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The extraction of phytochemicals from Mimosa pudica Linn (MPL) using supercritical CO_2(SC-CO_2) has been studied, and the results are presented in this article. The significant variables affecting this extraction were investigated by design of experiment analysis. The effect of temperature and pressure on phytochemical yields was explored by measuring the total phenolic content (TPC), total flavonoid content (TFC), and total alkaloid compound (TAC). The maximum yield of TPC, TFC, and TAC extracted from MPL was 74.83 mg gallic acid equivalent (GAE)/g dry MPL, 30.93 mg quercetin equivalent/g dry MPL, and 14.23 mg hyoscyamine (HE)/g dry MPL, respectively. The work of Chrastil was used to correlate the experimental data. It was found that temperature and pressure greatly affected the yield of phytochemicals, whereas the effect of extraction time on the extraction yield diminished after 2 h. Qualitative analysis of the phytochemicals extracted from MPL was performed by high-performance liquid chromatography (HPLC). The SC-CO_2 extraction was more selective than Soxhlet extraction based on the HPLC spectra.

Keywords: Alkaloid; Extraction; Flavonoid; *Mimosa pudica* Linn; Phenolic; Supercritical CO_2

Introduction

Different kinds of plant varieties can be found in Indonesia, and they can be used for various kinds of applications such as the raw materials for specialty chemicals and biofuel (Zhou et al., 2011). One of them is *Mimosa pudica* Linn (MPL). This plant is commonly known as a sensitive or sleepy plant since its leaves fold inward when touched. It is available throughout Indonesia as a wild bush. The plant originally migrated from Brazil, and its weed distributed throughout the tropics (Elango et al., 2012). The stem of MPL is erect as a young plant but trails along the ground with age. All parts of the MPL plant contain phytochemicals which have medicinal value. The benefits of phytochemicals in MPL have been investigated by various authors (Bendgude et al., 2012; Cheng et al., 2007; Saraswat and Pokharkar, 2012; Sowmya and Ananthi, 2011; Tanaka and Takeshi, 2004). Saraswat and Pokharkar (2012) extracted the phytochemicals from MPL leaves using methanol as the solvent, and the extract was found to be an antidiabetic agent. Extracts of MPL leaf extract also have anthelmintic (Bendgude et al., 2012) and anti-inflammatory properties (Cheng et al., 2007). The aqueous extract of this plant (using decoction method) has an anti-nociceptive benefit (Tanaka and Takeshi, 2004), and the root extract contains high levels of antioxidants (Sowmya and Ananthi, 2011).

Previous extraction studies of phytochemicals from MPL have been conducted using the Soxhlet extraction method which used polar organic solvents such as methanol (Saraswat and Pokharkar, 2012; Sowmya and Ananthi, 2011), ethanol (Sowmya and Ananthi, 2011; Tanaka and Takeshi, 2004), and even water (Cheng et al., 2007; Tanaka and Takeshi, 2004). Even though these extractions are quite simple, they require a complex separation process to obtain high purity of active compounds. Supercritical fluid extraction (SFE) is an attractive alternative for obtaining valuable compounds for use in pharmaceuticals (McHugh and Krukonis, 1986; Huang et al., 2004), nutraceutical (Riha and Brunner, 2000; Senorans et al., 2001), and flavor (Doker et al., 2010; Haas et al., 1989; Kim et al., 2008; Monteiro et al., 1997; Rodrigues et al., 2003). Supercritical fluids have unique properties that support effective extraction. The density of such fluid is much greater than gases and slightly less than organic solvents; the viscosity is close to the typical gases and less than organic solvents. The excellent mass transfer property of supercritical fluids is easily controlled by temperature and pressure or a modifier. For most SFE, carbon dioxide is the most widely used gas as the solvent since it has a relatively low critical temperature and pressure, is nontoxic, inexpensive, and environmental friendly.

Until now, there is no information available on the extraction of phytochemicals from MPL using supercritical carbon dioxide (SC-CO_2). In this work, the effect of

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temperature, pressure, CO_2 flow rates, and extraction times on phytochemical yields has been studied. The results are presented as total phenolic compound (TPC) and total alkaloid content (TAC), and the optimum extraction condition was obtained using 2^4 full factorial experimental designs. The SC-CO_2 extraction of MPL was modeled using density-based correlation (Chrastil, 1982).The correlation of pressure, temperature, and density on TPC and TAC is useful for the design of SC-CO_2 extraction process. The identification of phytochemical components in the extract was conducted by high-performance liquid chromatography (HPLC).

Materials and Methods

Raw Material and Sample Preparation

The whole plants of MPL were collected from a local area in Surabaya, Indonesia. They were collected in November 2012 and dried in an oven (Memmert type UNB 500, Büchenbach, Germany) at 323.15 K for 48 h until the moisture content was about 8.5%. The raw materials were then crushed using a domestic grinding mill and then sieved with a vibrating screen (Retsch AS 200, Haan, Germany) to 80/100 mesh. The dried MPL powder was stored in a desiccator containing silica gel to maintain the moisture content at the desired level (8.5%).

Chemical Reagents

Food grade carbon dioxide (>99.9% purity) was supplied as liquid CO_2 by Aneka Gas Pty Ltd, Indonesia. Analytical grade ethanol (>99.5%) and Folin–Ciocalteu reagent were supplied by Merck (Darmstadt, Germany). Gallic acid (98% purity), sodium carbonate, and DPPH (1,1-diphenyl-2-picrylhydrazyl-hydrate) were obtained from Sigma-Aldrich (Singapore, Singapore). Bromocresol green (American Chemical Society reagent with a purity of 95%), mimosine (CAS 500-44-7, 3-hydroxy-4-pyridine-1-yl L-alanine, 98% purity), catechin (CAS 18829-70-4, 98% purity), quercetin (CAS 117-39-5, 2-(3,4-dihydroxyphenyl)-3,5,7-trihydroxy-4H-1-benzopyran-4-one, 95% purity), and potassium acetate (99% purity) were obtained from Sigma-Aldrich.

Supercritical Fluid Extraction Procedure

Extraction of phytochemicals from MPL was performed in a SC-CO_2 extractor system. The extraction system consists of high-pressure pump (Eldex AA-100-S-2-CE, Napa, CA, USA), a pressure transducer (Druck PTX 611, Boston, MA, USA), and an oven (Memmert type UNB 500, Büchenbach, Germany). All valves and tubes were made from 316 SS Grade (Swagelok, Solon, OH, USA). A known mass of MPL powder (±10 g) was placed in SC-CO_2 stainless steel extractor. The heating chamber was heated to the desired temperature (313.15 K, 323.15 K, or 333.15 K). Liquid CO_2 was then pumped then into the system until the desired pressure was reached (10–28 MPa) using high-pressure pump with constant CO_2 flow rate (6–8 mL/min). When the extraction conditions were reached, the dynamic extraction was started. Finally, the CO_2-containing phytochemicals were introduced into a collector containing a known amount of ethanol (200 mL) and the CO_2 was released into the air. A schematic of the experimental setup is given in Figure 1.

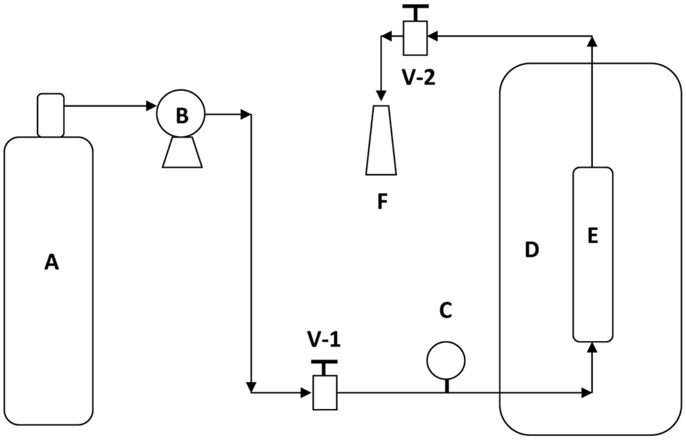

Fig. 1. Schematic diagram of the experimental setup. A, deep tube CO_2 cylinder; B, high-pressure pump; C, pressure transducer; D, oven; E, extraction column; F, sample collector.

Design of Experiment (DOE)

In this study, 2^4 full factorial of design of experiment (DOE) analysis was used to investigate the effect of significant variables on the response of the TPC. The controlled variables in this study were temperature (T), pressure (P), CO_2 flow rate (C), and dynamic extraction time (X). Each of the controlled variables was kept at three levels—high level (+1), the center point (0), and lower level (−1)—as listed in Table I. Based on the experimental design, 33 experiments were carried out in duplicate. The results were analyzed by analysis of variance (ANOVA) technique using MINITAB 16.0 software to a 95% confidence level.

Total Phenolic Content Assay

The determination of total phenolic content of the MPL extract was based on Habila et al. (2010) and Cavalcanti et al. (2012) using a Folin–Ciocalteu assay. Briefly, 5 mL of Folin–Ciocalteu reagent (1:10; v/v) was added to 1 mL of the extract to oxidize it. After an interval of 1–5 min, 4 mL of sodium carbonate (7.5%, w/v) was added to the mixture solution to stop the oxidation, and subsequently the solution was allowed to stand in a dark place at room

Table I. Variables and levels used in the design of experiment with TPC response

Variables	Low level (−1)	Center point (0)	High level (+1)
Temperature (°C) (T)	40	50	60
Pressure (MPa) (P)	12	20	28
CO_2 flow rate (mL/min) (C)	6	7	8
Dynamic extraction time (h) (X)	2	6	10

temperature for 60 min. The absorbance was measured at a wavelength of 739 nm with a UV/Vis spectrophotometer (Shimadzu mini UV 1240, Kyoto, Japan). Using gallic acid as standard; the TPC was expressed as mg GAE/L of extract. Data are reported for three replications.

Total Flavonoid Content Assay

Total flavonoid content (TFC) of each extract was determined in triplicate by aluminum chloride colorimetric method as modified by Woisky and Salatino (1998). Briefly, 0.5 mL of extract was mixed with 0.1 mL of 10% aluminum chloride, 0.1 M potassium acetate, and 2.8 mL of distilled water. The mixtures were allowed to stand at room temperature for 30 min. The absorbance of standard and extract solutions was measured at 415 nm using a spectrophotometer (Shimadzu UV mini 1240). The flavonoid content was expressed as milligram quercetin equivalents (QE)/gram sample.

Total Alkaloid Content Assay

TAC was determined by titrimetric method (Evan and Partridge, 1952) using bromocresol green as an indicator. About 1 mL of each extract was dissolved in 2.0 mL distilled water and 2 mL buffer, then the mixture solution was transferred into a conical flask, and three drops of bromocresol green indicator were added to the solution. The mixture was titrated with 0.005 N H_2SO_4 until the appearance of a light green color (end point). Each milliliter of acid used is equivalent to 0.00145 g of alkaloid calculated as hyoscyamine. Data are reported for three replications. The calculation of percentage total alkaloid is given below (Nuhu and Ghani, 2002):

$$\mathrm{TAC} = \frac{m_{\mathrm{alkaloid}}}{m_{\mathrm{sample}}}$$

HPLC Analysis

The identification of phytochemicals was conducted on a HPLC column using a modification of the procedure described by Tangendjadja and Wills (1980) and Weisz et al. (2009). The analysis of phytochemicals, namely gallic acid, catechin, mimosine, caffeic acid, epicatechin, p-Coumaric acid, ferulic, and quercetin, was conducted by HPLC (Jasco LC-Net II/ADC, Tokyo, Japan), in conjunction with a dual pump (Jasco PU-2089 plus) and a UV detector (Jasco PU-2077 plus). The column used for separation was an Enduro C-18 250 mm × 4.6 mm, with 5-µm particle diameters. The mobile phases were solvent A (water/acetic acid with a volume ratio 97/3) and solvent B (acetonitrile/acetic acid with a volume ratio 97/3) at a constant flow rate of 1 mL/min. The calibration curves were selected at a good detection wavelength of 280 nm. A single injection of the solvent was performed to specify the retention time of the solvent.

Result and Discussion

Effect of Variables on TPC

The extraction of phytochemicals using SC-CO_2 extraction is influenced by temperature, pressure, CO_2 flow rate, and time. Temperature and pressure significantly influenced the density, viscosity, and diffusivity of SC-CO_2, hence affecting the amount of phytochemicals in the extract. The flow rate of CO_2 also affected the yield of phytochemicals in some cases. The time for SC-CO_2 extraction greatly influenced the yield of phytochemicals extracted prior to equilibrium.

The determination of the significant variables affecting the extraction of phytochemicals using SC-CO_2 was carried out by performing two-way ANOVA. The statistical DOE was a simultaneous study of several process variables to determine the influence of controlled variables on the amount of phytochemicals extracted (presented as TPC) into a measurable response (Table II). The results were analyzed by ANOVA technique using Minitab 16.0. The estimated constants, coefficients of linear regression, and interaction effects are presented in Table III.

In ANOVA, a large regression coefficient and a small p-value indicate a significant effect on the respective response variables (Table III). Our results show that the individual effects of pressure and temperature and the

Table II. TPC results from the experimental runs of 2^4 full factorial design

Standard order	T	P	C	X	TPC response
5	−1	−1	1	−1	18.14
2	1	−1	−1	−1	27.20
9	−1	−1	−1	1	18.11
27	−1	1	−1	1	27.13
32	1	1	1	1	74.94
15	−1	1	1	1	27.77
10	1	−1	−1	1	27.48
31	−1	1	1	1	27.69
23	−1	1	1	−1	27.55
4	1	1	−1	−1	74.71
26	1	−1	−1	1	27.32
14	1	−1	1	1	27.59
16	1	1	1	1	74.85
25	−1	−1	−1	1	18.10
3	−1	1	−1	−1	27.18
30	1	−1	1	1	27.59
7	−1	1	1	−1	27.52
13	−1	−1	1	1	18.13
18	1	−1	−1	−1	27.48
17	−1	−1	−1	−1	18.05
1	−1	−1	−1	−1	17.76
12	1	1	−1	1	74.72
11	−1	1	−1	1	27.18
8	1	1	1	−1	74.94
24	1	1	1	−1	74.72
33	0	0	0	0	35.80
29	−1	−1	1	1	18.11
20	1	1	−1	−1	74.75
19	−1	1	−1	−1	27.18
22	1	−1	1	−1	27.48
21	−1	−1	1	−1	17.94
28	1	1	−1	1	74.73
6	1	−1	1	−1	27.68

Table III. The result of DOE analysis of SC-CO$_2$ extraction of MPL

Term	Effect	Coefficient	Standard error (SE) coefficient	T	P
Constant	—	36.8943	0.05000	737.94	0.000
T	28.4148	14.2074	0.05077	279.83	0.000
P	28.3364	14.1682	0.05077	279.06	0.000
C	0.2220	0.1110	0.05077	2.19	0.043
X	0.0746	0.0373	0.05077	0.74	0.472
$T \times P$	18.9769	9.4884	0.05077	186.89	0.000
$T \times C$	−0.0474	−0.0237	0.05077	−0.47	0.646
$T \times X$	−0.0407	−0.0203	0.05077	−0.40	0.694
$P \times C$	0.0768	0.0384	0.05077	0.76	0.460
$P \times X$	−0.0145	−0.0073	0.05077	−0.14	0.888
$C \times X$	0.0151	0.0076	0.05077	0.15	0.884
$T \times P \times C$	−0.1155	−0.0577	0.05077	−1.14	0.271
$T \times P \times X$	0.0143	0.0072	0.05077	0.14	0.889
$T \times C \times X$	−0.0104	−0.0052	0.05077	−0.10	0.920
$P \times C \times X$	0.0576	0.0288	0.05077	0.57	0.578
$T \times P \times C \times X$	−0.0263	−0.0132	0.05077	−0.26	0.798

$S = 0.287205$
$R^2 = 99.59\%$
$\%\ R^2$ (adjusted) $= 98.83\%$

interaction effect of pressure with temperature are very significant in the case of TPC in MPL extract ($p < 0.01$). There was a slightly significant individual effect of extraction time ($0.04 < p < 0.05$), which indicated that 2 h is sufficient to extract the phytochemicals from MPL solid matrices (Figure 2). There was no significant effect of CO$_2$ flow rate on TPC as individual variable and in the interaction with other variables ($p > 0.05$) (Figure 2).

The Taylor series of expansion can be used to express the regression model with the interaction terms:

$$\begin{aligned} Y = {} & b_0 + b_1x_1 + b_2x_2 + b_3x_3 + b_4x_4 + b_{12}x_1x_2 + b_{13}x_1x_3 \\ & + b_{14}x_1x_4 + b_{23}x_2x_3 + b_{24}x_2x_4 + b_{34}x_3x_4 \\ & + b_{123}x_1x_2x_3 + b_{124}x_1x_2x_4 + b_{134}x_1x_3x_4 \\ & + b_{234}x_2x_3x_4 + b_{1234}x_1x_2x_{3_{x_4}} \end{aligned} \tag{1}$$

where Y is TPC; b_i is model coefficients; and x_1, x_2, x_3, and x_4 are dimensionless coded factors for the controlled variables (i.e., temperature, pressure, CO$_2$ flow rate, and dynamic extraction time, respectively). Neglecting the coefficients of nonsignificant terms at 95% confidence level, the regression becomes:

$$\begin{aligned} Y = {} & 36.8943 + 14.2074x_1 + 14.1682x_2 + 0.1110x_3 \\ & + 0.0373x_4 + 9.4884x_1x_2 \end{aligned} \tag{2}$$

The yield of phenolic compounds (TPC) as a function of the controlled variables within the experimental range was generated by using the empirical model in Equation (2). The statistical model indicated that the TPC of the extract increased with temperature, pressure, and time.

Effect of Temperature and Pressure on TPC, TFC, and TAC

Based on a full factorial design analysis, the significant variables influencing the SC-CO$_2$ extraction of MPL were pressure and temperature. Since the extraction time slightly affected the TPC of extract, further investigation was carried out at constant dynamic extraction time (2 h). The flow rate of CO$_2$ was not a significant variable and was kept at 6 mL/min during the experiments.

The TPC of MPL extract significantly increased with pressure (12–28 MPa) at 313.15 K, 323.15 K, and 333.15 K. The lowest TPC was 17.76 mg GAE/g MPL at the lowest operating condition 12 MPa and 313.15 K and the highest TPC was 74.94 mg GAE/g MPL at the highest operating condition 28 MPa and 333.15 K. At a constant temperature, the density of SC-CO$_2$ increased, approaching the density of organic solvent with increasing pressure; as a result, the yield of extraction increases since its ability to dissolve phenolic compounds from the solid matrices increased.

The effect of SC-CO$_2$ pressure on TPC of MPL extract was more pronounced at higher temperature (Figure 3). At 313.15 K, the TPC increases from 17.76 mg GAE/g MPL at 12 MPa to 27.18 mg GAE/g MPL at 28 MPa, while at 333.15 K, the TPC increases from 27.21 mg GAE/g MPL at 12 MPa to 74.94 mg GAE/g MPL at 28 MPa. At constant pressure, the amounts of phenolic compounds dissolved in SC-CO$_2$ increased with temperature (Figure 3) because the solute vapor pressures, as well as the diffusivities of both solvent and solute, increased. The effect of pressure and temperature on the TFC of MPL extract was similar to TPC since flavonoid is one of the phenolic compounds (Figure 4). The TFC of MPL extract was expressed as milligram QE/g MPL, and the value was lower than TPC, which indicated that other types of phenolic compounds were also extracted.

The SC-CO$_2$ also extracted alkaloid compounds even though the result was lower than TPC and TFC. The TAC were presented as milligram hyoscyamine/g MPL. As expected, the lowest TAC was obtained at 313.15 K and 12 MPa (5.24 mg hyoscyamine/g MPL) and the highest was

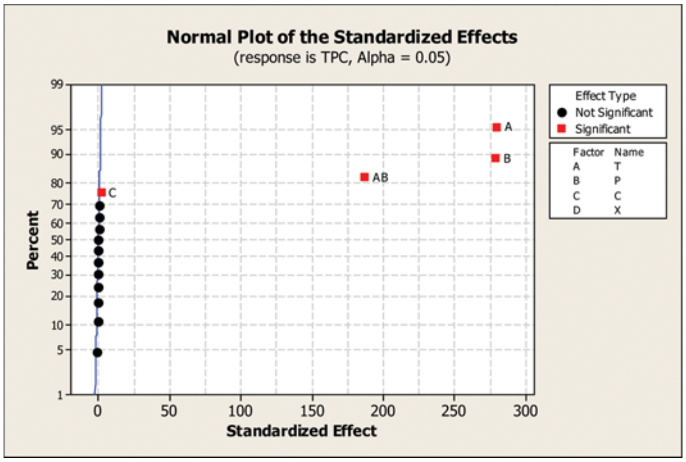

Fig. 2. Normal plot of the standardized effect.

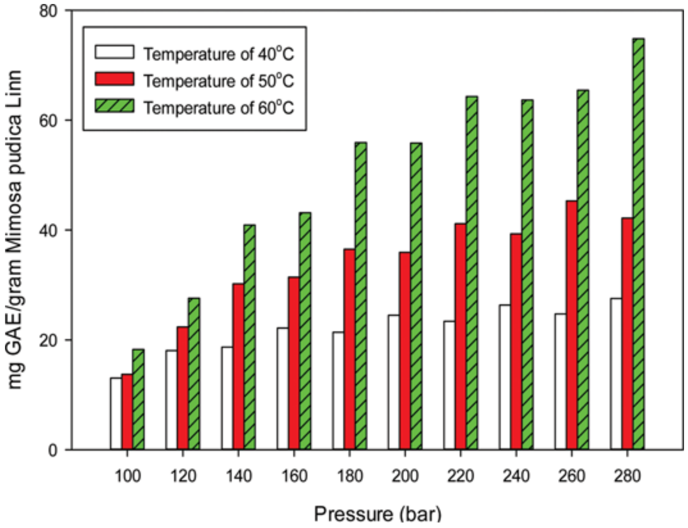

Fig. 3. The effect of pressure and temperature on TPC of MPL extract.

obtained at 333.15 K and 28 MPa (14.23 mg hyoscyamine/g MPL). The total content of alkaloids in MPL was obtained from Soxhlet extraction (29.83 mg hyoscyamine/g MPL), which is lower than the total content of phenolics (171.49 mg GAE/g MPL). The HPLC analysis (Figure 5) on the MPL extract from SC-CO_2 extraction showed that it contains gallic acid (phenolic compound) and mimosine (alkaloid), and the result from Soxhlet extraction using water ethanol as the solvent contained gallic acid and catechin (phenolic compounds) and mimosine (alkaloid).

The solubility of solutes in SC-CO_2 is affected by temperature and pressure and is generally a function of the density of SC-CO_2 as the solvent. The effect of temperature on solubility is complex due to the opposing factors of solute vapor pressure and solvent density. The solute vapor pressure increased with temperature and causes the solubility to increase, while the solvent density decreased resulting in decreased solubility. In this study, both factors influenced the yield of phenolic and alkaloid compounds. The yield was also influenced by the polarity of CO_2. Although CO_2 is nonpolar, it has a large quadrupole that is able to interact with other polar liquids and solids, especially at higher pressure; the proximity between CO_2 and solute molecules is shortened. This leads to the creation of substantial dipole (induced or not)—quadrupole interactions that explain the CO_2—philic character of many solutes (de Melo et al., 2012).

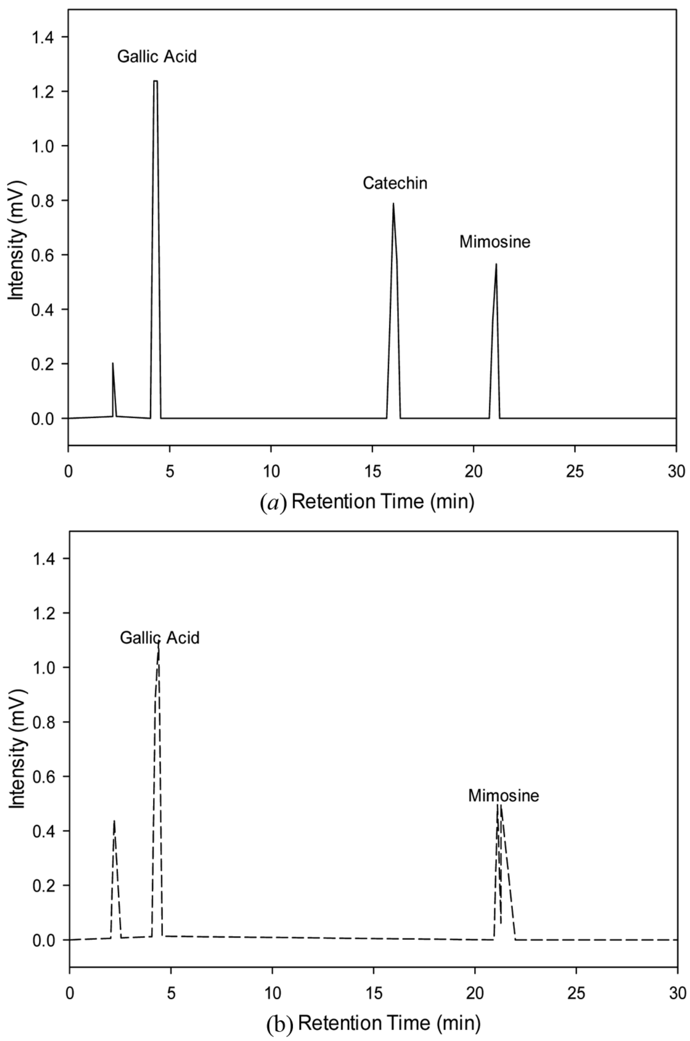

Fig. 5. HPLC chromatogram analysis of (a) Soxhlet and (b) SC-CO_2 extract from MPL at 280 nm.

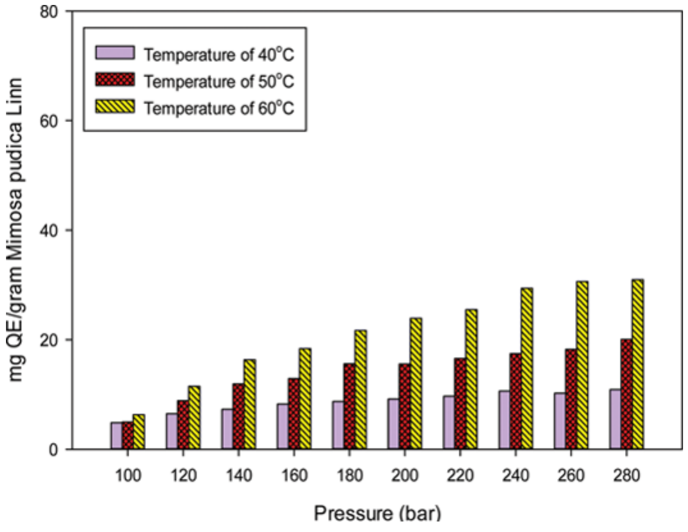

Fig. 4. The effect of pressure and temperature on TFC on MPL extract.

Density-Based Modeling

Density-based modeling is a semiempirical correlation of the logarithm of the solubility of the solute and the density. This approach is quite simple since it does not require knowledge of solute properties and permits easy analysis of correlations with existing solubility data. The Chrastil model was based on the formation of solvation complex model between the solvent and the solute molecules at an equilibrium state (Chrastil, 1982):

$$A + kB \leftrightarrow AB_k \tag{3}$$

This model assumes that one molecules of solute (A) is associated with k molecules of solvent (B) to form a solvation complex (AB_k) at equilibrium. The Chrastil model is a model which correlates the solubility of a solute in a supercritical solvent to the density of solvent and temperature process (Chrastil, 1982):

$$s = \rho^k \exp\left(\frac{a}{T} + b\right) \tag{4}$$

where s indicates the concentration of solute in SC-CO_2 (g/L), ρ indicates the density of SC-CO_2 (g/L), k indicates the number of CO_2 molecules performed in the complex solute of solvent, a is a function of the enthalpy of solvation ($\Delta H_{\text{solvation}}$) (J/mol) and enthalpy of vaporization ($\Delta H_{\text{vaporation}}$) (J/mol), and b is a function of the average association number that depends on the molecular weight of the solute and solvent. The mathematical form of a as a function of enthalpy of vaporization and solvation is

$$a = \frac{\Delta H_{\text{solvation}} + \Delta H_{\text{vaporation}}}{R} = \frac{\Delta H}{R} \tag{5}$$

where R is an ideal gas constant and ΔH (J/mol) is the enthalpy of solution or reaction of the substance in supercritical fluid.

The calculation of constants for the semiempirical density-based correlation (Chrastil) was conducted using multivariable nonlinear regression analysis. The quality of all data correlations was quantified by the sum of squared error, defined as follows:

$$\text{SSE} = \left[\frac{\left(\sum\left(s_{\text{exp}} - s_{\text{cal}}\right)\right)^2}{N}\right]^{0.5} \tag{7}$$

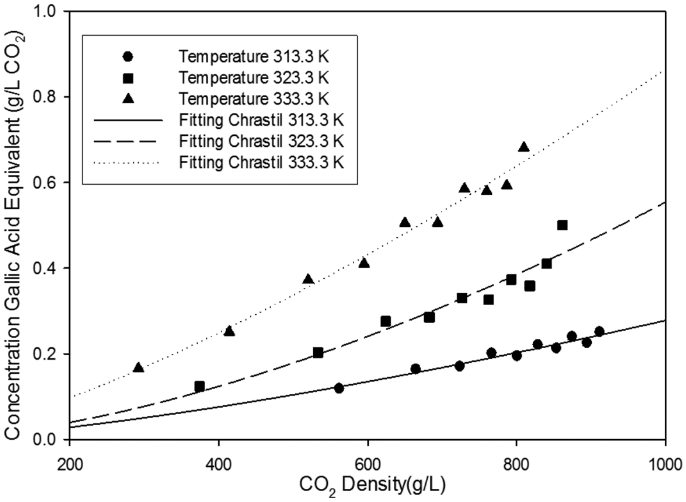

Fig. 6. The total phenolic compounds extracted at supercritical conditions and plots of Chrastil model.

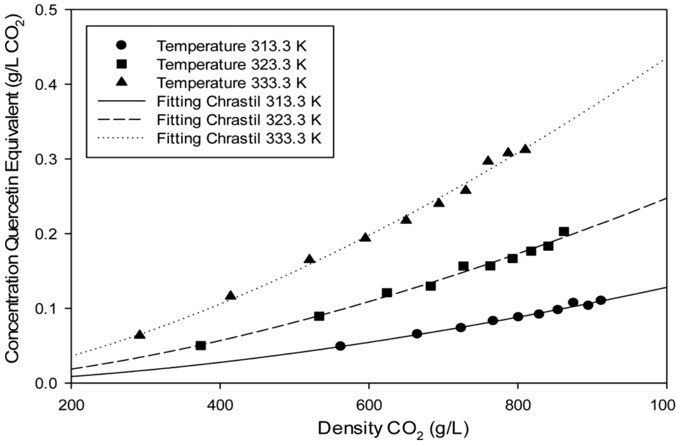

Fig. 7. The total flavonoid compounds extracted at supercritical conditions and plots of Chrastil model.

where s_{exp} is the actual data of TPC of the MPL extract, s_{cal} is the calculated data of TPC of MPL extract, and N is the number of experimental data. The multivariable nonlinear regression is applied with the iteration curve-fitting procedure using SigmaPlot 12.0. An initial estimation for each parameter was provided, and then iteration calculation was conducted until the convergence criteria for each final parameter were satisfied.

Figures 6–8 show the two-dimensional graphs of experimental data of phenolic, flavonoid, and alkaloid extracted in SC-CO_2 and the plot using Chrastil model. These figures clearly show that the model by Chrastil represents the data well.

The fitting parameters of Chrastil model obtained from nonlinear least-square method are summarized in Table IV. The value of k indicates the number of CO_2 molecules (B) associated with the solute (A) to form a solvation complex (AB_k). The enthalpy of solution for phenolic, flavonoid, and alkaloid in Chrastil model was −50,263.15 J/mol, −53,390.21 J/mol, and −53,286.28 J/mol, respectively.

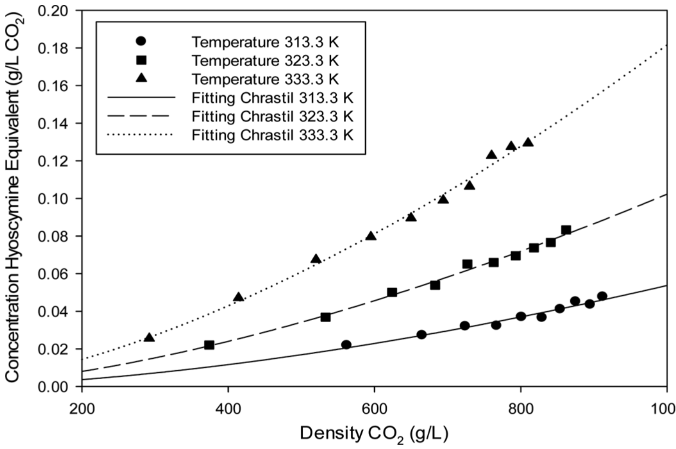

Fig. 8. The total alkaloid compounds extracted at supercritical conditions and plots of Chrastil model.

Table IV. Parameters of Chrastil model of phytochemical compounds from MPL

Chrastil model			
Parameter	Phenolic	Flavonoid	Alkaloid
k'	1.5512	1.5660	1.5853
b'	7.3493	7.7469	6.5755
a'	−6045.24	−6461.34	−6407.84
SSE	0.0918	0.0222	0.0094

Conclusion

According to the DOE results, the significant variables for the SC-CO_2 extraction of phytochemicals from MPL were temperature and pressure. The maximum amounts of TPC, TFC, and TAC were 74.83 mg GAE/g dry MPL, 30.93 mg QE/g dry MPL, and 14.23 mg HE/g dry MPL, respectively. The experimental data of phenolic, flavonoid, and alkaloid extracted from MPL were correlated by density-based models. The Chrastil model can represent the experimental data very well. From the identification of phytochemicals by HPLC, the SC-CO_2 extract is more selective than the Soxhlet extraction method, in which gallic acid and mimosine were detected in the SC-CO_2 extract of MPL.

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