

Heat, ultrasound, and microwave assisted extraction methods for recovering bioactive components from hawthorn fruit (*Crataegus monogyna* Jacq.)

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ABSTRACT

In this work, an assessment of effective solvents and extraction methods was carried out to recover the bioactive compounds from hawthorn fruit (*Crataegus monogyna* Jacq.). Extractions assisted by heat, microwave, and ultrasound were carried out using various organic solvents (methanol, ethanol, and isopropanol). pH differential, Folin–Ciocalteu's, and aluminum chloride methods were used to determine total monomeric anthocyanin (TMA), total phenolic compound (TPC), and total flavonoid content (TFC), consecutively. Ferric Reducing Antioxidant Power (FRAP), 2,2-Diphenyl-1-picrylhydrazyl Hydrate (DPPH), and 2,2'-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) assays were used to measure the antioxidant activity (AA) of the extracts. The outputs revealed that extraction methods and solvents significantly affect anthocyanin concentration, TPC, TFC, AA, and color values of hawthorn fruit extracts. Due to the highest recovered TMA (0.152 ± 0.002 mg ECy3Gl/g of dry weight), TPC (49.14 ± 0.38 mg gallic acid equivalents/g of dry weight), and TFC (18.38 ± 0.19 mg quercetin equivalents/g of dry weight) contents, the ultrasonic-assisted extraction is superior to heat and microwave-assisted extractions. Accordingly, it was also observed that the methanol solvent is more profound than ethanol and

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isopropanol. Further, the bioactive compounds' content and the extracts' antioxidant activity are shown to be highly correlated. Thus, hawthorn extracts are considered to have antioxidant properties because of their concentrated bioactive compounds.

KEYWORDS

hawthorn (*Crataegus monogyna* Jacq.), anthocyanin, heat-assisted extraction, ultrasound-assisted extraction, microwave-assisted extraction

INTRODUCTION

Food and beverage color is crucial for recognition and acceptance. Artificial colorants are often used to achieve the right hue, but their usage has decreased in the last few decades due to concerns about human health and potential cancer risks (Backes et al., 2018). As consumers demand natural products, food processors are now turning to naturally derived colorants like anthocyanins, water-soluble flavonoids found in vegetables and fruits (Backes et al., 2018). Depending on their pH, anthocyanidins can be reddish, pinkish, or orange (Piyapanrungrueang et al., 2016). Anthocyanidins are known for their health benefits as antioxidants and anti-inflammatories (Ribárszki et al., 2022; Joseph et al., 2014). Studies have shown that anthocyanins can reduce the occurrence of heart or blood vessel diseases, glucose-related issues, and cancer. Consuming anthocyanin-rich fruits has also been shown to improve clinical and biomedical outcomes in voluntary people with various health conditions (Fang, 2015).

Among its many health benefits, hawthorn is a fruit high in ascorbic acid, anthocyanins, and flavonoids (Liu et al., 2018). It has a long history of treating a variety of diseases, including cardiovascular conditions and liver damage (Kim et al., 2022). Generally, the extraction procedure is determined by the color's nature, the material's source, and the solvent used to extract it. All extraction methods aim to obtain maximum recovery of biologically active composites with minimum supplement and minimum disintegration or change in their natural form (Patil et al., 2009).

Besides the conventional extraction methods, several emerging techniques have been used such as ultrasound. Where UAE has been used as a new green technology to extract carotenoids from the by-products of fruits and increased the yield by around 143% compared to conventional extraction (Luengo et al., 2014). Another study showed that significant increase in yield for the extraction of natural color from Green wattle bark, Marigold flowers, and Pomegranate rinds (Sivakumar et al., 2011). In addition, UAE has been used to extract various compounds from vegetables and fruits such as lipids, aromas, antioxidants, and pigments (Geow et al., 2021). The increase in the extraction yield by UAE can be attributed to multiple mechanisms such as the generation and collapse of cavitation bubbles (Vilkhu et al., 2008), increased swelling index of the plant tissue matrix which helps in both the desorption and diffusion of solutes (Dezhkunov et al., 2004), increased the solvent absorption of the pomace thereby enhancing the accessibility of solvent to the bioactive compounds to be extracted (Pingret et al., 2012).

Microwave energy extraction (MAE) involves the direct impact of microwaves on molecules through dipole rotation and ion conduction. Microwaves are used for extracting phenolics, as



they align molecules according to the applied electric field as a result of the rotation of partial negative and positive charges present on the polar molecule (Cassol et al., 2019). This motion and friction transform electromagnetic waves into thermal energy, increasing plant matrices' temperature and disrupting cell wall structure. This enhances extraction yield with reduced solvent, time, and energy, making microwaves a potential tool for phenolic compound extraction. MAE has been applied to extract soybean and rice bran (Terigar et al., 2011). As well as for the extraction of polyphenols from olive tree leaves (Şahin et al., 2017). In addition, the microwave solvent-free method was used for the extraction of flavonoid content from onion (*Allium cepa* L.) (Zill-e-Huma et al., 2009). To evaluate the impact of extracting-solvent and techniques on the extraction process from hawthorn fruit, three methods: UAE, MAE, and HAE, together with three solvents (methanol, ethanol, and isopropanol) were compared in the current investigation.

RAW SUBSTANCES AND EXTRACTION TECHNIQUES

The fruits of hawthorn trees have been collected from several trees located throughout Hungary. Following the removal of the sticks, the fruits were washed, cleaned, wiped to remove excess water, and then shredded using (Retsch GM200 Blademill, Germany) pulverizers.

To prepare the working solvents, 80% (v/v) of each organic solvent, 19.9% (v/v) of water, and 0.1% (v/v) of hydrochloric acid (HCl) were mixed together. After that, each solvent was diluted with pure water to the concentration of 50% (v/v) before being used for extraction.

EXTRACTION METHODS

- Heat-assisted extraction (HAE)

The heat-assisted extraction process was carried out by (OS20-S Electric LED Digital Over-head Stirrer, Scilogex LLC). 10 g of ground hawthorn fruit was placed with 100 mL of prepared solvent in a double-walled tank connected to a (Lauda Ecoline E100 Immersion, Germany) thermostat to keep the temperature at 65 °C for 30 min was set by a timer.

- Microwave-assisted extraction (MAE)

Microwave extractions were conducted using home microwave ovens (Electrolux EMM 2005, Hungary) at a magnetron frequency of 2.45 GHz and microwave power of 800 W. To prevent superheating of the solvent and evaporation, microwaves were operated in pulse mode and cooled in between with icy water. Based on the pretest, 40 s on and 20 s off were considered, followed by 20 s on and 20 s off (until the time was up (10 min)

- Ultrasound-assisted extraction (UAE)

The ultrasound-assisted extraction (UAE) was carried out by power ultrasound (3.5 W cm^{-2} , 20 kHz) produced by a generator (Weber ULC 400 Premium Ultrasonic Generator, Germany) with a treatment time of 30 min, which was set by a timer. The ground fruit sample (10 g) was placed in a flask with the previously prepared solvent. To stabilize the heat distribution throughout the treatments, an icy water bath was used maintaining the temperature at around 25 °C.



TOTAL MONOMERIC ANTHOCYANIN CONTENT (TMA)

The TMA content in the unrefined extract was evaluated utilizing the differential-pH system reported by [Giusti and Wrolstad \(2001\)](#) to evaluate the TMA content in unrefined extracts. One buffer solution was prepared with (0.025 M potassium chloride) with a pH 1.0, while the other buffer solution was prepared with (0.4 M sodium acetate) with a pH 4.5. These two solutions were modified with (0.1) N of HCl. Genesys 5 UV-visible (MILTON ROY, USA) spectrophotometer has been utilized to evaluate the absorbance at 530 and 700 nm. All samples were analyzed three times. Equation (1) was used to calculate the TMA, and the units of outputs have been as milligrams of cyanidin-3-glucoside/gram of dry weight (ECy3Gl/g of dry weight).

$$TMA = \frac{A \times M_w \times DF \times L}{\epsilon} \quad (1)$$

“where $A = [(A_{530} - A_{700}) \text{ pH } 1.0 - (A_{530} - A_{700}) \text{ pH } 4.5]$, M_w is the molecular weight of anthocyanin (449.2 g per mole), DF is the dilution factor-100, ϵ is the molar absorptivity coefficient (26,900 centimeter / milligram for anthocyanin), and L is the path length cuvettes (one centimeter)”.

TOTAL PHENOLIC COMPOUNDS (TPC)

The TPC has been evaluated utilizing Folin–Ciocalteu’s reagent ([Mardokić et al., 2023](#); [Singleton and Rossi, 1965](#)). Gallic acid in 50% (v/v) methyl alcohol was used as a standard, and the calculation formula of the curve was as follows: $y = 0.1503x + 0.0606$; $R^2 = 0.9801$. The outputs are recorded as gallic acid equivalent/gram of dry weight (GAE/g dw).

TOTAL FLAVONOID CONTENT (TFC)

UV-Vis spectrometer analysis has been used to measure the TFC of food extracts ([Zin and Bánvölgyi, 2021](#); [Floegel et al., 2011](#)). Colored flavonoid–aluminum complex absorbance has been evaluated immediately at 510 nm against a blank. The quercetin has been used to create the calculation curve, and the calculation formula was ($y = 0.0664x + 0.1379$; $R^2 = 0.9811$). The TFC of the extraction-outputs has been expressed as mg quercetin equivalent/gram of dry weight (QUE/g dw).

ANTIOXIDANT ACTIVITIES (AA)

- Ferric Reducing Antioxidant Power (FRAP) assay

According to [Benzie and Strain \(1996\)](#), antioxidant activities were measured using the FRAP assay. An absorbance measurement was conducted at 593 nm compared with a blank. Using ascorbic acid ($C_6H_8O_6$) as a standard, the outputs have been recorded as mg of ascorbic acid equivalent/gram of dry weight (AAE/g dw). The calculation curve derived from the following calculation formula: ($y = 0.116x + 0.0797$; $R^2 = 0.9891$).



- 2,2-Diphenyl-1-picrylhydrazyl Hydrate (DPPH) assay

The DPPH assay has been carried out according to Blois's technique (1958). The absorbance has been evaluated against a blank at 515 nm utilizing a UV-Vis spectrometer analysis. The 2,2- Diphenyl-1- picrylhydrazyl radical has been evaluated as the percentage inhibition of 2,2- Diphenyl-1- picrylhydrazyl (I%).

- 2,2'- azino- bis (3-ethylbenzothiazoline-6- sulfonic acid) (ABTS) assay

ABTS assay has been carried out utilizing the technique of (Re et al., 1999). The required solution of 2,2'- azino- bis(3-ethylbenzothiazoline-6- sulfonic acid) has been prepared with an absorbance of 0.70 ± 0.02 at seven hundred and thirty-four nanometres. A control has been produced by blending ABTS solution with deionised water in the ratio of 1:1 mL. A UV-Vis spectrometer analysis has been used to evaluate absorbance at 734 nm. 2,2'- azino- bis(3-ethylbenzothiazoline-6- sulfonic acid) scavenging activity has been evaluated as % inhibition (I%) for each extract.

COLOR VALUE ANALYSIS

Various color systems can be used for instrumental color analyses. The system proposed by the International Commission on Illumination (CIE) in 1976, based on three-dimensional color space the three axes are L^* , a^* , and b^* (Fig. 1). The L^* value is a measure of the lightness of an object and is quantified on a scale such that a perfect black has an L^* value of zero and a perfect reflecting diffuser an L^* value of 100. The a^* value is a measure of redness (positive a^*) or greenness (negative a^*). The b^* value is a measure of yellowness (positive b^*) or blueness (negative b^*). The a^* and b^* co-ordinates approach zero for neutral colors (white, greys) and increase in magnitude for more saturated or intense colors (Joiner, 2004).

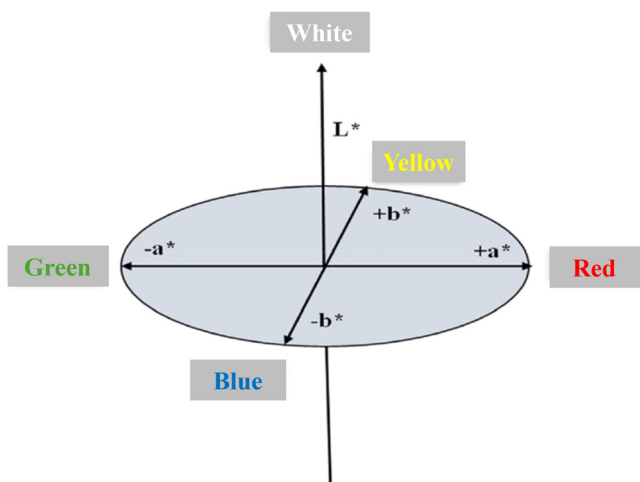


Fig. 1. CIE Lab colour space



Color determinations were made by a Minolta Chroma meter CR-400 at $20 \pm 2^{\circ}\text{C}$. A sample cup for reflectance measurements was used (5.9 cm internal diameter $\times$ 3.8 cm height) with a path length of light of 10 mm. Before the measurement, the colorimeter was standardized using a cup filled with distilled water against a reference white background. Color analyses were run in five replicates to obtain the results reported.

STATISTICAL ANALYSIS

Data have been taken from three separate investigations and expressed as mean $\pm$ standard deviation (SD). SPSS-IBM V.27.0 software has been used to determine significant differences ($P \leq 0.05$) between the means.

RESULTS

Impact of extraction technique and type solvent types on TMA content

For the significance test, two-way ANOVA was used, and then Tukey’s honest significance test was applied, taking (p) less than or equal to 0.05 as statistically significant. As plotted in (Fig. 2), both the extraction methods and solvent have significant effects on the total anthocyanins content. The optimal amount of TMA (0.152 ± 0.002 mg ECy3Gl/g of dry weight) was obtained via ultrasound extraction technique using methanol solvent while (0.125 ± 0.007 mg ECy3Gl/g of dry weight, 0.107 ± 0.007 mg ECy3Gl/g of dry weight) using extraction via microwave and extraction via heat as the extraction methods and methanol as solvent were used.

Similar findings have been reported by other studies. For example, ultrasonic extraction at the 1:30 solvent-to-liquid ratio was found to be a successful extraction method for saffron bioresidues. This method’s advantages over conventional solid-liquid extraction or microwave extraction included reduced extraction times and higher yields (Da Porto and Natolino, 2018). Furthermore, a significant difference ($P < 0.0001$) has been recorded in the anthocyanin yield of

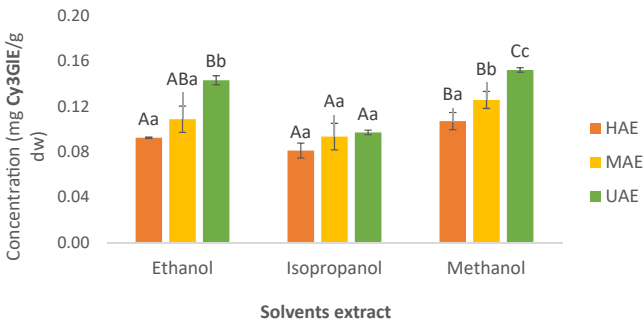


Fig. 2. TMA of the extracts expressed in mg Cy₃GlE/g dw obtained with different extraction methods and solvents

Upper cases for e.g. A, B, C... = significant differences between solvent with each extraction method.
Lower cases for e.g. a, b, c... = significant differences between extraction methods with each solvent



Australian blueberry among the three extraction methods (UAE, the Geno grinder, and the Dounce tissue grinder), where both the Geno and Dounce grinding methods showed a slightly lower anthocyanin yield than the UAE method (Singh et al., 2022). In addition, the anthocyanin concentration using UAE to extract blood fruit improved by 6.19%–10.28%, contrasted to that of conventional extraction (CE) (Sasikumar et al., 2021).

Impact of extraction technique and solvent types on the color values

Ultrasonic extracts were distinguished by darker color compared with both microwave and heat extracts using the same solvents, as the L^* values were found to be less for UAE with methanol (42.14 ± 0.19), ethanol (43.89 ± 0.23) and isopropanol (45.83 ± 0.015) solvents, respectively (Fig. 3).

Escalated a^* values of UAE were 24.56 ± 0.45 , 22.94 ± 1.16 , and 20.09 ± 0.29 for methanol, ethanol, and isopropanol indicating more intense color than MAE (18.05 ± 0.55 , 17.78 ± 0.02 , and 17.25 ± 0.6) and HAE (8.3 ± 0.2 , 6.91 ± 0.14 , and 3.25 ± 0.5) (Photo 1), shows the color differences between the samples.

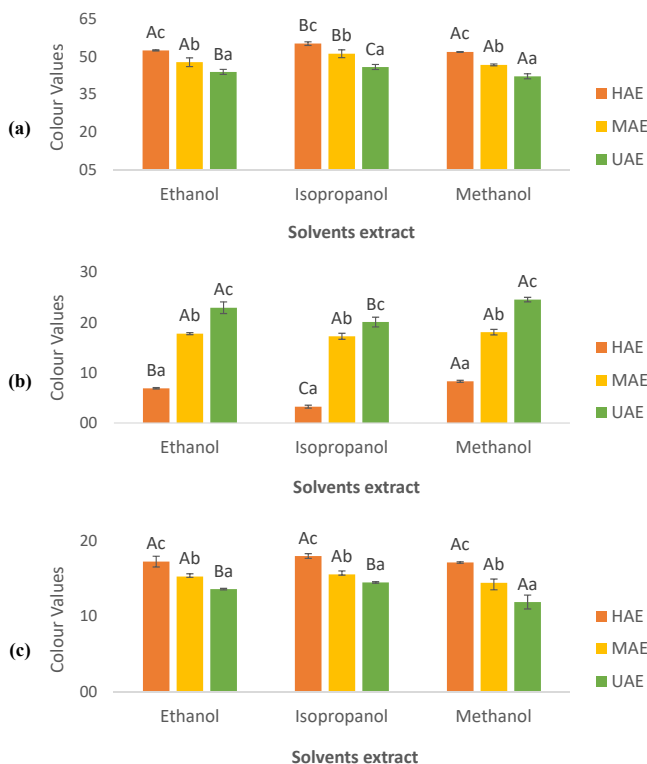


Fig. 3. Comparison of color values of hawthorn extract obtained with different extraction methods and solvents; (a) L^* , (b) a^* , (c) b^* .

Upper cases for e.g. A, B, C... = significant differences between solvent with each extraction method.

Lower cases for e.g. a, b, c... = significant differences between extraction methods with each solvent



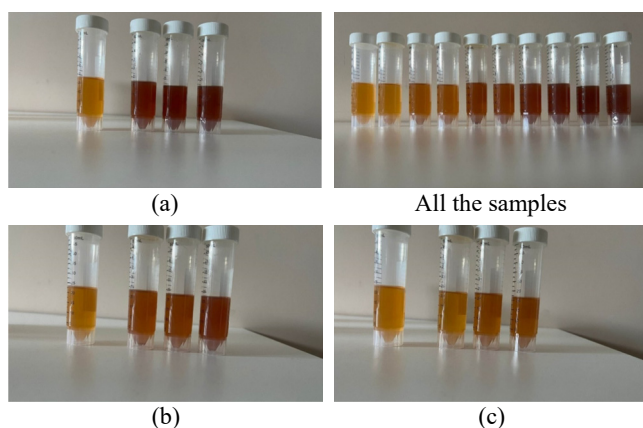


Photo 1. Hawthorn extracts by different extraction methods and solvents (a: UAE, b: MAE, c: HAE). The first sample from the left is the control sample followed by isopropanol, ethanol, and methanol samples

These results are accommodated by [Sasikumar et al. \(2021\)](#), who claimed that the ultrasonic blood fruit extracts with a variety of solvents were marked by a darker color than CE with the same solvents. Likewise, [Sharma et al. \(2021\)](#) also noted significant differences (p less than 0.05) between pumpkin (peel and pulp) extracts obtained from green extraction and conventional extraction. [Nguyen and Pirak \(2019\)](#) reported similar results for UAE versus CE for white dragon fruit peels.

Impact of extraction technique and solvent types on TPC and TFC

The combined impacts of various extraction techniques and utilized solvent kinds on extracted TPC and TFC are depicted in [Fig. 4](#). As is clear in the figure, the extracts using methanol solvent via UAE showed significantly (P less than 0.05) greater amounts of TPC (49.14 ± 0.38 mg GAE/g dw) and TFC (18.38 ± 0.19 mg QUE/g dw) contrasted to other extraction techniques and utilized solvents. At the same time, the lowest TPC (24.76 ± 0.27 mg GAE/g dw) and TFC (7.06 ± 0.48 mg QUE/g dw) have been observed utilizing Isopropanol solvent and extraction via heat technique. It can be due to the cavitation effect and strong shear forces ultrasound produces. This makes the extraction process more efficient by providing better mass transfer, reducing intracellular material, increasing solubility and solvent penetration of analytes, and enhancing the permeability of the plant tissue ([Altemimi et al., 2015](#)). The impact of various solvents could be regarded as being caused by the improver solubility of these components in CH_3OH than the other solvents carried out trials on because the yields of extraction depend on the differing polarity of the solvents and the nature of the bio-active components in each plant ([Do et al., 2014](#)). For example, the outputs revealed that CH_3OH was the best solvent for extracting bio-active components from *Severinia buxifolia* family plants ([Truong et al., 2019](#)). Conversely ([Do et al., 2014](#)), found that ethanol was superior to methanol and aqueous acetone in the extraction of bio-active components from *Limnophila aromatic* ([Zin et al., 2022](#)). Pure water solvent is the most effective in obtaining the highest amount of TPC (57.89×1.14 mg gallic-acid equivalent/gram of dry weight) and BC (17.12×0.37 mg/gram of dry weight). In this



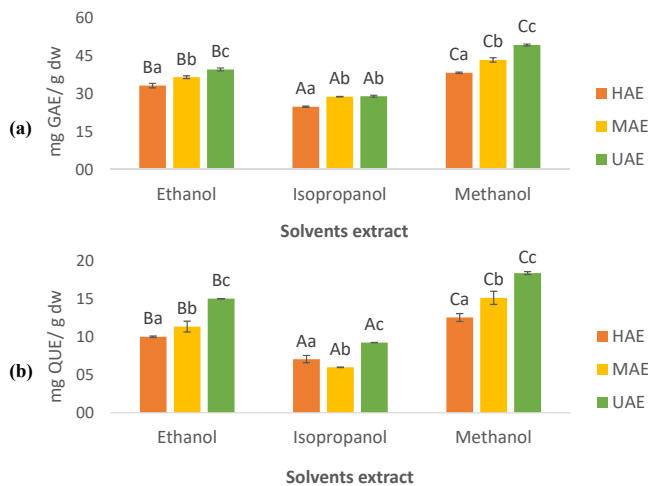


Fig. 4. Total phenolic content and total flavonoids content of the extract using different extraction methods and solvents, TPC (a), TFC (b).

Upper cases for e.g. A, B, C... = significant differences between solvent with each extraction method.
Lower cases for e.g. a, b, c... = significant differences between extraction methods with each solvent

case, any extraction techniques play a bigger role than the types of applied solvent or the combinatorial effects of emerging techniques.

Impact of extraction methods and applied solvent types on AA

As listed in Table 1, the percentage of inhibition of methanolic extracts of hawthorn using UAE, MAE, and HAE was slightly higher ($P < 0.05$) than that of ethanolic and isopropanol extracts by all of AA (FRAP, DPPH, and ATBS) assays. In addition, the UAE extraction method outperformed both MAE and HAE using the same solvents. AA values of the fruit extracts by UAE with

Table 1. Values of FRAP, DPPH, and ATBS resultant from hawthorn extracts

AA	Extraction methods	Methanol	Ethanol	Isopropanol
FRAP (mg AAE/g dw)	HAE	162.32 ± 0.93 ^{Ca}	155.97 ± 0.35 ^{Ba}	132.14 ± 0.82 ^{Aa}
	MAE	240.13 ± 0.82 ^{Cb}	234.22 ± 0.24 ^{Bb}	211.71 ± 0.65 ^{Ab}
	UAE	250.24 ± 1.46 ^{Cc}	242.21 ± 0.54 ^{Bc}	220.53 ± 0.52 ^{Ac}
DPPH (I %)	HAE	130.05 ± 1.0 ^{Ca}	122.01 ± 1.64 ^{Ba}	98.22 ± 0.45 ^{Aa}
	MAE	153.42 ± 0.95 ^{Cb}	135.3 ± 0.5 ^{Bb}	115.07 ± 1.24 ^{Ab}
	UAE	157.32 ± 0.39 ^{Bc}	156.22 ± 1.53 ^{Bc}	123.38 ± 1.43 ^{Ac}
ATBS (I %)	HAE	151.46 ± 0.9 ^{Ca}	143.29 ± 1.42 ^{Ba}	123.43 ± 0.69 ^{Aa}
	MAE	183.33 ± 1.17 ^{Cb}	171.06 ± 1.09 ^{Bb}	134.44 ± 0.88 ^{Ab}
	UAE	200.28 ± 0.39 ^{Cc}	182.4 ± 0.9 ^{Bc}	141.16 ± 1.21 ^{Ac}

Upper cases for e.g. A, B, C... = significant differences between solvent with each extraction method.
Lower cases for e.g. a, b, c... = significant differences between extraction methods with each solvent.



methanol solvent are as follows: (FRAP = 250.24 ± 1.46 mg AAE/g dw, DPPH = $157.32 \pm 0.39\%$, and ATBS = $200.28 \pm 0.39\%$) while those values decreased to 240.13 ± 0.82 mg AAE/g dw (FRRAP); 153.42 ± 0.95 and $183.33 \pm 1.17\%$ measured by DPPH and ABTS) via MAE. Followed by, the least amounts of AA were detected by methanolic HAE as FRAP = 162.32 ± 0.93 mg AAE/g dw, DPPH = $130.05 \pm 1.0\%$, and ATBS = $151.46 \pm 0.9\%$, respectively.

It has been reported that the extracts of red currant, black currant, and grape have a better antioxidant effect in CH₃OH extract than other solvents (Lapornik et al., 2005). Comparatively to Soxhlet extraction, grape seed’s extracts, which were gained by ultrasound extraction technique, recorded the greatest polyphenol concentration and anti-oxidant efficiency (Da Porto et al., 2013). Additionally, the antioxidant capacity of the UAE-derived gac peel extract was significantly greater than the conventional extraction with the identical solvent:substances ratio (Chuyen et al., 2018).

Correlation between TPC, TFC, and different AA assays

As determined by the Pearson correlation analysis, TPC, TFC, and radical scavenging assays (DPPH, ATBS) have a strong positive linear correlation [TPC-DPPH: $r = 0.924$,

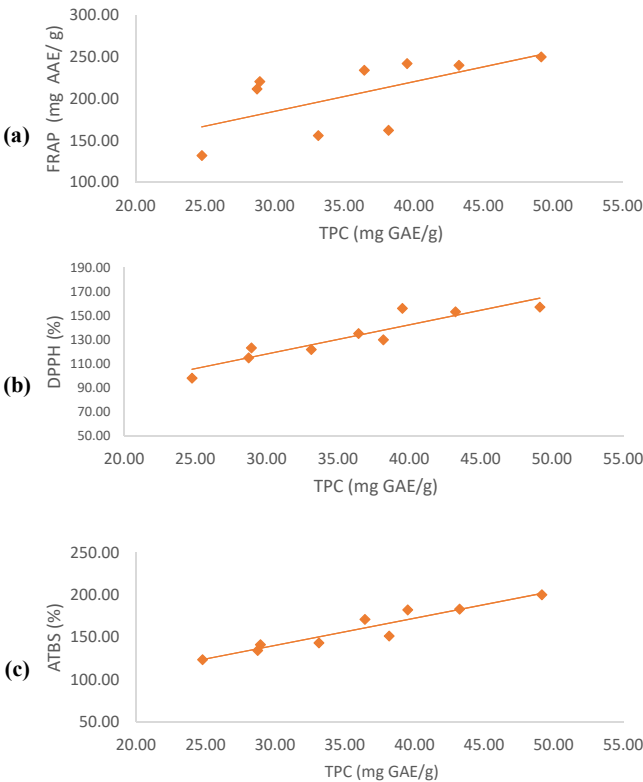


Fig. 5. The correlation of total phenolic content (mg of GAE/g) vis-antioxidant activity (a) FRAP, (b) DPPH, (c), ATBS



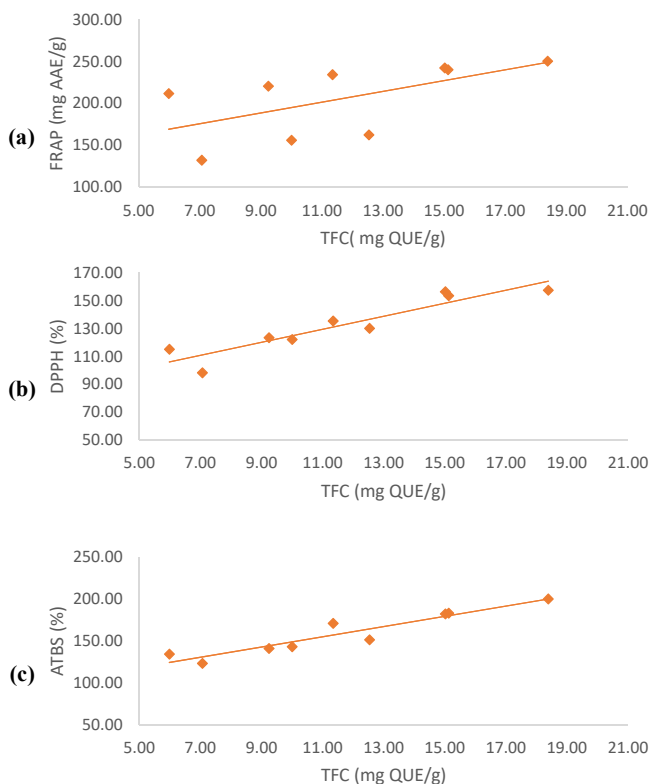


Fig. 6. The correlation of total flavonoids content (mg of QUE/g) vis-antioxidant activity (a) FRAP, (b) DPPH, (c), ATBS

TPC-ABTS: $r = 0.95$] (Fig. 5 (b), (c)), [TFC-DPPH: $r = 0.929$, TFC-ABTS: $r = 0.946$] (Fig. 6 (b), (c)). Meanwhile, the correlation was lower between the bioactive compounds and radical scavenging assay (FRAP) [TPC-FRAP: $r = 0.627$, TFC-FRAP: $r = 0.595$] (Figs. 5 (a), 6 (a)). It can be due to the differences in the principles of the AA assays, where assays such as FRAP measure the reducing capacity of ferric ions, which are irrelevant to antioxidant activity from a mechanistic and physiological standpoint. Considering these facts, one should be aware of selecting a method to estimate antioxidant activity and use more than one to have a complete idea (Ou et al., 2002). Variations have been also noticed among the two radical scavenging tests (DPPH and ABTS) by (Wootton-Bearda et al., 2011).

CONCLUSION

The extraction of hawthorn fruit utilizing three extraction techniques and three organic solvents is reported in the current study. With the maximum anthocyanin yield and the highest amounts of phenolics, flavonoids, and antioxidants among the extraction techniques and solvents examined, UAE with methanolic solvent proved to be the most effective option for extracting



bioactive components from hawthorn. Along the line, a powerful positive interconnection among the content of bio-active components and AA of the extracts. Although methanol extracts showed the greatest concentration of bioactive compounds and antioxidant activity, safer and environmentally friendly solvents are always recommended, when using hawthorn extracts as coloring agents or as ingredients in developing food products.

Declaration of competing interest: Authors declare that: “the work reported in this article was not influenced by competing financial interests or personal relationships”.

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