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Research Article

Comparison of green technologies for valorizing sugar beet (*Beta vulgaris* L.) leaves

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Abstract

This study is focused on valorizing sugar beet (*Beta vulgaris* L.) leaves, a prominent by-product wasted during the process of sugar beet. For doing so, Ultrasound-Assisted Extraction (UAE), Pressurized Liquid Extraction (PLE), and Maceration Extraction (ME) methods were compared by evaluating the extraction yield, Total Phenolic Compounds (TPC), Total Flavonoid Content (TFC), individual phenolic profile, and antioxidant activity (i.e., DPPH, FRAP, and ABTS). EtOH 70% was employed as the extracting solvent. The TPC for UAE and PLE methods was 4.90±0.083 and 6.46±0.33 mg GAE.mL^{-1}, respectively, differing significantly ($P<0.05$) from each other. Moreover, the extract obtained by the PLE method had the highest TFC (1.45±0.06 mg QE.mL^{-1}). The antioxidant activity of PLE extract was higher than other samples, differing significantly ($P<0.05$) in FRAP and ABTS methods. Benzoic acid was present only in the PLE extract, while PLE and ME recovered the highest quantity of syringic acid (0.35±0.00 mg.mL^{-1}) and pyrogallol (74.80±0.01 mg.mL^{-1}), respectively. However, the extraction yield obtained by the UAE method (19.58±0.33%) was significantly higher than the other methods. Therefore, despite the higher TPC and antioxidant activity achieved by the PLE method, it was proved that UAE yields higher polyphenolic compounds with a lower quantity of beet leaves.

Keywords: ultrasound-assisted extraction (UAE), pressurized liquid extraction (PLE), sugar beet leaves, polyphenols, antioxidant activity

Abbreviations: ABTS – 2,2-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid); DPPH – 2,2-diphenyil-1-picrylhydrazyl; FRAP – ferric reducing antioxidant power; GAE – gallic acid equivalent; ME – maceration extraction; PLE – pressurized liquid extraction; PPO – polyphenol oxidase; QE – quercetin equivalent; TE – trolox equivalent; TFC – total flavonoid content; TPC – total phenolic compounds; UAE – ultrasound-assisted extraction

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Introduction

The leaves of edible crops are mostly considered by-products discarded as waste during food processing (Fernandez et al. 2017). Since these food by-products could be principal sources for the recovery of bioactive compounds, many studies have proposed the strategy of valorizing them using extraction techniques (Tinello and Lante 2019; Cisneros-Yupanqui et al. 2021; Lante et al. 2020).

The sugar beet plant (*Beta vulgaris* L.), which constitutes one-fifth of world sugar production, generates large quantities of unexploited biomass (i.e., leaves). The by-product of the sugar beet comprises almost half of the entire plant (Bengardino et al. 2019; Pellegrini and Ponce 2020; Nutter et al. 2020). The sugar beet leaves are a rich source of bioactive compounds such as fatty acids, minerals (Biondo et al. 2014), proteins (Akyüz and Ersus 2021), and polyphenols (Nutter et al. 2020). Among these compounds, polyphenols are potent substances improving human health via their antibacterial, antifungal, anti-inflammatory, and anti-tumor properties. These compounds are a group of secondary metabolites synthesized in plants that own one or more phenolic rings with attached hydroxyl groups. They are regarded as natural antioxidants, improving the quality of food by retarding the oxidation of lipids (Ebrahimi and Lante 2021; Kolev 2022).

The recovery of polyphenols from food by-products is usually performed by conventional methods, i.e., maceration. However, these techniques are time-consuming and demand large amounts of solvents and energy. Hence, there is a growing demand for novel extraction methods without the mentioned drawbacks (Ebrahimi and Lante 2022). PLE and UAE are among the most studied emerging technologies developed in recent years (Oliveira et al. 2022).

PLE is an innovative extraction technology used on a laboratory and industrial scale (Cea Pavez et al. 2019). Many advantages could be attributed to this technique, such as short extraction time, elevated pressure and temperature, automatization of process, and low solvent consumption (Ebrahimi and Lante 2022). UAE is a prominent technique which involves physical and chemical forces different from those involved in conventional solvent extraction (Lante and Friso 2013). UAE achieves high-yield extractions in low temperatures and times with optimized energy, mass transfer, and start-up time, and it decreases solvent consumption (Zhou et al. 2017; Chemat et al. 2011).

The solvent could significantly impact the recovery yield of polyphenols (Wen et al. 2019). Ethanol is a green extraction solvent for obtaining bioactive compounds, which presents many advantages as it can be renewably produced from biotechnological processes and is non-toxic, with low flammability and environmental footprint (Cassiana Frohlich et al. 2022). As reported by many authors, the combination of ethanol with water in a ratio of 70% is an optimal proportion to increase the extraction yield of polyphenols (Ninčević Grassino et al. 2020; da Rosa et al. 2019; Motikar et al. 2020; Coelho et al. 2017) because adding water to pure ethanol provides better distribution of polyphenols and enhances the yield of this process (Ninčević Grassino et al. 2020). Therefore, ethanol 70% was used in the present study.

Many studies have been performed on how the extraction method affects the chemical composition and bioactive compounds of leaves (Keshavarzi et al. 2020; da Silva et al. 2020; Pellegrini and Ponce 2020; Bengardino et al. 2019). However, there is no research investigating the effect of novel extraction techniques on the bioactive compounds of sugar beet leaves. Thus, the aim of this study was to evaluate and compare the efficiency of UAE and PLE methods by measuring TPC, TFC, individual polyphenols, and antioxidant activity of extracts obtained from the sugar beet leaves.

Materials and Methods

Chemical and materials. The sugar beet leaves were acquired from a local farm in Padova, Italy.

All chemicals and solvents were of analytical grade and purchased from Sigma (Sigma Chemical Co., St. Louis, MO, USA). The deionized distilled water was employed for all the analyses.

Sample Preparation. The fresh sugar beet leaves were rinsed with distilled water and cleaned accurately by removing the soil and separating the stalks. The clean leaves were frozen with liquid nitrogen and ground using a mortar and pestle. The ground leaves were stored at -18°C until the following trials.

Maceration Extraction (ME). For the conventional extraction of polyphenols, 1 g of ground leaves was macerated in 25 ml EtOH 70% for 24 h at 25°C. The obtained extract was filtered with Whatman No. 1 filter paper and stored at -18°C until the analytical measurements.

Ultrasound-Assisted Extraction (UAE). The UAE of polyphenols from beet leaves was carried out using an ultrasound apparatus (HD 2200.2, Bandelin, Berlin, Germany) equipped with a probe model KE 76. The extraction condition was 200 W output power, 25% amplitude, 20 kHz frequency, and 15 min time. EtOH 70% with a solid to liquid ratio of 1:20 (w/v) was used as the extraction solvent. After ultrasonication, the obtained extract was centrifuged at 4°C and 5000 RPM for 10 min. The supernatant was filtered with Whatman No. 1 filter paper. The extraction was done in 2 cycles, and the combined filtrate of extracts was refrigerated at -18°C until the following analytical measurements.

Pressurized Liquid Extraction (PLE). The PLE was carried out according to the method reported by Lante et al. (2011). Briefly, 100 g of ground sugar beet leaf was placed into an automatic lab-scale PLE apparatus (NM LAB/M Depurex 88, Limena, Padova, Italy). The apparatus was filled with 535 mL EtOH 70%. The system was pressurized with a pressure of 10 bar at 25°C for 1.45 h. The extract after filtration using Whatman No. 1 filter paper was stored at -18°C until the analytical measurements.

Total Phenolic Content (TPC). The TPC was measured by Folin–Ciocalteu colorimetric method as reported by Azuma et al. (1999) with some modifications. For doing so, 0.5 ml of the diluted extract was added to a mixture of 1.25 mL sodium carbonate (Na_2CO_3) solution 7.5% and 0.25 mL of a two-time diluted Folin–Ciocalteu reagent. In the blank sample, 0.5 ml EtOH 70% was used instead of the extract. The obtained mixtures were kept at room temperature for 30 min in a dark place. Finally, the absorbance was recorded at 650 nm using a Varian Carry 50 Bio UV/Vis spectrophotometer (Agilent Technologies, Santa Clara, CA, USA). A standard curve of gallic acid ($R^2 = 0.996$) was used to calculate and report results as mg GAE.mL^{-1}extract.

Extraction yield (X_0). The X_0 was calculated using equation 1:

$$X_0 = \frac{C \times V}{F} \times 100 \quad (1)$$

Where c is the phenolic concentration of the extract (g.mL^{-1}), V is the volume of solvent in the extraction (mL), and F is the mass of sample in the extraction (g).

Total Flavonoid Content (TFC). The TFC was evaluated according to the method described by Mihaylova et al. (2021) with some modifications. In brief, 220 µL of the extract was added to a mixture of 45 µL 10% aluminum nitrate ($Al(NO_3)_3$) solution, 45 µL of a 1 M potassium acetate (CH_3CO_2K) solution, and 1.7 mL EtOH 70%. In the blank sample, 220 µL EtOH 70% was used instead of the extract. The obtained mixtures were kept at room temperature for 40 min, and the absorbance was measured at 415 nm. A standard curve of Quercetin ($R^2 = 0.999$) was used to calculate and report results as mg QE.mL^{-1}extract.

DPPH free radical scavenging activity. The scavenging effect of extracts was determined by the method described by Massini et al. (2016) with some modifications. A mixture of 1.8 mL of a DPPH$^{\cdot}$ solution (0.1 mM in EtOH 96%, v/v) and 0.2 mL of diluted extracts was prepared and kept at

room temperature for 30 min. The decrease in absorbance was recorded at 517 nm. In the blank sample, 0.2 mL EtOH 70% was used instead of the extract. A standard curve of trolox (R^2 = 0.999) was used to calculate and report results as mg TE.mL^{-1} extract.

Ferric Reducing Antioxidant Power (FRAP). FRAP assay was conducted based on the method of Stratil et al. (2006) with some modifications. The FRAP reagent was freshly prepared each day of analysis after mixing 38 mM of anhydrous sodium acetate buffer (pH 3.6), 20 mM of $FeCl_3$ in Milli-Q water and 10 mM of TPTZ in 40 mM HCl, in a ratio of 10:1:1. Then, 900 µL of prepared FRAP reagent was added into 100 µL of diluted extract/EtOH 70% (blank). The mixture was vortexed for 1 min and incubated for 30 min at 37°C. The absorbance was measured at 593 nm. A standard curve of trolox (R^2 = 0.999) was used to calculate and report results as mg TE.mL^{-1}extract.

ABTS radical scavenging activity. The ABTS radical scavenging effect of extracts was determined by the method described by Re et al. (1999). The radical cation ($ABTS^{\bullet+}$) was prepared by mixing 7 mM ABTS stock solution with 2.45 mM potassium persulfate (1/1, v/v) and leaving the mixture for 4-16 h until the reaction was completed and the absorbance was stable. The $ABTS^{\bullet+}$ solution was diluted with ethanol to an absorbance of 0.700 ± 0.05 at 734 nm for measurements. The photometric assay was conducted on 0.9 ml of $ABTS^{\bullet+}$ and 0.1 ml of tested extracts and mixed for 45 s, and measurements were taken at 734 nm after 1 min. A standard curve of trolox (R^2 = 0.999) was used to calculate and report results as mg TE.mL^{-1} extract.

Determination of Individual polyphenols. The profile of individual polyphenols of sugar beet leaves extracts was determined using a Thermo Finnigan SpectraSystem UV6000LP HPLC system (Thermo Finnigan, San Jose, CA, USA) with a diode array detector, as reported previously by Cisneros-Yupanqui et al. (2021). Firstly, the samples were filtered with a 0.22-µm cellulose acetate filter (Millipore, USA) prior to their injection into a SupelcosilTM LC-18 column. The compounds existing in the extracts were identified based on the retention time of some commercial standards, including pyrogallol, 4-hydroxybenzoic acid, benzoic acid, and syringic acid, previously solubilized in absolute methanol. The operating condition is detailed in Table 1.

Statistical analysis. All the analyses were carried out in triplicate. The experimental design of this project is shown in Fig. 1. The data obtained from the trials were processed using IBM SPSS Statistics (Version 20.0, SPSS Inc, Chicago, IL, USA). The obtained data were subjected to One-way ANOVA and the comparisons were made by Tukey's test with a significance level of 0.05.

Table 1. Operating condition of HPLC method for characterizing individual polyphenols present in the extracts

Mobile phase	Solvent A: 18 mL n-butanol Solvent B: 1.5 mL 50% v/v acetic acid
Flow rate	0.6 mL.min^{-1}
Wavelength	214 and 275 nm
Temperature	25 °C
Running time	60 min

Results and Discussion

Fig. 2 shows the TPC (a), extraction yield (b), and TFC (c) of the extracts obtained with different methods. The TPC for UAE and PLE methods was 4.90±0.08 and 6.46±0.33 mg GAE.mL^{-1}, respectively, differing significantly ($P<0.05$). The PLE method had the highest TPC and TFC among other extracts. The TFC of the extracts ranged from 1.057±0.00 to 1.45±0.06 mg QE.mL^{-1}, with PLE as the highest one, which differs significantly from other extracts ($P<0.05$). The results showed that both green technologies (e.g., UAE and PLE methods) extracted more phenolic content compared to the ME method. However, UAE had a

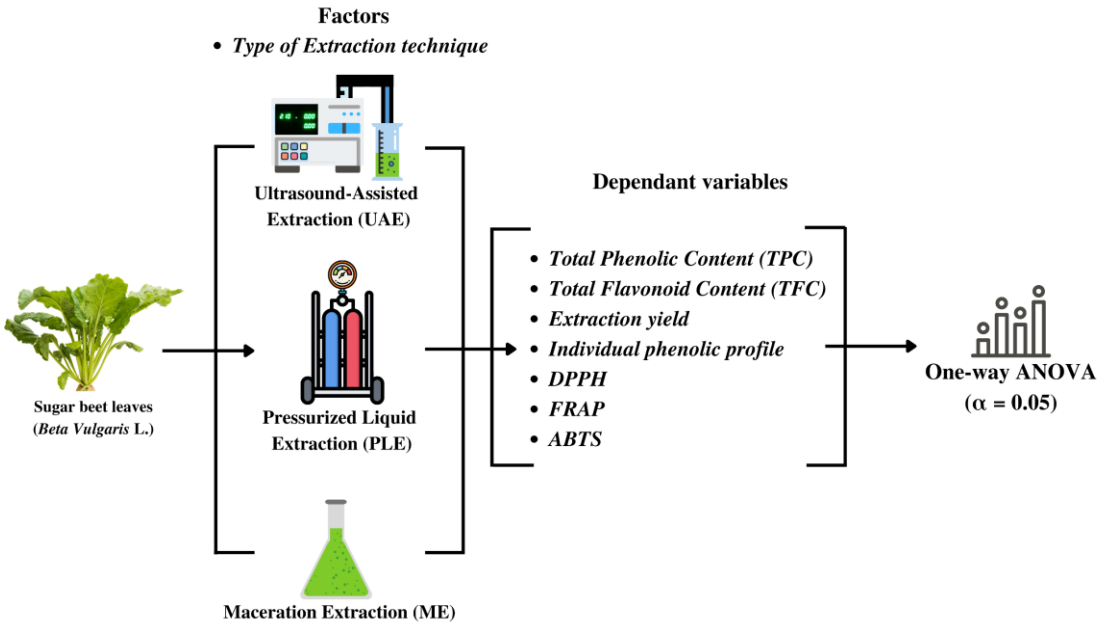

Figure 1. The experimental design of valorization of sugar beet leaves using UAE, PLE, and ME

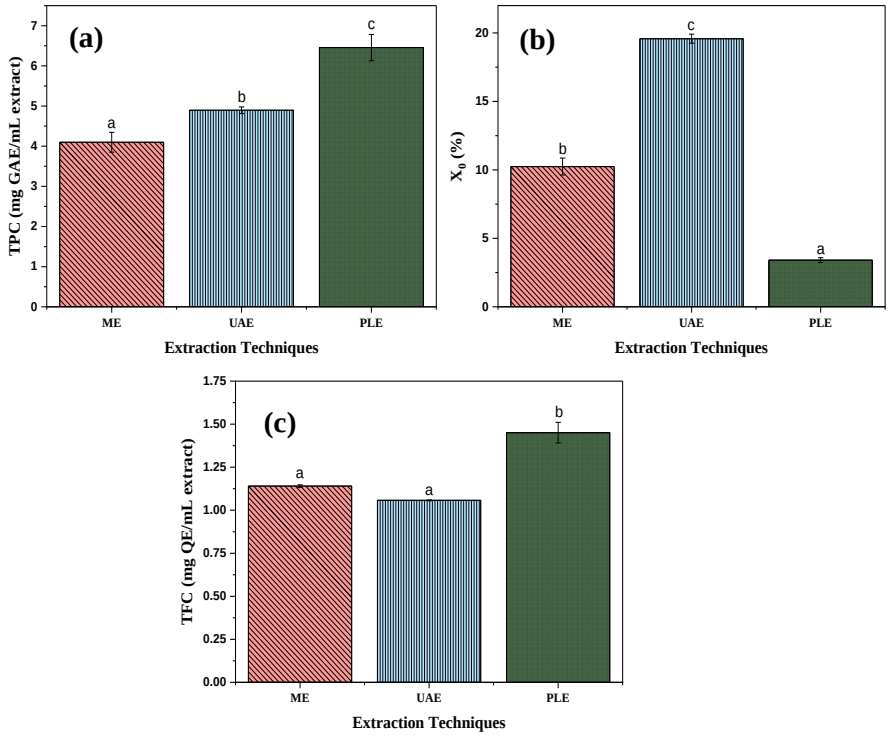

Figure 2. TPC (mg GAE.mL^{-1}) (a), Extraction yield (%) (b), and TFC (mg QE.mL^{-1}) (c) of extracts

ME: Maceration Extraction, UAE: Ultrasound-Assisted Extraction, PLE: Pressurized Liquid Extraction.
Data is indicated as mean ± SD of triplicate samples (n=3)

lower TFC compared to the other methods. According to figure 2 (b), the X_0 of extracts ranged from 3.40±0.17 to 19.58±0.33%, with UAE as the highest.

Table 2 shows the antioxidant activity of extracts measured using DPPH, FRAP, and ABTS methods. The antioxidant activity of extracts was significantly different as the PLE method yielded the highest antioxidant activity of all these methods.

Table 2. Antioxidant activity of extracts

Antioxidant activity, mg TE.mL^{-1}	Extraction method		
	ME	UAE	PLE
DPPH	0.60±0.02^{a}	0.86±0.05^{b}	0.97±0.06^{b}
FRAP	2.29±0.04^{a}	2.56±0.07^{b}	2.73±0.04^{c}
ABTS	2.15±0.03^{a}	2.12±0.01^{a}	2.71±0.01^{b}

Data is reported as mean ± SD of triplicate samples (n= 3). ME: Maceration Extraction, UAE: Ultrasound-Assisted Extraction, PLE: Pressurized Liquid Extraction

However, the DPPH method does not show any significant difference (P>0.05) between the extracts from UAE and PLE methods. The quantities from the FRAP method for all the extracts were different significantly (P<0.05). In counter to FRAP and DPPH assays, the extracts obtained by the UAE technique had the lowest antioxidant activity when measured by the ABTS method. The ABTS method does not show any significant difference (P>0.05) between the extracts from UAE and ME techniques.

Generally, the total content of bioactive compounds depends on many factors, including the extraction method, temperature, pressure, time, solvent, and the ratio between extraction solvent and sample (Setyaningsih et al. 2016; Supasatyankul et al. 2022; Chikane et al. 2021). The higher TPC, TFC, and antioxidant activity (DPPH, FRAP, and ABTS) obtained from the PLE method could be related to the higher extraction time and elevated solid to solvent ratio in this method. However, it was proved that UAE yields higher polyphenolic compounds with a lower quantity of sugar beet leaves. This could be justified as the cavitation bubbles generated by the ultrasound collaborate to rupture the wall of the extraction matrix, thus releasing more polyphenols (Rosa et al. 2021). In the UAE method, the sound waves cause high shear forces disrupting the cell walls, which leads to an increase in the solvent penetration into the plant cells resulting in the release of polyphenols (Rodsamran and Sothornvit 2019). Moreover, UAE could increase the polarity of the solvent when there is a growth in the cavitation or bubble formation, which can result in an enhanced extraction yield (Ninčević Grassino et al. 2020). In many cases, the UAE method is used as a supplementary practice to boost the yield, as proved by Xie et al. (2015) when they obtained a higher yield of oleuropein with UAE as a combined extraction technique. As reported by Giacometti et al. (2018), the yield of phenolic extraction using the UAE method was higher than the conventional method when they extracted polyphenols from olive leaves (*Olea europaea* L.) Nutter et al. (2020) optimized the condition of UAE of polyphenols from red beet leaves, and the optimized TPC obtained was 14.9 ± 0.6 mg GAE.g^{-1} of dried leaves. Machado et al. (2017) compared PLE and UAE techniques in extracting phenolic compounds from the residues of Blackberry, Blueberry, and Grumixama. Their results indicated that PLE gave a higher yield of phenolic compounds compared to the UAE method in all the samples. However, the extraction yield for the UAE method for blueberry samples was higher than the PLE method, which is consistent with our findings.

Spinelli et al. (2019) extracted bioactive compounds from Norway spruce bark using UAE and PLE, and they reported that the extracts obtained by the PLE method had the highest ABTS compared to the other methods. Also, the same as our results, they concluded that the TFC

for the UAE method is lower than the PLE method. As reported previously, flavonoids from sugar beet leaves act as hepatoprotective agents, which could prevent hepatic toxicity (El-Gengaihi et al. 2016). Moreover, flavonoids have several other activities, including anti-inflammatory, vasorelaxant, anticoagulant, cardioprotective, antidiabetic, chemoprotective, neuroprotective, and antidepressant properties (Rufino et al. 2021). Therefore, the sugar beet leaves could be considered a high-nutritional food product.

As highlighted by the results, the green technologies have higher TPC, TFC, and antioxidant activity compared to the ME method. Similarly, Alves et al. (2022) conducted research on extracting bioactive compounds from *Monteverdia aquifolia* leaves, and the same as our results, they reported that the UAE and PLE yield higher phenolic content and antioxidant activity in comparison to the conventional methods. Moreover, the extraction yield of the phenolic compound for the PLE method was the lowest among the other methods, which is consistent with the results obtained in the present paper. The higher TPC of extracts obtained by UAE and PLE techniques could be attributed to their ability to inactivate/decrease the amount of Polyphenol Oxidase (PPO). PPO is a copper-containing oxidoreductase enzyme that uses polyphenols as a substrate. The reaction between PPO and polyphenols gives rise to their oxidation resulting in a decrease in TPC, which has a negative impact on the extraction process of polyphenols from food by-products (Ebrahimi and Lante 2022).

Petkova et al. (2020) extracted polyphenols from Burdock roots with the same PLE method as the present paper using EtOH 70% as extracting solvent. The TPC they obtained was 12.13 ± 0.34 mg GAE.g^{-1}. Similarly, Mazzutti et al. (2018) used the PLE method for the extraction of polyphenols from cocoa (*Theobroma cacao*) bean hulls, and they reported that the yielded polyphenolic content was 9.6±0.3 mg GAE.g^{-1}. The high pressure in the PLE method maintains the solvent in the liquid state, allowing the improved solubility and mass transfer between the food matrix and the solvent, leading to better extraction of polyphenols. In addition, it could decrease the viscosity of the extractant, resulting in improved soaking of the food matrix. This causes the high solubility of the polar compounds (Ebrahimi and Lante 2022).

Table 3 details the quantity of the compounds found in the phenolic extracts using the HPLC method. Benzoic acid was present only in the PLE extracts with a quantity of 1.48±0.06 mg.mL^{-1}. PLE method had the highest syringic acid quantity (0.35±0.00 mg.L^{-1}), while there was no significant difference between the syringic acid content in ME and UAE techniques. There was no significant difference between the content of 4-hydroxybenzoic acid in the extracts obtained from PLE and ME methods. On the other hand, UAE had significantly lower amounts of hydroxybenzoic acid. Surprisingly, the extracts of the ME method showed the highest pyrogallol quantity, which was four times higher than the PLE method.

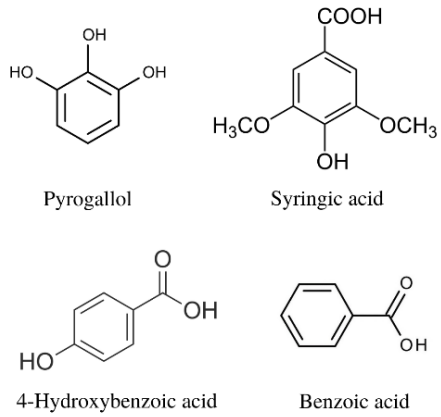

Figure 3. Structural characterization of the compounds found in the extracts using HPLC method

Fig. 3 shows the chemical structure of the compounds characterized using the HPLC method. As highlighted by the results, sugar beet

leaves are full of pyrogallol, which is an important polyphenol owning high antioxidant activity. According to the research conducted by Su et al. (2021), pyrogallol-containing natural products have an inhibitory effect against 3CL protease, which is a necessary compound for the replication of *coronavirus*. Thus, sugar beet leaves' extracts could be considered a potent food additive for preventing Covid-19 infection.

Table 3. The compounds characterized in the phenolic extracts using the HPLC method

Characterized compound, mg.mL^{-1} extract	**Extraction method**		
	ME	**UAE**	**PLE**
Pyrogallol	74.80±0.01^{c}	48.59±0.02^{b}	17.06±0.05^{a}
4-Hydroxybenzoic acid	0.52±0.00^{b}	0.45±0.02^{a}	0.53±0.02^{b}
Benzoic Acid	ND	ND	1.48±0.06
Syringic acid	0.19±0.00^{a}	0.19±0.01^{a}	0.35±0.00^{b}

Data is reported as mean ± SD of triplicate samples (n = 3).
ME: Maceration Extraction, UAE: Ultrasound-Assisted Extraction, PLE: Pressurized Liquid Extraction,
ND: Not Detected

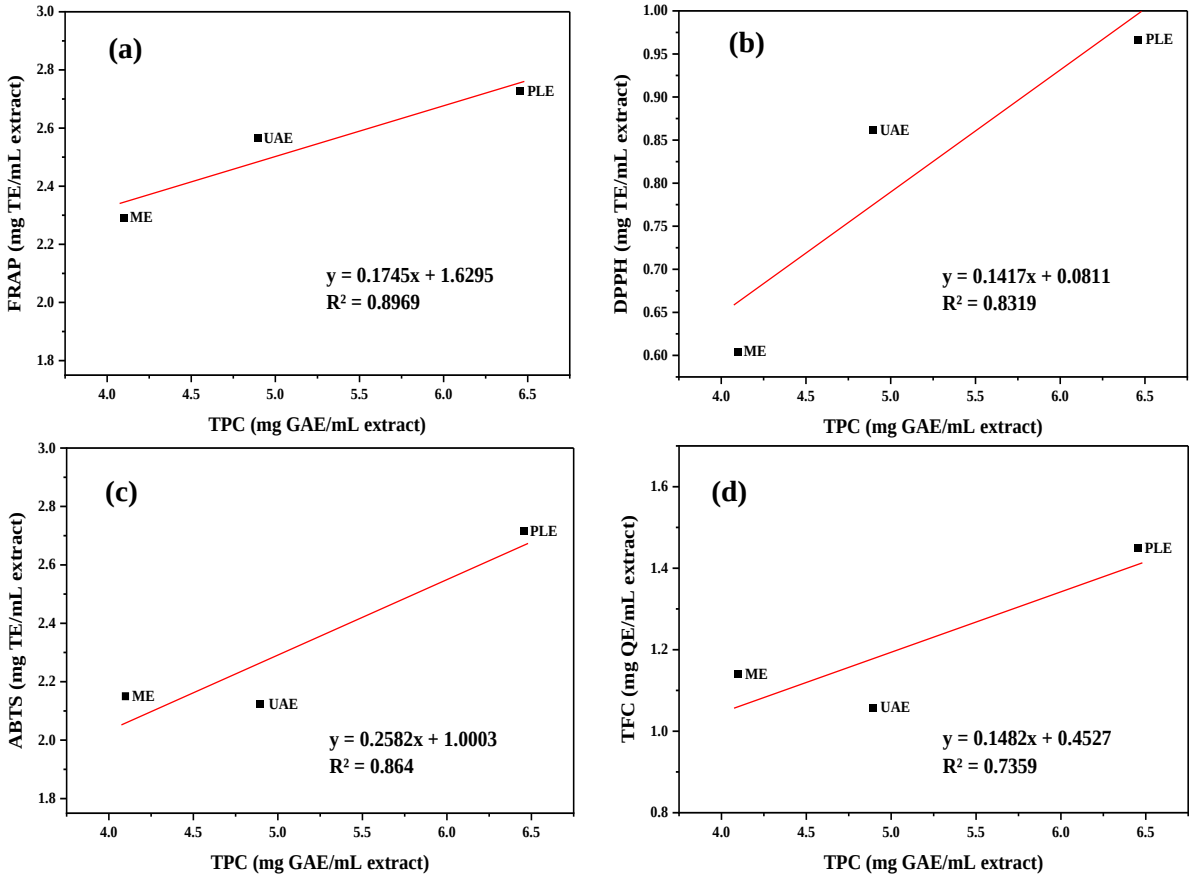

Figure 4. The relation (a) between FRAP and TPC, (b) between DPPH and TPC, (c) between ABTS and TPC, (d) between TFC and TPC

ME: Maceration Extraction, UAE: Ultrasound-Assisted Extraction, PLE: Pressurized Liquid Extraction

Fig. 4 shows the relation of TPC with DPPH, FRAP, ABTS, and TFC. The DPPH, FRAP, and ABTS of extracts were highly correlated to the TPC with the R^2 of 0.8319, 0.8969, and 0.864, respectively. According to the results, FRAP and DPPH assays have the highest and lowest relations with TPC, respectively. The relation between TPC and TFC is not as high as in other comparisons, which stems from the lower TFC of extracts obtained by the UAE method.

The high relation between TPC and antioxidant activity assays indicates the connection between TPC and their antioxidative abilities. This data is consistent with those obtained by Shofian et al. (2011), who evaluated the correlation between the TPC, DPPH, and FRAP for the extracts obtained from tropical fruits. In cases where the concentration of phenolic compounds is high, due to the increase in the number of hydroxyl groups in the reaction medium and the probability of hydrogen donation to free radicals, the radical scavenging ability is noticeable (Ebrahimi and Lante 2021).

Conclusions

The outcome of this work indicated that the utilization of PLE and UAE methods as green technologies could increase the quantity of extracted phenolic content. However, the extraction yield for the UAE method showed that this method is more reliable than the others. Moreover, the results proved that sugar beet leaves are a precious source of phenolic compounds with high antioxidant activity, making them a potential antimicrobial and anticancer agent. Therefore, this by-product could be valorized by adding it into the formulation of other food products to increase their functionality.

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