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ORIGINAL ARTICLE

Combination of ultrasound, microwave and conventional extraction techniques for roselle (*Hibiscus Sabdariffa. L.*) total anthocyanins and phenolics recovery: effect on antioxidant and structural properties

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Abstract
Roselle (*Hibiscus Sabdariffa* L.) has long been recognized as a source of natural food color additive. Microwave- and ultrasonic-assisted extraction are green and innovative processes widely used for natural dyes extraction. For this purpose, these two techniques as well as conventional extraction and their combination were proposed to extract total monomeric anthocyanins and total phenolics from roselle flowers. Ultrasonic-assisted extraction for 5 min yielded total monomeric anthocyanins of 13.47±0.22 mg/g and total phenolics of 163.23 ± 2.81 mg gallic acid/g of dried roselle flowers using 60% (V/V) aqueous ethanol and a solid/liquid ratio of 1/10 (g/mL). Microwave-assisted extraction required 10 min and microwave power of 180 W to extract higher monomeric anthocyanins and total phenolics. Conventional extraction required 24 h to obtain 14.46 ± 0.47 mg/g of total monomeric anthocyanins and 182.89 ± 2.49 mg gallic acid/g of total phenolics. The combination of ultrasonic- and microwave-assisted extraction reduced the extraction time from 24 h to 15 min and significantly ($p \leq 0.05$) increased total monomeric anthocyanin (17.36 ± 0.54 mg/g), total phenolics (208.28 ± 1.43 mg gallic acid/g), 2,2-diphenyl-1-picrylhydrazyl radical scavenging antioxidant activity and ferric-reducing antioxidant power by 28.88, 27.60, 73.41, and 85.77%, respectively, in comparison to ultrasound-assisted extraction. Fourier transform infrared showed that all extraction procedures did not affect the structural properties of roselle anthocyanins. This finding suggests the development of the use of green and innovative combined processes such as microwave and ultrasound for improving roselle pigment extraction for potential application in food and non food products.

Keywords *Hibiscus sabdariffa* L. · Anthocyanin · Microwave-assisted extraction · Ultrasound-assisted extraction · Phenolic compounds · Antioxidant activity

1 Introduction

Hibiscus sabdariffa L. also called roselle, *Jamaica hibiscus*, Jamaica sorrel or red sorrel and in Arabic, Karkade, is a plant of the Malvaceae family. It is widely cultivated in tropical and subtropical regions of Asia, Africa, Latin, and North America particularly in India, Saudi Arabia, China, Malaysia, Indonesia, the Philippines, Vietnam, Sudan, Egypt, Nigeria, and Mexico [1]. All parts of *Hibiscus sabdariffa* L. plant were used in food, pharmaceutical, and cosmetic industries and are recognized in traditional medicine and were successfully applied to treat inflammation, diabetes, and hypertension [1–3]. *Hibiscus sabdariffa* L. extracts are known as a rich source of anthocyanins, a family of pigments that are responsible for the bright red color of the flower and are the most important group of colorants [4]. Anthocyanins are found in vegetables, red fruits, and some cereals. The red, blue, and purple colors of most fruits, flowers, and leaves are due to anthocyanins [3]. The most common anthocyanins in the calyces of *Hibiscus sabdariffa* are cyanidin-3-sambubioside commonly known as

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gossypicyanin and delphinidin-3-sambubioside also called *Hibiscus*, and at lower levels delphinidin-3-glucoside and cyanidin-3-glucoside [5]. In vitro and in vivo studies showed that *Hibiscus sabdariffa* extracts possess important biological activities such as antibacterial [6], antioxidant [7], anticancer [8], antiobesity [9], antidiabetic, diuretic, hepatoprotective, hypocholesterolaemic, immunomodulatory, and antihypertensive [1, 10].

In the two past decades, extraction of natural dyes such as anthocyanins from fruits, vegetables, and plants increased because of their safety and health benefits. For this purpose, several extraction methods were used from conventional (hydrodistillation, maceration, agitation) to innovative: ultrasound-assisted extraction (UAE) [11], microwave-assisted extraction (MAE) [12], enzyme-assisted extraction, supercritical fluids extraction, pressurized liquid and subcritical water extraction, extraction with natural deep eutectic solvents, pulsed electric field pre-treatment [3–13]. More recently, to increase extraction yields and reduce extraction time and temperature, a new trend was developed the combination of these green and innovative methods [14, 15]. Thus, MAE, UAE, hydrodistillation, ohmic heating, and several other extraction techniques were used simultaneously or as successive treatment to extract bioactive compounds such as anise (*Pimpinella anisum* L.) seed essential oil [16], phenolic compounds of *Renealmia alpinia* (Rottb.) maas peel [17], total phenolics (TP), and flavonoid from bark [18]. Among these techniques, the combination of UAE and MAE offers advantages to highly shorten extraction time and to reduce energy consumption [19]. Wizi et al. [20] successfully used combined UAE-MAE to extract natural colorants from sorghum husk and improved the extraction yield 3.6 times in comparison to conventional shaking. Trujillo-Mayol et al. [19] improved the level of extracted avocado peel polyphenols by 1.3, 1.2, and 1.1 times in comparison to UAE, MAE, and maceration, respectively. Garcia-Vaquero et al. [21] showed that the combined UAE-MAE generated higher yields of bioactive compounds such as fucose-sulphated polysaccharides (FSPs) and total soluble carbohydrates from brown microalgae *A. nodosum* and in the meantime did not affect antioxidant properties of the extracts. Several studies performed the extraction of roselle (*Hibiscus sabdariffa* L.) anthocyanins, polyphenols, flavonoids, and other bioactive compounds from flowers, calyces, stems, leaves, and seeds using conventional processes [22] as well as innovative processes such as UAE [4, 11] and MAE [23]; however, to the best of our knowledge, only one paper reported combination of MAE and stirring at subcritical conditions for polyphenols extraction from roselle seeds [24].

Against this background, the aim of this study was to use two modern techniques, UAE and MAE to extract roselle (*Hibiscus sabdariffa* L.) anthocyanins. The effect of solvent concentration, time, solid-to-liquid ratio (S/L), and microwave power (*W*) was investigated. The potential combination of UAE, MAE, and conventional extraction (CE) methods was studied on the basis of the determination of total monomeric anthocyanins (TMA), TP, and antioxidant activities using 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging capacity and ferric reducing antioxidant power (FRAP).

2 Materials and methods

2.1 Materials

Commercial dry roselle (*Hibiscus sabdariffa* L.) flowers were used for anthocyanin extraction. Roselle flowers samples were purchased from a local market in Béchar in south Algeria. Dried roselle flowers were ground with a domestic blender and then sieved (0.200 mm) to obtain a fine powder and then stored at 4°C for further analysis or processing. All chemicals were purchased from Sigma-Aldrich and Merck and were of analytical grade.

2.2 Roselle anthocyanins extraction

Roselle anthocyanins were extracted using UAE, MAE, and CE, and their combination as described below.

2.2.1 Ultrasonic-assisted extraction (UAE)

UAE was performed using an ultrasonic bath (Isolab, Germany), at a frequency of 20 kHz. Anthocyanins were extracted from roselle flowers powder with water, pure ethanol, or aqueous ethanol at different concentrations 30, 50, 60, 70%. The effect of S/L ratio (1/10, 1/20, 1/30, to 1/40 (w/V)) was investigated. The mixtures were sonicated for 5, 10, 15, or 20 min. The temperature was maintained below 30°C. The samples were then filtered on a Buchner funnel through a Whatman filter paper n°4 and stored in amber glass containers at 4°C for further analysis. Extraction was repeated three times.

2.2.2 Microwave-assisted extraction (MAE)

Roselle anthocyanins were extracted using MAE in a domestic microwave oven (Samsung model ME73A, Malaysia 2450 MHz) equipped with a cooling system as condenser. The effect of extraction conditions: Solvent type and concentration (water, pure ethanol, and aqueous ethanol at different concentrations 30, 50, 60, 70% (V/V)), heating power (100, 180 and 300 W), time (5, 10, 15, and 20 min), and S/L ratio (1/10, 1/20, 1/30, to 1/40 (w/V)) were investigated. After extraction, the obtained mixtures were filtered through Whatman paper n°4 and the extracts were stored in amber

glass containers at 4°C for further analysis. All extractions were repeated at least three times.

2.2.3 Conventional extraction (CE):

CE was performed at room temperature in erlenmeyer flasks covered with aluminum foil, according to the method of Chumsri et al. [25] using the following conditions: S/L ratio of 1/10 and 60% (V/V) aqueous ethanol. The mixture was stirred at 750 rpm using a magnetic stirrer for 24 h. The samples were filtered on a Buchner funnel through Whatman filter paper n°4 and stored at 4°C in amber glass containers for further analysis. Extraction was performed at least in triplicate.

2.2.4 Combination of extraction methods

The three previous extraction processes (MAE, UAE, and CE) were combined at optimal conditions. The combination of ultrasound and conventional methods (UAE-CE) were performed as follows. First, ultrasonication of 1.5 g of roselle flower powder was performed in 15 mL of 60% (V/V) aqueous ethanol at a frequency of 20 kHz for 5 min. The obtained sample was then stirred (750 rpm) at room temperature for 30 min, 1 h, 2 h, 3 h, 6 h, or 24 h, the combined UAE and CE processes were labeled as follow UAE-CE30min, UAE-CE1h, UAE-CE2h, UAE-CE3h, UAE-CE6h, and UAE-CE24h, respectively. The microwave and conventional method (MAE-CE) were combined as described below. MAE of roselle anthocyanins was performed using 60% (V/V) aqueous ethanol at the same S/L ratio (1/10), and 180 W for 10 min then the mixture was stirred at 750 rpm at room temperature for 30 min, 1 h, 2 h, 3 h, 6 h or 24 h, the combined MAE and CE extraction methods were labeled as follow MAE-CE30min, MAE-CE1h, MAE-CE2h, MAE-CE3h, MAE-CE6h, and MAE-CE24h, respectively.

UAE-MAE consisted of a combination of ultrasonication and microwaves in the same previously described conditions. UAE was first performed using 60% (V/V) aqueous ethanol at a frequency of 20 kHz for 5 min, and then MAE of the obtained utrasonicated extract was carried out for 10 min using 60% (V/V) of aqueous ethanol and a power of 180 W.

2.3 Total monomeric anthocyanins pigment contents

TMA were evaluated using a modified differential pH method as previously described by Giusti and Wrolstad [26]. Two aliquots of buffer solutions of pH 1 (potassium chloride, 0.025 M) and pH 4.5 (sodium acetate, 0.4 M) were placed into separate glass tubes. One hundred microliters of roselle extract were then added and well mixed. The absorbances at 520 and 700 nm were measured using a visible spectrophotometer (Optizen 1412v, Korea). The TMA were calculated as follows:

$$A = (A_{520} - A_{700})_{\mathrm{pH=1.0}} - (A_{520} - A_{700})_{\mathrm{pH=4.5}} \quad (1)$$

$$\mathrm{TMA}\left(\frac{\mathrm{mg}}{\mathrm{g}}\right) = \frac{A \times \mathrm{MW} \times \mathrm{DF} \times V \times 1000}{\varepsilon \times L \times m} \quad (2)$$

where A_{520} and A_{700} are the absorbance measured at 520 and 700 nm at pH=1 or pH=4.5, MW is molecular weight of cyanidin-3-O-glucoside (449.2 g.mole^{-1}), DF is the dilution factor (total volume/volume of extract), V (L) volume of solvent, ε is the molar absorptive (26,900 L.mole^{-1}.cm^{-1}), L is the light path through the quartz cell (1 cm), and m (g) weight of the roselle flower.

2.4 Total phenolics

TP was determined as described by Singleton and Rossi [27] with some modifications. This method is based on the quantification of the total concentration of hydroxyl groups present in the extract. Briefly, in test tubes, 0.1 mL of roselle flower extract was added to a mixture of 3 mL of distilled water, 1 mL of Folin-Ciocalteu reagent diluted 10 times, and 0.2 mL of a 20% sodium carbonate solution. The tubes were shaken and kept in the dark for 30 min. The absorbance at 765 nm was measured using a visible spectrophotometer (Optizen 1412v, Korea). A calibration curve was performed under the same operating conditions using gallic acid as standard at different concentrations (10 to1000 µg/mL). Thereafter, TP in roselle flower extracts was calculated from the calibration equation established with gallic acid and was expressed as mg gallic acid/g of dried roselle flowers (mg GAE/g drf). The analysis was carried out in triplicate.

2.5 Antioxidant activity

Antioxidant activity of the obtained extracts was measured using two methods DPPH radical scavenging and FRAP.

2.5.1 DPPH radical scavenging activity

The antioxidant activity of roselle flower extract was evaluated using a combination of two methods previously described by Menaceur et al. [28] and Ghaliaoui et al. [28]. One thousand and nine hundred seventy-five microliters of DPPH solution (0.0024%) were added to 25 µL of roselle extract diluted 50 times. The mixture was incubated at room temperature for 30 min in the dark. The Absorbance at 517 nm was determined using a visible spectrophotometer (Optizen 1412v, Korea). All measurements were repeated at least

three times. The percentage of DPPH radical scavenging activity (RSA (%)) was calculated as follows:

$$\mathrm{RSA}(\%) = \frac{A_b - A_s}{A_b} \times 100 \quad (3)$$

where A_b is the absorbance of the extract at 517nm before reaction and A_s is the absorbance of the extract at 517 nm after 30 min. A standard curve was prepared using trolox with different concentration ranging from 0.1 to 1 mM. The DPPH activity was expressed as µM trolox equivalent per g of dried roselle flower (µM trolox eq/g drf).

$$\mathrm{DPPH}\left(\frac{\mu\mathrm{Mtroloxeq}}{\mathrm{g}}\right) = \frac{\mathrm{RSA} \times \mathrm{DF} \times V \times 1000}{m \times 100.55} \quad (4)$$

DF is the dilution factor,

V (L) is the volume of the solvent, m (g) is the weight of the roselle flower

100.55 is the slope of the standard curve.

2.5.2 Ferric reducing antioxidant power (FRAP)

According to Benzie and Strain [30], the FRAP method is used to measure the capacity of an extract to reduce iron, by recording the variation of absorbance at 593 nm of 2,4,6-tripyridin-2-yl-1,3,5-triazine-Fe^{3+} complex (TPTZ-Fe^{3+}) when reduced to the ferrous-TPTZ complex (TPTZ-Fe^{2+}) form in the presence of an antioxidant compound. The FRAP reagent was freshly prepared by mixing three different solutions in ratio of 10:1:1: sodium acetate buffer (300 mM, pH 3.6), 10 mM of TPTZ solution prepared in HCl (40 mM), and iron(III) chloride solution (20 mM). The obtained FRAP reagent was stored at 37°C until use. Then, 100 µL of roselle extract were added to 3 mL of FRAP reagent and incubated at 37°C for 4 min, TPTZ-Fe^{2+} was quantified at 593 nm using a visible spectrophotometer (Optizen 1412v, Korea). The standard curve was made using $FeSO_4$ solution, and the results were expressed as µmol of Fe (II)/g of dried roselle flower (µmol Fe/g drf).

2.6 Fourier transform infrared spectroscopy

A FTIR spectrometer (Spectrum two L1600301, Perkin Elmer, USA) equipped with an universal attenuated total reflectance (ATR) sampling accessory was used to identify structural changes of roselle anthocyanins extracted with the three previous methods (UAE, MAE, and CE) and their combination (UAE-CE, MAE-CE, and UAE-MAE). The spectra were recorded at 1 cm^{-1} resolution within the frequency range of 4000–450 cm^{-1}. Air in the empty crystal was used as background.

2.7 Statistical analysis

Statistical Analysis System R 4.0.2 was used to analyze the average of at least three repetitions. One-way analysis of variance (ANOVA) was performed with a Tukey multiple comparison procedure on a 5% significance level [31]. Supplemental ANOVA significant levels were presented ***$p \leq 0.001$, **$p \leq 0.01$, * $p \leq 0.05$. Results were presented as mean ± standard deviation.

3 Results and discussion

3.1 Ultrasonic-assisted extraction (UAE)

UAE has been widely used for bioactive compound extraction. It is considered as one of the easiest and most effective method that can accelerate heat and mass transfer in a shorter time [14].

Figures 1(a), 1(b), and 1(c) show the effect of UAE operating conditions on TMA contents in *Hibiscus sabdariffa* L. extract. Figure 1(a) illustrates the effect of solvent type and aqueous ethanol concentration on TMA. The mixture of water and ethanol gave significantly higher results than water alone or ethanol alone, in the meantime, water was found to be a better solvent than ethanol alone, which has been demonstrated in several previous studies [4]. Moreover, according to several studies, water and ethanol were the most used green solvents in UAE because they are the least harmful and most efficient [32]. The best solvent system for roselle pigment extraction was 60% (V/V) aqueous ethanol with TMA content of 13.46 ± 0.22 mg/g. Ethanol concentration lower than 50% or higher than 60% were significantly different ($p \leq 0.05$) and yielded lower TMA content than 60% aqueous ethanol.

Figure 1(b) shows the effect of various S/L ratios on TMA content in *Hibiscus sabdariffa* L. extracted by UAE. No statistical differences in TMA contents ($p > 0.05$) were identified when decreasing S/L ratio from 1/10 to 1/40. On this basis, S/L ratio of 1/10 was chosen for further experiments. Redzuan et al. [33] and Chumsri et al. [25] reported similar S/L ratio when extracting anthocyanins from *Hibiscus* petals or calyces; however, they obtained lower TMA contents than the present study: 71mg/L and 502.33 ± 0.52 mg/100g corresponding to only 0.71 and 5.02 mg/g, respectively. This is probably due to their use of a CE method and pure water as solvent. Whereas, Aryanti et al. [34] compared the level of total anthocyanin contents obtained from red or purple roselle calyces by CE and UAE methods and obtained lower results (0.65–0.75 mg/g of TMA) than the current study when using UAE, water as solvent for 30 min and 40 kHz of frequency. These differences could be due to the higher cavitations time along with higher ultrasound frequency which

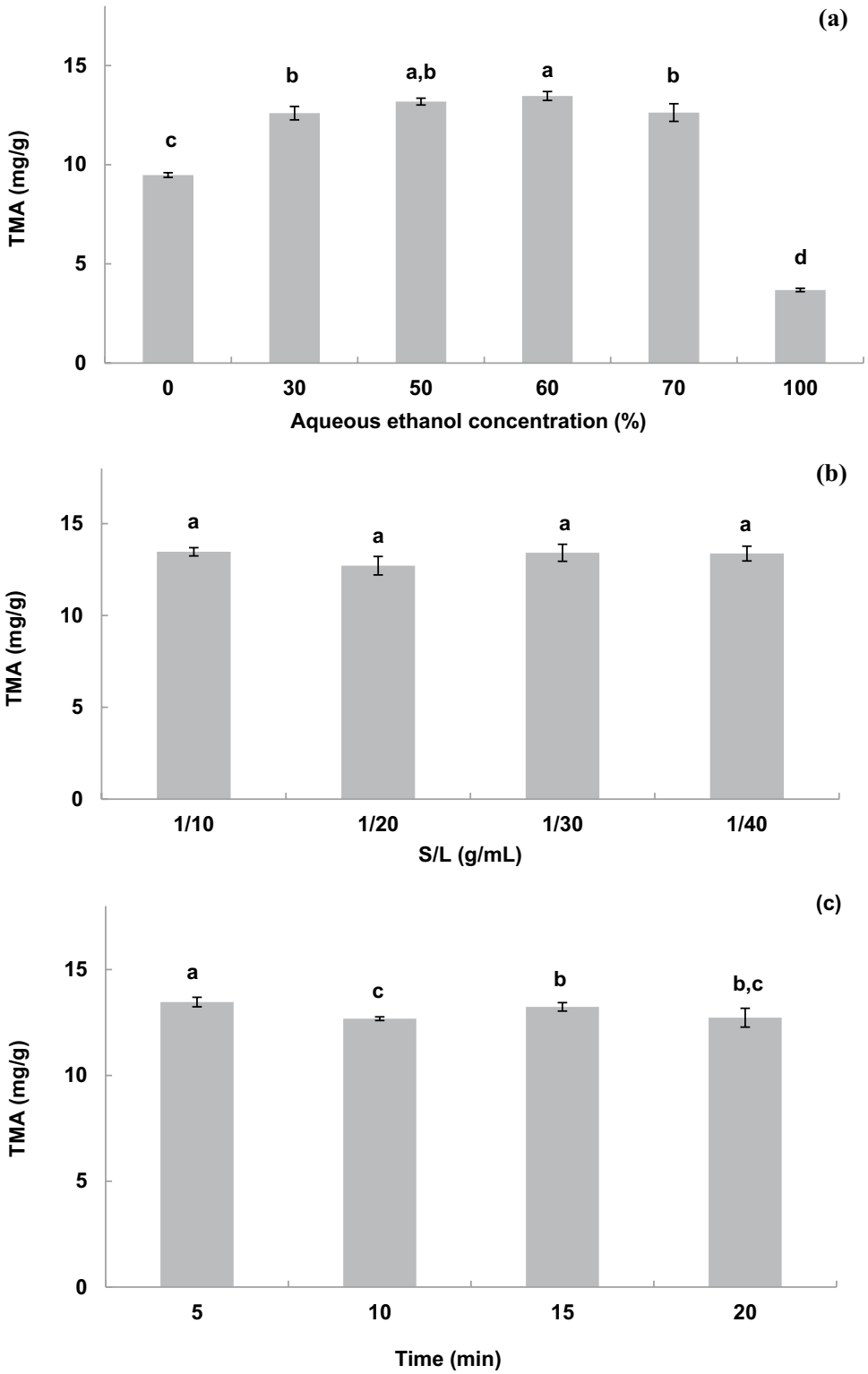

Fig. 1 Effect of operating conditions on UAE extraction of total monomeric anthocyanins contents in roselle *Hibiscus sabdariffa* L. extract. (**a**) Effect of solvent concentration on UAE extraction of total monomeric anthocyanins (TMA) contents in roselle *Hibiscus sabdariffa* L. extract. S/L=1/10; time =5 min, frequency 20 kHz. (**b**) Effect of solid to liquid ratio on UAE extraction of total monomeric anthocyanins contents in roselle *Hibiscus sabdariffa* L. extract. Ethanol concentration= 60%, time = 5 min, frequency 20 kHz

probably led to a destruction of roselle anthocyanins. More recently, Yuniati et al. [35] used UAE with distilled water as solvent for 5 min and at low frequency of 24 kHz, but with higher S/L ratio of 1/25, they obtained approximately 25 times lower anthocyanin contents than the present study.

Figure 1(c) shows the effect of ultrasonication time of roselle flowers extracts on TMA. The experiment was conducted at increasing times (5, 10, 15, 20 min) using 60% (V/V) aqueous ethanol, S/L ratio of 1/10, and temperature was maintained below 30°C to avoid anthocyanin thermal

degradation. Ultrasonic extraction time of 5 min allowed extracting significantly higher TMA ($p \leq 0.05$) than the other times, this is possibly due to the fact that, increasing ultrasonication time led to an almost constant TMA contents and may cause anthocyanins degradation in roselle flower extracts due to prolonged sound irradiation. Therefore, a time of 5 min was selected for the UAE process, providing an advantageous roselle extract quality in terms of TMA contents. Similar results were obtained by Yuniati et al. [35] who extracted anthocyanin pigment from *Hibiscus sabdariffa* L. by UAE for 1 to 5 min and stated that short sonication times lower or equal to 10 min are preferred to avoid the bioactive compounds degradation. In addition as hypothesized by Chemat et al. [14], UAE enhances water absorption favoring solvent access to the plant cells and then rapid diffusion of the bioactive molecules.

3.2 Microwave-assisted extraction (MAE)

Microwave is based on two oscillatory electromagnetic fields that allow the solvent to enter inside the roselle cells. A transfer of energy is induced by the excited water molecules that act as an accelerating and indirect heating source. Due to the rapid heating, the roselle cell structure breaks down allowing anthocyanins liberation in the solvent [36]. According to Silva et al. [3], polar solvents are more appropriate for anthocyanin extraction. Thus, various concentrations of aqueous ethanol, water, and pure ethanol were investigated as solvent in this study.

Figures 2(a), (b), (c), and (d) depict the effect of ethanol concentration (%), MAE power (W), time (min) and S/L ratio on TMA content in roselle flower extract, respectively. As shown in Fig. 2(a), aqueous ethanol allowed extracting higher TMA content than water and ethanol alone. The TMA contents were high significantly affected by solvent concentration ($***p \leq 0.05$). Moreover, TMA increased significantly ($***p \leq 0.05$) with increasing ethanol concentration; however, when ethanol concentration exceeded 60%, TMA decreased rapidly. The best solvent system for roselle pigments extraction was found to be 60% (V/V) aqueous ethanol which yielded 16.34 ± 0.48 mg/g of TMA contents. Similarly, Alara et al. [37] stated

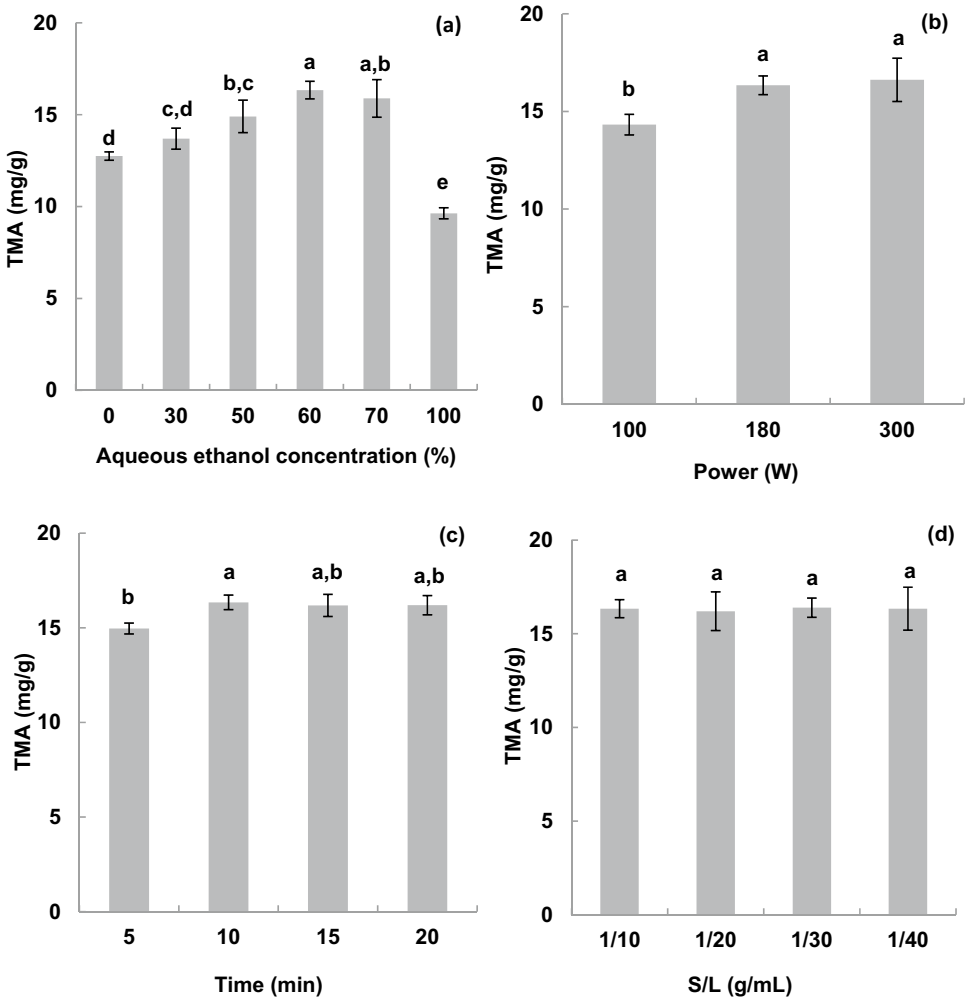

Fig. 2 Effect of operating conditions on MAE extraction of total monomeric anthocyanins contents in roselle *Hibiscus sabdariffa* L. extract. (**a**) Effect of ethanol concentration: S/L=1/10; power = 180 W; time = 10 min. (**b**) Effect of power: ethanol concentration= 60%; S/L=1/10; time = 10 min. (**c**) Effect of time: ethanol concentration= 60%, power= 180 W, S/L=1/10. (**d**) Effect of solid to liquid ratio: ethanol concentration= 60%, power= 180 W, time = 10 min

that higher ethanol concentration reduced solvent polarity and then leads to a decrease in the polyphenols solubility. For that reason, 60% (V/V) aqueous ethanol was selected as best ethanol concentration for MAE of roselle flowers anthocyanins.

Figure 2(b) illustrates the effect of increasing MAE power from 100 to 300 W on TMA contents, at fixed irradiation time (10 min) and ethanol concentration (60%). MAE power high significantly affected TMA contents (*** $p \leq 0.05$), while MAE power of 180 W and 300 W were not significantly different from each other ($p > 0.05$). TMA contents increased when MAE power increased from 100 to 180 W and reached a maximum at 180 W. This is probably due to the faster propagation of a large amount of electromagnetic energy on the particles by their dipolar rotation, which led to energy dissipation in the extract and then particles mobility and heating increase which break the cell and disperse the extract in the solvent [22, 37]. More recently, Alara et al. [23] investigated the effect of increasing MAE power from 200 to 600 W on flavonoids contents in *Hibiscus sabdariffa* calyces extract, they showed that flavonoids decreased when MAE power was higher than 400 W. According to Alara et al. [37] and Wen et al. [38], the use of a high microwave power reduces the duration of the process and increases anthocyanins solubility; nevertheless, it contributes to temperature raise and viscosity decrease leading to rapid anthocyanins degradation. In the present study, MAE irradiation power of 180 W was the best with a peak in TMA contents of 16.34 ± 0.48 mg/g in roselle extract; moreover, no significant difference was observed at 300 and 180 W. For all these reasons, 180 W was used to investigate other process parameters effect. Comparable result was found by Pham et al. [12] during MAE of *Rhodomyrtus tomentosa* anthocyanin using increasing power irradiation from 100 to 500 W, where 200 W was the most efficient power in term of TMA contents.

Figure 2(c) shows the effect of MAE time on TMA contents in roselle flower. TMA contents increased significantly ($p \leq 0.05$) with extraction time from 5 to 10 min and reached a maximum value of 16.34 ± 0.48 mg/g at 10 min. Further increase in time resulted in a non-significant increase ($p > 0.05$) in TMA contents in roselle extract. Thus, excessive MAE irradiation time reduced the concentration of TMA in the extract and had the ability to break down anthocyanins by promoting their degradation and reduced the efficiency of the process as previously reported by Pimentel-Moral et al. [39] for MAE of bioactive compounds from *Hibiscus sabdariffa*. Similar observations were reported by Pham et al. [12] where the best extraction duration for anthocyanins MAE from *Rhodomyrtus tomentosa* was 5 min. Similarly, Liazid et al. [40] found that 5 min of MAE are sufficient for high extraction yield of anthocyanins from grapes skins. Thus, MAE time of 10 min was chosen as the best time for TMA contents in roselle extract.

The S/L ratio is an important factor that can maximize anthocyanin recovery yields from the plant matrix and minimize solvent use [41]. Figure 2(d) shows the effect of varying S/L ratio from 1/10 to 1/40 (g/mL) on TMA contents in roselle extract at fixed values of microwave power 180 W, for an irradiation time of 10 min and ethanol concentration of 60%. TMA content were not significantly ($p > 0.05$) affected by S/L ratio. These results were in close agreement to those found by Alara et al. [23] for MAE of flavonoids from roselle. Then, S/L ratio was set to 1/10 g/mL for further experiments.

3.3 Combined extraction methods

CE requires large amount of organic solvents, high-energy expenditure, and is time consuming. For that reason, interest for new, clean, and green technologies increased. Recently, a new trend in bioactive compounds extraction appeared the combination of conventional and innovative extraction techniques which led to better extraction performances at lower time [15].

In the present study, UAE, MAE, and CE techniques were combined and compared to extract bioactive compounds from *Hibiscus sabdariffa* L. flowers. UAE and MAE were conducted for 5 and 10 min, respectively. However, the best of CE time was investigated in the combined UAE-CE and MAE-CE. Figures 3(a) and (b) show the effect of increasing CE time from 30 min to 24 h when combined with UAE or MAE, on TMA contents. As shown in Fig. 3(a), increasing CE extracting time in UAE-CE had no significant effect on TMA ($p > 0.05$). Similarly, Vega-Arroy et al. [17] stated that the combination of UAE and a 2-h Soxhlet extraction led to TMA increase; however, longer Soxhlet extraction times did not significantly affect TMA contents in x´kijit peel. Thus, in the present study, 30 min of CE was set for the combined UAE-CE.

In the same way, the results of roselle TMA extracted by MAE-CE performed at different time of CE showed that CE extracting time exceeding 30min did not significantly improve TMA contents when combined with MAE. An insignificant ($p > 0.05$) decrease in TMA contents was observed when MAE-CE was conducted at times 30 min to 6h, while at higher time 24h a significant decrease was observed. Consequently, the best MAE-CE combination was set at 10 min of MAE followed by 30 min of CE in term of TMA contents.

TMA, TP, and antioxidant activities were evaluated. Table 1 shows the effect of different extraction procedure (UAE, MAE, CE, UAE-CE, MAE-CE, UAE-MAE) on TMA and TP in roselle flowers extract. The use of UAE alone or combined with CE (UAE-CE) allowed extracting the lowest content of TMA (13.47 ± 0.22 and 13.61 ± 0.27 mg/g) and TP (163.23 ± 2.81 and 168.70 ± 6.96 mg GAE/g drf),

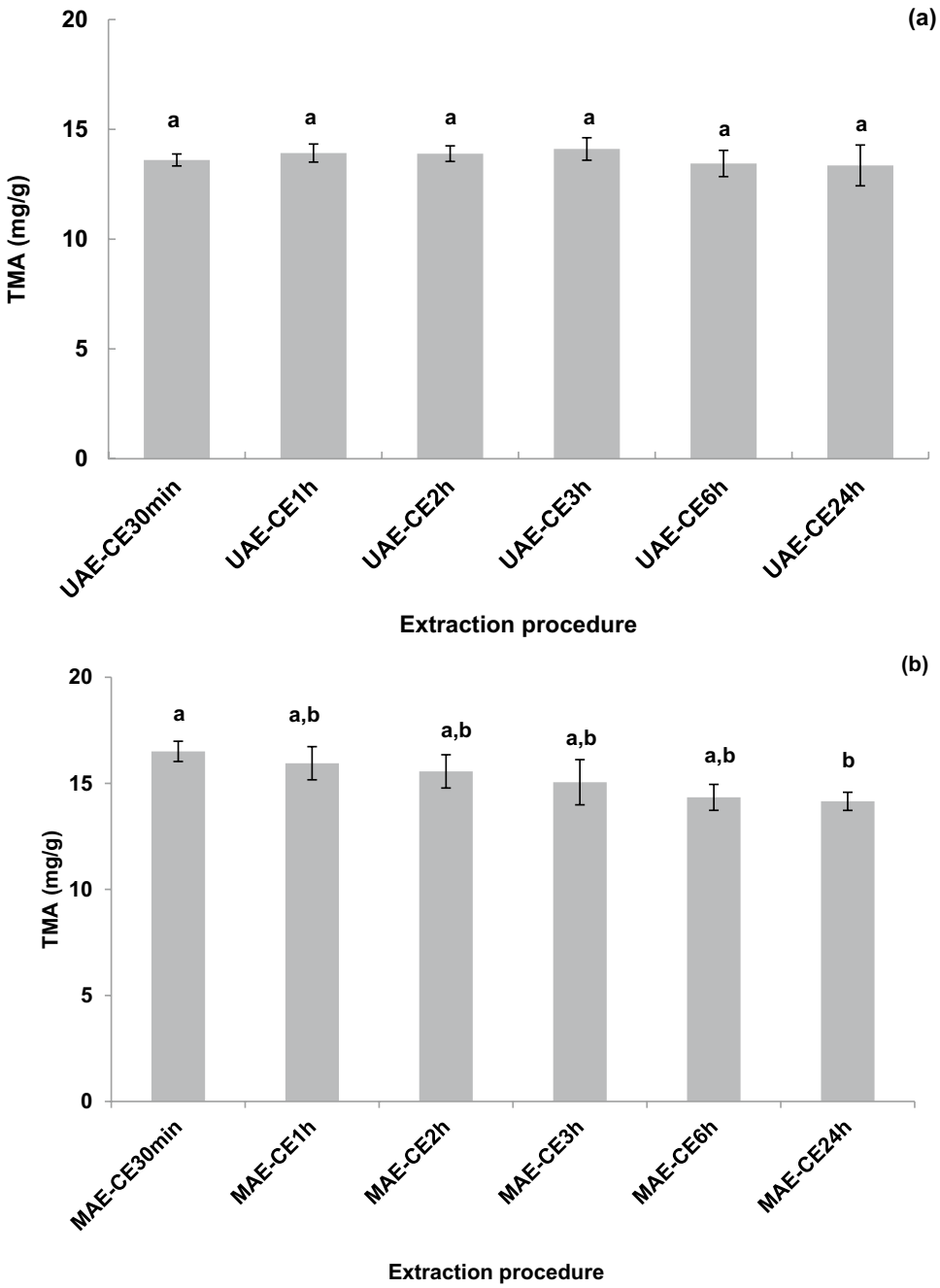

Fig. 3 Effect of increasing time of conventional extraction when combined with ultrasound and microwave-assisted extraction: (**a**) UAE-CE and (**b**) MAE-CE

compared to all the other extraction procedure. TMA contents obtained by MAE, MAE-CE, and UAE-MAE were not significantly different ($p>0.05$). However, in term of TP, UAE-MAE significantly ($p\leq0.05$) allowed producing the highest TP (208.28 ± 1.43 mg GAE/g drf), directly followed by MAE-CE and CE. In comparison to all three extraction processes UAE, MAE, and UAE-CE, MAE-CE yielded a significant ($p\leq0.05$) increase in TP. This may be a good indicator that roselle flower contains other bioactive compounds and that additional agitation promotes their extraction. On the other hand, the following extraction procedure (MAE, MAE-CE) did not significantly ($p^{>}0.05$) increased the TMA and TP. Same behavior was observed for UAE alone or combined UAE-CE. Similar results were obtained by Vega-Arroy et al. [17]; they stated that ultrasonication or additional stirring for 30 min after sonication irradiation did not significantly increase anthocyanins and polyphenol contents extracted from *Renealmia alpinia* Rottb. Maas peels in comparison to CE.

In the current study, the highest concentration of TMA (17.36 ± 0.54 mg/g) and TP (208.28 ± 1.43 mgGAE/g drf) were obtained by the combined UAE-MAE. These high

Table 1 Effect of extraction procedure on total monomeric anthocyanins and total phenolics of roselle

Extraction procedure	Total monomeric anthocyanins (mg/g) ***	Total phenolics (mg GAE/ g) ***
UAE	13.47 ± 0.22b	163.23 ± 2.81d
MAE	16.34 ± 0.48a	176.99 ± 9.11b,c
CE	14.46 ± 0.47b	182.89 ± 2.49b
UAE-CE	13.61 ± 0.27b	168.70 ± 6.96c,d
MAE-CE	16.51 ± 0.78a	182.13 ± 5.37b
UAE-MAE	17.36 ± 0.54a	208.28 ± 1.43a

UAE ultrasound-assisted extraction, *MAE* microwave-assisted extraction, *CE* conventional extraction, *UAE-CE* ultrasound-assisted extraction followed by conventional extraction, *MAE-CE* microwave-assisted extraction followed by conventional extraction, *UAE-MAE* ultrasound-assisted extraction followed by microwave-assisted extraction

Values indicate the mean of three triplicate ± standard deviation, Values in one column followed by different letters are significantly different ($p \leq 0.05$ Tukey's HSD test). ***0.001, **0.01, * 0.05

results indicated that UAE-MAE had a positive effect on the extraction of TMA and TP. Successive ultrasonic and microwave irradiations improved the roselle anthocyanin extract quality and reduced extraction time from 24 h when using CE to 15 min using UAE-MAE which consisted of 5 min UAE followed by 10 min MAE. As previously reported by Chemat et al. [14], CE did not affect the cells structure but bioactive compounds are only transferred in the solvent, this extraction mechanism is time-consuming; however, ultrasonication induces a gradual cell walls degradation at shorter time and allowed solvent accessibility and then better extractability. Meanwhile, MAE quickly heats the plant extract and enhances the migration of dissolved molecules. These two techniques are cost effective and offer fast bioactive compound recovery and they provide synergic effect for improving extraction yields.

Figures 4(a) and (b) depict the effect of different extraction techniques (UAE, MAE, CE, UAE-CE, MAE-CE, UAE-MAE) on the antioxidant activities of roselle anthocyanins evaluated by two analytical methods: DPPH and FRAP. The extraction procedures had a significant effect ($p \leq 0.05$) on antioxidant capacity. The DPPH antioxidant activity of roselle anthocyanins extracted by combined UAE-MAE were significantly ($p \leq 0.05$) the highest (253.76 ± 8.71 µM trolox eq/g drf) compared UAE, MAE, and UAE-CE procedure. UAE-MAE, CE, and MAE-CE DPPH antioxidant activities were not significantly ($p > 0.05$) different from each other (Fig. 4(a)). While, as shown in Fig. 4(b), FRAP antioxidant activity of UAE-MAE was significantly ($p \leq 0.05$) higher than all the other extraction procedure (679.13 ± 49.81 µmole Fe/g drf) as previously observed in TP.

The combined extraction procedure UAE-MAE gave the best results in term of time which did not exceed 15 min, as well as TMA, TP, and antioxidant activities. The increase in antioxidant activity could be considered as a result of the presence of other bioactive compounds in roselle flowers as stated by Vega-Arroy et al. [17]. In the meantime, the lowest DPPH and FRAP antioxidant activities were detected when using UAE alone with only 146.33 ± 1.66 µM trolox eq/g drf and 365.57 ± 30.77 µmol Fe/g, respectively or when using UAE-CE (167.27 ± 2.96 µM trolox eq/g drf and 428.85 ± 6.92 µmol Fe/g).

Consequently, the combination of these innovative and green extraction procedures UAE-MAE contributed to improve TMA, TP contents, DPPH and FRAP antioxidant activities by 28.88, 27.60, 73.41, and 85.77%, respectively, when compared to UAE alone. While, when compared to MAE, lower improvement was observed in term of TP contents, DPPH and FRAP antioxidant activities by 17.68, 29.41, and 25.86%, respectively. Similarly, the lowest improvements of UAE-MAE were observed in comparison to CE; thus, TMA content increased by 20.06%, while TP and FRAP antioxidant activities increased by 13.88 and 17.11%, respectively. However, the combined UAE-MAE allowed gaining extraction time by 96 times. The advantage of using UAE-MAE is probably related to UAE energy dissipation by cavitation and bubbles collapse that induce solvent penetration into vegetal tissues and improved phytochemicals mass transfer. Then, rapid heat of MAE provokes hydrogen bonds destruction by molecules dipole rotation and accelerates molecule dissolution in the solvent [13]. To the best of our knowledge, this is the first report about the efficiency of using combined extraction procedure for Rroselle flowers anthocyanin extraction, several reports compared using UAE, CE, MAE, or other innovative procedures but none to date investigated their combination [4, 5, 24]. In a similar study, Wizi et al. [20] compared CE and combined UAE-MAE to extract phenolic colorants from sorghum husk, UAE-MAE improved extraction yield 3.6 times and increased TP contents by 89.3% when using 60% aqueous ethanol at 55°C for 20 min. In the present study, UAE-MAE allowed lower increment of TP (13.88%); this is probably due to extraction of roselle bioactive compounds performed at room temperature to avoid their thermal degradation [43]. The present findings are in good agreement with those obtained by Trujillo-Mayol et al. [19] who studied and compared extraction of avocado peel polyphenols by UAE-MAE, UAE, MAE, and CE. UAE-MAE yielded better level of polyphenols, directly followed by CE, MAE, and then UAE. in the present study, UAE-MAE was the best extraction procedure in term of TP, DPPH, and FRAP antioxidant activities in comparison to UAE and MAE.

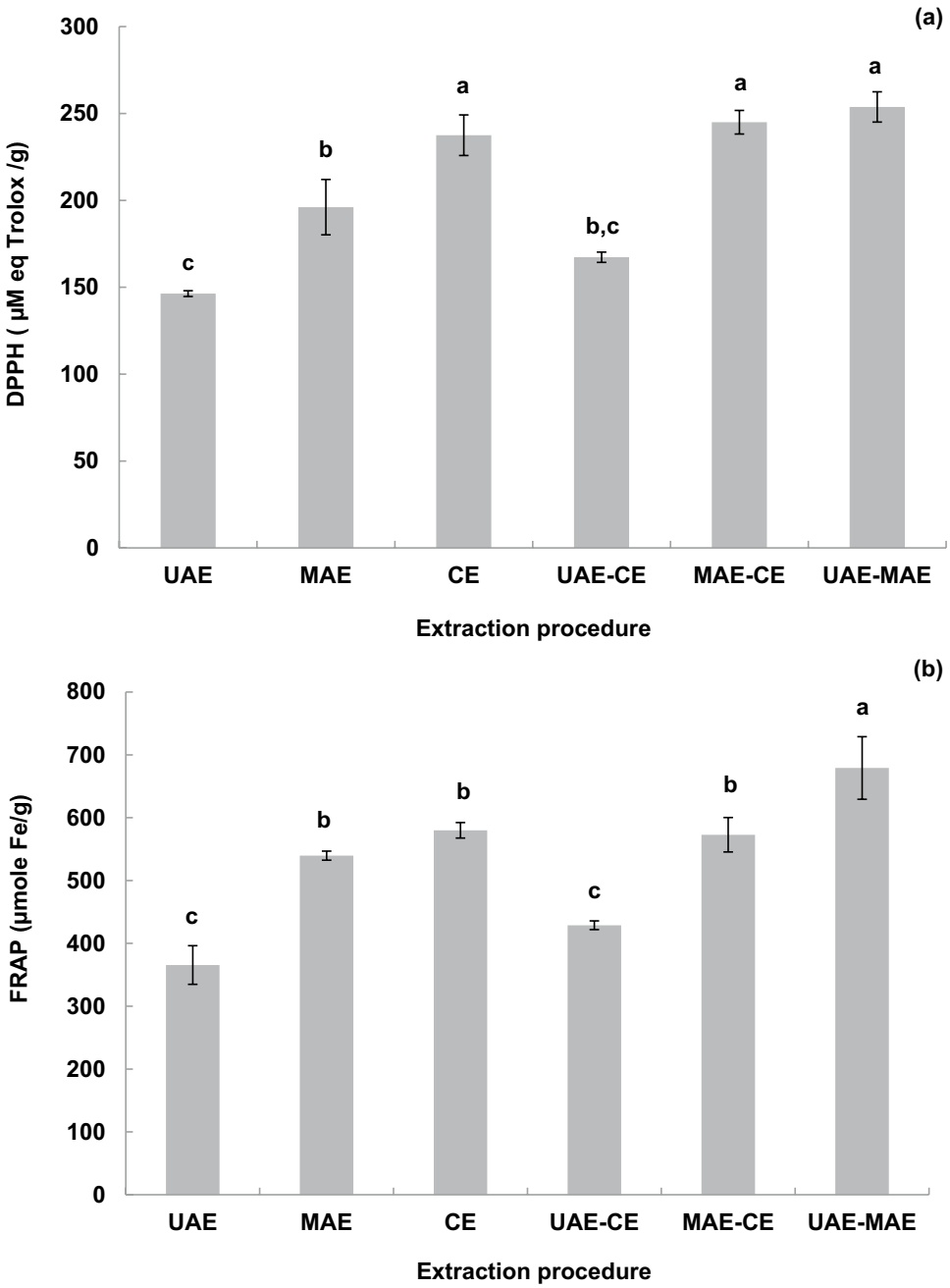

Fig. 4 Effect of extraction procedure on antioxidant activity of Roselle anthocyanin extract. (**a**) DPPH antioxidant activity. (**b**) FRAP antioxidant properties: UAE, ultrasound-assisted extraction, MAE, microwave-assisted extraction; CE, conventional extraction; UAE-CE, ultrasound-assisted extraction followed by conventional extraction; MAE-CE, microwave-assisted extraction followed by conventional extraction; UAE-MAE, ultrasound-assisted extraction followed by microwave-assisted extraction

3.4 Fourier transform infrared spectroscopy

The molecular structure of roselle anthocyanin extracts obtained by different extraction methods (UAE, MAE, CE, UAE-CE, MAE-CE, UAE-MAE) was determined by FTIR. The obtained spectrums indicated the presence of absorption peaks at different wave numbers as shown in Fig. 5. All extracts showed characteristic absorption peaks at 3346, 2936, 1644, 1046, and 890 cm^{-1}.

The broadband and strong intensity in the 3346 cm^{-1} region likely corresponds to the stretching of the O–H intermolecular bond in the hydroxyl function group indicating the presence of high concentration of phenolics in roselle anthocyanin extract [5, 35]. While, the peak at 2936 cm^{-1} is probably due to the bending vibration C-H and $-CH_2$ stretching of a methyl group [5]. The bands at 1644 cm^{-1} and 1416 cm^{-1} showed a strong absorption peaks and were possibly due to the stretching vibration of the C=C double bond of the aromatic ring in flavonoid compounds which is a characteristic absorption peak of phenyl [42]. Another significant band at 1046 cm^{-1} was related to the stretching vibrations of the C-C rings. An intense peak at 890 cm^{-1} could be attributed to aromatic ring vibration [37].

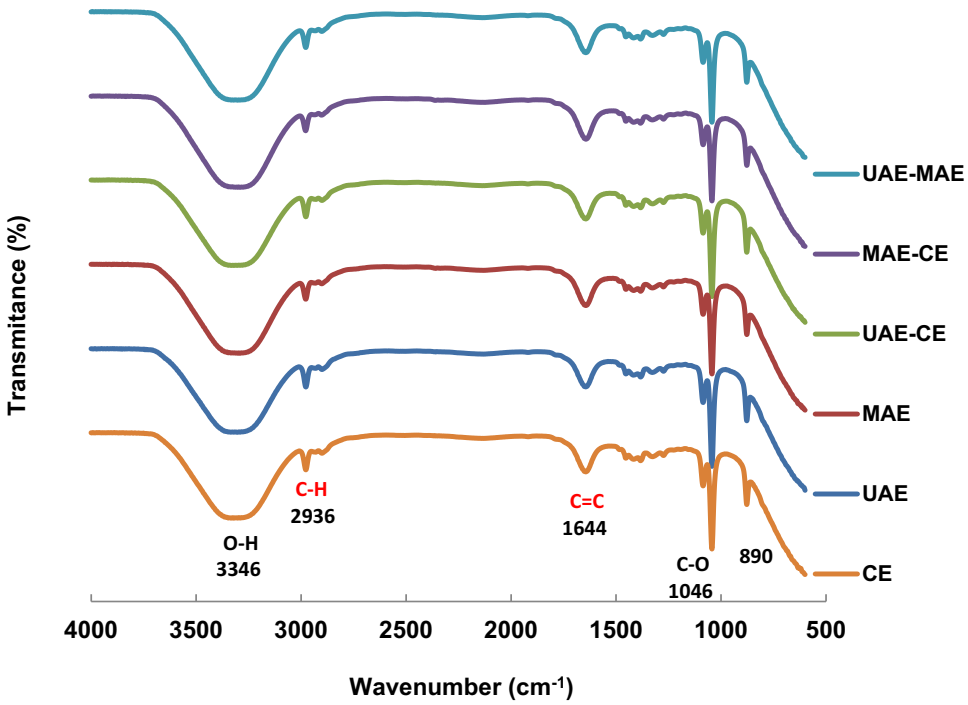

Fig. 5 Effect of extraction procedure on Roselle anthocyanin extract structural properties obtained by FTIR. UAE, ultrasound -assisted extraction; MAE, microwave-assisted extraction; CE, conventional extraction; UAE-CE, ultrasound-assisted extraction followed by conventional extraction; MAE-CE, microwave-assisted extraction followed by conventional extraction; UAE-MAE, ultrasound-assisted extraction followed by microwave-assisted extraction

The appearance of the intense peaks mentioned above proved the presence of phenolic and flavonoid components in anthocyanin roselle extract obtained by different extraction procedure. The functional groups obtained corresponded to the basic structure of anthocyanins. Moreover, the above results showed that the extraction procedure as well as the combination of two extraction methods did not influence the basic structure of roselle anthocyanins. Consequently, the combined UAE-MAE could be used as fast and efficient process for anthocyanins extraction from roselle flowers.

4 Conclusion

Nowadays, natural colorants become efficient alternatives to the extensively used artificial ones. Several innovative and conventional technologies were used to improve their extraction. Roselle flowers, calyces, stems, leaves, and seeds are promising sources of anthocyanins. In the present study, UAE, MAE, and CE and their combination UAE-CE, MAE-CE, UAE-MAE were successfully used for roselle flowers anthocyanins extraction. The best performances of UAE procedure were obtained with 60% aqueous ethanol, 5 min, and S/L ration of 1/10 (g/mL). MAE procedure required higher time 10 min and MAE power of 180 W using the same ethanol concentration and S/L ratio to obtain significantly ($p \leq 0.05$) higher TMA (16.34 ± 0.48 mg/g), TP (176.99 ± 9.11 mg GAE/g drf), and antioxidant activities. In the same condition, CE consisting of 24 h of continuous stirring at room temperature allowed obtaining 14.46 ± 0.47 mg/g of TMA and 182.89 ± 2.49 mg GAE/g drf of TP. In comparison to UAE and UAE-CE tried in this study, the combined UAE-MAE increased significantly ($p \leq 0.05$) TMA, TP, DPPH, and FRAP antioxidant activities, yielding 17.36 ± 0.54 mg/g of TMA, 208.28 ± 1.43 mg GAE/g drf of TP with the better antioxidant activities using DPPH (253.76 ± 8.71 µM eq trolox/g drf) or FRAP methods (679.13 ± 49.81 µmole Fe/g drf). When compared to CE, UAE-MAE reduced extraction time from 24 h (CE) to 15 min and significantly increased TMA contents by 20.06%, TP by 13.88%, and FRAP antioxidant activity by 17.11%.

FTIR showed that the structural properties of roselle anthocyanins obtained by UAE, MAE, CE, UAE-CE, MAE-CE, and UAE-MAE were preserved. Thus, a combination of two modern UAE-MAE extraction methods resulted in roselle extracts with both the highest concentration of bioactive compounds and the highest antioxidant activities, while reducing extraction time and increasing energy efficiency. This study could promote the widespread use of combined eco-friendly extraction such as UAE-MAE procedures for *Hibiscus sabdariffa* L. flowers anthocyanin extraction.

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Author contribution All authors contributed to the study conception and design. Material preparation, data collection, and analysis were performed by Habiba Boukerche, Nora Ghaliaoui, Nawel Saidji,

Ahmed Bensalem, and Fatiha Malki. The first draft of the manuscript was written by Habiba Boukerche. Writing—review and editing: Hind Mokrane and Habiba Boukerche. All authors commented on previous versions of the manuscript. All authors read and approved the final manuscript. Supervision: Hind Mokrane.

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Data availability All data and findings of this study are available from the authors upon reasonable request.

Declarations

Ethical approval Not applicable.

Competing interests The authors declare no competing interests.

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