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Antioxidant activity of spearmint (*Mentha spicata* L.) leaves extracts by Supercritical Carbon Dioxide (SC-CO_2) extraction

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Abstract: In this study, the effects of pressure (100–300 bar), temperature (40–60ºC) and co-solvent flow rate (3–9 g/min) on antioxidant activities of supercritical carbon dioxide (SC-CO_2) extracts of spearmint leaves were determined using full factorial in the frame of complete randomize design (CRD). The antioxidant activity of SC-CO_2 extracts was determined by DPPH radical scavenging method. The results showed that extraction pressure, temperature and co-solvent flow rate had significant effect ($P < 0.05$) on antioxidant activity of extracts obtained. The highest antioxidant activity (71.00 ± 2.65%) was obtained at 200 bar pressure, 50ºC and 6 g/min co-solvent flow rate. However, all of the extracts obtained in this study had significantly higher antiradical activities varying from 35.62 ± 0.34% to 72 ± 3.17% compared to butylated hydroxytoluene (BHT) as a reference. These results indicated that supercritical carbon dioxide is a promising alternative process for recovering compounds of high antioxidant activities from spearmint leaves.

Keywords: Supercritical carbon dioxide (SC-CO_2) extraction, spearmint (*Mentha sppicata* L.), antioxidant activity

Introduction

Spearmint belongs to the genus *Mentha* in the family *Labiateae (Lamiaceae)* (Reverchon 2006). This family is a rich source of polyphenolic compounds and hence could possess strong antioxidant properties (Palmer and Ting 1995; Bimakr *et al*., 2010). Undesirable changes in food quality due to oxidation reactions can be prevented by applying antioxidant compounds in to its formulation. Synthetic antioxidants such as butylated hydroxytoluene (BHT) and butylated hydroxyanisole (BHA) should be replaced by natural compounds due to their possible toxicity (Namiki 1990; Pizzale *et al*., 2002). By considering adverse effects of synthetic antioxidant on human health, alternative natural and safe sources of food antioxidant should be identified (Wanasundara and Shahidi, 1998; Goli *et al*., 2005). Plant extracts due to possess similar or even higher antioxidant activity can be natural alternatives to synthetic antioxidants, so they are strongly of interest in the food industry (Le Floch *et al*., 1998). Supercritical carbon dioxide extraction (SC-CO_2) is an attractive alternative to conventional liquid extraction due to the use of a supercritical fluid which is non-toxic, non-explosive, and easily removable from products (Kivilompolo and Hyotylainen, 2007; Liu and Zhu,2007).

Carbon dioxide (CO_2) is non-polar fluid which leads to difficulty in extraction of polar compounds. One effective way to overcome this problem is using small amounts of organic co-solvents (Cavero *et al*., 2006). Furthermore, SC-CO_2 extraction has been proposed for antioxidant extraction from rosemary leaves, sage, and herbaceous matrixes. Comparing to the conventional methods of extraction, the antioxidant activity of extracts by supercritical fluid extraction (SFE) was significantly higher (Lafka *et al*., 2007). Reactive oxygen species (ROS) are associated with aging, atherosclerosis, and carcinogenesis for the damaging of lipids, proteins, enzymes, DNA and RNA (Lopez-Sebastian *et al*., 1998; Hills *et al*., 1991; Heim *et al*., 2002). Therefore, studies on herbs have become the top issue at present time for their great potential of antiradical activities. In this study the effect of SC-CO_2 extraction parameters namely co-solvent flow rate (3, 6 and 9 g/min), temperature (40, 50 and 60ºC) and pressure (100, 200 and 300 bar) was investigated to obtain the highest antioxidant activity.

Materials and Methods

Raw material and reagents

The fresh leaves of spearmint (*Mentha spicata* L.) were obtained from Cameron Highland, Pahang,

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Malaysia. After harvesting, the leaves were washed under tap water. Leaves were dried at 40°C in a ventilated drying oven (1350FX, USA) for 24 h and then stored at ambient temperature in the dark. The samples were ground in grinder mill (MX-335, Panasonic, Malaysia) for 10 s to produce a powder with an approximate size of 0.525 mm. The following chemicals were used: Carbone dioxide (SFE grade), contained in a dip tube cylinder, was purchased from MOX Company in Malaysia. Ethanol (EtOH, 99.5%, analytical grade) was obtained from Scharlau Chemical, European Union. Sodium methoxide, butylated hydroxytoluene (BHT) and 1,1-diphenyl-2-picrylhydrazyl (DPPH) was purchased from Fisher (Pittsburgh, PA, USA).

Supercritical carbon dioxide (SC-CO_2) extraction

Supercritical carbon dioxide (SC-CO_2) extraction was performed on a supercritical fluid extractor (ABRP200, Pittsburgh, PA, USA) with the extractor volume 500 ml. Before liquid CO_2 passed into the extraction vessel, filled with the samples, by the means of a pump (P-50, Thar designs, Inc. Pittsburg, PA, USA), it was pressurized to the desired pressure and heated to the specified temperature in order to reach the supercritical state. The supercritical CO_2 flow rate was maintained at 15 g/min and also, the duration of static extraction time was fixed to 30 min. The powdered plant material (35 g) was mixed with 90 g glass beads (2.0 mm in diameter), placed into the extractor vessel. Ethanol was used as modifier. Extractions were performed at three different pressure levels (100, 200, 300 bar), three different temperatures (40, 50 and 60°C) and three different co-solvent flow rate (3, 6 and 9 g/min). The extraction was then performed under various experimental conditions in accordance with the Complete Randomize Design (CRD) full factorial.

Determination of antioxidant activity

The spearmint (*Mentha spicata* L.) leaves extract obtained under the SC-CO_2 conditions was subjected to screening for its possible antioxidant activity. The antioxidant activity was assessed using 1, 1-diphenyl-2-picrylhydrazyl (DPPH) radical-scavenging assay (Sanchez-Moreno *et al*. 1998; Turkmen *et al*. 2006; Lafka *et al*. 2007). The assay is based on the color change caused by reduction of the DPPH• radical which was determined by measuring absorbance at 515 nm. The reaction time for the assay was determined as 60min by preliminary experiments. This assay was carried out as described by Qiuhui *et al*. (2007) with some modifications (Qiuhui *et al*. 2007). An ethanolic solution of DPPH radicals was freshly prepared at a concentration of 0.2 mM. Butylated hydroxytoluene (BHT) was prepared at the concentration of 0.2 mg mL^{-1} in ethanol as reference. Extracts were dissolved in ethanol to give the final concentration of 5 mg/ml. Then 0.1 ml of extract solutions or BHT solution were mixed with 1.5 mL of ethanolic solution of DPPH radicals and the mixture was vortex for 20 s at room temperature. The blank test was conducted with 0.1 ml ethanol instead of extracts. Absorbance measurements commenced immediately in a 1-cm quartz cell after 1, 30, and 60 min using a UV-260 visible recording spectrophotometer (Thermo 4001/4 UV–Vis Spectrophotometer, Thermo Fisher Scientific). The inhibition percent of each sample was calculated according to the following equation:

$$\% \text{ inhibition} = [(\text{absorbance of control} - \text{absorbance of test sample})/\text{absorbance of control}] \times 100$$

All the data were the averages of triplicate determinations of three independent tests.

Statistical analysis

In the present study the process of extraction was investigated using Complete Randomize Design (CRD) full factorial for a higher antioxidant activity from spearmint leaves. Data were subjected to analyses of variance (ANOVA) and multiple comparison tests were formed using a Least Significant Difference (LSD) at 95% of confidence level. All the analyses were carried out using the statistical software, Minitab V. 14.0 statistical software (Minitab Inc. State College, PA, USA).

Results and Discussion

It was shown that the antioxidant activities of the experimental runs in terms of inhibition expressed as percentage of DPPH free radical removed after absorption of the mixture reached a plateau. The higher inhibition, the stronger the antioxidant activity of extracts is. All the extracts obtained using SC-CO_2 exhibited a high antioxidant activity. In comparison, butylated hydroxytoluene (BHT) scavenged 28.63±1.42 % of the initial DPPH free radicals, those were much lower than that of extracts obtained by SC-CO_2 extraction. But the difference in concentration of extracts and BHT should be considered (5 mg mL^{-1} and 0.2 mg mL^{-1}, respectively).

By considering to ANOVA analysis it can be concluded that all three independent variables namely extraction pressure, temperature and co-solvent flow rate had significant ($P< 0.05$) effect on antioxidant activity. Selection of the supercritical fluid must be taken into consideration due to its importance for development of successful supercritical fluid extraction. The variations of the antioxidant activities as a function of change in different levels of the factors studied were shown in Figure 1. The mean values of the extraction yields for the corresponding parameters at each level were calculated according to the assignment of the experiment. In Table 1 the average response of each level about antioxidant activity was presented. Also, *R* value which mentioned in Table 1 means range between three average responses of each level about antioxidant activity. Based on the *R* value, it can be concluded that the effect of variables on the crude extraction yield followed the decreased order: extraction pressure (*R*= 16.48) > extraction temperature (*R*= 8.21) > co-solvent flow rate (*R*= 6.21).

Table 1. Results obtained at the experimental condition using Complete Randomized Design (CRD) full factorial

Parameter	Antioxidant activity (% Inhibition) L1 [a]	Antioxidant activity (% Inhibition) L2 [a]	Antioxidant activity (% Inhibition) L3 [a]	Range [b]
Pressure	44.09 ± 5.18	60.57 ±6.55	59.13 ± 4.72	16.48
Temperature	53.00 ± 8.66	59.50 ± 10.05	51.29 ± 6.74	8.21
Co-solvent flow rate (g/min)	50.57 ± 9.74	56.44 ± 8.73	56.78 ± 8.12	6.21

[a] Average responses of each level about antioxidant activity.
[b] Range value means range between three average responses of each level about antioxidant activity.

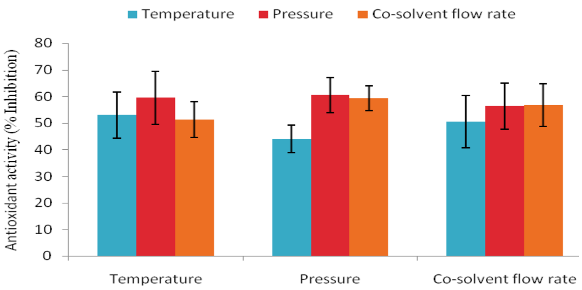

Figure 1. Antioxidant activities of crude extract under Complete Randomize Design (CRD) full factorial

Figure 2 showed the effect of pressure on the antioxidant activity of SC-CO_2 spearmint leaves extracts at constant co-solvent flow rate. It can be concluded that at 100 bar the antioxidant activity was increased with increasing co-solvent flow rate to 9 g/min min. But, at higher pressures (200 and 300 bar), the antioxidant activity was increased with increasing co-solvent flow rate up to 6 g/min, after which the antioxidant activity was reduced. This is due to the volatility of different compounds which attributed in antioxidant activity of spearmint leaves extract (Nilufer *et al*. 2009). It was clear that the highest antioxidant activity was achieved at 200 bar extraction pressure.

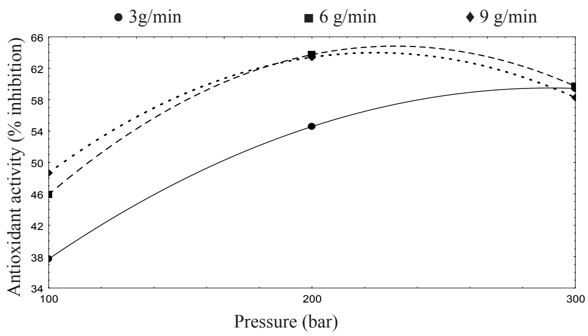

Figure 2. Effect of pressure on the antioxidant activity of SC-CO_2 extracts at the constant co-solvent flow rate

Figure 3 showed the effect of temperature on the antioxidant activity of spearmint (*Mentha spicata* L.) leaves in SC-CO_2 extraction at three temperature levels of 40, 50 and 60°C. The density of CO_2 at constant pressure is reduced with increasing temperature and leading to reduce the solvent power of supercritical CO_2. The effect of temperature on solute solubility is different at pressures in the critical range. Near the system critical pressure, the fluid density is very sensitive to temperature.

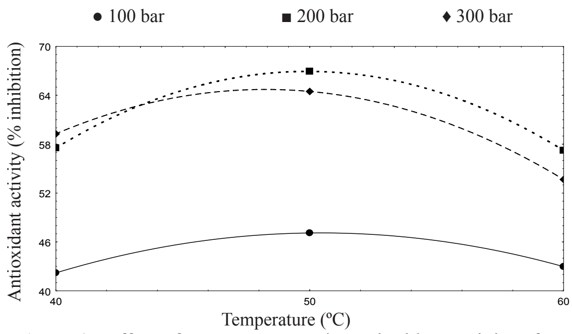

Figure 3. Effect of temperature on the antioxidant activity of SC-CO_2 extracts at the constant pressure

This might be the reason that antioxidant activity was changed significantly when temperature was changed over the range of 40–60ºC. Based on the obtained results, the antioxidant activity of SC-CO_2 spearmint extracts increased up to 50 ºC after which, decreased due to the thermo-sensitivity of antioxidants. About the effect of temperature also, it is interesting to know that at lower temperature (40ºC) the antioxidant activity was increased up to 9 g/min co-solvent flow rate but at higher extraction temperature (60ºC) it was decreased with further increase after 6 g/min co-solvent flow rate. Therefore, the moderate extraction temperature (50ºC) was preferred compare to 40 and 60ºC to save thermo labile compounds.

Figure 4 presented the effect of co-solvent flow rate on the antioxidant activity of spearmint leaves extracts by SC-CO_2 extraction. It was clear that the highest antioxidant activity was obtained by using

9 g/min (63.13%). The obtained SC-CO_2 extracts antioxidant activity using 9 comparing to 6 g/min was higher but the difference between them is too low (1.46%) and it is not significant according to a 2-sample t-test (P value (0.667) > α (0.05)). Therefore, 6 g/min co-solvent flow rate is preferable due to its better effect on the antioxidant activity of obtained SC-CO_2 extracts and economic cost. By considering to the polarity of bioactive compounds such as phenolic compounds which contributed greatly on the antioxidant activity of spearmint leaves extracts by increasing co-solvent flow rate (ethanol) the antioxidant activity was increased. Finally, it was concluded that the highest antioxidant activity (71.00 ± 2.65%) was obtained at moderate pressure, temperature and co-solvent flow rate (200 bar, 50ºC and 6 g/min).

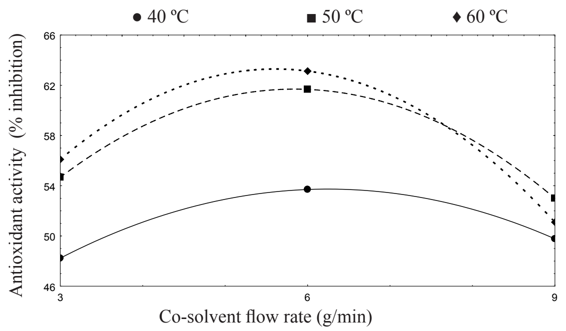

Figure 4. Effect of co-solvent flow rate on the antioxidant activity of SC-CO_2 extracts at the constant temperature

Conclusion

The extraction parameters on the antioxidant activity of the supercritical carbon dioxide (SC-CO_2) extracts from spearmint (*Mentha spicata* L.) leaves were discussed in this study to find the best extraction condition in order to obtain the highest antioxidant activity. Almost all the SC-CO_2 extracts exhibited high antioxidant activity. In principle, by manipulating the extraction conditions, it may be possible to concentrate the bioactive compounds from spearmint (*Mentha spicata* L.) leaves. The highest antioxidant activity (71.00 ± 2.65%) of supercritical carbon dioxide (SC-CO_2) extracts from spearmint leaves was obtained at 200 bar, 50ºC and 6 g/min. The obtained results showed that the spearmint (*Mentha spicata* L.) leaves are potential source of antioxidant compounds and also, supercritical carbon dioxide (SC-CO_2) extraction is a promising alternative technique to conventional extraction methods.

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