Supercritical fluid extraction of murici leaves (Byrsonima crassifolia): Global yield, total phenolic compounds, antioxidant activity, and linear correlations

Extracção de fluido supercrítico de folhas de murici (Byrsonima crassifolia): Rendimento global, compostos fenólicos totais, actividade antioxidante, e correlações lineares

DOI:10.34117/bjdv7n12-054

Recebimento dos originais: 12/11/2021
Aceitação para publicação: 03/12/2021

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ABSTRACT
The objective of this study was to obtain extracts from Byrsonima crassifolia leaves by supercritical CO2 (CO2-SFE) in order to determine the experimental data, global yield isotherms, total phenolic compounds, antioxidant activity, and linear correlations. Moisture, particle diameter, apparent and true density, bed porosity, and morphological characterization of murici leaves were analyzed. CO2-SFE was conducted at 313.15 K–323.15 K, and at 10 MPa to 30 MPa. The bed parameters agreed with those used in CO2-SFE, and the particles presented irregular flat shape. The isotherms showed an inflection point, and the highest global yield was obtained at 323.15 K and 30 MPa (1.24% d.b.). The highest values of phenolic compounds (68.85 mg GAE/g d.b.) and antioxidant activity (174.35 μM trolox/g d.b.) were obtained at 313.15 K and 30 MPa, in which a strong positive linear relationship was observed between these responses.

Keywords: Byrsonima crassifolia, Supercritical fluid extraction, Dynamic extraction period, Total phenolic compounds, Antioxidant activity, Linear correlations.

RESUMO
O objectivo deste estudo era obter extractos de folhas de Byrsonima crassifolia por CO2 supercrítico (CO2-SFE) a fim de determinar os dados experimentais, isotermas de rendimento global, compostos fenólicos totais, actividade antioxidante, e correlações lineares. Foram analisadas a humidade, o diâmetro das partículas, a densidade aparente e verdadeira, a porosidade do leito, e a caracterização morfológica das folhas de murici. O CO2-SFE foi conduzido a 313,15 K-323,15 K, e a 10 MPa a 30 MPa. Os parâmetros do leito concordaram com os utilizados no CO2-SFE, e as partículas apresentavam forma plana irregular. As isotermas apresentaram um ponto de inflexão, e o maior rendimento global foi obtido a 323,15 K e 30 MPa (1,24% d.b.). Os valores mais elevados de compostos fenólicos (68,85 mg GAE/g d.b.) e actividade antioxidante (174,35 μM trolox/g d.b.) foram obtidos a 313,15 K e 30 MPa, nos quais foi observada uma forte relação linear positiva entre estas respostas.

Palavras-chave: Byrsonima crassifolia, Extracção de fluidos supercríticos, Período de extracção dinâmico, Compostos fenólicos totais, Actividade antioxidante, Correlações lineares.

1 INTRODUCTION

Murici (Byrsonima crassifolia) is a plant from the tropical region, which can also be found in South and Central America, and Mexico. Leaves of the genus Byrsonima are used worldwide, and are known for their antifungal and antibacterial effects, being effective against Leishmaniasis [1]. Besides, they have other medicinal properties like anti-inflammatory, anti-hyperalgesic, antiplatelet [2], antidepressant [3], gastric and duodenal anti-ulcer, antidiarrheal [4], analgesic, antioxidant [5], and photochemoprotective effects [6].

Studies on the therapeutic properties of B. crassifolia leaves associate such effects with their antioxidant activity. This is related to their content of phenolic compounds,

which are responsible for the elimination of free radicals associated with various diseases [7].

Some studies on the application of B. crassifolia leaf extracts using organic solvents have been developed. In the study by Souza et al. [8], the main phenolic compounds found in the ethanolic extract of B. crassifolia are gallic acid, epicatechin, epigallocatechin gallate, quercetin 3-O-β-D-glucopyranoside, and catechin, which are associated with increasing antioxidant capacity in the human skin. Whereas according to Herrera-ruiz et al. [3], flavonoids rutin, hesperidin, and quercetin may be involved in the antidepressant effect of B. crassifolia leaf extract obtained with hexane and methanol.

The use of organic solvents to obtain natural products precludes some applications in food and pharmaceutical industries, since the products obtained by such techniques present toxic residues, and additional purification processes are required for total solvent separation, increasing cost and time to obtain the final product [9].

Thus, supercritical CO2 extraction is an alternative "green" technology, which provides high selectivity, yields, and purity, as well as shorter extraction times [10,11]. In addition, CO2 is safe, non-flammable and non-explosive, and is immediately removed after extraction [12].

Due to the multifunctional characteristics of B. crassifolia leaf extracts, this study aims to collect them by supercritical CO2 extraction, as well as to obtain experimental data, global yield isotherms, total phenolic compounds, antioxidant activity, and linear correlations, and finally evaluate the solvent flow rates over the dynamic period of CO2-SFE.

2 MATERIAL AND METHODS

2.1 SAMPLE PREPARATION

B. crassifolia leaves from adult plants during flowering were collected in Terra Alta city (1°02'25.9"S 47°54'12.3"W) (Pará, Brazil). Then, the samples were stored in plastic bags, and transported at room temperature. The leaves were dried in an air-conditioned room for three days at 291.15 K, and then ground in a knife mill (Tecnal, model TE-631/3, Brazil).

2.2 PHYSICAL AND MORPHOLOGICAL CHARACTERIZATION OF THE SAMPLE

For the physical characterization, the moisture content was first determined according to Jacobs [13], using the method of immiscible solvent distillation, with sample immersed in xylol. Crushed B. crassifolia leaves were subjected to particle size analysis in a sieve shaker, for 1200 s using 20 to 60 mesh Tyler sieve series. To determine the average particle diameter (dap), fractions from 28 to 35 mesh were used. The average particle diameter was calculated according to ASAE Standard method [14]. True density (ρt) was obtained using an automatic helium gas pycnometer (model Ultrapyc 1220e, Quantachrome, USA). Apparent density (ρa) was calculated from the mass/volume ratio of the test sample (kg/m3). The bed porosity (ε) was determined by the mathematical relationship between true density and apparent density [15]. The morphological characterization of the crushed leaves was evaluated by electron micrographs obtained by scanning electron microscopy (SEM) (model TM 300, Hitachi, Japan). The sample was sprinkled on double sided adhesive tape mounted on a base of 0.01 × 0.01 m diameter/height, and then gold plated under vacuum to obtain the electron beam-reflective surface.

2.3 SUPERCRITICAL CO2 EXTRACTION

2.3.1 Apparatus and extraction conditions

Supercritical extractions were performed on a Spe-ed™ SFE (model 7071, Applied Separations, USA) in the Supercritical Extraction Laboratory (LABEX/UFPA). The equipment was coupled to a compressor (Schulz, model CSA 7.8, Joinville, SC, Brazil), a 99.9% purity CO2 cylinder (White Martins, Belém, PA, Brazil), a recirculator (model F08400796, Polyscience, USA), and a flow meter (model M 5SLPM, Alicat Scientifc, USA). The extraction bed was composed of a stainless-steel extraction cell of 2.27×10-6 m3 (0.02 m internal diameter and 0.33 m internal height), which was packed with approximately 0.01 kg of crushed B. crassifolia leaves, occupying bed height of 0.19 m. The top and bottom ends of the cell were filled with small cotton balls and glass beads. The global yield isotherms were obtained at temperatures of 313.15 and 323.15 K, and at pressures of 10, 20, and 30 MPa. The global extraction yield was calculated from the mathematical ratio between the extract mass and the initial sample mass (on dry basis - d.b.). The determinations were performed in duplicate. Results were expressed as %

(d.b.). CO2 densities were calculated using the National Institute of Standards and Technology (NIST), that applies Span-Wagner equation of state [16].

2.3.2 Evaluation of the solvent flow rates over the dynamic period of CO2-SFE

The evaluation of solvent flow rates over the dynamic period was performed according to Pires et al. [17]. Extractions were conducted at pressure of 30 MPa, temperature of 323.15 K, CO2 density of 878 kg/m3, static period of 1800 s, and CO2 mass of 0.96 kg. This procedure was executed in two stages: SFE 1 and SFE 2. In SFE 1, CO2 flow rate of 8.85×10-5 kg/s ($Q_{CO_{2_1}}$), and dynamic period of 10800 s (t_1) were used. Whereas in SFE 2, CO2 flow rate of 1.33×10-4 kg/s ($Q_{CO_{2_2}}$), and dynamic period of 7200 s (t_2) were used. The extracts were collected and weighed every 3600 s to obtain the accumulated extraction masses. The other extractions were performed under the conditions that obtained the highest accumulated yield achieved in this evaluation. The experiments were conducted in duplicate.

2.4 EXTRACT CHARACTERIZATION

2.4.1 Total phenolic compounds (TPC)

TPC determination was performed using Folin-Ciocalteu methodology, according to Singleton et al. [18], and Georgé et al. [19]. 3.5×10-5 kg of B. crassifolia leaf extract, and 2.5×10-5 m3 of 70 % ethanol were homogenized for 120 s. Then, the sample was diluted with water until an ethanol concentration of 7% (w/v). For the reaction, 5.0×10-7 m3 of this solution was homogenized with 2.5×10-6 m3 of 10 % Folin-Ciocalteu reagent (v/v) for 120 s, to which were added 2.0×10-6 m3 of 7.5 % sodium carbonate solution (w/v). The reaction occurred for 3600 s at room temperature in the absence of light. Water was used for the blank preparation. Absorbances were performed in a spectrophotometer (Thermo Scientific, model Evolution 60, USA), at 760 nm. For quantification, gallic acid at concentration of 0.02-0.1 kg/m3 was used as standard to construct the analytical curve. TPC content was calculated from the equation of a line y=0.0111x - 0.0038), where y is the absorbance, and x is the concentration, with R2=0.9941. Analyses were performed in triplicate. Results were expressed as mg GAE/g (d.b.).

2.4.2 Trolox equivalent antioxidant capacity (TEAC)

Antioxidant capacity by TEAC method was determined according to the procedure proposed by Rice-Evans and Miller [20], in which was used the radical ABTS·+, obtained from the reaction, in aqueous solution, of 7 µM ABTS (2,2-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) and 140 µM potassium persulfate. The mixture was kept steady, in the dark, at room temperature (295.15 K) for 57600 s. Once ABTS·+ radical was formed, it was diluted in ethanol (P.A.) until absorbance of 0.7 ± 0.05 was reached. Aliquots of 3.0 × 10-8 m3 of extract reacted with ABTS·+. After 360 s of reaction, the absorbance reading was conducted at 734 nm. As a reference, an analytical curve was prepared with trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) at concentrations of 1.0 × 10-5 - 2.0 × 10-4 kg/m3. Antioxidant activities were calculated from the equation of a line y=-0.0002x - 0.6734, where y is the absorbance, and x is the concentration, with R2 = 0.9991. The determinations were performed in triplicate, and the results were expressed in µM trolox/g (d.b.).

2.5 STATISTICAL ANALYSIS

Supercritical extractions and global yield determinations were conducted in duplicate, and the analyses were done in triplicate. Means and standard deviations were calculated for all results. Data were submitted to Tukey test, when necessary, using a significance limit of $p < 0.05$. Correlations were performed using linear regression and Pearson's correlation coefficient (r). The softwares Excel 2000 SR-1 (Microsoft, Troy, NY) and Statistica Kernel Release 7.1 (StartSoft Inc., Tulsa, OK) were used as tools.

3 RESULTS AND DISCUSSION

3.1 SAMPLE CHARACTERIZATION

The moisture of crushed B. crassifolia leaves (9.59 % d.b.) was adequate for the process, as it was within the range used for CO2-SFE (4 - 14% d.b.), which prevented the simultaneous extraction of this wastewater together with the extract. This, consequently, reduced the Joule – Thompson effect on the system output. dap was equal to 3.5 × 10-4 m, and was within the expected range for natural products, which varies from 2.5×10-4 m to 1.8×10-3 m [21,22]. ρt and ρa values were equal to 1300 kg/m3 and 300 kg/m3, respectively, and the relationship between these variables allowed to obtain a relatively high ε (0.77), which reduced the formation of preferential paths during extraction, and increased the mass transfer area. The particles morphology showed varied shapes and

sizes, predominantly presenting an irregular flat shape (Figure 1). Knowledge of the flat morphology of B. crassifolia leaf particles has fundamental importance for the understanding of the mass transfer process during supercritical CO2 extraction, since for this particle type, mass transfer from solute to solvent occurs by linear driving force. In addition, the type of morphology influences the choice of mathematical models to predict the solubility of extracts in supercritical CO2, and Goto model is the most suitable for this particle format in the experimental data setting [23,24].

3.2 EVALUATION OF THE SOLVENT FLOW RATES OVER THE DYNAMIC PERIOD OF CO2-SFE

The accumulated yields of SFE 1 and SFE 2 extractions are shown in Figure 2. SFE 1 had global yield of 1.53% (d.b.), whereas the yield of SFE 2 was 1.93% (d.b.). It was possible to observe a 26% increase in the global extraction yield, when a 50% higher Q_{CO_2} and a 33% shorter dynamic period were used. The increment of extraction capacity by increasing solvent flow rates was possible due to increased surface velocity of the solvent over the particles, which also increased the convective and diffusive extraction rates [18][25]. This behavior was also observed by Daneshvand et al. [26], and by Pires et al. [17]. However, it proved to be contrary to that observed by Johner et al. [27] for extraction with supercritical CO2 using static period of 600 s. This difference was possibly due to the use of a longer static period in this study (1800 s), since an increase in static period may also increase compound solubility due to longer exposure time of solutes to the extractor solvent [28], which consequently increases the global extraction yield in a shorter dynamic period.

3.3 EXTRACT CHARACTERIZATION

3.3.1 Global yield

GY of B. crassifolia leaf extracts varied from 0.45 % (d.b.) ± 0.03 % (d.b.) to 1.24 % (d.b.) ± 0.17 % (d.b.) (Figure 3). The operating condition that obtained the highest GY was 323.15 K, 30 Mpa, and 878 kg/m3, which was similar to the result found by Fernández-Ponce et al. [29] with Mangifera indica leaf extract obtained by supercritical CO2 (1.22 % d.b ± 0.13 % d.b.). Overall, GY values increased with increasing pressure in both isotherms. The increase in temperature, under isobaric conditions, reduced GY at pressures of 10 and 20 MPa, and increased at 30 MPa, thus presenting an inflection point.

This shows that, at lower pressures, the predominant behavior of compound solubilization was controlled by increasing ρCO2. For higher pressures, solubility was controlled by increasing vapor pressure of the solutes due to the decreased partition coefficient caused by the increment of solute concentration in the supercritical fluid [30,31]. Similar behaviors were observed for Rosmarinus officinalis [32] and Copaifera sp. [33] leaf extracts obtained by supercritical CO2.

3.3.2 Total phenolic compounds (TPC)

TPC content of B. crassifolia leaf extracts varied from 28.42 mg GAE/g (d.b.) ± 0.50 mg GAE/g (d.b.) to 68.85 mg GAE/g (d.b.) ± 4.52 mg GAE/g (d.b.) (Figure 4). These values were higher than those found by Ameer et al. [34] for Stevia rebaudiana leaf extracts (23.78 mg GAE/ g d.b.) obtained with supercritical CO2. The ideal extraction condition was 313.15 K, 30 MPa and 927 kg/m3. Under isothermal conditions, the increase in TPC presented more significant differences at 313.15 K, with the increase in pressure and, consequently, in ρCO2. This was possibly due to the minimization of thermal degradation of these compounds with the use of milder temperatures. Under isobaric conditions, TPC did not show significant differences at 10 and 20 MPa with increasing temperature and ρCO2. Similar behavior was observed for Odontonema strictum leaf extract obtained with supercritical fluid [35]. At 30 MPa, TPC content increased with decreasing temperature, and increasing ρCO2. These results demonstrate that the selectivity during TPC extraction of B. crassifolia leaves is mainly caused by pressure and density of the fluid, being the same behavior found for the leaf extract of Raphanus sativus L. obtained with supercritical CO2 [36].

3.3.3 Trolox equivalent antioxidant capacity (TEAC)

TEAC results varied from 80.64 µM trolox/g (d.b.) ± 0.43 µM trolox/g (d.b.) to 174.35 µM trolox/g (d.b.) ± 2.53 µM trolox/g (d.b.) in B. crassifolia leaf extracts (Figure 5). The extraction condition with highest TEAC was 313.15 K, 30 MPa, and 927 kg/m3. It was evidenced that increasing pressure and density also increased the antioxidant activity of the extracts, indicating that these parameters caused an increment of the extracts efficiency in sequestering the $ABTS^{\cdot+}$ radical [20]. This behavior is related to increased solvation power of CO2 under these conditions [37,38], being similar to that reported by Salazar et al. [39] for CO2-SFE of leaves and stem of Cissus sicyoides. The use of low processing temperatures (313.15 K) prevented the degradation of bioactive

compounds responsible for antioxidant capacity. The same behavior was observed for TPC contents. Thus, the TEAC results of B. crassifolia leaf extracts are related to the TPC results, since their highest contents were obtained under the same extraction condition. This corroborates the understanding that the antioxidant capacity of plants is related to the amount of phenolic compounds [40], and that supercritical CO2 extraction can be used to maximize the extraction of bioactive compounds, and minimize thermal and oxidative degradation, given that it enables the extraction of these compounds without exposure to high temperatures and oxygen, in addition to obtaining a 100%-solvent free organic extract.

3.4 LINEAR CORRELATIONS

Pearson correlation coefficients (r) between the parameters varied from -0.09 to 0.97 (Table 1). According to the influence of the process parameters on the responses, it can be observed that only pressure and density of CO2 presented a strong positive linear relationship with TPC, TEAC, and GY. This certifies that B. crassifolia leaf extract can be obtained at the lowest temperature in order to reduce process costs. In correlations between responses, a strong positive linear relationship between TPC × TEAC, and a moderate positive relationship between TEAC × GY and TPC × GY were observed. This explains the similar behavior of these responses under different extraction conditions. The moderate positive correlation between TEAC × GY and TPC × GY shows that the highest mass extract obtained does not correspond to the extract with the highest bioactive compound content, which confirms the selectivity of supercritical CO2 under different extraction conditions.

4 CONCLUSION

The experiments of dynamic period reduction allow to affirm that it was possible to potentiate the extraction with supercritical CO2 of B. crassifolia leaves by modifying CO2 flow/time ratio. The extraction condition that enabled the highest GY was 323.15 K, 30 MPa, and 878 kg/m3, whereas the ideal condition to obtain TPC and TEAC was 313.15 K, 30 MPa, and 927 kg/m3. Pressure and CO2 density were the process parameters that presented the strongest positive linear correlations on all results, being the relationship between TPC × TEAC the strongest among the responses. According to the results, the use of supercritical technology proved to be effective and advantageous to obtain of B. crassifolia leaf extracts containing bioactive compounds and high antioxidant

capacity, which are related to several therapeutic properties that can be applied in food, cosmetic, and pharmaceutical industries.

ACKNOWLEDGEMENTS

This study was financed in part by the Coordenação de Aperfeiçoamento de Pessoal de Nível Superior-Brasil (CAPES)-Finance Code 001. The authors also thank LABEX/UFPA for their scientific contribution and the family members of Almeida MM (in memoriam).

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ANNEX

Figure 1. Scanning electron microscopy images of the extraction bed of crushed leaves of B. crassifolia (25 ×).

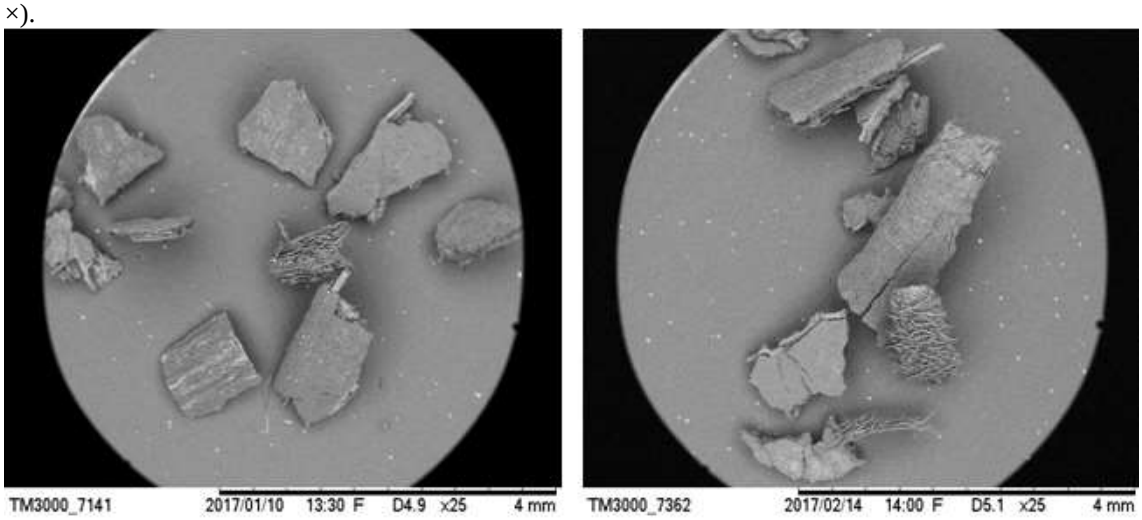


Figure 2. Accumulated yields for different solvent flow rates and dynamic periods of supercritical extraction of B. crassifolia leaves.

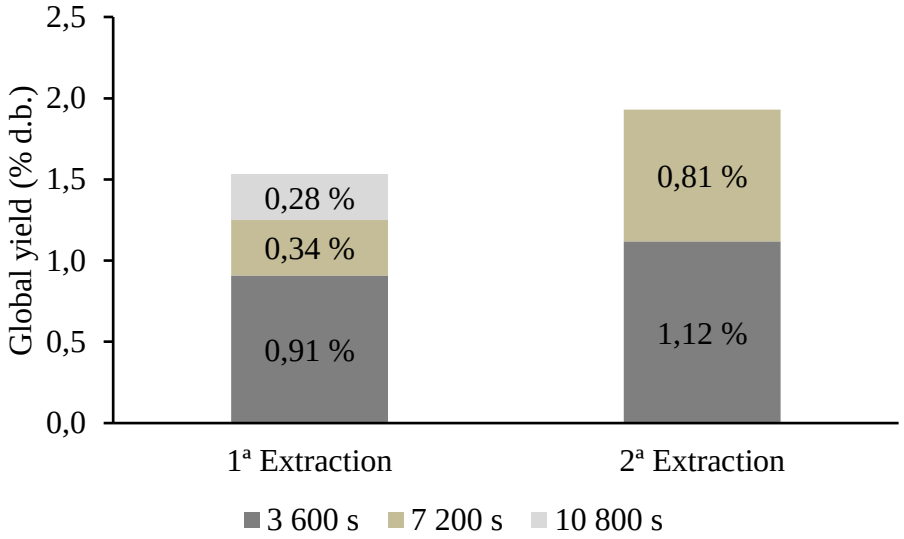


Figure 3. Global yield (GY) isotherms of *B. crassifolia* leaf extracts obtained with supercritical CO_2.

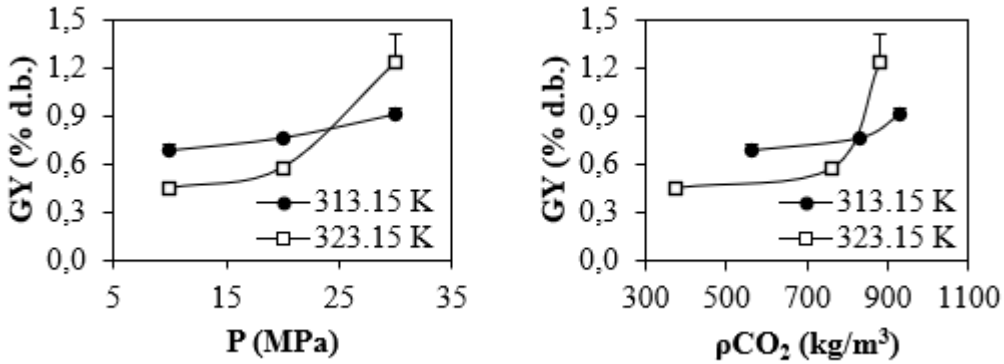


Figure 4. Total phenolic compounds (TPC) isotherms of *B. crassifolia* leaf extracts obtained with supercritical CO_2. * Same letters indicate no significant difference between responses by Tukey test ($p < 0.05$).

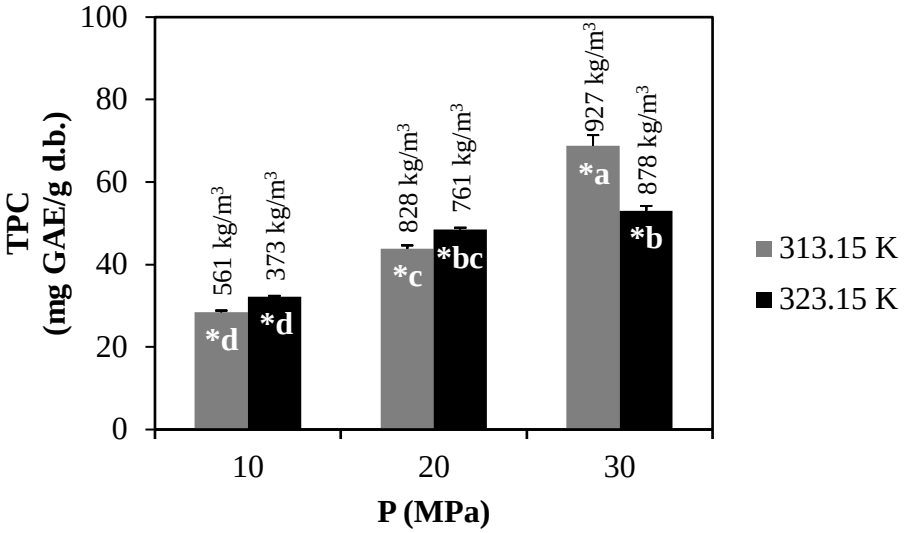


Figure 5. Trolox equivalent antioxidant capacity (TEAC) isotherms of *B. crassifolia* leaf extracts obtained with supercritical CO_2. *Same letters indicate no significant difference between responses by Tukey test ($p < 0.05$).

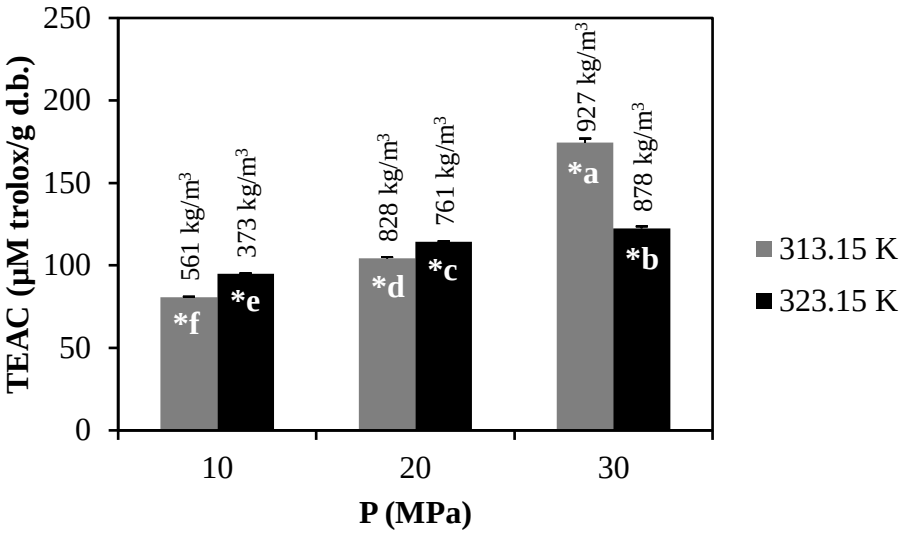


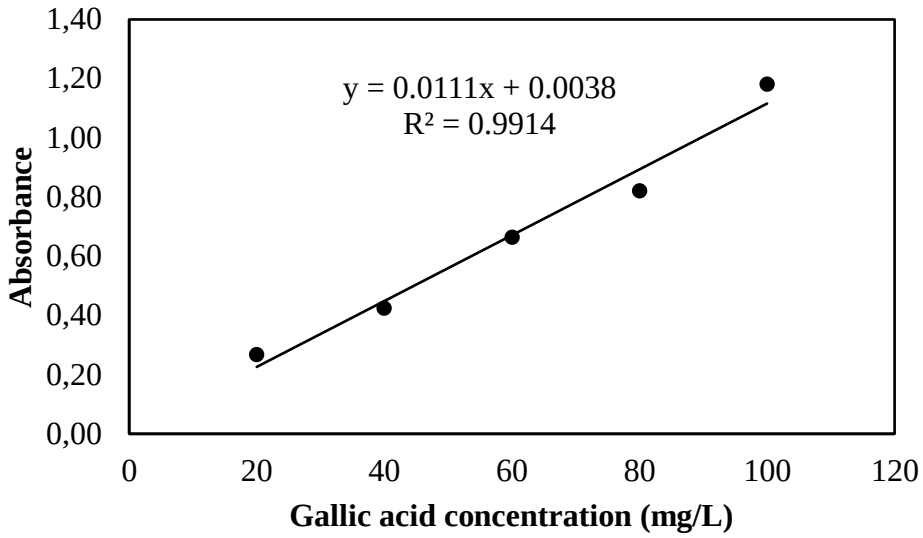
Table 1. Pearson correlation coefficients (r) between variables (N = 6) for supercritical CO_2 extraction of *B. crassifolia* leaves.

	T[a]	P[b]	ρ CO_2[c]	TPC[d]	TEAC[e]	GY[f]
TPC	-0.09	0.93	0.85	1.00		
TEAC	-0.15	0.83	0.71	0.97	1.00	
GY	-0.06	0.81	0.74	0.58	0.47	1.00

[a] T = temperature (K); [b] P = pressure (MPa); [c] ρ CO_2 = CO_2 density (kg/m^3); [d] TPC = total phenolic compounds (mg eq. Gallic ác./g d.b.); [e] TEAC = trolox equivalent antioxidant capacity (µM trolox/g d.b.); [f] GY = global yield (% d.b.).

S1a. Digital data of phenolic-compounds calibration curve.

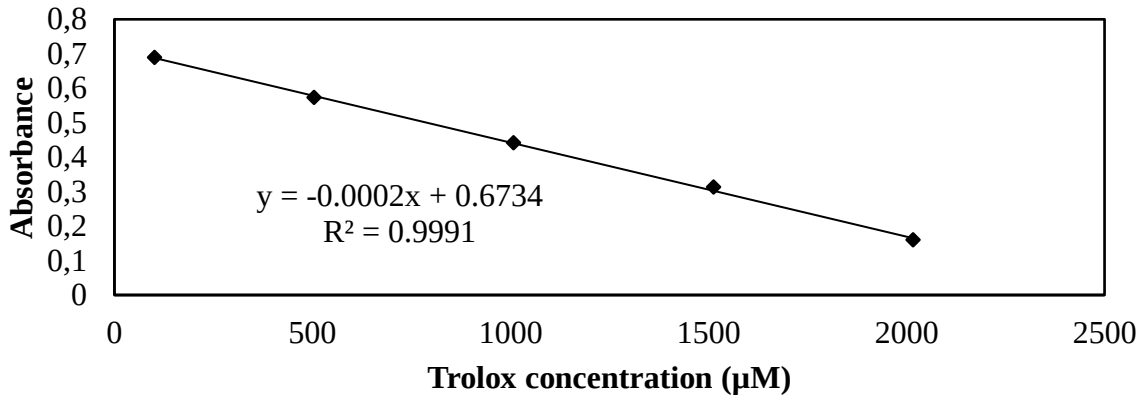
Gallic acid concentration (mg/L)	Absorbance
20	0.218
40	0.292
60	0.536
80	0.668
100	1.316



S1b. Calibration curve for phenolic compounds.

S2a. Digital data of TEAC calibration curve.

Trolox concentration (µM)	Absorbance
101	0.649
507	0.524
1014	0.398
1521	0.258
2028	0.109



S2b. Calibration curve for TEAC.