

Physicochemical and antioxidant evaluation of watercress (*Rorippa nasturtium aquaticum* L.) leaf extracts

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Abstract: This research aimed to evaluate the type of solvent (80% methanol, ethanol, and water), solute/solvent ratio (1 : 10 and 1 : 15 w/v), maceration time (0 h, 24 h and 7 d) and stage of leaf maturity [vegetative (VW) and generative (GW)] on the physicochemical and antioxidant properties of watercress leaf extracts. The leaf was characterised by determining the chemical composition, the phytochemical profile, and the colour. The GW presented the highest moisture content [93.25 g·(100 g)⁻¹], carbohydrates [70.74 g·(100 g)⁻¹], and lightness ($L^* = 59.66$), and the presence of alkaloids, phytosterols, phenols, and flavonoids. VW had the highest protein content [26.52 g·(100 g)⁻¹] and the lowest presence of phytochemicals. The best solvent for the extraction was distilled water at a 1 : 15 w/v ratio, GW at 24 h rest centrifuged at 2 300 × g for 15 min, obtaining the highest values of phenols [2 077 mg GAE·(100 g)⁻¹], of dust and an inhibition power of 85.09% by the 2,2'-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) radical cation (ABTS+) radical method. Therefore, cress leaves in the generative stage can be considered a potential source of bioactive compounds, and using water as an extracting agent for these compounds makes it a viable and economical method to be used in the food industry, and in addition, to be friendly to the environment.

Keywords: antioxidant capacity; extraction; maceration; phytochemical compounds; wild plants

Recently, in Mexico and internationally, the number of chronic degenerative diseases has been increasing, affecting adults as well as children (Ávila-Escalante et al. 2020). This is mainly a consequence of poor diet and low consumption of foods with nutraceutical compounds. It is the reason why alternatives are sought in natural foods that contain 30 substances beneficial to health. Watercress (*Rorippa nasturtium aquaticum* L.) belonging to the family *Brassicaceae* is a plant that can grow wild in swampy places in Mexico; it is cultivated commercially for its edible leaves in the

vegetative state (green), since the leaves in the generative state are discarded for their physical appearance (yellow and withered) (Al-Snafi 2020).

However, in both states of maturity of the leaves, this plant contains important compounds such as proteins, vitamins (A, B1, B2, B3, C, E), minerals (Fe, Ca, K), and bioactive compounds, among which are included phenolic acids, flavonoids (catechins, quercetins, flavonols), glucosinolates, isothiocyanates, terpenoids and tannins, which have been identified in watercress by various authors (Pinela et al. 2020), and which are

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responsible for the antioxidant capacity, the main function of which is to help prevent various chronic degenerative diseases (Al-Snafi 2020).

In this sense, to extract these bioactive compounds with biological activity, it is important to establish a viable, practical, environmentally friendly, and economic extraction method. In recent years, various extraction methods have been used for this purpose; these methods include conventional methods such as Soxhlet extraction, percolation, and maceration, as well as unconventional methods like ultrasound, microwaves, and high hydrostatic pressures. Another factor in the extraction of bioactive compounds is the use of a solvent, since it has been reported that ethanol, methanol, acetone and their mixtures with water at different ratios have had favourable results. However, there is no defined method and solvent as this will depend, among other factors, on the chemical structure of the compounds to be extracted, the concentration of the solvent, temperature, rest time with the sample, and mass-solvent ratio, as well as on the nature of the sample. Therefore, the aim of this investigation was to evaluate the impact of the type and ratio of solvent (80% methanol, ethanol, and water, 1 : 10 and 1 : 15 w/v), maceration time (0 h, 24 h, and 7 d), and stage of leaf maturity (vegetative and generative) on the physicochemical and antioxidant properties of watercress (*Rorippa nasturtium aquaticum* L.) leaf extracts.

MATERIAL AND METHODS

Collection and selection of raw material

The watercress leaves were purchased from the local market of San Juan Bautista Tuxtepec, Oaxaca, Mexico. Two batches of cress leaves were obtained depending on the stage of maturity. The first batch comprised leaves in the vegetative growth stage (VW) which presented intense green colour because they are new leaves produced by the plants during growth for root and foliage development. The second batch consisted of leaves in the generative growth stage (GW) that were yellow in colour; this characteristic is a consequence of the fact that the plant enters a stage of reproduction.

Physicochemical characterisation of the leaves

The physicochemical properties were determined according to the following AOAC (2005) methods (pH, soluble solids, moisture, ash, fats, fibre, proteins and total carbohydrates by difference). The energy content and phytochemical profile were determined according to Rodríguez-Miranda et al. (2022). Colour

was determined using a HunterLab tristimulus colorimeter (model WR-10QC, 45/0L, Reston, Virginia, USA) determining the parameters of luminosity [$L^* = 100$ light/0 dark), a^* (+ red/ – green), b^* (+ yellow/– blue), chromaticity ($C^* = ((a^{*2} + b^{*2})^{1/2})$) and hue angle ($h^\circ = ((a^{*2} + b^{*2})^{1/2})$).

Conditioning the leaves and obtaining leaf powder

The leaves were dried at $60 \pm 2^\circ\text{C}$ for 4 h, ground and an 80 mesh (180 μm) sieve was used.

Obtaining the extracts of watercress leaves

Leaf extracts in VW and GW stages were made by maceration at 1 : 10 and 1 : 15 w/v (solute/solvent) with three different solvents (80% methanol, ethanol, and water, respectively), at different rest times [0 h (T_0), 24 h (24h) and 7 days (7d)] and centrifuging at $6\,100 \times g$ for 10 min and $2\,300 \times g$ for 15 min. An extract was also made by infusion in distilled water (98°C for 15 min) (Figure 1).

Characterisation of the extracts

Total phenols. An amount 250 μL of the extract, 625 μL of the 10% Folin-Ciocalteu reagent, and 500 μL of 7.5% sodium carbonate solution were taken. It was kept in the dark for 120 min and the absorbance was measured at 760 nm (Rodríguez-Miranda et al. 2022). The values of total phenolic compounds were expressed as mg gallic acid equivalents GAE $\cdot(100\text{ g})^{-1}$ of watercress powder.

Total flavonoids. Two standards were used, catechin and quercetin. For catechin, 1 mL of the extract, 100 μL of 5% sodium nitrite, 100 μL of 10% aluminium chloride, and 200 μL of 4M sodium hydroxide were allowed to react for 5 min and read at a wavelength of 510 nm, expressing the results in mg catechin equivalents CE $\cdot(100\text{ g})^{-1}$ of watercress powder. For quercetin, 1 mL of the extract and 100 μL of 10% aluminium chloride were taken. The absorbance was read at 430 nm, expressing the results in mg quercetin equivalents QE $\cdot(100\text{ g})^{-1}$ of watercress powder. The results were added to obtain total flavonoids and expressed in mg $\cdot(100\text{ g})^{-1}$ of watercress powder (Rodríguez-Miranda et al. 2022).

Antioxidant capacity

DPPH $^{\bullet}$. The antioxidant capacity was measured by two methods: 1,1-diphenyl-2-picrylhydrazyl (DPPH $^{\bullet}$), 2,2-diphenyl-1-picrylhydrazyl (DPPH $^{\bullet}$), according to the methodology described by Rodríguez-Miranda et al. (2022), which is based on the reduction of the absorbance of the radical DPPH $^{\bullet}$ 100 μM (3.9 mL) dissolved

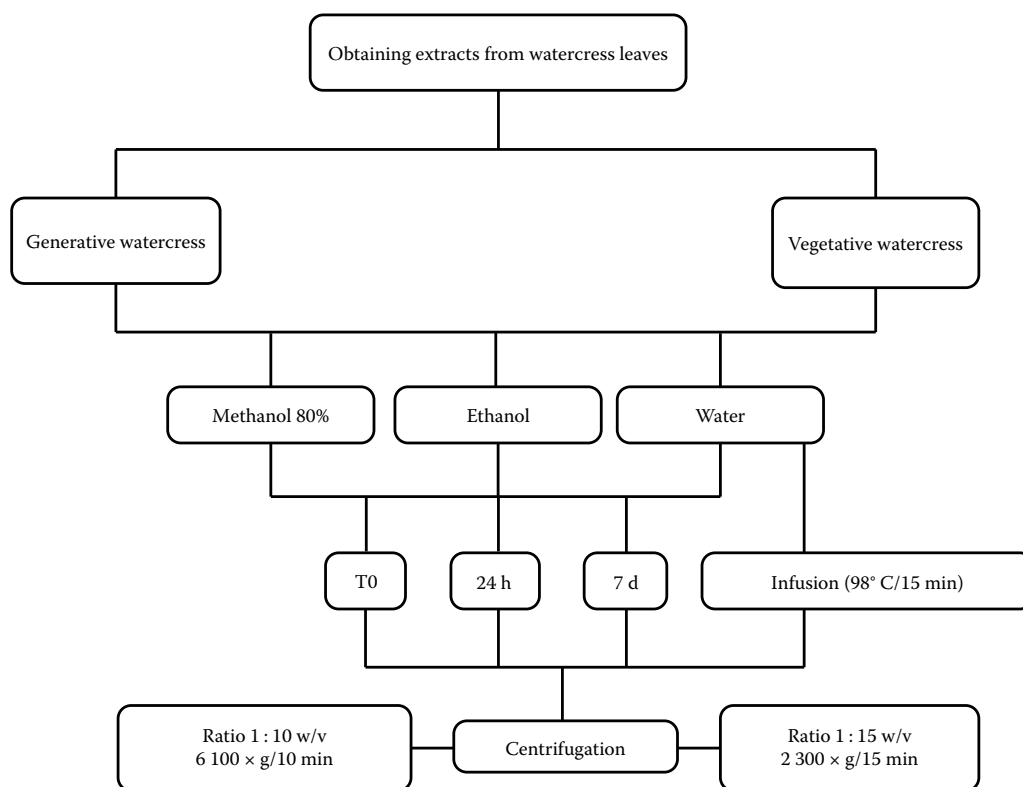


Figure 1. Diagram for obtaining watercress leaves extracts by maceration depending on the state of maturity, using different solvents

in methanol adjusting to an absorbance of 0.7 ± 0.02 , at 515 nm. 50 μL of the sample or standard and 1 050 μL of the radical were used, and kept in the dark for 30 min. The results were expressed in % antiradical activity of the watercress leaf extract.

ABTS+ radical. The ABTS+ radical (7 mM) solution was prepared with potassium persulfate (2.45 mM) (Almulaiky et al. 2020), when 1 mL of the ABTS+ radical was diluted with 45 mL of ethanol until obtaining an approximate absorbance of 0.7 ± 0.02 at 732 nm. An aliquot of 50 μL was taken from the extract and 1050 μL of the ABTS+ radical solution was added. It was kept in the dark for 30 min and its absorbance was measured at 732 nm. The results were expressed in % antiradical activity of the watercress leaf extract.

Statistical analysis

The results are the means of three replicates $\pm$ standard deviation. A one-way ANOVA and a mean comparison test were performed using the Tukey method with a confidence level of 95%, using the Statistica software (version 10, StatSoft, Inc., 2011, USA).

RESULTS AND DISCUSSION

Physicochemical characterisation of watercress leaves

The major component of the watercress leaves is moisture, when significant differences ($P < 0.05$) were obtained in different stages of maturity (Table 1). This increase in moisture is due to the mature leaf absorption of moisture from the environment, as the plant grows in water. So, most of the plant weight is moisture due to such water absorption, and also due to the loss of turgor stopping the plant transpiration mechanism, which causes them to lose their rigidity; the bound water contained in their compounds is released as a consequence of their degradation (Larridon et al. 2020).

They did not present any significant differences ($P > 0.05$) in the ash and fat content (Table 1). These results are lower than those reported by Pinela et al. (2020) in watercress leaves ($1.04 \text{ g} \cdot (100 \text{ g})^{-1}$ of ash and fats $2.46 \text{ g} \cdot (100 \text{ g})^{-1}$), due to the agronomic conditions of the crop.

On the other hand, high levels of the protein content were found in both GW and VW (Table 1). However, significant differences ($P < 0.05$) were observed

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Table 1. Physicochemical characterisation of watercress leaves in generative (GW) and vegetative (VW) stages

Parameters	Samples	
	GW	VW
pH	6.82 ± 0.00 ^a	6.53 ± 0.01 ^b
Soluble solids (°Brix)	4.00 ± 0.00 ^b	5.00 ± 0.00 ^a
Moisture [g·(100 g) ⁻¹]	93.25 ± 0.20 ^a	90.42 ± 0.21 ^b
Ash [g·(100 g) ⁻¹]	1.18 ± 0.11 ^a	1.35 ± 0.10 ^a
Fat [g·(100 g) ⁻¹]	3.69 ± 0.30 ^a	3.49 ± 0.37 ^a
Proteins [g·(100 g) ⁻¹]	24.37 ± 0.05 ^b	26.52 ± 0.91 ^a
Fibre [g·(100 g) ⁻¹]	15.51 ± 0.40 ^a	15.09 ± 0.07 ^a
Carbohydrates [g·(100 g) ⁻¹]	70.74 ± 0.44 ^a	68.62 ± 0.62 ^b
Total energy content [Kcal·(100 g) ⁻¹]	413.68 ± 1.11 ^a	412.02 ± 2.22 ^a
<i>L</i> [*]	59.66 ± 0.28 ^a	58.31 ± 0.24 ^b
<i>a</i> [*]	1.71 ± 0.67 ^a	−4.51 ± 0.18 ^b
<i>b</i> [*]	23.88 ± 0.53 ^a	21.63 ± 0.42 ^b
<i>C</i> [*]	23.95 ± 1.50 ^a	22.10 ± 0.38 ^b
<i>h</i> [°]	85.92 ± 1.51 ^a	78.23 ± 0.67 ^b

Data expressed as the mean of three repetitions ± standard deviation; ^{a,b}different letters between rows indicate a significant difference ($P < 0.05$); † values expressed on a wet weight; *L*^{*} – luminosity; *a*^{*} – +red/−green; *b*^{*} – +yellow/−blue; *C*^{*} – chromaticity, *h*[°] – hue angle

between them. This is a consequence of the protein degradation due to the effect of the stage of maturity, thus producing other types of compounds such as secondary metabolites (flavonoids and carotenoids) and decreasing proteins due to leaf senescence during the degradation of chloroplasts for the formation of gerontoplasts (Larridon et al. 2020). Pinela et al. (2020) observed lower values in watercress leaves proteins (23.18 g·(100 g)⁻¹) than those of this investigation.

In the fibre content there were no significant differences ($P > 0.05$) between the GW and VW, which can be explained by the carbohydrates that constitute the structure of the leaves. The stage of maturity does not affect the fibre, and therefore the same content was found in both batches of watercress leaves.

The fibre content was higher than that reported by Pereira et al. (2011) in wild watercress green leaves (26.08 g·(100 g)⁻¹). This may be due to the chemical structure of the leaves of the plants, as well as to the season (spring or winter) when the raw material was collected. Carbohydrates presented higher values in GW as a consequence of the presence of lower values of ash,

fat, and protein compared to VW (70.74 g·(100 g)⁻¹ and 68.62 g·(100 g)⁻¹, respectively).

The energy content was below (Table 1) the level than what is proposed by the Recommended Dietary Allowances (RDA in 2022). The calories suggested for daily consumption according to age and gender are as follows: children (1 300–2 000 Kcal), women (1 400–2 200 Kcal) and men (2 200–3 000 Kcal); therefore, the consumption of these leaves can be included in the daily diet, obtaining part of the calories necessary for our daily activities.

Significant differences ($P < 0.05$) were observed in the analysed colour parameters [*L*^{*}, *a*^{*}, *b*^{*}, *C*^{*}, and *h*[°] (Table 1)]. The values suggest that the WG presents a light brown colour (Figure 2b), while the VW presents a greyish-green colour (Figure 2a). This is because at the beginning of drying, the leaves of GW were yellow and those of VW were intense green. This phenomenon is known as leaf senescence and it results in the transformation of the plant cell chloroplasts to gerontoplasts, and subsequently in the degradation of chlorophyll, producing carotenoids and flavonoids (characteristic of yellow-orange colour).

In the phytochemical profile, the sample that presented the highest colour intensities during the qualitative tests was the GW, especially in the alkaloid, phytosterol, phenol, and flavonoid tests (Table 2). Alkaloids are compounds to which therapeutic potentials have been attributed, e.g. against multiple sclerosis, rheumatoid arthritis, and they also show anti-inflammatory properties (Núñez et al. 2018; Xu et al. 2020) while phytosterols are responsible for reducing lipids in the body. Some properties such as antitumor, anti-inflammatory, fungicidal, and bactericidal ones are also attributed to these compounds (Nattagh-Eshstivani et al. 2022). On the other hand, polyphenol compounds that encompass a large number of compounds, among others phenolic acids, hydroxybenzoic acids, and flavonoids, are responsible for the antioxidant and antimicrobial capacity (Zhang et al. 2022). It has been reported that watercress contains a large variety of beneficial compounds, such as polyphenols, glucosinolates, isothiocyanates, carotenoids, which are responsible for the antioxidant capacity, and thus for the reduction of chronic degenerative diseases like cancer, diabetes, cardiovascular diseases (Engelen-Eigles et al. 2006; Aires et al. 2013; Boligon et al. 2013; Klimek-Szczykutowicz et al. 2018; Chis et al. 2020).

Characterisation of the extracts

Total phenols. Significant differences ($P < 0.05$) were observed between the extracts. In the extracts

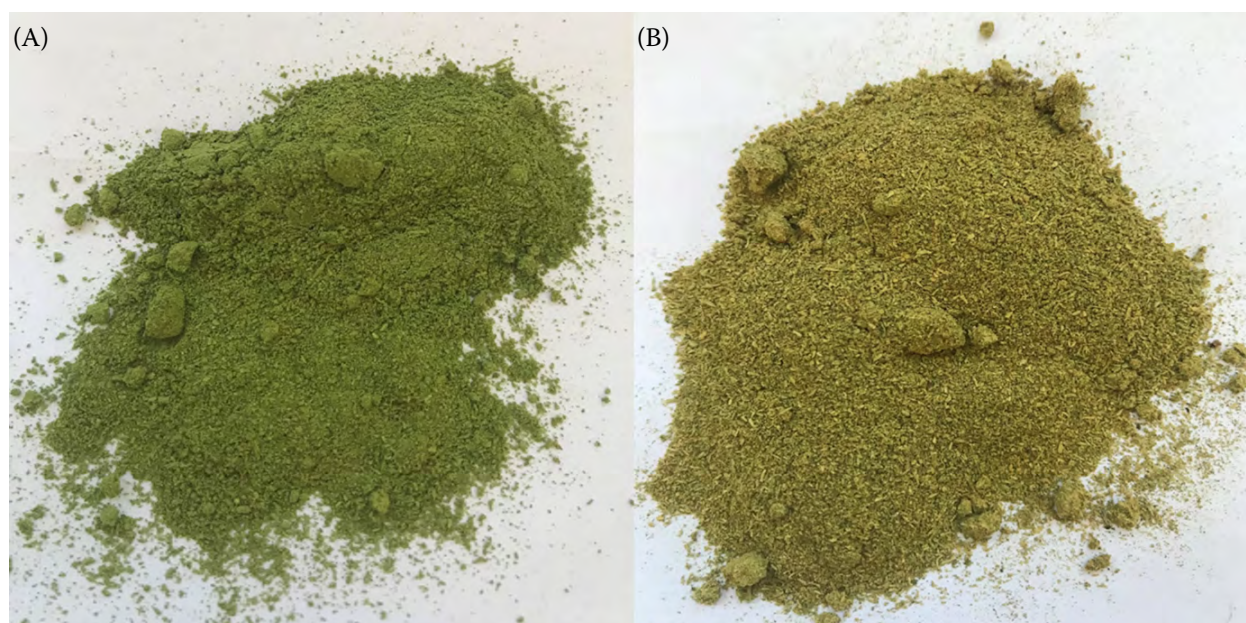


Figure 2. (A) Vegetative watercress leaf powder sample and (B) generative watercress leaf powder sample

(VW and GW) obtained at the watercress leaf powder to solvent ratio of 1 : 10 w/v, methanolic and aqueous extracts with the rest for 7 d and 24 h, presented the highest amount of phenols (2 522 mg GAE·(100 g)⁻¹ and 2 435 mg GAE·(100 g)⁻¹, respectively) (Figure 3a). While the ethanolic extracts were the ones with the lowest amount of phenolic compounds. This may be due to the low affinity of the bioactive compounds present in watercress to the solvent (Figure 3a). On the other hand, when the 1 : 15 w/v ratio was used, the aqueous extracts with 24 h of rest (VW

and GW) presented the highest content of polyphenols. A similar effect was observed by Paladino and Zuritz (2011), who reported the highest extraction using water as the solvent, followed by methanol and ethanol in the last place.

In the case of the infusion treatment, the highest phenolic content was expected to be obtained in this treatment (Figure 3a) since Sánchez-Chino et al. (2019) reported that more phenolic compounds are extracted by the infusion method applied for the maceration using water as a solvent, but everything will also

Table 2. Phytochemical profile of watercress leaves as a function of maturity stage

Phytochemical compound	Method	Sample	
		GW	VW
Alkaloids	Dragendorff	+++++	+++
	Hager's	+++++	+++
Carbohydrates	Benedict's	+	+
Glycosides	Borntrager's	+	+
Saponins	foam formation	+	+
Phytosterols	Salkowski	+++++	+++
	Liebermann-Burchard	+++++	+++
Phenols	ferric chloride	+++++	+++
Flavonoids	alkaline reagent	+++++	+++
Terpenoids	copper acetate	+	+

Data expressed as the mean of three repetitions ± standard deviation; + indicates presence of phytochemicals; +++ shows a moderate concentration; +++++ shows a high concentration VW – vegetative watercress; GW – generative watercress

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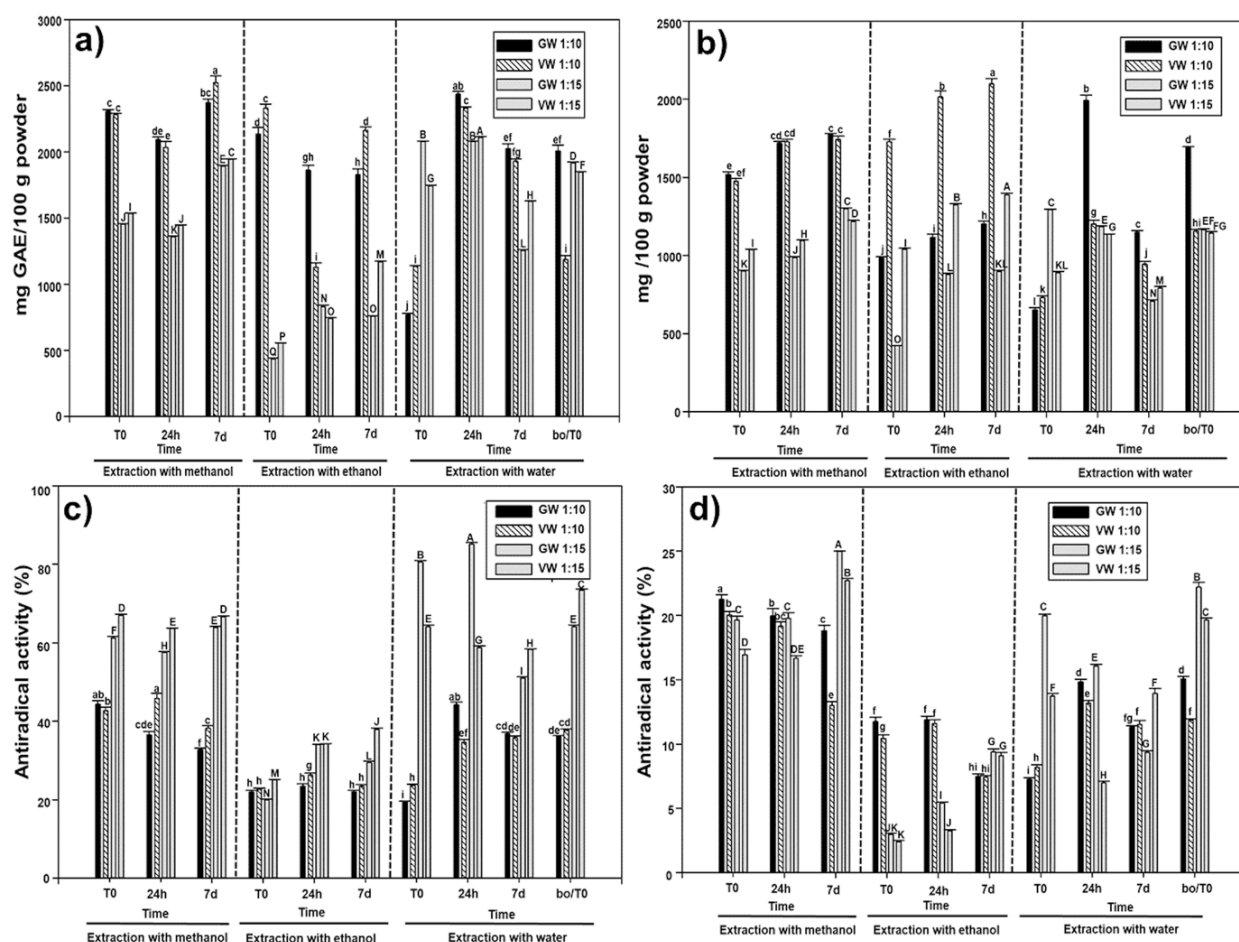


Figure 3. (A) Total phenols; (B) total flavonoids; antiradical activity: (C) ABTS+ radical; and (D) DPPH* in the different extracts of watercress leaves obtained by infusion in boiling water (bo) or maceration at different maturity stages—generative (GW) and vegetative (VW) – using different solvents (80% methanol, ethanol, and water), solute/solvent ratios (1 : 10 and 1 : 15 w/v), and resting times (T0 = zero time, 24 h, and 7 d)

depend on the chemical composition of the raw material. Mazandarani et al. (2013) obtained the values of 803–935 mg gallic acid equivalent (GAE)·(100 g)^{−1} for VW extract and 650–765 mg GAE(100 g)^{−1} for GW extract, concluding that these variations are due to different concentrations of the used raw material, as well as to the extraction methods used, and the variety and origin of the plant. Therefore, the greatest amount of phenolic compounds from watercress leaves can be extracted with water and methanol, and this method could be used in products in the pharmaceutical and food industries due to their beneficial effects on health, especially the aqueous extract due to its zero toxicity.

Total flavonoids. The highest amount of flavonoids was found in the ethanolic extracts prepared by maceration with the rest for 7 d in the VW with a ratio of 1 : 10 and 1 : 15 w/v (Figure 3b). It is so because

phenolic compounds, especially flavonoids, are more soluble in organic solvents such as ethanol (Rodríguez-Miranda et al. 2022). In the methanolic extracts with a 1 : 10 w/v ratio, no significant differences were found between GV and VW ($P > 0.05$) (Figure 3b). However, the aqueous extract obtained by infusion was more affected in the VW, since the content of total flavonoids decreased at both concentrations; the reason is that the boiling breaks the cell matrix, releasing bioactive compounds; however, when they are exposed to boiling for a long time, they tend to degrade. The impact of the rest time on the maceration was favoured by the methanol and ethanol solvents, since the longer the days of the rest, the greater amount of flavonoids was extracted, while with water and rest for 24 h, the content decreased mainly at the ratio of 1 : 10 w/v. This is due to the senescence of the leaves in which the chlo-

roplasts of plant cells are transformed into the gerontoplasts, and the degradation of chlorophyll producing carotenoids and flavonoids (characteristic of yellow-orange colours) occurs at the same time (Matile 2001; Krupinska 2006; Díaz-Mendoza et al. 2016). In addition, plant growth conditions, climatic conditions, soil types (nutrients) and the harvest period of raw materials can also be attributed to the extraction conditions of the above-mentioned compounds (Al-Snafi 2020; Pinela et al. 2020). The values found in this investigation are below those reported by Al-Snafi (2020): VW 2.881 mg quercetin equivalent (QE)·(100 g)⁻¹ and GW 3.977 mg QE·(100 g)⁻¹.

Antioxidant capacity

ABTS⁺. The results showed significant differences ($P < 0.05$) in all the extracts. The highest percentage of antiradical activity was at 1 : 15 w/v ratios in aqueous extracts (50.97–85.09% inhibition), followed by methanolic extracts (57.54–66.90% inhibition), with the highest antiradical activity in the extract from GW for 24 h (Figure 3c).

This is due to the type of compounds that watercress contains that are highly hydrophilic, such as flavonoids (quercetin-3-O-rutinoside, 7-O-glucoside, quercetin-3-O-triglucoside, kaempferol-3-O-(caffeoyldiglucoside)-7-O-rhamnoside, isorhamnetin) and catechin derivatives, followed by phenolic acids (caffeic acid, gallic acid, chlorogenic acid, ferulic acid, p-coumaric acid, p-hydroxybenzoic acid, sinapic acid, caftaric acid, vanillic acid, dicaffeoyltartaric acid, caffeoylmalic acid) and some glucosinolates [2-phenethyl glucosinolate (gluconasturtiin), glucosibarin, 4-methoxyglucobrassicin, glucobrassicin, 4-hydroxyglucobrassicin, glucohirsutin, glucotropaeolin and isothiocyanates] (Al-Snafi et al. 2020; Pinela et al. 2020).

These compounds present a large number of hydroxyl groups (-OH) in their chemical structures, responsible for granting this antioxidant capacity, due to the availability of donation of their hydrogen atoms to free radicals (Chaudhary et al. 2018). The amount of total phenols and flavonoids is not a determining factor of the antioxidant capacity of the extract, since, as can be seen in Figure 3c, the extracts prepared at a 1 : 10 w/v ratio that presented higher total phenols are the ones that are now below the antioxidant capacity of the extracts at a 1 : 15 w/v ratio.

DPPH[•]. In methanolic extracts of GW at the ratios of 1 : 10 and 1 : 15 w/v at times 0 h and 7 days, a higher percentage of inhibition was observed (Figure 3d). The lowest percentages were obtained with the ethan-

olic extracts with an inhibition range of 2.42–11.88%. This is because some phenolic compounds can react more quickly than others, such as quercetin and catechin, being more reactive than vanillic and p-coumaric acids (Oliveira et al. 2016). Therefore, the selection of the solvent for the extraction of active compounds is of great importance in complex food matrices, since it will determine the type of polyphenols that are extracted, while their amount also depends on polarity and solubility. The solubility of polyphenols increases with the addition of water because water weakens the hydrogen bonds of polyphenols with proteins (Safdar et al. 2017). That is why the results obtained in the antioxidant capacity by DPPH are low because the compounds extracted with water are mostly hydrophilic in nature, therefore these radicals react with lipophilic compounds (Ozdemir et al. 2024). While the ABTS⁺ radical acts on the compounds of hydrophilic and lipophilic nature, i.e. it can react with molecules that have a single hydroxyl group (Cerretani and Bendini 2010).

CONCLUSION

The watercress leaves in both stages of maturity showed important bioactive compounds, mainly phenols, flavonoids, phytosterols, and alkaloids. The aqueous extract of GW watercress leaves macerated for 24 h at a 1 : 15 w/v ratio had a higher antioxidant capacity, making this extract the most viable to be used as an alternative in food products. In addition, considering the extraction method and the type of solvent that makes it a viable and economical extraction method, it is recommended to carry out more research to protect the active compounds present in watercress, since they tend to be easily degraded by external factors such as light, pH, oxygen and temperature.

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