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Effect of ultrasound on the concentration of phenolic compounds and antioxidant capacity during the preparation of *Berberis vulgaris* leaves extracts

Elvis Gilmar Gonzales-Condori, D.Sc.[1], Juan Carlos Garcia-Cari, B.Sc.[2], Giovanna Garcia-Cari, B.Sc.[2], Jonathan Gonzales-Condori, B.Sc.[3], Rosa Alvarez-Gonzales, B.Sc.[2].

[1]Universidad Tecnológica del Perú (UTP), Av. Tacna y Arica 160, Arequipa, Perú, elvgonzalesc@gmail.com

[2] *Centro de Investigación Kodama S.A.C., Urb. Leoncio Prado U10, Paucarpata, Arequipa, Perú,* juagarcari@gmail.com, giovannaruthgarcar@gmail.com, rosalizethag@gmail.com

[3]Universidad Católica de Santa María (UCSM), Urb. San José s/n Umacollo, Arequipa, Perú, jogonzaco@gmail.com

Abstract: Berberis vulgaris is popularly known as Agracejo in Peru. This species has been shown to have important biological properties such as hypolipidemic, antidiabetic, cholagogue, and hepatoprotective effects. Its effects would be related to the varied phytochemical composition obtained after extraction with different solvents or extraction techniques. Therefore, it is necessary to determine the best extraction conditions to obtain the most significant effects according to each extraction technique used. The objective of this research was to evaluate the effect of ultrasound application on the concentration of total phenols and antioxidant capacity of ethanolic, hydroalcoholic, and aqueous extracts of Berberis vulgaris leaves, extracts were obtained by ultrasound application for 0, 5, 10, 20 and 30 minutes at 40 kHz in an ultrasonic bath. The results indicate that the application of ultrasound affects the extraction process of phenolic compounds in ethanolic and hydroalcoholic extracts, it was also demonstrated that an application longer than 20 minutes leads to a decrease in the concentration of these bioactive compounds. On the other hand, ultrasound positively affects the antioxidant capacity of ethanolic and hydroalcoholic extracts up to 20 minutes; however, in aqueous extracts, ultrasound increases the antioxidant capacity up to 10 minutes of application, since exceeding this time this antioxidant capacity decreases. In conclusion, ultrasound does affect the extraction process of phenolic compounds from Berberis vulgaris leaves, and the solvent that favors extraction, guaranteeing a greater antioxidant capacity, is water.

Keywords: Ultrasound, leaves, Berberis vulgaris, total phenols, antioxidant capacity.

I. Introduction

Free radicals are produced naturally by normal cellular metabolism, disease processes, or exposure to xenobiotics [1]. Therefore, studies to counteract free radicals are developed by the food, pharmaceutical, and cosmetic industries, promoting efforts in the search for natural compounds with antioxidant properties [2, 3]. Natural products have been used for the prevention and treatment of human diseases for thousands of years [4, 5, 6]. For this reason, there is much interest today in finding natural antioxidants for use in food, medicines, or cosmetic products [7]. Plants have in their composition various compounds with biological properties useful for treating diseases [5], therefore, many medicinal plants are used in folk medicine as an alternative treatment for some diseases [6]. This is because medicinal plants contain phenolic compounds in their composition [8] which are mainly natural organic chemicals, but also synthetic or semi-synthetic, including phenolic acids, flavonoids, stilbenes, and lignans [9]. These compounds would be responsible for the antioxidant capacity and biological effects of medicinal plants.

Agracejo whose scientific name is *Berberis vulgaris* [10] presents in its composition berlambine, Hydroxyxicanthine, Isocoridine, (-) Tejedine, Lupeol, Oleanolic acid, Stigmasterol steroids and Stigmasterol glycoside [11]. Because of this, it has demonstrated antioxidant capacity and phenolic compounds in important concentrations in fruits, stems, roots, and leaves, showing biological activities that benefit human beings. [12, 13, 14, 15, 16, 17, 18]. Among its main biological effects, *Berberis vulgaris* has been shown to significantly reduce total cholesterol in humans [13], on the other hand, it was shown to significantly reduce insulin levels [14], also regulate glucose metabolism and lipid profile in patients with type 2 diabetes [15] and has no significant side effects [16]. On the other hand, it proved to be a cholagogue (it provokes the evacuation of bile) and hepatic protector [10].

Considering the above, *Berberis vulgaris* is a plant with important properties, however, there are currently no studies related to the preparation of extracts of this species since it is known that the type of solvent and the extraction technique are determinant in the biological properties of an extract. This is because the extraction of biologically active compounds from biological matrices depends largely on the efficacy and efficiency of the extraction technique used [19]. Nowadays, several studies are being developed related to extraction procedures such as solid-liquid extraction, solid-phase extraction, and liquid-liquid extraction, but more recently, the introduction of supercritical fluid extraction, microwave-assisted extraction, and ultrasound-assisted extraction (UAE) have received great interest to overcome the disadvantages of

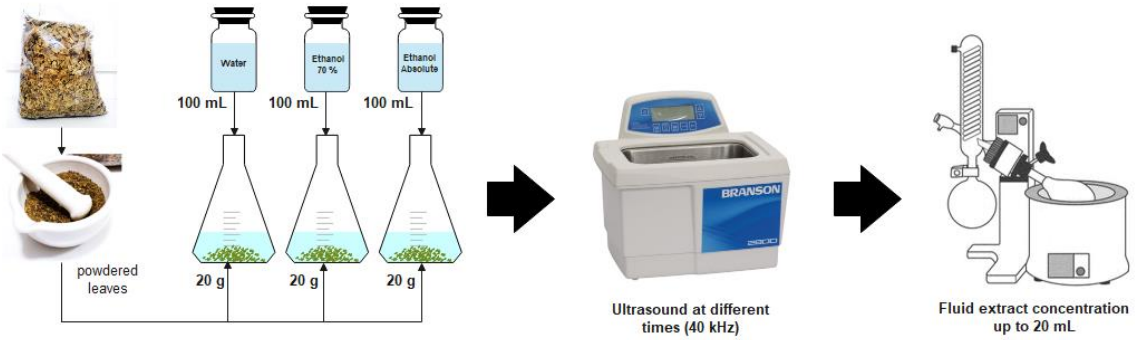

Fig. 1 Preparation process of fluid extracts of *Berberis vulgaris* leave

classical extractions [20]. Thus, studies have demonstrated the efficiency of extract preparation using ultrasound (US) [21] improving extraction performance [22] and achieving higher scavenging capacity of DPPH (2,2-Diphenyl-1-Picrylhydrazyl), ABTS (2,2'-azinobis[3-ethylbenzothiazoline-6-sulfonic acid]-diammonium salt) and hydroxyl radicals [23]. In addition, UAE improves extraction time and medicinal properties such as total phenol and flavonoid content of the extracts [24]. UAE presents an excellent extraction option due to the considerable reduction of time and energy used in the extractions [2]. However, the application of the UAE must be studied in each particular case since some components present in the plants during extraction can be degraded by sonochemical processes [25].

Therefore, in the present research, we sought to contribute to the current literature with the effect of ultrasound application time on the concentration of total phenols and antioxidant capacity during the preparation of ethanolic, hydroalcoholic, and aqueous extracts of *Berberis vulgaris* leaves obtained by UAE.

II. Materials and Methods

A. Reagents and equipment

The reagents Folin 2 N, calcium carbonate, gallic acid, Trolox, DPPH (2,2-Diphenyl-1-Picrylhydrazyl), and absolute ethanol were obtained from Merck. A 40 kHz Branson ultrasonic bath was used for extraction. The Buchi R-210 Rotavapor System was used for solvent recovery and/or extract concentration. Spectrophotometric measurements were performed on the UV/Vis Genesys 150 spectrophotometer.

B. Sample collection

The *Berberis vulgaris* leaves were acquired from a store dedicated to the sale of medicinal plants in the San Camilo market in the city of Arequipa, Peru (-16.4030251,-71.5349644). The leaves were washed with abundant distilled water and dried at room temperature in the shade for 7 days. Once dried, they were pulverized using a porcelain mortar and stored in Kraft paper until further use.

C. Obtaining the extract

The procedure for the preparation of the extracts is shown in Fig. 1. We weighed 20 g of powdered *Berberis vulgaris* leaves in three 250 mL flasks, then 100 mL of distilled water, 70% ethanol, and absolute ethanol were added to each flask to obtain hydroalcoholic and ethanolic aqueous extracts, respectively. Once the solvents were added, they were mixed for 5 minutes to homogenize the suspension. Then ultrasound was applied at times of 0, 5, 10, 20, and 30 minutes to determine the effect of ultrasound application [26, 27]. Different extracts were prepared for each ultrasound application time. The extracts were filtered and evaporated at a rotary evaporator to obtain 20 mL of fluid extract. The extracts were stored in amber glass vials until further analysis.

D. Total phenol determination

For the determination of total phenols by the Folin-Ciocalteu method, a calibration curve was prepared with 0.5 to 10 mg/L gallic acid standard solutions. The procedure consisted of adding appropriate volumes of a 100 mg/L solution of gallic acid to a 10 mL flask, then, 200 µL of Folin's reagent was added followed by 2 mL of 7.5% sodium carbonate. Then, it was made up to the mark with distilled water and it was taken to the dark for 2 hours. Once the color developed, the absorbance reading was taken at 765 nm. The calibration curve was then evaluated by plotting the concentration of gallic acid vs. absorbance at 765 nm. The concentration of total phenols was calculated using the linear equation. For the analysis of the extracts, 0.1 mL of each extract was measured in 10 mL flasks, then Folin's reagent was added followed by 7.5% sodium carbonate in the same amounts as in the standard solutions. Then, it was made up to volume with distilled water and placed in the dark for 2 hours and the absorbance was read at 765 nm. The concentration of total phenols was expressed in milligrams of gallic acid equivalents per liter of fluid extract (mg GAE/L) and was calculated with the linear equation.

E. Antioxidant capacity determination

For the quantification of the antioxidant capacity, calibration solutions were prepared by reacting the DPPH reagent with standard solutions of Trolox at concentrations of

0.2 to 4 mmol/L prepared from a 40 mmol/L solution of Trolox. The procedure consisted of measuring in test tubes 10 µL of these standard solutions where 3 mL of a 0.05 mg/mL DPPH solution was added. This mixture was left to react for 15 minutes in the dark and the absorbance was analyzed in a spectrophotometer at a wavelength of 517 nm. Then, the calibration curve was plotted by plotting the concentration of Trolox vs absorbance of DPPH at 517 nm. The extracts were diluted by measuring 0.5 mL in 10 mL flasks. Antioxidant capacity was analyzed in the same way as the standards by measuring 10 µL of each diluted extract and reacting with 3 mL of a 0.05 mg/mL DPPH solution. [28]. Antioxidant capacity was calculated using the linear equation. Antioxidant capacity was expressed as millimoles of Trolox equivalent per liter of extract (mmol ET/L).

III. Results and Discussion

A. *Extractos de Berberis vulgaris*

Fig. 2 shows the results of the process of obtaining extracts by applying ultrasound at different times. In this figure, a clear difference in the coloration is observed in the ethanolic and hydroalcoholic extracts as the coloration increases as the ultrasound time increases. In contrast, no clear difference is observed in the coloration of the aqueous extracts as a function of ultrasound time. According to Louie *et al.* [29] ultrasound uses high-frequency pulses to generate local hot spots at a macroscopic scale with high shear stress and temperature by producing cavitation bubbles generating advantages of simplicity achieving a more efficient interaction between the plant material with the solvent and extracting the plant components more efficiently. This could explain that as the ultrasound time increases the color of the extract increases significantly when using absolute ethanol and 70 % ethanol. The aqueous extracts show an almost imperceptible increase.

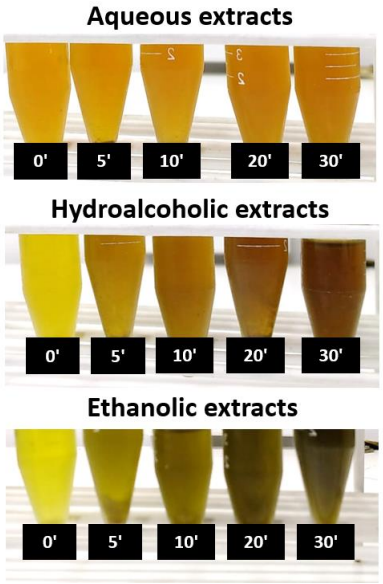

Fig. 2 Aqueous, hydroalcoholic, and ethanolic extracts of *Berberis vulgaris* leaves obtained by ultrasound application at different times.

B. *Total Phenols in Berberis vulgaris*

Fig. 3 shows the calibration curve for the determination of total phenols by the Folin-Ciocalteu method, in this graph the coefficient of determination R^2 is 0.9987.

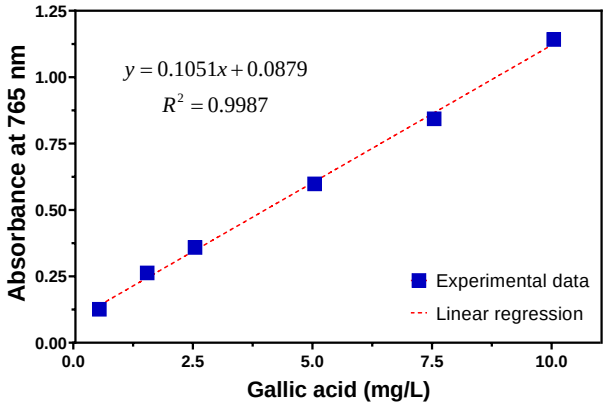

Fig. 3 Calibration curve for the determination of total phenols by the Folin-Ciocalteu method using gallic acid as reference standard.

The linear equation of the line is given in Equation 1:

$$y = 0.1051x + 0.0879 \quad (1)$$

With the above equation, the concentrations of total phenols in the extracts were determined taking into account that "*x*" corresponds to the concentration of total phenols expressed in milligrams of gallic acid equivalents (mg GAE/L) and "*y*" is the absorbance "*Ab*" at 765 nm. The dilution adjustment of 0.1 mL in 10 mL was added to the formula, leaving the formula (equation 2) for the calculation of total phenols as follows.

$$Total\ phenols\,(mg\ GAE/L) = \frac{Ab_{765nm} - 0.0879}{0.1051} \times \frac{10}{0.1} \quad (2)$$

Fig. 4 shows the results of the determination process of total phenols in *Berberis vulgaris* leaves extracts obtained at different ultrasound application times where color development is presented.

It is observed that the ethanolic extracts present ascending color intensities concerning the ultrasound time, likewise, it is noted that when ultrasound is not applied with this solvent the color change is minimal. Similar results were obtained with the hydroalcoholic extract. On the other hand, the aqueous extract does not show a clear difference in coloration, since in the absence or presence of ultrasound the color intensity is the same.

Table I shows the absorbances at 765 nm and the concentration of total phenols of the aqueous, hydroalcoholic, and ethanolic extracts that were calculated using Equation 2.

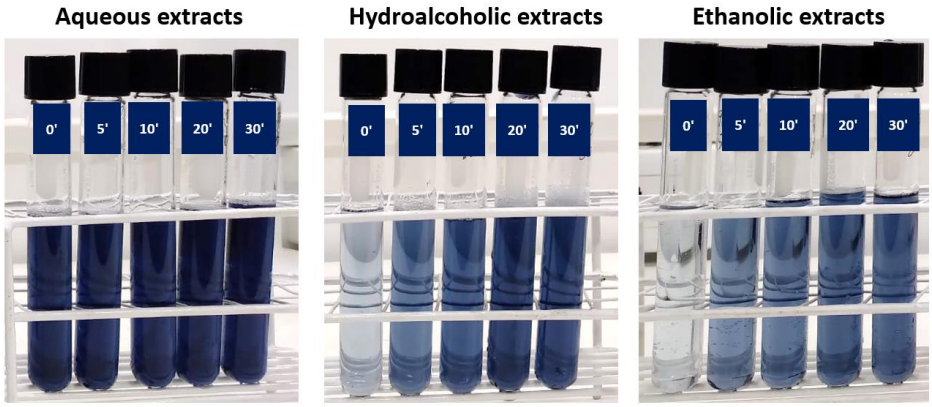

Fig. 4 Results of the determination process of total phenols in *Berberis vulgaris* leaves extracts obtained at different ultrasound application times.

TABLE I
THE CONCENTRATION OF TOTAL PHENOLS IN *Berberis vulgaris* LEAVES EXTRACTS WAS OBTAINED AT DIFFERENT ULTRASOUND APPLICATION TIMES.

US time (minutes)	Absorbance at 765 nm			Total phenolics (mg GAE/L)		
	Aqueous extracts	Hydroalcoholic extracts	Ethanolic extracts	Aqueous extracts	Hydroalcoholic extracts	Ethanolic extracts
0	1.262	0.313	0.167	1117.13	214.18	75.26
5	1.269	0.724	0.573	1123.79	605.23	461.56
10	1.307	0.873	0.662	1159.94	747	546.24
20	1.290	1.071	0.834	1143.77	935.40	709.90
30	1.261	1.006	0.83	1116.18	873.55	706.09

US: Ultrasound, **GAE:** gallic acid equivalents

Fig. 5 shows graphically the results of Table I. It can be observed that the aqueous extract shows higher concentrations of total phenols than the hydroalcoholic and ethanolic extracts.

The aqueous extracts showed a decrease in the concentration of total phenols after 20 min. On the other hand, it is observed that in the ethanolic extract, the concentration of total phenols increases with the application of ultrasound, but it becomes constant at 20 and 30 minutes.

In the case of the hydroalcoholic extract, the maximum concentration of total phenols is achieved at 20 minutes of ultrasound application, since at 30 minutes there is a decrease in the concentration of total phenols. Quintero *et al.*[30] indicates that ultrasound-assisted extraction creates a cavitation of small bubbles in the solvent due to the passage of the ultrasound waves, which allows greater penetration of the solvent inside the material increasing the surface area and allowing more phenolic compounds to be extracted, however, ultrasound seems to degrade or reduce the concentration of total phenols after 30 min.

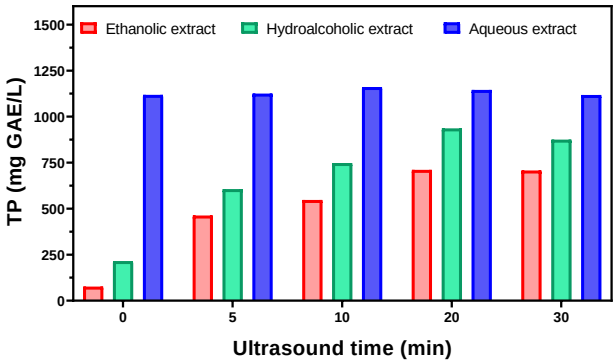

Fig. 5 Total phenol content in *Berberis vulgaris* leaves extracts obtained at different ultrasound application times.

Likewise, in the aqueous extract, the increase in the concentration of total phenols occurs up to 10 minutes. Still, this increase is minimal and it is also observed that after 20 minutes a decrease in this concentration is observed. The analysis of variance is presented in Table II where it is noted that the ultrasound time does affect the extraction process of phenolic compounds with a $p<0.05$, on the other hand, the type of solvent also affects the extraction process with a $p<0.05$, finding a higher concentration of total phenols in the aqueous extract.

Quintero *et al.*[30] in his study found that ultrasound accelerated the ethanol extraction process, reduced energy expenditure, and increased the yield of bioactive compounds present in annatto seeds. Also, the decrease in total phenol content in each case could be due to the oxidation process, as explained by Al Jitan *et al.* [31] which indicates that, when cavitation bubbles burst on the surface of the plant sample matrix, the shockwave-induced damage to the plant cell wall increases the mass transfer of phenolic compounds across the cell membranes into solution, however, ultrasonic waves have been reported to cause degradation of some phenolic acids and the creation of highly reactive hydroxyl radicals within the gas

bubbles. This could explain the decrease in the concentration of total phenols in each extraction.

TABLE II
TWO-WAY ANALYSIS OF VARIANCE OF THE CONCENTRATION OF TOTAL PHENOLS IN *Berberis vulgaris* LEAVES EXTRACTS OBTAINED AT DIFFERENT ULTRASOUND APPLICATION TIMES.

Source	Sum of squares	df	Average of squares	F	*p*
US time	409754.352	4	102438.588	4.271	*0.039*
Solvent	1065855.39	2	532927.697	22.218	*0.001*
Error	191888.051	8	23986.0064		
Total	1667497.8	14			

C. Antioxidant capacity in Berberis vulgaris

Fig. 6 shows the results of the elaboration of the calibration curve to determine the antioxidant capacity by the DPPH method, where the coefficient of determination R^2 is 0.9967. In addition, the equation of the straight line with which the antioxidant capacity was determined in the extracts expressed in Trolox equivalents (mmol TE/L) at 517 nm is observed.

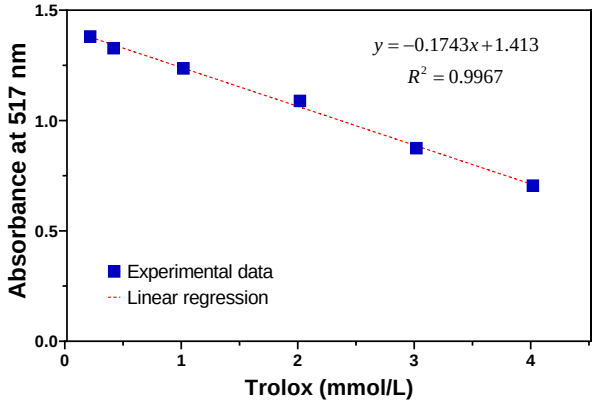

Fig. 6 Calibration graph for the determination of antioxidant capacity by DPPH method

The equation of the straight line is shown below (Equation 3)

$$y = -0.1743x + 1.413 \quad (3)$$

Where "x" and "y" corresponding to the antioxidant capacity equivalent to Trólox (mmol TE/L) and absorbance at 517 nm (Ab_{517nm}) respectively. Replacing, equation 4 is obtained where it is observed that the dilution factor (10/0.5) was added.

$$Antioxidant\ capacity\ (mmol\ TE/L) = \frac{Ab_{517nm} - 1.413}{-0.1743} \times \frac{10}{0.5} \quad (4)$$

Fig. 7 shows the results of the process of determining the antioxidant capacity in the extracts of *Berberis vulgaris* leaves obtained at different times of ultrasound application where the decrease of the DPPH color is presented and it is observed that the ethanolic extracts present descending color intensities as a function of the ultrasound time, likewise, it is noted that when ultrasound is not applied with this solvent the color change is minimal. Similar results were obtained with the hydroalcoholic extract. On the other hand, the aqueous extract does not show a clear difference in coloring, since both with the non-application of ultrasound and with its application the color intensity seems to be the same.

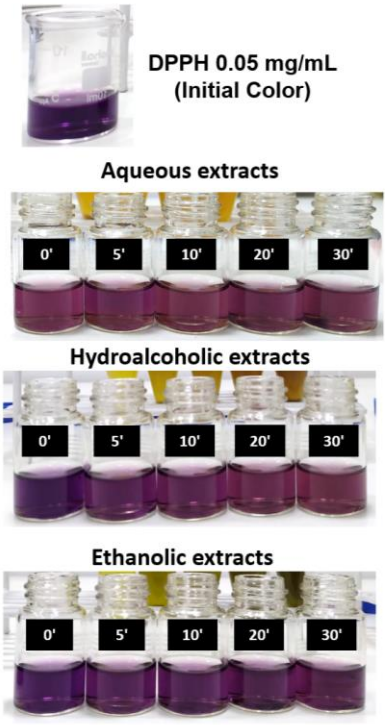

Fig. 7 Results of the process of determining the antioxidant capacity of *Berberis vulgaris* leaves extracts obtained at different ultrasound application times.

Table III and Figure 8 show that the aqueous, ethanolic, and hydroalcoholic extracts have antioxidant capacity. Regarding the effect of ultrasound on the antioxidant capacity, it is observed that in the ethanolic and hydroalcoholic extracts, this capacity increases according to the ultrasound time up to 20 minutes. Regarding this, Quintero *et al.*[30] indicates that ultrasound facilitates the extraction of bioactive compounds by increasing the interaction of the extracting solvent and the plant material. In the aqueous extract, the increase in the antioxidant capacity occurs in up to 10 minutes of ultrasound application. Still, this increase is minimal and it is also observed that after 20 minutes there is a decrease in this antioxidant capacity. The same is observed in the ethanolic and hydroalcoholic extracts after 30 minutes of ultrasound application, decreasing the antioxidant capacity.

The two-way analysis of variance is presented in Table IV where it is noted that the ultrasound time would not significantly affect the antioxidant capacity with a p>0.05 at the different times studied in the different extracts, on the

other hand, the type of solvent would significantly affect the antioxidant capacity of the extracts with a $p<0.05$, finding greater antioxidant capacity in the aqueous extract up to 10 minutes of ultrasound application. As for the ethanolic and hydroalcoholic extracts, the highest antioxidant capacity was obtained after 20 minutes of ultrasound application.

TABLE III
RESULTS OF ANTIOXIDANT CAPACITY IN *Berberis vulgaris* LEVES EXTRACTS OBTAINED AT DIFFERENT ULTRASOUND APPLICATION TIMES.

US time (minutes)	Absorbances at 517 nm			Total phenolics (mg TE/L)		
	Aqueous extracts	Hydroalcoholic extracts	Ethanolic extracts	Aqueous extracts	Hydroalcoholic extracts	Ethanolic extracts
0	0.805	1.263	1.336	69.76	17.21	8.84
5	0.780	1.087	1.208	72.63	37.41	23.52
10	0.774	0.989	1.183	73.32	48.65	26.39
20	0.835	0.878	1.137	66.32	71.49	43.03
30	0.890	0.790	1.038	60.01	61.39	31.67

TE: Trolox Equivalents

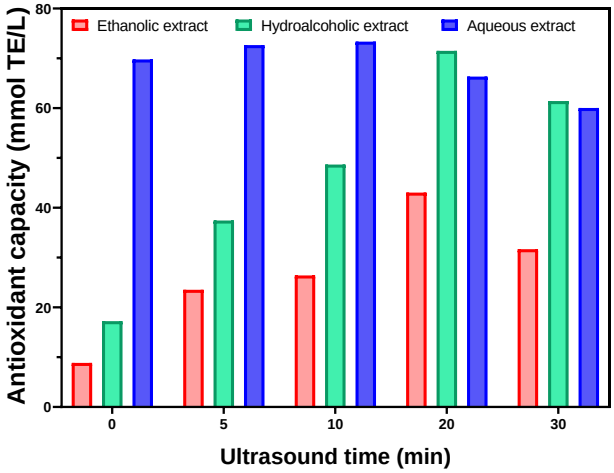

Fig. 8 Antioxidant capacity in *Berberis vulgaris* leaves extracts obtained at different ultrasound application times.

Several researchers have studied Berberis vulgaris, as is the case of Nuralın *et al.* [17] which evaluated the extraction of rutin and apigenin-rich oil from *Berberis vulgaris* fruits using the supercritical carbon dioxide extraction method, finding concentrations of 173 ± 14.97 µg/g and 2.91 ± 0.11 µg/g respectively. On the other hand, Zovko Končić *et al.*[18] found higher total phenol content and antioxidant capacity in leaves than in roots in ethanolic extracts. Likewise, Hosseinihashemi *et al.* [32] analyzed the antioxidant activity of *Berberis vulgaris* inner bark extracts using different solvents and found that acetone extracted the highest amount of antioxidant compounds. On the other hand, another study in fruits by Eroğlu *et al.* [33] both ethanol and water extracts of the fruits showed a higher level of antioxidant activity tested by β-carotene, DPPH, and $ABTS^{+}$ tests, and ethanol extracts showed a higher level of antioxidant activity compared to water extract.

TABLE IV
TWO-WAY ANALYSIS OF VARIANCE OF ANTIOXIDANT CAPACITY IN *Berberis vulgaris* LEAVES EXTRACTS OBTAINED AT DIFFERENT ULTRASOUND APPLICATION TIMES.

Source	Sum of squares	df	Average of squares	F	*p*
US time	1291.80	4	322.95	2.09	0.174
Solvent	4351.98	2	2175.99	14.08	0.002
Error	1236.00	8	154.50		
Total	6879.78	14			

US: Ultrasound

IV. CONCLUSIONS

It was demonstrated that the application of ultrasound significantly affects the content of total phenols in aqueous, hydroalcoholic, and ethanolic extracts of *Berberis vulgaris*. Water extracts a greater amount of phenolic compounds, which correlates with the antioxidant capacity obtained. With the results obtained, it could be said that to achieve the highest concentration of total phenols and antioxidant capacity in aqueous extracts, 10 minutes of ultrasound should be applied and for the preparation of hydroalcoholic and ethanolic extracts, 20 minutes of ultrasound is recommended.

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