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ORIGINAL RESEARCH PAPER

EFFECTS OF ULTRASOUND-ASSISTED EXTRACTION CONDITIONS ON THE RECOVERY OF PHENOLIC COMPOUNDS AND IN VITRO ANTIOXIDANT ACTIVITY OF JUJUBE (*Ziziphus jujuba Mill*.) LEAVES

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The optimum conditions for the Ultrasound-Assisted Extraction (UAE) of total phenolics and antioxidant activity from jujube leaves were studied. The effect of the solvent nature (acetone, methanol, ethanol and water), solvent concentration (25-100%; v/v), ultrasound intensity (25-100%), solid/solvent ratio (1/50-1/300; w/v) and extraction time (1-15 min) were also determined. The total phenolic content was used to determine the phenolic compounds. Ferric-reducing power and 2,2-diphenyl-1-picrylhydrazyl (DPPH) were used to evaluate the antioxidant activity. All the ultrasound-assisted extraction parameters that were investigated had from a statistically point of view significant effects ($p<0.05$) upon the phenolic compound extraction and antioxidant activity. The best extraction conditions were obtained by using methanol 60 %, a sonication intensity of 75 %with a solid/solvent ratio of 1/200(w/v), for 10 min. As such, the obtained values were 6.0g gallic acid equivalents/100g for total phenolic content, 3.886 g ascorbic acid equivalents/100g for free radical scavenging activity and 2.587 g ascorbic acid equivalents/100g for ferric reducing power. The phenolic content was positively correlated with the antioxidant activities.

Keywords: jujube leaves, ultrasound, extraction conditions, phenolics, antioxidant activity

Introduction

Most of the beneficial properties of fruits, vegetables and whole grains have been attributed to bioactive chemical compounds commonly named phytochemicals. Among these, phenolic compounds have been extensively studied due to their diverse health benefits as antioxidants, and also for preventing cardiovascular diseases, cancer and diabetes (Aybastier *et al.*, 2013).

Extraction is the first step in isolating the phenolic compounds from plant materials and its aim should be to extract the maximum amount of the targeted compounds, of the highest quality antioxidant power of the extracts). Regarding the extraction of all plant phenolics, there are no universal methods to develop a procedure that takes into account the compositional diversity of the natural sources of phenolics, as well as the structural interaction with other components that are present in the plant matrices and the physicochemical properties of these compounds. Thus, specific processes must be designed and optimized for each phenolic source. The extraction of bioactive compounds and the antioxidant capacity of plant material extracts are affected by different factors, such as their chemical nature, temperature, type of solvent, solid-to-solvent ratio, contact time (Bachir Bey *et al.*, 2013).

Ultrasonic-Assisted Extraction (UAE) is one of the most inexpensive, rapid, simple and efficient techniques compared to conventional extraction (Liu *et al.*, 2013).The application of the ultrasound power (10-100 W/cm^2)in sonochemistry is attributed to the cavitation phenomena, which is defined as the formation, growth, and subsequent collapse of bubbles, by releasing a large magnitude of energy to the reaction environment which results in the disruption of cell walls, reduction of particle size, and enhancement of mass transfer across cell membranes (Şahin *et al.*, 2013; Tiwari, 2015; Yu *et al.*, 2016).

Jujube (*Ziziphus jujuba Mill*.), which belongs to the Rhamnaceae family, is a native fruit tree of China being one of the most important *Ziziphus* species. It presents a highly economic value not only in the food industry, but also in medicine. *Ziziphus jujuba* leaves have been shown to possess such benefits as antioxidant and anti-inflammatory activities (Kumar *et al.,* 2004; Anideh *et al.,* 2016).

To the best of our knowledge, there are no undertaken studies to maximize the phenolic compound extraction and antioxidant activity of jujube leaves. Hence, the objectives of the present investigation were to determine the best ultrasound-assisted extraction conditions (type and concentration of solvent, ultrasound amplitude, solid-to-solvent ratio and extraction time) to obtain the highest total phenolic content (TPC) of jujube (*Ziziphus jujuba* Mill.) leaves and the best antioxidant activity(DPPH free radical-scavenging activity – FRSA; and ferric reducing power - FRP),by using an one-factor-one time approach, also known as a single factor experiment, where only one factor is variable at one time, while all others are constant.

Materials and methods

Chemical reagents

Folin-Ciocalteu reagent, trichloroacetic acid (≥99.0% purity), sodium carbonate and methanol (99.8% purity) were acquired from Biochem, Chemopharma (Montreal, Quebec); potassium ferricyanide (≥99.5% purity) was from Biochem, Chemopharma (Georgia, USA); gallic acid from Prolabo (Montreuil, France); and 2-2-diphenyl-1-picrylhydrazyl (DPPH) from Sigma-Aldrich GmbH (Sternheim, Germany).

Sample preparation

Ziziphus jujuba leaves were harvested in the region of Bejaia (Kherrata, North East of Algeria) during the September-October period (2015). The leaves wereallowed to air dry at room temperature. After drying, the leaves were ground until a granulometry lower than 250µm. Thus, the obtained powder was stored in glass jars, sealed, labeled, and stored away from light.

Extraction procedure

An ultrasonic apparatus (Vibracell.VCX 130 PB, USA) was used for UAE with the working frequency fixed at 20 kHz. The energy input was controlled by setting the amplitude of the probe sonicator. For the extraction, an aliquot of jujube leaves powder (12 mg) was placed in a 25-mLglass vial with 12 mL of solvent. The temperature was controlled continuously by circulating external cold water. The solvent type and concentration, sonication intensity, solid-to-solvent ratio and extraction time were set according to the single factor experiment. The extracts were centrifuged at 3000g (Nüve NF 200, Ankara, Turkey) for 5 min and filtered through paper. The supernatants were subsequently used for the determination of total phenolic content (TPC) and antioxidant activities: ferric reducing power (FRP) and DPPH free radical-scavenging activity (FRSA).

Experimental design

In the present study, to determine the optimum conditions for extracting the antioxidant phenolic compounds from jujube leaves, single factor experiments were used. A total of five parameters namely the solvent type (ethanol, methanol, acetone and water), methanol concentration (20-100%; v/v), ultrasound intensity (20-100%), solid-to-solvent ratio (1/50 to 1/300; w/v) and extraction time (1-15 min) were studied so that only one parameter varied while the other parameters were constant. The best extraction conditions were selected according to the values of main assessed parameters (TPC, FRP, and FRSA).

Solvent type

The samples were extracted with 100% acetone, 100% ethanol, 100% methanol and water by fixing the solid-to-solvent ratio (1/100; 12 mg/12 mL), sonication intensity (50%) and extraction time (5 min).

Solvent concentration

After selecting the extraction solvent, the samples were extracted with different solvent concentrations, namely, 25%, 50%, 75% and 100% (v/v) by fixing the solid-to-solvent ratio (1/100; 12 mg/12 mL), sonication intensity (50%) and the extraction time around 5 min.

Ultrasound-intensity

The samples were extracted using the most potent solvent at the most appropriate concentration. The extracts were prepared by varying the ultrasound intensity (25%, 50%, 75% and 100%) while fixing the extraction time (5 min) and 1/100; 12 mg/12 mL (solid-to-solvent ratio).

Solid-to-solvent ratio

The extraction procedure was repeated by varying the solid-to-solvent ratio (1/50, 1/100, 1/150, 1/200, 1/250 and 1mg/300 mL) using, afterwards, the most suitable solvent type, solvent concentration and the best ultrasound amplitude while adjusting the extraction time (around 5 min).

Extraction time

After choosing the solvent type, solvent concentration, the best ultra-sound intensity and the best ratio, the samples were extracted by varying the sonication time (1, 2, 3, 5, 10 and15 min).

Total phenolic content (TPC)

The extracts TPC was determined according to the method of Singleton and Rossi (1965). Aliquots (200µL) of extract were mixed with 1mL of Folin-Ciocalteu reagent and 800µL of sodium carbonate (6%). After 60min, the absorbance was measured at 740nm (UV mini 1240 spectrophotometer, Shimadzu, Suzhou, Jiangsu, China). The total phenolic content was expressed as g of gallic acid Equivalents per 100g dry weight (g GAE/100 g DW).

Antioxidant activity

Ferric reducing power (FRP)

The ferric reducing power was determined by the method of Oyaizu (1986). Samples (1 mL) were mixed with 2.5 mL of phosphate buffer (0.2M, pH 6.6) and 2.5 mL of potassium ferricyanide. After an incubation of 20 min at 50°C, 2.5 mL of trichloroacetic acid was added to the mixtures. After centrifugation at 3000 rpm for 10 min, 2.5 mL of the upper layer were diluted with 1 mL of distilled water and, afterwards, 0.5 mL of 0.1% ferric chloride was added. The absorbance was measured at 700nm. Ascorbic acid was used as a standard and ferric reducing power was expressed as g Ascorbic Acid Equivalent per 100g dry weight (g AAE/100 g DW).

DPPH free radical-scavenging activity (FRSA)

The DPPH free radical-scavenging activity was determined according to the method of Brand-Williams et al. (1995).An aliquot (50 µL) of the extract was added to 1 mL of the methanolic solution of DPPH (60µM).After 30 min of incubation at room temperature, the absorbance was recorded against a blank sample at 517nm. Radical scavenging activity was expressed as g Ascorbic Acid Equivalent per 100g dry weight (g AAE/100g DW).

Statistical analysis

The results were analyzed using STATISTICA software (version 5.5.fr; StatSoft, Inc, Tulsa, USA). For the undertaken repeated measurements, the significant differences were evaluated by analysis of variance (ANOVA) ($p<0.05$).

Results and discussion

Effect of solvent nature

The present study showed that all the used solvents were able to extract the phenolic compounds from jujube leaves (Figure 1). Methanol extract showed the highest phenolic content with a value of 3.57g GAE/100 g DW, followed by water, ethanol and acetone extracts (Figure 1A). Also, as illustrated in Figure 1B, the methanol extract exhibited the best antioxidant activity with values of 2.42 g AAE/100g DW and 1.32 g AAE/100g DW for FRSA and FRP, respectively, followed by water, ethanol and acetone extracts.

A)
B)

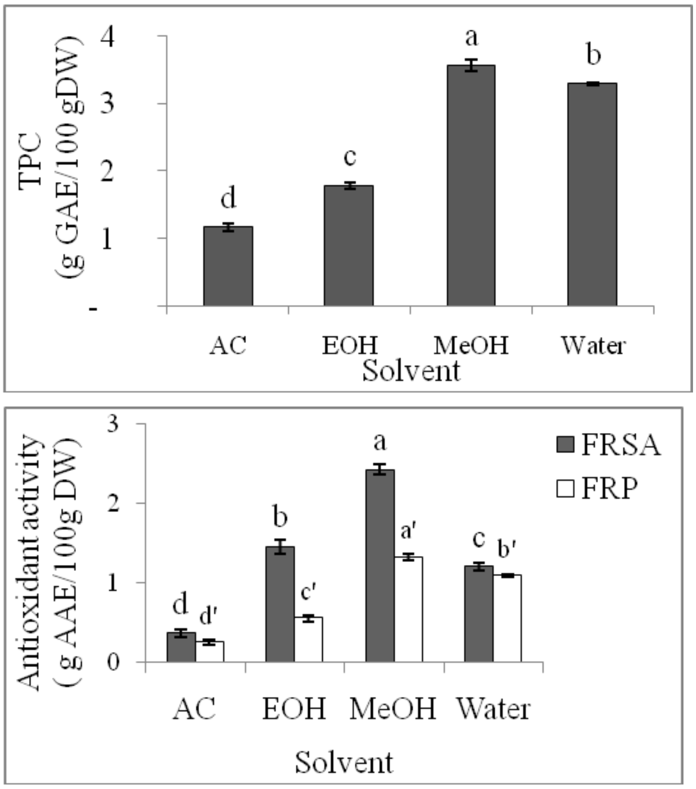


Figure 1. Effect of solvent nature on the recovery of phenolic compounds (A) and the antioxidant activities (B) of jujube leaf compounds. AC, acetone; EOH, ethanol; MeOH, methanol

Effect of solvent concentration

After determining the best type of solvent for the antioxidant extraction, different concentrations of methanol (25%, 50%, 75% and 100%, v/v) were tested in order to choose the most suitable concentration for the extracting solvent. The results revealed that methanol concentration had a significant effect ($p< 0.05$) on TPC, FRSA and FRP of jujube leaves (Figure 2). Phenolic contents and the antioxidant activities increased by increasing the proportion of methanol as the extracting solvent up to 60% with values of 5.61g GAE/100g DW (Figure 2A), 3.40 g AAE/100g DW and2.0 g AAE/100g DW (Figure 2B) for TPC, FRSA, and FRP, respectively. The lowest phenolic content and antioxidant activities were obtained with methanol 25% and methanol 100%.

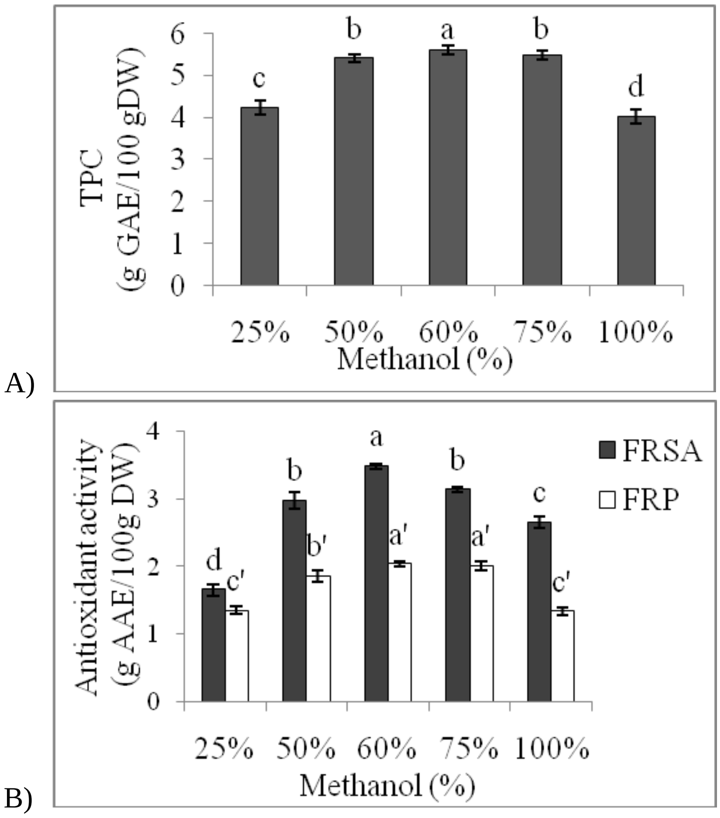
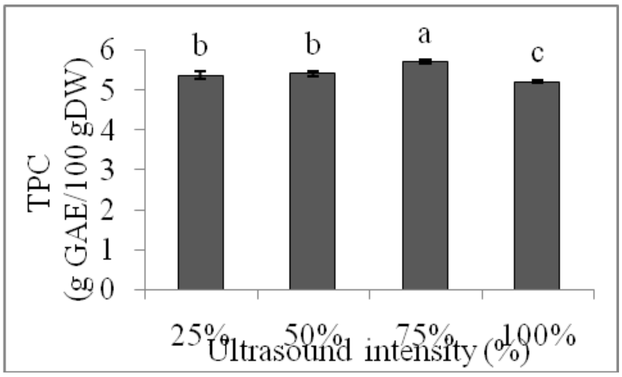


Figure 2. Effect of the solvent concentration on the recovery of phenolic compounds (A) and antioxidant activity (B) of jujube leaves compounds

Effect of ultrasound intensity

To evaluate the effect of ultrasound power on the extraction efficiency of jujube leaves phenolics and their antioxidant activity, the extraction was carried out by varying the ultrasound power (25%, 50%, 75% and 100%) using the best selected solvent for the extraction (60% methanol) as assessed in the previous steps. As depicted in Figure 3, the ultrasound intensity had a significant effect ($p<0.05$) on TPC, FRSA and FRP. These three parameters increased with the intensification of the ultrasound intensity (up to 75%) with values of 5.705 g GAE/100g DW (Figure 3A), 3.533g AAE/100g DW, 2.122g AAE/100g DW (Figure 3B), respectively.

A)


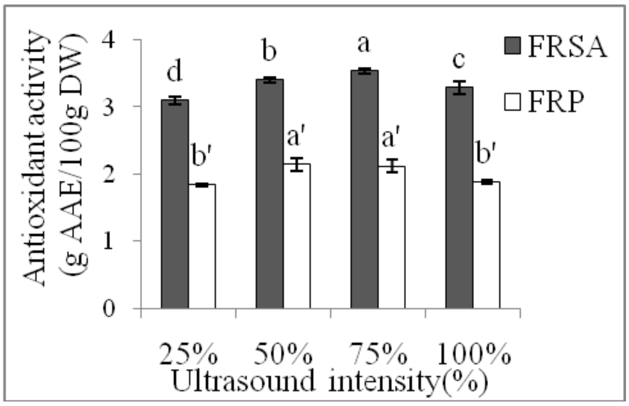
B)


Figure 3. Effect of the ultrasound intensity on the recovery of phenolics (A) and antioxidant activity (B) of jujube leaves compounds

Effect of solid-to-solvent ratio

The total phenolic content and the antioxidant activity of jujube leaves bioactive compounds extracted by 60% methanol, 75% ultrasound power, using six solid-to-solvent ratios 1/50, 1/100, 1/150, 1/200, 1/250 and 1/300 are shown in Figure 4. Sample-to-solvent ratio had a significant effect ($p<0.05$) on TPC and the antioxidant activities, the best sample-to-solvent ratio being 1/200 (0.06 mg/12 mL) with TPC of 5.889 g GAE/100g DW (Figure 4A), 3.778 g AAE/100g DW and an antioxidant activity of 2.426 g AAE/100g DW for FRSA and FRP, respectively (Figure 4B).

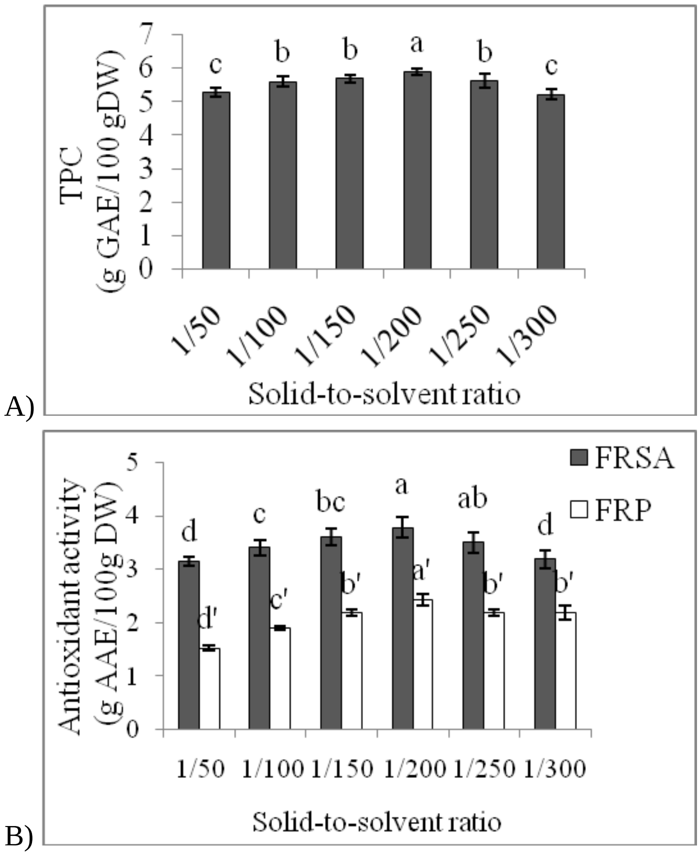


Figure 4. Effect of solid/solvent ratio on the recovery of phenolics (A) and the antioxidant activity (B) of jujube leaves compounds

Effect of extraction time

As it can be seen in Figure 5, the sonication time had a significant effect ($p<0.05$) on TPC, FRP and FRSA of jujube leave extracts. TPC, FRSA and FRP increased when the sonication time was increased from 1 to 10 min. After 10 min, a further increase of the process duration did not significantly ($p<0.05$) improve the recovery of phenolics and the antioxidant activities providing values of 6.0g GAE/100g DW (Figure 5A) for TPC, 3.886 g AAE/100g DW and 2.587 g AAE/100g DW for FRSA and FRP, respectively (Figure 5B).

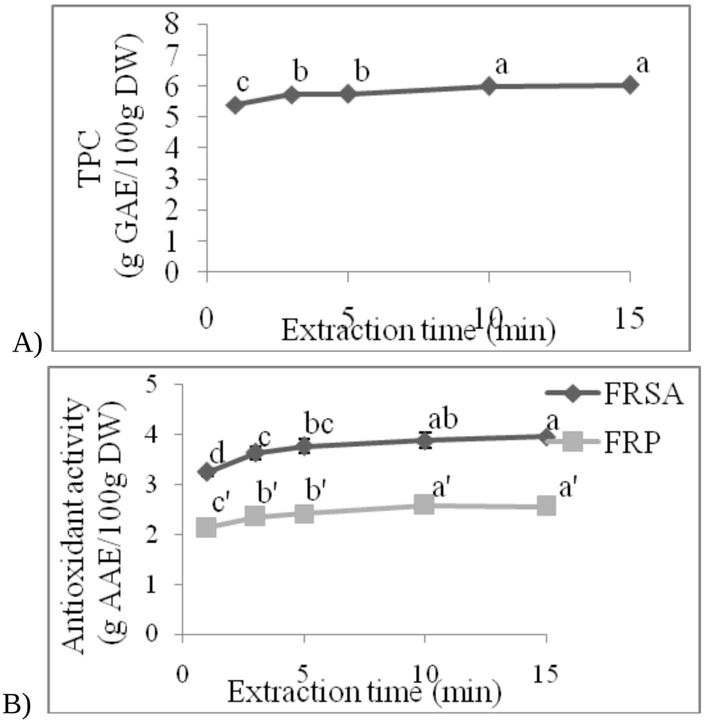


Figure 5. Effect of extraction time on the recovery of phenolic compounds (A) and the antioxidant activity (B) of jujube leaves compounds

Discussion

The extraction protocol for the preparation of antioxidant extracts was found to be the key factor in regards to the antioxidant performance of the extracts. The type of solvent has been the most investigated factor and several studies were carried out to determine the effect of solvents on the extraction efficiency. As shown previously, methanol was the best solvent for extraction. Similar results were obtained by Chirinos et al. (2007) who attested that methanol is the best solvent for the extraction of phenolic compounds and the study of antioxidant activity from *Tropaeolum tuberosum*.

Concerning the effect of solvent concentration, the lowest values were obtained with methanol 100%, whereas the highest TPC and antioxidant activity were obtained with methanol 60%. Similar result was reported by Aybastier *et al.* (2013) for blackberry leaves.

The use of water in combination with organic solvents contributes to the creation of a moderately polar medium that ensures the extraction of phenolics (Naczk and Shahidi, 2006; Chirinos *et al.*, 2007). Aqueous methanol system is capable to destroy cell membranes and to release and stabilize some phenolic sub-groups. It was also reported that methanol inhibits the activity of polyphenol oxidases which are present in plants, this way reducing the phenolic degradation (Vajić *et al.*, 2017). Aqueous methanol, due to its polarity, is more effective to extract polyphenols that are linked to polar fibrous matrices (Tabart *et al.,* 2007). Changing the solvent polarity alters the ability to dissolve a selected group of antioxidant compounds and influences the antioxidant activity estimation (Turkman *et al.,* 2006). The type of solvents and polarity may affect the single electron transfer and the hydrogen atom transfer, which are key aspects in the measurements of antioxidant capacity.

Ultrasound intensity has a significant effect on the recovery efficiency of TPC and antioxidant activity (FRSA and FRP). The TPC and antioxidant activities (FRSA and FRP) increased with the increasing ultrasound intensity until 75 % and decreased at a very high intensity (100%). Deng et al. (2017) reported that UAE could increase the recovery of phenolics compared to conventional extraction from fresh olive.

The advantages of UAE in extracting bioactive compounds from different materials due to its high reproducibility at shorter time, and its simplified manipulation, facilitate a more effective mixing, a significant reduction of solvent consumption and a temperature and lower energy input (Azmir *et al.,* 2013; Liu *et al.,* 2013; Prokopov *et al.,* 2017).

Ultrasonic assisted extraction (UAE) is a fast, effective extraction technique that utilizes ultrasonic waves to generate a cavitation effect on the solvent, resulting in a faster movement of molecules and a higher penetration of the solvent into raw plant materials (Teng *et al.,* 2014). Sound waves can create bubbles in a liquid and produce a negative pressure. The bubbles form, grow, and finally collapse. Close to a solid boundary, the cavity collapse is asymmetric and produces high-speed jets of liquid. The liquid jets have a strong impact on the solid surface. Therefore, it can increase the mass transfer of some compounds due to increase of cell wall permeability, and so, it facilitates the mass transfer of solutes to the extraction solvent (Shirsath *et al.,* 2012).

Solid-liquid extraction is a mass transport process that can be enhanced by variations of diffusion coefficients in response to ultrasound stimulation (Teng *et al.,* 2014). It can accelerate the extraction process at low temperature, causing less damage to the structural and molecular properties of compounds in plant materials.

The TPC and antioxidant activity (FRP and FRSA) increased with the increase of solid-to-solvent ratio from 1/50 to 1/200 and was consistent with the mass transfer principles, the driving force during mass transfer being a concentration gradient between solid and liquid which is greater when a higher solvent to solid ratio is used (Al-Farsi and Lee, 2008). However, the 1/250 and 1/300 ratios had no effect on the extraction efficiency of TPC and antioxidant activity. The presence of an excess of solvent may influence the cavitation effect of UAE, thereby leading to a smaller extraction efficiency (Li *et al.,* 2015). A high solid to solvent ratio determines an incomplete extraction and the solvent becomes saturated before the substrate exhaustion (Bachir Bey *et al.,* 2013).

The results showed a significant effect of the extraction time on TPC and upon the antioxidant activities (FRP and FRSA) of the extracts, from 1 to 10 min, when the values increased, by reaching the maximum at 10 min. Orphanides *et al.* (2014)

reported that UAE reduce the time of extraction and improves the extraction efficiency of spearmint polyphenols compared to the conventional extraction.

Table 1 showed that a good linear positive correlation exists between TPC and FRSA (R2 = 0.78) and between FRSA and FRP (R2 = 0.84), and a strong correlation between TPC and FRP (R2 = 0.96). The correlation between the antioxidant activity and the total phenolics may be explained in numerous ways, but in fact the total phenolic content does not incorporate all the antioxidants. In addition, the synergistic effect between the antioxidants in the mixture makes the antioxidant activity dependent not only on the concentration, but also on the structure and the interaction between antioxidants. Also, it can be concluded that the antioxidant activity of plant extracts is not the result of these compounds but may be related to the presence of some individual active phenolic compounds (Djeridane *et al*., 2006).

Table 1. Correlation between total phenolic contents and antioxidant activities

Correlation	**correlation coefficient**	**Equation**	***P***
TPC-FRP	0.96	y = 0.4444x – 480.61	***
TPC-FRSA	0.78	y = 0,7304x – 808.52	***
FRP-FRSA	0.84	y = 0,4127x + 561.24	***

*** p< 0.001 – significant correlation

TPC - total phenolic content; FRP - ferric-reducing power; FRSA - free radical-scavenging activity

Conclusions

In conclusion, the optimum ultrasound-extraction conditions for the recovery of antioxidants from jujube leaves were determined. An extraction with 60% methanol, at 75% ultrasound intensity using 1/200 solid-to-solvent ratio, for 10 min as the sonication time, were assessed as the optimum conditions for TPC recovery and for the antioxidant activities (FRP and FRSA). These conditions allowed a recovery of 6.0 g GAE/100g of TPC, 3.886 g AAE/100g for FRSA and 2.587 g AAE/100g for FRP. Phenolic contents were found to be well correlated with the antioxidant activities. Furthermore, it was determined that the compounds extracted from jujube leaves possess high antioxidant activity and that the source can be considered a good source from a phyto-pharmaceutical point of view.

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