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Effect of different extraction techniques on total phenolic content and antioxidant activity of *Quercus infectoria* galls

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Abstract

In this work, the bioactive compounds which was obtained by extracting *Quercus infectoria* via two extraction methods; Soxhlet and supercritical carbon dioxide (SC-CO_2) extraction, were analyzed using total phenolic content and DPPH (2,2-diphenyl-1-picryl hydrazyl) free radical scavenging activity analysis. The aim of this study is to compare the total phenolic content and antioxidant activity of *Quercus infectoria* extract acquired from SC-CO_2 extraction with those from Soxhlet extraction method. The results showed the used of SC-CO_2 extraction give the lowest extraction yield as compared to Soxhlet extraction. The selectivity of *Q. infectoria* extracts using SC-CO_2 extraction was better which in contrast with Soxhlet extraction method since it shows higher total phenolic content (143.75 ± 1.06 mg GAE/g sample). This study also revealed that the extracts from both extraction methods can posses' antioxidant activity and comparable to those obtained from commercial antioxidant.

Introduction

The extraction of bioactive compounds from natural materials has gain many interest from researchers as the demand for functional ingredients obtained via natural processes keep increasing due to consumers are getting more interested in functional foods. The commonly used conventional extraction method is Soxhlet extraction, which apply in extraction of several plant materials, including sweet grass (Grigonis *et al*., 2005), *Pinus radiata* bark (Aspé and Fernández, 2011), *Artemesia* sp. (Karabegović *et al.*, 2011) and *Lamii albi* flos (Wjciak-Kosior *et al*., 2013). Unfortunately, Soxhlet extraction has some drawbacks that caused limitation in the application of it. This extraction method only can be applied for compounds that can endure the boiling temperature of the solvent used, but not applicable for thermolabile compounds as prolonged heating may direct to compound degradation (de Paira *et al*., 2004). Other than that, the extraction process take a lot of time and large volume of solvent is needed in order to extract the product could contribute to disadvantage of Soxhlet extraction.

In practice, solvent extraction offers good recovery of antioxidant phytochemicals from various samples, such as fruits and vegetables. Extraction of phytochemicals from plant matrix with supercritical fluid extraction using carbon dioxide (CO_2) as a solvent has gain many interests as it is priority in finding alternative way to replace conventional extraction methods (Choi *et al*., 1997). This is due to the gas-like characteristics help the fluid diffuse to the matrix and access the phytochemicals, and liquid-like characteristics provide good salvation power. Many reviews have been reported that supercritical carbon dioxide (SC-CO_2) extraction gave high recovery than typical organic solvents (Tena *et al.*, 1997). Theoretically, supercritical fluid extraction based on the utilization of a fluid under supercritical conditions, is a technology suitable for extraction and purification of a variety of valuable compounds, especially for those that have low volatility or susceptible to thermal degradation since it did not pollute and harm the biological active compounds in herbs (Kawahito *et al*., 2008). Furthermore, supercritical fluids have higher diffusivity and lower density, surface tension and viscosity which can be varied by altering the operating conditions, subsequently can give advantages to the extraction process.

Quercus infectoria plant, known as oak tree, is a small tree native to Greece, Asia Minor and Iran (Basri and Fan, 2005). The irregular plant growth of the galls is stimulated by the reaction between plant hormones and powerful chemicals that regulate growth produced by insect called *Cynips quercufolii* by lays its eggs in the bark. *Q. infectoria* galls are greatly used as medicinal plant since ancient time because it has been reported to contain large amount

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of bioactive constituents such as tannins, gallic acid, syringic acid, ellagic acid, β-sitosterol, amentoflavone, hexamethyl ether, isocryptometrin, methyl betulate, methyl oleanate, hexagalloyglucose and others (Dar *et al*., 1976; Ikram and Nowshad, 1977; Hwang *et al.*, 2000). The main constituents found in the galls of *Q. infectoria* are tannin (50-70%) and small amount of free gallic acid and ellagic acid (Ikram and Nowshad, 1977; Wiart and Kumar, 2011). Tannin which is derived from phenolic compounds was proved to have antioxidant activity and has the ability to be antimicrobial, antibacterial and the antifungal agent (Everest and Ozturk, 2005; Hamid *et al.*, 2005; Yamunarani *et al.*, 2005). Besides, the galls also contain important minerals (calcium, phosphorus, potassium and magnesium) which play an important role in enhancement of bone health and osteoporosis prevention (Hapidin *et al*., 2012).

A study has been conducted to compare the ability of different extraction techniques including SC-CO_2 extraction and Soxhlet extraction to extract *Quercus infectoria* galls. The extracts were evaluated by total phenolic content and antioxidant activity analysis to select better technique in extracting the galls.

Materials and Methods

Preparation of plant material

The galls of *Q. infectoria* (purchased from the local market in Johor Bharu, Malaysia) were first crushed by blender and rinsed using tap water to remove any unwanted materials from the galls. The galls were dried in an oven at 60 overnight. The prepared samples stored in dark place at room temperature for further analysis.

Supercritical carbon dioxide (SC-CO_2) extraction

The extraction of *Q. infectoria* galls was performed using SC-CO_2 system which comprises of 50 ml extraction vessel, high-pressure pump, automated back pressure regulator and oven. Liquid CO_2 was supplied from a gas cylinder. A total of 10 ± 0.0010 g of grounded plant material was loaded into extraction vessel and the remaining volume is filled with cotton in the bottom and upper of the cell. The introduction of some firm materials with the grounded samples was able of maintaining suitable CO_2 flow rate and maintained the wanted permissibility of the particle throughout the extraction process (Chemat *et al*., 2004; Wang and Weller, 2006). The cotton is placed at the end to avoid any possible residue of solid material. The vessel is placed in an oven to maintain operating temperature. Liquid carbon dioxide was pumped into the vessel after required temperature was obtained. The extract was collected in a vial and placed in an oven to allow for evaporation of solvent. The conditions of the extraction process were a pressure of 26.84 MPa, a temperature of 49.6°C, particle size of 0.50 mm and a flow rate of 2 mL/min. The extraction process was done for 120 minutes.

Soxhlet extraction

5.00 ± 0.05 g of powdered *Qercus infectoria* galls was inserted in the thimble while 150 ml of methanol (100%) placed in the flask of Soxhlet apparatus. The temperature of the process was corresponded to boiling point ofsolvent used and the extraction time was set for 6 hours. Lastly, the solvent was removed from the yield by using rotary evaporator at temperature of 40. All of the steps were repeated by using 70% methanol, 100% ethanol, 70% ethanol and acetone as the extraction solvent.

Yield calculation

The yield of the extract was calculated by using the following equation:

$$\text{Percentage extraction yield} = \frac{m_1}{m_0} \times 100$$

Where m_1 is mass of the extract in gram and m_0 is mass of sample in gram.

Total phenolic content determination

Total phenolic content (TPC) in the extracts is determined by using Folin-Ciocalteu (FC) reagent (Mandana *et al*., 2012). 20 µl of 1 mg/ml plant extract, 1.58 ml of distilled water and 100 µl of FC reagent (diluted ten-fold) were mixed in a test tube. It was left at room temperature in order to the reaction take place. After 7 minutes, 300 µl of 75 g/l sodium carbonate (Na_2CO_3) solution was added to the sample solution. The tube then kept in a dark place for 30 minutes at room temperature. The absorbance of the colour changes was measured at 765 nm. The calculation of TPC was done on the basis of the gallic acid standard curve which construct by using the same procedure and concentrations of 0, 50, 100, 150, 250 and 500 mg/ml. The results were expressed as gallic acid equivalents (mg GAE/g extract sample).

Antioxidant activity determination

The antioxidant activity assay of the extract was evaluated using DPPH (2,2-diphenyl-1-picrylhydrazyl) radical scavenging method (Kaur *et al*., 2004; Akowuah *et al.*, 2005), by dissolving 77 µl of 2.5 mg/ml extracts in 3 ml of 6 x 10^{-5} M methanolic DPPH solution. DPPH is a stable free radical, which forms a purple-coloured solution when dissolved in

methanol. Antioxidant components can scavenge this stable free radical and therefore the purple colour will be bleached. The mixture was vortex at room temperature for 30 s. The control sample absorbance ($A_{control}$) which contains methanolic solution of DPPH was also measured. All of the mixtures were placed in a dark place for 30 minutes at room temperature. The absorbance of all sample solutions was measured at 517 nm using UV-Vis spectrophotometer. Radical scavenging activity was calculated by using the following equation:

$$DPPH\ radical\ scavenging\ (\%) = \frac{A_{control} - A_{sample}}{A_{control}} \times 100$$

Where A_{blank} is the absorbance of the blank; A_{sample} is the absorbance of the sample.

Statistical analysis

Results were expressed as the mean ± S.D. of duplicate independent experiments. Data was analyzed using SPSS 16.00 for windows (SPSS Inc., Chicago, IL). The significance differences between the data were analyzed by using one-way analysis of variance (ANOVA) at 95% confidence level. P values < 0.05 were considered to be significant.

Results and Discussion

Comparison of yield from soxhlet extraction method with supercritical carbon dioxide (SC-CO_2) extraction

Soxhlet extraction method was done in order to compare its properties with SC-CO_2 extraction method. The experimental result of extraction yield for *Q. infectoria* galls was presented in Table 1. The extraction yield using SC-CO_2 showed low ($p < 0.05$) percentage yield (0.17%) compared to those using Soxhlet extraction method. This occurrence mainly due to short extraction time applied when extracting the compound from the galls. Generally, every solvent used for Soxhlet extraction method gave a high percentage yield in the range between 45.71% and 80.03%. Our findings showed that the use of 100% aqueous as a solvent shows the highest percentage yield for the extraction of *Q. infectoria* by using Soxhlet extraction method followed by 70% methanol, 70% ethanol, pure methanol and pure ethanol. On the other hand, the lowest extraction yield for *Q. infectoria* galls extract using Soxhlet extraction resulting from 100% acetone suggesting that polar compounds in biological plant is easier to extract with more polar solvents. It conform the theory of 'likes dissolve likes'. The results specify that the mixture of the organic solvents give higher extraction yield than the pure solvent while the pure solvent of methanol gives higher yield compare to ethanol because of the higher polarity of the solvents. The finding was in agreement with the results of percentage of extraction yield from *Tamarix aphylla* (L.) where the highest yield was obtained when water was used as the solvent (8.07%) (Mohammedi and Atik, 2001). However, the extraction yield has been shown to vary for different raw material used. Opposite result was found that pomegrated peel was extracted effectively using methanol followed water, ethanol and acetone (Adnan *et al*., 2011). Nevertheless, high extraction yield does not necessarily demonstrate high antioxidant activity of the sample as proved by previous researchers (Mohammedi and Atik, 2001; Adnan *et al.*, 2011).

Table 1. Extraction yield of different extraction methods from *Q. infectoria* galls

Extraction method	Type of solvents	Extraction yield (%)
Supercritical fluid extraction	CO_2	0.17 ± 0.01^{a}
Soxhlet extraction	100% Methanol	69.54 ± 6.95^{b}
	70% Methanol	76.34 ± 4.90^{b}
	100% Ethanol	45.71 ± 13.00^{b}
	70% Ethanol	71.44 ± 6.64^{b}
	100% Acetone	43.57 ± 19.47^{b}
	100% Aqueous	80.03 ± 6.87^{b}

^{a,b} shows significantly different ($P < 0.05$)

Table 2. Total phenolic content shows by different extraction methods

Extraction method	Type of solvents	Total phenolic content (mg GAE/g sample) ± SD
Supercritical fluid extraction	CO_2	143.75 ± 1.06^{d}
Soxhlet extraction	100% Methanol	95.86 ± 2.02^{a}
	70% Methanol	112.29 ± 3.03^{c}
	100% Ethanol	$109.79 \pm 6.57^{b,c}$
	70% Ethanol	$99.43 \pm 2.02^{a,b}$
	100% Acetone	$107.65 \pm 0.50^{b,c}$
	100% Aqueous	95.86 ± 1.01^{a}

SD : standard deviation

^{a,b,c,d} shows significantly different ($P < 0.05$)

Total phenolic content

Total phenolic content (TPC) of extracts was measured to investigate its contribution in antioxidant activity of the galls. Results of the phenolic content in *Q. infectoria* galls were presented in Table 2. The *Q. infectoria* galls extract using SC-CO_2 extraction give the highest content of phenolic compound (143.75 ± 1.06 mg GAE/g sample) compared to TPC of Soxhlet extraction method. Other than that, for Soxhlet extraction method, better content of phenolic compound was found in 70% methanol extract (112.28 mg GAE/g sample) compared to the other solvents and it is well-matched with the previous findings where aqueous alcohol solvents were effective towards *Tamarix aphylla* extraction due to the advantage of alcohol and water mixture in adjusting the polarity of the solvents (Mohammedi and Atik, 2001). The differences between the content of phenolic compound between each solvents and extraction methods were statistically significant ($p < 0.05$). The presence of high phenolic compound

Table 3. The effect of different solvents on the DPPH radical scavenging activity compared to positive control (BHT)

Extraction method	Type of solvents	DPPH scavenging ± SD (%)
Supercritical fluid extraction	CO_2	98.05±0.18[a]
Soxhlet extraction	100% Methanol	93.38±0.19
	70% Methanol	94.36±0.64[a]
	100% Ethanol	92.60±1.29
	70% Ethanol	92.86±0.18
	100% Acetone	92.83±1.61
	100% Aqueous	94.55±0.37[a]
Positive control (BHT)		90.74±2.49

[a] $p < 0.05$ compared with control

content in the extract from SC-CO_2 extraction proved that the quality of the extracts using this extraction method was better since less impurity were extracted due to high selectivity of the process. This occurrence can be explained by clear colour attained from the extraction process for the plant matrix. Basically, the extracts from both extraction methods contain high amount of phenolic compound and it could be useful for the prevention of oxidative activities of the plant's extract.

Antioxidant activity

DPPH assay is one of the most widely used methods to investigate the antioxidative activity of the sample due to its stability, simplicity and short time required for analysis. The antioxidant activity assay was conducted to investigate the ability of *Q. infectoria* to scavenge free radicals *in vitro* by the improved of scavenging activity percentage. Based on Table 3, it shows that all of the extract using both extraction methods give significantly ($p < 0.05$) higher activity (%) than BHT (positive control). The table clearly shows that the highest antioxidant activity was attained by using SC-CO_2 as a solvent which was 98.05 ± 0.18% compared to other solvents used in Soxhlet extraction technique. In addition, the extracts of Soxhlet extraction shows 70% methanol (94.35%) gives the highest DPPH scavenging activity followed by 100% methanol with slight difference in scavenging activity (93.38%). Still, other solvents also indicated high free radical scavenging varies between 92.60% (100% ethanol) and 92.83% (100% acetone).

These results can be explained by the fact that these extract enriched with phenolic compounds which give positive correlation with the antioxidant activity of the plant (Pourmorad *et al*., 2006). Generally, the presence of hydroxyl (-OH) group and double bond between the carbon atom (C=C) in phenolic compound are responsible for antioxidant activity since they can enhance the radical scavenging activity of the compounds. The capability of antioxidant activity in *Q. infectoria* extracts also has been proved previously where the concentration of the extracts needed to inhibit 50% of DPPH radical, hydroxyl radical and lipoxygenase activity were 0.98 ± 0.05 µg/ml, 415.00 ± 15.00 µg/ml and 31.77 ± 0.54 µg/ml respectively (Pithayanukul *et al*., 2009). All of these values smaller than concentration of their reference standard which means low concentration of the extracts already can inhibit 50% of oxidative reaction.

Conclusions

The result of total phenolic content and antioxidant activity obtained from supercritical carbon dioxide extraction revealed the advantage of this extraction method against Soxhlet extraction. In improving the yield of the extract, the used of methanol as co-solvent with CO_2 can be applied since polar compound need to be extracted. Overall, the extract from both extraction methods posses antioxidant activity as they capable of quenching free radicals to inhibit the free radical chain reaction, and acting as reducing agents. Besides, a linear relationship was found between the total phenolic content in the extracts and their antioxidant activity; indicate that phenolic compound could be major contributors to antioxidant activity of the *Q. infectoria* galls extract.

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