_{ii}, β_{ij} were regression coefficients for intercept, linear, quadratic and interaction terms, respectively. X_i and X_j were coded values of the independent variables, while k equaled to the number of the tested factors (k=3).

Results and Discussion

Response surface methodology (RSM) model fitness and verification of models

Based on the ranges set for the identified parameters, 20 trails of each parameter, including six replicates of the center points that influence yield, TPC and DPPH-IC_{50} were selected. In this study, the lower and upper values for the variables were set at +alpha (+α=1.633) and –alpha (-α=1.633) and, hence all the factor levels were chosen within the limits that were practical and desirable for SFE (above critical temperature of 31°C and critical pressure of 72 bar). The experimental and predicted values for responses under the different combinations of extraction conditions via SC-CO_2 extractions were as in Table 1. The results indicated that yield, TPC and DPPH-IC_{50} obtained ranged from 0.4 to 5%, 15 to 128.9 mg GAE/g extract and 0.065 to 0.300 mg/mL, respectively. By utilizing multiple regression

Table 1. Yield, TPC and DPPH-IC_{50} values obtained at different extraction conditions via SC-CO_2 and EtOH

	Parameter				Response					
	Run	X_1	X_2	X_3	Yield (%)		TPC (mg GAE/g extract)		DPPH-IC_{50} (mg/ml)	
					[a]experimental	predicted	[a]experimental	predicted	[a]experimental	predicted
SC-CO_2 Extraction	1	15	40	300	4.96 ± 0.20	5.748	101.5 ± 1.41	88.5	0.140 ± 0.03	0.126
	2c	20	50	200	4.92 ± 0.14	4.422	110.2 ± 1.43	122.6	0.101 ± 0.00	0.097
	3	25	60	100	0.66 ± 0.02	0.550	16.4 ± 0.70	17.6	0.245 ± 0.03	0.241
	4	15	40	100	3.78 ± 0.04	3.114	39.9 ± 0.12	53.7	0.300 ± 0.10	0.271
	5	25	40	100	1.32 ± 0.08	1.712	20.5 ± 0.30	28.3	0.270 ± 0.07	0.271
	6	25	40	300	2.18 ± 0.01	4.344	61.3 ± 0.71	62.9	0.107 ± 0.30	0.126
	7c	20	50	200	4.36 ± 0.06	4.422	128.9 ± 2.83	122.6	0.112 ± 0.00	0.097
	8	15	60	100	2.02 ± 0.12	1.952	52.4 ± 1.41	43.3	0.221 ± 0.01	0.241
	9	25	60	300	3.50 ± 0.01	3.182	55.8 ± 1.41	52.5	0.080 ± 0.01	0.096
	10c	20	50	200	4.00 ± 0.10	4.422	124.0 ± 3.54	122.6	0.114 ± 0.07	0.097
	11	15	60	300	5.00 ± 0.14	4.586	84.0 ± 0.72	78.2	0.102 ± 0.04	0.096
	12c	20	50	200	4.48 ± 0.02	4.422	122.0 ± 0.85	122.6	0.065 ± 0.03	0.097
	13	11	50	200	4.20 ± 0.18	4.372	78.1 ± 0.72	85.2	0.094 ± 0.01	0.103
	14c	20	50	200	4.50 ± 0.08	4.120	102.6 ± 0.81	108.8	0.090 ± 0.02	0.103
	15	28	50	200	2.10 ± 0.14	2.082	48.5 ± 0.70	43.3	0.105 ± 0.02	0.103
	16	20	50	363	5.00 ± 0.16	4.926	52.0 ± 0.91	63.1	0.085 ± 0.01	0.068
	17c	20	50	200	4.20 ± 0.05	4.120	122.2 ± 1.41	108.8	0.110 ± 0.07	0.103
	18	20	33	200	4.26 ± 0.0.4	3.906	58.1 ± 1.22	50.5	0.261 ± 0.07	0.277
	19	20	50	78	0.40 ± 0.06	0.628	15.0 ± 0.05	6.5	0.294 ± 0.07	0.304
	20	20	66	200	1.50 ± 0.04	2.008	24.0 ± 0.72	33.5	0.253 ± 0.08	0.229
Solvent Extraction	EtOH	-	-	-	9.00 ± 0.24	-	112.7 ± 1.95	-	0.250 ± 0.02	-

[a]Means of duplicate values ± standard deviations; c: center point; X_1: CO_2 flow rate (g/min); X_2: Temperature (°C); X_3: Pressure (bar); TPC: total phenolic content; DPPH: diphenylpicrylhydrazyl; IC_{50}: inhibition concentration to 50% ; SC-CO_2: supercritical carbon dioxide; EtOH: ethanol.

analysis, relationships between the tested parameters and the responses were explained from the following regression equations (2, 3, and 4 for yield, TPC and DPPH-IC_{50}, respectively) showing the final reduced models.

$$\text{Yield} = -3.33 + 0.142\ X_1 + 0.164X_2 + 0.00735X_3 - 0.00669X_1^2 - 0.00218\ X_2^2 - 0.000025\ X_3^2 \quad \text{Eq (2)}$$

$$\text{TPC} = -909 + 25.4\ X_1 + 25.6\ X_2 + 1.54\ X_3 - 0.668\ X_1^2 - 0.250\ X_2^2 - 0.00278\ X_3^2 \quad \text{Eq (3)}$$

$$\text{DPPH-IC}_{50} = -0.604\ X_2 - 0.0177\ X_3 + 0.00559\ X_2^2 + 0.000031\ X_3^2 \quad \text{Eq (4)}$$

The fitness of response function and experimental data were evaluated from the linearity, quadratic and regression coefficients of independent variables as shown in Table 2. The ANOVA of regression model showed that the models were noticeably significant due to the extremely low probability value (p<0.001). The coefficient of determination (R^2) and significance of lack of fitness was further evaluated to check the fitness and model adequacy. The R^2 equal to the unity or ≥ 0.8, was desirable and the R^2 values for the regression model of yield, TPC, and DPPH-IC_{50}, were 0.935, 0.95, and 0.96, respectively (Table 2). Thus, indicating that the predicted second order polynomial models fitted well with the system. The values of adjusted R^2 (corrected value for R^2 after the elimination of the unnecessary model terms) of yield, TPC and DPPH-IC_{50} were also very high, hence suggesting the high significance of the model (0.897, 0.92 and 0.93). The simultaneous increase of both R^2 and adjusted R^2 plus the absence of any lack of fit (p>0.05) in our data has proven its credibility and model adequacy. The multiple regression results and the significance of regression coefficients yield, TPC and DPPH-IC_{50} models were as shown in Table 3. It was observed that both the linear and quadratic term of all parameters significantly (p<0.05) effected the yield, TPC and DPPH -IC_{50}. However, CO_2 flow rate did not significantly affect the DPPH-IC_{50} where temperature effect on TPC was only significant in the quadratic manner to remain in the model (Table 3).

For verification, the appropriateness of the response surface equation was tested by the evaluation of experimental and predicted values from the reduced response regression models. A close agreement between the experimental and predicted values (Table1) was noted. No significant difference was obtained between those values. Therefore, suggesting the adequate fitness of the response equations.

Influence of pressure, CO_2 flow rate and temperature on SC-CO_2 extraction efficiency

Figure 1(a) showed the three-dimensional response surface plots by presenting the response as the function of two factors and keeping the temperature at its mid level (50°C). It showed a higher yield in the region of extraction pressure between 190 to 300 bars and at CO_2 flow rate of 12 to 17 g/min. Both extraction pressure and CO_2 flow rate exhibited significant linear and quadratic effects on yield as shown in Table 3. The yield was optimum at about 14.8 g/min CO_2 flow rate and at the pressure of 320 bars. Extraction pressure had more influence on the yield than CO_2 flow rate as reflected by its higher linear and quadratic coefficients (β_3=0.65819; β_{33} = -0.25168) compared to the latter (β_1= -0.35060; β_{11}=-0.16731). Díaz-Reinoso *et al*. (2008) had also reported that instead of just increasing CO_2 flow rate alone, increased pressures with modifier (ethanol) resulted in increased solvent density and power of the solvent fluid which may lead to higher extraction

Table 2. Analysis of variance (ANOVA) of the second-order polynomial model for Yield, TPC and DPPH-IC_{50} of CLE

	source	DF	Sq SS	AdjSS	AdjMS	t- value	*p*-value
[a]Yield	Block	1	0.1104	0.11041	0.11041	1.86	0.198
	Regression	6	10.1543	10.15434	1.69239	28.49	0.000
	Linear	3	8.5405	8.54045	2.84682	47.92	0.00
	Square	3	1.6139	1.61389	0.53796	9.05	0.002
	Residual Error	12	0.7129	0.71293	0.05941		
	Lack-of-Fit	8	0.5313	0.53128	0.06641	1.46	0.377
	Pure Error	4	0.1816	0.18165	0.04541		
	Total	19	10.99777				
[b]TPC	Blocks	1	925.7	923.9	923.88	8.11	0.015
	Regression	6	26206	26206	4367.67	38.33	0.000
	Linear	3	6616.8	6610.6	2203.55	19.34	0.000
	Square	3	19589.2	19589.2	6529.74	57.3	0.000
	Residual Error	12	1367.4	1367.4	113.95		
	Lack-of-Fit	8	978.7	978.7	112.34	1.26	0.439
	Pure Error	4	388.7	388.7	97.19		
	Total	19	28499.2				
[c]DPPH-IC_{50}	Blocks	1	0.0022	0.0021	0.0021	0.59	0.456
	Regression	4	0.1243	0.1242	0.3105	83.90	0.000
	Linear	2	0.6993	0.6990	0.0349	94.42	0.000
	Square	2	0.0543	0.0542	0.0271	73.34	0.000
	Residual Error	14	0.0052	0.0051	0.0003		
	Lack-of-Fit	4	0.0018	0.0018	0.0004	1.30	0.307
	Pure Error	10	0.0033	0.0033	0.02961	0.0003	
	Total	19	0.1296				

[a]Coefficient of determination (R^2) = 0.93; R^2-adjusted = 0.89
[b]Coefficient of determination (R^2) = 0.95; R^2-adjusted = 0.92
[c]Coefficient of determination (R^2) = 0.96; R^2-adjusted = 0.90

Table 3. Regression coefficients of the predicted second-order model for the response variables, yield, TPC and DPPH-IC_{50} of CLE

Model parameter	Yield		TPC		DPPH-IC_{50}	
	Regression coefficient	S.E	Regression coefficient	S.E	Regression coefficient	S.E
Constant	2.13545[a]	0.0997	115.751[a]	4.369	0.0985[a]	0.006
Linear						
CO_2FR	-0.35[a]	0.0667	-12.82[a]	2.923	RM	RM
T	- 0.29[b]	0.0667	-5.184[A]	2.923	-0.003[c]	0.004
P	0.65[a]	0.0667	17.448[a]	2.923	-0.071[a]	0.005
Quadratic						
CO_2	-0.16[c]	0.0670	-16.689[a]	2.937	RM	RM
T	-0.29[b]	0.0667	-25.033[a]	2.937	0.057[a]	0.005
P	-0.25[b]	0.0670	-27.823[a]	2.936	0.032[a]	0.005

S.E.: Standard error; CO_2R: CO_2 flow rate (g/min); T: temperature (°C); P: pressure (bar). Values with lower case superscripts were statistically significant at [a]$p < 0.001$, [b]$p < 0.01$, [c]$p < 0.05$. Values with uppercase superscripts were not statistically significant at $p > 0.05$; RM: Neither its linear nor quadratic was significant and thus reduced from the model.

yield.

Figure 1(b) showed the effects of extraction pressure and extraction temperature on yield at constant CO_2 flow rate of 20 g/min. Extraction pressure had a very significant ($p<0.001$) effect on the yield in linear and quadratic manner as also shown in Table 3. At pressure of ≥140 bars and temperature not exceeding 47°C, the yield was increased. However, with further increase in the temperature, the yield showed a decrease which was most probably due to the reduced density of CO_2.

The relationship of CO_2 flow rate and extraction temperature with yield was plotted in Figure 1c. Both the parameters exhibited significant linear and quadratic effect ($p<0.05$) on the yield. The yield increased rapidly with decreasing CO_2 flow rate up to 13 g/m and this was followed by a slight decrease thereafter. By combining all the results presented in Figure 1, it was obvious that the extraction pressure had the most critical impact on yield of the extract followed by CO_2 flow rate and extraction temperature.

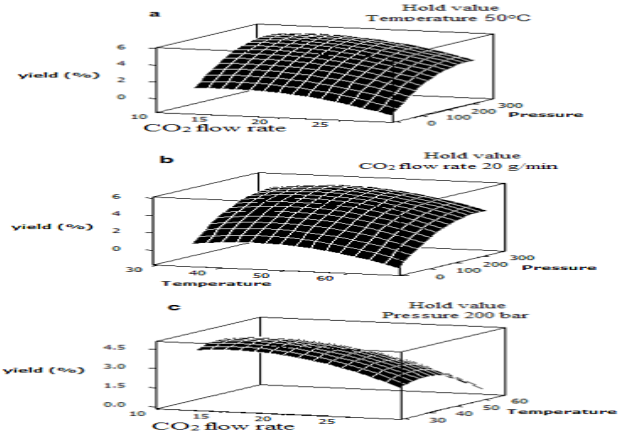

Figure 1. Response surface plot corresponding to yield of *C. hystrix* leaf extract as a function of (a) CO_2 flow rate (g/min) and extraction pressure (bar); (b) extraction temperature (°C) and extraction pressure; and (c) CO_2 flow rate and extraction temperature

Total phenolic content (TPC)

The TPC of the extract was as shown in Figure 2. Depending on the pressure, temperature and CO_2 flow rate, the TPC of the extract ranged from 15.0 to 128.9 mg GAE/g extract. No available literature

report could be used for comparison for the SC-CO_2 extraction method; however, Idris *et al*. (2008) reported that TPC of the extracts was about 103.2 mg GAE/g extract which was slightly lower than our EtOH extracted TPC (112.7 mg GAE/g extract) Moderate levels of the selected independent variables of SC-CO_2 extracts (run order 7, 10, 12, and 17) as in Table 1 reflected higher TPC of the *C. hystrix* leaf extracts than our EtOH extraction as well as Idris *et al*. (2008), which may be due to partial degradation of the extracted compounds due to long extraction time when conventional extraction methods are to be used. With SC-CO_2 method, the extraction time (90 min) was significantly shorter than that of EtOH extraction (>20 h).

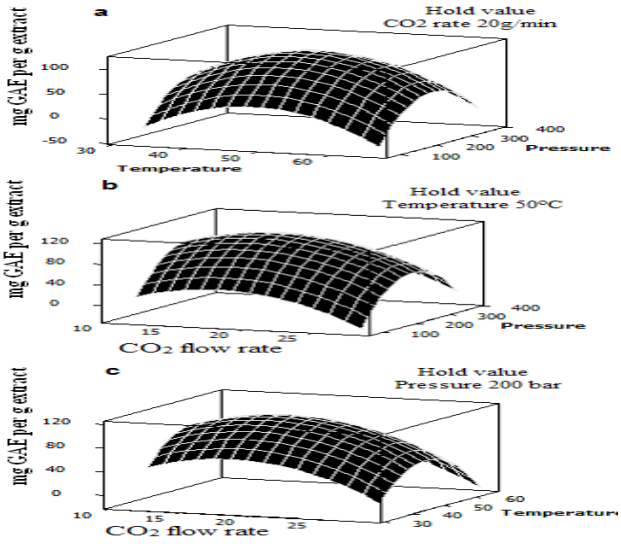

Figure 2. Response surface plot corresponding to TPC of *C. hystrix* leaf extracts as a function of (a) extraction temperature (°C) and extraction pressure (bar); (b) CO_2 flow rate (g/min) and extraction pressure; and (c) CO_2 flow rate and extraction temperature

Free radical scavenging activity

Figure 3 demonstrated the effect of temperature and pressure on the scavenging property of the *C. hystrix* leave extracts. The antioxidant activity of the extracts, determined by the IC_{50} of the radical scavenging properties of diphenylpicrylhydrazyl (DPPH-IC_{50}) was found to be gradually decreased with the increase of extraction temperature and pressure up to 50°C and 314 bars, respectively. The optimum value of IC_{50} at 0.0585 was inversely related to the DPPH-IC_{50} i.e. the lesser the IC_{50}, the stronger is the activity of DPPH-IC_{50}. In this study, the IC_{50} of BHA and α-tocopherol acted as positive controls and their corresponding values were 0.023 mg/ml and 0.031 mg/ml, respectively. Run orders 12, 9, and 16 (Table 1) possessed greater DPPH radical scavenging activities with the lower IC_{50} values of 0.065, 0.08 and 0.085 mg/ml, respectively. This was in agreement to the findings of Idris *et al*. (2008), where the activity of BHA was found to be higher than the sample. Compared to conventional solvent extraction method with the IC_{50} of 0.250 mg/ml (Table 1), it was observed that SC-CO_2 extracts had high DPPH radical-scavenging activity remarkably greater than that of traditional extraction method. The IC_{50} values for *C. hystrix* leaf extracted by SC-CO_2 ranged from 0.065 - 0.300 mg/ml depending on pressure and temperature where an increase in the pressure relatively resulted in an increase in its antioxidant capacity.

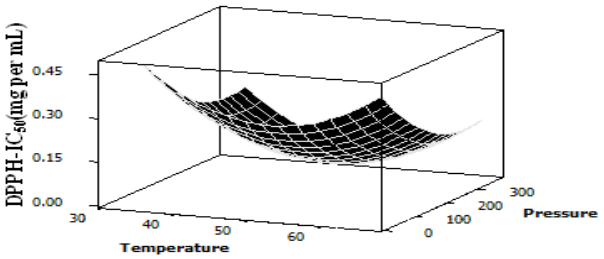

Figure 3. Response surface plot corresponding to DPPH-IC_{50} of *C. hystrix* leaf extracts as a function of extraction temperature (°C) and extraction pressure (bar)

Identification and quantification of phenolic acids of extracts

Out of seven standard phenolic acids, six have been detected in SC-CO_2 extracts (Table 4). Higher recovery of phenolic acids was found in EtOH extracts when compared to that of SC-CO_2 extracts. The number of polar function groups, e.g. hydroxyl groups, may have influenced volatility of the solutes

Table 4. Phenolic acids recovery (mg/ml) of SC-CO_2 optimum conditions and EtOH extraction of *C. hystrix* leaves

	Optimum SC-CO_2 Extraction conditions				Solvent Extraction
Phenolic acids	[a]Retention time(min)	yield	TPC	DPPH-IC_{50}	EtOH Extracts
Vanillic acid	2.18 ± 0.01	10.25 ± 0.35	9.15 ± 0.21	0.98 ± 0.00	67.00 ± 1.41
p-Coumaric acid	2.99 ± 0.04	2.40 ± 0.14	5.10 ± 0.14	[b]ND	12.87 ± 0.17
Sinapic acid	3.28 ± 0.02	0.23 ± 0.02	0.15 ± 0.00	0.18 ± 0.01	1.42 ± 0.03
m-Coumaric acid	3.70 ± 0.03	21.75 ± 0.25	14.25 ± 0.35	19.25 ± 0.35	134.86 ± 2.83
Benzoic acid	4.78 ± 0.09	2.95 ± 0.07	0.82 ± 0.02	2.10 ± 0.14	10.94 ± 0.08
Cinnamic acid	8.66 ± 0.12	87.70 ± 1.70	93.50 ± 2.12	70.65 ± 1.20	121.31 ± 1.69

[a]Values were means ± standard deviations; [b]ND: not detected

thus determining their optimum extractability with SC-CO_2 (Lang and Wai, 2001). For example, Stahl and Glatz (1984) successfully extracted steroids with three hydroxyl groups below 300 bars but failed to extract those steroids consisting of four hydroxyl groups, or three hydroxyls and one acid group, or one phenolic hydroxyl with two other hydroxyl groups. Despite the difference in quantity, the type of phenolic acids existing in the extracts for both EtOH and SC-CO_2 extraction methods remained the same. Trans-cinnamic, *m*-coumeric and vanillic acids were the predominant phenolic acids, while *p*-coumaric, benzoic and sinapic acids were detected in lesser amounts.

Conclusion

For yield, TPC and DPPH-IC_{50} of *C. hystrix* leave extracts using SC-CO_2 extraction, the optimum conditions needed were pressure at 265 bars, temperature at 50°C and CO_2 flow rate at 18 g/min. Of the three independent variables studied, extraction pressure was the most significant factor influencing yield, TPC and DPPH-IC_{50}, which was followed by CO_2 flow rate and extraction temperature. Solvent extraction gave higher yield but similar phenolic acids profile when compared to those of SC-CO_2. SC-CO_2 extractions gave better antioxidant activities measured by IC_{50} of 1,1-Diphenyl-picrylhydrazyl (DPPH) and total phenolic content (TPC). On the overall, SC-CO_2 extraction was faster and better for extracting active components of *C. hystrix* leaves.

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