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EXTRACTION OF BIOACTIVE COMPOUNDS OF LEAVES OF *Duguetia furfuracea* (ANNONACEAE) USING GREEN AND ORGANIC SOLVENTS

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Abstract - *Duguetia furfuracea* (Annonaceae) is a shrub found in the Brazilian Cerrado that is used in popular medicine as an antirheumatic, for wound healing, treatment of kidney pain and against pediculosis. The aim of this study was to analyze the extraction of leaves of *Duguetia furfuracea*, using supercritical CO_2, ethanol and hexane as solvents. For the supercritical extraction, an experimental planning 2^3 with triplicates of the central point was used to evaluate the effects of temperature (313-333 K), pressure (15-23 MPa) and volumetric flow rate (3-6 mL min^{-1}) on the extraction yield, total phenolic content and antioxidant activity compared with extracts obtained by Soxhlet extraction. The effects of the variables on the extraction yield were positive and the pressure had greater influence. However, the highest performances, total phenolic content and antioxidant activities were obtained by the Soxhlet method. The mathematical model based on the Brunauer-Emmett-Teller theory of adsorption correlated satisfactorily the experimental values of the supercritical extraction.
Keywords: *Duguetia furfuracea*; Supercritical CO_2; Phenolic compounds; Antioxidant activity.

INTRODUCTION

The Cerrado is a term commonly used to describe the group of ecosystems (savannas, forests, veredas and gallery forests) of the plateau in the center of Brazil (dos Santos et al., 2016, Cassino et al., 2015). It is the second largest Brazilian biome, occupying 23 % of the national territory (Borges et al., 2015). The Cerrado is the most diverse tropical savanna in the world, totaling more than 12,600 species (4,215 Brazilian endemics) (Forza et al., 2012). Despite this, about half of the 2 million km² of the Cerrado were turned into planted pastures, annual crops and other uses (Klink and Machado, 2005, Lambin et al., 2013) and then considered one of the world's biodiversity hotspots (Myers et al., 2000, Silva and Bates, 2002). Due to the high deforestation, large taxonomic diversity and its still understudied flora, it needs more research with native species on the conservation of natural resources and phytotherapics resources offered by medicinal plants (Guarin-Neto and Moraes, 2003).

Duguetia furfuracea (A. St.-Hil.) Benth and Hook. f. specie belongs to the Annonaceae family. It is a shrub found in the Cerrado and considered a weed by farmers for invading pastures (Lorenzi and Matos, 2002), popularly known as "sofre-do-rim-quem-quer," "araticum-bravo," "araticum-do-campo", "araticum-do-cerrado", "ata-brava", an d "ata de lobo" (Agra et

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al., 2007, Silva et al., 2012). It is used in traditional medicine as an antirheumatic, for wound healing, treatment of kidney pain and against pediculosis (Silva et al., 2007).

The phytochemical analysis of the essential oil from leaves and branches of *D. furfuracea* revealed the presence of sesquiterpenes (Carollo and Hellman-Carollo, 2005), flavonoids and several alkaloids (Carollo and Hellman-Carollo, 2006, Carollo et al., 2006). The alkaloid extracts obtained from the tree barks of the underground stems have antitumor, trypanocidal and leishmanicide activities (Silva et al., 2009). The ethanol extract shows cytoprotective activity with the bacterium *E. coli* against mercuric chloride, indicating that *D. furfuracea* is a promising source of cytoprotetors to combat heavy metals (Lima et al., 2014). Hexane and ethanol extracts of *D. furfuracea* have high larvicidal activity against larvae of *Aedes aegypti* (Rodrigues et al., 2006).

In this context, the aim of this study was to analyze the extraction of leaves of *Duguetia furfuracea* using supercritical CO_2, ethanol and hexane as solvents. The effects of temperature, pressure and volumetric flow rate of CO_2 were evaluated in the kinetics and the performance of supercritical extraction. The extracts were analyzed and compared to extracts obtained by Soxhlet using ethanol and hexane as solvents in terms of extraction efficiency, total phenolic content, antioxidant activity, and chemical composition.

MATERIALS AND METHODS

Raw materials

Samples of *Duguetia furfuracea* leaves were collected in the morning in April 2015, at the University of Rio Verde located in Rio Verde in the southwestern state of Goias, Brazil. The leaves were dried in a drying oven with forced circulation (Nova Etica 400 / 4ND) at 313 K for 24 hours. The samples were crushed in a knife mill (Solab SL-30) and then sieved through a set of W.S Tyler sieves (Mentor, OH, USA). For the extraction a sample with a grain size between 20 and 42 mesh was used.

Extraction Methods

Soxhlet extractions

The extractions with organic solvent were conducted according to the AOAC method (1990) using Soxhlet extractor with ethanol (Nuclear, minimum purity 99.8 %) and hexane as solvents (Nuclear, minimum purity 99.6 %). About 5 g of the crushed leaves of *Duguetia furfuracea* were put in a filter paper cartridge and inserted into the extractor coupled to a flask containing 150 mL of solvent recycling over the sample. The sample was subjected to extraction for a period of 6 hours. The extractions were performed in triplicate and the results were expressed as the average ± standard deviation.

Supercritical fluid extraction (SFE)

The extraction experiments with supercritical fluid were conducted in a laboratory scale unit as in earlier studies (Lemos et al., 2012, Silva et al., 2014, Santos et al., 2015). The unit consists of a reservoir of solvent (CO_2, White Martins SA, with 99.9 % purity), one syringe pump (Isco model 500D), two thermostatic baths (Julabo F25-ME and Quimis Q214M2) and a stainless steel extractor 17 cm long with an internal diameter of 2.85 cm. The experimental procedure consisted of introducing about 30 g samples of *Duguetia furfuracea* leaves inside the extractor, and then selecting the temperature and pressure desired for the experiment. After 30 min, time to stabilize the system, it is necessary to adjust the flow rate through the micrometric valve at the bottom of the extractor and measure the extract weight every 10 min for a period of 3 hours.

Experimental design

A complete factorial planning 2^3 with triplicate of the central point was used to evaluate the influence of pressure (P), temperature (T) and volumetric flow rate (Q) of CO_2 on the extraction performance (Y). The encoded and uncoded values used are shown in Table 1.

The volumetric flow rate was determined at 278 K and pressure of the experiments. Under these conditions the CO_2 density is approximately 1 g mL^{-1} resulting in mass flow rates of 3 and 6 g min^{-1}.

The analysis of variance was performed using the Design-Expert software (Stat-Ease, Inc., Minneapolis, USA) by response surface methodology considering 95% of confidence by the F-Test. The general model used for variable performance response is the polynomial given by Eq. (1):

$$Y = b_0 + \sum_{i=1}^{3} b_i X_i + \sum_{i,j=1;i<j}^{3} b_{ij} X_i X_j \qquad (1)$$

where b_0 is the constant fixing the performance at the central point of the experiment, b_i are the regression coefficients for linear effects, b_{ij} are the coefficients of the effects of interactions, X_i and X_j are the normalized independent variables.

Table 1. Operating conditions used in the extraction with supercritical CO_2.

Variables	-1	0	1
Pressure (MPa)	15	19	23
Temperature (K)	313	323	333
Flow rate (mL min^{-1})	3.0	4.5	6.0

Total phenolic content (TPC)

The determination of total phenolic content of *Duguetia furfuracea* extracts was performed by spectroscopy using the Folin-Ciocalteu method with modifications according to the methodology of Singleton and Rossi (1965). For analysis, the solutions were prepared by dissolving 0.1 g of extract in 10 mL of methanol, and then an aliquot of 0.1 mL of this solution collected and added to 7.4 mL of distilled water and 0.5 mL of Folin-Ciocalteu reagent. After three minutes, 2 mL of 15 % aqueous solution of sodium carbonate was added and the solution left standing for 2 hours away from light, and then the absorbance measured at 760 nm. The total phenolic content was determined through a calibration curve constructed with gallic acid used as standard and expressed in mg of GAE (gallic acid equivalents)/g extract.

Antioxidant activity

The determination of antioxidant activity was performed by the ABTS method (2,2-azino-bis (3-ethylbenzthiazoline-6-sulfonic acid)) with some modifications according to Re et al. (1999). The $ABTS^+$ radical is prepared from the reaction of 5 mL of 7 mM ABTS aqueous solution with 88 µL of 140 mM potassium persulphate solution. The mixture was kept in the dark for 16 hours. Then, 1 mL of this mixture was diluted in ethyl alcohol until the measured absorbance at 734 nm was 0.70 (± 0.05). With three different dilutions of the extract, a 30 µL aliquot of these dilutions was mixed with 3 mL of $ABTS^+$ and its absorbance measured 6 minutes after mixing. The total antioxidant activity was determined from a standard curve of Trolox and its value expressed in µM TEAC (Trolox equivalent antioxidant activity)/g extract.

Gas chromatography

The analysis of the chemical composition of the extracts was performed on a gas chromatograph (Agilent Technologies, Model 7890A) coupled to a mass spectrometry detector (Agilent Technologies, Model 5975 C). The capillary column used was a VF-WAXMS 30 m x 0.25 mm x 0.25 µm. The initial column temperature was 328 K rising 15 K/min to 413 K, then 10 K/min to 453 K, maintaining this for 2 min, and 5 K/min to 513 K, keeping this temperature for 8 min. Helium was used as carrier gas with a flow rate of 1 mL/min. The temperatures were 553 K in the injector, and 553 K and 473 K in the ion source and interface, respectively. The component identification was performed by comparing their mass spectra with those from the National Institute of Standards and Technology and Kovat's indices (Adams, 1995).

Modeling of the experimental data

A model described by Pardo-Castaño et al. (2015) was used to correlate the experimental values of the supercritical extraction. The model is based on the absorption theory of Brunauer-Emmett-Teller and it expresses the extraction yield (Y) as a function of time (t) with three parameters; y^*, extract solubility in the SFE, K, the relationship between the solute adsorption equilibrium constant of the first monolayer and the subsequent layers and x_m, the ratio of the mass of solute present in the first monolayer and the initial mass of solute, that can be derived according to Eq. (2):

$$t = \frac{m_0}{2\dot{m}_f y^*}\left\{x'_0 - x' + (2-K)\left[Y - x_m \ln\left(\frac{\alpha}{\beta}\right)\right] + Kx_m \ln\left[\frac{\alpha'}{\beta'(1-Y)^2}\right]\right\} \quad (2)$$

where:

$$x'_0 = \sqrt{a+b+c} \quad (3)$$

$$x' = \sqrt{a(1-Y)^2 + b(1-Y) + c} \quad (4)$$

$$a = K^2 \quad (5)$$

$$b = 2(2-K)Kx_m \quad (6)$$

$$c = (Kx_m)^2 \quad (7)$$

$$\alpha = x' + (2-K)(1+Y) + Kx_m \quad (8)$$

$$\alpha' = x' + (2-K)(1-Y) + Kx_m \quad (9)$$

$$\beta = x'_0 + K + (2-K)x_m \quad (10)$$

$$\beta' = x'_0 + (2-K) + Kx_m \quad (11)$$

$$y^* = y_{sat}\left[1 - \exp\left(-\frac{KL}{u\varepsilon}\right)\right] \quad (12)$$

where m_0 is the extractable initial mass of the solute in the packed bed, m_f is the mass flow of CO_2, L is the extractor length, u is the speed of CO_2, y_{sat} is the solute mass fraction in a saturated SCF phase and ε the porosity of the bed.

The objective function, defined as the average absolute relative deviation (AARD), is expressed by Eq. (13):

$$AARD = \frac{100}{n}\sum_{t=1}^{n}\left(\frac{t_{t,exp} - t_{t,cal}}{t_{t,exp}}\right) \quad (13)$$

where n is the number of experimental values, $t_{t,exp}$ and $t_{t,cal}$ are experimental times and calculated by the model, respectively.

RESULTS AND DISCUSSION

Extraction yield

Table 2 shows the experimental conditions for the extraction, extraction yield, the antioxidant activities and the total phenolic content of *Duguetia furfuracea* extracts obtained by supercritical CO_2 and Soxhlet extractions with the organic solvents hexane and ethanol.

The analysis of variance was used to check the influence of the variables temperature, pressure and volume flow rate on the performance of the supercritical extraction at a level of 95 % of confidence and to adjust the polynomial regression model as shown in Eq. (1). The results of the regression model in terms of encoded levels were significant for the F-Test and are given by Eq. (14):

$$Y = 0.77 + 0.23P + 0.041T + 0.066Q + 0.053PT + 0.035TQ \quad (14)$$

The significant effects ($p<0.05$) were the pressure, temperature, and volumetric flow rate of CO_2 and the interactions pressure-temperature, and temperature-flow rate. The effect of the pressure interaction with the flow was not significant ($p>0.05$).

The adjusted regression coefficient was 98.6 % indicating, that the proposed model and the experimental values have good agreement. Fig. (1) shows the response surface of the extraction efficiency as a function of temperature and pressure with a constant volumetric flow rate of 6.0 mL min^{-1}.

The positive pressure effect can be seen in both Fig. (1) and Table 2 for experiments 5 and 6, for which the yields were 0.60 and 0.92 %, respectively. This effect can be explained by an increase of CO_2 density and consequently the solvating power of the solvent (Paviani et al., 2012) promoted by an increase in the pressure at constant temperature. The positive effect of temperature observed in experiments 6 and 8 can be explained by the fact that, at higher temperatures, the solvent viscosity is lower and the vapor pressure of the extract greater, and consequently there is an increase in the solvent diffusivity (Goleroudbary and Ghoreishi, 2016). While the pressure and temperature affect the thermodynamic properties, such as density and solubility, and transport diffusivity, the flow rate increases the convective mass transfer coefficient and decreases the concentration of the solute in the bulk of the supercritical phase, thus obtaining a positive effect on the extraction yield (de Melo et al., 2014). The extraction by Soxhlet showed the highest results, with significant difference at the 95 % confidence level. The highest yield was obtained with ethanol, which is a polar solvent. This behavior can be attributed to the higher temperature of the recirculating solvent and solute-solvent interactions (Benelli et al., 2010).

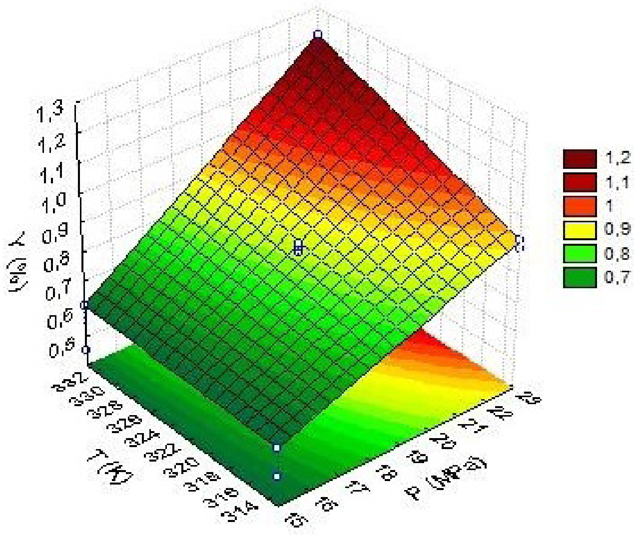

Figure 1. Influence of temperature and pressure on the SFE yield at constant solvent flow rate (Q_{CO2} = 6.0 mL min^{-1}).

Table 2. Yields, total phenolic compounds and antioxidant activity of supercritical and Soxhlet extractions of leaves of *Duguetia furfuracea*.

Exp.	P (MPa)	T (K)	*Q* (mL min^{-1})	Y (%)	TPC (mg GAE g^{-1} extract)	TEAC (µmol Trolox g^{-1} xtract)
1	15	313	3	0.51	53 ± 3[b]	166 ± 2[b]
2	23	313	3	0.89	42 ± 13[b]	130 ± 1[b]
3	15	333	3	0.45	30 ± 3[b]	67 ± 0.5[b]
4	23	333	3	0.97	63 ± 120[b]	170 ± 1[b]
5	15	313	6	0.60	28 ± 4[b]	98 ± 1[b]
6	23	313	6	0.92	44 ± 1[b]	102 ± 0.3[b]
7	15	333	6	0.61	32 ± 6[b]	96 ± 0.4[b]
8	23	333	6	1.21	38 ± 4[b]	97 ± 0.3[b]
9*	19	323	4.5	1 ± 0.01[c]	31 ± 10[b]	90 ± 18[b]
10*	Hexane			2 ± 0.2[b]	210 ± 32[a]	531 ± 123[a]
11*	Ethanol			9 ± 0.7[a]	253 ± 45[a]	606 ± 168[a]

Different letters indicate statistically significant difference at 5 % of significance.
* Average value of three extractions; N.A: not available.

Modeling of the experimental data

Table 3 shows the values of the parameters y^*, K and x_m adjusted to the experimental values. According to Pardo-Castaño et al. (2015), a single value of x_m encompasses all the experimental conditions studied due to its weak dependence on these conditions. This occurs because this property is dependent on the solid-solute interaction. The values of y^* are associated with solubility and can be noted that higher values, 8.04 and 5.48 for the experiments 6 and 2, respectively, are obtained for the experimental conditions of higher pressure, 23 MPa, and less temperature, 313 K, consequently higher density of CO_2.

Fig. 2 shows the correlation of experimental values and the adjusted model according to Pardo-Castaño et al. (2015).

Table 3. Adjustable parameters for the model of Pardo-Castaño et al. (2015).

Exp.	$y^* \times 10^3$	K	x_m
1	1.60	66.7	0.591
2	5.48	31.9	0.591
3	1.85	198	0.591
4	2.09	2.05	0.591
5	1.53	100	0.591
6	8.04	90.1	0.591
7	1.90	89.6	0.591
8	2.75	2.22	0.591
9	2.97	21.1	0.591

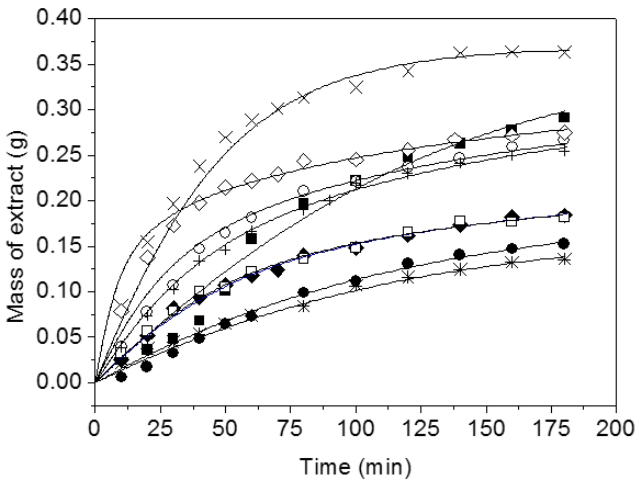

Figure 2. Calculated and experimental kinetic curves of extraction from leaves of *Duguetia furfuracea*: (—) model proposed by Pardo-Castaño et al. (2015); (•) Exp 1; (○) Exp 2; (*) Exp 3; (■) Exp 4; (□) Exp (5); (◊) Exp 6; (♦) Exp 7; (×) Exp 8, (+) Exp 9.

Gas chromatography

Table 4 shows the chemical composition of *Duguetia furfuracea* extract obtained by SFE and Soxhlet extraction with ethanol and hexane by GC-MS. In the composition of the extract obtained by SFE the sesquiterpenes (+)- spathulenol and β-caryophyllene oxide prevail with 39.53 and 11.68 % of relative area, respectively. For the ethanol extract, the main component was 3-deoxyestradiol with 28.41 % of relative area.

Table 4. Chemical composition of supercritical (SFE) and Soxhlet extracts of leaves of *Duguetia furfuracea*.

Compound	Relative Area %		
	SFE*	Ethanol	Hexane
Alloaromadendrene oxide-1	2.33		
β-Caryophyllene oxide	11.68	5.18	4.95
(+)-Spathulenol	39.53	20.35	13.80
Ethyl palmitate		3.61	
Spathulenol	1.64		
(-)-Caryophyllene oxide	3.08		
Methyl elaidate	2.19	10.47	2.63
Aromadendrene oxide-2	3.63		
Alloaromadendrene oxide-2	2.34		
(-)-Spathulenol	2.53		
Isoaromadendrene epoxide	3.22		
2-Methylenocholestan-3-ol	3.15	5.33	
α-Tocoferol		2.87	
Palmitic acid	6.32		
3-Deoxyestradiol		28.41	7.12
2-Methylhexadecan-1-ol	3.55		
Not identified	14.81	23.79	71.54

* Average value of three extractions at the center point (19 MPa, 323 K and 4.5 mL min^{-1}).

In the three extracts, it is important to emphasize the presence of spathulenol, a component that has been shown to have multiple biological activities, including antimicrobial, insecticidal and cytotoxic (Cantrell et al., 2005). This component also predominates in the chemical composition of the essential oil of *Duguetia furfuracea* leaves, according to Valter et al. (2008).

Total phenolic content (TPC)

The values of total phenolic content of SFE and Soxhlet extracts are shown in Table 2. In the supercritical extraction model, the results presented no significant difference, showing no dependence of the total phenolic content with pressure, temperature and volumetric flow. The values of TPC ranged from 28 to 62 mg GAE g^{-1} of extract and showed no statistical difference at the 5% significance level. The TPC values for ethanol and hexane extracts differed significantly at 5 % from the extracts obtained by SFE, and presented values of 253 and 210 mg GAE g^{-1} of extract, respectively. The TPC of the ethanol extract agrees with the results of other species of the Annonaceae family, such as the methanol extract of the twigs of the species *Duguetia riparia* (Cunha, 2009) and the ethanol extract of *Annona cherimoya Mill*. (Rabelo et al., 2014).

Antioxidant activity

Table 2 shows the values of the antioxidant activity of *Duguetia furfuracea* extracts performed by the ABTS method. The highest values obtained were 606 and 531 μM Trolox g^{-1} extract for ethanol and hexane extracts, respectively. For the supercritical extraction the antioxidant activity values were not significantly different, showing no dependence of the antioxidant activity with pressure, temperature and volumetric flow. The ABTS values for the extracts ranged from 67 to 170 μmol Trolox g^{-1} of extract. As expected, the extracts with higher total phenolic content showed the highest antioxidant activity. Pereira et al. (2013) also obtained higher values of ABTS for ethanolic extract of *Myrtus communis L*. in comparison with the supercritical extract.

CONCLUSION

The best extraction yield with supercritical CO_2 of *Duguetia furfuracea* was 1.21 %, with the highest pressure (23 MPa), highest temperature (333 K), and highest volume flow rate (6 mL min^{-1}). The values of yield were adjusted to a polynomial model with all the positive effects of variables, so that the pressure was the variable that had the greatest effect. The regression coefficient of 98.6 % indicates that the polynomial model can be used under the evaluated conditions. The total phenolic content and antioxidant activity showed no significant difference between the experiments with SFE. However showed higher values for TPC, 253 and 210 mg GAE g^{-1} of extract, and TEAC, 606 and 531 μM Trolox g^{-1} extract, for ethanol and hexane extracts. The chemical profile of the extracts obtained showed high levels of spathulenol, especially for SFE. The simple mathematical model proposed by Pardo-Castaño et al. (2015) satisfactorily correlated the experimental data with the calculated values.

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