

# Optimisation of asarone removal from *Piper sarmentosum* Roxburgh leaves using supercritical carbon dioxide extraction: the Box-Behnken design

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*Piper sarmentosum* is a herbaceous shrub with numerous pharmacological benefits. However, the presence of two toxic phenylpropanoids ( $\alpha$ - and  $\beta$ -asarone) limits the medicinal usage of the plant. In this study, the extraction of three asarone isomers, namely  $\alpha$ -,  $\beta$ -, and  $\gamma$ -asarone was optimised using supercritical carbon dioxide extraction (SC-CO<sub>2</sub>) combined with Box-Behnken experimental design. Comparison of asarone contents in different conventional solvent extracts of *P. sarmentosum* leaves prior to and after SC-CO<sub>2</sub> extraction was performed. The SC-CO<sub>2</sub> method successfully maximised the extraction of  $\alpha$ -,  $\beta$ -, and  $\gamma$ -asarone at  $P = 81.16$  bar,  $T = 50.11^\circ\text{C}$ , and  $t = 80.90$  min, yielding 13.91%  $\alpha$ -asarone, 3.43%  $\beta$ -asarone, and 14.95%  $\gamma$ -asarone. The SC-CO<sub>2</sub> residue of the leaves re-extracted with conventional solvents showed a significant decrease of asarone ranging from 45% to 100% ( $p < 0.001$ ) compared to their counterparts without SC-CO<sub>2</sub> treatment.  $\alpha$ -,  $\beta$ -, and  $\gamma$ -asarone were completely removed in the ethanol extract of the residue. These findings suggested that the optimised SC-CO<sub>2</sub> extraction parameters may serve as a quick treatment step for the selective removal of asarone from *P. sarmentosum* to develop safer extracts for the food and nutraceutical industries applications.

**Keywords:** *Piper sarmentosum*. Supercritical fluid extraction. Asarone. Optimisation. Box-Behnken. HPLC.

## INTRODUCTION

*Piper sarmentosum* Roxburgh, which belongs to the Piperaceae family, is a herbaceous shrub widely distributed in Southeast Asia with several pharmacological activities such as antioxidant (Sumazian *et al.*, 2010), anti-amoebic (Sawangjaroen, Sawangjaroen, Poonpanang, 2004), antibacterial (Masuda *et al.*, 1991), neuromuscular blocking (Riditid *et al.*, 1998), anti-malaria (Rahman *et al.*, 1999), hypoglycaemic (Steinrut, Itharat, 2014), anti-tuberculosis (Hussain *et al.*, 2009), anticancer (Ariffin

*et al.*, 2009), and anti-angiogenic activity (Hussain *et al.*, 2008). Several amide alkaloids, phenylpropanoids ( $\alpha$ -,  $\beta$ , and  $\gamma$ -asarone, Figure 1), lignans, sterols, and flavonoids were identified from the plant (Parmar *et al.*, 1997; Subramaniam *et al.*, 2003). Among the chemical constituents reported, two phenylpropanoids, i.e.,  $\alpha$ - and  $\beta$ -asarone, were reported to exhibit insecticidal, fungicidal, and neuroprotective activities (Cho *et al.*, 2002; Park, Kim, Ahn, 2003; Shenvi *et al.*, 2011). Nonetheless, they were also reported having carcinogenic, cytotoxic, and genotoxic compounds (Unger, Melzig, 2012; Cartus, Schrenk, 2016). Both asarones are regulated in food and herbal products to ascertain product safety (European Medicine and Health Agency, 2005). Therefore, an extraction technique to reduce the asarone isomers in herbal material is important for developing safer extracts in herbal remedy preparation.

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preliminary screening of asarone, three extracts were obtained using different pressure values. Experimental parameters were set as follows: (1) 50°C/100 bar/120 min dynamic extraction time; (2) 50°C/300 bar/120 min dynamic extraction time; and (3) 50°C/700 bar/120 min dynamic extraction time. Optimisation conditions were subsequently designed as follows: pressure (80, 115, and 150 bar), temperature (40°C, 50°C, and 60°C), and dynamic extraction time (30, 75, and 120 min). Static extraction time was fixed at 30 min, with a constant flow rate at 30 g/min and plant mass of 50 g for all conditions. The collection vessel and chiller temperature were set at 50°C and 0°C, respectively. SC-CO<sub>2</sub> crude extracts were collected in a vial (10 mL) and kept at 4°C prior to analysis. The experiments were performed in triplicate.

### Conventional extraction

Approximately 10 g of ground leaves were mixed with 200 mL (1:20) n-hexane, chloroform, ethyl acetate, acetone, methanol and ethanol and macerated at 50°C for 24 hours. The extracts were filtered and concentrated using a rotary evaporator and kept at 4°C before analysis. The same method was applied for the extraction of SC-CO<sub>2</sub> residue. The experiments were performed in triplicate.

### Isolation of $\gamma$ -asarone

Approximately 18 g of SC-CO<sub>2</sub> extract was subjected to flash column chromatography using the increasing ratio of ethyl acetate in n-hexane (0:100 to 100:0) to obtain 30 fractions. The asarone-rich fraction (F12) was further purified using Shimadzu LC-20AP preparative HPLC (Shimadzu Corporation, Japan) on PrepHT Phenyl-Hexyl Preparative Cartridge (21.2 × 250 mm, 5  $\mu$ m) column (Agilent Technologies, USA) with 0.1% ortho-phosphoric acid, acetonitrile and methanol as mobile phase. Meanwhile,  $\gamma$ -asarone was characterised using UV-Vis, FT-IR, GC-MS, and NMR.

### HPLC analysis

Quantification of asarone in the *P. sarmentosum* extracts was performed using HPLC described by

Hamil *et al.* (2016). Briefly, the analysis was performed on Agilent Technologies 1260 Infinity HPLC system (USA). Elution was achieved using an isocratic mobile phase consisting of 0.1% ortho-phosphoric acid:acetonitrile:methanol (50:40:10 v/v/v), with a flow rate of 1 mL/min on Zorbax Eclipse Plus C-18 column (250 × 4.6 mm, 5  $\mu$ m; Agilent Technologies, USA). The injection volume was 10  $\mu$ L, the column temperature was maintained at 30°C, and detection was fixed at 210 nm.

### Box-Behnken Design

The experiment was performed based on the developed design using Design Expert® (Version 7.1.5, Stat-Ease Inc, Minneapolis). In this study, the Box-Behnken design consisting of 17 runs, 3 factors, and 3 levels were employed for constructing a polynomial model for optimisation of the maximum extraction of  $\alpha$ -,  $\beta$ -, and  $\gamma$ -asarone.

## RESULTS AND DISCUSSION

### Identification of $\gamma$ -asarone

Compound **3** (21.4 mg) was obtained as a viscous liquid. UV-Vis showed maximum absorption at  $\lambda_{\text{max}}$  205, 234, and 291 nm. The FT-IR spectra showed vibrational signals at 2926, 1710, 1641, and 912 cm<sup>-1</sup> associated with the methylene group of CH<sub>2</sub> and CH<sub>3</sub>; 1610, 1512, 860, and 754 cm<sup>-1</sup> attributed to 2,4,5-tetra substituted moiety; and 1205, 1178, and 1037 cm<sup>-1</sup> due to the C-O-C stretching of phenolic ether. <sup>1</sup>H NMR (CDCl<sub>3</sub>) showed signals at  $\delta$  6.71 (1H, s, H-6), 6.55 (1H, s, H-3), 6.01 (1H, m, H-2'), 5.08 (2H, m, H-3'), 3.90 (3H, s, 2-OCH<sub>3</sub>) 3.85 (3H, s, 4-OCH<sub>3</sub>), 3.82 (3H, s, 5-OCH<sub>3</sub>), and 3.35 (2H, d,  $J$  = 6.5 Hz, H-1'); the <sup>13</sup>C NMR (CDCl<sub>3</sub>) spectra showed signals at  $\delta$  151.34 (C-2), 147.92 (C-4), 143.04 (C-5), 137.32 (C-2'), 120.07 (C-1), 115.18 (C-3'), 114.02 (C-6), 98.08 (C-3), 56.62 (4-OCH<sub>3</sub> & 5-OCH<sub>3</sub>), 56.25 (2-OCH<sub>3</sub>), and 33.65 (C-1'); EIMS analysis indicated a molecular formula C<sub>12</sub>H<sub>16</sub>O<sub>3</sub> ( $m/z$  208.1 [M]<sup>+</sup>), and fragmentation ions at 193, 181, 165, 124, 91, and 69. Based on the spectral data and comparing with reported literature (Sinha, Acharya, Joshi, 2002; Varma *et al.*,

2002), **3** was identified as  $\gamma$ -asarone (Data presented in supplementary section).

### Extraction yield of asarone using SC-CO<sub>2</sub> and conventional solvent extraction

The extraction yield of  $\alpha$ -,  $\beta$ -, and  $\gamma$ -asarone using SC-CO<sub>2</sub> was compared with those using n-hexane, chloroform, acetone, ethyl acetate, methanol, and ethanol as extraction solvents. A total of nine extracts were obtained. Asarone was quantified using HPLC, and the results are listed in Table I. Our findings showed that  $\alpha$ -asarone was the dominant isomer in *P. sarmentosum*, followed by  $\gamma$ -asarone. In contrast,  $\beta$ -asarone was found in a smaller amount in all extracts analysed. For the conventional solvent extraction,  $\alpha$ -,  $\beta$ -, and  $\gamma$ -asarone were extracted at the highest amount using n-hexane (7.48%, 1.22%, and 3.81%), followed by acetone, chloroform, ethyl acetate, methanol, and ethanol. Total asarone content extracted from the conventional

solvents ranged from 2.03% to 12.51%. For the SC-CO<sub>2</sub> extraction, a set of different pressure values were studied to determine the ideal condition for the highest asarone extraction. A negative correlation between pressure and asarone was observed, whereby asarone recovery was increased as the pressure decreased. This effect was observed by manipulating the pressure as a variable, and other parameters were fixed. SFE100 yielded the highest asarone content with 13.52%, 3.03%, and 13.84% for  $\alpha$ -,  $\beta$ -, and  $\gamma$ -asarone. Total asarone yield for SC-CO<sub>2</sub> ranged from 26.11% to 30.39%, more than two-fold compared to other solvent extraction methods. Asarone is a volatile compound that belongs to the phenylpropanoid group. The compounds showed favourable solubility in non-polar solvent compared to solvent at a higher polarity (Hamil *et al.*, 2016). From the preliminary screening, we found that SC-CO<sub>2</sub> extraction at a lower pressure was the most effective method to maximise recovery of asarone; thus, further optimised using the response surface model.

**TABLE I** - The comparison of the extraction yield of  $\alpha$ -,  $\beta$ -, and  $\gamma$ -asarone in *P. sarmentosum* extracted using SC-CO<sub>2</sub> and conventional solvents

Solvent	Extraction yield (%)	$\alpha$ -asarone (%)	$\beta$ -asarone (%)	$\gamma$ -asarone (%)	Total asarone content (%) <sup>a</sup>
Hexane	1.70 ± 0.03	7.48 ± 0.06	1.22 ± 0.001	3.81 ± 0.36	12.51 ± 0.42
Chloroform	3.30 ± 0.02	6.69 ± 0.01	0.42 ± 0.02	1.53 ± 0.002	8.64 ± 0.03
Acetone	2.68 ± 0.03	5.26 ± 0.01	1.03 ± 0.01	2.23 ± 0.002	8.52 ± 0.001
Ethyl acetate	2.80 ± 0.12	3.14 ± 0.001	0.15 ± 0.003	1.05 ± 0.003	4.34 ± 0.004
Methanol	9.58 ± 0.02	2.71 ± 0.01	0.15 ± 0.001	0.68 ± 0.001	3.54 ± 0.01
Ethanol	7.11 ± 0.05	1.66 ± 0.002	0.08 ± 0.003	0.29 ± 0.002	2.03 ± 0.003
SFE100	1.15 ± 0.02	13.52 ± 0.03	3.03 ± 0.01	13.84 ± 0.05	30.39 ± 0.10
SFE300	2.22 ± 0.01	13.27 ± 0.01	2.79 ± 0.06	12.85 ± 0.004	28.91 ± 0.01
SFE700	2.89 ± 0.01	11.43 ± 0.02	2.00 ± 0.01	12.68 ± 0.07	26.11 ± 0.10

SFE100: SC-CO<sub>2</sub> at 100 bar; SFE300: SC-CO<sub>2</sub> at 300 bar; SFE700: SC-CO<sub>2</sub> at 700 bar; <sup>a</sup>: sum of  $\alpha$ -,  $\beta$ - and  $\gamma$ -asarone.

### Optimisation of SC-CO<sub>2</sub> using Box-Behnken experimental design

The Box-Behnken experimental design was developed to optimise the SC-CO<sub>2</sub> extraction for maximum recovery of  $\alpha$ -,  $\beta$ -, and  $\gamma$ -asarone from *P. sarmentosum* leaves. The various parameters of design and asarone yield are summarised in Table II. Several variables that could potentially influence the extraction efficiency were chosen, such as pressure ( $P$ ), temperature ( $T$ ), and dynamic extraction time ( $t$ ). The results obtained from the Box-Behnken experimental design provided a statistical model used to identify asarone solubility patterns from the extraction process. The equation below illustrated the relationship between the three variables:  $P$ ,  $T$ , and  $t$  with asarone content.

Equation for  $\alpha$ -asarone:

$$Y = 15.98 - 0.85(P) - 0.086(T) - 0.019(t) + 0.58(P)(T) - 0.54(P)(t) + 0.075(T)(t) - 3.11(P^2) - 2.83(T^2) - 3.03(t^2) \quad (1)$$

Equation for  $\beta$ -asarone:

$$Y = 3.07 - 0.57(P) + 0.20(T) - 0.026(t) + 0.12(P)(T) - 0.097(P)(t) - 0.055(T)(t) - 0.21(P^2) - 0.52(T^2) - 0.32(t^2) \quad (2)$$

Equation for  $\gamma$ -asarone

$$Y = 10.75 - 2.69(P) + 0.63(T) - 0.24(t) - 0.22(P)(T) - 0.87(P)(t) - 0.22(T)(t) + 1.41(P^2) - 0.22(T^2) + 0.28(t^2) \quad (3)$$

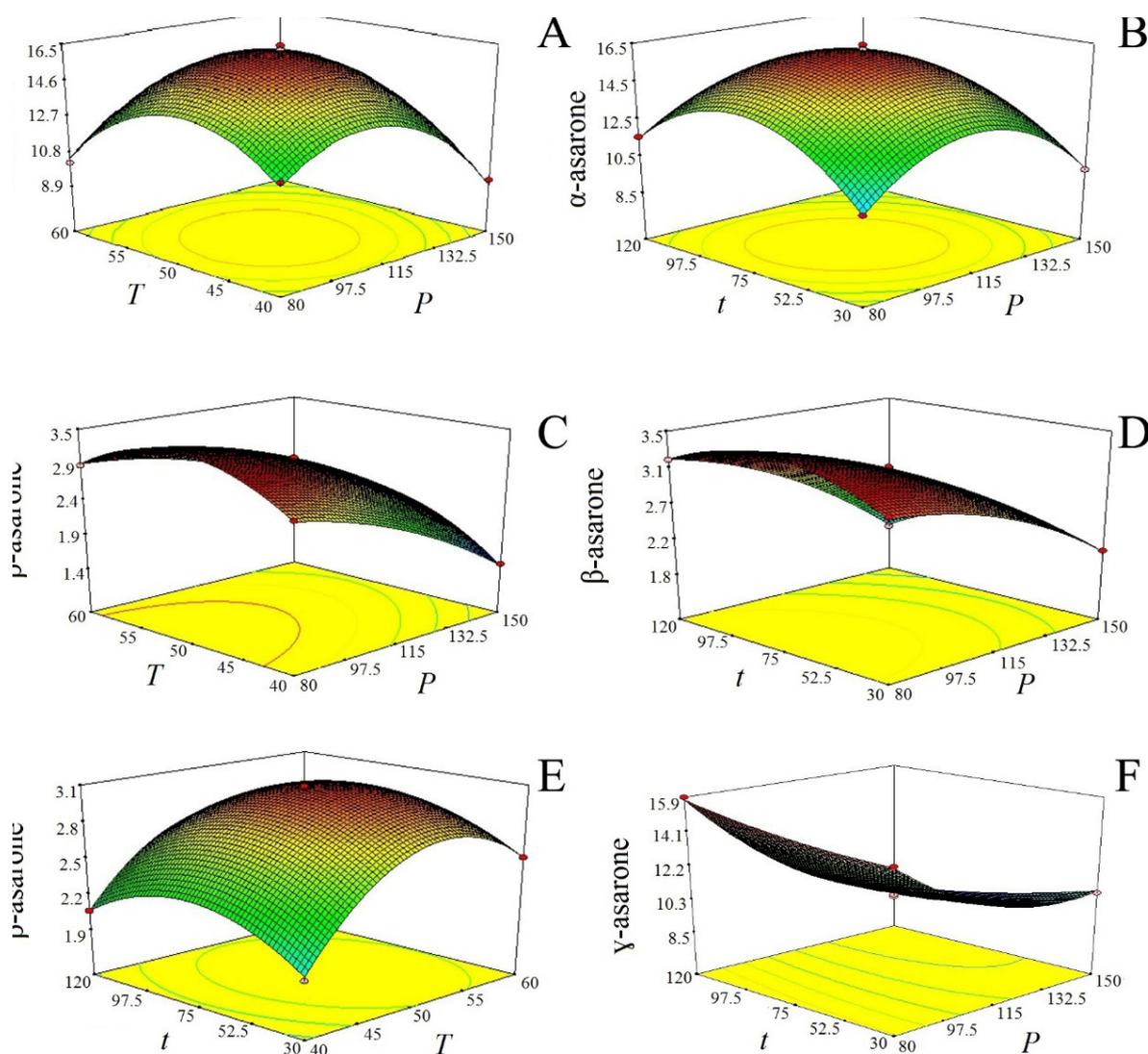
**TABLE II** - Box-Behnken experimental design order for optimisation of  $\alpha$ -,  $\beta$ -, and  $\gamma$ -asarone in *P. sarmentosum*

Run Order	Pressure (bar)	Temperature (°C)	Time (min)	Yield (%)	$\alpha$ -asarone (%)	$\beta$ -asarone (%)	$\gamma$ -asarone (%)
1	115	50	75	1.13	16.14	3.05	11.01
2	115	60	30	0.93	10.24	2.51	12.10
3	115	40	30	0.93	10.07	1.97	10.07
4	115	50	75	1.12	15.72	3.09	10.62
5	80	50	30	0.29	10.20	3.06	14.54
6	80	40	75	0.48	11.77	2.85	13.86
7	80	50	120	0.64	11.28	3.17	15.87
8	150	50	120	2.09	8.40	1.81	8.60
9	115	60	120	1.33	10.32	2.38	11.10
10	115	50	75	1.12	15.93	3.07	10.53
11	150	60	75	2.01	9.49	2.08	9.58
12	115	40	120	1.60	9.85	2.06	9.96
13	150	50	30	1.11	9.49	2.09	10.75
14	115	50	75	1.10	16.04	3.06	10.81
15	115	50	75	1.08	16.09	3.08	10.77
16	80	60	75	0.45	9.94	2.97	15.23
17	150	40	75	1.83	8.99	1.47	9.08

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By computation, the optimal points to maximise the extraction of three asarone isomers were predicted as follows:  $P = 81.16$  bar,  $T = 50.11^\circ\text{C}$ , and  $t = 80.90$  min, which yielded 13.91%  $\alpha$ -asarone, 3.43%  $\beta$ -asarone, and 14.95%  $\gamma$ -asarone. The percentage yield of SC-CO<sub>2</sub> extract at the optimised parameter was 0.54%. The predicted optimal points were validated by running the extraction using these conditions in triplicate. The percentage yield of the extract was 0.55%. A mean value of 13.99%, 3.44%, and 14.93% ( $\alpha$ -,  $\beta$ -, and  $\gamma$ -asarone) were obtained, which were in agreement with the predicted values of the asarone isomers at  $p > 0.05$ . The experimental results confirmed

that the response model was adequate for reflecting the expected optimisation with satisfactory accuracy. Figure 2 shows the contour plot of the percentage yields of  $\alpha$ -,  $\beta$ -, and  $\gamma$ -asarone against the independent variables analysed. At the constant temperature of  $50^\circ\text{C}$ ,  $\alpha$ -asarone in the extracts increased from 14.03% to 15.99%, as the pressure increased to 115 bar. After this point,  $\alpha$ -asarone was observed to decrease gradually as the pressure was increased. However, the opposite trend was observed for  $\beta$ - and  $\gamma$ -asarone, which showed decreasing recovery from 3.43% to 3.08% and 14.79% to 10.75% for the same operating parameters.



**FIGURE 2** - Contour plot of (A) pressure against temperature for  $\alpha$ -asarone, (B) pressure against extraction time for  $\alpha$ -asarone, (C) pressure against temperature for  $\beta$ -asarone, (D) pressure against extraction time for  $\beta$ -asarone, (E) temperature against time for  $\beta$ -asarone, (F) pressure against extraction time for  $\gamma$ -asarone.

The increase in temperature showed that the improved extraction was successful for all three asarone isomers until the optimal temperature at 50.11°C was reached. There was a decrease in the percentage at a constant optimal pressure of 81.16 bar after this point. Temperature plays an important role in SC-CO<sub>2</sub> extraction. Generally, an increase in temperature tends to reduce the CO<sub>2</sub> density; thus, decreasing the efficiency of SC-CO<sub>2</sub> extraction. However, as the temperature rises, the vapour pressure of highly volatile asarone increased, resulting in increased solubility until an optimal temperature was achieved (Dai, Ha, Shen, 2008). After the optimal temperature, a further increase in temperature did not improve asarone extraction. This could be due to the reduced solubility as the density decreased. On the other hand, a higher temperature can also be attributed to the degradation of thermally labile compounds (Ahmadian-Kouchaksaraie, Niazmand, 2017).

At the constant pressure of 81.16 bar, the increase of dynamic extraction time until 80.90 min enhanced  $\alpha$ -,  $\beta$ -, and  $\gamma$ -asarone percentage from 10.43% to 13.92%, 3.05% to 3.43%, and 14.35% to 14.95%. As the extraction time was prolonged, the percentage of  $\alpha$ -asarone was decreased gradually while  $\beta$ -asarone was slightly decreased. In contrast, the percentage of  $\gamma$ -asarone continue to increase to 120 min (15.51%). An excessive time is not efficient in maximising the extraction of all the asarone isomers. According to Chen, Zhao, and Yu (2003), a longer extraction time was not favourable for asarone, as other compounds will be co-extracted with it. Therefore, an optimised extraction time is crucial to maximising the extraction of asarone with minimal effect on other chemical compounds. We managed to obtain

32.29% of total asarone from the predicted optimised conditions, with an extraction yield of less than 0.6%.

### Statistical analysis

The analysis of variance (ANOVA) was performed to fit the model for each variable. The statistical analysis for the regression of coefficients of different factors in all models is shown in Table III. According to the ANOVA, the F-values indicated that the regression equation might explain most response variations. This indicated that the model term is significant at a 95% confidence interval. It was observed that  $P$  significantly influenced the recovery of  $\alpha$ -,  $\beta$ -, and  $\gamma$ -asarone ( $p < 0.0001$ ) in their linear models with negative coefficient values. On the other hand,  $T$  and  $t$  significantly influenced the extraction of  $\beta$ - and  $\gamma$ -asarone with  $p < 0.0001$  and  $p < 0.05$  in their linear forms. The model adequacy was calculated using the coefficient of determination and lack-of-fit test. The model was statistically significant, with a satisfactory coefficient of determination ( $R^2 = 0.9945-0.9989$ ). In addition, the values of adjusted determination of coefficients ( $_{adj}R^2 = 0.9874-0.9974$ ) also indicate that the model adequacy is highly significant. The lack-of-fit test did not show significant differences for all three asarones. The interaction effects of  $P$  and  $T$  significantly influenced the extraction of  $\alpha$ - and  $\beta$ -asarone in the quadratic forms. Significant interactions were observed between  $P$  and  $t$  for all asarone isomers ( $p < 0.01$  to  $p < 0.001$ ). The interaction between  $T$  and  $t$  was significant ( $p < 0.01$ ) for the extraction of  $\beta$ -asarone only. Furthermore, the coefficient of variation (CV) was within the acceptable range (1.11–2.52), indicating that the model exhibited better reproducibility.

**TABLE III** - Statistical variable for optimisation of *P. sarmentosum* SC-CO<sub>2</sub> extraction

Variables	$\alpha$ -asarone		$\beta$ -asarone		$\gamma$ -asarone	
	<i>F</i>	<i>p</i>	<i>F</i>	<i>p</i>	<i>F</i>	<i>p</i>
Model	170.60	<0.0001	685.45	<0.0001	140.54	<0.0001
Linear						
<i>P</i>	66.20	<0.0001	3262.56	<0.0001	989.80	<0.0001

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**TABLE III** - Statistical variable for optimisation of *P. sarmentosum* SC-CO<sub>2</sub> extraction

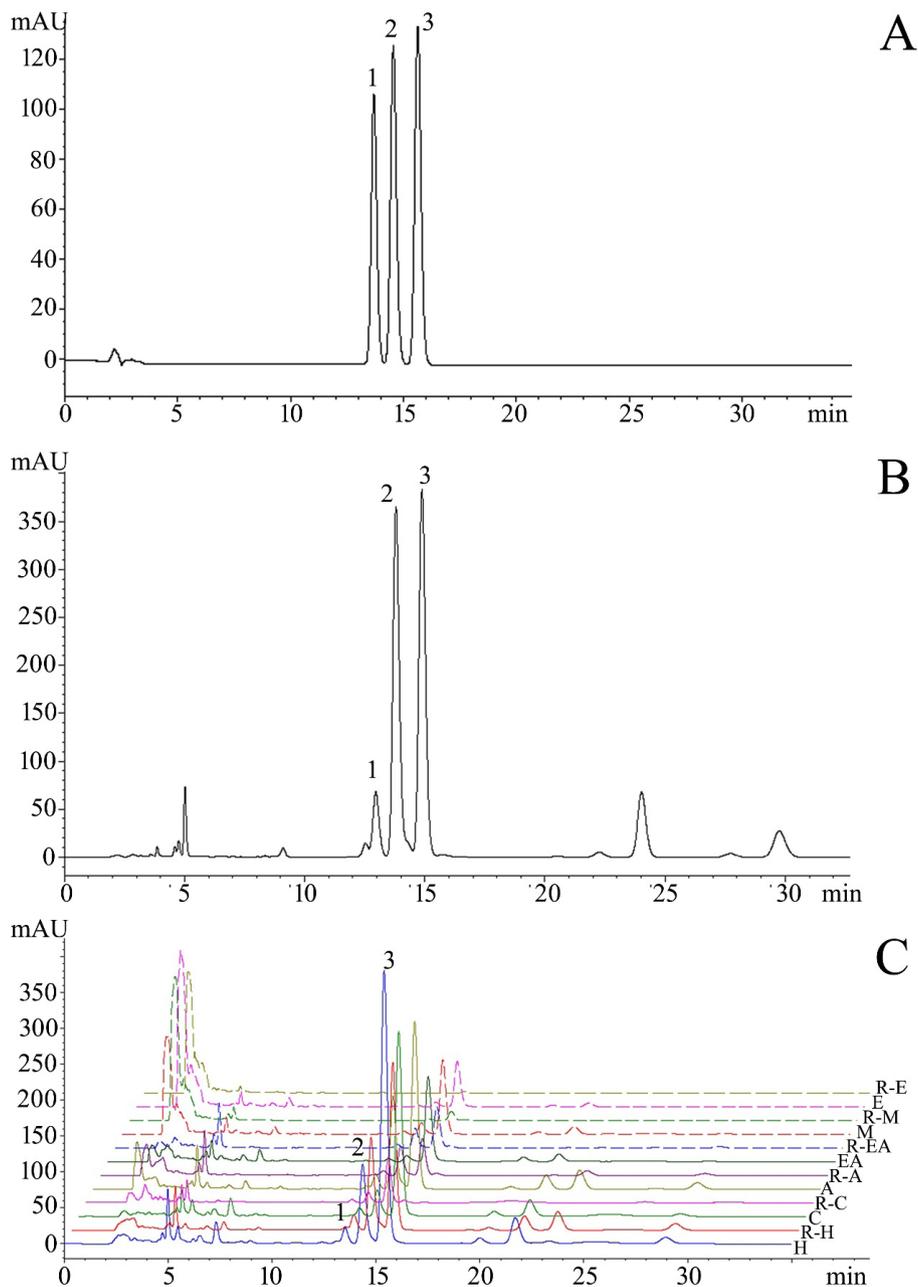
Variables	$\alpha$ -asarone		$\beta$ -asarone		$\gamma$ -asarone	
	<i>F</i>	<i>p</i>	<i>F</i>	<i>p</i>	<i>F</i>	<i>p</i>
<i>T</i>	0.68	0.4375	389.80	<0.0001	54.44	0.0002
<i>t</i>	0.032	0.8630	6.80	0.0350	7.98	0.0256
Quadratic						
<i>P</i> <sup>2</sup>	462.83	<0.0001	234.52	<0.0001	143.73	<0.0001
<i>T</i> <sup>2</sup>	383.85	<0.0001	1377.47	<0.0001	3.54	0.1019
<i>t</i> <sup>2</sup>	441.48	<0.0001	548.57	<0.0001	5.70	0.0483
Interaction						
<i>PT</i>	15.45	0.0057	74.04	<0.0001	3.24	0.1147
<i>Pt</i>	13.40	0.0081	46.90	0.0002	51.91	0.0002
<i>Tt</i>	0.26	0.6283	14.93	0.0062	3.40	0.1079
<i>R</i> <sup>2</sup>	0.9955	-	0.9989	-	0.9945	-
<i>R</i> <sub>adj</sub> <sup>2</sup>	0.9896	-	0.9974	-	0.9874	-
<i>Lack of fit</i>	6.03	0.0576	6.23	0.0547	2.64	0.1855
<i>CV%</i>	2.52	-	1.11	-	2.11	-

*P*: pressure; *T*: temperature; *t*: extraction time; *F*: Fisher-test value; *p*: significant level.

### HPLC analysis of SC-CO<sub>2</sub> residue

The content of asarone in the extracts varies, as depicted in Figure 3. In this study, *P. sarmentosum* powder residue from the optimised SC-CO<sub>2</sub> was extracted with conventional solvents to compare the quantity of asarone with their relative extracts without SC-CO<sub>2</sub> extraction. Extraction yields are slightly decreased from the relative solvent extracts; however, there is a significant decline in asarone contents ( $p < 0.001$ ), with the percentage of removal ranging from 45% to 100% in the extracts (Table

IV).  $\beta$ -asarone was removed successfully in the residue re-extracted with methanol and ethanol. Interestingly, the optimised SC-CO<sub>2</sub> extraction could remove all three asarone isomers in R-ethanol. This indicated the potential of SC-CO<sub>2</sub> to remove asarone from *P. sarmentosum* leaves effectively. In addition, the yield of optimised SC-CO<sub>2</sub> extract was about 0.5%, indicating minimal changes in the constituents and properties of the plant. However, further study is needed to compare the metabolite profile and pharmacological activities between SC-CO<sub>2</sub> residue and the extracts without SC-CO<sub>2</sub> treatment.



**FIGURE 3** - HPLC chromatogram of (A) mix asarone standard; 1=β-asarone, 2=γ-asarone, 3=α-asarone; (B) optimised SC-CO<sub>2</sub> extract; (C) overlay chromatogram of *P. sarmentosum* extracts with and without SC-CO<sub>2</sub> treatment; H=hexane, R-H=R-hexane, C=chloroform, R-C=R-chloroform, A=acetone, R-A=R-acetone, EA=ethyl acetate, R-EA=R-ethyl acetate, M=methanol, R-M=R-methanol, E=ethanol and R-E=R-ethanol.

**TABLE IV** - Percentage removal of  $\alpha$ -,  $\beta$ - and  $\gamma$ -asarone from *P. sarmentosum* SC-CO<sub>2</sub> residue as compared to the respective solvent extracts

Parameter	Yield (%)	Asarone content (%)				Percentage removal (%)			
		$\alpha$ -asarone	$\beta$ -asarone	$\gamma$ -asarone	Total asarone	$\alpha$ -asarone	$\beta$ -asarone	$\gamma$ -asarone	Total asarone
Optimised SC-CO <sub>2</sub>	0.55±0.02	13.99±0.01	3.44±0.005	14.93±0.01	32.36±0.01	NA	NA	NA	NA
R-Hexane	0.96±0.06	4.49±0.004*	0.61±0.01*	1.78±0.001*	6.88±0.01*	39.97±0.50	50.00±0.87	53.28±4.53	45.00±1.80
R-Chloroform	2.55±0.04	1.42±0.004*	0.16±0.002*	0.52±0.001*	2.10±0.001*	78.77±0.02	61.90±1.56	66.01±0.003	75.69±0.06
R-Acetone	2.43±0.06	1.03±0.001*	0.09±0.005*	0.24±0.001*	1.36±0.001*	80.42±0.003	91.26±0.01	89.24±0.03	84.04±0.001
R-Ethyl Acetate	2.61±0.04	0.60±0.002*	0.04±0.003*	0.39±0.004*	1.03±0.01*	78.57±0.05	73.33±0.28	62.86±0.49	74.06±0.16
R-Methanol	9.16±0.09	0.33±0.01*	NA	0.08±0.01*	0.41±0.01*	87.82±0.26	100±0.00	88.24±0.88	88.42±0.37
R-Ethanol	6.97±0.04	NA	NA	NA	NA	100±0.00	100±0.00	100±0.00	100±0.00

NA: not available; R: SC-CO<sub>2</sub> residue; \* indicated significant different as compared to the solvent extracts without SC-CO<sub>2</sub> treatment at  $p < 0.001$ . Percentage removal was calculated using the following formula:  $[1 - (\text{organic solvent extract after SC-CO}_2 / \text{organic solvent extract before SC-CO}_2)] \times 100$ .

Ethanol is a very polar molecule due to its hydroxyl group, with a high electronegativity of oxygen that allows hydrogen bonding to occur with other molecules. Ethanol is the best solvent for extracting phenolics, flavonoids, and alkaloids from a sample matrix (Ivanovska, Philipov, 1996; Do *et al.*, 2014). In the SC-CO<sub>2</sub> system, the addition of ethanol as a co-solvent is commonly used to increase the solubility of polar compounds (Dobbs *et al.*, 1987). In this study, we compared the solubility of asarone in organic solvents with different polarities. As we increased the polarity of solvents from n-hexane to ethanol, the asarone content in the extracts was gradually decreased. After the SC-CO<sub>2</sub> treatment, the amount of asarone and non-polar components in the sample were substantially decreased as CO<sub>2</sub> removed them. According to Vatai, Škerget, and Knez (2009), the high-pressure SC-CO<sub>2</sub> could break the plant cell walls, resulting in an abundant release of phenolic compounds. As more metabolites are available in the samples, they readily available to be extracted by ethanol, which produces an asarone-free extract.

Several studies have reported that  $\alpha$ - and  $\beta$ -asarone possess psychoactive, carcinogenic, genotoxic, and cytotoxic properties. Their presence was reported in *Acorus calamus*, *Acorus gramineus*, *Asarum europaeum*, *Guatteria gaumeri*, and *Piper sarmentosum* up to 95% in various extracts and essential oils (Authority, 2009; Hamil

*et al.*, 2016). The administration of products with a high level of asarone could pose health issues among consumers with observed side effects such as tachycardia, dizziness, tremor, irregular breathing, pallor, anxiety, nausea, and vomiting. (Zuba, Bryska, 2012). Several regulatory bodies developed the guidelines on the maximum intake of asarone to ascertain safe asarone-related products. The cut-off value for  $\beta$ -asarone in alcoholic products that contained calamus was limited to 0.5 mg/kg (Council of Europe, 2005). According to the European Medicine and Health Agency (2005),  $\alpha$ - and  $\beta$ -asarone should be reduced as minimum as possible. Permissible daily intake of herbal products containing  $\beta$ -asarone should be less than 115  $\mu\text{g/day}$  or 2  $\mu\text{g/kg bw/day}$ .

## CONCLUSION

In this study, SC-CO<sub>2</sub> extraction was optimised using the Box-Behnken experimental design for maximum removal of  $\alpha$ -,  $\beta$ -, and  $\gamma$ -asarone from *P. sarmentosum* leaves. Optimised extraction conditions using Box-Behnken experimental design was achieved at  $P = 81.16$  bar,  $T = 50.11^\circ\text{C}$ , and  $t = 80.90$  min, which yielded 13.91%  $\alpha$ -asarone, 3.43%  $\beta$ -asarone, and 14.95%  $\gamma$ -asarone. The HPLC data indicated that all three asarone isomers were reduced significantly ( $p < 0.001$ ) in the residue extracted

with conventional solvents compared to their respective extracts without SC-CO<sub>2</sub> treatment. The asarone-free extract was obtained from SC-CO<sub>2</sub> residue extracted using ethanol. It can be concluded that the optimised SC-CO<sub>2</sub> technique may serve as a quick treatment step for the removal of asarone from *P. sarmentosum* to develop safer extracts for the food and nutraceutical industry applications.

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