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Effects of process conditions on the ultrasonic extraction of phenolics scavenger from *Curcuma caesia* rhizome

[2]Jemain, S.F.P., [1,2,*] Jamal, P., [2]Raus, A. R., [2]Amid, A. and [2,3]Jaswir, I.

[1]*Bioenvironmental Engineering Research Centre (BERC),*[2]*Bioprocess and Molecular Engineering Research Unit (BPMERU), Biotechnology Engineering Department, Faculty of Engineering, International Islamic University Malaysia, P. O. Box 10, 50728, Kuala Lumpur, Malaysia.*
[3]*International Institute for Halal Research and Training (INHART), Kuala Lumpur. Malaysia*

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Abstract

Medicinal properties of Malaysian *Curcuma caesia* have not been studied extensively, even though it has been used as a traditional remedy. This study examined the effects of various extraction temperatures (30, 40, 50, 60, 70°C) using a high frequency (40 kHz) ultrasonic extraction method, time (30,60,90 and 120 minutes), pH (1,2,3,4,5,6,7,8,9,10) on the extraction yield of total phenolics and DPPH (2,2-diphenyl-1-picrylhydrazyl) scavenging activities from *C. caesia* rhizome. Extraction was most efficient at pH 6.0, while the extraction time of 30 minutes and temperature of 60°C was the best in terms of total phenolics content and DPPH scavenging activity. This study is important due to its ability to improve extraction of total phenolics compound using ultrasonic extraction method while maintaining a relatively high DPPH scavenging activity of the extracts.

Introduction

According to a survey by the World Health Organization, 80% of the world's population relies on traditional herbal medicines for their basic health care (Ekor, 2013). Malaysia has more than 25,000 plant species; thus, it is not surprising that plants of various species have been used as a source of remedy for various ailments among the Malays. Many of these traditional prescriptions were not properly documented but were passed orally from generations of traditional Malay herbal medicine experts. One such plant that has been used for generations in the Malay culture is the 'kunyit hitam'. Kunyit hitam has a strong camphoraceous sweet smell and its rhizome has a bluish black colour. Its scientific name is, *Curcuma caesia* and it belongs to the Zingiberaceae family.

A research done on *Curcuma caesia* grown in Manipur, India, found that the rhizome contains 30 mg/g weight of flavonoids content, 104.2 mg/g dry weight alkaloid, 47.5 mg/g fresh weight soluble protein and 60 mg/g phenolic compounds (Sarangthem, 2010), and a later research done on the Curcuma caesia rhizome grown in Dindhori district of Madhya Pradesh, India, also found that the methanolic extract of *Curcuma caesia*, contains flavonoids, alkaloids, phenolics, tannin and also protein (Pritesh Paliwal, 2011). In India, *Curcuma caesia* or 'Kali haldi' has been used to cure leprosy, asthma, cancer, epilepsy, wound, menstrual disorder, aphrodisiac, inflammation and gonorrhoeal discharges, smooth muscle relaxant and haemorrhoids (Das Sonjit, 2013). Methanolic extract of *Curcuma caesia* were found to show DPPH scavenging activity (Mangla Mohit, 2010; Indrajit Karmakar *et al.*, 2011) and the crude extract of *Curcuma caesia* exhibited stronger DPPH scavenging activity than the enzymatic extract (Yogamaya Dhal, 2012). Methanolic extract of Curcuma caesia were also shown to have muscle relaxant effect and antidepressant effect on mice and were also shown to exhibit antitumour and antiulcer activity in mice (Indrajit Karmakar *et al*., 2011; Das *et al.*, 2012; Indrajit Karmakara, 2013).

The genus *Curcuma* consists of over 70 species of rhizomatous herbs (Zou *et al*., 2011). *Curcuma caesia* has been used by the locals to treat erectile dysfunction and as a general energy booster and helps increase blood circulation for both men and women. The Malays also used the rhizome as a treatment for piles, bruises, sprains, and hernia, and also to treat drug addiction. Its usage among the locals provide an indication that it could contain phytochemicals that could increase blood circulation and it could contain phosphodiesterase-5 inhibitors (PDE-5 inhibitors) and anti-inflammatory substances due to its usage in

*Corresponding author.
Email: *jparveen@iium.edu.my, jparveen3@yahoo.com*

treating erectile dysfunction and piles.

Numerous extraction techniques have been developed for the extraction of antioxidant secondary metabolites including ultrasound-assisted extraction, supercritical fluid extraction, and microwave-assisted extraction and accelerated solvent extraction. Ultrasound-assisted extraction is a simple, efficient and inexpensive method. It is effective at extracting secondary metabolites due to the acoustic cavitation effect produced in the solvent by the passage of ultrasonic waves which can lead to the destruction of cells and enhance the contact surface area between solid and liquid phases. These effects permit better penetration of the solvent into the sample increasing the extraction yield of secondary metabolites (Wang *et al.*, 2008). The efficiency of an extraction process is influenced by solvent composition, extraction temperature, extraction time and solvent to solid ratio (Hammi *et al.*, 2015) and these variables can be studied effectively by incorporating response surface methodology (RSM), which is a useful tool for optimizing the extraction process; it is an efficient mathematical and statistical technique for analysis of empirical models that describes the effect of independent variables and their interactions on response variables (Myers, 2002). One of the major advantages of the RSM is reduction in the number of experimental trials required for evaluation and analysis. It is quicker than previous approaches and has been successfully used in optimizing ingredients and process variables or both. Thus far, the extraction of metabolites from *Curcuma caesia* were done using maceration and the sohxlet apparatus and no optimization study have been done on the extraction process. In the present study, several solvents were screened for the extraction process. Subsequently, the effects of various pH, extraction time and temperature on the total phenolic contents and DPPH scavenging activity of CC using ultrasonic extraction method were studied. Further optimisation study of extraction process were also done by response surface methodology (RSM) using face-centered central composite design (FCCD) in order to optimize the DPPH scavenging activity (but results are not included in this article).

Materials and methods

Plant material

Curcuma caesia rhizomes were collected from an organic *Curcuma caesia* plantation in Johor, in southern Peninsula Malaysia. The rhizomes were washed, sun-dried and grinded into powder and kept at 4°C until further use.

Chemicals and reagent

DPPH (2,2-diphenyl-1-picrylhydrazyl), Folin-Ciocalteau reagent, sodium carbonate and Gallic acid were purchased from Sigma-Aldrich (USA), Ultrapure water was obtained from the Millipore system

Extraction solvent screening

Absolute hexane, ethanol, methanol and deionised water was used to screen the solvent that would be used for further study and optimisation of DPPH scavenging activity. Solid-to-solvent ratio was 1:15 where 1 g of dried *Curcuma caesia* was extracted with 15 mL of solvent (Tan *et al*., 2011).

Ultrasound-assisted extraction of antioxidant compounds

Extractions were carried out in an ultrasonic bath at 40 kHz (screening was done earlier on the frequency value, but the results are not included in this article). Dried and powdered Curcuma caesia rhizomes (1 g) were placed into Erlenmeyer flasks (250 mL) and 15 mL of ultrapure water was added. Samples were extracted at different extraction time (30, 60, 90 and 120 minutes), at different pH (2, 3, 4, 5, 6, 7, 8 and 9) and at different temperatures (30, 40, 50, 60, 70°C). The mixtures were centrifuged at 2000 x g for 20 min at 4°C and the supernatants were collected for DPPH scavenging activity determination.

Analytical methodology

Total phenolic content (TPC) of the extracts were determined using Folin-Ciocalteu colorimetric method described by (Khoo, 2009) with slight modifications. First, appropriate dilution of the extracts was prepared (1 mg/mL). Then, 20 µL of diluted crude extract was added to 1.58 mL of deionized water and 100 µL of Folin-Ciocalteu reagent (FCR) in a 15 mL test tube that has been wrapped with an aluminium foil. After 10 minutes, 300 µL sodium carbonate (Na_2CO_3) (20%) was added into the test tube. The mouth of the test tube was then covered with parafilm and aluminium foil and vortex for about 10-15 seconds. Next, the mixture was incubated for 2 hours in a dark environment for colour development. Then, the absorbance was measured at 765 nm against the blank reagent by using UV-VIS spectrophotometer. The measurements were done triplicates for more accurate results. Gallic acid was used for calibration of a standard curve with different concentrations and the results were expressed as mg/L Gallic acid equivalent (GAE) (graph not included in this article).

DPPH free radical scavenging assay

The DPPH free radical scavenging assay was carried out according to the method described by Khoo (2009) with slight modifications. The extract was mixed with ethanol to prepare an ethanolic test solution at concentrations of 1mg/mL. Next, 100 µL of the test solution was mixed with 3.9 mL of ethanolic DPPH (60 µM) in a 15 mL test tube that has been wrapped with aluminium foil. Then, the mouth of the test tube was covered with parafilm and vortex mixer was used to mix the mixture for about 10 seconds. The mixture was then allowed to stand for 30 minutes at room temperature in dark environment. Absolute ethanol is used as the blank. After that, the absorbance of blank, control and also sample were measured at 517 nm by using UV-VIS spectrophotometer. All the experiments were carried out in triplicates to obtain an accurate result. The DPPH free radical scavenging activity (%) is calculated as:

$$\% \text{ DPPH scavenging} = [(Ac - As)/Ac] \times 100\%$$

Where As and Ac is the absorbance (at 517 nm) of crude extract and control solution respectively.

Results and discussion

Screening of solvent for extraction

There are many steps to obtain phytochemicals from plant such as milling, grinding, homogenization, and extraction. Among these steps, extraction is the main step for recovering and isolating phytochemicals from plant materials. Extraction efficiency is affected by the chemical nature of phytochemicals, the extraction method used, sample particle size, the solvent used, as well as the presence of interfering substances (Stalikas, 2007). The yield of extraction depends on the solvent with varying polarity, pH, temperature, extraction time, and composition of the sample. Under the same extraction time and temperature, solvent and composition of sample are known as the most important parameters. In this work, *Curcuma caesia* extracts were obtained using deionized water, absolute ethanol, absolute methanol and absolute hexane as solvents. Extraction yields ranges from 1.20 mg/g to 2.05 mg/g (Figure 1). The highest DPPH scavenging activity (86.91%) and the highest phenolics (909.04 mg/L GAE) (Figure 2) content were shown when absolute methanol was used as the solvent.

When deionized water was used as the solvent, the extraction yield was only 1.65 mg/g with a total phenolics content of 592.86 mg/L GAE and a DPPH scavenging activity of 67.03%. Methanol and

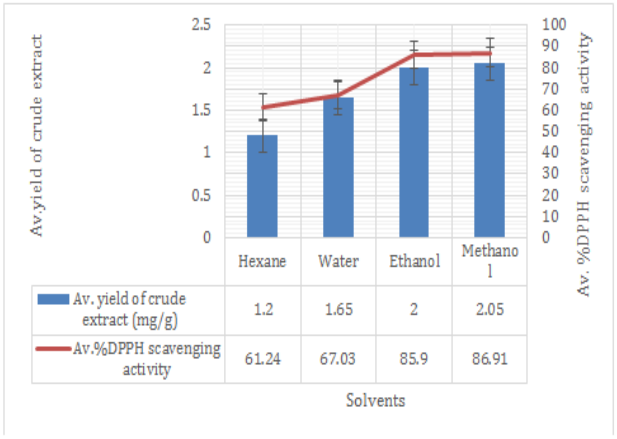

Figure 1. Average value of %DPPH scavenging activity and the extraction yields from *Curcuma caesia* (CC) rhizome using selected solvents.

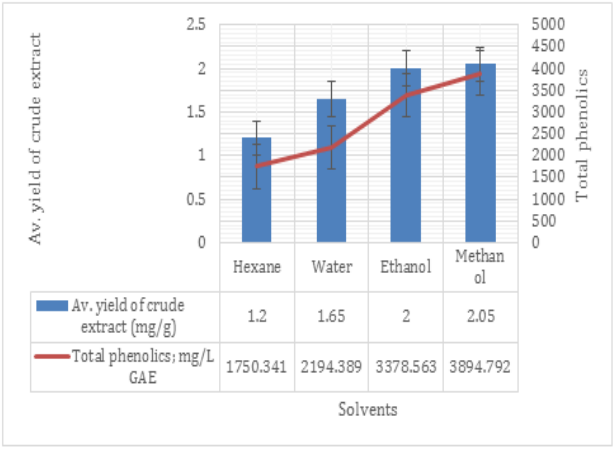

Figure 2. Average value of total phenolics content and the extraction yields from *Curcuma caesia* (CC) rhizome using selected solvents

ethanol have been known to exert toxic effects to living tissues (Tephly, 1991; Kołaciński and Rusińsk, 2003), thus, deionized water was chosen in this study for further optimisation of DPPH scavenging activity of the crude extract of *Curcuma caesia*. This is aligned to the long-term objective of this study, which is, developing a halal nutraceutical and cosmeceutical product from local *Curcuma caesia.*

Ultrasound-assisted extraction of antioxidant compounds

The extraction yields of phenolics from *Curcuma caesia* (CC) rhizome increased as the extraction temperature increased between 30°C - 70°C, (Figure 3) which suggested that *Curcuma caesia* phenolics are relatively stable at high temperature. Significant differences existed among 30, 40, 50 and 60°C. However, at 70°C, the phenolic contents decrease. Thus, in practice, an extraction temperature of 60°C would be suitable due to the observed combined effects of good extraction yield of phenolics and

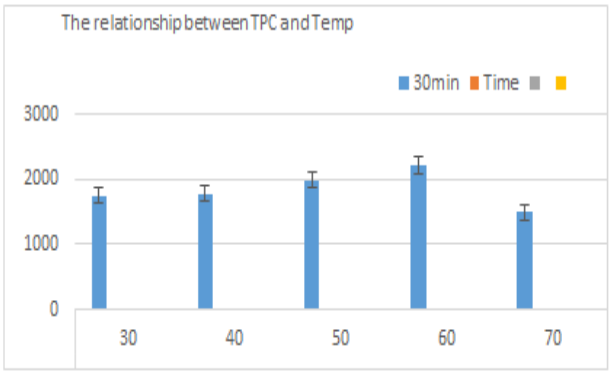

Figure 3. The total phenolics content from *Curcuma caesia* (CC) rhizome crude extract increased as the extraction temperature increased between 30°C - 70°C.

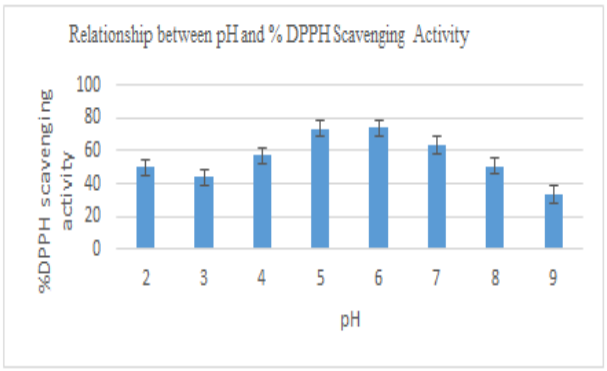

Figure 5. There was a higher scavenging DPPH radical activity at pH 4-6, which was significant compared with pH 3 or 9

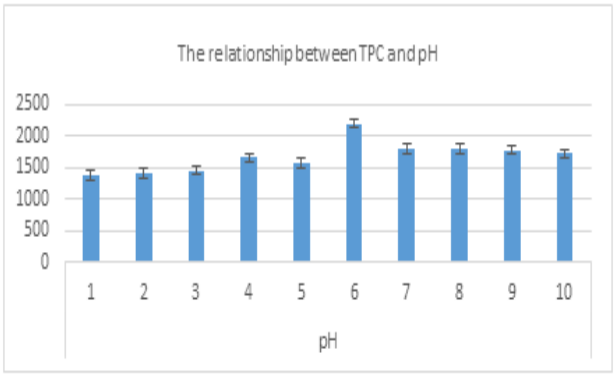

Figure 4. The extraction yields of phenolics increased as the pH values increased from 1 to 6, but it decreased as the pH values higher than 6 were used.

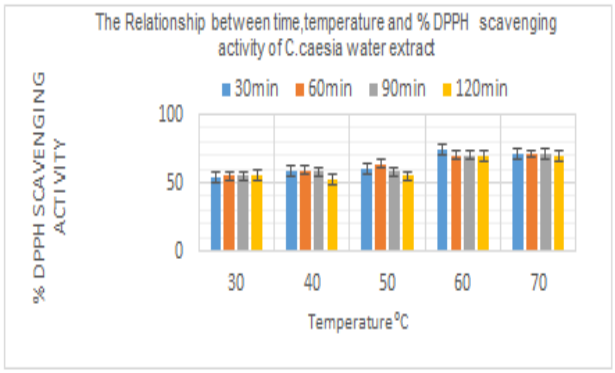

Figure 6. The DPPH radical scavenging activity was enhanced as the incubation time was extended from 30 to 60 min

the stability of *Curcuma caesia* antioxidants which is depicted by a high DPPH scavenging ability. A similar range of temperature was also used to optimize the extraction conditions of phenolic and flavonoids compounds from quinoa *(Chenopodium quinoa)* seeds using ultrasound assistance technology, where the best extraction conditions obtained by simultaneous maximization of the responses were, an extraction temperature of 60°C and 80% ethanol was used as solvent (Ramiro *et al*., 2015). Similar high temperature extraction was also studied on *Curcuma longa* (turmeric) and *Zingiber officinale* (ginger) (Vinod, 2011), in *Citrus unshiu* (tangerine) peel extract and the antioxidant activity, increased as heating temperature increased (Jeong *et al*., 2004). The free phenolic acids of grapefruit *(Citrus paradisi)* extract increased after heat treatment and another study reported that total phenolic content of orange peel was increased by a higher drying temperature (70°C – 100°C) (Chen *et al*., 2011). Naturally existing phenolic compounds in fruits and vegetables are usually covalently bound to insoluble polymers. Therefore, heat treatment may be used to release bound phenolic compounds from citrus as well as increasing their antioxidant activity (Choi *et al*., 2011).

Extraction efficiency and antioxidant stability depend largely on the combined effects caused by temperature and pH of extraction solution (John Owusu, 2015). There is an observed 59.2% increase in the extraction yield of phenolics as the pH values increased from 1 to 6 and the yield peaked at pH 6 and gradually decreased as the pH became more alkaline, however the yield at alkaline pH is relatively higher compared to a more acidic extraction pH (Figure 4). Similar optimum pH of between 6 and 7 was also preferred for the extraction of phenolic compounds from flaxseed meal, higher pH will result in the extraction of protein (Engy *et al*., 2017).

There is an observed 48.99% increase from pH 2 to pH 6. The DPPH scavenging activity peaked at pH 6 with a recorded 73.71% scavenging activity. A similar high DPPH scavenging activity was also observed in tea infusions, with a pH of 5.8 and an acidic pH, resulted in a lower DPPH scavenging activity (Pekal and Pyrzynska., 2015). In a study done with palm wine and palm vinegar, *Borassus flabellifer*, showed that total phenolics concentration and antioxidant activity increase as pH increases, with an optimum pH of 6.5 (Ghosh *et al*., 2014).

Temperature affects solute diffusivity and solute solubility. An increase in temperature will result in an increase of solute diffusivity, thus the rate of extraction of bioactive compounds would increase as well. As a result, the DPPH scavenging activity was observed to reach a peak at 60°C (Figure 6). There is an observed increase of 37.07% DPPH scavenging activity when the temperature was increased from 30°C to 60°C with an extraction time of 30 minutes. However, an increase in extraction time and temperature did not result in a further significant increase in DPPH scavenging activity. This is also accompanied by a decrease in total phenolic contents when an extraction temperature of 70°C was used (Figure 3). This might be due to degradation of the phenolic compounds at elevated temperatures.

Conclusion

Various temperatures and pH significantly influenced the extraction yield of phenolics from CC and the DPPH scavenging activity of the extracted bioactive compounds. Therefore, 60°C is a suitable extraction temperature. Application of pH 6.0 exhibited the most efficient extraction while the extraction temperature of 60°C could be used in terms of the combined effects of the extraction yield of phenolics and the stability of the extracted CC DPPH scavengers. Furthermore, the temperatures from 50-60°C and pH values from 5 to 6 exhibited a relatively high DPPH scavenging activity. As the extraction efficiency and the antioxidant activity of the extracted antioxidants depend largely on the combined effects of temperature and pH, further investigation and optimisation would be required. Since this present study focuses on ultrasonic assisted extraction DPPH scavengers of *Curcuma caesia* rhizome; three influencing factors in the aqueous extraction which are pH, time and extraction temperature were investigated. Taking DPPH radical scavenging capacity of antioxidants as the index, the extraction processing will be further optimized using RSM on the basis of the single factor method.

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