Extraction and fractionation of anthocyanins from red cabbage: ultrasonic-assisted extraction and conventional percolation method

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Abstract

We extracted the anthocyanins from red cabbage through ultrasonic-assisted extraction and conventional percolation method. The effect of ultrasonic output power, ultrasonic operation time and temperature, as well as ultrasonic pulse duration on the extraction efficiency was investigated. As a conventional method, red cabbage leaves were percolated in water to obtain the crude extract. We showed that ultrasonic irradiations enhanced the extraction of anthocyanins significantly in comparison to the conventional percolation method. The optimum extraction efficiency was obtained at ultrasonic output power of 100 W, pulse mode of 30 s ON: 30 s OFF, at 15 °C for 90 min. Fractionation of crude extract was further carried out using three different concentration of ethanol in ion-exchange column chromatography HP_{20}. We demonstrated that the fraction obtained through the fractionation with ethanol/water (1:1) showed the highest antioxidant activity, anthocyanin content and phenolic compounds.

Graphical abstract

Extended author information available on the last page of the article

Practical application

The present study investigate the extraction of anthocyanins from red cabbage through conventional extraction method and ultrasonic-assisted extraction method. The results of optimum extraction parameters can be applied to food and drug industry to achieve the maximum extraction efficiency and higher quality of anthocyanins from the red cabbage waste. Moreover, the results of fractionation of the extracted anthocyanins can be applied as a valuable reference for food and drug industry, in order to substitute the synthetic preservatives and/or colorants with a natural anthocyanin source.

Introduction

Natural colorants are widely used in foods and beverages due to increasing consumer concerns about the safety of synthetic colorants [1]. Natural colorants are extracted from fruit and vegetable sources, such as saffron, berries, annatto, and beetroot [2–4]. Anthocyanin, a water-soluble flavonoid derived from red and blue parts of plants such as red cabbage leaves (RCL) [5], berries [6–8], and black carrots [9, 10], is one of the most commonly used colorants because of its vibrant color [11, 12]. Moreover, the phenolic compounds such as anthocyanins are reported to quench oxygen-derived free radicals, as well as the substrate derived free radicals. Thus, they can be applied in the food systems contained labile compounds to preserve their shelf life [13–16]. There is a growing demand for developing the techniques to increase the extraction efficiency and shelf life of bioactive compounds from the natural sources such as vegetables. For example, people use encapsulation techniques to preserve the labile bioactive compounds and increase their stability [17, 18]. One of these techniques is exploiting the ultrasonic irradiations, which increase the mass transfer of the bioactive compounds from the solid vegetable to the surrounding solution [19]. Other studies use different methods and different solvents to extract the anthocyanins from red cabbage, such as sandy porous medium enriched with clay, and acetic acid aqueous solution [20, 21].

In this study, the conventional extraction method and extraction using ultrasonic irradiations were applied to extract anthocyanins from red cabbage. We present the optimum extraction parameters such as time, temperature, ultrasonic output power, and pulse duration. We further fractionate the extract using ion exchange column chromatography to obtain a fraction with high antioxidant activity, phenolic compounds and anthocyanin content.

Materials and methods

Materials

All reagents were obtained from Merck Chemical Co., Darmstadt, Germany.

Experimental design

Extraction of anthocyanins from red cabbage using conventional percolation method

Red cabbage (*Brassica oleracea* L. Var. Capitata f. Rubra) leaves, obtained from the local market (Shiraz-Iran), were used for the extraction of anthocyanins. To extract the anthocyanins through the conventional percolation method, the RCL were lyophilised (Zirbus, Germany), and ground into powder (mesh < 18, 1000 microns). The lyophilised red cabbage powder (50 g) was soaked with 300 mL water under stirring to obtain a crude extract. To extract the anthocyanins through the ultrasonic-assisted extraction method, the lyophilized red cabbage powder in water was treated with ultrasonic system (Bandelin, GM 3200, Germany, 20 kHz and 150 W).

Investigation of ultrasound effect on the anthocyanin extraction from red cabbage

The effect of extraction time (0, 30, 60, 90, 120, 150 and 180 min), ultrasonic output power (50, 100 and 150 W), ultrasonic pulse duration (1 s on: 1 s off, 30 s on: 30 s off and 60 s on: 60 s off) and temperature (15, 30 and 45 °C) was investigated.

Fractionation of anthocyanins using ion exchange column chromatography

In order to fractionate the crude extract, a portion of crude extract (40 g) was dissolved in 80 mL distilled water and loaded on ion exchange column chromatography (HP$_{20}$) (length of 80 cm, internal diameter of 4.5 cm and external diameter of 5 cm). Elution was performed by water (4.3 L), ethanol/water (1:1) (4 L) and pure ethanol (12 L), respectively with the flow rate of 2 mL/min. All samples were concentrated using rotary-evaporation and lyophilized.

Anthocyanin quantification

The spectrophotometric pH differential method [5] was used to quantify the anthocyanins in the crude extract and the

fractions. Briefly, 0.4 mL of sample was mixed with 3.6 mL of 0.025 M potassium chloride (pH 1.0) and 0.4 M sodium acetate (pH 4.5). The absorbance at 519 nm against a distilled water blank was measured using a UV–Visible spectrometer (T-80+, PG Instruments Ltd, Korea). Anthocyanin content was calculated with the following equation:

$$A = A519 \, (pH \, 1.0) - A519 \, (pH \, 4.5)$$

$$Anthocyanin \ content \left(\frac{mg}{L}\right) = \frac{A \times MW \times DF \times 1000}{\varepsilon \times l}$$

where A is the absorbance, MW is the average molecular weight of anthocyanin (433.2 g/mol), DF is the dilution factor (10), ε is the extinction coefficient (31,600 L cm^{-1} mol^{-1}) and l is the path length (1 cm).

Determination of the antioxidant properties [nitric oxide (NO) radical scavenging]

Sodium nitroprusside solution (10 mM) was prepared using 20 millimolar phosphate buffer (pH 7.4). Crude extract and fractions (3200 µg/mL) was diluted to 12.5 µg/mL and 50 µL of each concentration was mixed with 50 µL sodium nitroprusside solution in 96-well micro-plate. The plate was incubated at 27 °C for 150 min and 100 µL Greiss solution was added immediately. The absorbance was read at 542 nm. Control was prepared using 50 µL nitroprusside solution, 50 µL solvent and 100 µL Greiss solution. The blank was involved just 50 µL of each sample [17].

$$\%Inhibition \ of \ NO \ production = \frac{A0 - A}{A0} \times 100$$

Here A_0 is the control absorbance and A is the sample absorbance–blank absorbance.

Determination of total phenolic content (TPC)

TPC of crude extract and fractions was determined spectrophotometrically using the Folin & Ciocalteu assay [22, 23]. 1 mL of crude extract and fractions (10 g/L) was mixed with 1 mL of 6 M HCl and 5 mL of 75% methanol/water solution in a screw-capped tube. The tube was vortexed and placed in a 90 °C water bath and shaken for 2 h. Then, the

mixture was diluted to 100 mL with distilled water. The absorbance versus prepared blank was read at 760 nm until it reached steady state. The same procedure was applied for six standard solutions of Gallic acid (0.03125, 0.0625, 0.125, 0.25, 0.5, 1, 2, 4, 8 and 16 mg of Gallic acid/mL of water). Final results were expressed as mg of Gallic acid per 1 g of sample.

Yield of extracts

In order to find out the yield of total extract from red cabbage, samples were filtered and placed in a clean, dried and pre weighed petri dish. The samples were dried in an oven at 105 ± 1 °C until all the water was evaporated. The petri dish was cooled in a desiccator prior to weighing. The weight of the extract obtained per gram of the red cabbage was evaluated. The percentage yield of total extract was determined by the following formulation:

$$\% \ Yield \ of \ total \ extract = \frac{Total \ extract \ obtained \ (g) \times 100}{Amount \ of \ red \ cabbage \ used \ (g)}.$$

The yield of extracted anthocyanin was established by the following formulation:

$$\% \ Anthocyanin \ yield = \frac{Anthocyanin \ obtained \ (g) \times 100}{Amount \ of \ red \ cabbage \ used \ (g)}.$$

Maximum extractable anthocyanin (MEA)

In order to assess the MEA, 50 g of red cabbage was extracted in a high shear mixer at ambient temperature. The solid was washed with 1000 mL of distilled water to extract as much anthocyanins as possible in the RCL. Total amount of anthocyanin per gram of red cabbage was determined using spectrophotometric pH differential method [24]. This was taken as MEA. The extraction efficiency therefore is evaluated as:

$$Extraction \ efficency \ (\%) = \frac{\% \ Anthocyanin \ yield \times 100}{\% \ MEA \ yield}$$

The enhancement due to ultrasound (US) in % anthocyanin yield as compared to water bath (WB) process was calculated using the following formula:

$$Enhancement \ in \ \% \ yield \ of \ anthocyanin = \frac{\% \ Anthocyanin \ yield \ for \ US}{\% \ Antocyanin \ yield \ for \ WB} \times 100$$

tube was allowed to cool to room temperature and diluted to a 10 mL volume with distilled water. One millilitre of this solution was mixed with 5 mL of previously tenfold diluted Folin & Ciocalteau reagent. 15 mL of Na$_2$CO$_3$ (7 g/100 mL) was added to this mixture to produce basic conditions. The

Statistical analysis

Data was subjected to analysis of variance (ANOVA) using SPSS software package version 15.0 for windows. Mean comparisons were performed using one sample T-test and Post

Hoc Multiple Comparison the Duncan test to examine if differences between samples were significant at $p < 0.05$. Pearson correlation 2-tailed between the measured parameters was investigated. All experiments were performed in triplicate.

Result and discussion

Anthocyanin extracts obtained from ultrasound-assisted extraction

The red cabbage extract was purple at pH of 6.3 ± 0.2. The UV–Vis spectrum of red cabbage extract is shown in supplementary Fig. 1. The main peak (λ_{max}) at pH = 6.3 ± 0.2 was obtained at 554 nm. The result was in agreement with the finding of other investigators [25]. Absorbance at the maximum wavelength ($\lambda_{max} = 554$ nm) was recorded. Then the amount of anthocyanin present in the extract was calculated from pH differential method. The calibration curve for the red cabbage anthocyanins was then plotted (Supplementary Fig. 1). From the calibration curve, a linear fit with equation y = 0.008x + 0.002 was obtained, where, Y axis is the absorbance value at 554 nm and X axis is total anthocyanin content in mg/L, and the anthocyanin content in the extracts (in mg/L) was calculated. The results indicated that MEA obtained from
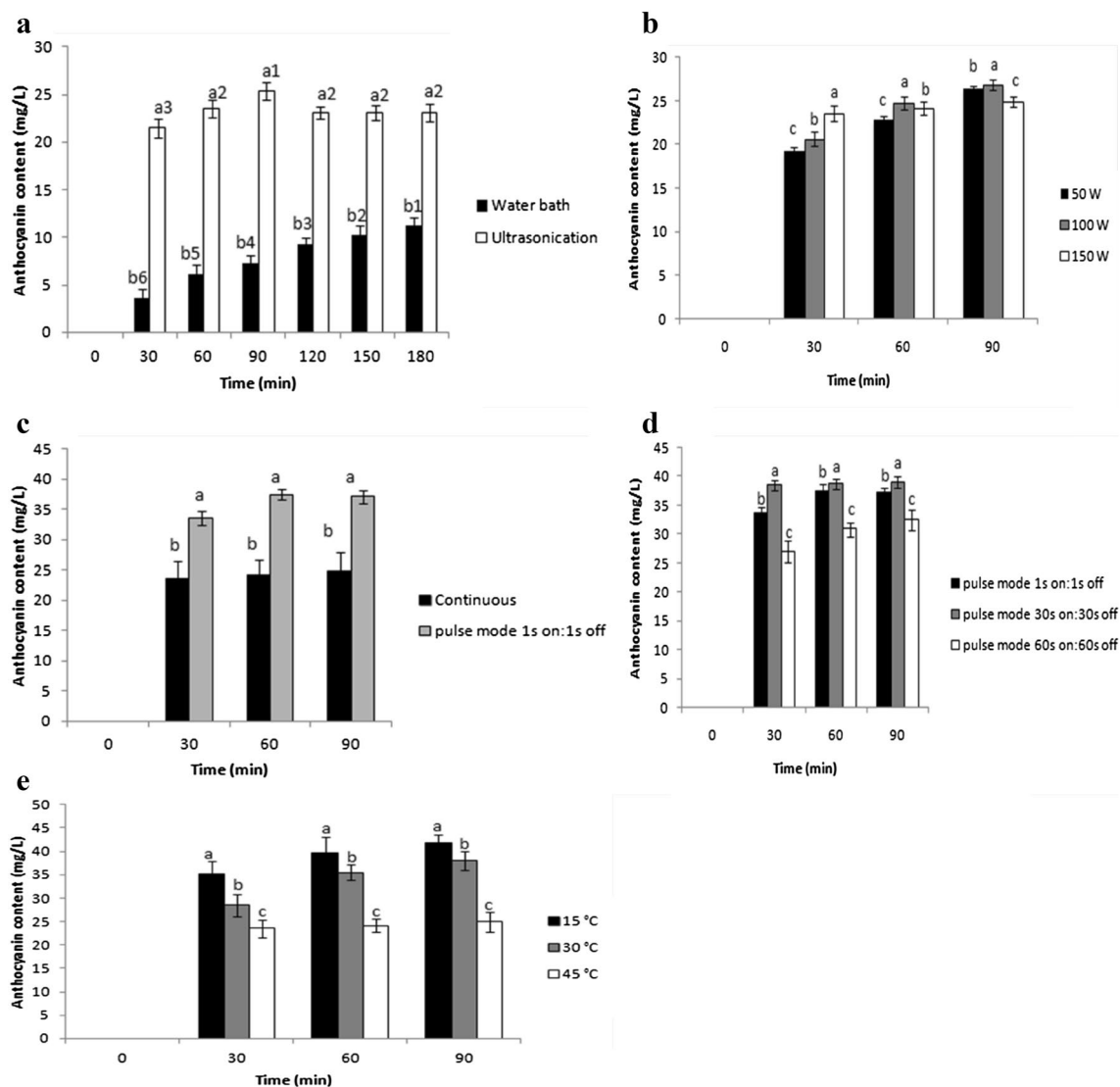

Fig. 1 **a** The effect of ultrasound (150 W, 45 °C, 180 min, continuous pulsation), **b** ultrasonic output power (45 ± 1 °C, 90 min, continuous pulsation), **c** ultrasonic pulse mode (45° ± 1 °C, 90 min, 150 W), **d** pulsation duration (45 ± 1 °C, 90 min, 150 W), **e** temperature (90 min, 150 W, continuous pulsation) on the extraction of antho-cyanin from red cabbage. Data are the means ± SD, ($p < 0.05$). Significant difference ($p < 0.05$) between data is expressed by different letters "a", "b" and "c". Increasing the number of letters in Fig. 1a shows the order of anthocyanin content from the highest to lowest level, respectively

2 g of red cabbage through the WB extraction (45 °C) is 31.08 ± 0.02 mg/L (data not shown). The results indicate that there is a significant enhancement in anthocyanin yield and extraction efficiency when complementing the extraction process with the ultrasound (150 W output power, 45 °C) (Fig. 1a). The anthocyanin yield was almost doubled in ultrasonic assisted process in comparison with WB extraction method (Supplementary table 1). This increase could be because of rupturing the membrane of anthocyanoplasts by ultrasonic irradiations and simple release of anthocyanins, while with regard to hot WB extraction, anthocyanins might be discharged through the concentration gradient. Sivakumar et al. [26] demonstrated a significant increase in betalain (red coloured cyanins) extraction from beetroot in comparison with the magnet stirrer extraction method. The results show that increasing the extraction time increases the anthocyanin yield, and the maximum yield was obtained at 90 min for ultrasound-assisted extraction (Fig. 1a). Longer extraction resulted in reduction of anthocyanin yield, since the phenolic compounds such as anthocyanins could decompose by increasing the exposure time to ultrasonic irradiations (Fig. 1a). Thus, we continued all experiments to maximum time of 90 min. The effect of various ultrasonic power 50–150 W on extraction was also studied. As it is shown in supplementary table 2, the yield of total extract increased as power increased. The kinetics of extraction for different ultrasonic power is shown in Fig. 1b. The anthocyanin yield and extraction efficiency were maximum at 100 W output power. There is excessive energy dissipation in the form of heat when 150 W is applied, which in turn may lead to degradation of anthocyanins. The anthocyanin content achieved at 100 W output power was very close to MEA of 31.08 (mg/L). Sivakumar et al. [26] reported the power of 100 W as the best power of betalains extraction from beetroot. In order to reduce the electrical energy consumption, experiments were conducted with ultrasonic pulse mode operation of one second ON, one second OFF (1 s ON: 1 s OFF). Interestingly, ultrasonic pulse mode is better in terms of anthocyanin extraction as compared to continuous mode as shown in Fig. 1c. Extraction efficiency in the case of applying pulse mode is almost 1.5 fold more than that of the continuous mode (Supplementary table 3). The reason for the improvement in extraction with ultrasonic pulse mode can be due to non-steady state mass transfer as compared to continuous mode. Hence pulse mode operation of ultrasound is found to increase the extraction efficiency as well as the energy efficiency of the process. Experiments were carried out to find the effect of pulsation duration in relation to extent of extraction efficiency. Three duration of ultrasonic pulsation were used: 1 s ON: 1 s OFF, 30 s ON: 30 s OFF, 60 s ON: 60 s OFF. The total anthocyanin content in the extract obtained

from red cabbage increased by increasing the duration of pulsation from 1 s intervals to 30 s intervals, as shown in Fig. 1d. Two explanations may be offered to discuss these results. On the one hand, increasing the pulsation duration can permit non-steady state mass transfer from anthocyanoplasts to solvent. On the other hand, increasing the pulsation duration can decrease the temperature rise particularly on the sonotrode head. Sonotrode high temperature, in turn, may cause destruction of anthocyanins. The extraction efficiency, anthocyanin yield and yield of total extract for three modes of pulsation are given in supplementary table 4. When the ultrasound system was set to 60 s intervals the anthocyanin extraction decreased (Fig. 1d). Therefore, pulsation duration more than 30 s On: 30 s Off, has an offensive effect, because the exposure time of ultrasonic irradiations is not enough to break down anthocyanoplasts and release anthocyanins. In order to compare the thermal effect of the ultrasonic assisted extraction process, experiments were conducted in temperatures of 15, 30, and 45 °C. The highest extraction efficiency and yield of anthocyanin were achieved at low temperature of 15 °C (Fig. 1e). It was previously reported that at higher operating temperatures the yield of total extract is improved [26]. In this study, the % yield of total extract increases by increasing the temperature, while the higher temperature decreased the extraction efficiency and anthocyanin yield (Supplementary table 5). This is due to high sensitivity of anthocyanin to heat and probable degradation of anthocyanins through increasing the temperature. Anthocyanin is rather unstable in varying temperature and pH [25].

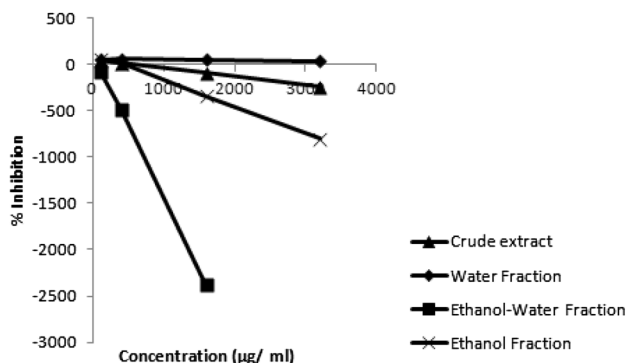## Anthocyanin extracts obtained from conventional percolation method

RCL contains $92.11 \pm 0.21\%$ moisture (wet-based). Freeze drying process reduced the moisture content to $9.8 \pm 0.2\%$. The yield of dried leaves subsequent to freeze drying was determined ~ 8.7%. We reported in our previous work that the extraction using percolation method resulted in crude extract with a yield of ~ 57.5% [5]. Moreover, fractionation of crude extract through chromatography (HP_{20} resin) using water, water–ethanol (50:50) and ethanol resulted in the yield of ~ 92.4, 3.6 and 3.9%, respectively [5]. We also reported that the water–ethanol fraction was determined as the most concentrated fraction which was rich in anthocyanin. Total anthocyanin content of ethanol–water fraction was about fourfold higher than that of crude extract. The yield of anthocyanin in crude extract, water fraction, ethanol–water fraction and ethanol fraction was ~ 1.0, 0.06, 4.13 and 0.17%, respectively.

The results of NO radical scavenging related to crude extract and three fractions have been shown in Table 1. In

Table 1 IC_{50} calculated for NO radical scavenging, and TPC in crude extract and fractions from red cabbage

Sample	IC_{50} (µg/mL) $\pm$ SD	Total phenolic compounds (mg Gallic acid/g of sample) $\pm$ SD
Crude extract	128.21 ± 5.97^b	28.23 ± 4.91^b
Water fraction	1813.74 ± 167.21^d	4.59 ± 0.78^d
Water–ethanol fraction	42.33 ± 6.23^a	425.23 ± 9.64^a
Ethanol fraction	215.90 ± 40.87^c	23.46 ± 5.21^c

The small letters show a significant difference at level of $p < 0.05$

Fig. 2 NO radical scavenging% for crude extract and three fractions from red cabbage

Table 2 Pearson's correlation coefficients (r) among some investigated parameters of red cabbage

	Anthocyanins	Total phenolic compounds	IC_{50}
Anthocyanins	1	0.973**	-0.751**
Total phenolic compounds	0.973**	1	-0.651*
IC_{50}	-0.751**	-0.651*	1

*Correlation is significant at the 0.05 level (2-tailed)

**Correlation is significant at the 0.01 level (2-tailed)

order to calculate IC_{50}, the graph of radical scavenging% was drawn versus concentration of each sample (Fig. 2). The inhibition% of NO production decreased by increasing the concentration. The most NO radical scavenging was related to ethanol–water fraction and crude extract, respectively (Table 1). Ethanol fraction revealed lower NO radical scavenging and water fraction antioxidant effect was negligible (Table 1). These results were in agreement with the results of DPPH radical scavenging in our previous work [5]. Fractionation could improve the purity of the bioactive compounds. The most antioxidant properties were in water–ethanol fraction, which contained the most anthocyanin. Chigurupati et al. [25] reported that the most bioactive compounds in red cabbage are anthocyanins. The antioxidant activity of phenolic compounds is mainly due to their redox properties, which can play an important role in adsorbing and neutralizing free radicals, quenching singlet and triplet oxygen or decaying peroxides [27]. The water fraction was observed to have the highest amount of sugar in comparison with the other fractions. Water fraction and ethanol fraction could not exhibit any valuable radical scavenging (Fig. 2). Recent researches have reported IC_{50} of several plants. Few studies indicated that active biomolecules are distributed in defined fractions by several kinds of fractionation procedure [28]. The crude extract of *Zhumeria majdae* showed the IC_{50} of 400 ± 15 µg/mL and this was 40 ± 4 µg/mL for ethyl-acetate fraction as the most active fraction [29].

Total phenolic compound was measured using the Gallic acid standard curve (Supplementary Fig. 2). Table 1 shows the TPC of crude extract and three fractions from RCL. The highest TPC was found in ethanol–water fraction (425.238 ± 9.646 mg Gallic acid/g of sample) and this amount was about 15-fold higher than that of crude extract (28.239 ± 4.918). Moein et al. [30] investigated total phenolic compounds in *Salvia mirzayanii* and reported that in the most active fraction, TPC was 55.23 ± 4.20 mg/g of sample. RCL is an excellent source of TPC. The amount of TPC extracted from RCL is almost 8 times that of *Salvia mirzayanii*. The yield of total phenolic compounds in crude extract, water fraction, ethanol–water fraction and ethanol fraction was ~2.82, 0.458, 42.51, and 2.341%, respectively. Pearson's correlation coefficients between the means of each variable were computed (Table 2). Statistically significant correlation coefficient ($p < 0.01$), ($p < 0.01$) and ($p < 0.05$) were found between anthocyanin and total phenolic compounds ($r^2 = 0.973$), and between IC_{50} of DPPH radical scavenging and total anthocyanin content ($r^2 = -0.751$), as well as between TPC and IC_{50} of DPPH radical scavenging ($r^2 = -0.651$). Correlation coefficients were significantly high among antioxidant capacity values (IC_{50}) and anthocyanin content (Table 2). Therefore, anthocyanins play an important role in exhibiting antioxidative properties. High correlation coefficient of total anthocyanin content and total phenolic compounds indicates that major phenolic compounds in RCL are anthocyanins. In this study, it was found a significant correlation between total phenolic compounds and antioxidative properties, and increasing phenolic compounds resulted in increasing antioxidant activity.

The relationship between the antioxidant activity and phenolic compounds depends on several factors such as chemical structure of individual component, synergistic interaction among them and specific conditions applied in different assay [31]. The results were in agreement with findings of Abeysinghe et al. [32] and Anagnostopoulou et al. [33]. Moein and Moein [29] found that antioxidant property of *Zhumeria majdae* was correlated with phenolic compounds. Correlation coefficient ($r^2 = -0.65$) between phenolic compounds and DPPH radical scavenging found in the present study was in agreement with the results of another study on *Zhumeria majdae* [30].

Conclusion

RCL is an excellent source of phenolic compounds, anthocyanins and antioxidants. Ultrasonic irradiations can be a first-class extraction method, as it has the potential of making cavitation in cells. Therefore, it is anticipated that the active compounds such as anthocyanins extracted from anthocyanoplasts more efficiently. Ultrasound influences significantly on the extraction process, while the best anthocyanin yield is obtained at 15 °C for 90 min, with an output power of 100 W in pulsation mode of 30 s ON: 30 s OFF.

Ethanol–water fraction has the highest antioxidant activity, expressed by its capacity to scavenge free radicals of DPPH and NO, as well as phenolic compounds and anthocyanin content. Thus, red cabbage is an excellent source of anthocyanins which may be used as natural colour and preservative in the food and drug industries.

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