Antioxidant and anti-inflammatory polyphenols in ultrasound-assisted extracts from salvilla (*Buddleja scordioides* Kunth)

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**ABSTRACT**

Salvilla is a widely distributed plant used in treatments against gastrointestinal disorders due to its phenolic antioxidant and anti-inflammatory potential. Major yield and quality of bioactive polyphenols must be obtained with no degradation during suitable processes such as Ultrasound-Assisted Extraction (UAE), which allows an efficient extraction of metabolites at appropriate parameter conditions. Salvilla extractions were made using UAE and aqueous ethanolic solutions. Variables used in UAE were sonication time, wave amplitude and percentage of ethanol in solvent. Extracts were tested for total flavonoids, antioxidant activity (ABTS, FRAP and ORAC) and an identification and quantification of phenolic compounds was carried out by UPLC-PDA-ESI-MS/MS. Once elected the better extraction conditions, an anti-inflammatory test was performed for this treatment. As a result, total flavonoids content in extracts was 147 to 288 µg catechin equivalents/mg of dry salvilla extract. All extracts have shown good antioxidant activity (86 to 280 mM Trolox eq/mg dry salvilla extract). Flavonoids contents by chromatography were higher than hydroxybenzoic and hydroxycinnamic acids specially the flavone, flavanol and flavanone groups. Treatment T6 (75% ethanol, 30% amplitude and 10 min extraction time) was the best extract in terms of significant flavonols, antioxidant activity, and higher anti-inflammatory potential.

1. Introduction

The obtention of natural extracts has been possible probably since the discovery of fire. Archaic civilizations developed revolutionary extraction methods such as maceration or alembic distillation used for different proposes. However, most solvents used in mentioned procedures are hazardous and not recognized as ecofriendly processes [1]. Particularly, ethanol is categorized as a green solvent since it is obtained by fermenting renewable resources like sugars, starches or lignocellulosics; it is a low-cost solvent compared with other solvents and is very important because of its non-toxicity [2]. In this sense, it is necessary also to take appropriate measures to assure that potential bioactive constituents will not be lost, modified, or degraded during the process of extraction [3]. Latest trends in extraction methods have largely centered on developing solutions that lessen the use of organic solvents and energy, such as ultrasound-assisted extraction, supercritical fluid extraction, controlled pressure drop process, subcritical water extraction, pulsed electric field, and microwave extraction [1].

One of the most important emergent technologies is the ultrasound assisted extraction (UAE). It consists of pressure waves transferred throughout any medium as compression and rarefaction cycles at high frequency (20–100 kHz) [4]. These fluctuating pressure modifications produce the formation and, eventually after several cycles, the collapse of bubbles inside a liquid medium. The process of formation, enlargement, and implosive collapsing of microbubbles in ultrasound-treated liquids is identified as “acoustic cavitation” [5]. Cavitation collapse on solid surfaces causes microjets that ease the extraction operation; in addition, this surface action can displace particles and split them into smaller sizes [6].

There are many variables that can affect the process of ultrasound

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Some of them are intrinsic of the equipment while others are external factors. The intrinsic factors are wave amplitude related with ultrasound intensity, power, ultrasonic frequency, and extraction temperature; while external factors are the extraction time, the solvent properties and the matrix used as the source of extracts [5]. Amplitude is one of the factors that can impact directly in the cavitation process. High amplitudes can promote a faster deterioration of the ultrasonic transducer, having as a result a liquid agitation instead of cavitation phenomenon and poor transmission of the ultrasound through the liquid media [7]. The time of extraction is a very important factor, as short times can result in incomplete extractions and longer times can generate unfavorable reactions and less selective extractions [4]. The solvent to use can affect the cavitation phenomena throughout viscosity, vapor pressure and surface tension; a viscous solvent can decrease cavitation, while vapor pressure and surface tension influence this phenomenon. In other aspects, volatile solvents may be evaporated if the ultrasound extraction is accomplished at higher temperatures for longer times, and finally, the solvent needs to be compatible with the polarity and solubility of the target compound [5]. As an example, Chemat et al. [6] proposed to prove how the factors temperature and extraction time can affect the extraction yield of artemisinin crystals from Artemisia annua leaves. Dadi et al. [9] reported that ultrasound improved the extraction yield of bioactive compounds and their antioxidant activity. This technology has been in use in the extraction of phenylpropanoids like verbascoside and oleuropein; as well in flavonoids like luteolin and apigenin from Olea europaea leaves [10]; and quercetin, catechin, nar- ingenin, neohesperidin and mangiferin in Coffea arabica leaves [11]. Consequently, it is a promising technology to be used in the extraction of bioactive compounds of Buddleja scordioides Kunth leaves.

Many plants are used by Mexican population to help in the treatment of many disorders, one of them is the Buddleja scordioides Kunth or salvilla [12]. The salvilla is a plant classified in the Loganiaceae family, its localization is widely spread from Southern United States to Central Mexico [13]. This plant, also known as salvilla, escobilla, butterfly-bush, mato or salvia real, has been reported with antispasmodic properties [14], antibacterial [15], sunscreen properties [16] anti diarrheic and gastrointestinal disorders treatment [17], gastric and intestinal antiinflammation [18].

Inflammation is a physiological process that begins because of harmful agents such as microbial infection, physical damage, oxidative stress, or other type of phenomenon [19]. This process includes synthesis of inflammatory mediators induced by cyclooxygenase-2 enzyme (COX-2), which metabolizes free arachidonic acid to prostaglandins, being those molecules correlated with acute and chronic inflammatory disorders [20,21].

Polyphenols, specifically flavonoids, have been linked in the resolution of the inflammatory process due to their antioxidant activity and the inhibition of a variety of enzymes involved in this process, such as COX-2 enzyme [22]. Salvilla has a phenolic profile rich in flavonols such as flavones like apigenin, acacetin and luteolin, flavanones as neo- hesperidin and naringenin and flavonols; in this regard, its antiinflammatory effect is ascribed to the presence of those polyphenols, particularly to quercetin [23,24].

Salvilla bioactive compounds has been extracted in different ways, from infusions to traditional macerations with a sort of solvents in successive extractions such as hexane, ethyl acetate and methanol [16], chloroform [14] or acetone–water mixtures [24]. Although there is information on the extraction with these solvents, there are no reports on the use of ultrasound assistance to extract salvilla bioactive compounds. Thus, the aim of this study was to obtain ethanolic salvilla extracts using UAE, describe their phenolic composition and to prove their antioxidant and anti-inflammatory properties.

2. Material and methods

2.1. Chemical reagents

Catechin, trans-cinnamic acid, coumaric acid, caffeic acid, quinic acid, ferulic acid, sinapic acid, 4-O-cafeoylquinic, chlorogenic acid, benzoic acid, vanillic acid, shikimic acid, 4-hydroxybenzoic acid, 2,4,6-trihydroxybenzaldehyde, protocatechuic acid, syringic acid, apigenin, luteolin, acacetin, eriodictyol, naringenin, naringin, neohesperidin, quercetin, myricetin, kaempferol, kaempferol 3-O-glucoside, quercetin-O-glucoside, quercetin glucuronide and rutin (reagent grades), acetone-nitrite and methanol (UPLC grade), ethanol (reagent grade), 2,2′-azino-bis-(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS), 2,2′-azobis(2-amidinopropan) dihydrochloride (AAPH), 2,4,6-tris(2-pyridyl)-s-triazine (TPTZ), 3,6-dihydroxypyri(soobenzofuran-1[3H],9′[9H]-xanthen)-3-one (fluorescein), Trolox®, ferric chloride hexahydrate (FeCl₃·6H₂O), sodium hydroxide (NaOH), aluminum chloride (AlCl₃), sodium nitrite (NaNO₂), potassium persulfate (K₂S₂O₈), hydrochloric acid (HCl), monobasic potassium phosphate, dibasic potassium phosphate, glacial acetic acid, sodium acetate (reagent grades), were purchased from Sigma Aldrich® (Toluca, Mexico).

2.2. Plant material collection

Wild plants of Buddleja scordioides Kunth were collected in 2017 in Guadalupe Victoria, Durango, México (24°18′59″N, 104°5′3″W). Salvilla leaves were separated from the stems and the leaves dried at room temperature (25 °C) protected from light, milled in a knife lab mill with a #2 sieve (IKA®, Staufen, Germany), and stored in hermetic bags until use.

2.3. Ultrasound assisted extraction

Extraction was performed according to Chemat et al. [8] with some modifications. The milled salvilla leaves were hydrated in a hydro-alcoholic solution at 1:50 ratio, after that, treatments were placed into ice bath and the horn (90.5 in.) of sonicator Branson® 250 (maximum power 250 W, amplitude 21–145 μm, Danbury, Connecticut, USA) was immersed 30 mm inside the sample. Then, a different amplitude (30, 50%) was applied to the system at three extraction times (10, 15 and 20 min). Temperature was controlled with the ice bath preserving the process at 35 ± 2 °C. After finishing the extract process, all treatments were filtered, and organic solvent was removed in a Buchi® rotavapor (New Castle, USA). Finally, the treatments were lyophilized in a freeze dryer (Labconco® Kansas City, Missouri, USA) and kept in a dry place until use.

2.4. Extraction yield

A gram of dry salvilla leaves was subjected to the UAE process, followed by lyophilization. The extraction yield was calculated as the extract percentage of dry leaves as follows:

\[ \text{Extraction yield} \% = \left( \frac{W_{\text{extract}}}{W_{\text{leaves}}} \right) \times 100 \]

Where \( W_{\text{leaves}} \) is the initial dry leaves weight and \( W_{\text{extract}} \) is the final lyophilized extract weight.

2.5. Total flavonoid content

Total flavonoid content was tested according to Park et al. [25] with some modifications. A sample of 20 μL, blank or standard were mixed with 7.5 μL of 5% (m/V) of NaNO₂, 15.0 μL of 10% (m/V) AlCl₃, 50.0 μL of 1 M NaOH and 157.0 μL of distilled water. The mix was kept in the dark for 5 min and the absorbance measured at 515 nm in a microplate reader Daigger® (Vernon Hills, Illinois, USA). As standard, (-)-catechin was used and results were shown as microgram equivalent of...
2.6. ABTS (2,2′-azino-bis-(3-ethylbenzothiazoline-6-sulphonic acid)) assay

ABTS antioxidant assay was done according to Re et al. [26] with some modifications. The ABTS+ radical cation was generated by the reaction of ABTS (7 mM) and K3Fe(CN)6 (2.42 mM) 16 h before the assay. Once the ABTS+ radical cation was ready, a 10 µL of sample, blank or calibration standard was mixed with 190 µL of the prepared cation and the mix was kept in dark for 10 min, measuring the absorbance of the reaction at 750 nm in a microplate reader Daigger® (Vernon Hills, IL, USA). Trolox was used as standard. Results were shown as millimoles of Trolox equivalent per milligram of dry salvilla extract (mM TE/mg of SE).

2.7. Ferric ion reducing antioxidant power (FRAP) assay

Finally, FRAP assay was performed according to Benzie and Strain [27] with some modifications. A fresh working solution was prepared as follows: 2.5 mL of 0.03 mM TPTZ acid solution (1.0% HCl solution), 2.5 mL 0.06 mM FeCl3⋅6H2O solution and 25 mL of acetate buffer (pH 3.6). An aliquot of 180 µL from this solution was mixed with 20 µL of sample, blank or standard and kept in dark for 10 min. After that, the treatments were read at 593 nm in a microplate reader Daigger® (Vernon Hills, Illinois, USA). Trolox was used as standard. Results were shown as millimoles of Trolox equivalent per milligram of dry salvilla extract (mM TE/mg of SE).

2.8. Oxygen radical absorbance capacity (ORAC) assay

This assay was done according to Ou et al. [28] with some modifications. The peroxy radical was generated from AAPH and fluorescein as a fluorescent probe. A 20 µL of sample, blank or calibration solutions were mixed with 200 µL of 1.09 µM fluorescein. The treatments were incubated at 37 °C for 15 min in a Synergy HT® multi-detection microplate reader (Bio-Tek, Winooski, VT, U.S.A.). After that 75 µL of 79.65 mM AAPH radical were added and mixed. The detector was adjusted to 485 and 535 nm of excitation and emission wavelengths, respectively. The fluorescence was measured every 1.5 min for 2.5 h. Trolox was used as standard. ORAC values were calculated using area under the curve of each treatment and expressed as millimoles of Trolox equivalent per milligram of dry salvilla extract (mM TE/mg of SE).

2.9. Chemical characterization by UPLC-PDA-ESI-MS/MS

The phenolic characterization was performed according to Díaz-Rivas et al. [23]. An Ultra-High-Performance Liquid Chromatography (Waters Corp., Milford) system coupled to a tandem Photodiode Array-Electric Ionization-Triple Quadrupole (Xevo TQS, Waters Corp., Wexford) (UPLC-PDA-ESI-MS/MS) was used, including a sample manager (6 °C) and an Agilent® C18, 150 × 2.1 mm, 1.7 µm column. The elution profile was composed of acidified MilliQ water (formic acid 7.5 mM) or solvent A and LC-MS grade acetonitrile or solvent B in the following gradient: initial – 3% B, 1.88 min – 9% B; 5.66 min – 16% B; 16.90 min – 50% B; 19.62 min – 3% B; and finally 20.0 min – 3% B for column stabilization at 0.210 mL/min. Ionization was realized using methanol with 0.1% of formic acid (v/v) as co solvent at 0.2 mL/min using an isocratic solvent manager (Waters Corp., Wexford). Multiple reactions ionization mode (MRM) was used for MS/MS assays. Electrospray ionization (ESI) was operated in negative mode and is described as follows: capillary voltage 2.25 kV, desolvation temperature 450 °C, source temperature 150 °C, desolvation gas flow 800 L/h, and cone gas flow 150 L/h; collision gas flow was 0.13 mL/min, MS mode collision energy 2.0, and MS/MS mode collision energy 20. To identify and quantify phenolic compounds, a mixture of standards was prepared at 20 ng/µL to monitor retention times, m/z values and MS/MS transitions. UPLC-PDA-ESI-MS/MS management and data processing were performed using MassLynx v. 4.1 Software (Waters Corp., Milford, Massachusetts) [29].

2.10. Election of the best extraction conditions

A contingency table was prepared to weight the responses and choose the best conditions of extraction that had the highest quality and antioxidant activity according to total flavonols. First, the dependent variables were kept from 40 to 10 points according with their importance, also a value going from 1 to 5 was assigned for each result interval of every dependent variable. The size of each interval was calculated with the following formula:

\[ \text{Interval} = \frac{(M_v - M_m)}{\# \text{of interval}} \]

Where \( M_v \) is the maximum value of the obtained result of the variable, \( M_m \) is the minimum value of the obtained result of the variable and \# of interval are the desirable intervals, in this case were five.

After this assignment, real values were substituted by the corresponding interval and multiplied by the assigned points of the variable and finally all products from each treatment were added. The highest value showed the better treatment as well the better conditions to obtain a high quality and antioxidant activity.

2.11. Determination of inhibition of COX-2 enzyme

Once the best extraction condition was chosen, the anti-inflammatory activity test of the elected treatment was carried out using a commercial kit of COX-2 inhibition (Cayman Chemical®, Ann Arbor, MI, USA). The results were managed in the resource provided by Cayman Chemical® (Elisa Double Worksheet, www.caymanchem.com/analysis/eia) and expressed as inhibition percentage (%).

2.12. Statistical analysis

The experimental design of the UAE was a completely randomized unbalanced factorial with three factors of two and three levels, having in total 18 treatments with three repetitions (Table 1). The data analysis was performed with factorial ANOVA, \( p = 0.05 \) and mean analysis by Fisher test (\( p = 0.05 \)). Results are shown as the mean ± their standard deviation. The analysis was carried out using Statistica, v. 12.0 software, (TIBCO Software Inc, Palo Alto, CA, USA) [30].

The data analysis for the best extraction conditions was performed with one way ANOVA (\( p = 0.05 \)) and mean analysis by Fisher test (\( p = 0.05 \)) was performed to the selected five extraction conditions. Results

### Table 1

| Treatment | Time (min) | Ethanol (%) | Wave amplitude (%) |
|-----------|------------|-------------|--------------------|
| T1        | 10         | 25          | 50                 |
| T2        | 30         |             |                    |
| T3        | 50         | 50          | 30                 |
| T4        | 30         |             |                    |
| T5        | 75         | 50          | 30                 |
| T6        | 30         |             |                    |
| T7        | 15         | 25          | 50                 |
| T8        | 30         |             |                    |
| T9        | 50         | 50          | 30                 |
| T10       | 30         |             |                    |
| T11       | 75         | 50          | 30                 |
| T12       | 30         |             |                    |
| T13       | 20         | 25          | 50                 |
| T14       | 30         |             |                    |
| T15       | 50         | 50          | 30                 |
| T16       | 30         |             |                    |
| T17       | 75         | 50          | 30                 |
| T18       | 30         |             |                    |
are shown as the mean ± their standard deviation. The analysis was carried out using Statistica, v. 12.0 software, (TIBCO Software Inc, Palo Alto, CA, USA) [30]. In the measurement of each response variable, for total flavonoids by colorimetric assay, ABTS, FRAP and ORAC there were three measurements per repetition of treatments; for phenolic profile by UPLC-PDA-ESI-MS/MS quantification there was one measurement per repetition of treatments; and finally, the COX-2 assay was performed taking two measurements per repetition of the selected treatment.

3. Results and discussion

3.1. Yield and total flavonoids of UAE extracts

Once obtained the UAE extracts, they were lyophilized and their yield average calculated, being 10.58 ± 0.33 to 12.92 ± 0.40%, compared with a salvilla infusion of 15.27 ± 0.64% (Table 2). In that sense, the resulted yields are lower than the reported by Herrera-Carrera et al. [12] who made a salvilla infusion with 20.31 ± 0.61% of yield extraction, Avila-Acvedo and Romero-de-Vivar [15] and Avila-Acvedo et al. [16] reported a 15.15% in a successive hexane – ethyl acetate – methanol salvilla extract. Our extracts have a higher yield extraction than the reported by Perez-Gutierrez and Vargas-Solis [31] in a successive hexane – chloroform - methanol salvilla extract, where they obtained 7.5% and it is also higher than Alarcón-Herrera et al. [32] who in a successive hexane – methanol extract obtained 10% of yield. In the extract yield all the independent variables in ethanol (p < 0.05), time (p < 0.05) and amplitude (p < 0.05) influenced the response variable, as well as the interactions of the time and the solvent (p < 0.05), time and the amplitude (p < 0.05), and the time, solvent, and amplitude (p < 0.05) (see supplementary information, Table S1). The treatments having higher extraction yields were T4 and T15.

A total flavonoids quantification was estimated, obtaining values were from 107.3 ± 3.0 to 224.9 ± 6.3 µg CE/mg of SE (catechin equivalent/mg of dry salvilla extract) (Fig. 1). According with the statistical analysis, the three factors (time, % solvent, % amplitude and all their interactions) influenced the response variable (p < 0.05), being Treatment T6 and T11 the ones with the highest content of total flavonoids (Table S2). Those treatments corresponded to the conditions 10 min, 75% of ethanol and 30% of amplitude and 15 min, 75% of ethanol and 50% of amplitude, respectively, coinciding in the ethanol percentage. In this instance, all treatments were significantly higher than the reported by Pan et al. [33], who reported in Buddleja officinalis extracts values of 62.56 ± 0.35 to 75.33 ± 0.42 µg CE/mg of dry extract, and as higher than the reported by Díaz-Rivas et al. [34], where the salvilla infusion and concentrated salvilla infusion had a concentration of 10.76 ± 0.05 and 7.06 ± 0.37 µg CE/mg of SE, respectively. Comparatively, ethanol enhances the extraction of salvilla metabolites such as flavonoids due to its polarity and affinity [35]. Besides, ultrasound helps solvent diffusion through broken cell membranes, rinsing and extracting the cell content [36].

3.2. Antioxidant activity

Phenolic compounds are characterized for possessing a high antioxidant capacity due to their ability of donate hydrogen atoms from their aromatic hydroxyl group to a free radical and the resonance effect (charge delocalization) in the aromatic ring - double bond system [37,38]. That is why they can be used as part of a nutraceutical product, when this product is ingested, it supports the endogenous antioxidant system and helps to stabilize free radicals formed in the body as a product of poor nutrition, and environmental or stressing factors. Three antioxidant assays were performed to evaluate samples activity, two assays based in electron donation (ABTS and FRAP), and one based in the hydrogen donation (ORAC) mechanisms. The ABTS assay is based on the quantification of discoloration of the blue/green ABTS•+ chromophore by antioxidant molecules, monitoring the reaction at 734 nm. ABTS•+ cation is previously produced from the reaction of ABTS and K2S2O8, and its discoloration is directly proportional to the interaction with hydrogen or electron donor molecules (i.e., phenolic antioxidants), which reduces the cation ABTS•+. This reduction is dependent of the antioxidant activity and treatments concentration [26]. It is recommended to be used in water-soluble, lipid-soluble antioxidants as well as pure compounds or food extracts, as it was possible to test the salvilla extract treatments.

The FRAP assay measures the capacity of phenolic antioxidants, through the reducing effect of ferric to ferrous ions (Fe3+ to Fe2+). This ion reduction carried out at low pH produces an intense blue colored ferrous-tripyridyltriazine complex that can be read at 593 nm. This assay offers an assumed index of antioxidant activity, which may be used in studies of oxidative stress and its effects. The developing of color proves that there are antioxidant molecules (i.e., polyphenols) present in the treatments [27]. Finally, one of the most important antioxidant assays is the ORAC assay, which is based in the hydrogen atom transfer mechanism, and assesses antioxidant activity against peroxyl radical induced by AAPH using fluorescein as fluorescent probe. When this molecule mislays its fluorescence means that it has been transformed as consequence of its peroxyl radical interaction. In this case, the protective effect of an antioxidant molecule is directly proportional to the extent of fluorescence, measuring the area under the fluorescence curve of the sample compared to the blank without antioxidant molecules [28].

3.2.1. ABTS assay

The treatments tested with this assay showed values from 100.9 ± 5.4 to 224.2 ± 11.9 mM TE/mg of SE (Trolox equivalent/mg of dry salvilla extract) (Table 3). The three factors (time, % solvent, % amplitude and all their interactions) influenced significantly (p < 0.05) the antioxidant activity, being treatments T6 and T13 the ones showing the highest antioxidant activity (Table S3). However, these do not present significant differences with treatments 9, 10 and 11. The mentioned treatments corresponded to the conditions 10 min, 75% of ethanol and 30% of amplitude and 20 min, 25% of ethanol and 50% of amplitude, respectively. Estrada-Zúñiga et al. [39] found values for wild, micro propagated and greenhouse plants of Buddleja cordata with values too much lower than the reported in this paper, with values of 0.8–2.2 mM TE/g of sample, this in a methanolic extract.

3.2.2. FRAP assay

The values related with this assay exhibited activities from 86.3 ± 5.3 to 249.7 ± 15.2 mM TE/mg of SE extract (Table 3). In this case the treatment with higher antioxidant activity was T6. The statistical
Díaz-Rivas et al. [23] reported values in 1% infusion of elicited salvilla showing antispasmodic properties, with values of 3870.2 ± 10.2 µmol TE/g. The treatments with higher total phenolic content were T1, T2, T10, and the solvent in their linear and interaction terms did not have any effect on the response (p > 0.05) (Table S4). According with these results, treatment T6 is the one showing the highest antioxidant activity from the three assays, which could be explained by the solvent concentration and its polarity. Additionally, statistical analysis showed that time and amplitude had an influence, at the lowest time and amplitude, the highest antioxidant activity; at larger time extractions, molecules could be degraded, and at higher amplitude levels, treatments seem to have a poorer ultrasound transmission [7]. Flavonoids with unsubstituted hydroxyl groups or sugars such as flavonols and flavones are considered mild polar compounds for what they become soluble in polar solvents such as ethanol. Furthermore, the ultrasound assisted extraction allowed that ethanol provided a better rinse of the vegetal cells, increasing the content of phenolic compounds into the medium [45].

### 3.2.3. ORAC assay

All the treatments tested by ORAC assay exhibited values from 98.2 ± 0.7 to 280.4 ± 2.1 mM TE/mg of SE (Table 3). The treatments with higher antioxidant activity were T6 and T16, where the ANOVA analysis confirmed that the time, % solvent, % amplitude and all their interactions influenced the results (p < 0.05) (Table S5). About this assay, Díaz-Rivas et al. [23] reported values in 1% infusion of elicited salvilla with salicylic acid of 0.000038 ± 0.000003 to 0.000045 ± 0.000002 mM TE/g, and lower antioxidant activity than all the extracts with ultrasound assistance. The use of ultrasound enhances the antioxidant activity of extracts, as confirmed by Rosales-Villarreal et al. [11]. They reported 166.17 ± 9.82 mM TEq/mg of extract in a Coffea leaves infusion, showing lower activity than the obtained by ultrasound assisted extraction. However, extraction conditions were different, 40% methanol, 6 min and 40% of wave amplitude. This could mean that the solvent and the ultrasound treatment improved the antioxidant activity. The ultrasound can achieve an intensification of mass transfer due to the formed bubbles collapse (cavitation) nearby cell walls, breaking down this wall and allowing the contact between solvent and bioactive compounds in plant material [41,42].

Polyphenols are highly competent in breaking free radical chain reactions [43]. The results obtained in all assays are explained by the proposed action mechanisms such as the radical scavenging activity of reactive oxygen species (ROS) or reactive nitrogen oxide species (RNOS). Due to their phenolic hydroxyl groups polyphenols can reduce free radicals by hydrogen atom transfer, electron donation or by metal chelation. In this way, they can also prevent the production of reactive species catalyzed by transition metals [43,44].

According with these results, treatment T6 is the one showing the highest antioxidant activity from the three assays, which could be explained by the solvent concentration and its polarity. Additionally, statistical analysis showed that time and amplitude had an influence, at the lowest time and amplitude, the highest antioxidant activity; at larger time extractions, molecules could be degraded, and at higher amplitude levels, treatments seem to have a poorer ultrasound transmission [7]. Flavonoids with unsubstituted hydroxyl groups or sugars such as flavonols and flavones are considered mild polar compounds for what they become soluble in polar solvents such as ethanol. Furthermore, the ultrasound assisted extraction allowed that ethanol provided a better rinse of the vegetal cells, increasing the content of phenolic compounds into the medium [45].

### 3.3. Phenolic characterization by UPLC-PDA-ESI-MS/MS

In this study it was possible to identify five groups of phenolic compounds present in all salvilla ultrasound assisted extracts: hydroxybenzoic acids, hydroxycinnamic acids, flavones, flavanones and flavonols. The treatments with higher total phenolic content were T1, T2, T13 and T14 (Fig. 2). The ANOVA analysis showed that wave amplitude did not have any effect on the response (p = 0.789), however, the time and the solvent in their linear and interaction terms did (p < 0.05) (Table S6). As Fig. 2 shows, the major content of total phenolic acids was extracted with 25% ethanol, 20 min and 30 and 50% of wave amplitude corresponding to treatments T13 and T14, while the major content of flavonoids was extracted with 25% of ethanol, 10 min and 30 and 50%
of wave amplitude corresponding to treatments T1 and T2. The phenolic profile was like the obtained by Díaz-Rivas et al. [23] who reported the same phenolic groups in elicited plants of salvilla.

In the group of hydroxybenzoic acids were found vanillic, shikimic, protocatechuic, benzoic, syringic, 4-hydroxybenzoic acids and 2,4,6 trihydroxybenzaldehyde, being vanillic acid the most abundant at concentrations from 75.55 ± 6.5 to 225.11 ± 19.3 ng/mg of SE. The total concentration of hydroxybenzoic acids was from 95.27 ± 8.4 to 261.9 ± 22.8 ng/mg of SE. The statistical analysis indicated that wave amplitude did not have any effect on the response (p = 0.657), while the time and the solvent, in their interaction, as well the interaction of the solvent and the amplitude did (p < 0.05 for both factors and their interactions) (Table S7). The treatments with higher concentration of hydroxybenzoic acids were T13 and T14 corresponding to 20 min of extraction, 25% of ethanol and 30 and 50% of wave amplitude, respectively. In Fig. 3 are shown the total concentration of hydroxybenzoic acids. The phenolic profile of all treatments coincided in almost all the components obtained by salvilla infusion reported by Herrera-Carrera et al. [12].

Another type of acids found in the characterization were hydroxycinnamic acids specifically caffeic, coumaric, trans cinnamic, ferulic, synaptic, chlorogenic, quinic and 4-O-caffeoylquinic, being trans cinnamic acid the most abundant with concentrations going 22.04 ± 6.3 ng/mg of SE of the treatment T9 to 195.73 ± 55.8 ng/mg of SE of the treatment T13 (Fig. 4). The wave amplitude did not have any effect on the response (p = 0.323), while the time and the solvent in their interaction did (p < 0.05) (Table S8). The treatments with major concentration of hydroxycinnamic acids were T13 and T14 corresponding to 20 min of extraction, 25% of ethanol and, 30 and 50% of wave amplitude, respectively. In Fig. 4 is shown the total concentration of hydroxycinnamic acids. In this sense, the chemical profile coincides with the phenolic acids (i.e., caffeic, synaptic, coumaric, chlorogenic and 4-O-caffeoylquinic acids) from elicited salvilla infusions by Díaz-Rivas et al. [23].

In addition to phenolic acids, another important group of phenolic compounds are the flavonoids. This group is divided into many subgroups such as flavones, flavanones, flavan-3-ols, flavonols, chalcones and anthocyanidins [46]. The sub-groups found in the salvilla ultrasound assisted extracts are flavones, flavanones and flavonols.

In first place, the flavones found in all the treatments were luteolin, apigenin and acacetin with total concentrations from 19.52 ± 3.0 to 206.45 ± 31.4 ng/mg of SE (Fig. 5). The wave amplitude did not have any effect on this response (p = 0.393), while time and solvent, in their simple and interaction effects did (p < 0.05) (Table S9). The extracts with the highest concentration were T1, T2 and T14. They correspond to 10 min of extraction, 25% of ethanol and, 50 and 30% of wave amplitude for T1 and T2, respectively; and 10 min of extraction, 25% of ethanol and 30% of wave amplitude for T14. Díaz-Rivas et al. [34] reported the same metabolites in salvilla infusions, but at lower concentrations.
concentrations due to the different extraction technology and solvent, which was only water.

In the sub-group of flavanones were found naringenin, eriodictyol
and neohesperidin with concentrations from 228.23 ± 46.2 to 1141.48 ± 224.1 ng/mg of SE (Fig. 6). In this case like in other sub-groups, the
wave amplitude did not have any effect on the response ($p = 0.903$),
while the time and the solvent in their linear and interaction terms did $(p < 0.05)$ (Table S10). The highest concentration of flavonones was found in treatments T1 and T2, which were significantly similar at the extraction conditions of 10 min, 25% of ethanol and 50 and 30% of wave amplitude, respectively.

Finally, several flavonols were found, such as kaempferol, quercetin, myricetin, rutin, kaempferol 3-O-glucoside, quercetin 3-glucoside, and kaempferol 3-O-glucoside. In this case, all factors and their interactions had influence on the yield of flavonols $(p < 0.05)$ (Table S11). The treatment having the highest content of flavonols was T3 with $431.84 \pm 35.3$ ng/mg of SE (Fig. 7). Villegas-Novoa et al. [24] reported a high concentration of flavonols profile in their salvilla acetonic extracts, solvent with a similar polarity than ethanol. All treatments with 25% of ethanol (except T1) are statistically similar at the lowest concentration.

3.4. Best extraction conditions

Once all results were obtained, it was not clear which condition parameters were the best to identify the treatment with the highest yield and antioxidant activity. From one side, there is a treatment with the highest results in colorimetric assays (T6); in the other, there are four treatments with the highest phenolic compound concentration tested by chromatography (T1, T2, T13 and T14). Thus, it was decided to weight first each variable by importance order and then to compare the five mentioned treatments in a contingency table. This importance order was assigned to find the extract that will contain the major concentration of bioactive flavonoids in the antioxidant assays as this activity is closely related with anti-inflammatory activity.

In this analysis, the variables were weighted as follows: 10 points to total phenolic acids, total flavones, total flavanones, total flavonoids, and total phenolic compounds, all previously determined by UPLC-PDA-ESI-MS/MS; 20 points were assigned to total flavonoids in microplate; 30 points, to the yield, ABTS and FRAP; and finally, 40 points, to the total flavanols and ORAC outputs. Different assignations were given to antioxidant activities by ABTS and FRAP because they are synthetic radicals non-existing in the human body, although they provide information on the potential power and action mechanisms of tested antioxidant molecules. Meanwhile, ORAC has more physiological significance since it uses AAPH radical, which is hydrolyzed into two peroxyl molecules that occur in live organisms under oxidative stress [26–28]. The assignment of values to the intervals is shown in Table 4.

After these assignations, original values of each selected treatment were substituted for their interval value (1 to 5), multiplicapt by their assigned importance value (10 to 40) and summarized to obtain a global arbitrary value, shown in Table 5. This data analysis showed significant differences $(p = 0.018)$, being treatment T6 the one that showed the highest arbitrary value $(900 \pm 124.9)$; it meant that T6 had the highest antioxidant activity by all the assayed methods, total flavonoids and total flavanols, group formed mainly for quercetin an its glycosylated derivate and distinguished for their high antioxidant and anti-inflammatory activity. In this way, the antioxidant activity of flavonoids is linked to the structure and substitution of their hydroxyl groups, being the incidence of this group in position 3 of the ring C favorable for this property, allowing phenolic compounds to show a scavenging power of reactive oxygen species and to have the capacity of inhibiting free radical-producing enzymes [37,38].

3.5. Inhibition of COX-2 enzyme

Antioxidant activity is linked with anti-inflammatory activity, to give support to this result election an in vitro anti-inflammation test was carried out to the selected treatment. As a result, the treatment T6 presented a COX-2 inhibition of 99.35 ± 0.6 % at a concentration of 10 mg/mL (data not shown), this probably due to the high concentration of flavonols, specifically quercetin (Table 5). Muñoz-Velázquez et al. [20] tested COX-2 inhibition of different commercial infusions, having values from 25 to 45% versus a 100% of the epigallocatechin gallate at a concentration of 50 μg/mL. They also reported that the lowest COX-2 inhibition was shown in those infusions lacking quercetin in their phenolic profile. Quercetin is well known for presenting a higher radical scavenging capacity due to its free hydroxyl groups and COX-2 inhibition activity [18,47]. In this case, D’Andrea [48] recommended that quercetin must be included at concentrations of 0.008–0.5% or 10–125 mg/serve of nutraceutical product or functional food. Therefore, treatment T6 could be consider a rich nutraceutical source of flavonoids, particularly flavonol like quercetin.

4. Conclusions

Ultrasound assisted extraction proved to be a good alternative for getting phenolic compounds from leaves of Buddleja scordoides Kunth. It was demonstrated that varying extraction time was possible to obtain higher concentrations of flavonoids (at 10 min) and phenolic acids (at 20 min). Furthermore, with 75% of ethanol, 30% of amplitude and 10 min of extraction conditions, it was feasible to get the best treatment with extracts rich in flavonols (T6), showing the highest antioxidant capacity by three reported assays and a high anti-inflammatory activity. The use of emergent technologies resulted effective and recommendable to the extraction of bioactive compounds, shortening the extraction time and decreasing the required energy versus the conventional extractions.
Table 4
Assignments to dependent variables and their importance order.

| IV  | DV    | Assigned value to the interval |
|-----|-------|--------------------------------|
| 40  | TFlav | 130.3-170.6                    |
|     | ORAC  | 163.1-187.0                    |
| 30  | ABTS  | 177.6-189.3                    |
|     | FRAP  | 164.0-184.2                    |
|     | Yield | 11.09-11.38                    |
| 20  | MPTF  | 172.7-184.3                    |
| 10  | TPA   | 124.8-258.9                    |
|     | TFlav | 231.6-458.4                    |
|     | TFn   | 223.3-412.4                    |
|     | TPC   | 540.0-786.4                    |
|     | 665.7-991.2 |

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CRediT authorship contribution statement

Elizabeth Macías-Cortés: Investigation, Formal analysis, Methodology, Writing – original draft. José Alberto Gallegos-Infante: Conceptualization, Data curation, Formal analysis. Nuria Elizabeth Rocha-Guzman: Conceptualization, Investigation, Validation. Marta Rocío Moreno-Jiménez: Methodology, Project administration, Resources. Veronica Cervantes-Cadóza: Data curation, Methodology. Resources. Gustavo Adolfo Castillo-Herrera: Methodology, Resources.

Rubén Francisco González-Laredo: Conceptualization, Funding acquisition, Supervision, Writing – review & editing

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

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