ORIGINAL PAPER

CrossMark

Profiling of phenolic compounds and antioxidant activities of *Cissus rotundifolia* (Forssk.) as influenced by ultrasonic-assisted extraction conditions

Wedad Q. AL-Bukhaiti[1] · Anwar Noman[1,2] · Amer Ali Mahdi[1] · Abdelmoneim H. Ali[1] · Sherif M. Abed[1] · Marwan M. A. Rashed[1] · Hongxin Wang[1]

Abstract

Cissus rotundifolia (Forssk.) is a wild plant belongs to the Vitaceae family, containing high levels of minerals, vitamins and amino acids. It has been used as a traditional medicine for the treatment of several diseases. In this work, the effects of different ultrasound-assisted extraction parameters including solvent type and concentration, sonication time, power, temperature, and sample to solvent ratio on the total phenol compounds (TPC) and antioxidant activities of *C. rotundifolia* leaves extract were investigated. The leaves extract showed high phenolic compounds content and antioxidant activity. Under the optimal conditions (acetone as a solvent, with a concentration of 40%, sonication time of 40 min, power of 150 W, temperature of 40 °C, and sample to solvent ratio of 1:50 w/v), TPC of 15.68 gallic acid equivalents/gram dry weight (g DW) was attained. Regarding the antioxidant activities, 1,1-diphenyl-2-picrylhydrazyl (DPPH) and 2,2-azino-bis-(3-ethylbenzothioline-6-sulphonic acid)-di-ammonium salt (ABTS) were 59.35% and 20.34 mg Trolox/g DW, respectively. There was a significant correlation between TPC, DPPH, and ABTS with the extraction conditions in particular solvent type and concentration. The scanning electron microscopy revealed that the ultrasound affected the walls of plant cells, and thus facilitated solvent penetration for phenolic compounds extraction. Liquid chromatography–mass spectrometry analysis showed that *C. rotundifolia* leaves extract contained various phenolic compounds characterized by their potential applications in several medical and pharmaceutical purposes.

Keywords *Cissus rotundifolia* · Ultrasound · Extraction conditions · Total phenol compound · Antioxidant activity · Liquid chromatography–mass spectrometry

Introduction

Many wild plants have the ability to grow without any agricultural treatments. Due to their higher contents of minerals, vitamins, fatty acids, amino acids, and dietary fibers, wild plants can be used as a food in poor rural communities around the world [1]. They considered as an important source of human food, especially in cases of disaster and famine, because they remain the cheapest source of food for those people [2]. *Cissus rotundifolia* (Forssk.) Vahl, is a wild plant, climber, evergreen, and a species of Cissus belonging to the Vitaceae family (grape family). It is known as a common Arabian wax Cissus, and locally called Halas or Alfaq in Yemen. The edible part of this plant is the leaves after cooking. *C. rotundifolia* has been used in traditional Yemeni medicine to treat the gastrointestinal diseases and fever. It also showed antibacterial activity [3], and has been used for the treatment of malaria, liver diseases, and otitis [4].

The protective effect against diseases arises from the antioxidant properties that the body obtains from eating fruits and vegetables [5]. Several studies have revealed that the intake of phenolic and flavone compounds decreased the risk of coronary heart disease, and they can work as anti-cancers [6]. It was reported that the addition of polyphenols to the diets reduced cancer, neurological diseases, diabetes,

✉ Hongxin Wang
  hxwang@jiangnan.edu.cn

[1] State Key Laboratory of Food Science and Technology, School of Food Science and Technology, Jiangnan University, Wuxi 214122, China

[2] Department of Agricultural Engineering, Faculty of Agriculture, Sana'a University, Sana'a, Yemen

cardiovascular diseases, and osteoporosis [7, 8], in addition to their potential resistance to the diseases related to oxidative stress [9]. Antioxidants are the compounds that neutralize free radicals generated naturally in the body through metabolism or by external factors, such as radiation and air pollution that damage cells, caused inflammation, and might cause different types of cancer [10]. The use of industrial antioxidants in food applications is not safe because they may be toxic and cause cancer. Therefore, it is necessary to find sources of safe and economical natural antioxidants. These sources are not limited to the common known vegetables and fruits, but also medicinal plants and herbs are considered important sources of many natural antioxidants [11].

The utilization of ultrasonic technique has an important role in increasing the efficiency of biologically active compounds extraction from plant sources. The enhancement of these compounds extraction by ultrasonic technique can be attributed to the concentration of mass transport due to the breakdown of the internal bubbles resulting by ultrasound, which facilitated the flow of solvent in the tissues and increased the extraction process [12]. *C. rotundifolia* has been extensively used in traditional nutrition and therapeutic aspects; however, and to the best of our knowledge, no previous studies reported the extraction of phenolic compounds and the antioxidant activities under optimal conditions. Therefore, the present study aimed to evaluate the effectiveness of ultrasonic technique application for total phenolic compounds (TPC) extraction and antioxidant activities of *C. rotundifolia* leaves extract under different extraction conditions including solvent type, solvent concentration, sonication time, power, temperature, and sample to solvent ratio.

Materials and methods

Material

Cissus rotundifolia leaves were collected during the spring season (March–April, 2017) from Al-Sobod mountainous region, elevation above sea level by 1100 m. Haifan directorate, southern of Taiz city, Yemen, at geographic coordinates: Latitude (13°16′06″N) and Longitude (44°18′16″E). 1,1-diphenyl-2-picrylhydrazyl (DPPH), 2,2-azino-bis-(3-ethylbenzothioline-6-sulphonic acid)-di-ammonium salt (ABTS), Folin–Ciocalteu reagent, 3,4,5-trihydroxybenzoic acid (gallic acid) and ferrozine were purchased from Sigma-Aldrich chemicals company (Germany). All other solvents, chemicals, and reagents used in the experiments were of high purity and analytical grade.

Plant preparation for extraction

Cissus rotundifolia leaves were cut into small pieces (4–6 mm), and dried for 15 days at room temperature (23–25 °C) in a shaded place by air stream. The leaves were packaged in plastic bags emptied from air, and then sealed and transported to the laboratory, where the drying process was completed at 50 °C. The dry material was milled and sifted through a sieve of 40 mesh, and kept in plastic bags until used for the preparation of the extracts.

TPC extraction

In order to obtain the optimal extraction conditions of *C. rotundifolia* leaves, the influence of extraction conditions was firstly studied in a single-factor test, and multiple experiments (solvent type, solvent concentration, time, power, temperature, and sample to solvent ratio) were applied as shown in Table 1. The extracts were prepared according to the method described by Zhang et al. [13] with some modifications. One gram of the dried material was mixed with the solvent, and the mixture was placed in a sealed glass vial and subjected to ultrasound-assisted extraction using an ultrasonic cleaning bath (YIJING YQ-920 D, Shanghai Yi Jing ultrasonic instrument Co., Ltd. Kunshan, China) operated at a frequency of 40 kHz under the extraction conditions used. After ultrasonic treatment, the volume was made up to 100 mL with the same solvent, and centrifuged at 3500 rpm for 20 min. Then, the supernatant was filtered through Whatman filter paper, and the resulting extracts were used to determine TPC and antioxidant activities. All

Table 1 Different parameters applied to obtain the optimal extraction conditions of *Cissus rotundifolia* leaves by using ultrasonic

Parameters	Units	Symbol	Levels				
			1	2	3	4	5
Solvent type	mL	L	AC	MT	ET	WT	MIX
Solvent concentration	%	SC	20	40	60	80	100
Sonication time	min	t	10	20	30	40	50
Power	W	P	100	150	200	250	300
Temperature	°C	T	20	30	40	50	60
Sample to solvent ratio	mL/g	S/L	1:20	1:30	1:40	1:50	1:60

AC acetone, *ME* methanol, *ET* ethanol, *WT* water, *MIX* mixture

experiments were carried out in triplicate, and the average values were reported.

Determination of TPC

TPC were determined by using Folin–Ciocalteu method [14] with a slight modification. 200–600 μL of the extracts were added to 5 mL of 1:10 diluted Folin–Ciocalteu reagent. After 5 min, 4 mL of saturated sodium carbonate solution (70 g/L) was added, and then incubated for 105 min in the dark at room temperature. The absorbance of the mixture was read at 765 nm using the respective solvent as a blank sample. Gallic acid was used as a standard for a calibration curve, and the results were expressed as mg of gallic acid equivalents per gram of the dry weight (mg GAE/g DW).

Antioxidant activities

DPPH radical-scavenging activity (DPPH-RSA)

DPPH• scavenging activity assay was determined according to the method reported by Rashed et al. [12] with some modifications. Briefly, 70 μL from each extract were mixed with 3.5 mL of DPPH (60 mM). The mixture was left in the dark at room temperature for 30 min, and the absorbance values were measured at 517 nm. The DPPH-RSA was calculated as a percentage by using the following equation:

$$\%DPPH\text{-}RSA = \left[(A_C - A_S)/A_C \right] \times 100$$

where A_C is the absorbance of the control and A_S is the absorbance of the extract.

The IC_{50} values were determined according to the method described by Rashed et al. [12] with some modifications. Different dilutions (crude extract: solvent; 20:80, 40:60, 60:40, 80:20 and 100:0, v:v) were prepared and mixed with DPPH-RSA. Ascorbic acid was used as a reference sample based on total phenolic content in the sample extract at the same dilutions.

ABTS·+ scavenging ability

The ABTS·+ scavenging ability was estimated according to the method described by Zhang et al. [15] with slight modifications. The ABTS·+ work solution was prepared by mixing 7 mM of ABTS stock solution (dissolved in distilled water) with 2.45 mM potassium persulfate by reaction (1:1 v/v). The mixture was left in the dark for 16 h at room temperature, and diluted with ethanol (98%) to an absorbance of 0.7 ± 0.2 at 734 nm before use. Then, 70 μL of the diluted extracts were mixed with 3.5 mL of ABTS·+ working solution and kept at room temperature for 10 min. The absorbance was measured at 734 nm after incubation time, and the ABTS·+ scavenging ability of extracts was calculated as

mg Trolox equivalent per gram of the dry weight of plant sample ($R^2 = 0.9987$). To measure the IC_{50} values, different dilutions of extracts (20:80, 40:60, 60:40, 80:20 and 100:0, v:v) were mixed with ABTS·+ as previously described after the volume was made up to 100 μL using the same solvent. Based on the phenolic compounds content in the extract, ascorbic acid was used as a reference sample at the same dilutions of sample extract.

Reducing power assay

The reducing power was determined according to Lou et al. [16] with slight modifications. Different dilutions of sample extracts (crude extract:solvent; 20:80, 40:60, 60:40, 80:20 and 100:0, v:v) were mixed with 2.0 mL of phosphate buffer 0.2 M (pH 6.6) and 2.0 mL of potassium ferricyanide $K_3[Fe(CN)_6]$ 1% was added, then incubated at 50 °C for 20 min. After incubation time, 2.0 mL of 10% trichloroacetic acid was added, and the reaction mixture was then centrifuged at 700 rpm for 10 min. 2.0 mL from this mixture was mixed with 2.0 mL deionized water and 0.4 mL $FeCl_3$ (0.1%). The absorbance of the final mixture was measured at 700 nm. Increased absorbance of the reaction mixture indicated increased reducing power. To compare, ascorbic acid was used as a reference sample according to the content of the TPC in the extract at the same previous dilutions.

Ultra-performance liquid chromatography analysis of phenolic compounds

Cissus rotundifolia leaves extract obtained under the optimal conditions was subjected to UPLC system with BEH C18 column (2.1–100 mm, 1.7 μm) (Waters, Acquity, PDA). Acetonitrile was used as the mobile-phase A, while formic acid solution (0.1%, v/v) was used as the mobile-phase B in a gradient mode as follow: 0–0.1 min, 5% acetonitrile; 0.1–25 min, 5–25% acetonitrile; 25–27 min, 25–100% acetonitrile; 27–27.1 min, 100–5% acetonitrile; 27.1–30 min, 5% acetonitrile. The flow rate was 0.3 mL/min, and the volume of the injected sample was 1 μL. The column temperature was set at 45 °C, and wavelength of the detector was fixed at 200–600 nm. The MS analysis was performed in the negative ionization mode with the mass range set at m/z 20–1500 Da in full scan resolution mode. The electrospray ionization conditions were as follow: source and desolation gas temperature of 100 °C and 400 °C, respectively. Nitrogen and argon as cone and collision gases were used. The flow rate of cone gas was set at 50 L/h and flow rate of desolation gas at 700 L/h. The voltage of capillary, 3 kV; the voltage of cone 20 V; and the collision energy (eV) 6/20 V. The processes and analysis of data were controlled by using MassLynx (V 4.1) software.

Scanning electron microscopy (SEM)

The morphological structure of *C. rotundifolia* powder before and after the treatment by ultrasound was observed using a Hitachi High-Technologies Corp., SEM SU 1510 (Tokyo, Japan). The accelerating beam voltage was 5.00 kV, and the magnification coefficient 3 k with working distances of 8.1 and 8.2 mm was used.

Statistical analysis

All experiments were carried out in triplicate. The values were expressed as mean $\pm$ SD, and significant differences at ($P < 0.05$) between mean values were evaluated by Fisher's least significant difference test using SPSS version 20.0.0 (SPSS IBM, Chicago, IL, USA). The Pearson correlation test was used to compare the different results of assays obtained in the different extracts. Microsoft Excel 2010 was used to calculate IC_{50} values.

Results and discussion

Optimization of extraction conditions

Effect of solvent type

The impact of solvent type on the phenolic compounds and antioxidant activities of *C. rotundifolia* leaves was investigated, and the results are displayed in Fig. 1. Several types of solvents (acetone, methanol, ethanol, water and mixture from equal proportions of these solvents) with a concentration of 60% were used for the extraction of the phenolic compounds and estimating the antioxidant activities of *C. rotundifolia* extract. All extracts contained phenolic compounds at different rates depending on the type of the solvent used (Fig. 1a). Among the different solvents, acetone exhibited the highest phenolic content (11.14 mg GAE/g DW), which was significantly higher ($P < 0.05$) than the values obtained by using other solvents. By using a mixture of solvents, TPC content was 9.02 mg GAE/g DW. There was no significant variations in case of using ethanol and methanol (8.03 and 7.61 mg GAE/g DW), respectively. Finally, water was the lowest effective solvent to extract the phenolic compounds (4.43 mg GAE/g DW). This may be attributed to that the using of water leads to extract a high content of impurities, such as soluble proteins in water, organic acids, and sugars, which can affect the identification of phenols and the other extracted compounds [17]. The solubility of phenols greatly depends on the polarity of the solvent, thus this finding can explained that the solvent used (acetone) and the phenolic compounds in the sample both have low polarity, which facilitated the solubility and extraction of
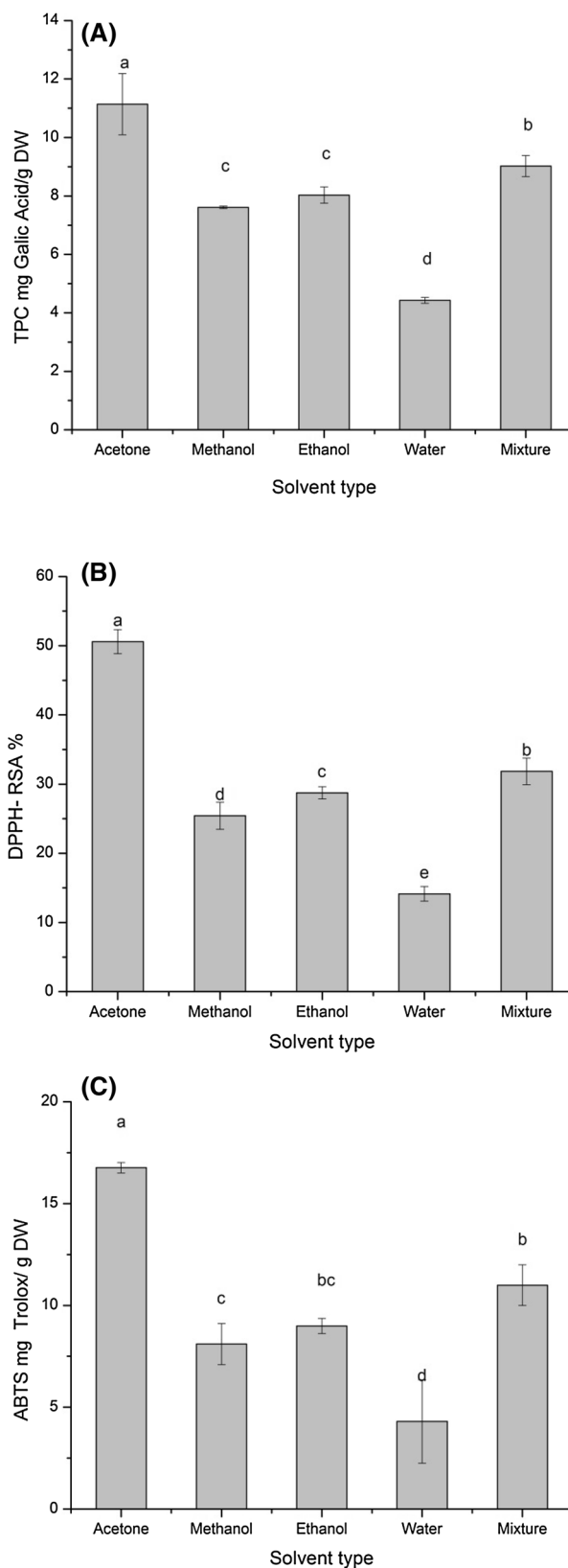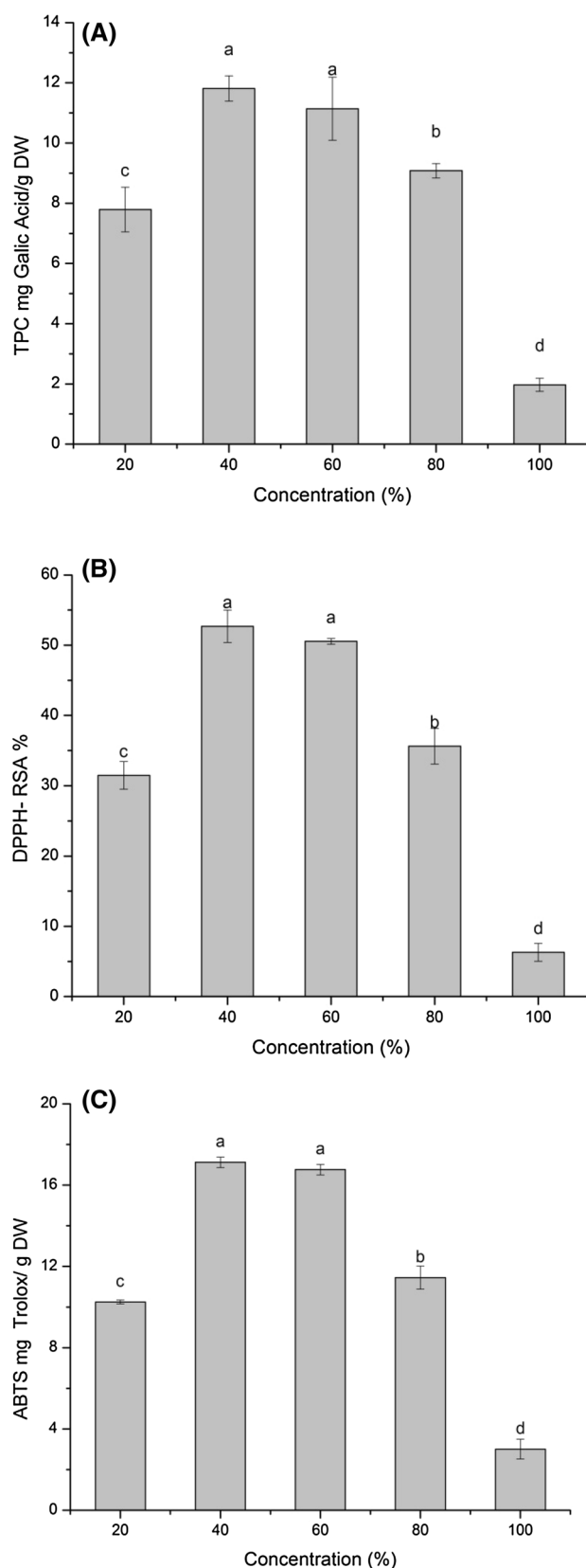

Fig. 1 Effect of solvent type on the extraction of TPC (**a**), DPPH-RSA (**b**), and ABTS (**c**) from *C. rotundifolia* extracts. Data expressed as mean $\pm$ S.D. of triplicate determinations. Different letters indicate significant difference at $P < 0.05$

phenols. Mokrani and Madani [7] reported that solvent type and sample solubility have an obvious effect on the phenolic compounds, including the antioxidant activities. Therefore, the extraction of all phenolic compounds classes from the sample required different solvents. Generally, acetone is characterized by its low polarity and high molecular weight. Thus, it is more efficient for the extraction of phenolic compounds with high molecular weights. Moreover, it has been proven as the most effective solvent for the extraction of antioxidant phenols from various plant types, such as berries, buckwheat, apples, strawberries, barley seeds, onions, pistachio, fenugreek, soybeans, figs, and eggplant products.

Figure 1b, c showed that acetone was the optimal solvent when estimating DPPH and ABTS in *C. rotundifolia* extracts as compared to the other solvents. DPPH values obtained by using acetone, methanol, ethanol, water, and the mixture of solvents were 50.58, 25.42, 28.73, 14.14, and 31.84%, respectively. The statistical analysis showed a significant difference ($P < 0.05$) between DPPH values of the different extracts. ABTS values obtained by using acetone, methanol, ethanol, water, and the mixture were 16.76, 8.10, 8.99, 4.30, and 10.99 mg Trolox/g DW, respectively. It was observed that there was a significant difference ($P < 0.05$) between the values of acetone extract and the other solvents extracts. While, there were no significant differences between ABTS values of methanol and ethanol extracts, as well as between the values of ethanol and solvent's mixture extracts. Generally, solvent efficiency in the extraction of phenolic compounds differs from a plant material to another; therefore, the optimal solvents for particular phenolic classes' extraction should be selected and improved separately in order to attain extracts with the highest antioxidant activities.

Effect of solvent concentration

Different concentrations of acetone (20, 40, 60, 80, and 100%) have been tested. The statistical analysis showed significant differences ($P < 0.05$) between the total phenols content obtained by using different concentrations of acetone as shown in Fig. 2a. The optimal concentration for total phenols extraction was 40% (11.81 mg GAE/g DW), followed by the concentration of 60% (11.14 mg GAE/g DW) with non-significant difference ($P < 0.05$). The using of pure acetone recorded the lowest value (1.97 mg GAE/g DW), while the acetone concentrations of 80 and 20% recorded intermediate values (9.08 and 7.79 mg GAE/g DW), respectively. Increasing water ratio in solvents mixture increased the extraction efficiency. This may be due to that water is a polar solvent and phenols compounds are polar compounds attracted to each other. Similar results were reported by Tan et al. [18], who revealed that the optimum concentration for phenols extraction from *Lawsonia inermis* stems was 40% of acetone. The content of TPC obtained with the optimal
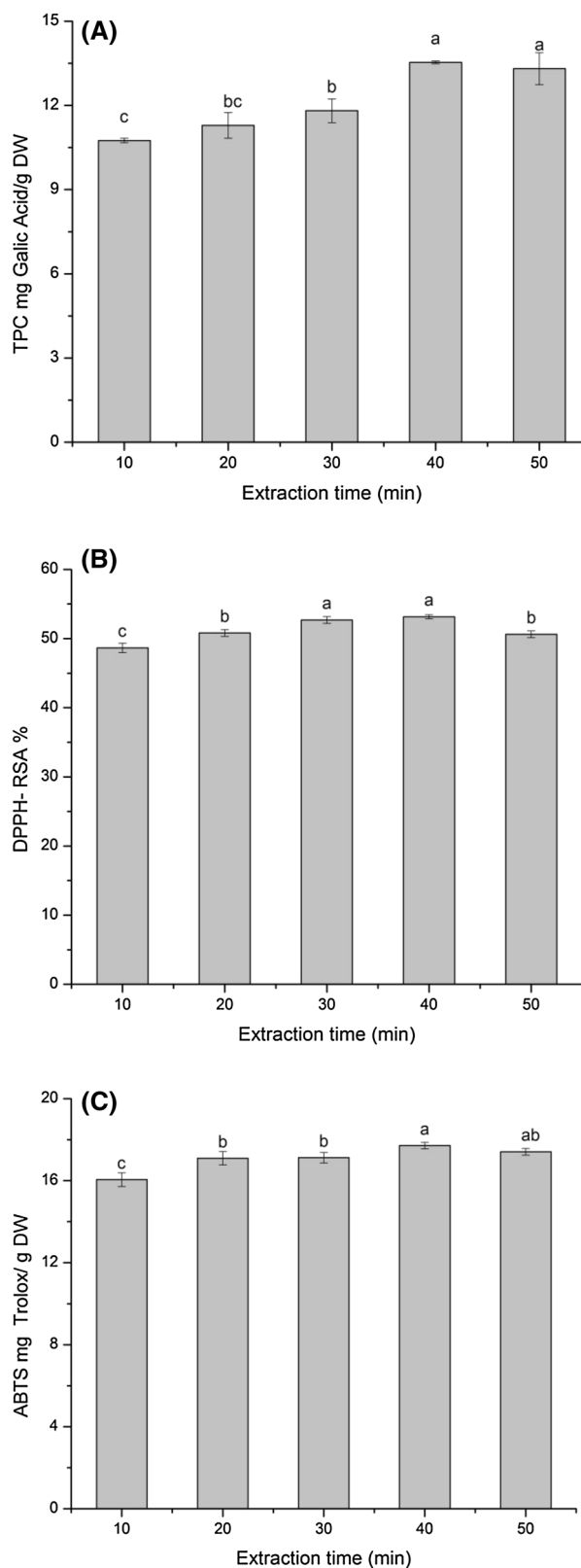

Fig. 2 Effect of solvent concentration on the extraction of TPC (**a**), DPPH-RSA (**b**), and ABTS (**c**) from *C. rotundifolia* extracts. Data expressed as mean ± S.D. of triplicate determinations. Different letters indicate significant difference at $P < 0.05$

concentration in this study was higher than that reported by Dahmoune et al. [19], who found that the content of total phenols in apples by using acetone (50%) and microwave was 9.18 mg GAE/g DW. While, higher results were reported by Vu et al. [20], who used an acetone concentration of 60% in an ultrasonic bath with banana peels. These differences may be attributed to plant species, treatments and other conditions used, as well as the status of the sample used fresh or dried. As can be seen in Fig. 2b, c, the acetone concentration (40%) showed a significant increase in DPPH and ABTS values (52.70% and 17.12 mg Trolox/g DW), respectively, compared to the other concentrations, except the concentration 60% since the difference was not significant (50.55% and 16.76 mg Trolox/g DW), respectively. The lowest values of DPPH and ABTS were detected with the pure acetone extracts (6.30% and 3.01 mg Trolox/g DW), respectively. Based on these results, the concentration of acetone 40% was chosen as the optimal concentration for extraction in the following experiments since the utilization of more than 40% does not achieve any economic feasibility.

Effect of ultrasonic time

The effects of extraction time on TPC and antioxidant activities (DPPH and ABTS) were assessed within the time ranges of 10–50 min. As shown in Fig. 3a, the total phenols significantly increased with the progress in the extraction time from 10 to 40 min (10.75–13.53 mg GAE/g DW), respectively. However, by prolonging the time to 50 min, a slight decrease in the TPC was observed. This may be due to that the majority of plant cells in the sample used have been destroyed during the previous time, and the increase in treatment time did not lead to any significant impact. Numerous studies on different species of plant materials have reported similar results regarding the effect of time on the extraction of TPC and antioxidant activities. It was reported that the extraction time had a significant impact on the TPC of *Prunus persica* and *Carica papaya* leaves [7, 21]. Concerning the antioxidant activities, it was shown that DPPH value obtained at 40 min was significantly higher (53.15%) than the values obtained after 10, 20, and 50 min (Fig. 3b). While, there was non-significant difference between the values of 30 and 40 min. As well, the ABTS value at 40 min (17.71 mg Trolox/g DW) was significantly higher ($P < 0.05$) than the values of other time ranges except the extract obtained after 50 min (Fig. 3c). The increase in extraction time did not significantly increase the TPC and their antioxidant activities. This may be attributed to the release of other compounds that are not required, which could accelerate the degradation of phenolic compounds [19]. According to the obtained results of total phenols and ABTS, 40 min was selected as the optimal extraction time for the following investigations.
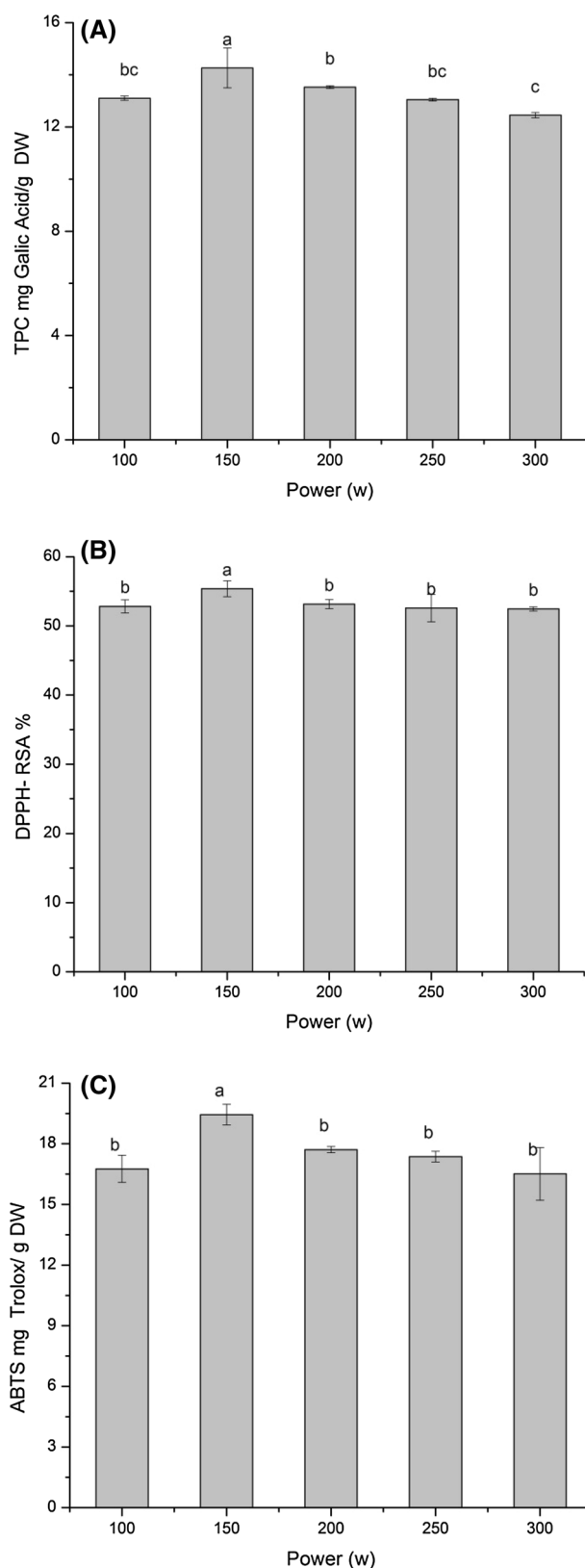

Fig. 3 Effect of extraction time on the extraction of TPC (**a**), DPPH-RSA (**b**), and ABTS (**c**) from *C. rotundifolia* extracts. Data expressed as mean ± S.D. of triplicate determinations. Different letters indicate significant difference at $P < 0.05$

Effect of power

In order to obtain the optimal extraction power, five levels of power (100, 150, 200, 250 and 300 W) were tested. As shown in Fig. 4a, the power level of 150 W achieved the optimal result for the extraction of TPC (14.26 mg GAE/g DW), followed by the levels of 200 W (13.47 mg GAE/g DW), 100 W (13.10 mg GAE/g DW) and 250 W (13.04 mg GAE/g DW). The extract of 300 W showed the lowest value (12.45 mg GAE/g DW) with non-significant difference with the power level of 250 W. This result indicates that most of the cells have been destroyed and were emptied of their phenol contents using the power of 150 W. Therefore, the increase in power used did not achieve any positive effect. Vu et al. [20], found that the optimal power for TPC extraction from banana peel was 150 W by using an ultrasonic bath. The values of DPPH and ABTS obtained at the power level of 150 W were 55.37% and 19.44 mg Trolox/g DW, respectively, which were significantly higher than those of the other power levels (Fig. 4b, c). In light of the obtained results, the power of 150 W was selected as the optimal extraction power level.

Effect of temperature

The extraction temperature had a great impact on the TPC of *C. rotundifolia* leaves extract, since temperature coupled with ultrasound power leading to the disruption of cell walls, which by the way facilitates solvent penetration and phenolic compounds extraction as shown in Fig. 8a, b. The temperature was set at 20, 30, 40, 50 and 60 °C to evaluate the influence of extraction temperature on the total phenols and antioxidant activities. Figure 5a showed that the increase in extraction temperature from 20 to 40 °C significantly increased the total phenols from 12.25 to 14.26 mg GAE/g DW. However, by further increasing the extraction temperatures to 50 and 60 °C, a slight descend in TPC was observed. Accordingly, 40 °C was selected as the optimal extraction temperature to save time and power used. The results obtained in this study were higher than those reported by Altemimi et al. [22], who found that TPC in pumpkin and peach ranged between 38–44 and 50–55 mg GAE/100 g DW, respectively under the optimal temperature of 41.45 and 41.53 °C, respectively by using the ultrasonic technique. Li et al. [11] reported that by using a water bath and deionized water as a solvent at 100 °C for 30 min, the contents of TPC in *Morus alba* L., *Perilla frutescens* L., and *Ginkgo biloba* L. leaves were 2.29, 5.24 and 2.50 mg GAE/g DW, respectively. As shown in Fig. 5b, c, the optimal results for DPPH and ABTS were attained at 40 °C (55.36% and 19.44 mg Trolox/g DW) respectively, which were significantly higher than the other temperature levels.

Fig. 4 Effect of power levels on the extraction of TPC (**a**), DPPH-RSA (**b**), and ABTS (**c**) from *C. rotundifolia* extracts. Data expressed as mean ± S.D. of triplicate determinations. Different letters indicate significant difference at $P < 0.05$
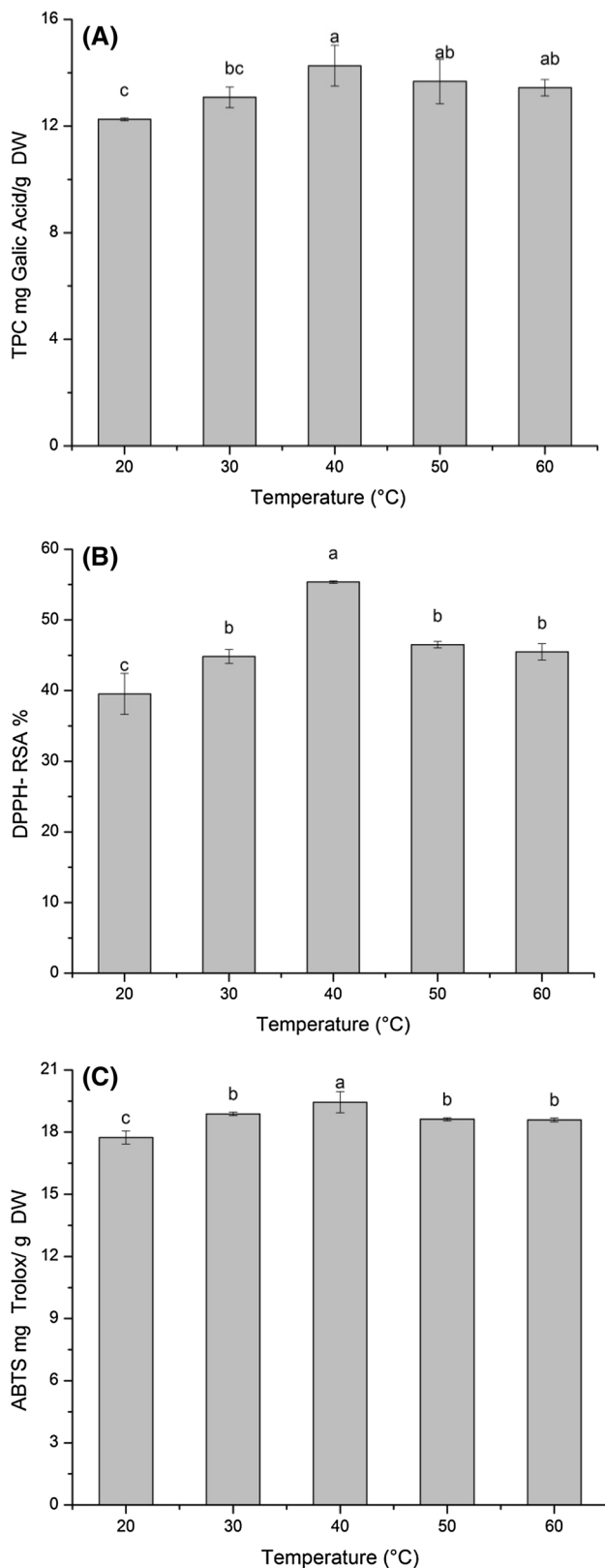
Fig. 5 Effect of temperature on the extraction of TPC (**a**), DPPH-RSA (**b**), and ABTS (**c**) from *C. rotundifolia* extracts. Data expressed as mean ± S.D. of triplicate determinations. Different letters indicate significant difference at $P < 0.05$

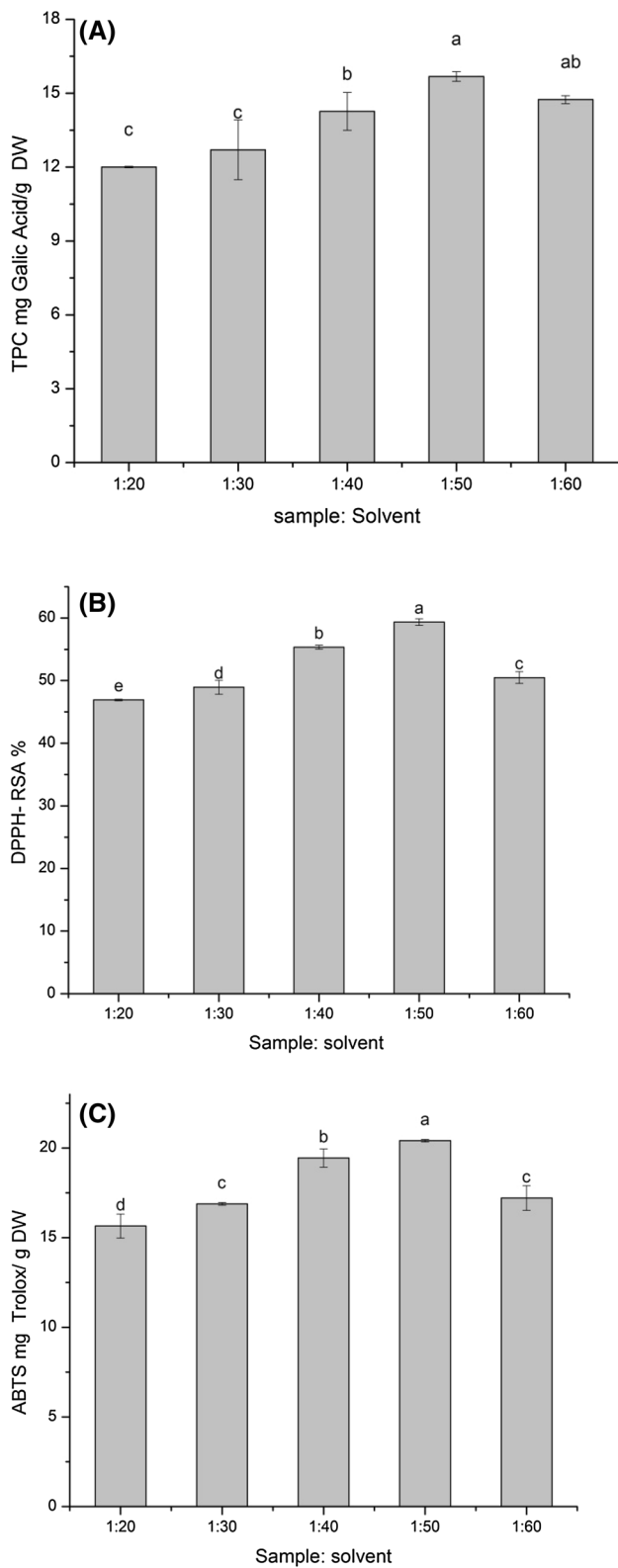### Effect of sample to solvent ratio (w/v)

The influence of sample to solvent ratio on the TPC of *C. rotundifolia* was evaluated within the range from 1:20 to 1:60 (w/v), and the results are displayed in Fig. 6a. The optimal mixing ratio was found to be 1:50 (w/v), in which the obtained TPC was 15.68 mg GAE/g DW. TPC at this ratio was significantly higher than the mixing ratios from 1:20 to 1:40 (w/v). This may be attributed to that increasing the solvent ratio to a specific extent increased solvent penetration, which allowed further extraction of phenolic compounds from cells. However, with a further increase in the mixing ratio to 1:60, the TPC decreased to 14.75 mg GAE/g DW. The obtained results are in agreement with those of Fang et al. [23], who investigated the effects of sample to solvent ratio on TPC and antioxidant activity from *Eclipta prostrate* L. by using an ultrasonic probe system, and found that the increase in mixing ratio between the sample and solvent did not show positive results. The highest DPPH and ABTS values (59.35% and 20.34 mg Trolox/g DW), respectively were detected by using the optimal sample to solvent ration (Fig. 6b, c). ABTS values of the mixing ratios 1:30 and 1:60 w/v (16.89 and 17.20 mg Trolox/g DW) respectively, were not significantly different. Subsequently, the ratio of 1:50 (sample to solvent, w/v) was considered as the optimal ratio.

IC$_{50}$ can be defined as the concentration of phenols required to inhibit 50% of DPPH or ABTS. The antioxidant activities of *C. rotundifolia* extracts obtained under the optimal conditions were compared with ascorbic acid activity as a reference sample as shown in Fig. 7a, b. The activity of ascorbic acid was higher than the sample extract in the IC$_{50}$ DPPH test, where the used volumes from ascorbic acid and sample extract to reach the IC$_{50}$ were 50.98 and 61.75 μL, respectively. On the other hand, the sample extract was more active than the ascorbic acid in the IC$_{50}$ ABTS test, where the IC$_{50}$ was achieved at 46.15 and 50.78 μL, respectively. This activity might be attributed to the concentration of phenolic compounds in the crude sample extract.
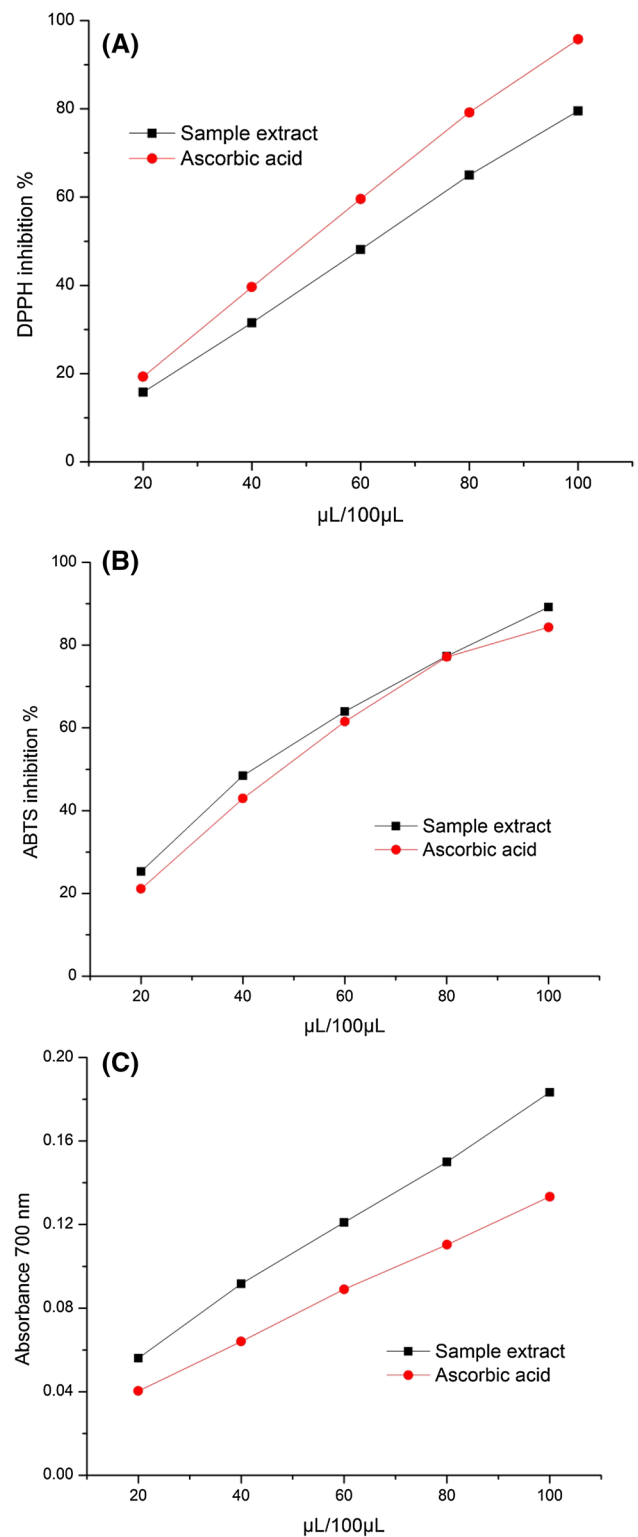
Reducing power

The power-reducing assay depends on the reduction of ferricyanide (Fe^{3+}) complex to the ferrous (Fe^{2+}) form in the presence of an antioxidant in the samples; therefore, the increase in absorbance at 700 nm indicates higher reducing power [19]. The results showed that the reducing power increased when the concentration of the extract was increased (Fig. 7c). The sample activity depends on its antioxidants content, where the free radicals are neutralized by donating an electron from agent donate an electron to a free radical, and then the reduced species acquire a proton from the solution. Therefore, the reducing power indicates the potential antioxidant activity of phenolic compounds. The

Fig. 6 Effect of sample/solvent ratio on the extraction of TPC (**a**), DPPH-RSA (**b**), and ABTS (**c**) from *C. rotundifolia* extracts. Data expressed as mean $\pm$ S.D. of triplicate determinations. Different letters indicate significant difference at $P < 0.05$

Fig. 7 Comparison of IC_{50} values between the final *C. rotundifolia* extract and ascorbic acid as a reference; DPPH-RSA (**a**) and ABTS (**b**). Reducing power assay (**c**). Data expressed as mean $\pm$ S.D. of triplicate determinations

obtained results are in agreement with those reported by Boulekbache-Makhlouf et al. [24] and Jayanthi and Lalitha [25], who found that the reducing power increased with the increase in extract concentration. On the other hand, the results of Fig. 7c showed that the phenolic extract at optimal conditions was more active than the reference sample (ascorbic acid) when different dilutions were used, which might arise from the several active phenolic compounds in the sample (Table 3).

Correlation between TPC and antioxidants activities

To more assess the relationships between TPC and antioxidant activities of *C. rotundifolia* leaves extract, the correlations between the obtained values were analyzed under the different experimental conditions as shown in Table 2. Under solvent type and solvent concentration, the correlation of TPC, DPPH, and ABTS was significant ($P < 0.01$). This indicated that solvent type and concentration correlated with TPC and antioxidant activities of the extracts in consistent with the results reported by Hou et al. [26]. The correlation between TPC, DPPH, and ABTS was not significant under the effect of extraction time. On the other hand, the correlation between TPC, DPPH, and ABTS was significant at ($P < 0.05$) under the influence of power and temperature. From these results, it can be concluded that power and temperature have a sensitive effect on plant sample extracts. The correlations between TPC and DPPH, TPC and ABTS under the impact of sample to solvent ratio were not significant except for DPPH and ABTS.

Scanning electron microscopy

SEM of *C. rotundifolia* samples showed a change in the treated cells by ultrasound compared with the untreated samples (Fig. 8a, b). The images showed that the ultrasound affected the walls of plant cells, and thus facilitated the penetration of the solvent to these cells and then outlet the contents from the cells including phenols. Dahmoune et al. [19], reported that ultrasound influenced the plant cells

via emptying the cell contents by cavitation phenomena, but this effect varied according to the resistance of particles to the applied energy. Decreasing the particle size of the plant under ultrasound conditions might cause an increased collapse of the cellular material, which facilitated greater permeation of the solvent into the plant matrix, and thereby
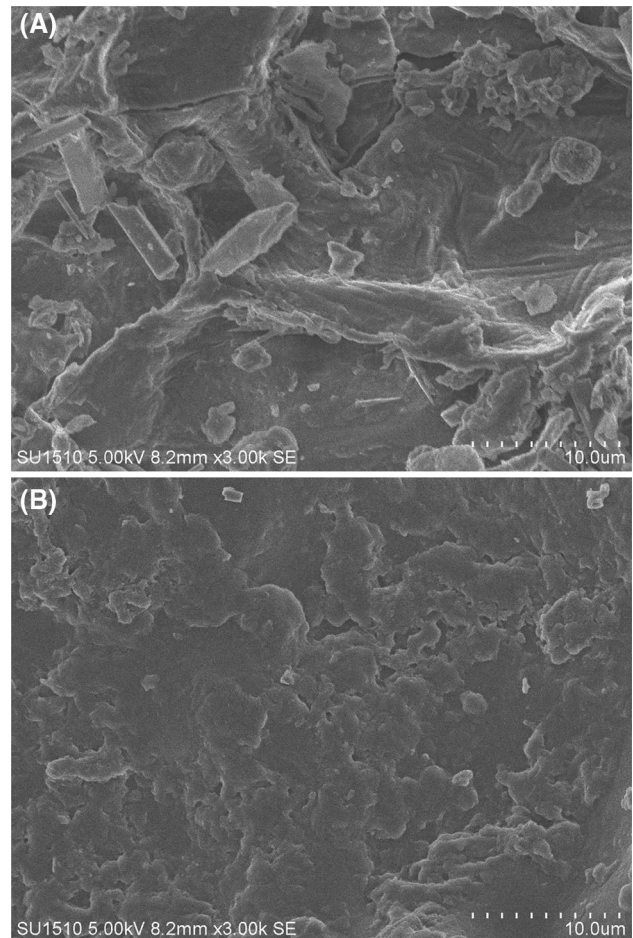
Fig. 8 Scanning electron microscope images of *C. rotundifolia* leaves powder before (**a**) and after extraction process by using ultrasound-assisted extraction (**b**)

Table 2 Pearson correlations coefficient between various assays under the effect of different extraction conditions

	Solvent type		Solvent concentration		Extraction time		Power		Temperature		Sample to solvent ratio	
	TPC	DPPH	TPC	DPPH	TPC	DPPH	TPC	DPPH	TPC	DPPH	TPC	DPPH
DPPH	0.967**		0.993**		0.606[NS]		0.917*		0.944*		0.865[NS]	
ABTS	0.972**	0.997**	0.986**	0.999**	0.866[NS]	0.822[NS]	0.949*	0.951*	0.899*	0.927*	0.844[NS]	0.990*

TPC total phenolic compounds, *DPPH* 1,1-diphenyl-2-picrylhydrazyl, *ABTS* 2,2-azino-bis-(3-ethylbenzothioline-6-sulphonic acid)-di-ammonium salt, *NS* non-significant

*Correlation is significant at $P < 0.05$ level

**Correlation is significant at $P < 0.01$ level

Table 3 Phenolic compounds in *C. rotundifolia* leaves extract as analyzed by LC–MS

RT (min)	Content %	[M–H]⁻ (m/z)	Molecular formula	Phytochemical compound
10.239	3.27	457.1135	$C_{23}H_{22}O_{10}$	Daidzein 7-(6-*O*-acetyl-β-D-glucoside)
11.190	8.36	353.0873	$C_{16}H_{18}O_9$	Chlorogenic acid
				1-*O*-caffeoylquinic acid
12.090	23.27	609.1397	$C_{27}H_{30}O_{16}$	Isoorientin 2″-*O*-glucoside
				Kaempferol 3,7-di-*O*-β-D-glucoside
				Quercetin 3-*O*-rhamnoside-7-*O*-glucoside
		609.1244	$C_{30}H_{26}O_{14}$	Gallocatechin-(4α→8)-epigallocatechin
		609.1761	$C_{35}H_{30}O_{10}$	Guangsangon (M, N)
		489.1033	$C_{23}H_{22}O_{12}$	1,3,6-Trihydroxy-2-hydroxymethyl-9,10-anthraquinone-3-*O*-(6′-*O*-acetyl)-β-D-glucopyranoside
		429.0822	$C_{21}H_{18}O_{10}$	(2S,3S,4S,5R,6S)-3,4,5-trihydroxy-6-[4-(7-hydroxy-4-oxo-1-benzopyran-3-yl)phenoxy]-2-oxanecarboxylic acid
12.851	12.36	285.0610	$C_{12}H_{14}O_8$	2,3-Dihydroxybenzoic acid 3-*O*-β-D-xyloside
			$C_{16}H_{14}O_5$	Brazilin
				2′,4′,6′-Trihydroxy-3′-formyldihydrochalcone
				Lichexanthone
				3-Deoxysappanone B
				2,3-Dihydrobiochanin A
				Vestitone
				Bauhinoxepin I
				Sappanchalcone
			$C_{15}H_{10}O_6$	Maritimetin
				Aureusidin
				Asphodelin A
13.439	5.09	327.0716	$C_{14}H_{16}O_9$	Bergenin
		327.0869	$C_{18}H_{16}O_6$	Hyperxanthone E
				3′-Hydroxy-4,6,4′-trimethoxyaurone
14.010	28.00	593.1295	$C_{30}H_{26}O_{13}$	(+)-Gallocatechin-(4α→8)-(+)-catechin
				(+)-Gallocatechin-(4α→8)-(−)-epicatechin
				(+)-Catechin-(4α→8)-(−)-epigallocatechin
				(−)-Epigallocatechin-(4β→8)-(−)-epicatechin
		593.1870	$C_{28}H_{34}O_{14}$	β-(4-Hydroxyphenyl)ethyl-4-*O*-E-caffeoyl-*O*-[β-D-apiofuranosyl-(1→2)]-β-D-glucopyranoside
		593.0931	$C_{29}H_{22}O_{14}$	7,4′-Di-*O*-galloyltricetiflavan
		413.0873	$C_{21}H_{18}O_9$	Premithramycinone
		563.1612	$C_{26}H_{28}O_{14}$	6-C-(α-L-arabinosyl)-8-C-(β-L-arabinosyl)chrysoeriol
		563.1612	$C_{26}H_{28}O_{14}$	Glucofrangulin B
		293.0814	$C_{18}H_{14}O_4$	Dynasore
14.840	6.18	577.1557	$C_{27}H_{30}O_{14}$	Kaempferol 3,7-di-*O*-α-L-rhamnoside
				1,3,6-Trihydroxy-2-methyl-9,10-anthraquinone-3-*O*-α-L-rhamnopyranosyl-(1→2)-β-D-glucopyranoside
		577.1346	$C_{30}H_{26}O_{12}$	Procyanidin (B1 to B6 and B8)
				(−)-Epicatechin-(4α→8)-(−)-epicatechin
		577.1193	$C_{26}H_{26}O_{15}$	Tricin 7-*O*-(6″-*O*-malonyl)-β-D-glucopyranoside
15.341	5.09	411.1444	$C_{23}H_{24}O_7$	11β,13-Dihydrolactucopicrin

Table 3 (continued)

RT (min)	Content %	[M–H]$^-$ (*m/z*)	Molecular formula	Phytochemical compound
17.780	5.82	301.0348	$C_{15}H_{10}O_7$	Bracteatin
		301.0712	$C_{16}H_{14}O_6$	Haematoxylin
				Decussatin
				Marsupsin
				Ferreirin
				Homoeriodictyol chalcone
				2,5,7-Trihydroxy-4′-methoxyisoflavanone
29.041	2.55	153.0188	$C_7H_6O_4$	3,4-Dihydroxybenzoic acid

increasing the diffusion rates of polyphenols into the solvent [27]. Therefore, the positive effects of using ultrasound might arise from cell damage, and thus increasing the rate of mass transfer for the bioactive compounds including phenols.

LC–MS analysis of phenolic compounds

Phenolic compounds possess a common chemical structure, containing an aromatic ring with one or more hydroxyl substituents. The phenol groups include phenolic acids, flavonoids, stilbenes, tannins, and lignans [9]. Table 3 indicates the different TPC detected in *C. rotundifolia* leaves after LC–MS analysis based on the retention time and mass to charge values. The most abundant phenolic compounds were detected at 14.01 min with a relative abundance of 28%, including (+)-gallocatechin-(4α→8)-(+)-catechin, (+)-gallocatechin-(4α→8)-(−)-epicatechin, (+)-catechin-(4α→8)-(−)-epigallocatechin, (−)-epigallocatechin-(4β→8)-(−)-epicatechin, β-(4-hydroxyphenyl)ethyl-4-*O*-E-caffeoyl-*O*-[β-D-apiofuranosyl-(1→2)]-β-D-glucopyranoside, 7,4′-di-*O*-galloyltricetiflavan, premithramycinone, 6-C-(α-L-arabinosyl)-8-C-(β-L-arabinosyl) chrysoeriol, glucofrangulin B, and dynasore. Furthermore, isoorientin 2″-*O*-glucoside, kaempferol 3,7-di-*O*-β-D-glucoside, quercetin 3-*O*-rhamnoside-7-*O*-glucoside, gallocatechin-(4α→8)-epigallocatechin, guangsangon (M, N),1,3,6-trihydroxy-2-hydroxymethyl-9,10-anthraquinone-3-*O*-(6′-*O*-acetyl)-β-D-glucopyranoside, and (2S,3S,4S,5R,6S)-3,4,5-trihydroxy-6-[4-(7-hydroxy-4-oxo-1-benzopyran-3-yl) phenoxy]-2-oxanecarboxylic acid were detected at 12.09 min with a relative abundance of 23.27%. These different phenolic compounds can be used in various nutritional and therapeutic applications, such as (+)-catechin, (−)-epicatechin, and procyanidins compounds (B1, B2 and B4), which can be applied as potential compounds to complement conventional diabetes medication and prevention. As well, these compounds have been effectively reported as antiglycation agents [28]. Catechin, epicatechin, procyanidin (B1, B2, B3, and B4), and epigallocatechin have the ability to prevent lipid and protein oxidative damage, and the potentiality to decrease the incidence of degenerative illnesses associated with oxidative stresses [29].

Higdon and Frei [30], reported that chlorogenic acid has been used in the inhibition of chemically induced hepatic carcinogenesis in animal models. In addition, 1-*O*-caffeoylquinic acid was considered as an antineoplastic agent and prevented their proliferation [31]. Table 3 showed that *C. rotundifolia* leaves extract contained the brazilin compound, which had been used as a dye to textile dyeing. It also had many medical uses, such as blood clotting inhibiting, lowering blood glucose levels, inhibiting or preventing the proliferation of neoplasms, anti-inflammatory, anticonvulsant therapy, and decreasing gluconeogenesis in hepatocytes [32]. Bauhinoxepin I belongs to the bioactive compounds reported by Boonphong et al. [33], can be used in the treatment of malaria, antimycobacterial, antifungal and anti-inflammatory. As well, LC–MC analysis revealed the detection of sappanchalcone. This compound has been suggested to suppress oral cancer cells growth [34]. In another context, Jung et al. [35], found that sappanchalcone had attenuating effects from rheumatoid arthritis collagen-induced. Hyperxanthone E compound presented moderate cytotoxicity for the human tumor cells [36]. *C. rotundifolia* leaves extract also contained compounds with various therapeutic applications, since Silva et al. [37], reported that kaempferol 3,7-dirhamnoside reduced glycemia in diabetic mice. This compound can be considered as an exemplary antiosteoporotic candidate [38]. Cassani et al. [39] evaluated the effect of kaempferitrin isolated from *Justicia spicigera* on the antidepressant-like, and found positive effects. Moreover, kaempferitrin showed immunostimulatory effects in the experiments on mice [40].

Conclusions

In the present study, *C. rotundifolia* leaves extract was successfully prepared by ultrasonic-assisted extraction; in addition, TPC and antioxidant activities were determined. The using of single factor experiments showed that the optimal

extraction conditions were as follow; acetone as a solvent, solvent concentration of 40%, sonication time of 40 min, power level of 150 W, temperature of 40 °C, and sample to solvent ratio of 1:50 w/v. The results showed that TPC and the antioxidant activities were affected by different extraction conditions. Under the optimal conditions, TPC, DPPH, and ABTS were 15.68 mg GAE/g DW, 59.35%, and 20.34 mg Trolox/g DW, respectively. Clear positive correlation coefficients were found between TPC, DPPH-RSA, and ABTS, in particular solvent type and concentration. In addition, LC–MS analysis showed that *C. rotundifolia* leaves extract contained numerous compounds with potential nutritional and therapeutic applications. The results of the present study could provide a valuable knowledge to the food and pharmaceutical industries, particularly in the field of safe and healthy natural antioxidants. However, the comprehensive identification of the phenolic compounds should be further studied to assess their medical and therapeutic uses. Moreover, the supplementation of novel food products with *C. rotundifolia* leaves extract and its nutritional characteristics need further investigations.

Compliance with ethical standards

Conflict of interest The authors declare no conflict of interest.

References

1. M. Korish, Nutritional evaluation of wild plant *Cissus rotundifolia*. Ital. J. Food Sci. **28**, 43–49 (2016)
2. A. Afolayan, F. Jimoh, Nutritional quality of some wild leafy vegetables in South Africa. Int. J. Food Sci. Nutr. **60**, 424–431 (2009)
3. M. Al-Fatimi, M. Wurster, G. Schröder, U. Lindequist, Antioxidant, antimicrobial and cytotoxic activities of selected medicinal plants from Yemen. J. Ethnopharmacol. **111**, 657–666 (2007)
4. R.A. Mothana, S. Kriegisch, M. Harms, K. Wende, U. Lindequist, Assessment of selected Yemeni medicinal plants for their in vitro antimicrobial, anticancer, and antioxidant activities. Pharm. Biol. **49**, 200–210 (2011)
5. H.R. de Moraes Barros, T.A.P. de Castro Ferreira, M.I. Genovese, Antioxidant capacity and mineral content of pulp and peel from commercial cultivars of citrus from Brazil. Food Chem. **134**, 1892–1898 (2012)
6. O. Benavente-Garcia, J. Castillo, Update on uses and properties of citrus flavonoids: new findings in anticancer, cardiovascular, and anti-inflammatory activity. J. Agric. Food Chem. **56**, 6185–6205 (2008)
7. A. Mokrani, K. Madani, Effect of solvent, time and temperature on the extraction of phenolic compounds and antioxidant capacity of peach (*Prunus persica* L.) fruit. Sep. Purif. Technol. **162**, 68–76 (2016)
8. A. Scalbert, C. Manach, C. Morand, C. Rémésy, L. Jiménez, Dietary polyphenols and the prevention of diseases. Crit. Rev. Food Sci. Nutr. **45**, 287–306 (2005)
9. G.-L. Chen, S.-G. Chen, Y.-Y. Zhao, C.-X. Luo, J. Li, Y.-Q. Gao, Total phenolic contents of 33 fruits and their antioxidant capacities before and after in vitro digestion. Ind. Crops Prod. **57**, 150–157 (2014)
10. K. Krishnaswamy, V. Orsat, Y. Gariépy, K. Thangavel, Optimization of microwave-assisted extraction of phenolic antioxidants from grape seeds (*Vitis vinifera*). Food Bioprocess Technol. **6**, 441–455 (2013)
11. S. Li, S.-K. Li, R.-Y. Gan, F.-L. Song, L. Kuang, H.-B. Li, Antioxidant capacities and total phenolic contents of infusions from 223 medicinal plants. Ind. Crops Prod. **51**, 289–298 (2013)
12. M.M. Rashed, Q. Tong, M.H. Abdelhai, M.A. Gasmalla, J.B. Ndayishimiye, L. Chen, F. Ren, Effect of ultrasonic treatment on total phenolic extraction from *Lavandula pubescens* and its application in palm olein oil industry. Ultrason. Sonochem. **29**, 39–47 (2016)
13. H. Zhang, G. Xie, M. Tian, Q. Pu, M. Qin, Optimization of the ultrasonic-assisted extraction of bioactive flavonoids from ampelopsis grossedentata and subsequent separation and purification of two flavonoid aglycones by high-speed counter-current chromatography. Molecules **21**, 1096–1112 (2016)
14. G.-L. Chen, S.-G. Chen, Y.-Q. Xie, F. Chen, Y.-Y. Zhao, C.-X. Luo, Y.-Q. Gao, Total phenolic, flavonoid and antioxidant activity of 23 edible flowers subjected to in vitro digestion. J. Funct. Foods **17**, 243–259 (2015)
15. L. Zhang, Z. Tu, H. Wang, Z. Fu, Q. Wen, H.-x. Chang, X.-q. Huang, Comparison of different methods for extracting polyphenols from *Ipomoea batatas* leaves, and identification of antioxidant constituents by HPLC-QTOF-MS2. Food Res. Int. **70**, 101–109 (2015)
16. Z. Lou, J. Chen, F. Yu, H. Wang, X. Kou, C. Ma, S. Zhu, The antioxidant, antibacterial, antibiofilm activity of essential oil from *Citrus medica* L. var. sarcodactylis and its nanoemulsion. LWT-Food Sci. Technol. **80**, 371–377 (2017)
17. R. Chirinos, H. Rogez, D. Campos, R. Pedreschi, Y. Larondelle, Optimization of extraction conditions of antioxidant phenolic compounds from mashua (*Tropaeolum tuberosum* Ruíz & Pavón) tubers. Sep. Purif. Technol. **55**, 217–225 (2007)
18. M. Tan, C. Tan, C. Ho, Effects of extraction solvent system, time and temperature on total phenolic content of henna (*Lawsonia inermis*) stems. Int. Food Res. J. **20**, 3117–3123 (2013)
19. F. Dahmoune, L. Boulekbache, K. Moussi, O. Aoun, G. Spigno, K. Madani, Valorization of *Citrus limon* residues for the recovery of antioxidants: evaluation and optimization of microwave and ultrasound application to solvent extraction. Ind. Crops Prod. **50**, 77–87 (2013)
20. H.T. Vu, C.J. Scarlett, Q.V. Vuong, Optimization of ultrasound-assisted extraction conditions for recovery of phenolic compounds and antioxidant capacity from banana (*Musa cavendish*) peel. J. Food Process. Preserv. **41**, e13148 (2017)
21. Q.V. Vuong, S. Hirun, P.D. Roach, M.C. Bowyer, P.A. Phillips, C.J. Scarlett, Effect of extraction conditions on total phenolic compounds and antioxidant activities of *Carica papaya* leaf aqueous extracts. J. Herb. Med. **3**, 104–111 (2013)
22. A. Altemimi, D.G. Watson, R. Choudhary, M.R. Dasari, D.A. Lightfoot, Ultrasound assisted extraction of phenolic compounds from peaches and pumpkins, PLoS ONE **11**, e0148758 (2016)
23. X. Fang, J. Wang, Y. Wang, X. Li, H. Zhou, L. Zhu, Optimization of ultrasonic-assisted extraction of wedelolactone and antioxidant polyphenols from *Eclipta prostrate* L using response surface methodology. Sep. Purif. Technol. **138**, 55–64 (2014)
24. L. Boulekbache-Makhlouf, L. Medouni, S. Medouni-Adrar, L. Arkoub, K. Madani, Effect of solvents extraction on phenolic content and antioxidant activity of the byproduct of eggplant. Ind. Crops Prod. **49**, 668–674 (2013)

25. P. Jayanthi, P. Lalitha, Reducing power of the solvent extracts of *Eichhornia crassipes* (Mart.) Solms. Int. J. Pharm. Pharm. Sci. **3**, 126–128 (2011)

26. W. Hou, W. Zhang, G. Chen, Y. Luo, Optimization of extraction conditions for maximal phenolic, flavonoid and antioxidant activity from *Melaleuca bracteata* leaves using the response surface methodology, PLoS ONE **11**, e0162139 (2016)

27. K. Papoutsis, P. Pristijono, J.B. Golding, C.E. Stathopoulos, M.C. Bowyer, C.J. Scarlett, Q.V. Vuong, Screening the effect of four ultrasound-assisted extraction parameters on hesperidin and phenolic acid content of aqueous citrus pomace extracts. Food Biosci. **21**, 20–26 (2017)

28. K. Sun, A. McIntyre, P.S. Saleem, J.T. Haddad, Arnason, The relationship between antiglycation activity and procyanidin and phenolic content in commercial grape seed products. Can. J. Physiol. Pharmacol. **90**, 167–174 (2012)

29. G. Scola, D. Conte, P. Wilmsen Dalla-Santa, C. Spada, R. Dani, C. Vanderlinde, M. Funchal, Salvador, Flavan-3-ol compounds from wine wastes with in vitro and in vivo antioxidant activity. Nutrients **2**, 1048–1059 (2010)

30. J.V. Higdon, B. Frei, Coffee and health: a review of recent human research. Crit. Rev. Food Sci. Nutr. **46**, 101–123 (2006)

31. M.K. Khan, I.A. Ansari, M.S. Khan, J.M. Arif, Dietary phytochemicals as potent chemotherapeutic agents against breast cancer: inhibition of NF-κB pathway via molecular interactions in rel homology domain of its precursor protein p105. Pharmacogn. Mag. **9**, 51–57 (2013)

32. R. Dapson, C. Bain, Brazilwood, sappanwood, brazilin and the red dye brazilein: from textile dyeing and folk medicine to biological staining and musical instruments. Biotech. Histochem. **90**, 401–423 (2015)

33. S. Boonphong, P. Puangsombat, A. Baramee, C. Mahidol, S. Ruchirawat, P. Kittakoop, Bioactive compounds from *Bauhinia purpurea* possessing antimalarial, antimycobacterial, antifungal, anti-inflammatory, and cytotoxic activities. J. Nat. Prod. **70**, 795–801 (2007)

34. Y.-M. Lee, Y.-C. Kim, B.-J. Choi, D.-W. Lee, J.-H. Yoon, E.-C. Kim, Mechanism of sappanchalcone-induced growth inhibition and apoptosis in human oral cancer cells. Toxicol. In Vitro **25**, 1782–1788 (2011)

35. E.-G. Jung, K.-I. Han, H.-J. Kwon, B.B. Patnaik, W.-J. Kim, G.M. Hur, K.-W. Nam, M.-D. Han, Anti-inflammatory activity of sappanchalcone isolated from *Caesalpinia sappan* L. in a collagen-induced arthritis mouse model. Arch. Pharmacal Res. **38**, 973–983 (2015)

36. N. Tanaka, Y. Takaishi, Y. Shikishima, Y. Nakanishi, K. Bastow, K.-H. Lee, G. Honda, M. Ito, Y. Takeda, O.K. Kodzhimatov, Prenylated Benzophenones and Xanthones from *Hypericums cabrum*. J. Nat. Prod. **67**, 1870–1875 (2004)

37. L.M. Silva, M.C. Casanova, J.M. Marcondes, L.P. Espindola-Netto, G.O. Paixão, P. Melo, M. Zancan, S.S. Sola-Penna, Costa, Antidiabetic activity of *Sedum dendroideum*: metabolic enzymes as putative targets for the bioactive flavonoid kaempferitrin. IUBMB Life **66**, 361–370 (2014)

38. X.Q. Ma, T. Han, X. Zhang, J.Z. Wu, K. Rahman, L.P. Qin, C.J. Zheng, Kaempferitrin prevents bone lost in ovariectomized rats. Phytomedicine **22**, 1159 (2015)

39. J. Cassani, A.M. Dorantes-Barrón, L.M. Novales, G.A. Real, R. Estrada-Reyes, Anti-depressant-like effect of kaempferitrin isolated from *Justicia spicigera* Schltdl (Acanthaceae) in two behavior models in mice: evidence for the involvement of the serotonergic system. Molecules **19**, 21442–21461 (2014)

40. M. del Carmen Juárez-Vázquez, A.J. Alonso-Castro, A. García-Carrancá, Kaempferitrin induces immunostimulatory effects in vitro. J. Ethnopharmacol. **148**, 337–340 (2013)