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Microwave- and ultrasound-assisted extraction of phenolic and flavonoid compounds from konar (*Ziziphus spina-christi*) fruits

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Abstract

Konar (*Ziziphus spina-christi*) fruit is known to have antioxidant and anti-inflammatory properties. In the present work, we investigated the yields for microwave-assisted extraction (MAE), ultrasound-assisted extraction (UAE), and magnetic stirrer extraction (MSE) methods using different extraction parameters including type of solvent, solvent to sample ratio (SSR), microwave irradiation power, extraction temperatures for MSE, microwave irradiation time, sonication time for UAE, and extraction time for MSE on the total phenolic (TP) and total flavonoid (TF) contents of the underutilised nutritious konar fruit. The highest TP content was obtained by MAE using water as solvent [10.3 ± 0.1 mg tannic acid equivalent (TAE) per gram (g) of sample]. However, the highest TF contents were obtained using ethanol in MAE [1.9 ± 0.0 mg quercetin equivalent (QE) per g of the sample], and methanol in UAE and MSE [1.7 ± 0.0 mg QE per g of sample]. An SSR of 15 resulted in the highest TP content while no significant differences were observed in the TF contents. MAE was considered the best extraction method for the extraction of TP and TF from konar fruits as demonstrated in the present work.

Abbreviations

d.w.: dry weight basis; E: ethanol; M: methanol; MAE: microwave-assisted extraction; MSE: extraction using magnetic stirrer; P: microwave power (watt); QE: quercetin equivalent; SD: standard deviation; SSR: solvent to sample ratio; t: extraction time; T: temperature (°C); TAE: tannic acid equivalent; TF: total flavonoid; TP: total phenolic; UAE: ultrasound-assisted extraction; W: water.

Introduction

Phenolic compounds are amongst the most important antioxidants in nature with numerous health benefits (Vicente and Boscaiu, 2018). These compounds, found in a variety of fruits and vegetables, are secondary metabolites produced by plants and play an important role in defending against infections, injuries, and plant growth and development. The polyphenolic compounds contain an aromatic ring with one or more hydroxyl groups that can be classified into different categories such as phenolic acids, simple and complex flavonoids, and anthocyanins (Naczk and Shahidi, 2006). Consumption of foods containing phenolic compounds has been shown to reduce the risk of diseases such as cardiovascular disease and cancer (Aune *et al.,* 2017; Vitale *et al.,* 2017). Therefore, it is important to identify the available sources of these compounds and the most efficient methods for their extraction. Various fruits and vegetables have been recognized as primary sources of phenolic compounds; however, there are still other sources such as herbs and plants rich in phenolic compounds that have not yet been utilised. One of such sources is a fruit/herb of a tree known as *Ziziphus spina-christi* (konar) belonging to the family of Rhamnaceae. This fruit comes from an evergreen tree that can reach up to 10 m height (Saied *et al.,* 2008) and is widely spread in the Middle Eastern and Mediterranean regions, Africa, Australia and tropical America (Shahat *et al.,* 2001). The fruits of this tree can be consumed either fresh or dried, and can also be made into fine flours for other consumption. The *Ziziphus* genus has several medicinal effects and is known as hypoglycaemic, anti-inflammatory, antimicrobial, antioxidant, anti-tumour and liver protective agent (Said *et al.,* 2006). Additionally, there are several studies on the antimicrobial (Motamedi *et al.,* 2009) and antifungal benefits (Hadizadeh *et al.,* 2009) of konar leaves and fruits.

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Due to the health benefits of this fruit, it is therefore important to find the most efficient extraction method that can yield the highest amount of phenolic compounds.

A conventional extraction method of magnetic stirrer and relatively new method of ultrasound-assisted extraction (UAE) as well as microwave-assisted extraction (MAE) method have been used to extract phenolic compounds from plants (Pan *et al.,* 2003; Khan *et al.,* 2010). Among these extraction methods, MAE is used as an efficient method to extract polyphenolic compounds from green tea leaves (Pan *et al.,* 2003), apple pomace (Rezaei *et al.,* 2013), sour cherry (Garofulić *et al.,* 2012), and sage (Garofulić et al., 2012). MAE has also been used to extract essential oils from *Thymus vulgaris* L. (Golmakani and Rezaei, 2008a), *Zataria multiflora* Boiss. (Golmakani and Rezaei, 2008b), *Satureja hortensis* and *S. montana* (Rezvanpanah *et al.,* 2008), and *Bunium persicum* Boiss. (Mazidi *et al.,* 2012). MAE is a rapid extraction technique that also reduces the amount of solvent consumed (Eskilsson and Björklund, 2000). MAE uses microwave power as a source of energy that is provided almost uniformly to the matrix, thus improving the extraction efficiency. UAE is also considered as a cost-effective method that may be used in large-scale applications (Soria and Villamiel, 2010). Through UAE, ultrasound waves can produce cavitation inside the sample matrix, thus creating high shear forces that leads to an enhanced mass transfer, and as a result, a higher extraction yield (Chemat *et al.,* 2017).

Having a more concentrated product (pill or juice) containing exclusively the bioactive components is a practice that can provide such products with additional functional properties. Studies on other fruits high in phytochemicals have shown that the bioavailability of an extract is higher when compared to a fresh fruit as the body would metabolize them more efficiently. In the present work, MAE, UAE, and MSE were used to assess yield of total phenolic (TP) and total flavonoid (TF) contents from konar fruits while considering the effects of solvent type, solvent to sample ratio (SSR), microwave power, MSE temperature, and irradiation, sonication and extraction times.

Materials and methods

Materials

Sodium carbonate, Folin-Ciocalteau reagent, tannic acid, and quercetin were purchased from Merck (Darmstadt, Germany). Aluminium chloride was purchased from Riedel–De Haen Ag, Seelze (Hannover, Germany). Methanol (100%) and ethanol (99.6%) were purchased from Jahan Alcohol (Tehran, Iran).

Sample preparation

Fully ripened konar fruits were collected from Kāzerūn (Fars, Iran), dried at 37°C until a constant moisture, grounded using mortar and pestle to fine powder, stored in a sealed package, and maintained at -20°C until further use.

Microwave-assisted extraction

In order to extract the phenolic compounds, 5 g of konar fruit dried powder was placed in a flask inside a modified microwave apparatus (Model MC-175, frequency 2450 MHz; AEG, Nuremberg, Germany). The microwave system was equipped with a condensation section to allow for solvent recycle back to the flask. Effects of solvent type, SSR, microwave irradiation time, and microwave power on TP and TF extraction yields were evaluated. Extractions were performed using three different solvents (ethanol, methanol, and water), SSR (10 to 1, 15 to 1, and 20 to 1; mL of solvent per g sample), microwave power (90, 180, and 270 W) and irradiation time (5, 10, and 15 min). The applied irradiation times were based on preliminary experiments with MAE. The suspensions resulted from the extraction process were filtered through Whatman No. 1 filter paper and evaporated using rotary vacuum evaporator at 50°C. Applying lower pressures (i.e., that of vacuum) reduces the boiling temperature of the solvents that is additionally helpful to avoid compound deterioration. Since the TP and TF contents were evaluated on their solutions, there was no need to dry the extracts completely. Therefore, this step was used to make sure the extracts prepared using different extraction conditions have comparable volumes (for subsequent measurements). Extracts were used immediately or maintained at -20°C for further analysis. The conditions of the MAE were designed according to Taguchi's experimental design (Table 1).

Ultrasound-assisted extraction

Similar to MAE, 5 g of konar fruit dried powder was placed in a flask and placed in an ultrasonic bath (model DSA100-SK1, China). Effects of solvent types, SSR, and sonication time were studied using UAE. Extractions were performed using three different solvents (ethanol, methanol, and water), SSR (10 to 1, 15 to 1, and 20 to 1; mL of solvent per g sample), and sonication times (30, 60, and 90 min). The suspensions resulted from extraction process were filtered through Whatman No. 1

Table 1. Experimental design for extraction of TP and TF from konar using Taguchi's experimental design.

Run No.	MAE				UAE			MSE			
	S	SSR (g/ml)	P (watt)	t (min)	S	SSR (g/ml)	t (min)	S	SSR (g/ml)	T (°C)	t (min)
1	W	1:10	90	5	W	1:10	30	W	1:10	30	30
2	E	1:15	180	10	E	1:15	60	E	1:15	40	60
3	M	1:20	270	15	M	1:20	90	M	1:20	50	90
4	E	1:20	90	5	E	1:20	30	E	1:20	30	30
5	M	1:10	180	10	M	1:10	60	M	1:10	40	60
6	W	1:15	270	15	W	1:15	90	W	1:15	50	90
7	M	1:15	90	5	M	1:15	30	M	1:15	30	30
8	W	1:20	180	10	W	1:20	60	W	1:20	40	60
9	E	1:10	270	15	E	1:10	90	E	1:10	50	90

MAE: microwave-assisted extraction; UAE: ultrasound-assisted extraction; MSE: extraction using magnetic-stirrer; SSR: solvent to sample ratio; S: solvent; t: extraction time; T: temperature; P: microwave power; E: ethanol; M: methanol; W: water.

filter paper and evaporated using rotary vacuum evaporator at 50°C. Extracts were used immediately or kept at -20°C for further analysis. The conditions of UAE were designed according to Taguchi's experimental design (Table 1).

Magnetic-stirrer extraction

Five grams of konar fruit dried powder was placed in a beaker. The effects of solvent type, SSR, extraction temperature, and extraction time on the recovery of phenolic compounds in MSE were investigated. The applied temperatures were monitored and controlled throughout the extraction using a thermometer. Extractions were performed using three different solvents (ethanol, methanol, and water), SSR (10 to 1, 15 to 1, and 20 to 1; mL solvent per g sample), extraction temperatures (30, 40, and 50°C), and extraction times (30, 60, and 90 min). The applied temperatures in the present work were somewhat higher than the room temperature which could help improve the extraction efficiency without impacting the integrity of the extracted components. Some of the target compounds are heat labile and can be destroyed at higher temperatures. Suspensions resulted from extraction process was filtered through Whatman No. 1 filter paper and evaporated using rotary vacuum evaporator at 50°C. Extracts were used immediately or maintained at -20°C for further analysis. The conditions of MSE were designed according to Taguchi's experimental design (Table 1).

Total phenolic contents

The TP contents of the extracts were determined spectrophotometrically using Folin-Ciocalteau reagent (Singleton *et al.,* 1999). Briefly, 0.1 mL of sample extract was mixed with 0.5 mL of Folin-Ciocalteu reagent and 1 mL of saturated solution of sodium carbonate (35 g anhydrous Na_2CO_3 dissolved in 100 mL of water) in a 10-mL volumetric flask and was taken to the volume using distilled water. Following 30 min incubation at room temperature, the absorbance was determined at 760 nm using a UV-visible spectrophotometer (UNICO S2100 Series, China). The TP contents were expressed as mg tannic acid equivalents (TAE) per g of fruit samples on a dry basis (mg TAE per g sample).

Total flavonoid contents

The TF contents resulting from the different extraction conditions were determined by aluminium-chloride colorimetric method (Brighente *et al.,* 2007). Briefly, 1 mL of phenolic extract from each sample was mixed with 1 mL of aluminium chloride solution (2% w/v, in methanol) and placed in a 10-mL volumetric flask. The mixture was diluted to the mark using distilled water. Following 10 min of incubation at room temperature, the absorbance was determined at 415 nm using the UV-visible spectrophotometer. The TF contents were expressed as mg quercetin equivalent (QE) per g of fruit samples on a dry basis (mg QE per g sample).

Taguchi's experimental design and statistical data analysis

The design of the experiments for MAE, UAE, and MSE were performed according to Taguchi's experimental design (Taguchi, 1986). Efficacy of this method is improving the data quality, reducing the experimental cost, and providing robustness in experimental design. Means of the extraction yields for each treatment using different extraction conditions were determined following Taguchi's experimental design. One-way analysis of variance (ANOVA) with Tukey's multiple comparison tests

was used to compare mean differences among treatments using GraphPad Prism version 7.0 for Windows (GraphPad Software, San Diego, CA). The experiments were performed in duplicate. The TP and TF contents were expressed as means ± standard deviation (SD). The differences were considered statistically significant when the confidence level was above 95% (i.e., $p < 0.05$).

Results and discussion

Effect of solvent

In the present study, the effects of three different solvents on the extraction yields of phenolic and flavonoid compounds were investigated (Figure 1). In all studied extraction methods, methanol and water were able to extract larger amounts of phenolic compounds than ethanol (Figure 1A).

The TP contents of the extracts in water obtained by MAE were significantly higher ($p < 0.05$) than those obtained by other solvents. These results are due to the relative polarities of solvents and extracted phenolic compounds (Mokrani and Madani, 2016). In a study by Xi *et al.* (2009), higher TP contents were obtained from green tea leaves using acetone and methanol by high-hydrostatic pressure extraction. Also, Jakopič *et al.* (2009) reported higher TP contents from two cultivars of green walnut fruit (*Juglans regia* L.) (Elit and Franqette) after 45 min of ultrasonic extraction using methanol, as compared to ethanol. Similarly, Lapornik *et al.* (2005) showed that using methanol in water as solvent (70% v/v) resulted in the highest TP content from the by-products of black currant (*Ribes nigrum* var. *Rosenthal falch*), red currant (*R. rubrum* var. *Rondom*), and grape (*Vitis vinifera* var. *Pinot Noire*), when compared to pure water or ethanol in water (70% v/v). TP contents of the extracts depend on the type, polarity, and concentrations of the solvents used for the extraction. For instance, Lapornik *et al.* (2005) showed that methanol, compared to ethanol, is a better solvent for the extraction of phenolic compounds and anthocyanins from grape and black currant. Wang and Weller (2006) reported similar results on the efficiency of water, methanol, and ethanol in the extraction of plant materials. This can be attributed to the higher affinity of phenolic compounds toward more polar solvents (such as water). Furthermore, when using MAE technique, the polarity level of medium constituents is a crucial factor in enhancing the overall extraction yield.

In the extraction of flavonoids using the conditions mentioned above, ethanol and methanol yielded significantly ($p < 0.05$) higher TF content in MAE and UAE than did in MSE (Figure 1B). A similar trend has also been reported by

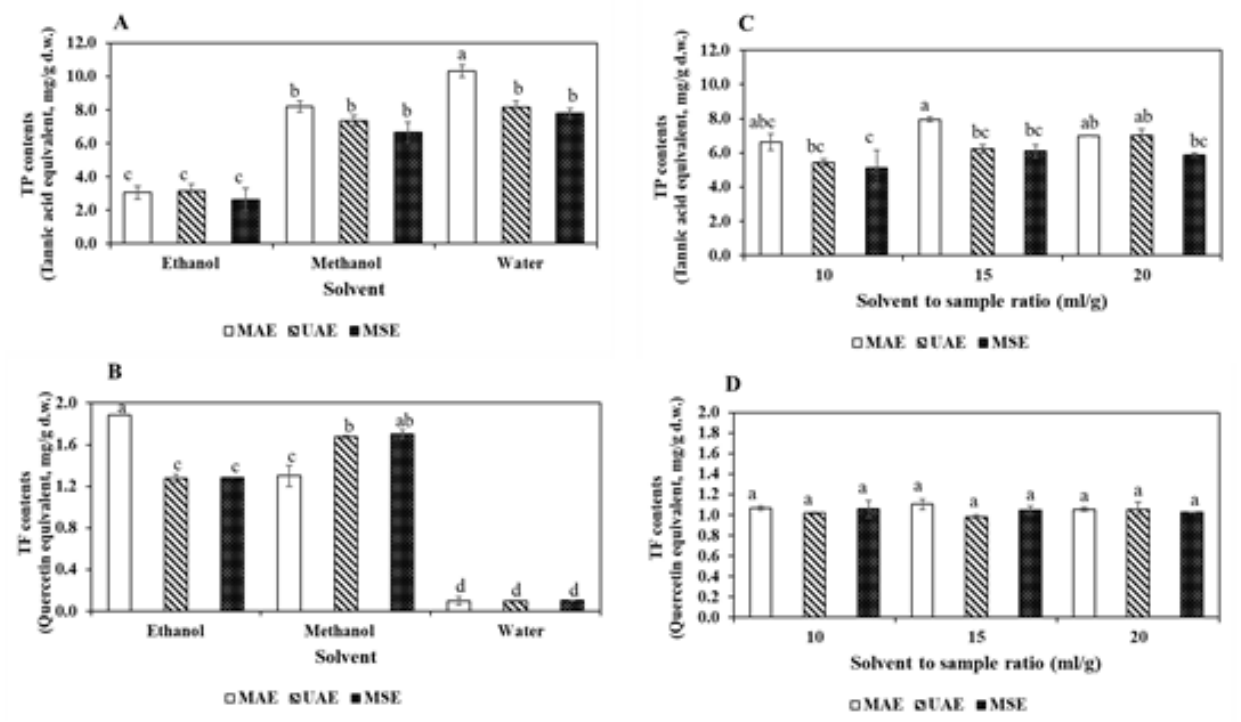

Figure 1. Effects of solvent (ethanol, methanol, and water) and solvent to sample ratio (10, 15, and 20) on the extraction yields of total phenolic contents (A and C) and total flavonoid contents (B and D) using microwave-assisted extraction (MAE), ultrasound-assisted extraction (UAE), and magnetic-stirrer extraction (MSE) methods based on Taguchi's experimental design.

Do *et al.* (2014). Water as a standalone solvent or in combination with other organic solvents yielded lower levels of TF content. This has been explained by the differences in the polarity of water and those of the target compounds and hence water is unable to extract all the flavonoids from the source material.

In the present work, ethanol and methanol, as compared to water, resulted in the highest amounts of flavonoid compounds in all extraction methods. Additionally, ethanol has resulted in significantly higher yield than methanol in MAE. However, with UAE and MSE, methanol is a better solvent than ethanol in extracting/maintaining the flavonoid contents (Figure 1B). The greater efficiency of methanol in extracting flavonoids observed in the present work is also consistent with the results reported by Mohdaly *et al.* (2010), where highest flavonoid and flavonol contents were related to methanol as solvent, when compared with acetone, ethanol, hexane, petroleum ether, and diethyl ether.

Effect of solvent to sample ratio (SSR)

To find the optimized conditions for the extraction of phenolic compounds, three different SSR (10 to 1, 15 to 1, and 20 to 1; mL of solvent for 1 g of sample) were studied (Figure 1C). It was observed that in all extraction methods, increasing the SSR from 10 to 15 slightly increased the TP contents but it was decreased when the ratio was increased to 20. The highest TP content was found when using SSR 15 to 1 in MAE (8 mg tannic acid equivalent per g sample). It appears that using the SSR of 15 was enough to extract TP contents, since increasing the ratio to 20 further diluted the extracts. In the UAE, increasing the ratio from 10 to 15 and then 20 did not significantly increase TP content ($p > 0.05$). Similarly, no significant differences were found among the three SSR levels applied with MSE. Considering the flavonoid extraction, using different SSR levels in the three methods of MAE, UAE, and MSE showed no significant differences in the TF contents obtained by these methods. Among these treatments, it seems that an SSR of 10 to 1 was high enough to extract most of the flavonoids in the sample (Figure 1D). The highest TF content was measured as 1.1 mg quercetin per g of sample on a dry basis in MAE, UAE, and MSE.

Generally, by increasing the SSR, the concentration gradient, which is the driving force for the extraction, will be higher in the source material and as a result the extraction yield is increased due to the higher diffusion rate for the solvents (Sahin and Samli, 2013). This phenomenon is consistent with the basic principles for mass transfer (Cacace and Mazza, 2003). Cacace and Mazza (2003) investigated the effect of temperature in the extraction of anthocyanins and found that the extraction yield increases as a function of temperature up to certain levels. Extraction at 74°C had a negative impact on the extraction yield. However, as observed here, increasing SSR decreased the amount of TP contents in MAE method, which can partly be related to the possible lower temperatures when a greater amount of solvent is used. They monitored the temperature in the extraction of anthocyanins, and it was shown that extraction yield increases as a function of temperature and time up to certain temperatures. In a previous study, Guo *et al.* (2001) reported similar findings where increasing the SSR from 5 to 30 in MAE initially increased the extraction yield of puerarin (an isoflavone) from a Chinese herbal medicine, *Radix puerariae*. However, increasing the SSR from 30 to 50 decreased the puerarin extraction yield. Elsewhere, it was also shown that extraction of flavonoid compounds was increased in MAE of *Radix astragali* (root of Astragalus) as a function of SSR from 10 to 30 and then decreased when SSR was increased from 30 to 40 using 90% ethanol as solvent (Xiao *et al.,* 2008).

Effect of microwave power

The results obtained indicated that the extraction yields of phenolic compounds significantly increased ($p < 0.05$) (Figure 2A) as the microwave power increased from 90 (6.3 ± 0.1 mg TAE per g sample) to 180 W (7.0 ± 0.1 mg TAE per g sample) and then to 270 W (8.3 ± 0.1 mg TAE per g sample). Higher levels of microwave power can result in a higher temperature at a shorter time, and as a consequence, accelerate the extraction process and reduce the extraction time. Our results agree with those of Wang *et al.* (2010) who showed that increasing microwave power up to 700 W increased the extraction yield of phenolic compounds from tea. Additionally, although TF content slightly increased as a function of microwave power, no significant differences ($p > 0.05$) were observed among the flavonoid contents when different power levels were used (Figure 2B).

Effect of temperature with magnetic stirrer extraction

The changes in the TP contents when using MSE at different temperatures are presented in Figure 3A. Increasing the temperature during the extraction using magnetic stirrer from 30 to 50°C resulted in slight increase in the TP contents from 4.9 ± 0.2 to 6.5 ± 0.7 mg TAE per g sample, respectively; however, the changes observed were not significantly different ($p > 0.05$). Similarly, the TF contents

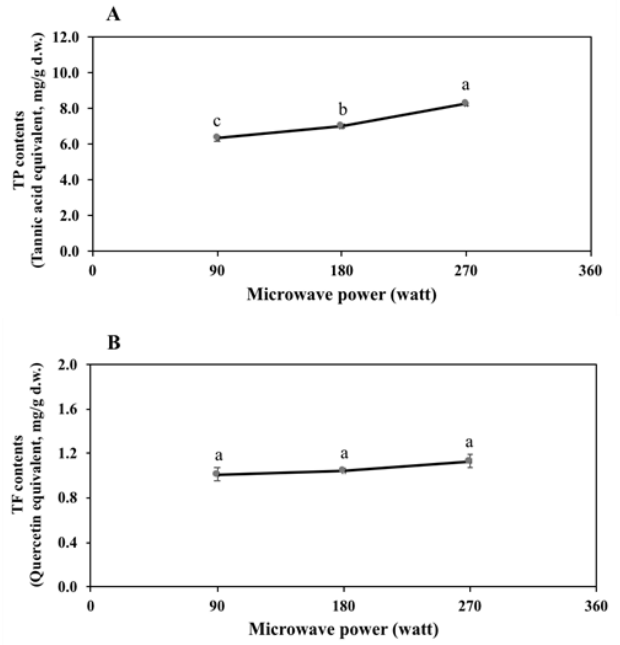

Figure 2. Effect of microwave power (90, 180, and 270) on the extraction yields of total phenolic (A) and total flavonoid (B) contents using MAE method. Data are presented as mean ± SD. Treatments with different letters are significantly different ($p < 0.05$).

were slightly, but not significantly ($p > 0.05$), increased from 1.0 ± 0.1 mg QE per g sample at 30°C to 1.1 ± 0.1 mg QE per g sample at 50°C (Figure 3B).

Effects of microwave irradiation, ultrasound sonication, and stirring time

The changes in the TP and TF contents during different treatments are shown in Figures 4A and 4B. The levels of phenolic compounds extracted at different extraction times were significantly different ($p < 0.05$) for MAE; however, it did not change with UAE. But a slight increase was observed for MSE (Figure 4A). The TP contents obtained using MAE following irradiation for 15 min, 8.3 ± 0.1 mg TAE per g sample, was significantly higher ($p < 0.05$) than that for the 90-min extraction time used in UAE and MSE. In a study by Pan *et al.* (2003), it was suggested that increased MAE irradiation time enhances the extraction yield due to increased temperature and molecular kinetic energy induced by the microwaves. For MSE, TP contents increased from 4.9 ± 0.2 to 6.5 ± 0.7 mg TAE per g sample after stirring for 30 to 90 min, respectively (Figure 4A).

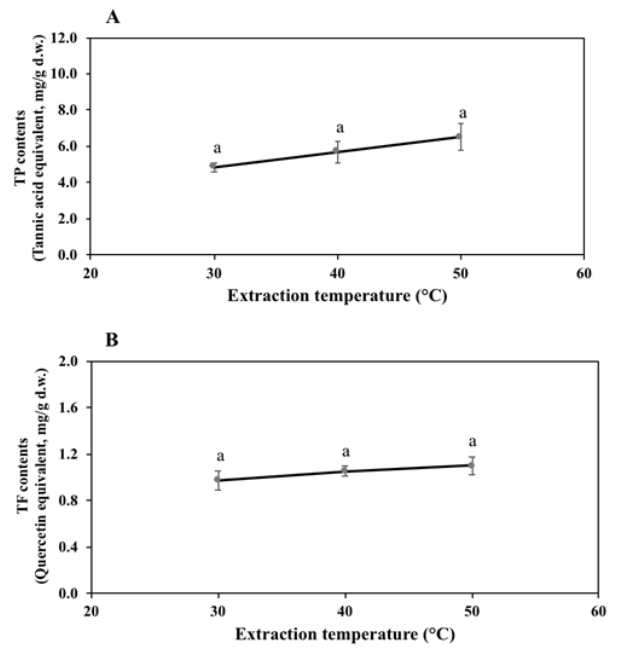

Figure 3. Effect of extraction temperature (30, 40, and 50°C) on the extraction yields of total phenolic (A) and total flavonoid (B) contents using MSE method. Data are presented as mean ± SD. Treatments identified with different letters are significantly different ($p < 0.05$).

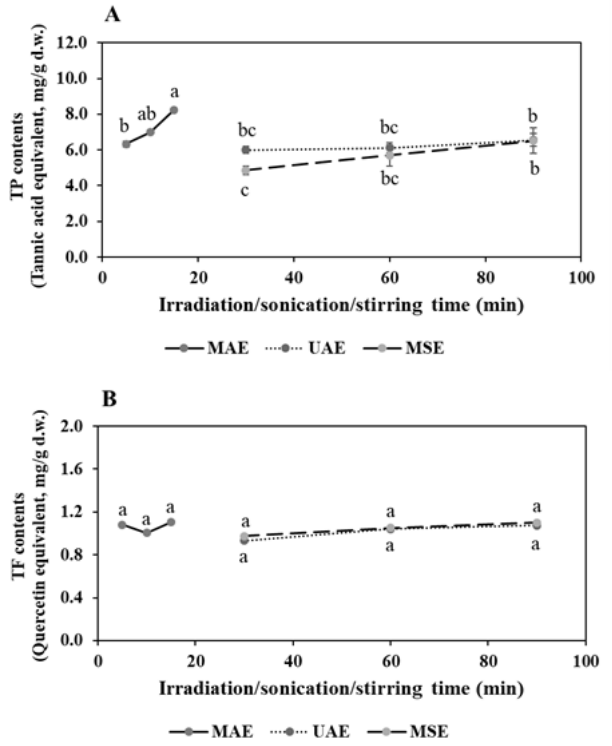

Figure 4. Effect of extraction time (MAE: 5, 10 and 15 min; UAE: 30, 60, and 90 min; MSE: 20, 60, and 90 min) on the extraction yields of total phenolic (A) and total flavonoid (B) contents using MAE, UAE, and MSE methods. Data are presented as mean ± SD. Treatments with different letters are significantly different ($p < 0.05$).

The TF contents obtained using MAE, UAE, and MSE at different extraction times showed no significant differences ($p < 0.05$) among the various treatments applied (for 30 min extraction, UAE: 0.9 ± 0.0; MSE: 1.0 ± 0.1 mg QE per g sample and for 90 min, UAE: 1.1 ± 0.0; MSE: 1.1 ± 0.1 mg QE per g sample) (Figure 4B). Also, 5 min of irradiation using MAE resulted in the extraction of flavonoids at 1.1 mg QE per g sample, which was comparable to those of the 90-min sonication and stirring in UAE and MSE methods. In a study by Thoo *et al.* (2010), it was shown that the extraction yield of phenolic compounds from mengkudu (*Morinda citrifolia*) using different ethanol concentrations using water bath shaking was not affected by increasing the extraction time from 20 to 100 min; though it significantly increased during 100 to 120 min of extraction. They also indicated that extraction time in the range of 20 - 120 min had insignificant effect on the flavonoid compounds.

Conclusion

One of the important factors in the efficacy of phenolic compounds with regards to human health is their bioavailability as well as their extraction yield from various food sources. In the present study, the effect of different extraction methods was investigated to understand the optimum extraction conditions such as solvent, SSR, time, and temperature in the extraction of TP and TF compounds from konar fruit. The results obtained showed that compared with the conventional extraction methods, MAE provided the highest extraction yield in a shorter period, and was less labour-intensive. As such, MAE can be used as an appropriate method of extraction of phenolic compounds from konar fruits. Food and medicinal industries could benefit from this emerging technology, as it is a rapid, safe, and eco-friendly method as compared to other conventional extraction techniques.

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