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SCIENTIFIC PAPER

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EVALUATION OF SUPERCRITICAL CARBON DIOXIDE EXTRACTION TO OBTAIN BIOACTIVE COMPOUNDS FROM *Vernonia amygdalina* DELILE LEAVES

Article Highlights

- Extracts from *Vernonia amygdalina* Delile were obtained with supercritical CO_2
- The overall extraction yield varied from 0.69 to 1.24%
- Spline model was used quite successfully to describe the extraction kinetics

Abstract

Literature reports have shown that supercritical extraction is a promising method to obtain bioactive compounds used to develop drugs based on natural products. This work investigated the yield, antioxidant activity, and phytochemical constituents of the extracts obtained from Vernonia amygdalina Delile leaves with supercritical CO_2. The supercritical extraction was examined at temperatures of 40, 50 and 60 °C and at pressures of 200 and 250 bar. The supercritical fluid extraction (SFE) method was compared with the classical Soxhlet using different solvents at atmospheric pressure. The overall yield obtained using SFE varied from 0.69 to 1.24%. The extraction conditions which favored the highest yield were 60 °C and 250 bar. The overall SFE curves were fitted using three mathematical models. All of them were able to describe satisfactorily the extraction kinetics. Spline model presented the best fit to experimental data and was able to characterize constant and decreasing extraction rate periods. Extracts were characterized by gas chromatography coupled with mass spectrometry technique and some major components were identified. The antioxidant activity tests showed that the SFE extracts had low antioxidant activity exhibiting the estimated concentration of extract required to reduce 50% of the stable free radical DPPH (EC_{50}) values to be between 622.62 and 937.88 μg mL^{-1}.

Keywords: antioxidant activity, bioactive compounds, mathematical modeling, phytochemical components, supercritical extraction, Vernonia amygdalina Delile.

Extracts of the several species of the genus *Vernonia* (Asteraceae) are used in folk medicinal form, mainly in South and Central America and Africa. In Brazil, *Vernonia amygdalina* Delile is used in folk medicine for gastrointestinal disorders, headaches, and diarrhea pain. The plant is grown in different regions of the country where it is known as “boldo”, “boldo-baiano”, “figatil” and “heparém” [1]. Phytochemical evaluations revealed high levels of antioxidant vitamins, mineral elements, and phytocomponents in various parts of the plant *V. amygdalina* Delile, especially in the leaves [2]. The phytocomponents present in the leaves are saponins, sesquiterpene lactones [3], and other compounds such as terpenes, coumarins, phenolic acids, lignans, xanthones and anthraquinones [4-6].

The literature reports some applications of extracts from *V. amygdalina* Delile in drug products. For instance, Atangwho *et al.* [2] showed in a study with rats that the plant extracts have anti-diabetic properties which may be explained by synergy of its

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compounds. The extracts were obtained by sequential maceration in water, methanol, chloroform and petroleum ether. Another example is the work of Imafidon and coworkers [7] which concluded that their extracts reduced Cd-induced liver damage and is a proper option in adjuvant therapy for heavy metal toxicity. Adefisayo *et al*. [8] presented results that suggest that *V. amygdalina* Delile extracts possess gastro-protective properties against aspirin-induced gastric ulcer. Achuba [9] showed the potential of extracts obtained from immersion in methanol. The study concluded that crude petroleum contaminated diets are deleterious to animal health and extracts are capable to avoid the renal dysfunction induced by crude petroleum contaminated diets.

Some recent studies point out the effect of extraction technique on the composition and the quality of *V. amygdalina* Delile extracts. Different extracts were obtained from this plant using Soxhlet and microwave assisted extraction (MAE) with water [10,11] or ethanol [10,12,13] as a solvent. Alara *et al*. [10,11] studied, by a factorial design, the extracts obtained using microwave assisted extraction with water as a solvent. The authors obtained yields lower than 23%. Alara *et al*. [11] identified the chemical components using GC-MS analysis using aqueous extracts using Soxhlet and MAE. The most prevailing compounds were phytol and terpenoids.

Several extraction methods have been employed to obtain bioactive compounds from plants. Among the unconventional methods, SFE is a technique that has attracted a lot of attention lately due to its reduced extraction time, higher quality of the extracts and reduced solvent use. In addition, Soxhlet low pressure extraction needs a greater volume of solvent and typically requires longer times of extraction, which can result in the degradation of phytocompounds present in the plants [14]. A literature analysis reveals that SFE of oils and resins from various plants have been extensively studied: *e.g.*, *Helichrysum italicum* (Roth) G. Don, *Angelica archangelica* L., *Lavandula officinalis* L., *Salvia officinalis* L., *Melilotus officinalis* L. and *Ruta graveolens* L. [15]; *Artemisia annua* L. [16], *Hibiscus sabdariffa* [17], *Salvia fruticosa* [18], *Lippia graveolens* [19], *pepper-rosmarin (Lippia sidoides Cham.)* [20] and several other plants were *st*udied by SFE. The conclusions about operation parameters and extractions of compounds are specific to each plant. In general, temperatures of extraction varied from 40-60 °C and pressures between 150-350 bar. SFE has been mainly employed for the recovery of low-polarity components from plants such as fatty-acids, phytosterols, carotenoids, and alkaloids among others [21]. However, to the best of authors' knowledge, there are no studies reported about the SFE of oleoresin from any part of *V. amygdalina* Delile plant. Except for the study published by King *et al*. [22], that evaluated SFE of *Vernonia galamensis* seed, no study related to the genus *Vernonia* could be accessed in the literature.

In this context, the aim of the present study was to investigate the overall performance of the extraction of bioactive compounds from *V. amygdalina* Delile using carbon dioxide in supercritical conditions (SC-CO_2). The extraction runs using SC-CO_2 were performed in a temperature range of 40 to 60 °C and pressures of 200 and 250 bar. The conventional Soxhlet extractions using organic solvents were carried out also, in order to evaluate and compare the mass percent yield and chemical profile of extracts obtained with SFE. Soxhlet extraction was chosen because it is a standard laboratory-scale extraction method that is often employed for comparison purposes [23]. The extraction kinetic curves of SFE were modeled using different functions proposed in the literature for this purpose: logistic model [24], diffusional model [25] and spline model [26-28]. The chemical profiles of all extracts were identified by gas chromatography coupled with mass spectrometry (GC-MS). In addition, the antioxidant activity and the total phenolic content of the *V. amygdalina* Delile extracts were evaluated.

MATERIALS AND METHODS

Plant material

The leaves of *Vernonia amygdalina* Delile used in this study were grown and collected near the Itaipu Hydroelectric Power Plant Foz do Iguaçu-PR, Brazil, in October 2014. The collected leaves were stored under room temperature for 24 h, packaged in an oven with recirculating air at 45 °C for 12 h in order to dry. The absolute humidity for the dried material was 68.7%. A voucher specimen of the species studied was deposited in the Herbarium Pharmacies Farmanguinhos Greens, Fiocruz, FFAR under registration number 639. After drying, the leaves were milled in an industrial processor. All dried and crushed material was sieved. For the classification, Tyler type sieves with 8-50 meshes (#8 = 2.38 mm and #50 = 0.297 mm) were employed using a sieve vibratory system (AS 200, Retsch, Germany). The leaves were retained between Tyler meshes of 16-32 (#16 = 1.19 mm and #32 = 0.635 mm). This granulometry of leaves allowed the completing of the supercritical

extraction without problems in maintaining the flow of the supercritical fluid.

Extraction methods

Supercritical extraction

The laboratory scale pressurized extraction system used in this study consisted of a CO_2 cylinder (99% purity), two thermostatic baths, a syringe pump (Model 500D, ISCO, US), and one extractor with an internal volume around 63 mL (height 20.5 cm and diameter of 1.98 cm bed). A schematic diagram of the experimental apparatus used in the CO_2 supercritical extraction experiments can be found in Silva *et al.* [29]. Approximately 10 g of dried and crushed leaves from the plant *V. amygdalina* Delile was placed in the extraction vessel. The remaining bed was filled with glass beads (Ø = 4 mm). The solvent was pumped at a constant flow rate of 3 mL min^{-1} into the extraction chamber and maintained in contact with the bed for 30 min to allow the solvent to penetrate into the solid, as well as the diffusion of the solute from inside the particles to the fluid phase. It is important to highlight that the volumetric flow is based on pump temperature (5 °C), which is different from the extraction temperature. The mass flows of CO_2 were calculated using a thermodynamic data for carbon dioxide [30], which corresponds to 3.007-3.0203 g min^{-1}. Additional information about flow calibration is available as Supplementary material.

The extractions were carried out at 40, 50 and 60 °C, and pressures of 200 and 250 bar. For sampling, the solvent feed was interrupted and a valve at the bottom of the system was opened. The extraction curves were obtained by weighing the extracted mass collected at the end of the apparatus every 10 min.

Conventional extraction with organic solvents

The conventional Soxhlet extraction with organic solvents was performed using ethanol (SYNTH, 99.5%), dichloromethane (SYNTH, 99.5%) and *n*-hexane (SYNTH, 98.5%). These solvents were selected because of the different polarities: ethanol is a protic solvent, dichloromethane is an aprotic solvent and *n*-hexane is an apolar solvent, and their dielectric constants are 25.0, 9.1 and 1.9, respectively, at 25 °C and 1.01325 bar [31]. For each conventional extraction, a mass of 4 g of dried leaves and 400 mL of solvent were used. The mixture of leaves and solvent was maintained above the boiling temperature of the selected solvent at ambient pressure and the condenser temperature was kept at 15 °C. The system was kept in reflux for 8 h. In order to eliminate the solvent, the obtained samples were placed in a rotary evaporator. Then, the materials were dried until constant weight (±0.01 g). The drying temperatures used for extraction performed with ethanol, dichloromethane, and *n*-hexane were 90, 50 and 80 °C, respectively. The results were given relative to the mass of the initial sample.

Gas chromatography-mass spectrometry (GC-MS) of *V. amygdalina* Delile extract

Extract derivatization

The derivatization of the extracts was necessary to use the gas chromatography technique, because the samples have non-volatile metabolites. The technique used for sample derivatization was silylation. 150 µL of derivatizing reagent *N*-methyl-*N*-(trimethylsilyl) trifluoroacetamide (MSTFA, MSTFA/1% TMCS, Acros Organics) and 20 mg of sample were added in a tube with a screw cap. The mixture was homogenized and placed in an oven for 1 h at 70 °C to react. The reagent was evaporated and the product of derivatization was further diluted in 300 µL of chloroform (PA F.MAIA ACS, 99.8% purity) and homogenized. The supernatant was injected in the GC-MS. Thus, the sample was analyzed as a trimethyl derivative.

Methodology for analysis

The extracts were analyzed in high-resolution gas chromatography coupled to mass spectrometry (GC-MS, Agilent 19091S-433 model) equipped with HP-5MS capillary column (0.25 mm×30 m×0.25 µm) and interfacing with a mass selective detector. The procedure used followed that applied by Atangwho *et al.* [2], and it is described here in a simplified way. The column heating schedule was started at 70 °C for 2 min and then the temperature was increased at a rate of 20 °C/min to 280 °C. Then, the temperature was maintained at 280 °C for 20 min. The injector temperature was 220 °C and split ratio was 10:1. The carrier gas was helium at a flow rate of 1.2 cm^3 min^{-1}. The injection volume was 1 µl of extract solution. The library applied was NIST 02. The fraction compositions of the recognized compounds were evaluated from the GC peak area without taking account response factors.

Mathematical modeling of the SC-CO_2 extraction kinetics

The overall extraction curves (OEC) obtained were fitted to logistic [24], simple single plate (SSP) [25], and spline models [26–28]. Eqs. (1)-(5) present the mathematical description of each model.

Logistic model

$$m_{extracted} = \frac{m_0}{\exp(Ct_m)}\left\{\frac{1+\exp(Ct_m)}{1+\exp\left[C\left(t_m - t\right)\right]} - 1\right\} \quad (1)$$

Logistic model [24] has two adjustable parameters: t_m and C. t_m is defined as the time when the extraction reaches its maximum rate, which can be an applicable index for process control. C is a parameter that has no physical meaning. In this work, the resinoid material was considered a pure pseudo-component. The logistic model depends on the m_0 value which is the initial solute mass corresponding to the extractable mass. This value was considered constant and equals to the maximum value obtained in each OEC, as adopted by Sovová *et al.* [32] to model the extraction of grape oil with supercritical CO_2

Simple single plate model

$$m_{extracted} = m_0\left\{\sum_{n=0}^{\infty}\frac{0.8}{(2n+1)^n}exp\left[\frac{-D(2n+1)^2\pi^2 t}{\delta^2}\right]\right\} \quad (2)$$

The SSP model [25] was derived from heat and mass transfer analogy. It assumes that the extractable material is initially uniformly distributed within the plate-like particles and mass transfer resistance in the fluid phase is negligible, among other assumptions. In this model, D is the matrix effective diffusivity, and δ is the thickness of particles (plates). Gaspar *et al.* [25] adopted δ as 24 μm for extraction of oil from oregano bracts with compressed CO_2. This value was also assumed by Campos *et al.* [33] for the extraction of marigold oleoresin. However, in this work, we included δ as an adjustable parameter, to obtain a bi-parametric model as the logistic model.

Spline model

When $t \le t_{CER}$, then:

$$m_{extracted} = b_0 m_{Alim} + Q_{sol} b_1 t \quad (3)$$

When $t_{CER} < t \le t_{FER}$, then:

$$m_{extracted} = b_0 m_{Alim} + Q_{sol}\left[b_1 t + b_2\left(t - t_{CER}\right)\right] \quad (4)$$

and when $t > t_{FER}$, then:

$$m_{extracted} = b_0 m_{Alim} + \\ +Q_{sol}\left[b_1 t + b_2\left(t - t_{CER}\right) + b_3\left(t - t_{FER}\right)\right] \quad (5)$$

The Spline model [26-28] subdivides the overall extraction curves into three straight lines to represent the three regions in the extraction curve: constant extraction rate (CER), falling extraction rate (FER), and diffusion controlled (DC). For many process applications, the extraction operation finishes briefly after CER period since the best operational conditions will be those in which a high amount of extract is obtained in a low operation time [26].

The CER, FER, and DC periods are delimited by the time-span periods of CER (t_{CER}) and FER regions (t_{FER}). In this model, m_{Alim} is the initial sample mass in the extractor, and Q_{sol} is the solvent mass flow. The adjustable parameters of this model are b_0, b_1, b_2 and b_3, which are globally optimized. The product $Q_{sol}b_1$ represents M_{CER}, which is the extraction rate for the CER period. b_1 represents the mass ratio of extract in the supercritical phase at the bed outlet at CER step (Y_{CER}). More precise models can supply a more consistent depiction of the mass transfer phenomena as, for example, Sovová's model [34]. However, the use of these complex models requires some supplementary data which not frequently are accessible or easily obtained. Thus, an advantage of the spline model is that the use of merely kinetic data is suitably to carry out the modeling of the OEC [28].

Except for the spline model, parameters of the kinetic models were calculated by non-linear regression using Statistica 10® software. We used the Marquardt-Levenberg least square fitting procedure with a convergence criterion of 10^{-6}. For the spline model, the mathematical modeling was performed using an alternative algorithm implemented in MS Excel, which was able to provide reliable fittings comparable to SAS software and it was recently published by Santana *et al.* [27].

The concordance between experimental data and calculated values was established by the coefficient of determination (R^2) and root mean square error (*RMS*) computed using Eq. (6):

$$RMS = \sqrt{\frac{1}{N_P}\sum_{i=1}^{N_P}\left[\frac{\left(m_{extractedExp} - m_{extractedCalc}\right)}{m_{extractedExp}}\right]^2} \quad (6)$$

where the subscripts *Exp* and *Calc* indicated experimental and calculated values, respectively. N_P is the number of experimental data points.

Antioxidant activity

DPPH free radical scavenging assay

The antioxidant activity was determined using the stable free radical DPPH (2,2-diphenyl-1-picrylhydrazyl) which undergoes a reduction in the presence of antioxidants according to a modification of the method described by Brand-Williams *et al.* [35]. The solutions with the antioxidant (Rutin) were

prepared in different concentrations of 0.8267 to 5.7863 μg mL^{-1} and the solvents were ethyl acetate in methanol (20%). The extracts obtained under supercritical conditions were similarly prepared with concentrations of 123.33 to 1213.25 μg mL^{-1}. The samples extracted by the addition of organic solvent were prepared with concentration varying from 8.66 to 1115.26 μg mL^{-1}. In order to determine the free antiradical activity first, 140 μL of an extract solution was placed in a transparent glass tube. Then, 140 μL of rutin standard was added to the extract solution, as a positive control and 140 μL of distilled water as a negative control, followed by 2860 μL of $DPPH^{\bullet}$ working solution. Next, this solution was placed in an environment without light for 1 h and the absorbance was measured.

Absorption measurements were taken in duplicate and the percentage of antioxidant activity (*%AA*) was calculated from Eq. (7). The experiments were recorded using a UV-VIS spectrophotometer (V-1200, Pro-Tools, Brazil) at 515 nm:

$$\%AA = 100\left(1 - Abs_{sample} / Abs_{negative\ control}\right) \quad (7)$$

where Abs_{sample} is the absorbance of the sample and $Abs_{negative\ control}$ is the absorbance of the negative control.

The values of the percentage of antioxidant activity as a function of extracts concentrations were plotted. From the equation of the curve, the estimated concentration in μg mL^{-1} of extract required to reduce 50% of the stable free radical DPPH (EC_{50}) was determined. Therefore, the lower the value, the greater is the antioxidant capacity of these substances.

Total phenolic content

The total phenolic content was determined according to the methodology of Meda *et al.* [36]. A solution containing 4 mg/L of extract diluted in a mixture of 20% of ethyl acetate in methanol was prepared for each sample obtained by CO_2 supercritical extraction. The same procedure was done for the extracts obtained by conventional Soxhlet methods, except for the ethanolic extract. In this case, a concentration of 0.4 $mg.L^{-1}$ was used. Next, 0.5 mL of the extract solution was added to 2.5 ml of Folin-Ciocalteu 10% solution in ultrapure water and allowed to stand for 5 min. Then, 2.0 mL of sodium carbonate solution in ultrapure water (14%) was added. The solution was kept in the dark for 2 h. The absorbance was measured by a spectrophotometer (V-1200, Pro-Tools, Brazil) at a wavelength of 760 nm. For negative control, the same mixture prepared for the extracts was used, but with pure methanol extract solution. Tests were performed in duplicate. The total phenolic content was determined from the standard calibration curve (gallic acid) at final concentrations from 0.4 to 3.2 μg mL^{-1} and expressed as g gallic acid equivalents (GAE)/100 g extract.

RESULTS AND DISCUSSION

Overall yield of SFE extractions

The effects of pressure and temperature on the overall yield of SFE extraction in the 160 min of extractions process are shown in Table 1. Experiments were performed in duplicate and results are presented as the mean value. For constant temperature, the total yield increased with pressure. At these experimental conditions, the estimated CO_2 density varied between 724.55 and 880.22 kg m^{-3}. It could be noticed that, with increasing pressure, the CO_2 density increased which caused an enhancement of the extraction power of the solvent. This type of behavior has been previously reported in the literature by several authors [37-39]. For instance, Rai *et al.* [33] also observed a similar effect while investigated the supercritical CO_2 extraction of *Moringa oleifera* seed oil.

For the extractions performed at 200 bar, the yield decreased with the temperature increase. On the other hand, for the experiments performed at 250 bar, the effect of temperature on the yield was positive. The temperature effect on the extraction yield is complex because the effects of temperature on solvent density and solute vapor pressure are opposite.

Table 1. Overall yield of SFE extraction of Vernonia amygdalina Delile leaves

Pressure, bar	Temperature, °C	CO_2 density[a], kg m^{-3}	Average yield[b], mass%
200	40	840.60	0.69
	50	785.17	0.94
	60	724.55	0.85
250	40	880.22	1.06
	50	834.94	1.12
	60	787.20	1.24

[a]Estimated values using a thermodynamic table of carbon dioxide [30] considering pressure (200 or 250 bar) and temperature (40-60 °C) of extraction; [b]Yield (%) = 100($m_{extracted} / m_{Alim}$); confidence interval = 0.1%

At higher temperature, the solvent density decreases which results in a reduction of the extraction capacity of the solvent (smaller interaction of the solvent and the solute). The solute vapor pressure increases with temperature, favoring the extraction due to an increase in solubility [38,40]. Finally, the highest overall mass yield (1.24%) was obtained for the highest operating pressure (250 bar) and temperature (60 °C).

Mathematical modeling of supercritical extraction

Table 2 shows the coefficients of determination (R^2), root mean squares, and constants for kinetic models fitted by regression to the experimental data. All models described the extraction kinetics very well, given the high R^2 values and low *RSM*. Figure 1 illustrates the goodness of fit to experiments performed at different conditions, representing mass extracted as a function of time of extraction (t). The fitting curves were extrapolated until 240 min (4 h). SFE curves indicated that extraction yield is still increasing after this time. This behavior is not common for supercritical fluid extraction of vegetable matrices [14]. One possible reason for this behavior can be linked to the intrinsic characteristic of the milled leaves. For example, it is well known that the extraction time could increase as a function of particle size. Gaspar *et al.* [25] showed for oregano leaves SFE extraction (70 bar, 300 K and 0.3 kg h^{-1} of supercritical CO_2), using particles of 0.7 mm, the extraction degree (mass/extractable mass) stabilized at ~90% at 200 min of extraction. However, for particles of 1.55 mm, the extraction degree achieved ~45% and the kinetic curve was still arising after 200 min. In our study, at the operation condition selected, it was not possible to reduce the particle size of leaves without getting into difficulties in keeping the flow of the supercritical fluid. Moreover, some studies endorse the fact that high extraction time can be obtained for some vegetable matrices. For example, Martinez-Correa *et al*. [16] got extracts from *Artemisia annua* L. leaves. The time necessary was ~417 min to finish the extraction from particles with 0.838 mm at 400 bar, 60 °C and 0.144 kg h^{-1} of supercritical CO_2. Despite of our study not being able to seek the end of some curves, all of them have three well delimited regions which is essential to mathematical modeling and future economic evaluation of the process.

When we used the logistic model, although we obtained good agreement, t_m values were negative in most regressions. Other authors [41–43] also have obtained negative values for t_m and, consequently, no physical meaning could be related to this parameter. Probably, t_m negative values occur due to the fact that this model does not represent the physical phenomena that happens in the extractive process. Despite the fact that the logistic model delivered an appropriate quantitative delineation of the OEC, the lack of physical meaning for t_m turns this model into an empirical model. Then, the adjustable parameters provided by this model do not have any practical physical meaning and they cannot be used for further studies [44].

Table 2. Coefficient of determination (R^2), root mean squares, and model constants for various kinetic models fitted by non-linear regression to the experimental data

Model	Value	Unit	40 °C, 200 bar	50 °C 200 bar	60 °C 200 bar	40 °C 250 bar	50 °C 250 bar	60 °C 250 bar
Logistic	R^2	[-]	0.9870	0.9966	0.9788	0.9920	0.9870	0.9802
	RSM	[-]	0.0774	0.8964	0.8888	0.9040	0.9031	0.9026
	C	[min^{-1}]	0.0113	0.0303	0.0252	0.0238	0.0187	0.0249
	t_m	[min]	-270.5	25.22	-66.82	6.050	-570.1	-525.3
SSP	R^2	[-]	0.9850	0.9717	0.9694	0.9739	0.9890	0.9897
	RSM	[-]	0.0144	0.8833	0.8840	0.8950	0.9007	0.9016
	$D\times10^{-13}$	[m^2 min^{-1}]	1.913	2.836	3.998	1.443	1.804	2.539
	$\delta\times10^{-6}$	[m]	41.812	41.502	42.066	30.551	31.112	31.924
Spline	R^2	[-]	0.9927	0.9977	0.9922	0.9985	0.9977	0.9972
	RSM	[-]	0.0684	0.0681	0.3408	0.0222	0.0490	0.0284
	b_0	[-]	1.054E-03	-4.757E-04	1.242E-04	8.685E-04	1.959E-03	2.938E-03
	$b_1 = Y_{CER}$	[g g^{-1}]	2.086E-04	4.793E-04	6.344E-04	3.373E-04	3.779E-04	4.928E-04
	b_2	[g g^{-1}]	-9.175E-05	-2.949E-04	-5.282E-04	-1.252E-04	-1.892E-04	-3.414E-04
	b_3	[g g^{-1}]	-1.031E-04	-1.826E-04	-1.060E-04	-1.720E-04	-1.588E-04	-1.369E-04
	t_{CER}	[min]	60.00	58.00	43.00	60.00	56.00	54.00
	t_{FER}	[min]	149.00	123.00	151.00	134.00	140.00	140.00
	S/F	[g g^{-1}]	18.00	17.40	12.90	18.00	16.80	16.20

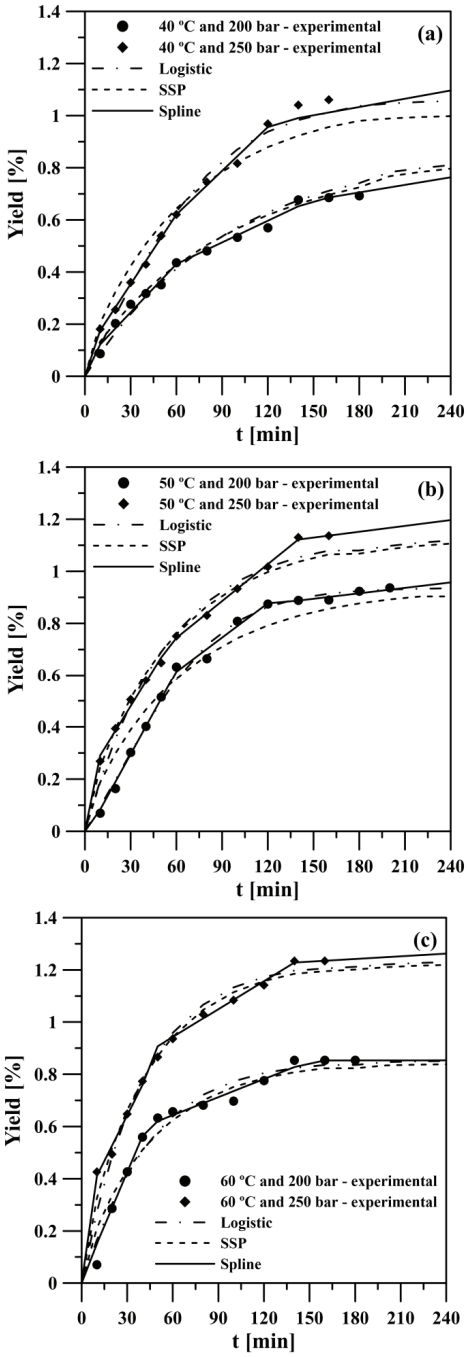

Figure 1. Kinetics of SFE of Vernonia amygdalina Delile leaves. Symbols are the experimental data points. Lines are simulated results obtained using logistic, SSP, and spline models. Experiments at: a) 40, b) 50 and c) 60 °C.

In relation to the SSP model, the values obtained for the effective diffusion coefficient are in the usual range obtained to others SFE of biomass modeled in literature, which indicate values between 10^{-11} and 10^{-14} [41,45,46]. It is interesting to notice that D values increased with temperature, which is expected because of the rise of temperature increases the kinetic energy and consequently the molecules motion. D values were smaller at 250 bar than at 200 bar, when comparing at the same temperature which can be interpreted as a sign of slower motion of particles due to pressure effects. The medium estimated value for δ was ±36.495 μm, which is similar to 24 μm used for other herbaceous matrices in presence of compressed CO_2 [25,33]. Estimated values of δ at 250 bar were smaller than that obtained at 200 bar, which can be explicated by particle compressibility.

The spline model described very well the OEC quantitative behavior, presenting the lowest RSM and highest R^2 values. The higher number of parameters guaranteed better performance than other models. The increase in temperature at the same pressure increased the yield in the extract and the Y_{CER} values. The parameters of this model can be used to develop the scale-up of laboratory data for industrial design purposes [27]. As indicated by Meireles [26], the best process time from the economic viewpoint is placed between t_{CER} and t_{FER}. In the studied kinetics, the CER and FER steps evidenced by their respective time--span periods, varied between 43-60 min and 123--151 min, respectively. These values are compatible with other materials that have shown to be economically viable at industrial scale [47,48].

Low-pressure extractions

The yield obtained during Soxhlet extractions was 10.4, 12.4 and 31.8% using hexane, dichloromethane and ethanol, respectively. Comparing the yields obtained from the SFE with the ones acquired using Soxhlet extractions, it can be seen that the SFE showed lower yield that the extracts obtained with organic solvents. Ethanol, which is a polar solvent, had the highest global yield, indicating that most of the compounds extracted have a polar nature also. The higher levels of total extraction yield by Soxhlet are also associated with extended extraction time, since it increases the contact time between the solvent and the solutes.

Although the yield values using organic solvents are superior, it should be noted that the extracts obtained by Soxhlet methods require the post-extraction purification process, in addition to heat applied to remove the solvent by evaporation stage, which can promote the degradation of some thermolabile antioxidants [49]. Some previous studies indicated that, due to long processing time associated with the high temperature, Soxhlet proved to be one of the most expensive methods of extraction [48,50]. Further studies are necessary to compare the manufacturing cost of SFE and Soxhlet extracts from *V. amygdalina* leaves. Therefore, choosing the best extraction pro-

cess should take into account the whole process, characterized by the extraction and separation. The supercritical fluid extraction offers several attractive features for processes with easy oxidation, resulting in several distinct characteristics, such as the easy recovery of the solute and the possibility of direct separation by the appropriated choice of the temperature and/or pressure conditions. The use of CO_2 as a solvent, fluid inert, non-toxic, non-flammable, non--aggressive to the environment and with low critical temperature, presents the possibility of carrying out the extraction and fractionation without the risk of leaving unwanted waste and/or thermally degrading desirable products. Therefore, the SFE technology is a viable alternative extraction for the food and pharmaceutical industries [51].

Identification of bioactive compounds

Table 3 shows the results regarding the composition of the substances extracted using CO_2 in supercritical conditions of *V. amygdalina* Delile. These compounds were obtained in different operating conditions, at temperatures of 40, 50 and 60 °C and pressures of 200 and 250 bar.

The presence of compounds with complex structures and high molecular weight was detected by gas chromatographic analyses of the *V. amygdalina* Delile extracts. The major compounds of the heavy fraction (SFE fraction) were: hexadecanoic acid or palmitic acid (10.02–19.01%), phytol (4.63–11.82%), 9,12-octadecadienoic acid (9.84–20.49%), α-linolenic acid (10.89–28.04%) and octadecanoic acid (2.37–15.20%). Compounds related to the retention times of 15.77, 16.42 and 19.12 min were also significant: 3.84–4.8%, 3.91–5.89% and 6.67–9.94%, respectively. However, the identification of these substances was not possible. It is very relevant to point out the presence of phytol in all the extracts obtained. Phytol is a constituent of chlorophyll commonly found in nature. This compound is widely used in cosmetics, shampoos, detergents and soaps. However, recently it was proven that this compound and several derivatives have pharmacological uses such as antimicrobial, antioxidant, anxiolytic, anti-inflammatory and antispasmodic, among other properties [52].

The sample extracts obtained by Soxhlet extraction (using hexane, dichloromethane and ethanol) were characterized in terms of composition (area%)

Table 3. Composition of the extracts (% area) of the plant in different extraction conditions (°C/bar); RT (retention time)

Compound	*RT* [min]	40 °C	50 °C		60 °C	
		250 bar	200 bar	250 bar	200 bar	250 bar
		SFE extraction				
Hexadecanoic acid[a]	11.349	11.14	19.01	10.02	10.39	12.92
Phytol	11.956	4.63	10.49	6.06	8.20	10.37
9,12-Octadecadienoic acid[b]	12.164	11.53	17.27	9.84	20.49	-
α-Linolenic acid	12.196	11.45	22.43	10.89	-	28.04
Octadecanoic acid[c]	12.242	2.71	4.04	2.13	2.37	2.85
Eicosanoic acid	13.134	1.55	-	1.52	1.37	1.47
Docosanoic acid	14.233	0.85	-	0.90	0.92	-
		Soxhlet				
		Hexane		Dichloromethane		Ethanol
2-Butenoic acid	3.728	-		-		0.25
Phosphoric acid	6.782	-		-		2.08
Butanedioic acid	7.070	-		-		0.45
L-proline	6.984	-		-		0.77
Propanoic acid	7.207	-		-		0.23
Inositol	10.991	-		-		5.73
Hexadecanoic acid[a]	11.358	20.00		11.97		-
2-Propenoic acid	11.840	-		-		0.71
Phytol	11.962	3.58		2.52		-
9,12-Octadecadienoic acid[b]	12.162	14.14		13.83		2.59
α-Linolenic acid	12.208	18.09		13.85		-
Octadecanoic acid[c]	12.265	3.37		4.28		-
α-D-glucopiranoside	14.518	-		-		10.44

[a]Palmitic acid; [b]linoleic acid; [c]stearic acid

and the results are shown in Table 3. The major compounds identified were: palmitic acid (C16: 0), stearic acid (C18: 0), linoleic acid (C18: 2n-6), *α*-linolenic acid (C18: 3n-3) and phytol, for the extracts obtained with the solvents hexane and dichloromethane. However, for the extract obtained with ethanol as solvent, the phyto-compounds were: phosphoric acid, inositol, and *α*-D-glucopyranose.

In the available literature, information about the composition of the extracts of *V. amygdalina* Delile leaves is quite limited. Therefore, it was not possible to compare and identify the compounds obtained in this work. However, there are studies reporting the presence of compounds obtained by conventional methods. In the study performed by Atangwho *et al.* [2], the compounds listed in Table 3 were obtained by the extraction of the leaves of *V. amygdalina* Delile by the organic solvent chloroform. Erasto *et al.* [53] also identified these phytochemicals in the extract of *V. amygdalina* Delile obtained by Soxhlet with a mixture of hexane/isopropanol (3:1). Alara *et al.* [11] identified phytol and fatty acids as the main compounds extracted by water as a solvent being applied Soxhlet or microwave extractor [11]. Except for Alara *et al.* [11] and Atangwho *et al.* [2], phytol had not been identified in great quantity in prior studies [53]. This can be due to the geographical place of the plant samples. Our results and previous study [2,11,53] point out high amounts of unsaturated fatty acids which suggest the anti-inflammatory and antioxidant potential of extracts of *V. amygdalina* leaves.

Comparing the composition of the extracts obtained by SFE and Soxhlet extraction, it can be seen that samples obtained by SFE and the ones using hexane and dichloromethane as solvents have high contents of palmitic acid. On the other hand, the relative amounts of phytol obtained using SFE were much higher than when using conventional extraction, regardless of the solvent used. The highest extraction yield was obtained using ethanol but the extract did not show the presence of phytol or palmitic acid. This shows that SFE can have an important role in the extraction industries since it can access molecules that are not obtained with conventional solvents.

Antioxidant activity: Method of DPPH• reduction and total phenolic content

The SFE extracts were obtained at different conditions of pressure and temperature. These parameters influence the physical properties of solute in the fluid phase (diffusivity, viscosity) and also have influence in the mechanism of mass transfer between the solute and the solvent. The variation of pressure of 200 to 250 bar showed a marked effect on the EC_{50} values according to the results presented in Table 4. The pressure increase at constant temperature caused a decrease in the EC_{50} values. These results indicate that as the pressure is increased, the concentration of the extracted compounds with antioxidant activity also increased. Then, apparently, higher pressure can lead to disruptions in the plant cells, allowing the release of compounds that were not previously accessible. However, EC_{50} values were lower than Soxhlet extracted.

According to Garmus *et al.* [20], the extracts can be classified as very active extracts with $EC_{50} < 50$ μg mL^{-1}. Then, in the experiments reported in this work, only ethanolic extract has a good antioxidant potential. High antioxidant activity was already reported for extracts of *V. amygdalina* Delile leaves obtained using water as solvent in a Soxhlet apparatus [11]. Garmus *et al.* [20] studied the extraction of phenolic

Table 4. Antioxidant potential evaluation of Vernonia amygdalina Delile leaves extracts: DPPH• method and phenolic content

Experiment	Extraction	EC_{50} / μg mL^{-1}	Phenolic content, g GAE/100 g extract
		Soxhlet	
1	Hexane	1091.44	10.88
2	Dichloromethane	381.93	15.05
3	Ethanol	31.84	81.80
		Supercritical	
4	40 °C/200 bar	929.70	-
5	50 °C/200 bar	916.53	8.77
6	60 °C/200 bar	920.13	8.57
7	40 °C/250 bar	622.62	11.29
8	50 °C/250 bar	649.99	10.13
9	60 °C/250 bar	814.30	10.02
		Standard	
	Rutin	4.24	-

content from pepper-rosmarin (*Lippia sidoides* Cham.) leaves using ethanol, water, and supercritical CO_2 as solvents. Ethanolic and aqueous extracts presented the best antioxidant activity, with EC_{50} lower than 50 μg mL^{-1}. Other studies [54–56] also showed ethanolic/aqueous extracts (by Soxhlet) with higher antioxidant activity than SC-CO_2 extracts. In contrast, some studies showed that SC-CO_2 extracts presented higher antioxidant activity than ethanolic/aqueous extracts [57–59]. This suggests that the antioxidant compounds extracted with SC-CO_2 and high polar solvents are of different types, with varying radical scavenging activities.

In our study, the best EC_{50} values were obtained for the extracts with polar solvents (dichloromethane and ethanol) instead of nonpolar (hexane and CO_2). These data indicate that the compounds responsible for the antioxidant activity present in the *V. amygdalina* Delile leaves have more affinity for polar solvents.

The low values of antioxidant activity for extracts obtained by SFE can be caused by factors such as: lack of donor substances, hydrogen or electrons, in plant extracts; the presence of a wide variety of compounds interacting with the environment containing DPPH•. Furthermore, the interaction of pure substances with each other can lead to donate electrons or hydrogen, rendering them unable to do so in a complex mixture. In complex systems the state of excitation of molecules interfere directly in antioxidant activity because of the presence of high molecular weight compounds and complex structural form, as in the case of triterpenes and sesquiterpenes which may have difficulty reacting with DPPH• due to steric hindrance and which gives a high stability (Ito *et al.* [60]).

Table 4 shows the average values of phenolic content expressed in g GAE/100 g of extract. The phenolic content in the extracts obtained by supercritical extraction showed an increase, though not significant, with temperature and pressure. With increasing pressure, maintaining a constant temperature, the concentration of gallic acid equivalents in extracts increased. However, the increase of temperature at a constant pressure led to a decrease in the concentration of gallic acid equivalents in extract. The results of the concentrations ranged from 6.89 to 11.29 g GAE/100 g of extract. Thus, the condition with a supercritical fluid which favors extraction of total phenols is 250 bar and 40 °C (11.29 g GAE/100 g of extract). In the case of the extracts obtained by extraction with organic solvents, ethanol showed higher phenolic content (81.80 g GAE/100 g of extract), followed by dichloromethane and hexane. The amount of total phenols concentration in the ethanolic extract was estimated as the solution was diluted 10 times with respect to other extracts.

CONCLUSIONS

This work reports the use of supercritical CO_2 as a solvent of bioactive compounds from dry leaves of *Vernonia amygdalina* Delile. The best yield extraction was 1.24% obtained at 250 bar and 60 °C. The overall SFE curves were fitted by logistic, simple single plate, and spline models. All of them were satisfactory in being able to describe the extraction kinetics obtaining coefficients of determination higher than 0.9694. SC-CO_2 and conventional low pressure Soxhlet extraction showed to be promising methods for obtaining extracts rich in bioactive compounds, with great potential for medical use. Some components of the obtained extracts could be identified, such as palmitic acid, which is a potentially powerful antimicrobial agent and phytol, that has important biological effects, such as thermogenic activity in mammals. Yields, antioxidant potential, and phenolic content were lower using SC-CO_2 extraction than using ethanol in a conventional Soxhlet apparatus. However, SFE technique requires less time of extraction and does not require a solvent separation step. Further studies are necessary to access the manufacture cost of *V. amygdalina* Delile extraction by both techniques (SFE and ethanol at low pressure) aiming to compare their feasibility.

Supplementary material

Additional data are available from the corresponding author upon request.

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Nomenclature

b_0, b_1, b_2 and b_3 – adjustable parameters of spline model
C- a parameter of SSP model
D - matrix effective diffusivity
m_{Alim} - initial sample mass in the extractor
$m_{extracted}$ – extracted mass
m_0 – extractable mass
M_{CER} - extraction rate for the CER period
N_P - number of experimental data points
Q_{sol} - solvent mass flow
t – extraction time variable
t_{CER} - time-span period of CER region
t_{FER} - time-span period of CER region

t_m - time when the extraction reaches its maximum rate in SSP model
Y_{CER} - the mass ratio of extract in the supercritical phase at the bed outlet at CER step
δ - thickness of particles (plates) in SSP model
%AA - antioxidant activity
$Abs_{negative\ control}$ - absorbance of the negative control
Abs_{sample} – absorbance of the sample
CER – constant extraction rate
DC - diffusion controlled period
DPPH$^{\bullet}$ - 2,2-diphenyl-1-picrylhydrazyl radical
EC_{50} - Estimated Concentration in $\mu g.mL^{-1}$ of extract required to reduce 50% of the stable free radical DPPH$^{\bullet}$
FER – falling extraction rate
GAE - gallic acid equivalents
GC-MS - gas chromatography coupled with mass spectrometry
OEC - overall extraction curves
R^2 - coefficient of determination
RMS - root mean square error
SC - supercritical
SFE – supercritical fluid extraction
SSP – Simple single plate

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NAUČNI RAD

EKSTRAKCIJA BIOAKTIVNIH JEDINJENJA IZ LIŠĆA *Vernonia amygdalina* DELILE POMOĆU NATKRITIČNOG UGLJENIK(IV)-OKSIDA

Do sada objavljeni naučni radovi pokazali su da je natkritična ekstrakcija obećavajuća metoda za dobijanje bioaktivnih jedinjenja koja se koriste za razvoj lekova na bazi prirodnih proizvoda. U ovom radu, istraženi su prinos, antioksidativna aktivnost i fitohemijski sastojci ekstrakata dobijenih iz lišća Vernonia amigdalina Delile sa natkritičnim CO_2. Superkritična ekstrakcija je istražena na temperaturama od 40, 50 i 60 °C i pritiscima od 200 i 250 bar. Ova metoda je upoređena sa klasičnom Sokhletom ekstrakcijom primenom različitih rastvarača pod atmosferskim pritiskom. Ukupni prinos dobijen natkritičnom ekstrakcijom varira od 0,69 do 1,24 %. Najveći prinos je dobijen na 60 °C i 250 bar. Kinetika ekstrakcije je uspešno opisana trima matematičkim modelima, ali je najbolje slaganje dobijeno sa modelom koji podrazumeva čestice štapićastog oblika. Ovaj model opisuje periode konstantne i opadajuće brzine ekstrakcije. Ekstrakti su okarakterisani gasnom hromatografijom u kombinaciji sa masenom spektrometrijm, pri čemu su identifikovane neke glavne komponente. Testovi antioksidativne aktivnosti pokazali su da dobijeni ekstrakti imaju malu antioksidativnu aktivnost pošto procenjena koncentracija ekstrakta koja je potrebna za smanjenje od 50% vrednosti stabilnih DPPH slobodnih radikala između 623 i 938 μg/mL.

Ključne reči: antioksidativna aktivnost, bioaktivna jedinjenja, matematičko modelovanje, fitokemijske komponente, natkritična ekstrakcija.