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<http://www.ifrj.upm.edu.my> Supercritical CO₂ extraction of bioactive compounds from *Stachytarpheta jamaicensis* (L) Vahl 1Calista, 1Tjpto, M.S., 1Putro, J.N., 1Nugraha, A.T., 1Soetaredjo, F.E., 2Ju, Y.H. and 1*Ismaadi, S. 1Department

5of Chemical Engineering, Widya Mandala Surabaya Catholic University, Kalijudan 37, Surabaya 60114, Indonesia 2Department of Chemical Engineering, National Taiwan University of Science and Technology, 43 Sec 4, Keelung Road, Taipei 106-07 Taiwan

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Supercritical CO₂ Extraction Phenolic compounds Phytochemicals Abstract

8Supercritical fluid extraction (SFE) has become a focus of interest for extraction of natural

material in the area of food, pharmaceutical and biotechnology industries due to its preeminent properties. The extraction of bioactive compounds from *Stachytarpheta jamaicensis* (L) Vahl using supercritical CO₂ was conducted in static mode. The extraction experiments were carried out at various pressures and three different temperatures (313, 323, and 333 K). The

14central composite design combined with response surface methodology (RSM) was used to construct the

experimental design. Pressure and temperature have a positive effect on the amount of phenolic compounds, flavonoid compounds, and alkanoid compounds extracted using supercritical CO₂.

20Chrastil model can represent the experimental data well.

Several compounds were identified in the SJL extract such as

2Gallic acid, catechin, caffeic acid, p-coumaric acid, and quercetin. The

concentration of bioactive compounds in SJL extract obtained by supercritical CO₂ extraction is: Gallic acid 4.43 mg/L, caffeic acid 93.64 mg/L, quercetin 19.72 mg/L, catechin 15.39 mg/L, and p-coumaric Acid 9.78

4mg/L. The amount of phenolic, flavonoid, and alkaloid compounds extracted increased with the

increase in pressure and temperature. Chrastil model could represent the solubility of the bioactive compounds in the extract quite well. © All Rights Reserved Introduction Phytochemical screening of *Stachytarpheta* reveals that these plants contain terpenes, flavonoids, arylpropanoids and iridoids (mainly of the ipolamiide type) (Chowdhury et al., 2003; Penido et al., 2006). Vicini in a previous study has reported that the bioactive compounds in *Stachytarpheta*

11possess several biological activities such as antimicrobial, antitumor, anti-inflammatory, antinociception, hepatoprotective and laxative (Vicini et al.,

2008). The studies of phytochemical compounds on the Genus of *Stachytarpheta* have been focused on the species of *Stachytarpheta agustifolia* (Awah et al., 2010), *Stachytarpheta urticaefolia*

11(Chowdhury et al., 2003), *Stachytarpheta glabra* (Vicini et al., 2008),

Stachytarpheta cayennensis (Penido et al., 2006) and rarely on *Stachytarpheta jamaicensis*. *Stachytarpheta jamaicensis* (L) Vahl (SJL) is a herbaceous plant from the Verbenaceae family. *Stachytarpheta jamaicensis* and *Stachytarpheta cayennensis* are commonly used as herbal medicines in Brazil, China, India, etc. (DeLuca et al., 1980). In Indonesia, SJL is

22traditionally used in folk medicine as a purgative, vermifuge, expectorant, diuretic, emmenagogue, fever, diabetes and sore-throat gargles

(Penido et al., 2006; Vicini et al., 2008; Abe and Ohtani, 2013). The studies and utilization of SJL for herbaceous medicine have been conducted by maceration using water (Cano and Volpato, 2004) and ethanol [Penido et al., 2006; Vicini et al., 2008; Awah et al., 2010]. The results of the previous studies reveal that *Stachytarpheta* species contain

16 **iridoid glycosides (Futuro and Kaplan, 1998), steroids (Ganapaty et al., 1998), flavonoids (Duret et al.,**

1976), and some other phenolic compounds (Awah

16 **et al., 2010, Duret et al., 1976).**

However, the drawbacks of using ethanol and water as extracting agents are low purity and a requirement for several stages of purification of the resulting product.

8 **Supercritical fluid extraction (SFE) has become a focus of interest for extraction of natural**

material in the area of food, pharmaceutical and biotechnology industries due to its preeminent properties (i.e. excellent mass transfer properties and ease of control using temperature, pressure or *Corresponding author. Email: suryadiismadji@yahoo.com a modifier). CO₂ has been used as the solvent for most SFE studies because

17 **it is inexpensive, nontoxic, non-flammable, environmentally acceptable, and has a low critical temperature and moderate critical pressure. To the**

present there is no information about the use of supercritical fluid technology to extract flavonoids from SJL. In this work, the supercritical extraction of bioactive compounds (i.e. phenolic compounds and flavonoids) from SJL

20 **by supercritical CO₂ (SC- CO₂) was conducted in a batch system. The**

effect of process variables such as pressure and temperature on the yield of phenolic and flavonoids compounds was investigated. In addition, the solubility of phenolic and flavonoid compounds on the supercritical CO₂ at various experimental conditions was correlated using Chrastil modelling (Chrastil, 1982). Materials and Methods Sample preparation *Stachytarpheta jamaicensis* (L) Vahl (SJL) was collected from Surabaya, Indonesia. It was collected in mid-month of November 2012. It was dried in an oven (Memmert type UNB 500, Germany) at 323 K for 24 h until its moisture content (MC) became $\pm 5\%$. Dry SJL was then crushed using a domestic grinding mill and sieved with a vibrating screen (Retsch AS 200, Germany) to obtain a fine powder (-80/+100 mesh). The powder of SJL was stored in a desiccator containing silica gel to avoid moisture adsorption. Chemicals Food grade liquid CO₂ (99.9% purity) was supplied by Aneka Gas Pty Ltd, Indonesia. Analytical grade ethanol 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). Bromocresol green (ACS

reagent with purity 95%), 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 (Singapore). Soxhlet Extraction The Soxhlet extraction process was carried out to ensure intimate contact of the sample matrix with the extraction solvent. The procedure for soxhlet extraction is as follow: 10 grams of finely ground SJL sample were placed in a porous bag or “thimble” made of strong filter paper. The ethanol was used as the extracting solvent. 250 ml of ethanol was placed in a round bottom flask. The flask, then was heated until the extracting solvent evaporated and the vapors condensed in the

1 condenser. The condensed extractant dripped **into the thimble containing the** SJL **sample** and extracted **it by contact. When the level of liquid in** the extraction **chamber** rose **to the top of the siphon tube, the liquid contents of the** extraction **chamber** siphon flowed **into the flask. This process** repeated many times **until a drop of solvent from the siphon tube** did not leave **residue when evaporated.**

SC-CO₂ extraction The supercritical fluid extraction equipment used in the experiment consisted of a 150 ml extraction tube (Swagelok, USA), sampling tube (1 ml), a high pressure pump (Eldex AA-100-S-2-CE, USA) and pressure transducer (Druck PTX 611, USA) with a digital process indicator (Druck DPI 280, USA). The uncertainties of the pressure measurement were ± 0.1 bar. An oven Memmert (Germany) was used as the heating system, and the accuracy of this heating chamber was within

101 K. The maximum working pressure and temperature **of the system** are 40 **MPa and 373. 15 K, respectively.**

All fitting and tubing used in the system were made of stainless steel 316 (Swagelok, USA). The supercritical extraction of bioactive compounds from SJL powder was carried out in static mode, and the extraction procedure is as follows: 10 g of SJL powder was placed in the extraction tube and allowed to equilibrate to a pre-set extraction temperature (313, 323 and 333 K). Liquid CO₂ was pumped into the system until the targeted pressure was achieved (10 – 25 MPa). After the equilibrium condition was achieved in 4 h (based on preliminary trials), the output valve was opened to release the supercritical CO₂ containing phytochemicals into a sample collector which contained a known amount of ethanol (250 ml). The phytochemicals were dissolved in ethanol, and the CO₂ was released into the atmosphere. At least three replications were carried out for every combination of temperature and pressure used.

9 Total phenolic content (TPC) test The total phenolic content in the extract **was determined using** the methods of **Folin-**

Ciocalteu (Habla et al., 2010) and Cavalcanti (Cavalcanti et al., 2012). The extract sample (1 ml) was oxidized with 5 ml Folin-Ciocalteu reagent (1:10, v/v), and the reaction was neutralized with 4 ml

47.5% sodium carbonate. The mixture was allowed to stand in the dark **for 30 min** before measuring **the absorbance at 722.5 nm using** spectrophotometer **UV/Vis (Shimadzu mini UV 1240, Japan). Gallic acid**

was used as standard for total phenolic content, and the latter was expressed as mg GA equivalent/ g SJL. Total flavonoid content (TFC) method The total flavonoid assay was conducted according to Woisky and Salatino (Woisky and Salatino, 1998). The determination of total flavonoid content was conducted using aluminum chloride colorimetric method. The brief procedure of the Woisky and Salatino method is as follows: 1 ml of extract solution

3was mixed with **3 ml** ethanol, **0.2 ml of 10% aluminum chloride, 0.2 ml 1 M potassium acetate and 5.6 ml of distilled water. The** mixtures were **allowed to stand at room temperature for 30** minutes. **The** observance of

standard and extract solutions were measured at 435 nm using a spectrophotometer (Shimadzu UV mini 1240, Japan). The flavonoid content was expressed as mg quercetin equivalents (QE)/g SJL. Total alkaloid content (TAC) Bromocresol green solution was prepared by dissolving 69.8 mg bromocresol green in 1 L of distilled water. The total alkaloid content in SJL leaf was determined by a titrimetric method by Evan and Partridge (Evan and Partridge, 1952). One

9ml of extract was mixed with 2 ml

distilled water, 5 drops bromocresol green as an indicator were added to the mixture, subsequently 1 ml of 2N NaOH solution was also added to the mixture. The mixture then was titrated with 0.005N sulfuric acid until the color change into light green. Each ml of titrant is equivalent to 1.45 mg Hyoscyamine. The alkaloid content was expressed with mg Hyoscyamine/ g SJL. HPLC analysis HPLC analysis was conducted

6for the analysis of the main phenolic compounds on

the extract of SJL. The analysis of phenolic compounds was conducted on the JASCO HPLC system. The system consists of Jasco PU-2089 Plus gradient pump, Jasco UV-2077 detector, and Enduro C18 column (250 x 4.6 mm) dp = 5 µm.

15A mixture of water and acetic acid (97:3)

was used as

15solvent A, and a mixture of acetonitrile/ acetic acid (97:3) was employed as solvent B.

The following gradient was used for the separation: 90% of

12A and 10% of B at 0 min, 80% of A and 20% of B at 15 min, 68% of A and 32% of B at 38 min, 54% of A and 46% of B at 50 min,

45% of A and 55% of

7B at 55 min, 100% of B at 60 min, 90% of A and 10% of B at 70 min. The identification of

7each compound was carried out by comparing the retention time of UV spectra peaks with those of the

standards. Results and Discussion Snakeweed (*Stachytarpheta jamaicensis* L. Vahl) is a plant which possesses many bioactive compounds. It contains carbohydrates, glycosides, flavonoids, tannins, saponins, terpenoids, and alkaloids. In this study, some of the bioactive compounds could be extracted using supercritical fluid extraction. Response surface methodology

6Phenolic compounds are a large and diverse group of molecules, which includes many different families of aromatic secondary metabolites in plants. Since the

phenolic compounds cover a diverse family of bioactive compounds, therefore, in this design of experiment we employed the phenolic compounds to represent all of bioactive compounds present in snakeweed. The variables potentially affect the extraction process and the yields of SJL are examined using a statistical method. In this study, there are three variables that needed to be further investigated by

14central composite design combined with response surface methodology (RSM).

The

13response surface methodology was applied to evaluate the effect of the pressure, temperature,

and extraction time on the TPC of SJL.

18A second order polynomial model was used to express the TPC as a function of independent variables: (1)

is pressure (bar), X2 is temperature (°C), and X3 is Where Y is the TPC (g/L) in the SJL extract, X1 extraction time and n is the number of variables. The effect of the three variables as the

13linear, quadratic, and interaction terms were

tested

13by analysis of variance (ANOVA), and the

results are given in Table 3. Inserting the parameter's value obtained from analysis of variance into Equation (1) gives the following result (2) The significance of each variable as indicated by p-value is also given in Table 1. When the p-value of a variable is less than 0.05, the variable has a significant influence on the extraction process. From Table 1, it can be seen that the linear term of pressure and the temperature had a significant effect on the TPC of SJL. The linear interaction of these parameters (pressure and temperature) also has a Figure 1. The effect of temperature and pressure on the yield of TPC strong influence on the yield of TPC in SJL extract. The extraction time, all in the form of linear, square interaction, and linear interaction has no significant effect on the TPC yield (p-value > 0.05). The response surface plot of the influence of pressure and temperature on the TPC of SJL is given in Figure 1. At a constant temperature, the TPC of SJL increases with increasing of the pressure. By increasing pressure, the density of CO₂ increases, leading to the increase of solvating power of CO₂, therefore, more bioactive compounds are extracted from the SJL. Since only pressure and temperature have a significant effect on the results of TPC, in the subsequent section we only discussed the effect of these variables on the yield of other bioactive compounds. Supercritical fluid extraction of SJL The effect of pressure and temperature on the yield of bioactive compound extracted from SJL is given in Figure 2. Figure 2a depicts the effect pressure on the amount of phenolic compounds (expressed as mg GAE/g extract) extracted from SJL at three different temperatures. The extraction used a supercritical fluid method, the amount of organic compounds extracted from solid matrices, highly depended on the balance between the density of supercritical fluid and vapor pressure of the solutes, which are controlled by fluid temperature and pressure. In most cases, increase of pressure significantly enhances the extraction yield due to the increase in the density of solvent. On the other hand, the temperature has a negative effect on the density of supercritical fluid, by increasing temperature the density of solvent decreases, but it is compensated by the increase of solute vapor pressure. As seen in Figure 2a, both of pressure and temperature have a positive effect on the amount of phenolic compounds extracted using supercritical CO₂. The yield of phenolic compounds (TPC) was in the

range of 4.7 mg GAE/g extract to 59.9 mg GAE/g Figure 2. The effect of supercritical CO₂ pressure and temperatures on the yield of (a) phenolic compounds, (b) total flavonoid compounds, and (c) total alkaloid compounds extract. The highest yield of TPC was obtained at 333 K and 250 bar. Flavonoids are a class of plant secondary metabolites and one of the most prominent families in phenolic compounds. During the extraction of SJL using supercritical CO₂, different kinds of bioactive compounds were extracted, and one of them is flavonoids. Figure 2b shows the effect of pressure on the yield of total flavonoids content (TFC) expressed as quercetin equivalent (QE) at three different temperatures. The amount of TFC extracted during the supercritical CO₂ extraction process also increased with the increase of pressure. The temperature also gave a positive effect on the amount of TFC extracted, by increasing temperature; the amount of TFC extracted also increased due the increase of vapor pressure of flavonoids compounds in SJL. The maximum amount of TFC extracted was 15.9 mg QE/g extract achieved at 333 K and 250 bar. One of valuable bioactive compounds in SJL is alkaloid compounds. The effect of pressure on the yield of TAC at three different temperatures is shown in Figure 2c. Both of pressure and temperature have a positive effect on the yield of TAC as also observed for TPC and TFC. The maximum alkaloid compounds extracted from SJL using supercritical fluid extraction was 4.2 mg Hyoscyamine/ g extract obtained at 333 K and 250 bar. Density based equation To obtain reliable supercritical extraction experimental data at various pressures and the temperature is tedious, time consuming, and expensive. Therefore, many mathematical models have been developed to overcome these experimental drawbacks. Some of the models are purely empirical, and some have fundamental background. One of the most widely used semi-empirical models is Chrastil equation (Chrastil, 1982), which has the form as follows: (3) Where Y is the concentration of bioactive compound in supercritical CO₂ (g/L), (ρ is the density of CO₂ at supercritical condition (g/L) while a and b are constants in Chrastil equation. The plots of the Chrastil model of the supercritical extraction

10 experimental data are given in Figure 3. The plot of Chrastil model

is given in 3D mesh diagram. The fitted parameters of a Chrastil model for the concentration of bioactive compounds in supercritical CO₂ are summarized in Table 2. From Figure 3, it can be seen that the Chrastil model can represent the concentration of bioactive compounds in supercritical CO₂. The Chrastil model is a semi- empirical equation which was developed based on the assumption that the interaction of solute molecule and gas molecule cause the formation of

19 solvato complex which is in equilibrium with the gas. The

parameter k indicates how many molecules of CO₂ associated with one molecule of solute

19 to form a solvato complex at equilibrium condition. (4) The

values of k for all the bioactive compounds are not integer as indicated in Table 4. This phenomenon due to the presence of more and less stable association complexes (BCO₂)_k simultaneously in the system (Chrastil, 1982). The heat of solution of the bioactive compounds (a) (b) (c) Figure 3. Chrastil plots of bioactive compounds in supercritical CO₂ (a) phenolic, (b) flavonoids, and (c) alkaloids in supercritical carbon dioxide can be obtained from parameter a according to the following correlation: $a = \Delta H/R$. The negative sign in the value of ΔH (Table 2) indicates that the dissolution of bioactive compounds into

supercritical CO₂ is an endothermic process. This phenomenon is consistent with the experimental results, the extraction yield increased with the increase of temperature. The parameter b in Chrastil model indicates the relation between molecular weight of both solute and solvent (in this case supercritical CO₂) on their melting point. The correlation between parameter b and molecular weight can be written in the following equation (5) If the phenolic compounds are assumed as gallic acid, flavonoid compounds as quercetin, and alkaloid compounds as hyoscyamine with a molecular weight of 170.12 g/mol, 302.2 g/mol, 289.375 g/mol, respectively, the constants q for phenolic, flavonoid and alkaloid is 7.618, 2.596, and 5.549. Here, the fitted parameters of k, a, and b are consistent with their physical meaning and have reasonable values and comparable to other systems. HPLC analysis The HPLC analysis was employed to identify and quantify the bioactive compounds in the extract of SJL. Identification was performed by comparison of the retention time and UV absorption spectra with those of the standards. Several compounds were identified in the SJL extract such as

2Gallic acid, catechin, caffeic acid, p-coumaric acid, and quercetin. The results

indicate that caffeic acid was the main phenolic compound in SJL extracts. This compound is the derivatives of hydroxycinnamic which frequently occur in food as simple esters similar to quinic acid or glucose, which also a key intermediate in the biosynthesis of lignin, one of the principal sources of biomass. The concentration of bioactive compounds in SJL extract obtained by supercritical CO₂ extraction is as follows: gallic acid 4.43 mg/L, caffeic acid 93.64 mg/L, quercetin 19.72 mg/L, catechin 15.39 mg/L, and p-coumaric acid 9.78 mg/L. Comparison between supercritical fluid extraction and Soxhlet extraction The amount of TPC, TFC, and TAC in SJL ethanol extract obtained by soxhlet extraction is 51.7 mg GAE/g extract, 14.8 mg QE/g extract, and 3.8 mg Hyoscyamine/ g extract, respectively. Some bioactive compounds found in supercritical fluid extract such as

2gallic acid, caffeic acid, quercetin, catechin, and p-coumaric acid

were also found SJL ethanol extract. The concentration of these bioactive compounds in SJL ethanol extract is as follow: gallic acid 3.92 mg/L, caffeic acid 81.77 mg/L, quercetin 17.54 mg/L, catechin 13.98 mg/L, and p-coumaric acid 8.08 mg/L. Other bioactive compounds such as tannin and saponin were also observed in SJL ethanol extract. It is obvious that the supercritical extraction process gave higher extraction efficiency than soxhlet extraction. From the experimental results it can be seen that supercritical extraction process is very attractive since the effectiveness of the process can be controlled by small changes in pressure as well in temperature. With the density much greater than those of typical gases and slightly less than those of organic liquids and viscosity nearer the typical gases and less than those of liquids, the supercritical carbon dioxide can easily penetrate the interior structure of SJL matrices, so more bioactive compounds were extracted from the plant matrices. Furthermore this process offers several advantages than the traditional soxhlet extraction such as energy savings (333 K in SCF extraction process and 351 K in soxhlet extraction), higher selectivity and purity, more rapid extraction (4 hours in SCF extraction process and 6 hours in soxhlet extraction) and phase separation, and solvent- free product. Conclusions The extraction of bioactive compounds from Snakeweed (*Stachytarpheta jamaicensis* L.Vahl) using supercritical CO₂ was conducted at various pressures and temperatures. The response surface methodology was applied to evaluate the effects of the pressure, temperature, and extraction time on the total phenolic compounds of *Stachytarpheta jamaicensis* L.Vahl. The amount of phenolic, flavonoid, and alkaloid compounds extracted

increased with the increase in pressure and temperature. The concentration of phenolic, flavonoid, and alkaloid compounds in supercritical CO₂ were correlated using Chrastil equation. Several compounds were identified in the SJL extract such as

2gallic acid, catechin, caffeic acid, p-coumaric acid, and

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