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Scientific Article

Use of Supercritical fluid extraction (SFE) as a Sustainable Strategy for Polyphenol Recovery from Salvilla (*Buddleja scordioides* Kunth)

Uso de la extracción por fluidos supercríticos (SFE) como estrategia sustentable en la recuperación de polifenoles de Salvilla (*Buddleja scordioides* Kunth)

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Abstract

Emerging extraction technologies gained position due to their advantages in obtaining high-quality metabolites. Supercritical fluid extraction (SFE) recovers bioactive molecules while preserving their integrity. In this study, SFE was applied to *Buddleja scordioides* Kunth (salvilla) bush leaves, a species rich in phenolic compounds with medicinal properties. A central composite design of three factors was used: co-solvent percentage (ethanol), pressure, and extraction time. Extracts were evaluated for yield, total flavonoid content, antioxidant activity (ORAC and ABTS) and quantification of phenolic compounds by UPLC-PDA-ESI-MS/MS. Extraction yields ranged from 3.63-6.27 %, total phenolic content varied from 219.17-314.72 µg CE/mg extract. Antioxidant activity was ranged from

53.54–81.88 mmol TE/mg of salvilla leaves extract (SE) for ABTS and 78.69-185.40 mmol TE/mg SE for ORAC. The total phenolic content determined by UPLC-PDA-ESI-MS/MS was ranged from 4188.1-13008.2 ng/mg SE with the co-solvent as the most influencer factor. According to the general linear model (GLM), the central points (120 min, 150 bar, 20 % co-solvent) yielded the highest results. This work is original in its application of SFE, to improve the extraction of bioactive molecules. This work provides novelty by applying SFE, to enhance the extraction and quality of bioactive compounds. These results enhance the SFE as a sustainable strategy for the valorization of medicinal plants.

Keywords: salvilla, emerging extraction, supercritical fluids, phenolic acids, flavonoids.

Resumen

Las tecnologías de extracción emergentes cobraron relevancia por sus ventajas en la obtención de metabolitos de calidad. La extracción con fluidos supercríticos (SFE) recupera moléculas bioactivas preservando su integridad. En este estudio, se aplicó SFE a las hojas del arbusto de *Buddleja scordioides* Kunth (salvilla), especie rica en compuestos fenólicos con propiedades medicinales. Se empleó un diseño central compuesto de tres factores: porcentaje de co-solvente (etanol), presión y tiempo. Los extractos se analizaron para rendimiento, flavonoides totales, actividad antioxidante (ABTS y ORAC) y cuantificación fenólica mediante UPLC-PDA-ESI-MS/MS. Los rendimientos oscilaron entre 3.63-6.27 %, los compuestos fenólicos totales entre 219.17-314.72 μg CE/mg de extractos de hojas de salvilla (SE), actividad antioxidante entre 53.54-81.88 mmol TE/mg SE para ABTS y 78.69-185.40 mmol TE/mg SE para ORAC y el análisis por UPLC-PDA-ESI-MS/MS osciló entre 4188.1-13008.2 ng/mg SE siendo el co-solvente la variable de mayor influencia. De acuerdo con el modelo lineal general (MLG), los puntos centrales (120 min, 150 bares y 20 % de co-solvente) tuvieron los mejores resultados. Este trabajo aporta originalidad al aplicar SFE, para mejorar la extracción de compuestos bioactivos y su calidad. Los resultados exponen a la SFE como estrategia para obtener compuestos fenólicos de salvilla con capacidad antioxidante relevante, respaldando su aplicabilidad en la valorización sostenible de plantas medicinales.

Palabras clave: salvilla, extracción emergente, fluidos supercríticos, ácidos fenólicos, flavonoides.

1. Introduction

The extraction of natural compounds has been an integral part of human culture since antique times. Early societies, like the Chinese, Egyptians and even Mayans, relied on rudimentary methods like maceration and steam distillation for the preparation of herbal medicines, perfumes, and food preservatives (Azmir et al., 2013). Over centuries, these techniques evolved, but solvent-based extractions remained predominant, often relying on large volumes of organic solvents and energy-intensive processes.

Despite their widespread use, traditional extraction methods present significant disadvantages when applied to bioactive compound extraction. Prolonged extraction times and high temperatures can cause the degradation of thermolabile molecules, such as phenolic compounds, diminishing their activity (Chemat et al., 2019). Moreover, the use of toxic solvents of organic nature causes alarms

about environmental contamination, operator protection, and solvent residues in the final product, directly contradicting the principles of green chemistry (Mustafa & Turner, 2011). These tasks highlight the necessity for more sustainable and efficient options.

Emerging extraction technologies have gained momentum in recent decades as part of a broader shift toward green chemistry. Techniques such as ultrasound-assisted extraction (UAE), microwave-assisted extraction (MAE), pressurized liquid extraction (PLE) and supercritical fluid extraction (SFE) have demonstrated significant rewards over conventional technologies, like improved yields of bioactive compounds, shorter extraction times, and reduced solvent use (Usman et al., 2023). They are closely associated with the 12 principles of green chemistry, offering cleaner, safer, and more energy-efficient approaches to phytochemical recovery (Chemat et al., 2015).

Among these, supercritical fluid extraction (SFE) stands out for its unique physico-chemical foundation. SFE uses the properties of fluids in their supercritical phase, beyond their critical pressure and temperature, where they demonstrate gas-like diffuseness and liquid-like dissolving power (Banafi et al., 2023). For this, carbon dioxide (CO_2), is the commonly utilized supercritical fluid since it is non-toxic, easily removed from extracts, inexpensive, and non-flammable, properties given by its low critical pressure (73.8 bar) and temperature (31.1 °C). The closed, oxygen-free, and low-temperature environment of SFE minimizes oxidation and thermal degradation, making of this technology particularly appropriate for obtaining thermosensitive bioactive molecules (Vinitha et al., 2022; Sheibani et al., 2024). In this way this technique allows the use of co-solvents, such as ethanol that offers several advantages in the obtention of bioactive compounds. By adjusting pressure, temperature, and the proportion of ethanol, the extraction process can be selectively directed to obtain more specific chemical profiles with fewer impurities, while requiring significantly smaller solvent volumes compared to conventional ethanolic extractions (Herrero et al., 2006; Reverchon & De Marco, 2006).

In this context, SFE offers notable potential for the obtention of phenolic molecules, key secondary metabolites that have anti-inflammatory, antimicrobial and antioxidant activities from medicinal plants. In this tenor, *Buddleja scordioides* Kunth, commonly known as "salvilla," is an endemic bush widely disseminated in Mexico and habitually used in traditional medicine for its healthy properties such as anti-inflammatory and wound-healing properties (Ávila-Acevedo & Romo-de-Vivar, 2002; Díaz-Rivas et al., 2015; Villegas Novoa et al., 2019). Phytochemical studies have revealed that salvilla is rich in polyphenols, particularly flavonols and phenolic acids, which confer its pharmacological activities (Macías-Cortés et al., 2022).

Given the sensitivity of polyphenolic compounds to heat and oxidation, SFE emerges as a particularly advantageous method for their recovery from salvilla. Previous research has demonstrated the successful application of SFE for extracting polyphenols and other bioactives from diverse plant matrices, including Amaryllidaceae alkaloids (Pilařová et al., 2025), rosemary (*Salvia rosmarinus*) (Ayyildiz et al., 2024), green tea (*Camellia sinensis*) (Dębczak et al., 2024), and grape seeds (*Vitis vinifera*) (Coelho et al., 2018), achieving high yields and preserving bioactivity. Such precedents highlight the promise of optimizing SFE conditions to maximize the yield and functionality of salvilla leaves extracts, paving the way for their potential use for pharmaceutical, nutraceutical, and cosmetic products. The aim of the present work is to evaluate the extraction of bioactive compounds from *Buddleja scordioides* Kunth (salvilla) using the supercritical fluid extraction, using a central composite design; this can help to determine the optimal conditions to maximize yield, phenolic content, and antioxidant activity of the salvilla leaves extracts.

2. Materials and methods

2.1 Chemical reagents

For total flavonoids catechin, (HPLC grade), potassium persulfate ($K_2S_2O_8$), aluminum chloride ($AlCl_3$), sodium hydroxide (NaOH), and sodium nitrite ($NaNO_2$) were used. Finally, for 2,2'-azino-bis (3-ethylbenzothiazoline-6-sulphonic) acid (ABTS) and Oxygen Radical Absorbance Capacity (ORAC) antioxidant techniques fluorescein, monobasic potassium phosphate, ABTS radical, dibasic potassium phosphate, 2,2'-azobis(2-amidinopropane) dihydrochloride (AAPH) radical and Trolox® were used. For the Ultra High-Performance Liquid Chromatography system coupled to a Xevo TQS triple-quadrupole detector with photodiode array and electrospray interfaces (UPLC-PDA-ESI-MS/MS) determinations, this reference standards and solvents were employed: luteolin, quinic acid, neohesperidin, ferulic acid, 4-hydroxybenzoic acid, apigenin, chlorogenic acid, acetonitrile (HPLC grade), syringic acid, protocatechuic acid, naringenin, caffeic acid, benzoic acid, eryodictiol, kaempferol 3-O-glucoside, ethanol, sinapic acid, shikimic acid, rutin, 2,4,6-trihydroxybenzaldehyde, vanillic acid, acacetin, quercetin-O-glucoside, trans-cinnamic acid, naringin, myricetin, methanol, 4-O-caffeoylquinic acid, quercetin, coumaric acid, quercetin glucuronide, kaempferol. All chemical reagents (reagent or LC grade) and standards (HPLC grade) were purchased from Sigma-Aldrich® (Merck KGaA, Darmstadt, Germany).

2.2 Methods

2.2.1 Collection of salvilla leaves

In 2018, wild bushes of *Buddleja scordioides* Kunth were harvested near Victoria de Durango, Durango, México (24°00′00″ N, 104°24′59″ W). After collection, the salvilla leaves were detached from the stems and air-dried at 25 °C under light-protected conditions. Once dehydrated (with a moisture content of 5.32 ± 0.3 %), the plant material was ground using a #2 sieve knife mill (IKA®, Staufen, Germany) and subsequently kept in sealed containers until further use.

2.2.2 Supercritical fluid extraction (SFE)

A portion of milled salvilla leaves (55 g) was introduced into the extraction chamber of a Thar® SFE system (Pittsburg, USA). Extractions were carried out at 40 °C, using CO_2 as the primary solvent and ethanol as co-solvent with a 10 mL/min flow, varying the co-solvent percent, pressure, and time (described in statistical analysis). To recover each extract, the ABPR valve was gradually opened in increments to prevent freezing, with the instrument software controlling the opening at a rate of 5 % per minute, enabling collection from the separator vessel. A total of fifteen SFE conditions were used (Table 1). After each extraction, the system was slowly depressurized, the collected material was retrieved from the vessel, and it was stored under refrigeration and protected from light until further analysis.

Table 1. Treatment conditions
Tabla 1. Condiciones de los tratamientos

Treatment	Run	Time (min)	Pressure (Bar)	Co-solvent (%)
1	13	162.4	185.4	16.5
2	4	162.4	114.6	23.5
3	14	77.6	185.4	23.5
4	1	77.6	114.6	16.5
5	7	60	150	20
6	15	180	150	20
7	9	120	100	20
8	6	120	200	20
9	2	120	150	15
10	5	120	150	25
11	3	120	150	20
12	10	120	150	20
13	8	120	150	20
14	11	120	150	20
15	12	120	150	20

2.2.3 SFE extracts pre-treatment to lyophilization

Because all extracts were generated using ethanol as the co-solvent, it was necessary to remove the organic phase before freeze-drying. For this purpose, distilled water equivalent to 25 % (v/v) of each extract was added, followed by solvent removal in a Büchi® rotary evaporator (New Castle, USA) at 40 °C. Once the alcohol had been eliminated, the aqueous extracts were frozen and subsequently lyophilized in a Labconco® freeze dryer (Kansas City, Missouri, USA). The resulting dry powders were kept under desiccated conditions until further use.

2.2.4 Extraction yield

The energy content of the supplement was estimated based on its nutritional composition, in accordance with Mexican Official Standard NOM-051-SCFI/SSA1-2010. Quantification of the total macronutrient content (proteins, lipids, and carbohydrates) was performed. Subsequently, Atwater's general conversion factors were applied, assigning 4 kcal per gram to proteins and carbohydrates, and 9 kcal per gram to lipids (Sánchez-Peña et al., 2017). Results were represented as kilocalories per 100 grams of supplement (kcal/100 g).

To determine the extraction yield, 55 g of dried salvilla leaves were exposed to the SFE procedure and later lyophilized. Extraction yield was expressed as the percentage of recovered extract relative to the dry mass:

$$Extraction\ yeld(\%) = \frac{lyophilized\ extract\ mass}{initial\ dry\ mass} \times 100$$

2.2.5 Total flavonoid content

Total flavonoids were assessed using the methodology reported by Rosales-Villarreal et al. (2022). For the calibration curve, catechin solutions (0–300 μg/mL) were prepared. Samples of the lyophilized SFE extracts were diluted in a milliliter (mL) of deionized water. Then, 20 μL of each standard, blank, or sample were blended with 5 % $NaNO_2$ (7.5 μL), 10% $AlCl_3$ (15 μL), 1 M NaOH (50 μL), and 157 μL distilled water. The reaction mixtures were protected from light for 5 min, and absorbance was recorded at 570 nm using a Daigger microplate reader (Buffalo Grove, IL, USA). Results were expressed as μg of catechin equivalents per mg of salvilla leaves extract (μg CE/mg SE).

2.2.6 Antioxidant activity of samples

For this project, both electron-transfer (ABTS) and hydrogen-donation (ORAC) assays were used to evaluate antioxidant capacity in all treatments.

The ABTS assay was performed according to the method reported by Macías-Cortés et al. (2022). After the $ABTS^{\bullet+}$ radical was activated by mixing equal quantities of ABTS solution (7 mM) and potassium persulfate solution (2.45 mM) and allowing them to react at room temperature for 16 h in the dark, this was diluted with 5 mM phosphate buffered-saline (PBS) buffer (pH 7.4) to achieve an absorbance of 0.700 ± 0.02 nm at 750, then 10 μL of diluted blank, sample, or Trolox® (50μM) were aggregated to 190 μL of diluted $ABTS^{\bullet+}$ radical. After 10 min in the dark, absorbance was measured at 750 nm using a Daigger® microplate reader (Vernon Hills, IL, USA). Results were reported as mmol Trolox equivalents per mg of salvilla leaves extract (mmol TE/mg SE). In other hand, ORAC assay was adapted from Rosales-Villarreal et al. (2022). Each well received 20 μL of diluted blank, sample or Trolox® (50μM), and then, 200 μL fluorescein (1.09 μM) were added. After a 15 min incubation at 37 °C using a Synergy HT® microplate reader (Bio-Tek, Winooski, VT, USA), 75 μL of 2,2'-azobis(2-amidinopropane) dihydrochloride (AAPH) radical (79.65 mM) were added. In this assay, fluorescence of each well was monitored each 1.5 min, finishing at 2.5 h. The readout was programmed at 485 nm for excitation and 535 nm for emission. Antioxidant activity was calculated from the area under each decomposition curve and reported as mmol TE/mg dry salvilla leaves extract.

2.2.7 Phenolic characterization by Liquid chromatography – mass spectroscopy (UPLC-PDA-ESI- MS/MS)

For phenolic characterization, profiling was performed following the Díaz-Rivas et al. (2018) method with some modifications. Analyses were performed using a Waters Ultra High-Performance Liquid Chromatography (UPLC) system coupled to a Xevo TQS triple-quadrupole detector with PDA and ESI interfaces (Xevo TQS, Waters Corp., Wexford). Samples were injected via a temperature-controlled manager (6 °C) into an Agilent® C18 column (150 × 2.1 mm, 1.7 μm). The elution involved solvent A composed of 7.5 mM formic acid and solvent B that is acetonitrile, this according to the following gradient: 97:3 (A:B) at 0 min; 91:9 at 1.23 min; 84:16 at 3.82 min; 50:50 at 11.40 min; returning to 97:3 at 13.24–15.00 min. The elution flow rate was 250 μL/min, while methanol/formic acid introduced at 0.210 mL/min as co-solvent was added post-separation to stabilize ion formation. Total

runtime was 15 min. Mass spectrometry was conducted in Multiple Reaction Monitoring (MRM) mode, operating in negative ESI with the following parameters: cone 30 V, capillary 2.25 kV, desolvation 400 °C, source 150 °C, desolvation gas 800 L/h, cone gas 150 L/h, collision gas 0.13 mL/min, MS collision energy 2.0 eV, and MS/MS 20 eV. Phenolic standards at 20 ng/µL were used to establish retention times, m/z values, and fragmentation patterns. Data acquisition and processing were performed using MassLynx v4.1 Software (Waters Corp., Milford, MA). Samples were prepared at 10 mg per milliliter (mg/mL), filtered through 0.45 µm acrodiscs, and placed into amber vials in the autosampler, wich injected 1 microliter for the assay. The results were expressed as ng of phenolic compound per mg of salvilla leaves extract.

2.2.8 Experimental design and data analysis

The use of a central composite design (CCD) is widely recommended for multivariable optimization in extraction technologies, particularly in SFE, where process efficiency and selectivity depend on synergistic effects between operational parameters (Herrero et al., 2006; Bezerra et al., 2008). For the experiment a CCD was chosen, small type, with 15 experimental runs: 10 without central points and 5 with central points, using an alpha (α) of 1.41421. The independent variables were extraction time, pressure and cosolvent percent according to the follow table 2:

Table 2. Independent variables of the experiment
Tabla 2. Variables independientes del experimento

Factor	- 1	- α	0	α	1
Time (min)	60	77.57	120	162.43	180
Pressure (bar)	100	114.64	150	185.36	200
Co-solvent (%)	15	16.46	20	23.54	25

Furthermore, a general linear model (GLM quadratic modeling) was employed to analyze the experimental data because it allows the simultaneous evaluation of the individual and interactive effects of the extraction variables included in the central composite design. Results are shown in RSM graphics. The analysis was performed using obtained data from statistical software Design Expert ® (Minneapolis, USA) and Statistica v. 12.0 software (Palo Alto, CA, USA).

3. Results and discussion

3.1 Extraction yield

Once the SFE salvilla leaves extracts were obtained, all treatments were lyophilized and their yield considered, where all samples were from 3.6 to 6.3 % (central points had an average of 4.90 ± 0.1 %). These results were fitted to a linear model in which the most influential factor according to their equation is ethanol (p = 0.0001) (Table 3).

Table 3. Modeling for each extraction parameter
Tabla 3. Modelamiento para cada parámetro de extracción

EP	Model	*p* value
Yield	$1.60 + 0.02a + 0.003b - \mathbf{0.07c}$	0.0001
TFM	$-295.23 - 1.10a + 3.37b - \mathbf{29.31c} - 0.01ab + 0.11ac - 0.05bc + 0.004a^2 + 0.001b^2 - 0.70c^2$	0.0011
ABTS	$-19.05 - 1.49a + 0.88b + \mathbf{7.89c} + 0.0007ab + 0.03ac - 0.04bc + 0.004a^2 + 0.00002b^2 - 0.10c^2$	0.005
ORAC	$-1749.04 + 5.17a + 9.43b + \mathbf{94.31c} + 0.01ab - 0.06ac - 0.28bc - 0.02a^2 - 0.01b^2 - 1.21c^2$	0.0005
TPC	$-152999 + 400.76a + 658.99b + \mathbf{9576.79c} - 0.37ab - 11.52ac - 24.71bc - 0.52a^2 - 0.58b^2 - 114.18c^2$	0.0004

**EP= extraction parameters; TFM = Total flavonoids (microplate); TPC = total phenolic compounds a= time b= pressure c= cosolvent*

Although the co-solvent is the most influential factor, it can be noticed that minimum yield corresponds to a 60 min of extraction, while 6.3 % corresponds to 162.4 min, treatment obtained at 180 min had a yield of 6.0 %. Fig. 1 showed the yield modeling in a 2D surface method response graphic. In this respect, the obtained results of yield were smaller than the observed by traditional technologies such as the reported by Avila-Acevedo & Romo-de-Vivar (2002), who made a successive extraction of salvilla, beginning with hexane, then ethyl acetate and finally methanol, reporting 15.15 % of yield. Another case is the salvilla extract obtained using an infusion method made (Herrera-Carrera et al., 2015), obtained a 20.31 ± 0.61 % of yield extraction. In the other hand, SFE showed lower yield extraction than the other one obtained by ultrasound assisted extraction (UAE), an emergent technology made by Macías-Cortés et al. (2022), who reported a yield extraction of 10.58 ± 0.33 to 12.92 ± 0.40 % in hydroalcoholic salvilla leaves extracts obtained with different conditions such as wave amplitude, time, and ethanol percent. The yield obtained in this work is similar to the described by Perez-Gutierrez & Vargas-Solis (2008) who reported a yield of 7.6 % in a successive extraction consisted of hexane, chloroform and methanol (in this order) salvilla extract. Fig. 1.

Despite the comparatively lower extraction yields obtained through supercritical fluid extraction (SFE), this behavior is consistent with the intrinsic characteristics of the technique. SFE typically produces smaller quantities of extract than conventional or solvent-intensive methods (Pourmortazavi & Hajimirsadeghi, 2007); however, it offers significant technological advantages that justify its use for the recovery of bioactive compounds. Because CO_2 is non-toxic, non-flammable, and easily removed from the final product, SFE generates cleaner extracts free of solvent residues, which is particularly valuable for applications in food, nutraceutical, and pharmaceutical formulations (Herrero et al., 2006; Reverchon & De Marco, 2006). Additionally, the process operates under mild temperatures, reducing thermal degradation of thermolabile compounds and improving the preservation of antioxidant constituents (Plaza & Turner, 2015). From an environmental perspective, SFE is considered a more sustainable alternative, as it minimizes the use of organic solvents and allows CO_2 to be recycled within the system, reducing waste generation and ecological impact. Therefore, although the yields obtained in this study were lower than those reported for traditional extraction techniques, the high purity, safety, and sustainability associated with SFE position it as a competitive and environmentally responsible method for salvilla bioactive compound recovery.

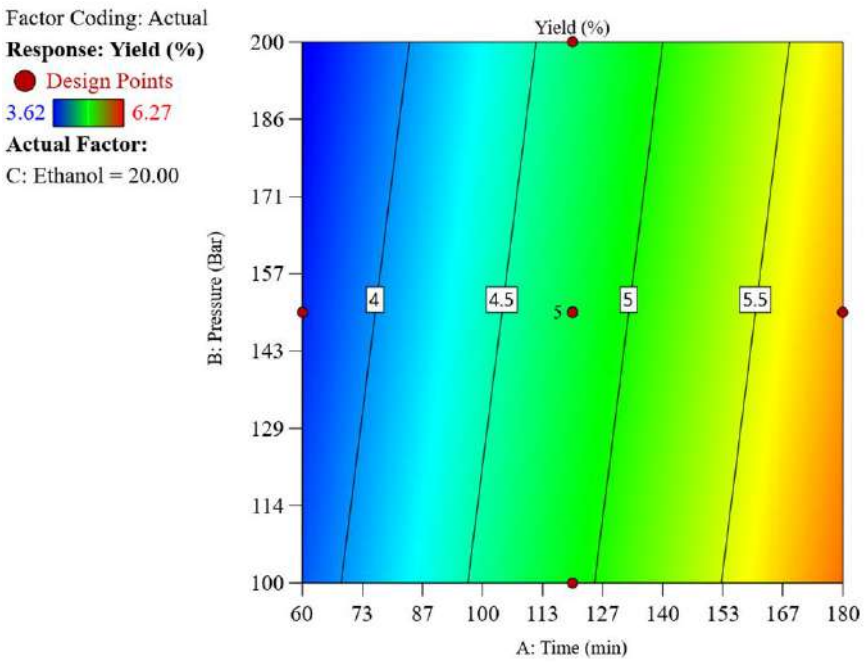

Figure 1. Yield of salvilla leaves extract (2D surface response model)
Figura 1. Rendimiento del extracto de salvilla (modelo de superficie de respuesta en 2D)

3.2 Total flavonoid content

The flavonoids are a group of secondary metabolites acknowledged for their anti-inflammatory, antioxidant, and protective properties against oxidative stress. Their quantification is a key indicator of the biological potential of plant extracts and serves as an important criterion to assess the efficiency of extraction methods. Therefore, determining the total flavonoid content provides valuable insight into the capacity of supercritical fluid to recover bioactive compounds with functional relevance (Herrero et al., 2006; Macías-Cortés et al., 2020).

In this tenor, a total flavonoid content by microplate was done for all treatments. These results were adapted to a quadratic model where the most influential factor according to their equation was ethanol ($p = 0.0011$) (Table 3). The achieved values ranged from 219.17 to 314.72 μg CE/mg SE, with the central points showing a mean value of 270.43 ± 1.2 μg CE/mg SE (Fig. 2) where it seemed that higher values can be found in higher ethanol percent. Despite the lower yields obtained, supercritical fluid extraction demonstrated to be a competitive emerging technology since flavonoid content was higher than the reported in other works, for example, Macias-Cortés et al. (2022) with values of 107.3 ± 3.0 - 224.9 ± 6.3 μg CE/mg SE in samples obtained with ultrasound assisted extraction, and the described by Díaz-Rivas et al. (2015), who informed quantities of 10.8 ± 0.05 μg CE/mg SE in a salvilla infusion and their work where the salvilla infusion and 7.06 ± 0.4 μg CE/mg SE in a concentrated salvilla infusion. These findings confirm that ethanol, in synergy with supercritical CO_2, enhances the recovery of metabolites like flavonoids as a consequence of its polarity and solvent affinity. Moreover, the supercritical CO_2 phase improves the solubilization and mass transfer of medium-polarity molecules, thereby increasing their concentration in the last extracts (Herrero et al., 2006).

Therefore, the ability of SFE to selectively enrich flavonoids positions this method as an auspicious green replacement for the obtention of functional molecules from salvilla and related medicinal plants.

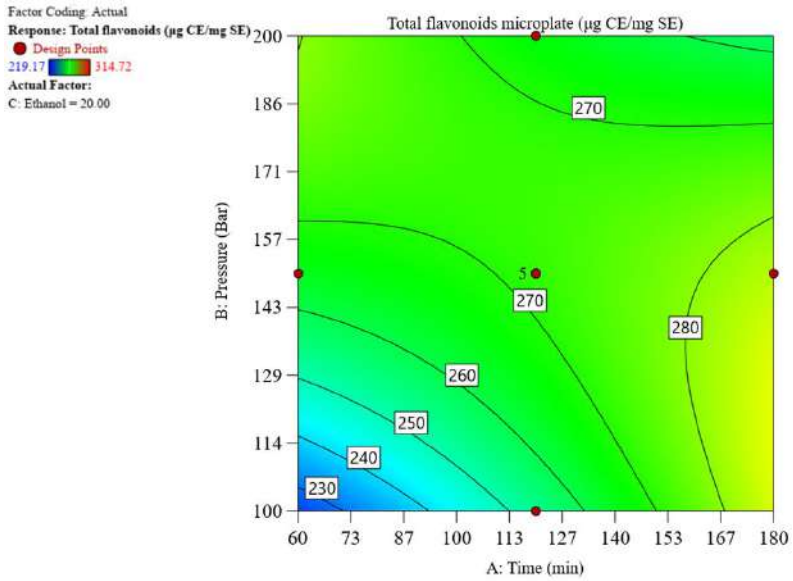

Figure 2. Total flavonoids content by microplate (2D surface response model)
Figura 2. Contenido de flavonoides totales por microplaca (modelo de superficie de respuesta en 2D)

3.3 ABTS assay

The production of reactive oxygen species and free radicals during oxidation processes in biological and food systems can damage DNA, proteins and lipids, leading to several chronic diseases and the deterioration of food quality. Therefore, evaluating the antioxidant capacity of natural bioactive compounds or plant extracts is essential to evaluate their functional potential, either as nutraceutical ingredients or for applications in functional foods. The ABTS assay has become a standard tool for this purpose due to its versatility. The $ABTS^{\bullet+}$ radical cation exhibits a characteristic absorption that decreases when an efficient antioxidant neutralizes it. Additionally, its rapid reaction and good reproducibility make it an assay of choice for screening and comparing antioxidant capacity between samples (Dawidowicz and Olszowy, 2013).

In this study, the antioxidant activity measured by the ABTS assay showed a clear dependence on the extraction conditions, particularly on ethanol concentration, as indicated by the quadratic model that best fitted the data ($p = 0.005$). For all the samples, quantities were going from 53.54 to 81.88 mmol TE/mg SE; in this tenor, central points had an average of 62.83 ± 0.8 mmol TE/mg SE. Ethanol percentage was the factor with the greatest influence on the results (Fig. 3), suggesting that solvent polarity played a key role in the extraction efficiency of antioxidant compounds.

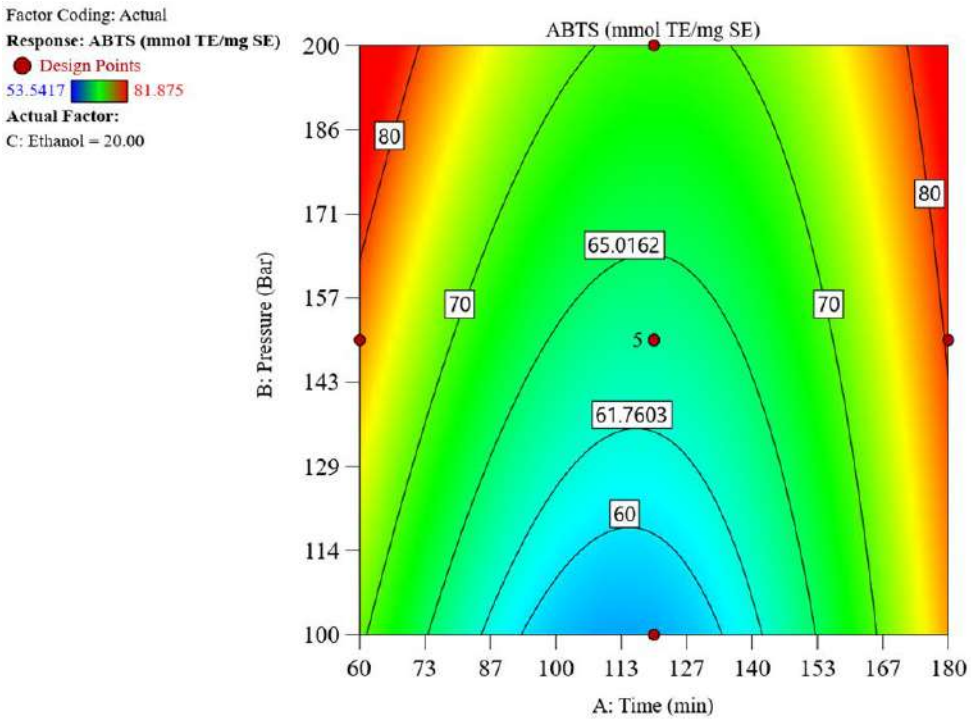

Figure 3. Antioxidant activity by ABTS assay (2D surface response model)
Figura 3. Actividad antioxidante por el ensayo ABTS (modelo de superficie de respuesta en 2D)

This result aligns well with previous findings in which the addition of ethanol as a co-solvent in supercritical-CO_2 based extraction enhanced the recovery of antioxidant phenolics or flavonoids, likely by improving the polarity match between solvent and solutes. In contrast to the performance obtained with ethanol as a co-solvent in supercritical CO_2 extraction, studies using methanol as a co-solvent have shown comparatively lower antioxidant efficiency. For example, Uwineza et al. (2021) reported that SFE extracts of *Lamium album* obtained with methanol as co-solvent exhibited values for ABTS assay ranging only from 0.043 to 0.045 µg TE/mg extract, indicating limited antioxidant capacity under those conditions. The influence of ethanol concentration aligns with the chemical nature of the bioactive compounds typically extracted from plant matrices. Phenolic compounds—such as phenolic acids, flavonoids, and tannins—are widely recognized for their strong radical-scavenging capacity due to their ability to donate electrons or hydrogen atoms, effectively neutralizing reactive species like $ABTS^{\bullet+}$ (Rice-Evans et al., 1997). Because these compounds exhibit intermediate polarity, ethanol can enhance supercritical CO_2 extraction. Moderate ethanol concentrations tend to favor the simultaneous solubilization of both polar phenolic acids and less polar flavonoid aglycones, which may explain the increased antioxidant activity observed at specific ethanol levels (Radzali, 2020). To support this, Lee et al. (2020) reported that increasing ethanol concentration during conventional extraction significantly elevated antioxidant activity in citrus peel extracts. supporting the idea that ethanol concentration modulates the extraction of compounds with high antioxidant potential.

Overall, the relationship between extraction conditions, bioactive compound solubility, and antioxidant activity underscores the importance of optimizing solvent composition to maximize the

functional potential of plant-derived extracts. However, excessively high ethanol proportions may diminish the supercritical CO_2 diffusivity or reduce selective extraction by co-extracting non-target constituents, which may dilute the specific antioxidant compounds (Radzali, 2020).

3.4 ORAC assay

The measurement of antioxidant activity by ORAC assay is a reliable and extensively used method in the evaluation of natural extracts. This is based on their capability to neutralize the peroxyl radicals. Unlike other assays that rely on single-electron transfer mechanisms, ORAC measures the hydrogen atom transfer capacity, providing a more physiologically relevant estimate of antioxidant performance. This assay has been widely used to evaluate plant-derived phenolics and flavonoids, specifically regarding their antioxidant capacity, allowing quantitative comparison of different extraction methods and treatments (Ou et al., 2001).

In Fig. 4 the response surface graphic, illustrates the effects of extraction time and pressure and with 20 % of ethanol as co-solvent, expressed in mmol TE/mg SE, and the results were fit to a quadratic model ($p = 0.0005$). In this sense, values were going from 78.69 to 185.40 mmol TE/mg SE, obtaining the central points the highest values, with an average of 183.60 ± 2.0 mmol TE/mg SE. According to the equation in Table 3, co-solvent was the most influential factor. In SFE, ethanol functions as an effective polarity modifier, enabling fine-tuning of the solubility of phenolic compounds, flavonoids, and other metabolites. Moreover, when used as a co-solvent, ethanol increases the polarity of the supercritical CO_2 phase, facilitating the recovery of polar bioactive compounds typically obtained through traditional ethanolic extraction, but with the added advantages of higher purity and reduced thermal or oxidative degradation (Machmudah et al., 2006). It can be seen that Supercritical fluid extraction can enhance the obtention of bioactive compounds. As an example, Picos-Salas et al. (2024) observed that supercritical fluid extract from Mexican oregano (*Lippia graveolens*) showed an ORAC activity of 6923.65 ± 57.2 μmol TE/g extract while the methanolic extract obtained from the same specie had 3577.08 ± 195.09 μmol TE/g extract, a 48.33 % less activity than the obtained with this suitable emerging technology. Supercritical CO_2, particularly when modified with a polar solvent, exhibits strong affinity for highly bioactive antioxidant molecules with elevated radical-scavenging capacity (Pilařová et al., 2024).

In summary, the response surface trends clearly demonstrate that the combination of moderate pressure and adequate extraction time maximizes the obtention of bioactive molecules, highlighting the strong influence of these parameters on the increase of antioxidant molecules recovery. The high values obtained at the central points of the present work, together with those observed by Picos-Salas et al. (2024), reinforce the idea that supercritical CO_2 can be used potentially as an efficient method for obtaining antioxidant molecules from salvilla.

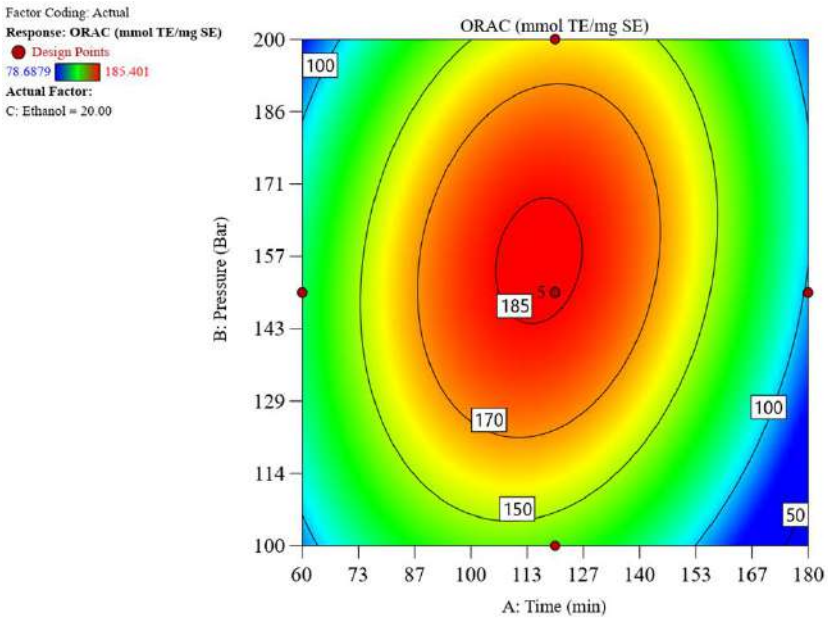

Figure 4. Antioxidant activity by ORAC assay (2D surface response model)
Figura 4. Actividad antioxidante por el ensayo ORAC (modelo de superficie de respuesta en 2D)

3.5 Identification and quantification of Total Phenolic compounds

As previously reported, salvilla is characterized by a rich and diverse phenolic profile, which contributes to many of its traditional and pharmacological properties. Its phenolic matrix typically includes flavonoids and their glycosides, along with phenylethanoid derivatives, and hydroxybenzoic and hydroxycinnamic acids reported in different plant parts (Macías-Cortés, et al., 2022). These molecules are acknowledged for their anti-inflammatory, antioxidant and cytoprotective activities, making salvilla a relevant source of bioactive metabolites. Under this idea, it is important to determine whether bioactive compounds can be extracted, identified, and quantified from supercritical fluid extracts.

Using UPLC-PDA-ESI-MS/MS five groups of phenolic compounds were identified in the extracts. In this study, as hydroxybenzoic acids were discovered protocatechuic, benzoic, syringic, 4-hydroxybenzoic, shikimic, quinic and vanillic acids, giving a total hydroxybenzoic acids from 338.6 to 622.0 ng/mg of SE, while in the hydroxycinnamic acids group were found coumaric, caffeic, ferulic, caftaric, 4-O-cafeoilquinic, chlorogenic and trans cinnamic acids, with a total content going from 123.8 to 238.6 ng/mg of SE. For total flavones, apigenin, luteolin and acacetin were detected, with total values ranging from 374.30 to 848.00 ng/mg of SE; sample 7 obtained at 120 min, 100 Bar and 20 % of co-solvent had the highest values. Another identified group was flavanones, containing eriodyctiol, naringenin and naringin, with total values from 4.30 to 19.60 ng/mg of SE, this the result obtained at 180 min, 150 Bar and 20 % of ethanol (sample 6). Finally, many molecules of flavonols group were found, such as mirycetin, kaempferol and its glucoside, rutin, and the most important, quercetin and its glucosides, quercetin glucuronide and quercetin-3-O-glucoside, quercetin is known as a powerful anti-inflammatory molecule (Villegas-Novoa et al., 2019). This group had the highest concentration

in sample 7, who showed 11586.70 ng/mg of SE. This profile is consistent with the reported by Macías-Cortés et al. (2022) who performed an ultrasound-assisted extraction of salvilla using a hydroalcoholic solvent.

Fig. 5 presents the response surface plot evaluating the effects of pressure and time of extraction on the content of total phenolic compounds (ng/mg of SE), while keeping the ethanol concentration constant at 20 %. In this sense, values ranged from 4185.10 to 13008.20 ng/mg of SE, with sample 7 showing the highest values. These discoveries are consistent with previous reports showing that adequate extraction times and the use of polar co-solvents such as ethanol can improve the recovery of bioactive molecules using supercritical or pressurized fluid-based technologies (Díaz-Rivas et al., 2015; Macías-Cortés et al., 2022; Rosales-Villarreal et al., 2022).

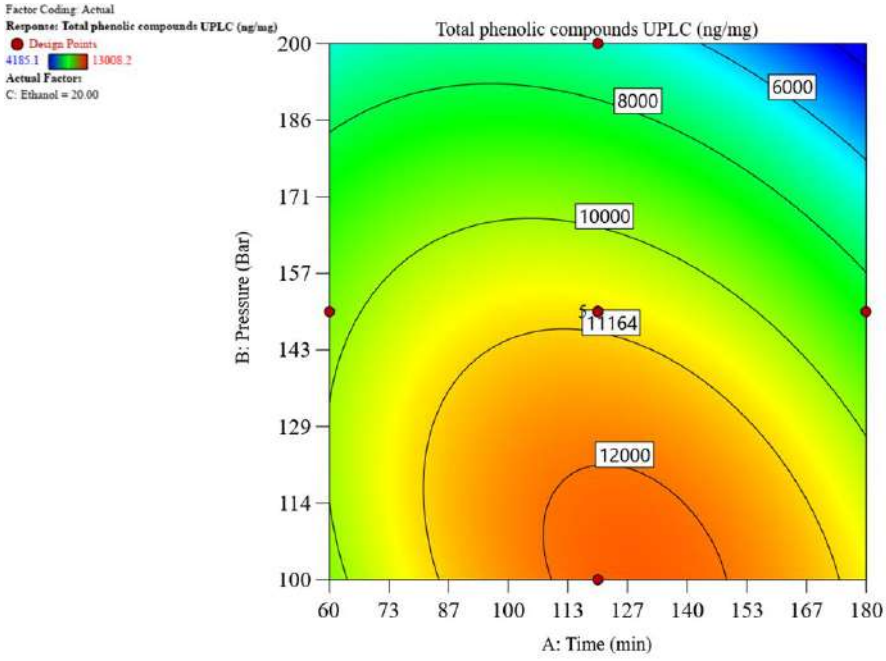

Figure 5. Total phenolic compounds by UPLC-PDA-ESI-MS/MS (2D surface response model)
Figura 5. Compuestos fenólicos totales por UPLC-PDA-ESI-MS/MS (modelo de superficie de respuesta en 2D)

3.6 General Linear Modeling of treatments

Regarding optimal conditions for salvilla leaves extraction, a General linear Modeling (GLM) was used with a quadratic fit, and a comparison between factors was performed, revealing a good fit. According with this, in Fig. 6a can be observed that higher values of bioactive compounds and its antioxidant activity can be obtained up to 120 minutes, no matter how much pressure is applied on the system (desirability 8.0), the same behaviour can be observed in Fig. 6b (desirability 7.5), were no matter how much pressure were added to the system, it did not have any influence on the results, getting a good extract beginning in 20 % of ethanol. Finally, in Fig. 6c it can be observed that from 20 % of ethanol and 120 minutes of extraction it is possible to obtain good results, with a good quality

extract, this can be a benefit since the process can be expensive compared to other emerging technologies.

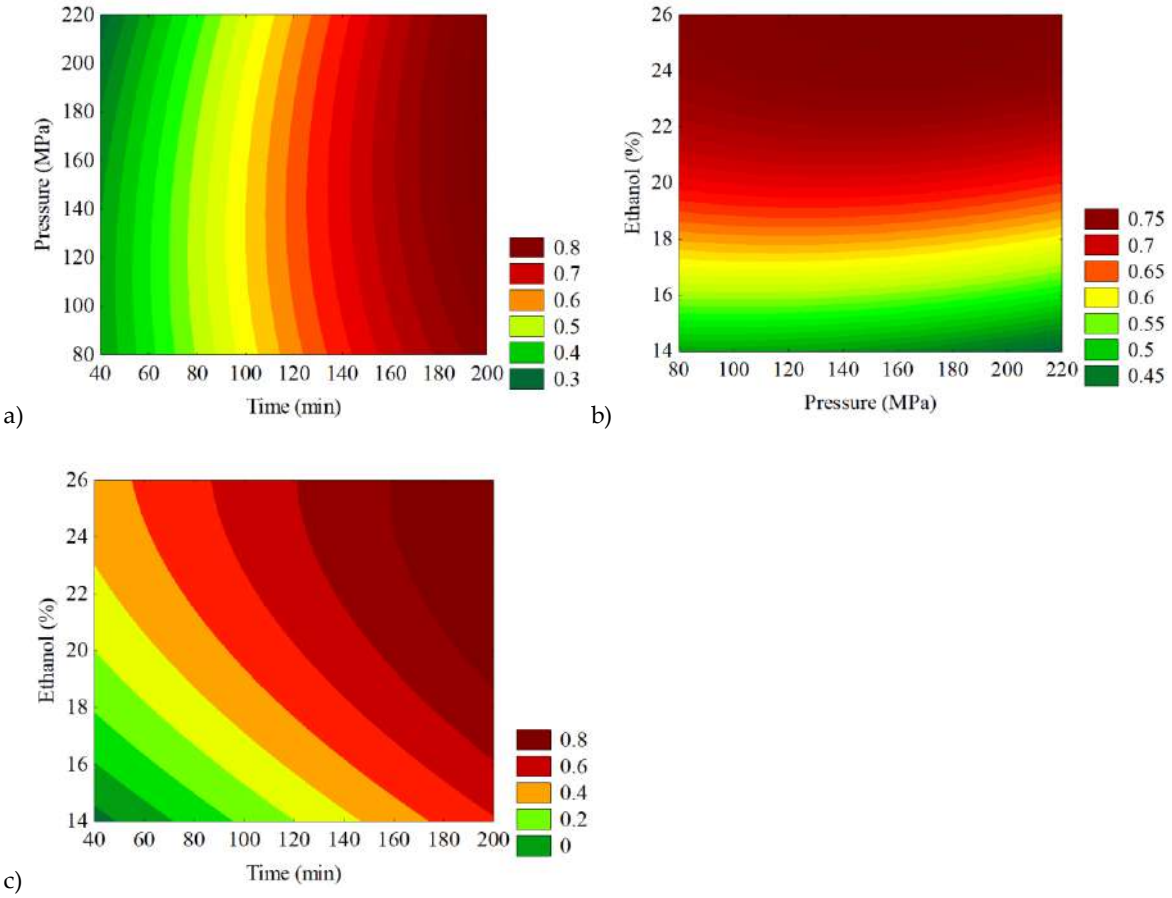

Figure 6. a) General Linear Modeling desirability contours (Quadratic fit) of pressure vs time; b) General Linear Modeling desirability contours (Quadratic fit) of ethanol vs pressure; c) General Linear Modeling desirability contours (Quadratic fit) of ethanol vs time

Figura 6. a) Contornos de deseabilidad del Modelo Lineal General Linear (Quadratic fit) de la presión vs tiempo; b) Contornos de deseabilidad del Modelo Lineal General Linear (Quadratic fit) del etanol vs presión; c) Contornos de deseabilidad del Modelo Lineal General Linear (Quadratic fit) de etanol vs tiempo

4. Conclusions

In the present work, the extraction of bioactive compounds from salvilla was evaluated using supercritical fluid extraction, with a central composite design. Supercritical fluid extraction (SFE) of *Buddleja scordioides* Kunth (salvilla) has demonstrated to be an effective, environmentally friendly, and promising green emerging technology for the recovery of phenolic compounds, antioxidant metabolites, and other bioactive molecules, despite achieving lower extraction yields compared to conventional or alternative emerging extraction methods. Ethanol concentration consistently

emerged as the most influential factor on extraction yield and the recovery of phenolic and flavonoid compounds, as confirmed by statistical modeling, highlighting its key role in modulating solvent polarity and enhancing solubilization of medium-polarity compounds. On the other hand, supercritical fluid extraction (SFE) enables the recovery of compounds with antioxidant capacity and other biologically relevant activities, often surpassing the performance of extracts obtained with conventional solvents; antioxidant assays confirmed this behavior, while UPLC-PDA-ESI-MS/MS profiling revealed diverse phenolic groups, predominantly flavonols such as quercetin derivatives, widely recognized for their biological and pharmacological relevance.

General linear modeling further indicated that extraction times around 120 minutes combined with ≥20 % ethanol are sufficient to achieve extracts with high bioactive quality, regardless of pressure increments. This finding is especially relevant considering the energetic and operational costs associated with high-pressure systems. Overall, the integration of chemical, antioxidant, and modeling results supports the use of SFE as a selective, cleaner, and scalable alternative for obtaining functional compounds from salvilla. These insights not only strengthen the technological relevance of SFE but also open the door for its future application in the development of nutraceutical, cosmetic, or functional-food ingredients derived from this traditionally valued species. Future studies should explore process scale-up, fine-tuning of co-solvent dynamics, and integration with downstream purification steps to further enhance compound recovery and commercial feasibility. In this sense, the promising results obtained here provide a solid foundation for consolidating SFE as a sustainable and efficient technology for the industrial exploitation of bioactive potential from salvilla plants.

Author Contributions

Conceptualization, E.M.C. and M.G.R.A.; methodology, R.M.H.R; software, J.A.V.N and J.A.G.I.; validation, R.F.G.L. and E.M.C.; formal analysis, R.F.G.L. and N.E.R.G.; investigation, E.M.C.; resources, G.A.C.H. and J.A.G.I.; data curation, R.F.G.L.; writing—original draft preparation, E.M.C.; writing—review and editing, M.G.R.A.; visualization, J.A.V.N.; supervision, R.F.G.L.; project administration, R.F.G.L. All authors have read and accepted the pubished version of the manuscript.

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Conflict of interest

The authors declare that they have no conflicto of interests.

Nomenclature

CO_2	Carbon dioxide
ESI	Electrospray ionization
MAE	Microwave-assisted extraction
mL	Milliliter
mL/min	Milliliters per minute
RSM	Response surface methodology
#	Number
%	Percent
≥	Major or equal than
®	Registered trademark
°C	Celsius degree
μmol TE/g	Micromole of Trolox equivalent per gram of extract
AAPH	2,2'-azobis(2-amidinopropane) dihydrochloride
ABTS	2,2'-azino-bis (3-ethylbenzothiazoline-6-sulphonic) acid
$ABTS^{\bullet+}$	2,2'-azino-bis (3-ethylbenzothiazoline-6-sulphonic) radical
$AlCl_3$	Aluminum chloride
Bar	Bars
CCD	Central composite design
DNA	Deoxyribonucleic acid
Fluorescein	3′,6′-dihydroxyspiro[isobenzofuran-1[3H],9′[9H]-xanthen]-3-one
FRAP	Ferric Reducing Antioxidant Power
g	Grams
GLM	General linear model
h	Hours
HPLC	High performance liquid chromatography
$K_2S_2O_8$	Potassium persulfate
kV	Kilovolts
L/h	Liters per hour
LC grade	Liquid chromatography grade
M	Molar
m/V	Ratio mass – volume
min	Minutes
mL/min	Milliliter per minute
mm	Millimeters
mM	Millimolar
mmol TE/mg of SE	Millimoles of Trolox equivalent per milligram of salvilla leaves extract
MRM	Multiple reactions ionization mode
$NaNO_2$	Sodium nitrite
NaOH	Sodium hydroxide
ng/mg SE	Nanograms per milligram of salvilla leaves extract
ng/μL	Nanograms per microliter
nm	Nanometers
Greek symbols	
α	Statistical alpha

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