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COMPARISON OF DIFFERENT EXTRACTION METHODS FOR THE EXTRACTION OF TOTAL PHENOLIC COMPOUNDS FROM WINTER MELON (*Benincasa hispida*) SEEDS

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ABSTRACT

Conventional Soxhlet extraction (CSE) and Ultrasound-assisted extraction (UAE) methods were applied for the extraction of total phenolic compounds (TPC) from winter melon (*Benincasa hispida*) seeds. Effects of different UAE variables such as amplitude (25-75%), temperature (45-65 °C) and sonication time (20-60 min) on the TPC of extracts was determined using complete randomised design (CRD). The results showed that the TPC of extracts obtained UAE process significantly affected by independent variables. The maximum value of TPC (22.12 ± 1.23 mg GAE/g extract) was obtained at the combined treatment conditions of 75%, 55 °C and 40 min. The UAE results obtained were compared with those achieved by using conventional Soxhlet extraction (CSE) method. It was found UAE allowed extraction at lower temperature and the extracts obtained posses higher quality compare with CSE. UAE is a promising environment friendly technique for the extraction of bioactive compounds from winter melon (*Benincasa hispida*) seeds.

Keywords: *Winter melon (Benincasa hispida), ultrasound-assisted extraction, conventional Soxhlet extraction, complete randomised design, total phenolic content.*

INTRODUCTION

Finding the alternative natural and safe sources of antioxidant compounds is the main objective of various researchers due to adverse effects of synthetic antioxidant on human health and environment [1, 2]. Polyphenols are one of the most used groups of biological systems which have been extensively applied for decades as food additives because of their abilities for free radicals scavenging (i.e., antioxidant capacity). Plant materials are important source of natural antioxidants like phenolic compounds. However, it has been reported that seeds of tropical and sub-tropical plants contain high amount of polyphenolic compounds [3]. Plant materials crude extracts which contain high amount of phenolic compounds lead to reduce the rate of lipid oxidation and improve the quality of food products are strongly favored in food industry [4]. Dietary phenolic compounds have a variety of health promoting effects including antioxidant, anti-apoptosis, anti-aging, anti-carcinogen, anti-inflammation, anti-atherosclerosis and cardiovascular protection [5]. *Benincasa hispida* which belongs to the family *Cucurbitaceae* is commonly known as Kundur in Malay. *Benincasa hispida* is also called winter melon, ash pumpkin, tallow gourd, white pumpkin, wax gourd, and Chinese watermelon or Chinese preserving melon in English [6]. This fruit is very large and seedy with white color and spongy flesh. Index of Nutritional Quality (INQ) data showed that *Benincasa hispida* has been valued as a high-quality vegetable [6].

Conventional Soxhlet extraction (CSE) is considered as a standard procedure and main reference to assess the performance of different extraction techniques [7]. The application of this method is limited due to high amount of organic solvents usage which has adverse effects on human health and environment. Furthermore, natural bioactive valuable compounds are thermo sensitive and may decompose at higher temperatures for long extraction time which is common in CSE [8]. In past decades, environmental friendly techniques are being interested to develop the ‘‘Green Chemistry” concept. Therefore, an improved or better extraction technique is necessary and favored. Ultrasound waves can be applied as either low intensity with high frequency (above 100 kHz) or high-intensity with low frequency (18-100 kHz) [9]. Interaction of ultrasound waves with material leads to increase the rate of mass transfer and easier access of solvent to cell compounds. During sonication, high energies around the solvent molecules generated as a result of cavitation phenomenon leading to disruption of cell walls for better penetration of solvent into the cell [10]. UAE has been employed to extract various active compounds like phenolic compound [11], flavonoids [12], hydrocarbons [13] and antioxidants [14] from plant materials.

So far, there is no report on UAE of valuable compounds from *Benincasa hispida* seeds by using food grade solvent. Therefore, this work aimed to outline the potentiality of UAE in the fast preparation of extracts rich in bioactive phenolic compounds from winter melon seeds. Several parameters such as amplitude, temperature and sonication time that could potentially affect the total phenolic content (TPC) of seeds extract were investigated and the results were compared with that obtained from CSE.

MATERIALS AND METHODS

Materials and Reagents

Whole winter melon (*Benincasa hispida*) fruits were purchased from a local market in Serdang, Selangor, Malaysia. The fruits were cut, and then seeds were separated manually and washed under tap water. Seeds were dried at 40 °C in a ventilated drying oven (1350FX, USA) for 24 h. The samples were ground in grinder mill (MX-335, Panasonic, Malaysia) for 10 s to produce a powder with an approximate size of 1.5-2.5 mm before extraction. Ethanol (EtOH, 99.5%, analytical grade) obtained from Scharlau Chemical, European Union. Nitrogen gas was purchased from MOX Company in Malaysia. Gallic acid and Folin-Ciocalteu reagent (FCR) was purchased from Fisher (Pittsburgh, PA, USA).

Conventional Soxhlet Extraction Method

About five gram (5 g) of ground *Benincasa hispida* seeds were put into extraction thimble and covered with wool. Approximately, 150 mL of selected solvent (ethanol, 99.5%) were added to each flask which was connected to the extractor. Each extraction was performed in triplicate during 6 h. The temperature of extraction corresponded with the boiling point of the solvent in use. After conventional Soxhlet extraction (CSE), solvent was removed at 40 ºC under reduced pressure using a rotary evaporator (Eyela, A-1000S, Japan). Subsequently, the residual solvent was removed by drying in an oven at 40 ºC for 1 hr and by flushing with nitrogen (99.9%). The obtained extracts were weighed gravimetrically using an analytical Mettler Toledo balance (± 0.0001 g) (Mettler Toledo GmbH, Greinfensee, Switzerland) and then the crude extraction yield (CEY) was calculated according to the following equation:

$$CEY = \frac{m_e}{m_s} \times 1000 \qquad (1)$$

The results of CEY were expressed as mgg^{-1} sample. Where m_e is the crude extract mass (g) and m_s is the extracted sample mass (g). Finally, the winter melon seeds extract were kept for further analysis.

Ultrasound-Assisted Extraction

About five gram (5 g) of ground *Benincasa hispida* seeds sample was extracted in a beaker (100 ml) containing a volume of extraction solvent. The extraction solvent was ethanol (99.5 %) as a food grade solvent which is recommended by the US Food and Drug Administration for extraction purposes [15]. The solid/solvent ratio was 1:10 (g/ml). The extraction was performed by a 500 W ultrasound equipment (Sonics and Materials Inc., Model VC505, Danbury, CT, USA) with a titanium ultrasonic probe (13 mm diameter). The beaker placed in a temperature control water-bath (Memmert, WNE14. Memmert GmbH Co. KG, Germany). Temperature was verified with a digital thermometer (Ellab CTD-85, Ellab, Denmark) and a thermocouple (1.2 mm needle diameter constantan type T) which no significant increase in temperature (below 2 ºC) was observed during extraction. The applied power levels were adjusted to 25%, 50% and 75% of the maximal equipment power (500 W). The immersed seeds in extraction solvent were subjected to ultrasonic waves for 20 to 60 min. The variables and levels investigated were reported in Table 1. All experiments were performed in triplicates. After extraction, the crude extracts were filtered through the Whatman No. 1. Then, ethanol was removed from the extracts by evaporation under vacuum at 40 ºC on a rotary evaporator (Eyela, A-1000S, Japan). Subsequently, the residual solvent was removed by drying in oven (1350FX, USA) at 40 ºC for 1 hr and by flushing with nitrogen (99.9%). Gravimetric measurement (Eq. 1) was used to obtain the amount of total crude extract weight.

Table 1: Experimental levels of the variables used in complete randomized design (CRD) full factorial

Independent Variables	Levels		
	1	2	3
Amplitude (%)	25	50	75
Temperature (ºC)	45	55	65
Sonication time (min)	20	40	60

Determination of Total Phenolic Content

This method is based on measuring the color change caused by reduction of the Folin-Ciocalteu reagent by phenolates in the presence of sodium carbonate. The total phenol content (TPC) of the seed extracts was determined using Folin-Ciocalteu reagent (FCR) according to the procedure reported by Singleton et al. [16] with some modifications [17]. Three replications were done for each sample.

RESULTS AND DISCUSSIONS

Effect of UAE Variables on the Total Phenolic Content

Based on ANOVA results, all three independent variables including amplitude, temperature and sonication time significantly (p<0.05) affected total phenolic content (TPC) of *Benincasa hispida* seed extracts. The K and R values were calculated and listed in Table 2. L value is the average extracting efficiency of each variable at different levels. R value means range between three average responses of each level of TPC.

Table 2: TPC (mg GAE/g extract) results obtained at the experimental condition using complete randomized design (CRD) full factorial

Variable	L1 [a]	L2 [a]	L3 [a]	R [b]
Amplitude	11.82 ± 3.69	14.60 ± 3.39	17.34 ± 3.12	5.52
Temperature	13.43 ± 4.01	17.69 ± 3.78	12.63 ± 2.23	5.06
Sonication Time	11.47 ± 3.58	15.73 ± 3.48	16.55 ± 3.22	5.08

[a] Average responses of each level of TPC.
[b] *R* value means range between three average responses of each level of TPC.

From the results it was clear that amplitude is the most important variable followed by temperature and sonication time. The TPC of extracts obtained using UAE varied widely from 5.55 ± 0.84 to 22.12 ± 0.31 mg GAE/g extract. The mean values of TPC for the corresponding variables at different levels were calculated and presented in Fig. 1.

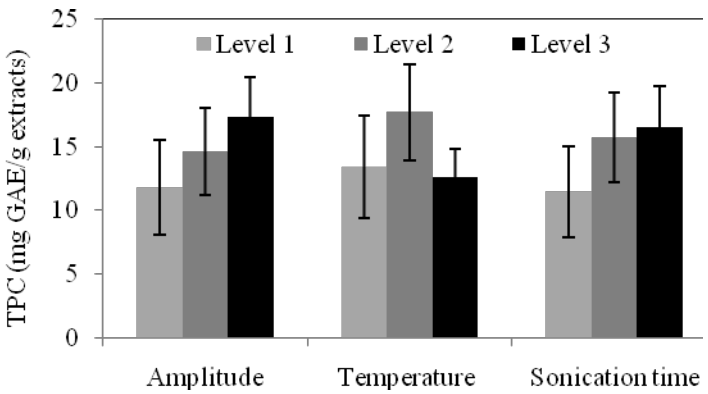

Fig. 1: Mean values of TPC under full factorial in a frame of complete randomize design (CRD)

It is obvious that the highest mean value of TPC was obtained at 75% (17.34 ± 3.12 mg GAE/g extract), 55 °C (17.69 ± 3.78 mg GAE/g extract) and 60 min of sonication time (16.55 ± 3.22 mg GAE/g extract). However, the difference between 40 and 60 min of sonication time was too low (0.82 mg GAE/g extract). At lower amplitude and temperature, the TPC increased until 60 min of sonication time. But at higher amplitude and temperature due to the higher extraction rate of the phenolic compounds, extraction was completed during 40 min of sonication time. Therefore, the highest mean value of TPC was obtained at 60 min but in each response of TPC in experimental design the highest TPC was achieved during 40 min of sonication time.

In Fig. 2 (a-c) the effect of temperature levels at 25, 50 and 75% amplitude on the TPC of extracts during 60 min sonication time using UAE was presented. By using lower amplitude (25%) at temperature of 45 and 55 °C the TPC of extracts increased significantly ($p<0.05$) with increasing sonication time up to 60 min reached to value of 14.26 ± 0.42 and 18.26 ± 0.76 mg GAE/g extract, respectively (Fig. 2 a). The lower value of TPC at 65 °C (12.00 ± 0.87 mg GAE/g extract) compare to 55 °C (18.26 ± 0.76 mg GAE/g extract) could be due to thermo degradation of phenolic compounds at higher temperature. The current finding is associated with higher efficiency of acoustic cavitation at 55 ºC. A powerful and effective collapse of bubbles was pointed out by Hemwimol et al. [18] at 55 ºC. Although the number of bubbles is higher at 65 ºC, their collapse are less efficient due to the fact that the bubbles collapse with lower intensity as a result of smaller internal/external pressure difference. This finding is consistent with those obtained by Boonkird et al. [19] for extraction of capsaicinoids from *Capsicum frutescens* using 95% ethanol. It was revealed that capsaicinoid recovery increased with increasing of temperature from 30 to 45 ºC. In addition, the percentage of recovery was decreased by increasing temperature from 45 to 60 ºC. In the current study, by using moderate amplitude (50%) the targeted response increased up to 60 min at temperature of 45 ºC (17.00 ± 1.01 mg GAE/g extract) which completed during 40 min using higher temperature of 55 and 65 ºC (19.40 ± 0.77 and 13.30 ± 0.74 mg GAE/g extract, respectively). This could be due to the positive effect of temperature in isolating bioactive compounds from sample matrix. When the temperature increased the solubility and diffusivity increased and caused higher mass transfer [8]. Results revealed that the maximum recovery of phenolic compounds was obtained using 75% of amplitude (mean value of 17.34 ± 3.12 mg GAE/g extract). Worthy of note, using the highest amplitude under the range of study (75%) at constant temperature resulted in reduction of TPC after 40 min of extraction time. It was mentioned that by using high amplitude during long extraction time the degradation of valuable compounds is common. Herrera et al. [20] studied on UAE of phenolic compounds from strawberries and found that by using high amplitude the degradation of phenolic compounds occurred during long extraction time.
In the current study, this phenomenon was observed by using 75% amplitude for more than 40 min of extraction time. This phenomenon was more obvious at higher temperature (65 ºC) which the value of TPC decreased from 16.37 ± 0.31 to 14.17 ± 0.74 mg GAE/g extract with extending sonication time from 40 to 60 min. It was due to thermo sensitivity of valuable compounds. It seems that the phenolic compounds extraction was occur very rapidly at the first 40 min of sonication time. The results revealed that ultrasound was more effective during first 40 min sonication to separate phenolic compounds from *Benincasa hispida* seeds. As can be seen the maximum value of TPC (22.12 ± 0.31 mg GAE/g extract) can be obtained using 75% amplitude, 55 °C and 40 min sonication time.

Comparison between UAE and CSE methods

In association with the further effectiveness in evaluating the UAE method the results are compared with those achieved from CSE. From our findings, more total phenolic compounds could extract using UAE (22.12 ± 0.31 mg GAE/g extract) compare to ethanolic CSE (11.34 ± 1.30 mg GAE/g extract). During CSE the thermo degradation of natural bioactive compounds is inevitable due to using high temperature (79 °C) for long extraction time. While it is possible to extract more phenolic compounds using UAE due to the lower extraction temperature (55 °C). Furthermore, CSE is a very time consuming technique and not always accepted from the industries so it was necessary to replace new green techniques. The extraction time was reduced from 6 h to 40 min by using UAE method. It should kept in mind that solvent consumption in UAE method (solid: solvent ratio was 1:10) was less than used in CSE (solid: solvent ratio was 1:30). It could be suggested that UAE is an alternative novel and green extraction method to CSE for separation of bioactive phenolic compounds from *Benincasa hispida* seeds.

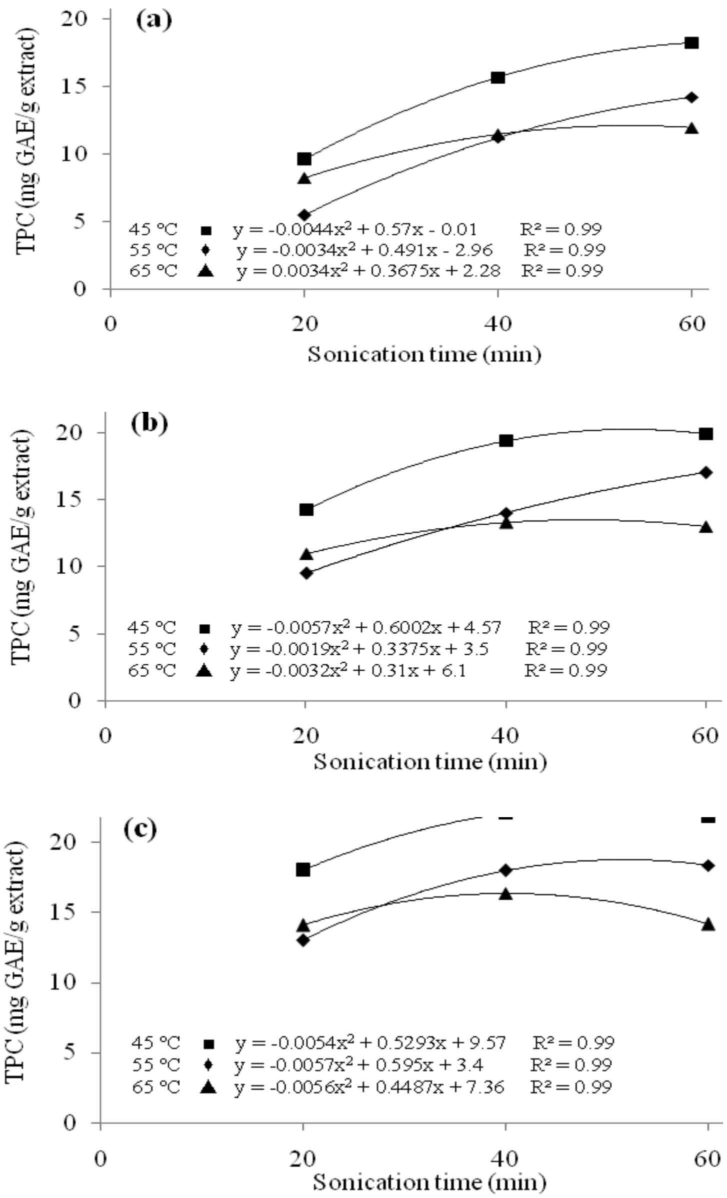

Fig. 2: Effect of temperature at (a) 25% amplitude; (b) at 50% amplitude and (c) 75% amplitude on TPC at different sonication time (standard deviation bars are smaller than the symbol size).

CONCLUSION

The findings of current study, for the first time, revealed the potentiality of ultrasound-assisted technique as a simple and effective extraction method for extraction of bioactive phenolic compounds from *Benincasa hispida* seeds. Amplitude, temperature and sonication time had significant effect ($p<0.05$) on the TPC of *Benincasa hispida* seed extracts. Compared with CSE method the application of ultrasound waves for the extraction of bioactive phenolic compounds from *Benincasa hispida* seeds provided higher value of TPC in shorter time reduced solvent consumption, easy operation and lower temperature. Therefore, UAE of winter melon seeds by using food grade solvent (ethanol, 99.5%) has strong potentiality for the preparation of rich extracts in phenolic compounds aimed at replacing synthetic antioxidants.

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