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Original research paper

Optimization of process variables for phyllanthin extraction from *Phyllanthus amarus* leaves by supercritical fluid using a Box-Behnken experimental design followed by HPLC identification

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The response surface methodology using the Box-Behnken design was established to describe supercritical carbon dioxide assisted extraction of phyllanthin from *Phyllanthus amarus* Schum and Thonn leaves prior to HPLC analysis. The effects of extraction pressure, temperature, modifier concentration and extraction time on the yield of phyllanthin were investigated. By solving the regression equation, the optimum conditions were as follows: extraction pressure 23.2 MPa, temperature 40 °C, methanol as modifier at a concentration of 10 % and time 90 min. Under these conditions, the phyllanthin yield was 12.83 ± 0.28 mg g^{-1}, which was in good agreement with the predicted values. Modifier concentration and extraction time showed a significant effect on the phyllanthin yield.

Keywords: phyllanthin, *Phyllanthus amarus* (*Euphorbiaceae*), supercritical carbon dioxide assisted extraction, Box-Behnken design, HPLC

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Phyllanthus amarus Schum and Thonn (*Euphorbiaceae*) has a long history in the traditional medicine in every tropical country. It is used in the treatment of diabetes, intestinal parasites and liver, kidney and bladder problems (1). *P. amarus* has been shown to possess anti-hepatitis B virus surface antigen activity in both *in vivo* and *in vitro* studies (2, 3). The lignan phyllanthin (Fig. 1) is the main therapeutically active constituent of *P. amarus*.

Pharmacological screening revealed that phyllanthin is a hepatoprotective (4, 5), antioxidant (6), antihyperuricemic (7), antimicrobial (8), antigenotoxic (9), anti-inflammatory (10), and vasorelaxant (11) compound. Very few studies have been reported on the extraction and analysis of phyllanthin. For phyllanthin extraction from *P. amarus*, numerous methods have been reported: maceration (12), hot percolation (13), cold per-

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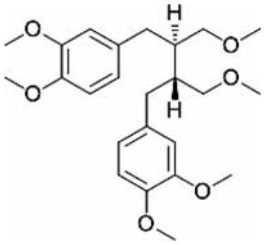

Fig. 1. Structure of phyllanthin.

colation (14) and ultra-sonication (15) with subsequent analysis by HPTLC (12, 13), HPLC (6, 13, 16), HPLC-MS (17). It is a well known fact that conventional solvent extraction methods are tedious and time consuming. Moreover, these processes may lead to thermal, oxidative and photo decomposition of active phytoconstituents (18, 19).

Supercritical carbon dioxide assisted extraction (SC CO_2E) has immediate advantages over traditional extraction techniques: it is a flexible process due to the possibility of continuous modulation of the solvent power/selectivity of the supercritical CO_2, it allows elimination of polluting organic solvents and expensive post-processing of the extracts for solvent elimination (20).

Hamrapurkar *et al*. (21) reported the Soxhlet assisted extraction (SAE), SC CO_2E, isolation and HPTLC analysis of phyllanthin from the whole plant of *P. amarus*. However, the effect of extraction parameters on the yield of phyllanthin and the optimum extraction conditions has not been subjected to a thorough study with response surface methodology. The phyllanthin content varies in plant parts. It was reported that the content of phyllanthin was higher in the leaves compared to other plant parts (13, 22). Until now, there have been no literature reports on the use of supercritical carbon dioxide extraction of phyllanthin from *P. amarus* leaves. This study was designed with the objectives to develop and optimize the SC CO_2E process, to compare the SC CO_2E yield with conventional SAE yield, to describe the optimized SC CO_2E process using the Box-Behnken design and to develop and validate a HPLC method for qualitative and quantitative analyses of phyllanthin.

EXPERIMENTAL

Plant material and reagents

The plant material was obtained from Vedashri Ayurved Bhandar (India) and authenticated by M. M. Sardesai (Botany Department, Dr. Babasaheb Ambedkar Marathwada University, Aurangabad, MH, India). A voucher specimen was deposited in the herbarium of the same department. Authenticated dried leaves of *P. amarus* were ground to a powder using a pulverizer (K. C. Engineers, India). Powder was sifted in a sieve shaker (CIP Machineries, India) with sieves of different sizes (1.68, 0.71, 0.354 and 0.210 mm,

Swastika electric and scientific works, India) for a period of 15 min. The powder passed through a sieve size 0.354 mm and retained on a sieve size 0.21 mm was collected and used for further extraction experiments.

Standard phyllanthin (purity 98 % by HPLC) was obtained from Natural Remedies Pvt. Ltd. (India). All solvents used for extraction and chromatography were of analytical grade (Finar Chemicals Ltd., India) and LC grade (Merck, Germany), respectively. CO_2 (99 % purity) was procured from M/S Jain Cylinders (India).

HPLC analysis

The HPLC system consisted of a Waters e2695 Separation Module with an auto-sampler and Waters 2489 ultraviolet spectrophotometric detector (Waters, USA) equipped with MassLynx data acquisition software, version 4.1. All samples and standards were filtered through 0.45-μm syringe filters (Millipore, India) and 20 μL was injected. Separation was achieved on a Grace Brava BDS C-8 column (250 mm × 4.6 mm, 5 μm particle size) (Grace, USA) at 40 °C with mobile phase consisting of acetonitrile and water (45:55, *V*/*V*) in the isocratic elution mode with 1 mL min^{-1} flow rate. The UV detection was carried out at 230 nm.

Calibration standards and quality control samples

A reference stock solution of phyllanthin was prepared by accurately weighing 5 mg of the standard. The weighed amount was transferred to a 5-mL volumetric flask, dissolved and diluted suitably with HPLC grade methanol. Reference stock solution was diluted suitably with HPLC grade methanol to achieve 6 calibration standards containing phyllanthin (expressed in μg mL^{-1}): CAL STD-1: 1, CAL STD-2: 2, CAL STD-3: 4, CAL STD-4: 8, CAL STD-5: 16, CAL STD-6: 32. Three quality control (QC) standards containing phyllanthin (expressed in μg mL^{-1}) (LQC: 4; MQC: 12 and HQC: 20) were prepared from the reference stock solution.

Method validation

The analytical method was validated to meet the acceptance criteria as per ICH guidelines (23). Recovery studies were performed using the standard addition method. The linearity and range were established using 6 calibration standards. The peak area *vs*. concentration plots were subjected to linear least square regression analysis. Intra- and inter-day accuracy was established from quality control standards by evaluating nominal and mean measured concentrations of quality control standards, which were compared, and relative error was calculated.

The intra- and inter-day precision (RSD, %) was established by analyzing 9 replicates, each of 3 quality control standards on day 1 and again on each of three consecutive days. The lowest concentration with acceptable accuracy and precision was noted as the limit of quantification (*LOQ*) for phyllanthin. The noise response was multiplied by the quantitation limit factor whereas for limit of detection (*LOD*), it was multiplied by the detection limit factor of 3.3. Both values were then converted to *LOQ* and *LOD* using the calibration curve.

Soxhlet assisted extraction (SAE) of P. amarus

Thirty grams of powdered leaves of *P. amarus* were placed in a thimble (Borosil, India), which was inserted into a Soxhlet apparatus and extracted with 300 mL methanol. Extraction was performed for 24 h. After extraction, the extract was concentrated at 40 °C using a rotary vacuum evaporator (Heidolph, Germany) and analyzed for phyllanhin content by HPLC.

Supercritical carbon dioxide extraction (SC CO_2E) of P. amarus

A bench top SC CO_2E unit (Model: SFE 2000 series, Jasco, Japan) was used for extraction purposes. A diagram of SC CO_2E system is presented in Fig. 2.

The extractor column was densely packed with 4.5 g of *P. amarus* powder. The column was carefully fixed in a column oven. The CO_2 from the cylinder was passed through the chiller unit (~277 K) *via* a siphon tube, delivered and compressed to the desired working pressure with a CO_2 delivery pump (PU 2080-CO_2 Plus, Jasco) equipped with a pressure regulator (BP-2080 Plus, Jasco). Methanol was introduced into system as an organic modifier using a solvent pump. The temperature and pressure of CO_2 were manipulated with a pressure regulator. The SC CO_2 was passed through an extraction column (150 mm length × 15 mm i.d.) placed in a thermostatically controlled oven (CO-2060 Plus, Jasco). After the pressure and the fluid flow rate reached the desired values, the six-port valve was opened so as to pass SC CO_2 through the extractor; this was taken as the start of the extraction cycle. The exit fluid from the extractor was expanded to ambient pressure with a pressure regulator. The extract was then concentrated by removing the solvent under vacuum and the concentrated extract was then diluted appropriately with methanol and analyzed for phyllanthin content by HPLC.

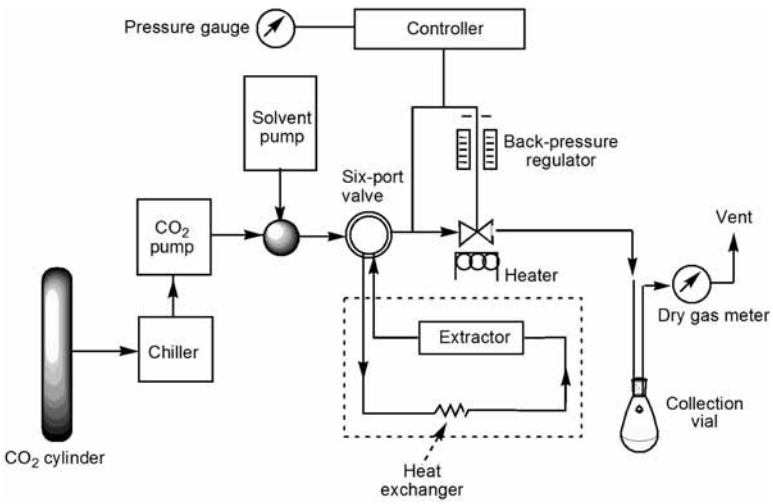

Fig. 2. Diagram of the SC CO_2E system.

Experimental design and evaluation

The Box-Behnken design is a second-order multivariate technique based on three--level incomplete factorial design that have a wide application for assessment of critical experimental conditions, that is, maximum or minimum of the response function. The number of experiments (N) needed for the development of Box-Behnken matrix is defined as $N = 2k\,(k\text{-}1) + C_0$, where k is the factor number and C_0 is the number of replicates of the central point (24–26). A Box-Behnken experimental design with four variables at three levels was used to determine the response pattern and the interaction effect of independent variables on the response. The four key variables, *viz.*, extraction pressure (X_1), extraction temperature (X_2), modifier concentration (X_3) and dynamic extraction time (X_4) were selected and their effect on the SC CO_2E of phyllanthin was evaluated at the different levels. Variables and levels tested are depicted in Table I. The experimental design used for the study is shown in Table II.

Second-order polynomial equation was used to express phyllanthin yield (mg g^{-1} *P. amarus* leaves) (Y) as a function of the independent variables, where X_1, X_2, X_3 and X_4 code represent the pressure, temperature, methanol concentration in CO_2 and extraction time, respectively:

$$Y = a_0 + a_1X_1 + a_2X_2 + a_3X_3 + a_4X_4 + a_{11}X_1^2 + a_{22}X_2^2 + a_{33}X_3^2 + a_{44}X_4^2 + a_{12}X_1X_2 + a_{13}X_1X_3 + a_{14}X_1X_4 + a_{23}X_2X_3 + a_{24}X_2X_4 + a_{34}X_3X_4$$

Design-Expert software (version 8.0.6.1, Stat-Ease, Inc., Minneapolis, USA) was used for the ANOVA analysis of the obtained experimental data. The quality of the fit of the polynomial model equation was expressed by the coefficient of determination R^2 and the values of the adjusted R^2 of models were evaluated to check model adequacies. The significance of each term in the equation is to estimate the goodness of fit in each case. The analysis of variance table was generated, and the effect and regression coefficients of individual linear, quadratic and interaction terms were determined. The p-values of less than 0.05 were considered to be statistically significant. The regression coefficients and regression models were used for statistical calculations and generation of three dimensional plots.

RESULTS AND DISCUSSION

HPLC analysis and validation

Optimum chromatographic separation of phyllanthin was achieved with acetonitrile/water (45:55, V/V) with a flow rate of 1 mL min^{-1}. The UV detection of analytes was carried out at 230 nm. The resulting chromatograms showed a retention time of 13.12 min for phyllanthin (Fig. 3a).

Phyllanthin content was determined by referring to the calibration curve established by running phyllanthin standards through the HPLC system under the same conditions. The calibration curve of phyllanthin was linear over the concentration range of 1

to 32 μg mL^{-1} (R^2 = 0.999). The recovery of phyllanthin was 97.3 ± 2.2 %, calculated by addition of known amounts of phyllanthin to the leaf extract. The intra-day inaccuracy in terms of relative error was in the range of –2.2 to +2.4 % whereas inter-day inaccuracy

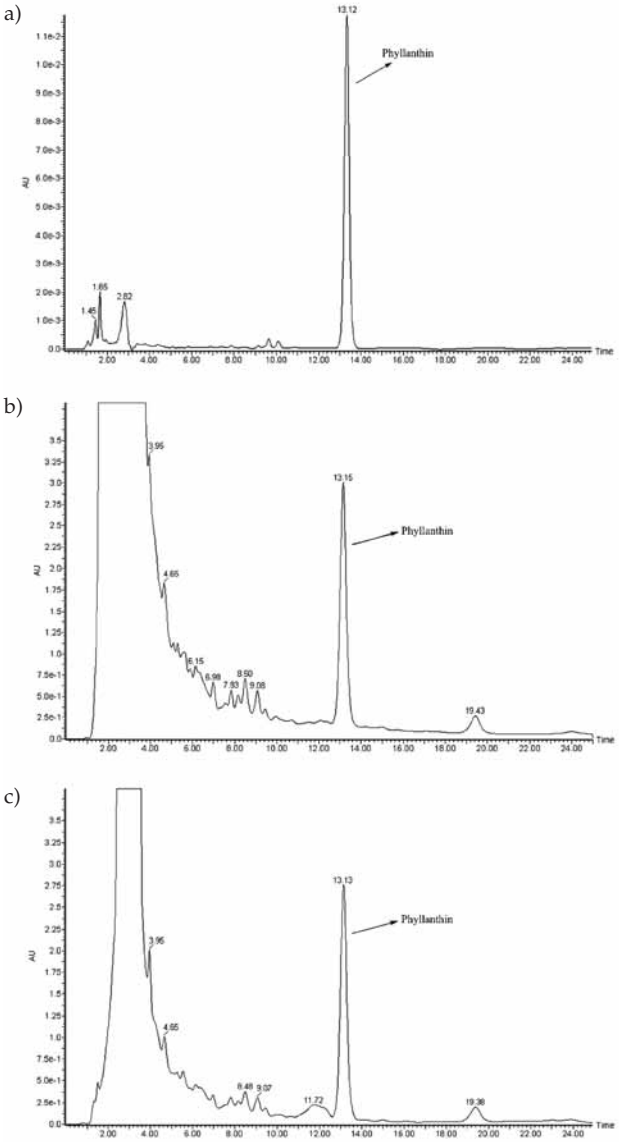

Fig. 3. HPLC chromatograms showing: a) standard phyllanthin, b) extracts obtained by SAE, and c) extracts obtained by SC CO_2E under optimized conditions.

was in the range of –3.4 to +4.0 %. Intra-day precision RSD was in the range of 1.7 to 2.9 % whereas inter-day precision was in the range of 0.8 to 3.1 %. *LOQ* and *LOD* for phyllanthin were 1 and 0.3 μg mL^{-1}, resp.

Typical HPLC chromatograms of the standard phyllanthin and sample extracts obtained by SAE and SC CO_2E are shown in Figs. 3a-c.

Soxhlet assisted extraction

The conventional SAE of *P. amarus* leaves was carried out to recover the maximum extractable amount of phyllanthin. After SAE, 10.62 ± 0.35 mg phyllanthin per gram of *P. amarus* leaves was obtained.

Experimental design applied to SC CO_2E extraction

An optimum process should be defined in order to obtain a high phyllanthin yield. The effects of four process variables, *viz.*, extraction pressure (X_1), extraction temperature (X_2), modifier concentration (X_3) and extraction time (X_4) were studied during experimentation. These conditions seemed to be varied depending on the response required. The results of 27 runs using the Box-Behnken design are presented in Table II. They include the design and experimental values. The Box-Behnken design with four factors and three levels, including three replicates at the center point, was used to fit a second--order response surface in order to optimize the extraction conditions. Three center point runs were carried out to measure the process stability and inherent variability.

Phyllanthin yield (mg g^{-1}) was selected as the response *Y*. The mathematical model describing the extraction yield of phyllanthin as a function of the coded independent variables (Table II) in the selected ranges was demonstrated by the following second--order polynomial equation:

$$Y = 7.81 - 0.38X_1 - 0.074X_2 + 5.04X_3 + 0.88X_4 - 1.69X_1^2 - 0.073X_2^2 - 2.05X_3^2 + 0.4X_4^2 - 0.28X_1X_2 - 0.65X_1X_3 + 0.13X_1X_4 - 0.072X_2X_3 - 0.43X_2X_4 + 1.16X_3X_4$$

where X_1, X_2, X_3 and X_4 are the coded variables for pressure, temperature, modifier concentration and extraction time, respectively.

Table I. Box-Behnken experimental design of variables

Independent variable	Coded symbol	Level		
		–1	0	1
Extraction pressure (MPa)	X_1	15	25	35
Extraction temperature (°C)	X_2	40	60	80
Modifier concentration (%)	X_3	0	5	10
Extraction time (min)	X_4	30	60	90

Table II. The Box-Behnken experimental design and the response for phyllanthin yield

Run No.	X_1	X_2	X_3	X_4	Yield (mg g^{-1}) [a]	
					Observed	Predicted
1	25	60	5	60	7.86 ± 0.27	7.80
2	15	40	5	60	6.17 ± 0.26	6.22
3	25	60	0	90	ND	0
4	25	80	10	60	10.67 ± 0.52	10.58
5	15	60	0	60	ND	0
6	35	60	10	60	7.44 ± 0.31	8.07
7	25	80	0	60	ND	0
8	15	60	5	90	6.03 ± 0.29	6.85
9	35	80	5	60	5.33 ± 0.21	5.31
10	25	80	5	30	7.07 ± 0.3	6.81
11	25	40	5	30	6.46 ± 0.19	6.09
12	25	60	10	90	12.99 ± 0.61	12.43
13	35	60	5	30	4.56 ± 0.2	4.31
14	35	60	0	60	ND	0
15	25	60	5	60	7.85 ± 0.38	7.80
16	25	80	5	90	7.92 ± 0.33	7.70
17	25	40	10	60	10.95 ± 0.46	10.87
18	15	60	5	30	5.04 ± 0.16	5.35
19	15	80	5	60	6.69 ± 0.24	6.63
20	25	60	10	30	8.37 ± 0.33	8.35
21	25	40	0	60	ND	0
22	35	60	5	90	6.09 ± 0.28	6.35
23	15	60	10	60	10.02 ± 0.47	10.13
24	25	60	0	30	ND	0
25	25	40	5	90	9.06 ± 0.43	8.72
26	25	60	5	60	7.71 ± 0.38	7.80
27	35	40	5	60	5.93 ± 0.25	6.01

[a] Mean ± SEM of three determinations.
ND – not detected

When a factor and an interaction among variables have a *p*-value lower than 0.05, it influences the process in a significant way at a confidence level of 95 % (27). The significance of the *F*-value depends on the number of degrees of freedom (DF) in the model.

The analysis of variance (ANOVA) (Table III) showed that this regression model was highly significant ($p < 0.0001$) with *F*-value of 58.55. The *F*-value of 73.15 for lack of fit

Table III. Analysis of variance (ANOVA) for the response surface quadratic model of P. amarus *extraction determined from the Box-Behnken experimental design*

	Sum of squares	DF[a]	Mean square	*F*-value	*p*-value
Model	357.77	14	25.56	58.55	< 0.0001
X_1	1.76	1	1.76	4.04	0.0674
X_2	0.066	1	0.066	0.15	0.7040
X_3	304.32	1	304.32	697.25	< 0.0001
X_4	9.35	1	9.35	21.43	0.0006
$X_1 X_2$	0.31	1	0.31	0.72	0.4140
$X_1 X_3$	1.66	1	1.66	3.81	0.0745
$X_1 X_4$	0.073	1	0.073	0.17	0.6902
$X_2 X_3$	0.02	1	0.02	0.047	0.8323
$X_2 X_4$	0.75	1	0.75	1.73	0.2136
$X_3 X_4$	5.35	1	5.35	12.27	0.0044
X_1^2	15.24	1	15.24	34.91	< 0.0001
X_2^2	0.028	1	0.028	0.064	0.8040
X_3^2	22.38	1	22.38	51.28	< 0.0001
X_4^2	0.87	1	0.87	1.99	0.1840
Residual	5.24	12	0.44		
Lack of fit	5.22	10	0.52	73.15	0.0136
Pure error	0.014	2	7.14×10^{-3}		
Cor total	363.01	26			
R^2	0.9856				
Adj R^2	0.9687				

[a] Degree of freedom.

implies that it is not significant comparing to the pure error. The fitness of the model was further confirmed by a satisfactory value of the determination coefficient, which was calculated to be 0.9856, indicating that 98.6 % of variability in the response could be predicted by the model. The value of the adjusted determination coefficient (adjusted R^2 = 0.9687) also confirmed that the model was highly significant. Also, the extraction yield predicted by the final quadratic model, along with the corresponding values, given in Table II, indicate that the agreement between the extraction yield predicted by the model and the experimental data is satisfactory, which suggests a good fit to the mathematical model.

As shown in Table III, variables with the largest effect were X_3 and X_4, followed by the other quadratic term coefficients X_1^2 and X_3^2, which were highly significant at $p <$ 0.0001. It is also evident from Table III that the interaction term coefficient X_3X_4 was also significant ($p < 0.05$). The other term coefficients did not influence the extraction yield significantly.

Effect of extraction conditions on phyllanthin yield

It is usually considered that the yield of target compounds with SC CO_2E is influenced by the extraction pressure, temperature, modifier concentration and time. The solubility of the solute in supercritical fluid depends on a complex balance between fluid density, solute vapour pressure and the repulsive solute-fluid interaction, which are controlled by temperature and pressure (27). When considering the effect of temperature on the solubility of solid compounds, two different effects can appear by changing the temperature. One is the increase in solid volatility with temperature rise, causing an increase of vapor pressure. In the case when vapor pressure is overwhelming, the solubility of solid compounds would increase with an increase in vapor pressure (28). However, if density effect is predominant, as the temperature increases at constant pressure, dissolving power decreases due to decreased density of supercritical CO_2 (27). Improvement of solubility by temperature is dependent on which effect prevails. As the pressure continues to increase, however, the repulsive solute-fluid interaction becomes stronger. When pressure reaches a certain value for some compounds, the repulsive solute-fluid interaction may become greater than the increase in solubility obtained from increased solvent density (by elevating pressure under constant temperature). In this situation, the solubility of compounds decreases. Lower solubility leads to a decrease in extraction yield. For example, under the optimized extraction conditions of 23.2 MPa pressure, modifier concentration of 10 % and extraction time of 90 min, the extraction yield of phyllanthin at 40 and 80 °C was 12.99 and 11.94 mg g^{-1}, respectively, while the supercritical CO_2 density was 0.87 and 0.66 g mL^{-1}, respectively (29). Increase in temperature at constant pressure resulted in a decrease in the supercritical CO_2 density, which led to a reduction in the extraction yield of phyllanthin.

Shorter extraction time could cause incomplete extraction and longer extraction time could be time and solvent wasting. Addition of a small amount of polar organic solvent (co-solvent) to the supercritical CO_2 can remarkably increase the extractability of target analytes. Various polar co-solvents have been tried over the years for the supercritical CO_2 extraction of polar constituents, but methanol has remained the most popular (30). Since the nature of the co-solvent may influence extraction yield and selectivity, three different co-solvents, *viz*,. methanol, ethanol and *n*-hexane were used during the preliminary SC CO_2E of *P. amarus*.

The relationship between the responses and experimental variables can be illustrated graphically to investigate interactions of the variables and to determine the optimal level of each variable for the maximum response by plotting three-dimensional response surface plots. Each plot shows a pair of factors by keeping the other factor constant at its middle level.

Fig. 4a is the three dimensional plot showing the effects of pressure and temperature on phyllanthin yield, while the modifier concentration and extraction time were fixed at their central levels. Higher yield was obtained at a pressure between 20 and 30 MPa. The highest yield of phyllanthin (12.99 mg g^{-1}) was attained at 25 MPa pressure and 60 °C temperature. When the temperature was increased from 40 to 60 °C at optimum values of pressure, modifier concentration and dynamic extraction time (23.2 MPa, 10 % and 90 min), the yield decreased from 12.99 to 12.42 mg g^{-1}. Increase in temperature from 60 to 80 °C did not enhance phyllanthin yield. In this study, the interaction between pressure and temperature was not found to be statistically significant.

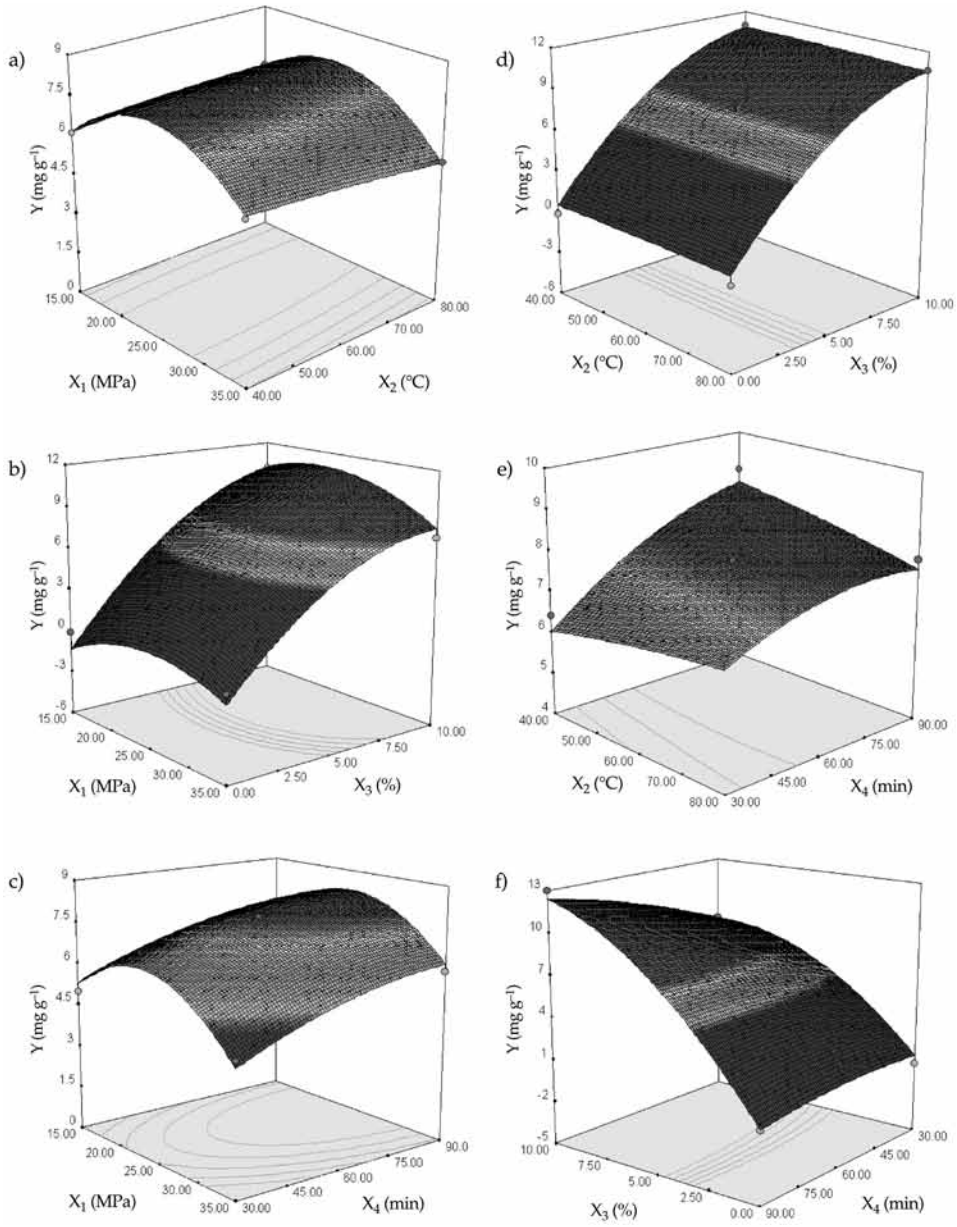

Fig. 4. Response surface plots showing the effects of two variables on the phyllanthin response yield (Y, mg g^{-1}), with the other two fixed at 0 level (X_1 – extraction pressure, X_2 – extraction temperature, X_3 – modifier concentration, X_4 – extraction time).

Considering the effects of pressure and modifier concentration on the yield of phyllanthin, higher yield was obtained at pressures below 30 MPa and modifier concentration above 5 %, as can be seen in Fig. 4b. Phyllanthin yield increased from 11.56 to 12.99 mg g^{-1} with an increase in pressure from 15 to 25 MPa, while keeping the temperature, modifier concentration and extraction time at optimum values (40 °C, 10 % and 90 min). The interaction between pressure and modifier concentration was not statistically significant for phyllanthin yield.

Fig. 4c indicates the effect of interactions between pressure and extraction time on phyllanthin yield. It can be observed from Fig. 4c that higher yield of phyllanthin was attained by setting pressure between 22.5 to 27.5 MPa. When the mathematical model was used to predict the yields that could be obtained using different extraction times, phyllanthin yield increased from 10.11 to 12.99 mg g^{-1} with an increase in extraction time from 50 to 90 min keeping the extraction pressure, temperature and modifier concentration at optimum values (23.2 MPa, 40 °C and 10 %). The interaction between pressure and extraction time was not statistically significant for phyllanthin yield.

It can be seen from Fig. 4d that higher yield of phyllanthin was attained by setting temperature between 50 and 70 °C and modifier concentration above 5 %. When the mathematical model was used to predict the yields that could be obtained using different modifier concentrations, phyllanthin yield increased from 8.74 to 12.99 mg g^{-1} with an increase in modifier concentration from 5 to 10 % keeping the extraction pressure, temperature and extraction time at optimum values (23.2 MPa, 40 °C and 90 min). The interaction between temperature and modifier concentration was not statistically significant for phyllanthin yield.

The 3D plot in Fig. 4e shows the effects of temperature and extraction time on phyllanthin yield. There was a rapid rise in phyllanthin yield with an increase in extraction time; however, phyllanthin yield was found to rise slightly with extraction temperature. The interaction between temperature and extraction time was not statistically significant for phyllanthin yield. Similarly, Fig. 4f shows the effects of modifier concentration and extraction time on phyllanthin yield. Phyllanthin yield was also significantly increased when increasing the modifier concentration above 5 % ($p < 0.05$). Phyllanthin yield was increased with extraction time increase above 50 min. In general, the interaction between modifier concentration and extraction time was found statistically significant for phyllanthin yield ($p < 0.05$).

Optimization of extraction parameters and model validation

Optimum values of selected variables were obtained using the response surface. In summary, the optimal conditions of SC CO_2E process for the highest phyllanthin yield were 23.2 MPa pressure, 40 °C temperature, 10 % modifier (methanol) concentration and 90 min extraction time. Suitability of the model equation for predicting the optimum response values was tested by executing three experiments under conditions of pressure, temperature, methanol concentration and extraction time. The experimental yield of phyllanthin from *P. amarus* (12.83 ± 0.28 mg g^{-1}) was close to the predicted yield (12.99 mg g^{-1}). The results indicate that the experimental values were in good agreement with the predicted values and that the regression model was accurate and adequate for the extraction process.

Comparison of SAE and SC CO_2E in terms of yield and extraction time

It was initially assumed that the conventional SAE would provide a maximum yield of phyllanthin. The Soxhlet assisted methanol extract of leaves of *P. amarus* powder resulted in 10.62 ± 0.35 mg g^{-1} phyllanthin yield after 24 h of extraction. The SC CO_2E showed 12.83 ± 0.28 mg g^{-1} recovery of phyllanthin after an extraction period of 90 min. Comparison of the yield and the time required for phyllanthin extraction demonstrated that the SC CO_2E technique is more efficient than the SAE technique. This could be attributed to the action of SC CO_2, which produces cell disruption leading to a larger contact area between solid and liquid phases and better access of the solvent to extractable components.

CONCLUSIONS

In this study, the effects of pressure, temperature, modifier concentration and extraction time were evaluated in order to develop an optimized SC CO_2E method. Response surface methodology using a Box-Behnken experimental design was successfully applied for optimization of supercritical carbon dioxide assisted extraction of phyllanthin from the leaves of *P. amarus*. High regression coefficients of the second-order polynomial of the response showed that the model fitted the data well. Modifier concentration and extraction time showed significant effects whereas the pressure above 30 MPa and temperature showed insignificant effects on phyllanthin yield.

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REFERENCES

1. K. M. Nadkarni, *Indian Materia Medica*, Popular Prakashan Pvt. Ltd., Vol. 1, Bombay 1976, pp. 946–948.
2. J. B. Calixto, A. R. S. Santos, V. C. Filho and R. A. Yunes, A review of the plants of genus *Phyllanthus*: Their chemistry, pharmacology and therapeutic potential, *Med. Res. Rev.* **18** (1998) 225–258; DOI: 10.1002/(SICI)1098-1128(199807)18:4<225::AID-MED2>3.0.CO;2-X.
3. S. Khatoon, V. Rai, A. K. S. Rawat and S. Mehrotra, Comparative pharmacognostic studies of three *Phyllanthus* species, *J. Ethnopharmacol*. **104** (2006) 79–86; DOI: 10.1016/j.jep.2005.08.048.
4. P. Padma and O. H. Setty, Protective effect of *Phyllanthus fraternus* against carbon tetrachloride-induced mitochondrial dysfunction, *Life Sci*. **64** (1999) 2411–2417; DOI: 10.1016/S0024-3205(99)00195-2.
5. H. Chirdchupunseree and P. Pramyothin, Protective activity of phyllanthin in ethanol-treated primary culture of rat hepatocytes, *J. Ethnopharmacol*. **128** (2010) 172–176; DOI: 10.1016/j.jep.2010.01.003.
6. R. Krithika, R. Mohankumar, R. J. Verma, P. S. Shrivastav, I. L. Mohamad, P. Gunasekaran and S. Narasimhan, Isolation, characterization and antioxidative effect of phyllanthin against CCl_4-induced toxicity in HepG2 cell line, *Chem. Biol. Inter.* **181** (2009) 351–358; DOI: 10.1016/j.cbi.2009.06.014.

7. V. Murugaiyah and K. L. Chan, Antihyperuricemic lignans from the leaves of *Phyllanthus niruri*, *Planta Med*. **72** (2006) 1262–1267; DOI: 10.1055/s-2006-947224.
8. A. Mazumder, A. Mahato and R. Mazumder, Antimicrobial potentiality of *Phyllanthus amarus* against drug resistant pathogens, *Nat. Prod. Res*. **20** (2006) 323-326; DOI: 10.1080/14786410600650404.
9. B. Gowrishanker and O. S. Vivekanandan, *In-vivo* studies of a crude extract of *Phyllanthus amarus* L. in modifying the genotoxicity induced in *Vicia faba* L. by tannery effluents, *Mutat. Res--Genet. Tox*. **322** (1994) 185–192.
10. C. A. L. Kassuya, D. F. P. Leite, L. V. De Melo, V. L. G. Rehder and J. B. Calixto, Anti-inflammatory properties of extracts, fractions and lignans isolated from *Phyllanthus amarus*, *Planta Med*. **71** (2005) 721–726; DOI: 10.1055/s-2005-871258.
11. M. Inchoo, H. Chirdchupunseree, P. Pramyothin and S. Jianmongkol, Endothelium-independent effects of phyllanthin and hypophyllanthin on vascular tension, *Fitoterapia* **82** (2011) 1231–1236; DOI: 10.1016/j.fitote.2011.08.013.
12. A. K. Tripathi, R. K. Verma, A. K. Gupta, M. M. Gupta and S. P. S. Khanuja, Quantitative determination of phyllanthin and hypophyllanthin in *Phyllanthus* species by high-performance thin layer chromatography, *Phytochem. Anal*. **17** (2006) 394–397; DOI: 10.1002/pca.936.
13. A. Annamalai and P. T. V. Lakshmi, HPTLC and HPLC analysis of bioactive phyllanthin from different organs of *Phyllanthus amarus*, *Asian J. Biotechnol*. **1** (2009) 154-162; DOI: 10.3923/ajbkr.2009.154.162.
14. J. Kasthuri, K. Kathiravan and N. Rajendiran, Phyllanthin-assisted biosynthesis of silver and gold nanoparticles: a novel biological approach, *J. Nanopart. Res*. **11** (2009) 1075–1085; DOI: 10.1007/s11051-008-9494-9.
15. V. Murugaiyah and K. L. Chan, Determination of four lignans in *Phyllanthus niruri* L. by a simple high-performance liquid chromatography method with fluorescence detection, *J. Chrom. A* **1154** (2007) 198–204; DOI: 10.1016/j.chroma.2007.03.079.
16. A. Alvari, M. S. Ohadi Rafsanjani, F. J. Ahmed, Mohd S. Hejazi and M. Z. Abdin, Rapid RP-HPLC technique for the determination of phyllanthin as bulk and its quantification in *Phyllanthus amarus* extract, *Int. J. Pytomed.* **3** (2011) 115–119.
17. K. Shanker, M. Singh, V. Srivastava, R. K. Verma, A. K. Gupta and M. M. Gupta, Simultaneous analysis of six bioactive lignans in *Phyllanthus* species by reversed phase hyphenated high performance liquid chromatographic technique, *Acta Chromatogr.* **23** (2011) 321–337; DOI: 10.1556/AChrom.23.2011.2.10.
18. C. W. Huie, A review of modern sample-preparation techniques for the extraction and analysis of medicinal plants, *Anal. Bioanal. Chem*. **373** (2002) 23–30; DOI: 10.1007/s00216-002-1265-3.
19. D. P. Fulzele and R. K. Satdive, Comparison of techniques for the extraction of the anti-cancer drug camptothecin from *Nothapodytes foetida*, *J. Chrom. A* **1063** (2005) 9–13; DOI: 10.1016/j.chroma.2004.11.020.
20. E. Reverchon and I. De Marco, Supercritical fluid extraction and fractionation of natural matter, *J. Supercrit. Fluids* **38** (2006) 146–166; DOI: 10.1016/j.supflu.2006.03.020.
21. P. Hamrapurkar, S. Pawar and M. Phale, Quantitative HPTLC analysis of phyllanthin in *Phyllanthus amarus*, *J. Planar. Chromatogr.* **23** (2010) 112–115; DOI: 10.1556/JPC.23.2010.2.4.
22. S. Khan, F. Al-Qurainy, M. Ram, S. Ahmad and M. Z. Abdin, Phyllanthin biosynthesis in *Phyllanthus amarus*: Schum and Thonn growing at different altitudes, *J. Med. Plants Res*. **4** (2010) 41–48.
23. International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use, *ICH Harmonised Tripartite Guideline, Validation of Analytical Procedures: Text and Methodology Q2(R1)*, Current Step 4 version, Geneva, November 2005; http://www.ich.org/fileadmin/Public_Web_Site/ICH_Products/Guidelines/Quality/Q2_R1/Step4/Q2_R1__Guideline.pdf; last access date February 1, 2012
24. D. C. Montgomery, *Design and Analysis of Experiments*, 4th ed., Wiley, New York 1997.

25. R. E. Santelli, M. A. Bezerra, O. D. Sant Ana, R. J. Cassella and S. L. C. Ferreira, Multivariate technique for optimization of digestion procedure by focused microwave system for determination of Mn, Zn and Fe in food samples using FAAS, *Talanta* **68** (2006) 1083–1088; DOI: 10.1016/j.talanta.2005.07.010.
26. S. Buco, M. Morgagues, M. Sergent, P. Doumenq and G. Mille, An experimental design approach for optimizing polycyclic aromatic hydrocarbon analysis in contaminated soil by pyrolyser-gas chromatography-mass spectrometry, *Environ. Res*. **104** (2007) 209–215; DOI: 10.1016/j.envres.2004.06.008.
27. A. A. Patil, B. S. Sachin, D. B. Shinde and P. S. Wakte, Supercritical CO_2 assisted extraction and LC–MS identification of picroside I and picroside II from *Picrorhiza kurroa*, *Phytochem. Anal.* **24** (2013) 97–104; DOI: 10.1002/pca.2383.
28. A. Erkucuk, I. H. Akgun and O. Yesil-Celiktas, Supercritical CO_2 extraction of glycosides from *Stevia rebaudiana* leaves: Identification and optimization, *J. Supercrit. Fluids* **51** (2009) 29–35; DOI: 10.1016/j.supflu.2009.07.002.
29. National Institute of Standards and Technology, *Thermophysical properties of fluid systems*, NIST, 2011; http://webbook.nist.gov/chemistry/fluid/; last access date May 29, 2012
30. M. L. Jeong and D. J. Chesney, Investigation of modifier effects in supercritical CO_2 extraction from various solid matrices, *J. Supercrit. Fluids* **16** (1999) 33–42; DOI: 10.1016/S0896-8446(99)00015-7.