Co-solvent Selection for Supercritical Fluid Extraction of Essential Oil and Bioactive Compounds from *Polygonum minus*

Masturah Markom [*,1]
Norsyamimi Hassim [1]
Nurina Anuar [1]
Syarul Nataqain Baharum [2]

[1]*Department of Chemical and Process Engineering, Faculty of Engineering and Built Environment, National University of Malaysia, 43600 UKM Bangi, Selangor, Malaysia.*
[2] *Institute of Systems Biology, National University of Malaysia, 43600 UKM Bangi, Selangor, Malaysia.*
[*]*e-mail :* masturah@eng.ukm.my

This study evaluated the biological activity (antioxidant assay) of *Polygonum minus* extracted using Supercritical Fluid Extraction (SFE) added with different types of co-solvents. The seven co-solvents employed were water, methanol, ethanol, 50% methanol, 50% ethanol, 70% methanol and 70% ethanol for selection of the best co-solvent prior to optimization of SFE. 70% methanol produced the highest total yield of extract (33.1%) compared to other co-solvents. The antioxidant capacity was then evaluated using four different assays: the total phenolic content (TP), the total flavonoid content (TF), the ferric reducing/antioxidant power (FRAP) and the free radical-scavenging capacity of 2,2-diphenyl-1-picrylhydrazyl (DPPH). The highest TP and TF were from 70% methanol extract (11.2 ± 0.15 mg GAE/g sample (mg GAE/g) and 11.9 ± 0.03 mg CAE/g sample (mg CEQ/g) respectively). 70% metanol extract also showed the highest FRAP value (346.7 ± 0.66 μmol Fe (II)/g sample) and the highest percentage of DPPH radical inhibition was also shown by 70% methanol extract (88.7 ± 0.40%). There was a positive correlation between the antioxidant capacity (FRAP and DPPH) with those of TP and TF contents. Therefore, the best co-solvent chosen for further optimization of SFE is 70% methanol.

Keywords: *Polygonum minus*, Supercritical Fluid Extraction, co-solvent, phelic content, antioxidant capacity, biological activity.

INTRODUCTION

The study of the solvent effects on the extraction of active components from herbs is very important for the screening and selection of the solvent for the extraction, fractionation and purification steps in the herbal processing. The extraction of plant

essential oils and components using solvents at high pressure, or supercritical fluids, has received much attention in the past several years, especially in food, pharmaceutical and cosmetic industries, because it presents an alternative to conventional processes such as organic solvent extraction and steam distillation (Pourmortazavi and Hajimirsadeghi 2007). Most of the studies showed that the quality of the essential oils extracted using SFE are better than steam distillation and solvent extraction (Povh *et al.* 2001, Gomez and Ossa 2002, Diaz-Maroto *et al.* 2002).

There have been several methods used to extract antioxidants from plant, such as solid–liquid extraction, aqueous alkaline extraction, extraction with aqueous solutions (Teiantaphyllou *et al.* 2001, Bergman *et al.* 2001), and supercritical fluid extraction (Djarmati *et al.* 1991, Nguyen *et al.* 1994). In general, products obtained by SFE from different plants have a higher antioxidant activity than extracts obtained by using solvent extraction with organic solvents (Snorans *et al.* 2000, Dauksas *et al.* 2001), probably due to a difference in composition deriving from the extraction conditions applied. Although solvent extraction is widely employed for phenolics extraction from aromatic plants, the use of SFE in the extraction of antioxidant compounds has increased because the co-solvent composition in SFE extraction had a great influence on extract yield and composition, for example, in terms of extraction of total phenolic compounds,

total flavonoids and antioxidant activity of elderberry pomace extracts (Seabra *et al.* 2010).

In SFE extraction using a co-solvent, carbon dioxide can be used in combination with water and/or an alcohol to form a gas-expanded solvent to extract desirable polar compounds, such as phenolic compounds. The addition of a small amount of a liquid co-solvent as a modifier can enhance significantly the extraction efficiency and, consequently, reduce the extraction time (Lang and Wai 2001). SFE using a co-solvent is better than solvent extraction because SFE can yield more extract and perform a faster extraction as well as produce a pure extract due to the use of a nontoxic carbon dioxide solvent. Piggott *et al.* (1997) reported that SFE afforded the highest yields of extractable material and total volatile compared with steam distillation, solvent extraction and liquid CO_2 extraction. Herbal plants are well known to be associated with many medicinal properties. In this study we chose a commonly grown herb plant in Malaysia, namely *Polygonum minus* (locally known as kesum). To date, there are no publications found on the antioxidant and antibacterial activity from SFE extracts of this plant. Therefore, the main objective of this study were to determine the antioxidant capacity and antibacterial activity of *Polygonum minus*, and to select the best co-solvent for optimization of SFE process by examining the efficiency of different solvent systems for the extraction of antioxidant compounds.

EXPERIMENTAL

Sample preparation and extraction

Fresh *Polygonum minus* samples was obtained from Ulu Yam, Selangor. The fresh samples were cleaned and washed using running tap water and then the leaf part was separated. The leaf samples were dried using an oven (Sheldon Manufacturing, Inc., FX2-2, USA) at 40°C and then ground for approximately 2-3 minutes using a grinder (*Multifunction disintegrator* SY-04, Golden Bull). Supercritical fluid extraction (SFE) system was used in extraction process for co-solvent study.

Supercritical Fluid Extraction

The SFE system consists of a CO_2 pump (JASCO, PU-2080, Japan), solvent pump (Lab Alliance, Series III, USA), a back-pressure regulator (BPR) (JASCO, BP-1580-81, Japan), an extractor vessel enclosed in an air-circulating oven (Sheldon Manufacturing Inc., FX2-2, USA), a pressure transmitter (Dwyer Instrument, Inc., 682-8, USA) and a sample collector. The CO_2 was chilled to -2°C using a chiller (Protech Electronic, Malaysia) to maintain its liquid state before it was pumped to the extractor. The extractor consists of a high pressure stainless steel vessel that was filled with the plant sample. A back-pressure regulator was employed to maintain the system pressure, while needle valves controlled the flow of the supercritical fluid extraction process. Several types of co-solvents (namely water, 50% (v/v) methanol, 50% (v/v) ethanol, 70% (v/v) methanol, 70% (v/v) ethanol, methanol and ethanol) were used based on the polarity values (Murov 2011). The parameters were set at temperature of 40°C, operating pressure of 150 bars (15.0 MPa), supercritical carbon dioxide (SC-CO_2) flow rate of 3 ml/min and co-solvents flow rate of 0.3 ml/min. 5 g samples were held in static extraction for 20 minutes, followed by a dynamic extraction for 240 minutes or 4 hours. The extract fractions were collected every 30 minutes. All the extracts were dried using an air-circulating oven at a temperature of 40°C until all the excess co-solvents were dried and then weighed to obtain the final mass.

Determination of total phenolic content (TP)

The total phenolic content (TP) of the *P. minus* extracts was determined using the Folin-Ciocalteu reagent (FC) as described by Singleton and Rossi (1965). A calibration curve was prepared using a standard solution of gallic acid (20, 40, 60, 80 and 100 mg/l, $R^2 = 0.960$). Properly diluted *P. minus* extract solution (20 μl) was mixed with 100 μl of FC reagent in the dark. After the reagent stood for 3-8 minutes at room temperature, 80 μl of sodium carbonate solution (7.5% w/v) was added. The solutions were mixed and allowed to stand in the dark for 2 hours at room temperature for the reaction to occur. The absorbance at 765 nm was measured. The results were expressed on a fresh weight basis as mg gallic acid equivalents (GAE)/g sample.

Determination of total flavonoid content (TF)

Total flavonoid contents (TF) of the extracts were determined according to the calorimetric assay developed by Zhishen *et al.* (1999) with a slight modification in term of solution volume. A calibration curve was

prepared using a standard solution of catechin (20, 40, 60, 80 and 100 mg/l, R^2 = 0.972). 20 µl of properly diluted extract was mixed with 80 µl of distilled water. At zero time, 6 µl of (5% w/v) $NaNO_2$ was added. After 5 min, 6 µl of (10% w/v) $AlCl_3$ was added. At 6 min, 40 µl of 1 M solution of NaOH were added. After that, the volume was made up to 200 µl, immediately by the addition of 48 µl of distilled water. The absorbance of the mixture was read at 510 nm. The results were expressed on a fresh weight basis as mg catechin equivalents (CEQ)/g sample.

Ferric reducing/antioxidant power (FRAP) assay

The FRAP assay was performed according to a modified method described by Benzie and Strain (1999). FRAP reagent was freshly prepared by mixing 5 ml 2,4,6-tris(2-pyridyl)-1,3,5-triazine (TPTZ) solution (10 mM) in 40 mM hydrochloric acid solution with 5 ml $FeCl_3.6H_2O$ solution (20 mM) and 50 ml acetate buffer solution (0.3 M, pH 3.6) and incubated at 37°C after the mixing. A calibration curve was prepared using an aqueous solution of ferrous sulphate ($FeSO_4.7H_2O$ at 200, 400, 600, 800 and 1000 µM, R^2 = 0.948). Properly diluted *P. minus* extract (50 µl) was mixed with 1.5 ml of FRAP reagent under dark conditions. The absorbance at 593 nm of 200 µL of the mixture was determined against a blank. FRAP values were expressed on a fresh weight basis as micromoles of ferrous equivalent Fe (II) per gram of sample.

DPPH free radical-scavenging assay

The antioxidant capacity was studied through the evaluation of the free radical-scavenging effect on the 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical. The determination was based on the method proposed by De Ancos *et al.* (2002). Diluted extract (20 µl) was mixed with 80 µl of methanol and 200 µl of 0.1 mM DPPH. The mixture was kept in the dark for 30 minutes before the absorbance at 515 nm was measured against a control solution of methanol and DPPH without extracts. The results were expressed as percentage of the DPPH radical. The percentage of the DPPH radical was calculated according to following equation:

$$\% \text{ inhibition of DPPH} = \frac{A_{control} - A_{sample}}{A_{control}} x100 \qquad (1)$$

where $A_{control}$ is the absorbance of DPPH without extract, while A_{sample} is the absorbance of the extracts.

RESULTS AND DISCUSSION

Extraction yield

The result of extraction yield for SFE with different co-solvents was shown in Figure 1. From the figure, 70% methanol gave the highest extraction yield while the pure methanol and ethanol produced the lowest extraction yields. The mixture of water-alcohol co-solvent appeared to increase the extract yield when compared with water or alcohol alone. Due to the low polarity and poor solvent of carbon dioxide for
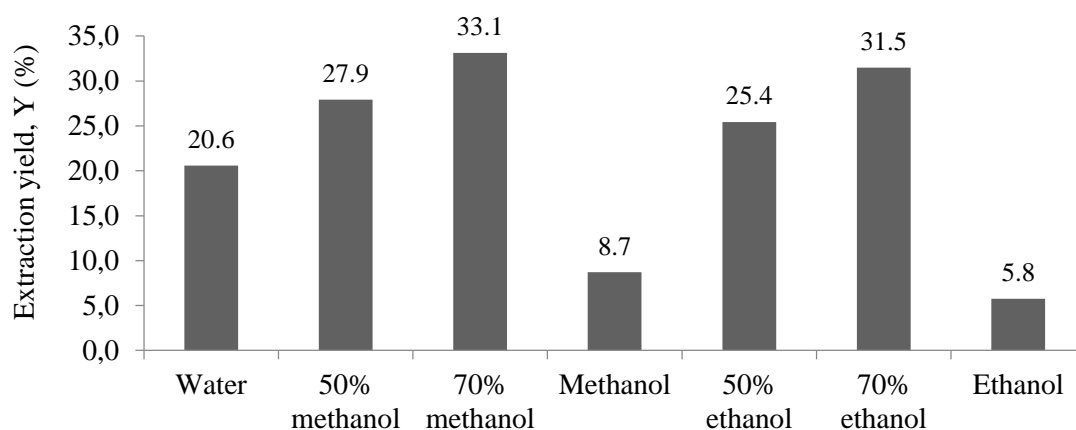
Fig.1: The extraction yield of of *P. minus* at 40°C and 150 bar

polar materials, the polar co-solvent such as water and alcohol may be useful for breaking down the polar materials so that the oil or trapped components are more accessible for extraction. The co-solvent was also suggested as the primary solvent, thus enhancing the diffusivity within the complex plant matrix (Kerrola and Kallio 1994). Besides, the mixture of water-alcohol co-solvent system increases the sample solubility in supercritical phase, where polarity changes of supercritical fluid increased the solvation power of solvents toward analytes (Dohrn and Buenz 1995). Thus, the result showed that changing co-solvent type and concentration could significantly affect the yield and selectivity of the compounds extracted.

Antioxidant assay

Four antioxidant assays were conducted on all SFE fractions. Table 1 showed the highest result obtained based on extraction time. Total phenolic content (TP) of the extracts measured using Folin-Ciocalteu's colorimetric method. The highest TP

Table 1. Antioxidant capability of SFE extracts

Co-solvent	TP (mg GAE/g) ± S.D[a]	TF (mg CAE/g) ± S.D[a]	FRAP (µmol Fe (II)/g) ± S.D[a]	DPPH (%) ± S.D[a]
Water	1.7 ± 0.05	4.7 ± 0.07	69.7 ± 0.18	46.5 ± 0.52
50% methanol	4.5 ± 0.11	8.9 ± 0.04	169.3 ± 0.70	66.2 ± 0.45
50% ethanol	3.0 ± 3.76	7.1 ± 0.04	154.7 ± 0.18	62.5 ± 0.86
70% methanol	11.2 ± 0.15	11.9 ± 0.03	346.7 ± 0.66	88.7 ± 0.40
70% ethanol	3.9 ± 0.18	9.5 ± 0.05	151.0 ± 0.33	72.6 ± 0.61
Methanol	2.0 ± 0.12	4.3 ± 0.12	99.4 ± 0.59	62.5 ± 0.50
Ethanol	1.2 ± 0.23	3.7 ± 0.24	89.0 ± 0.16	39.8 ± 0.17

[a] Standard deviation for 3 replicates

obtained from 70% methanol (11.2 ± 0.15 mg GAE/g sample). The total flavonoid content (TF) of this plant showed the same trend with TP result where the highest TF obtained from 70% methanol extracts. This correlation indicates that flavonoids are the important phenolic group in representing the antioxidant capacity of *P. minus*. The antioxidant capacity of the different *P. minus* extracts was studied using the ferric reducing/antioxidant power (FRAP) test and the DPPH free radical inhibition test. The FRAP value and percentage of DPPH inhibition showed the same trend where a positive correlation exists between the antioxidant activity and the reducing capability of the extracts. On the other hand, the antioxidant power is related to the phenolic antioxidant activity (Yildrim *et al.* 2000). Results also showed that FRAP and DPPH have a positive correlation with TP. The existence of this relationship demonstrates that the phenolic (flavonoid) compounds are the main components contributing to the antioxidant activity of the plant.

The solvent type and concentration are key factors in this extraction process where as solvent polarity will play a key role in increasing phenolic solubility (Naczk and Shahidi 2006). Besides, solvent polarity affects both the kinetics of phenolic release from the solid matrix and the antioxidant activity of the extract (Mussatto *et al.* 2011). The best results of TP and TF as well as antioxidant power (FRAP and DPPH) were achieved on methanol extracts. The use of methanol and ethanol gave better results than the use of only water since phenolic compounds are often more soluble in

organic solvents less polar than water (Kim and Lee 2002, Liu and Ang 2000). As can be seen in Table 1, concentration of 70% (v/v) for both methanol and ethanol in water extracted more phenolics and flavonoids and also exhibited the highest antioxidant power compared with other concentrations. Previous studies reported that the use of water in combination with an organic solvent contributing to the creation of a moderately polar medium that insures the extraction of phenolics, giving better results than when using a pure organic solvent (Lapornik *et al.* 2005, Musa *et al.* 2011, Mussatto *et al.* 2011).

CONCLUSION

The result of this study showed that the type of solvents and their concentrations affect the extraction yield and antioxidant activity. It can be said that the potential of SFE in the extraction of antioxidant and antibacterial compounds from plants is tremendous. This is supported by the previous study that stated SFE is capable of extracting a wide range of diverse compounds from variety of sample matrices. There was a positive correlation between total phenolic, total flavonoid and antioxidant capacity of the *P. minus* extracts. The higher the total phenolic and flavonoid contents, the higher were the FRAP and DPPH values. 70% methanol as co-solvent in SFE was observed to exhibit the best extraction yield and antioxidant capacity compared to other solvents. Methanol is the most commonly used extraction solvent due to its high polarity, which could usually produce high

extraction yields. However, its toxic characteristic could be judgemental when it was to be used in the food and pharmaceutical applications. Regardless of that, the use of methanol in the present study was very minimal and very useful to establish the optimum conditions of SFE in producing the high yield of *P. minus* extracts with high antioxidant capacity.

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