



Comparison of the effects of extraction method and solvents on biological activities of phytochemicals from selected violet and blue pigmented flowers

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Abstract

The flowers of many plant species are characterized by intense color and richness of bioactive compounds. They can be their valuable and curious sources that are worth extracting with the highest possible efficiency. The study compares the effects of different extraction methods and ultrasound (US) treatment on the physicochemical properties of dried petals of *Malva sylvestris* (MS), *Clitoria ternatea* (CT), *Centaurea cyanus* (CC), and *Trifolium pratense* (TP). The petals were ground and then extracted by three methods: (1) with H₂O, (2) with 70% methanol, (3) with 70% methanol + acetic acid solvents, additionally used US. Measured: color parameters in CIEL*a*b* system; total antioxidant activity (TAA) referred to the 1,1-diphenyl-2-picrylhydrazyl (DPPH) and 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) radicals activity; total phenolic content (TPC) by the Folin-Ciocalteu method; total anthocyanin content (TAC) by the pH differential method. No specific differences in color parameters founded. The highest TAA (in % of DPPH reductive- MS: 87.86; CT: 62.56; CC: 72.04; TP: 82.99), TPC (in mg gallic acid equivalent/100 g of sample- MS: 1072.86; CT: 849.85; CC: 912.07; TP: 873.77), and TAC (in mg of cyanidin-3-glucoside/100 g of sample- MS: 747.79; CT: 226.46; CC: 267.53; TP: 234.77) was reached by method (3). US treatment increased the effect.

Keywords Plant droughts · Extraction · Ultrasounds · Anthocyanins · Antioxidant activity

Introduction

Plants are a valuable source of bioactive compounds that have a wide range of applications. The isolation of these compounds from plant tissues is of particular interest due to their potential use as functional ingredients in food products. These compounds, when extracted and utilized as food ingredients, can provide various health benefits to consumers and align with the clean label concept, which is becoming increasingly popular among food technologists.

Bioactive compounds, also known as secondary metabolites, are a diverse group of substances found in plants that are not essential for the growth and development of the organism, but play a vital role in the plant's survival mechanisms. These compounds are involved in a wide range of functions such as signalling, defence against biotic and abiotic stress, and interactions with other organisms. They have diverse structural features and are synthesized through various metabolic pathways [1, 2]. This group includes polyphenols, carotenoids, phytosterols, glucosinolates, alkaloids, and saponins [3, 4]. These molecules have antioxidant, anticarcinogenic, antimicrobial, and anti-inflammatory properties [5, 6]. Inflammation is a complex biological response to tissue injury or disease, characterized by the activation of immune cells and the release of various signaling molecules. This response is essential for the repair and healing of damaged tissue, however, chronic or prolonged inflammation has been linked to the development of various chronic diseases such as cardiovascular disease, diabetes, and cancer. Furthermore, chronic inflammation is

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often accompanied by an increase in oxidative stress, which can further exacerbate cellular damage and contribute to the development of these chronic diseases [7].

Polyphenols are a large group of secondary metabolites commonly found in plants. Flavonoids, which include anthocyanins, are a subgroup of polyphenols. Anthocyanins are often found as glycosides, and can be used as natural food colorants. Studies have shown that they have a positive effect on obesity, diabetes, and cardiovascular diseases and can also have a glycemic control effect [8, 9].

The use of edible flowers and their petals in Europe has been known since prehistoric times and is gaining popularity thanks to French, Swiss, and Asian cuisine. Flowers are usually fresh, dried, or sugar-coated food additives for flavour and aesthetic appeal [10, 11]. Because of their primary evolutionary function to attract pollinators, they are particularly rich in anthocyanins, which give them an intense colour that increases the chance of dust [12].

Malva sylvestris L., also known as common mallow, is a plant of the *Malvaceae* family with a long history of traditional medicinal use dating back at least 3,000 years. The flowers of this plant have been utilized as a remedy for various ailments such as colds, coughs, burns, and wound healing. The traditional use of this plant in wound healing has been supported by animal studies. The active compounds found in *M. sylvestris* include mucilage, tannins, malvin, malvidin, oenine, and delphinidin. These compounds have been shown to have high levels of antibacterial and antioxidant activities, particularly the polyphenols and flavonoids [13, 14].

Clitoria ternatea L. also known as butterfly pea or blue pea is an herb belonging to family the *Fabaceae* [15]. It is native to tropical areas of Asia, and more than 60 species of *Clitoria* have been identified, of which *C. ternatea* is the most common [16]. It is used in traditional medicine, as well as a colorful food additive or decorative garden plant [17]. Petals' vibrant blue color comes from anthocyanins known as ternatins. The structure of this.

group of anthocyanins was determined as malonylated delphinidin 3,3',5'-triglucosides that has 3',5'-side chains with alternative D-glucose and p-coumaric acid units a total of 15 (poly)acylated delphinidin glucosides. Ternatins named A1, A2, B1, B2, D1, and D2 are the six main anthocyanins present in clitoria flowers [18].

Cornflower or *Centaurea cyanus* L. of the *Asteraceae* family is a popular plant in Europe and Asia. It was treated as agricultural waste, but interest is now increasing for its potential usefulness as an alternative green fertilizer [19, 20]. Its characteristic blue color is provided by a supramolecular pigment called protocyanin- cyanidin-3-O-(6-O-succinylglucoside)-5-O-glucoside [21]. The cornflower has proven anti-inflammatory,

antimicrobial, diuretic, gastroprotective, immunological, purgative, and many other bioactivities functions [22].

Red clover (*Trifolium pratense* L.) from the family *Fabaceae* first began to be deliberately cultivated in European areas about 1000 years ago. The clover flowers have an intense red colour. Anthocyanins that give colour to their petals aren't identified yet [7]. It was originally used as a natural fertilizer and animal feed, providing assimilable nitrogen. It lost its importance after the spread of nitrogen fertilizers, although it is receiving renewed.

attention due to the growing popularity of sustainability [23]. The phenolic content, total flavonoid content, and antioxidant properties of red clover flower extract indicate that it can be used as a natural source of biologically active ingredients in human nutrition, as well as in the pharmaceutical industry and others [24].

Extraction is an important step in obtaining plant-derived bioactive compounds for use in food or medicine. Traditional extraction methods are time-consuming and require large amounts of solvent. Newer techniques such as ultrasonic-assisted extraction (UAE) and solvent acidification can improve efficiency and preserve the structure and properties of the extracted compounds. UAE uses ultrasonic waves to increase the extraction of polyphenols and flavonoids, while acidifying the solvent can preserve the color and antioxidant properties of anthocyanins during processing. Factors such as temperature, pH, and type of solvent should also be considered when designing the extraction process [25–27].

This research aimed to compare the effects of extraction methods and solvents on the biological activity of solutions of dried flowers such as common mallow, clitoria, cornflower, and red clover. The scope of the study included color measurements in L*a*b* color space, evaluation of total antioxidant activity using DPPH (1,1-diphenyl-2-picrylhydrazyl radical) and ABTS (methanol, 2,2'-azino-bis(3-ethylbenzothiazoline)-6-sulfonic acid) radicals, and measurements of total anthocyanin and phenolic content.

Materials and methods

Preparation of plant droughts

The following flower petals - *Malva sylvestris*, *Clitoria ternatea*, *Centaurea cyanus*, *Trifolium pratense*, and - were used as raw material for dye extraction. All of them were purchased in 2022 in the form of conventionally dried in mild conditions petals from Planteon (Żelazków, Poland). The samples were tightly packaged in 100 g eco-friendly doypack packaging with a zipper by the manufacturer and opened directly before testing. The samples were tightly packaged by the manufacturer and opened only before

testing. Reagents used in the study 1,1-diphenyl-2-picrylhydrazyl radical (DPPH), methanol, 2,2'-azino-bis(3-ethylbenzothiazoline)-6-sulfonic acid (ABTS), potassium persulfate, Folin-Ciocalteu reagent, sodium carbonate and gallic acid were purchased from Sigma Aldrich (Saint Louis, USA).

Grinding

To standardize particle size before extraction, flower petals were ground using a Retsch ZM 200 laboratory hammer mill (Haan, Germany) on a 500 µm sieve.

Extraction methods

Three different extractions were used in the study based on methodologies described in detail by Rodriguez-Saona & Wrolstad [28]. In a 50 mL Falcon-type tube, 3 g of dried sample and 30 mL of extraction solution (I: water, II: 70% methanol in water, III: methanol/water/acetic acid mixture in a ratio of 40:60:0.5 v/v/v) were added. Samples were shaken for 15 min on a rotator (MyLab SLRM-3, Nano-EnTek Inc., Seoul, Republic of Korea), and then the tube contents were centrifuged for 10 min, 3500 g (MPW-251 laboratory centrifuge, MPW Med. Instruments). The supernatant was decanted into a 250 mL beaker. The pellet remaining after extraction was poured again with 30 mL of the extraction mixture, and the shaking and centrifugation steps were repeated. The extracts were decanted and stored at 4 °C. For each dried sample, extraction was carried out in triplicate. To study the effect of using ultrasound treatment on the content of antioxidant compounds, each group was additionally treated with ultrasound extraction (US) using an ultrasonic cleaner (Elmasonic S180H, Elma Schmidbauer GmbH, Germany). The process was conducted immediately after vortexing the extracts and lasted 10 min. The ultrasonic washer was operated at 21 ± 1 °C and ultrasonic power of 200 W.

Color in the L*a*b* system

The color of the samples was measured using a Minolta CR-400 colorimeter (Konica Minolta Inc., Japan) using the CIEL*a*b* measurement system (measuring area $\varnothing$ = 8 mm and observer 2°, light source D65). Parameters for color determination were L (analyzed sample was black when L=0 or white if L=100), a* (−a* means greenness and +a* redness), b* (−blue; +yellow) with a special attachment for liquid samples.

Based on the results, the average color difference was calculated according to the equation ΔE (1):

$$\Delta E = (\Delta L^*)^2 + (\Delta a^*)^2 + (\Delta b^*)^2 \quad (1)$$

The criterion for the interpretation of the calculated ΔE [29]:

- $\Delta E < 3$ - color difference unobservable.
- $3 \leq \Delta E \leq 5$ - color difference noticeable
- $5 < \Delta E$ - color difference evident

Total antioxidant activity: DPPH, ABTS

The total antioxidant activity (TAA) of the analyzed samples was measured as a reductive capacity referred to the 1,1-diphenyl-2-picrylhydrazyl radical (DPPH) according to Zhang et al. [30], with modifications. DPPH powder was dissolved in methanol. Absorbance was measured at a wavelength of 517 nm (Tecan Spark™ 10 M, Männedorf, Switzerland). A control sample was prepared without sample addition (the sample was replaced with ethanol). Flower petals extract (0.5 mL) was mixed with 3.5 mL of DPPH solution (600 µM) and incubated for 30 min in darkness. Measurements were performed in duplicate. The total antioxidant activity (TAA) expressed as a % reduction of DPPH was calculated as (2):

$$\text{TAA \%} = (1 - A_s/A_c) \times 100 \quad (2)$$

where:

A_s - the absorbance of tested sample; and

A_c - the absorbance of control (without extract).

The ABTS radical scavenging activity of the petals extracts was measured according to Moczowska et al. [31] with modifications. The ABTS solution was made 16 h before the beginning of the analyses by mixing 7 mM ABTS with 2.45 mM potassium persulfate. The mixture was incubated in the dark at room temperature. The ABTS solution was diluted with methanol to adjust absorbance to 0.700 ± 0.02 at 734 nm. A 2 mL of extract solution was added to 2 mL diluted ABTS solution and incubated in the dark at room temperature for 30 min. Absorbance was measured using a UV-Vis spectrophotometer (TecanSpark™ 10 M, Männedorf, Switzerland) at 734 nm. The results were expressed as mM TE/g extract (TE–Trolox equivalent).

Total phenolic content (TPC)

The total phenolic compounds content (TPC) was determined by the Folin-Ciocalteu (F-C) method [32]. To 0.1 mL of every extract, 6.0 mL of distilled water and 0.5 mL of F-C reagent (Sigma Aldrich Inc., USA) were added. After 3 min, 1.5 mL of saturated sodium carbonate of 200 mg/mL concentration (Sigma Aldrich Inc., USA) and 1.9 mL distilled water was added. The mixture was incubated for 30 min in the water bath (WNB 7 Memmert, Germany) at

a temperature of 40 °C. Absorbance was measured spectrophotometrically at the wavelength of 760 nm (TecanSpark™ 10 M, Männedorf, Switzerland). TPC was expressed as an equivalent gallic acid (GA) based on the prepared calibration curve. The results were provided as an average from two repeats in mg GA equivalent per kg of the sample.

Total anthocyanin content (TAC)

Total anthocyanin content (TAC) was measured spectrophotometrically using a pH differential method according to the procedure of Zhang et al. [30]. This method involves calculating the difference in absorbance of dyes in solutions with pH of 1.0 and 4.5. To the tubes, 100 µl each of the extracts was added and then diluted with 500 µl of 0.025 M potassium chloride solution, where the pH was 1.0, or 500 µl of 0.4 M sodium acetate, where the pH was 4.5. Then the samples were stored in a dark place for 30 min. After this time, the absorbance was measured at two wavelengths: $\lambda = 535$ nm and $\lambda = 700$ nm. All measurements were performed in triplicate. The TAC was calculated according to the formulas (3) and (4):

$$A = (A_{535} - A_{700})_{\text{pH}1.0} - (A_{535} - A_{700})_{\text{pH}4.5} \quad (3)$$

where:

A – the absorbance of the anthocyanin extract,

A_{535} – the absorbance measured at 535 nm,

A_{700} – the absorbance measured at 700 nm.

Anthocyanin concentration in the samples was expressed as cyanidin-3-glucoside equivalents, which were calculated according to the formula (4):

$$TAC = \frac{A \times MW \times DF \times 1000}{\varepsilon \times 1} \quad (4)$$

where:

Fig. 1 Cornflower (*Centaurea cyanus* L.) drought before and after milling



MW - (433.2 g/mol) the molecular weight of cyanidin-3-glucoside,

DF- dilution factor,

ε - the molar coefficient of absorbance 17,300 L/mol*cm for cyanidin-3-glucoside.

Statistics

A one-way analysis of variance (ANOVA) and Tukey's test was used to establish the significance of differences between the means at $p < 0.05$. Statistical analysis was conducted with Statistica 13 (TIBICO Software Inc., USA).

Results and discussion

Color properties

The detected differences between the color parameters differed statistically significantly, but according to the applied criteria, these differences were not noticeable to the observer (Fig. 1). This result means that between no samples of a particular species, the ΔE was higher than 3, which indicates that the extraction method has no potential effect on the color of the extracted intermediate product, which could find further application in the food industry (Table 1). The highest L^* value (brightness) was for clitoria (22.74) extracted with 70% MeOH without US treatment, and the lowest was also for clitoria extracted with H_2O and US treatment (21.73). The highest a^* (red color parameter) value was red clover (2.41) extracted by 70% MeOH with US treatment, and the lowest was clitoria (0.16) extracted by MeOH + acetic acid with US treatment. The highest b^* (yellow color parameter) value was cornflower (2.95) extracted by 70% MeOH without US treatment, and the lowest was common mallow (1.64) extracted by H_2O without US treatment. Red clover and common mallow extracts had the highest proportion of

Table 1 Color parameters in the L*a*b* system of extracts depending on the method used and the application of ultrasonic treatment

Plant material	Extraction method		Components of color		
			L*	a*	b*
MS	without US	H ₂ O	22.28 ± 0.01 ^{Aa}	1.47 ± 0.06 ^{Aa}	1.64 ± 0.05 ^{Aa}
		70% MeOH	22.08 ± 0.01 ^{Ca}	1.13 ± 0.05 ^{Ba}	2.53 ± 0.03 ^{Ca}
		MeOH + acetic acid	22.16 ± 0.01 ^{Ea}	1.63 ± 0.06 ^{Ca}	2.32 ± 0.04 ^{Ea}
	with US	H ₂ O	21.92 ± 0.02 ^{Bb}	1.31 ± 0.04 ^{Bb}	2.12 ± 0.04 ^{Bb}
		70% MeOH	21.96 ± 0.01 ^{Db}	1.16 ± 0.06 ^{Ca}	2.59 ± 0.06 ^{Db}
		MeOH + acetic acid	22.05 ± 0.01 ^{Fb}	1.44 ± 0.06 ^{Db}	2.32 ± 0.02 ^{Db}
CT	without US	H ₂ O	21.78 ± 0.02 ^{Aa}	0.72 ± 0.05 ^{Aa}	2.08 ± 0.07 ^{Aa}
		70% MeOH	22.74 ± 0.01 ^{Ca}	0.76 ± 0.04 ^{Aa}	2.32 ± 0.02 ^{CDa}
		MeOH + acetic acid	22.00 ± 0.01 ^{Da}	0.51 ± 0.05 ^{Ca}	2.36 ± 0.04 ^{CDa}
	with US	H ₂ O	21.73 ± 0.01 ^{Bb}	0.60 ± 0.04 ^{Bb}	2.19 ± 0.05 ^{Bb}
		70% MeOH	21.78 ± 0.01 ^{Ab}	0.75 ± 0.05 ^{Aa}	2.33 ± 0.04 ^{CDa}
		MeOH + acetic acid	21.74 ± 0.01 ^{Bb}	0.60 ± 0.06 ^{BDb}	2.26 ± 0.04 ^{BCb}
CC	without US	H ₂ O	21.81 ± 0.02 ^{Aa}	0.16 ± 0.02 ^{Aa}	2.24 ± 0.05 ^{Aa}
		70% MeOH	22.21 ± 0.01 ^{Ca}	1.26 ± 0.04 ^{Ba}	2.95 ± 0.06 ^{Ba}
		MeOH + acetic acid	21.69 ± 0.01 ^{Ea}	0.19 ± 0.04 ^{Aa}	2.15 ± 0.05 ^{Da}
	with US	H ₂ O	21.75 ± 0.01 ^{Bb}	0.17 ± 0.03 ^{Aa}	2.19 ± 0.02 ^{ADb}
		70% MeOH	22.15 ± 0.01 ^{Db}	1.10 ± 0.05 ^{Cb}	2.73 ± 0.03 ^{Cb}
		MeOH + acetic acid	21.69 ± 0.01 ^{Ea}	0.16 ± 0.03 ^{Aa}	2.16 ± 0.04 ^{Da}
TP	without US	H ₂ O	22.07 ± 0.01 ^{Aa}	1.46 ± 0.03 ^{Aa}	2.27 ± 0.05 ^{Aa}
		70% MeOH	22.02 ± 0.02 ^{Ba}	2.04 ± 0.05 ^{Ca}	2.64 ± 0.07 ^{Ba}
		MeOH + acetic acid	21.99 ± 0.02 ^{Ca}	1.63 ± 0.04 ^{Ea}	2.52 ± 0.04 ^{Da}
	with US	H ₂ O	22.01 ± 0.01 ^{BCb}	1.24 ± 0.03 ^{Bb}	2.32 ± 0.07 ^{Aa}
		70% MeOH	22.03 ± 0.01 ^{Ba}	2.41 ± 0.04 ^{Db}	2.77 ± 0.08 ^{Cb}
		MeOH + acetic acid	22.03 ± 0.02 ^{Bb}	1.76 ± 0.04 ^{Fb}	2.58 ± 0.05 ^{BDa}

Mean values bearing different letters (A, B, C, D, E, F; a, b) within each raw material differ significantly ($p < 0.05$).

MS – *Malva sylvestris* L.; CT – *Clitoria ternatea* L.; CC – *Centaurea cyanus* L.; TP – *Trifolium pratense* L.; US - ultrasounds.

red color parameter (a^*) due to the rose-red dyeing of this flower's petals and therefore higher content of pigments of this hue. Petals with a bluer hue (clitoria and cornflower) didn't show an indeed lower b^* parameter (blue color parameter) than this more red. Also, the expected color of the extract - blue - may not necessarily occur. It all depends on the pH and extraction conditions, but the composition of the pigments in a particular plant is just as important. The authors often reported a shift to a more red color during their work with extracts. [33, 34]. Significant differences in the extracts' color parameters obtained from plant material when comparing the method with and without ultrasound were reported before by Guandalini et al. [35] and Gadioli Tarone et al. [36]. They used fruit tissues, which responded to the drying process (ran before extraction) with Maillard reactions and the formation of brown pigments. Ultrasound increased the brown pigments flushing out from the samples tissues, and thus differences in color between extracts obtained by the method with and without ultrasound treatment were shown. The color of the extract can also be affected by reactions between anthocyanins and phenols and their degradation due to environmental conditions, but as Patras [37] reported-in plant extracts, this does not necessarily happen quickly, and often only after storage time.

Phenolic content and anthocyanin content

The results showed that the best method of obtaining phenolic compounds from petals is an acidified mixture of water and methanol. In the case of polyphenols, extractions are usually carried out under acidic conditions because polyphenols are more stable at low pH, and acidic conditions help them to remain neutral by protonating their molecular structures – it may be related to the increased electron donation capacity after deprotonation of the molecular structure. Thus, this facilitates extraction with organic solvents and uses a weak or low concentration of solid acid as a solvent additive. High acid concentrations can cause hydrolysis of glycosides or acylglycosides and thus can give different images of native polyphenol profiles [38]. This is supported by previous research which has also shown that extracts obtained from flower petals using acidified solvents have higher total phenolic content (TPC) compared to those obtained using non-acidified solvents, as demonstrated by Piovesana & Noreña [38] and Escher et al. [39]. For example (Table 2.), in red clover water extract TPC was 132.99 mg GAE/100 g of sample, but 70% methanol extract had almost 5 times higher TPC (654.27 mg GAE/100 g of sample) and acidified 70% methanol solvent was over 32% (866.32 mg GAE/100 g of sample) more effective than non-acidified. The US treatment increases the

Table 2 Total phenolic content (TPC) and total anthocyanin content (TAC) of extracts depending on the method used and the application of ultrasonic treatment

Plant material	Extraction method	Chemical quality parameters	
		TPC mg GA equivalent/100 g of sample	TAC mg of cyanidin-3-glucoside/100 g of sample
MS	with-out US	H ₂ O	778.01 ± 4.42 ^{Aa}
		70% MeOH	926.71 ± 1.80 ^{Ba}
		MeOH + acetic acid	1038.02 ± 5.32 ^{Da}
	with US	H ₂ O	784.54 ± 2.38 ^{Aa}
		70% MeOH	992.91 ± 2.29 ^{Cb}
		MeOH + acetic acid	1072.86 ± 6.68 ^{Eb}
CT	with-out US	H ₂ O	713.18 ± 8.88 ^{Aa}
		70% MeOH	792.47 ± 3.81 ^{Ca}
		MeOH + acetic acid	838.98 ± 6.03 ^{Da}
	with US	H ₂ O	731.54 ± 8.86 ^{Ba}
		70% MeOH	806.26 ± 5.96 ^{Cb}
		MeOH + acetic acid	849.85 ± 2.09 ^{Db}
CC	with-out US	H ₂ O	858.36 ± 3.95 ^{Aa}
		70% MeOH	875.14 ± 1.84 ^{Ba}
		MeOH + acetic acid	896.16 ± 4.37 ^{Ca}
	with US	H ₂ O	864.43 ± 5.33 ^{Aa}
		70% MeOH	883.18 ± 1.93 ^{Bb}
		MeOH + acetic acid	912.07 ± 2.95 ^{Db}
TP	with-out US	H ₂ O	132.99 ± 2.70 ^{Aa}
		70% MeOH	654.27 ± 9.78 ^{Ba}
		MeOH + acetic acid	866.32 ± 3.11 ^{Da}
	with US	H ₂ O	138.83 ± 1.69 ^{Ab}
		70% MeOH	718.46 ± 6.83 ^{Cb}
		MeOH + acetic acid	873.77 ± 2.58 ^{Db}

Mean values bearing different letters (A, B, C, D, E, F; a, b) within each raw material differ significantly ($p < 0.05$).

MS – *Malva sylvestris* L.; CT – *Clitoria ternatea* L.; CC – *Centaurea cyanus* L.; TP – *Trifolium pratense* L.; US - ultrasounds

efficiency of phenol extraction by destroying the petals' cell walls and thus facilitating the disentanglement of the phenolic compounds, which was confirmed in a study carried out (e.g., cornflower 896.16 mg GAE/ 100 g of sample for MeOH + acetic acid and 912.07 mg GAE/ 100 g of sample for MeOH + acetic acid + US; $p < 0.05$). The highest phenolic content in the extracts obtained by the most efficient method (70% MeOH + acetic acid + US) had common mallow (1072.86 mg GAE/ 100 g of sample), and the lowest - cornflower (849.85 mg GAE/ 100 g of sample).

The anthocyanins exist in the form of flavylum cations at highly acidic conditions (red color at $pH < 2$). The increase in pH results in the formation of anionic forms of anthocyanins, which are better electron donors, resulting in higher properties to autoxidation than the corresponding neutral forms. That means their stability can be defined as the inability of flavylum cations to convert into colorless carbinol pseudobases and chalcone forms [38]. Due this anthocyanins properties similar trend, like for TPC, was observed for TAC, where the statistically best method in most samples was acidified methanol with additional ultrasound treatment. For common mallow, a difference between the lowest effective method (H₂O without US: 489.92 mg of cyanidin-3-glucoside/100 g of sample) and the most effective (MeOH + acetic acid + US: 747.79 mg of cyanidin-3-glucoside/100 g of sample) method was over 50%. The research confirms Gamage et al. [15] that lowering the pH of the solvent increases the efficiency of extracting anthocyanins from clitoria flower, although among the species tested in this research showed the lowest content of anthocyanins (for H₂O: 149.90 mg of cyanidin-3-glucoside/100 g of sample; for MeOH + acetic acid + US: 226.46 mg of cyanidin-3-glucoside/100 g of sample), but US increase efficiency over 50% too. The effect of higher efficiency of clitoria extraction due to the use of ultrasound was also demonstrated by Srichaikul [40], in whose study this method was more effective than a few days of maceration. US treatment increased TAC in the cornflower extract by about 12% (without US: 238.59 mg cyanidin-3-glucoside/100 g of sample.; with US: 267.53 mg cyanidin-3-glucoside/100 g of sample) and red clover—almost 10% up (without US: 215.55 mg cyanidin-3-glucoside/100 g of sample; with US: 234.77 mg cyanidin-3-glucoside/100 g of sample) when was used with MeOH + acetic acid solvent. Granados-Balbuena [41] compared the anthocyanin content of fresh and dried dahlia petals. The results showed that the dried petals had up to a few dozen times the anthocyanin content of the same weight as fresh ones. This agreed with the results obtained because compared to Gamage's [42] research on fresh clitoria petals, the TAC, depending on the extraction method, is several tens of times higher in this study.

Antioxidants properties

Antioxidants and their antioxidant abilities are a valuable feature of plant extracts, which have the potential to help prevent diseases that affect millions of people each day [43]. The DPPH radical can react with most of the widely occurring natural antioxidants and allows assessment of their content in the extract examined [44]. The measurements carried out showed (Table 3.), in most cases, a statistically significant increase in the antioxidant activity of the extracts

Table 3 Total antioxidant activity: DPPH, ABTS of extracts depending on the method used and the application of ultrasonic treatment

Plant material	Extraction method	Chemical quality parameters	
		TAA (% DPPH)	ABTS (mg TE/g of extract)
MS	without US	H ₂ O	73.54 ± 0.79 ^{Aa}
		70% MeOH	81.78 ± 0.12 ^{Ca}
		MeOH + acetic acid	84.71 ± 0.59 ^{Da}
	with US	H ₂ O	75.27 ± 0.72 ^{Bb}
		70% MeOH	82.95 ± 0.13 ^{Cb}
		MeOH + acetic acid	87.86 ± 0.19 ^{Eb}
CT	without US	H ₂ O	53.22 ± 0.72 ^{Aa}
		70% MeOH	58.66 ± 0.93 ^{Ba}
		MeOH + acetic acid	60.65 ± 1.53 ^{CDa}
	with US	H ₂ O	55.69 ± 0.48 ^{ABb}
		70% MeOH	61.05 ± 0.31 ^{CDb}
		MeOH + acetic acid	62.56 ± 1.85 ^{Da}
CC	without US	H ₂ O	64.52 ± 1.27 ^{Aa}
		70% MeOH	69.23 ± 0.23 ^{Ca}
		MeOH + acetic acid	71.25 ± 1.09 ^{CDa}
	with US	H ₂ O	66.68 ± 0.87 ^{Aa}
		70% MeOH	70.97 ± 0.13 ^{CDb}
		MeOH + acetic acid	72.04 ± 0.56 ^{Da}
TP	without US	H ₂ O	67.36 ± 0.40 ^{Aa}
		70% MeOH	73.13 ± 0.86 ^{Ca}
		MeOH + acetic acid	79.44 ± 0.64 ^{Da}
	with US	H ₂ O	70.74 ± 1.33 ^{Bb}
		70% MeOH	74.04 ± 0.78 ^{Ca}
		MeOH + acetic acid	82.99 ± 0.68 ^{Eb}

Mean values bearing different letters (A, B, C, D, E, F; a, b) within each raw material differ significantly ($p < 0.05$).

MS – *Malva sylvestris* L.; CT – *Clitoria ternatea* L.; CC – *Centaurea cyanus* L.; TP – *Trifolium pratense* L.; US - ultrasounds

obtained using the method with the 70% MeOH solvent compared to the H₂O solvent. For common mallow, this was a difference of more than 11%; for clitoria, over 10%; for cornflower, above 7%; and for red clover, above 8%. Acidification of the solvent enhanced the stability of the extracted antioxidant compounds, and thus another statistically significant increase by several percent of their content in most of the analyzed extracts (the lowest 60.65% for clitoria and the highest 84.71% for common mallow).

Comparing methods with and without ultrasound treatment, a successive improvement in extraction efficiency and an increase in the antioxidant activity of the tested samples was observed. Even with the least effective solvent (H₂O),

the US treatment increased the TAA of the tested extract, for instance, in the case of red clover, where significantly changed – 67.36% (without US) vs. 70.74% (with US). Common mallow extract obtained with acidified MeOH and with US treatment showed the highest total antioxidant activity (87.86%) from among all the samples. Xu [45] obtained similar results that proved ultrasound-assisted extraction was more effective than maceration and Soxhlet extraction and allowed obtaining an extract with higher antioxidant capacity from *Jatropha integerrima* flowers.

Analogous tendencies were observed for the results obtained with the ABTS radical test. method, but the differences between samples were slightly lower than for the DPPH method. It may be because the ABTS assay is aqueous-based, thus hydrophilic compounds with antioxidant capacity are favored [40]. All flower species showed similar levels of TAA obtained by the most efficient method (MeOH + acetic acid + US)—from 56.40 to 56.98 mg TE/g of extract, where mallow extract was the richest. Higher antioxidant efficiency of extracts extracted with ultrasound assistance was also reported by Flieger when obtaining an extract from dried goutweed with a water-ethanol solvent [46].

Conclusion

The study proved that the extraction efficiency of antioxidant compounds, including phenols and anthocyanins, from the petals of common mallow, clitoria, cornflower, and red clover flowers varies depending on the method used. The most effective solvent was 70% MeOH acidified with acetic acid. Additional ultrasound treatment statistically significantly increased extraction efficiency by several percent in most samples. Mallow flower petals were the richest source of anthocyanins and phenols and showed the highest radical scavenging ability among the species tested. The studied flowers can be regarded as a potential source of antioxidant compounds, which should be extracted using methods that ensure efficient extraction and full utilization of the raw material, i.e., selecting an appropriate solvent that provides a stabilizing environment for bioactive compounds (like MeOH and acetic acid used) and completing the process with supporting methods such as ultrasound treatment.

Declarations

Conflict of interest The authors declare no conflicts of interest.

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