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Ultrasonic assisted extraction of phenolic and flavonoid content from *Orthosiphon stamineus* leaves

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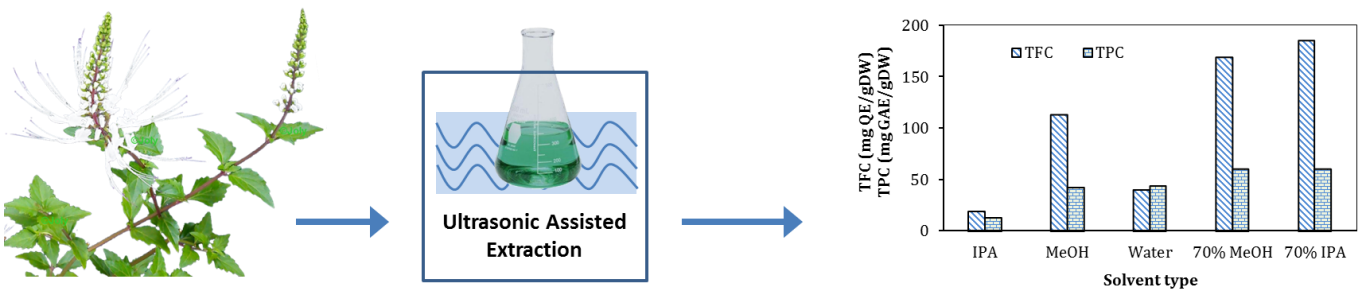
This paper presents a combined effect of ultrasonic assisted extraction and solvents of varying polarities on phenolic compound extraction from *O. stamineus*. The polyphenol content in the plant extracts was analysed using Singleton's method and aluminium chloride colorimetric assay. The results suggest that the polyphenol extraction from *O. stamineus* is affected by the solvent type. The highest phenolic content of 168.8 mg GAE/g DW was obtained from ultrasonic assisted extraction (UAE) using 70% aqueous methanol and 70% aqueous propanol solvent. The highest total flavonoid content of 185.3 mg QE/g DW was obtained using 70% aqueous propanol. The phenolic acid and flavonoid yield increased with extraction time, however, extraction beyond 120 min or at a temperature higher than 60°C induced degradation and hence reducing extraction yield.



Introduction

Orthosiphon stamineus (vernacular name: *'misai kucing'*) is traditionally used in Malaysia for treatment of bladder inflammation, eruptive fever, edema, hypertension, diabetes mellitus, rheumatism and diuretic[1]. Previous studies revealed that extract of *O. stamineus* contained many useful bioactive compounds such as terpenoids, polyphenols and sterols[2] leading to various activities such as antibacterial, antifungal, antimicrobial and antitumor.

The first step to recover and purify bioactive compounds from plant materials involves extraction. The solvent used, extraction method and condition affect the yield of bioactive compound in the extract. Conventional method such as maceration and soxhlet extraction is often performed at high temperatures for several hours, and thus may cause thermal degradation of polyphenols due to prolonged heat exposure. Other methods have been developed such as the ultrasonic-assisted extraction (UAE), accelerated solvent extraction and supercritical fluid extraction. Supercritical method requires higher capital and operating cost owing to its high pressure requirement and hence less favourable. The UAE is an inexpensive and efficient alternative compared with other extraction techniques such as microwave-assisted extraction, supercritical fluid extraction and conventional extraction techniques. The UAE technique reduced the inner and external mass transfer limitation and hence increases the yield of extraction from plant material. Furthermore, ultrasonic wave can break the cell membranes, which may enhance inner mass transport[3], thus UAE was employed in this work. Moreover, limited comprehensive study on phenolic compound extraction from *O. stamineus* leaves by UAE is available in the literature. Although Pang and coworkers[4,5] recently performed an UAE of *O. stamineus* as part of their work on phenolic compounds microencapsulation, however, details study on the extraction parameter is not presented.

It is known that phenolic and flavonoid content in the extracts from the same plant material may vary widely according to the polarity of the solvent used. A combined effect of UAE and varying solvent polarity to the phenolic and flavonoid extraction from *O. staminues* leaves has never been studied previously, and hence this is the objective of this work.

Materials and methods

Plant material

Sodium nitrite, methanol, isopropanol, sodium hydroxide and Folin–Ciocalteu reagent were obtained from Merck (Darmstadt, Germany). Aluminium hexachloride was obtained from Sigma Aldrich (St. Louis, MO). Leaves from a white-flowered *O. stamineus* similar to one that has been deposited at the Forest

Research Institute, Malaysia (voucher no. ZAS1113) were collected in Gambang, Pahang, Malaysia. The freshly collected leaves were washed with deionised water and dried at 37°C for 3 days before crushed to powder. The powder was kept in an air-tight plastic bag in a desiccator at room temperature to prevent moisture absorption prior to experiment.

Ultrasonic assisted extraction

The powdered plant material was weighed (1 wt.%) and mixed with 100 ml of solvent in a 250 ml sealed Erlenmeyer flask. UAE was carried out in an ultrasonic bath (CREST P1800D, United States) filled with 12 litres of water at 45 kHz and 133.33 W for 90 min and temperature was set at 50°C to study the effect of solvent type. Various solvent with polarity index (PA) ranged from 3.9 to 9.0 such as isopropanol (IPA) PA = 3.9, methanol PA = 5.1 and ultrapure water PA = 9.0 were used for the extraction process. Aqueous mixtures of IPA and methanol are also tested. The study was performed in this way because solubility of methoxylated and hydroxylated polyphenols in solvent is affected by its polarity. A suitable solvent that enabled a simultaneous extraction of both phenolic acid and flavonoid were chosen for the remainder of this work. The study on extraction time (30 to 180 min) and temperature (40 to 70°C) was performed using the selected solvent. The parameters were determined based on literature and according to the equipment limitation. The UAE supernatant was then separated from the residue by filtration using 0.45 µm nylon membrane filter.

Total phenolic content

Total phenolic content (TPC) was assessed using the Folin–Ciocalteu reagent, following Singleton's method[6]. A sample aliquot of 0.125 ml was added to a test tube containing 0.5 ml of ultrapure water and 0.125 ml of the Folin–Ciocalteu reagent. After 3 min, 1.25 ml of 7% Na_2CO_3 solution was added, and the final volume was made up to 3 ml with ultrapure water. The solution was mixed well and incubated for 60 min in the dark. The absorbance was measured against the prepared blank reagent at λ = 760 nm using a calibrated ultraviolet–visible spectroscopy (Hitachi U-1800, Japan). TPC of the leaves was expressed as mg gallic acid equivalents per gram dry weight (mg GAE/g DW) by comparing with the calibration curve for gallic acid.

Total flavonoid content

Total flavonoid content (TFC) was measured using the aluminium chloride colorimetric assay[7]. A sample aliquot (0.2 ml) was added to a 15 ml centrifuge tube containing ultrapure water (4.8 ml). $NaNO_2$ (0.3 ml, 5%) was then added and mixed using a vortex mixer for 5 min. Subsequently, $AlCl_3$ (0.3 ml, 10%) was added, followed by addition of NaOH solution (2 ml, 1M), and the total volume adjusted with ultrapure water to the final volume of 10 ml. The solution was mixed well, and absorbance measured against a blank reagent at λ = 510 nm using a calibrated ultraviolet–visible spectroscopy (Hitachi U-1800, Japan). The TFC of the sample solution was expressed as mg quercetin equivalents per gram dry weight (mg QE/ g DW) by comparing with the calibration curve for quercetin.

Statistical analysis

Each experiment was repeated in triplicates. Analysis of variance (ANOVA) was performed by using the data analysis tools in Microsoft Excel 2010, and a least significant difference (LSD) test was used to compare the means with a confidence interval of 95%.

Results and discussion

Effect of solvent type on phenolic compound extraction

The solubility of the bioactive component in a different solvent was directed by its structural characteristic. Highly methoxylated compounds such as flavanoids, which are lipophilic were more stable in lower polarity solvent such as methanol and isopropanol. However, highly hydroxylated compound such as phenolic compound is hydrophilic thus more soluble in water than in isopropanol. Similar findings are also reported by Akowuah and co-workers[8] which found that the amounts of flavonoid are higher in lower polarity solvent, i.e. chloroform extracts.

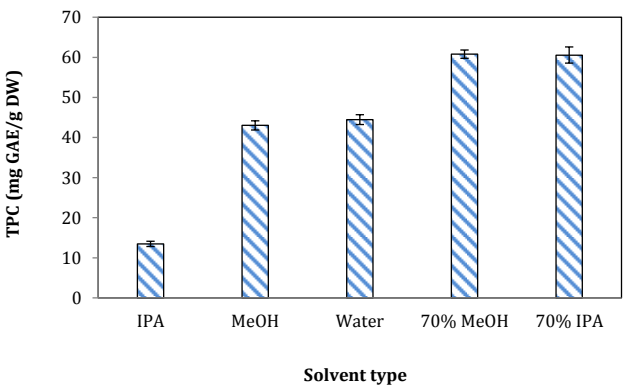


Fig. 1 Effect of solvent type of TPC obtained from UAE

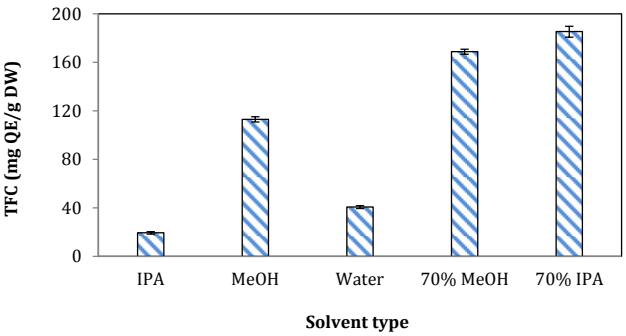


Fig. 2 Effect of solvent type of TFC obtained from UAE

The results (Figs. 1 and 2) show that aqueous alcoholic solvent has a significantly higher (> 30%) extracting capacity of total flavonoid and total phenolic content compared to pure solvent (100% methanol and 100% propanol). Similar findings are also reported by Wach and co-workers[9] who found that aqueous methanol ranged from 40 to 80% is preferable for rutin and quercetin extraction from *Hypericum perforatum*. The aqueous solvent has a wider range of polarity as opposed to the pure solvent, and hence enhances the simultaneous extraction of both lipophilic and hydrophilic compounds, resulting in a better extraction. This phenomenon can be seen clearly for the case of isopropanol and aqueous isopropanol, which increases extraction of flavonoid (methoxylated flavonoid) about10% from 167.7 to 185.3 mg QE/g DW without adversely affecting the extraction of the hydroxylated compound. Finding from this work suggests that aqueous alcoholic solvent (i.e. 70% MeOH and 70% IPA) enables a simultaneous extraction of hydrophilic component (phenolic) and lipophilic component (flavonoids). The highest simultaneous extractions of

both phenolic and flavonoid content were obtained using 70% IPA and thus this solvent will be used for the remainder of this work.

Effect of extraction time on phenolic compound extraction

UAE extraction time is an important parameter for the extraction process in order to maximize the phenolic and flavonoid content obtained from the sample when the equilibrium concentration reached before the apparent reduction due to polyphenol degradation[10]. Extraction time was studied by extracting *O. stamineus* dried leave powder at 50°C with the extraction time ranging from 30 to 180 min. The solvent, 70% propanol, was chosen due to its ability of extracting simultaneously both phenolic and flavonoid content. The effect of extraction time on the phenolic and flavonoid content is shown in Fig. 3. TPC was significantly reduced after 120 min, however, TFC still increasing even after 120 min of extraction. These dissimilarities might be due to differences in solubility of phenolics and interaction of phenolics with other components, which would lead to the difference in the times needed to reach equilibrium between the solid matrix (*O. stamineus* leaves) and the solvent (70% IPA). Similar findings are also reported by Thoo and co-workers[11], who studied extraction of *Morinda citrifolia*; they reported a different optimum extraction time for TPC and TFC.

Effect of temperature on phenolic compound extraction

Influence of temperature on polyphenol extraction for UAE extraction was studied by extracting *O. stamineus* dried leaves powder for 90 min using 70% aqueous propanol at temperature ranging from 40°C to 70°C. It was understood from previous finding, that extraction time longer than 90 min induces significant degradation of rosmarinic acid and eupatorin, thus time is fixed at 90 min for this study.

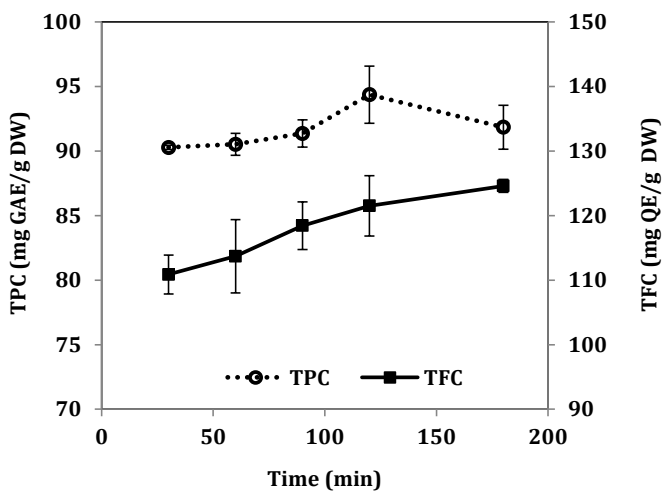


Fig. 3 Effect of UAE time on TPC and TFC at constant temperature of 50°C

The relationship of extraction temperature phenolic and flavonoid content are shown in Fig. 4. Extraction of both TPC and TFC from *O. stamineus* leaves increases with increasing temperature up to 60°C and followed by a slight decrease afterwards due to thermal degradation of polyphenols. Higher temperature may increase the diffusion rate and therefore, increases extraction yield. In addition, mild heating softens the plant tissue, weakening the cell wall integrity hence more polyphenol diffuses to the solvent. However, increasing temperature beyond a certain limit may induce polyphenol degradation which can be observed for UAE above 60°C. Similar findings are also reported by Tabaraki and Nateghi[12], in which was observed that TPC of rice bran extracts increases with increasing temperature up to 54°C and followed by a slight decrease thereafter. Similar findings are also reported by Ishak et al.[13], who studied the effect of temperature on extraction of *Habbatus sauda* seeds.

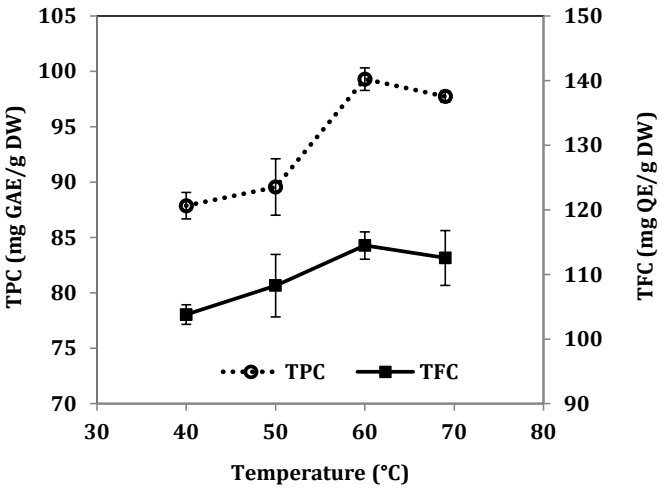


Fig. 4 Effect of UAE temperature on TPC and TFC after 90 minutes

Conclusions

The highest phenolic content of 168.8 mg GAE/g DW was obtained from UAE using 70% aqueous methanol solvent. Similar TPC are also obtained using 70% aqueous propanol solvent. The highest TFC of 185.3 mg QE/g DW was obtained using 70% aqueous propanol. Aqueous solvent provides a wider range of polarity as opposed to the pure solvent, and hence enhances simultaneous extraction of both methoxylated and hydroxylated compounds. Extraction beyond 120 min or at a temperature higher than 60°C induced degradation and hence reducing extraction yield. This work may be useful for obtaining higher polyphenol extract from *O. stamineus*.

Acknowledgements

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