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Box-Behnken design to study the bergenin content and antioxidant activity of *Endopleura uchi* bark extracts obtained by dynamic maceration

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Abstract: Brazil has one of the world's largest biodiversity in flora and a plant that has attracted attention is the *Endopleura uchi* (Huber) Cuatrec., Humiriaceae, which is native of the Brazilian Amazon. Among the many popular uses, this species is utilized in the treatment of woman´s genito urinary tract affections and also as anti-inflammatory. It is believed that their actions derive from the major constituent, bergenin. The objective of this work was to study the *Endopleura uchi* barks extraction using the dynamic maceration method and the effects of the extraction time, drug to solvent ratio and temperature. A Box-Behnken design was applied to study the influence of these factors and the respective response surfaces. The extract characterization was made by determination of its antioxidant activity by DPPH; total polyphenol content and bergenin content. In general, the extracts showed good antioxidant activity, with the IC50 ranging from 4.02 to 5.87 µg/mL. The polyphenol content ranged from 31.89 to 47.82%. High levels of chemical markers are observed in all extracts, with average bergenin content of 35.58%. The result showed that the multivariate study of extraction is key step in the development and standardization of extracts *Endopleura uchi.*

Article

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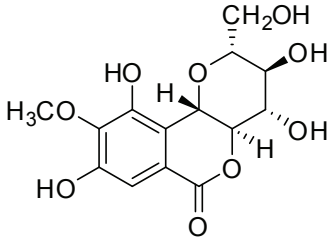
Introduction

Brazil has a very extense biodiversity in flora which is very little known or studied for medicinal purposes (Agra et al., 2007; Coelho-Ferreira, 2009; de Carvalho et al., 2008). One Brazilian plant that has recently raised strong interest is the *Endopleura uchi* (Huber) Cuatrec., Humiriaceae. This species originates from the Brazilian Amazon, is dispersed all over the Amazon River basin, and is popularly known as "uxi", "uxi-amarelo", "uchi-pucu", "uxi-liso" e "uchi" (Carvalho et al., 2007; Cuatrecasas, 1961; Politi et al., 2011).

In Amazonian folk medicine, the bark of the uxi is used to treat hypercholesterolemia, diabetes, arthritis, and as anti-inflammatory. Furthermore, the *Endopleura uchi* bark tea has been popularly used to treat uterine fibroids and disorders of woman´s genito urinary tract (Nunomura et al., 2009).

It is believed that the main active constituent of this plant is bergenin (**1**), a *C*-glycoside of 4-*O*-methyl gallic acid, found in the fruits and barks of *Endopleura uchi*. (Gu et al., 2009; Magalhães et al., 2007; Nunomura et al., 2009) The scientific literature shows several biological activities attributed to this glycoside: anti-inflammatory (Nunomura et al., 2009); antihepatotoxic (Kim et al., 2000), antifungal (Prithiviraj et al., 1997), anti-HIV (Piacente et al., 1996).



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One of the key steps in phytopharmaceutical technology studies is the extraction method that will be used for the extraction of a particular constituent or group from the plant raw material (Helou,1989).

One of the most used extraction methods is the maceration (Moura et al., 2011), due to its simplicity and suitability to different scales: from laboratory bench top to industry (Silva & Aragão, 2009; Tour & Talele, 2011). One of its variants, the dynamic maceration, has the advantage of steadily stirring, resulting in increased contact of the plant material with the solvent and thus shorter process times (De Paivai et al., 2004).

The aim of this work was to apply a design of experiment, DOE, to study the extraction of bergenin from *Endopleura uchi* barks using the dynamic maceration method and also to characterize the extracts, evaluating their polyphenol and bergenin content and antioxidant activity.

Materials and Methods

Plant

The plant material was the bark of *Endopleura uchi* (Huber) Cuatrec., Humiriaceae. The plant material was purchased from Flores e Ervas Ltda (Piracicaba, São Paulo, Brasil) with batch number 029071/SFS015. A sample of the *Endopleura uchi* bark was deposited at the "Herboteca Carlos Stelfled" at the Pharmacognosy Lab from the Universidade Federal do Paraná, under registry number No. 331-A. The pharmacognostic characterization of the plant material resulted in a maximum foreign elements of 2.0%; moisture content of 7.0% and total ash of 5.1%. The barks were subsequently subjected to a milling process, using a knife mill SL31 (Solab Ltda, Piracicaba, Brasil).

Dynamic maceration

The dynamic maceration runs were carried out in 250 mL sealed Erlenmeyer flasks and a 15-sample multipoint magnetic stirrer with digital speed control. The stirrer speed was calibrated with an optical tachometer TO404 (OpthoTako Ltd) and was kept constant at 600 rpm to all experiments. The extraction flasks were kept in a thermostatic air bath with digital temperature control AB-15 (Labmaq do Brasil Ltda, Ribeirão Preto, Brazil). The solvent chosen was ethanol 70 °GL and before the extraction experiments, the drug swelling index in this solvent, SWI, was determined and used to calculate the amount of solvent used in each experiment.

The factors chosen for the dynamic maceration study were the extraction time t (h), the temperature T (°C) and the plant material to the solvent ratio, P/S (%). After the extraction process, the extracts were filtered using qualitative filter paper.

Design of experiments

For better understanding of the dynamic maceration process, a Box-Behnken type factorial design was applied. This model of fractional factorial design has three variables and three levels but only 15 experimental runs (Box et al., 1978). The three factors were the extraction time t (h); the temperature T (°C) and the plant/solvent ratio P/S (%). The Box-Behnken design is shown in Table 1, where the chosen levels are presented in a coded form, using the following nomenclature: -1 (low level), 0 (intermediate level) and 1 (high level). The actual values for the three levels of the factors studied are presented in Table 2.

Table 1. Factorial design-Dynamic maceration.

Exp	t (h) coded	T(C) coded	P/S(%) coded
1	-1	-1	0
2	1	-1	0
3	-1	1	0
4	1	1	0
5	-1	0	-1
6	1	0	-1
7	-1	0	1
8	1	0	1
9	0	-1	-1
10	0	1	-1
11	0	-1	1
12	0	1	1
13	0	0	0
14	0	0	0
15	0	0	0

Table 2. Factors studied and their levels.

Factors	Levels		
	-1	0	1
t (h)	6	12	18
T (°C)	25	35	45
P/S (%)	10	30	50

Antioxidant activity

The evaluation of antioxidant activity was performed using the radical 2,2-difenilpicrilhidrazil (DPPH) method (Martins et al., 2012). The curves of absorbance measurements as function of extract concentration were prepared in triplicate with five concentrations for each of the fifteen extracts obtained in the Box-Behnken design. Besides the curves for *uchi* extracts, absorbencies were also measured for blank and positive control (C+) of the DPPH reaction. The samples (E) were prepared by dissolving 50 µL of extracts in 1.0 mL of acetate buffer (pH 5.5), 1.0 mL of ethanol, and 500 µL of DPPH (200 µM). The blank was a solution of 1.0 mL acetate buffer (pH 5.5), 1.5 mL of ethanol. The positive control, C+, was prepared with 1.0 mL acetate buffer (pH 5.5), 1.0 mL of ethanol, 50 µL of ethanol and 500 µL of DPPH (200 µM).

After 15 min in the dark, absorbance was measured at a wavelength of 520 nm in a spectrophotometer M330 (Camspec Ltd., Cambridge, UK). Absorbance

measurements were used to calculate % inhibition, with the aid of equation 1 (Martins et al., 2012).

$$\%\ Inhibition = 100 - \left(\frac{AbsE}{AbsC+} * 100\right) \qquad [1]$$

Where: *AbsE* = absorbance for extract and *AbsC+* = absorbance for positive control.

The results are expressed as inhibitory concentration, IC50 (µg/mL), needed to decrease by 50% the initial concentration of DPPH. The IC50 of gallic acid standard was also analyzed to serve as a reference in antioxidant activity.

Phenols analysis

The quantification of phenol was performed using the Folin Ciocalteu reagent (Marquele et al., 2006; Politi et al., 2011). This reagent is a mixture of two acids, with molybdenum and tungsten in oxidation state 6+. These two compounds change their oxidative state when they are in the presence of reducing agents, such as phenols, leading to formation of blue color. Consequently, the concentration can be measured by spectrophotometry. The more reducing agents, more intense is the blue color. The analytical curve was prepared with gallic acid, used as reference substance for analysis of phenols. Absorbance was measured at 760 nm (Marquele et al., 2006).

Bergenin analysis

The bergenin quantification was performed by high performance liquid chromatography (HPLC) in a Shimadzu LC-AT 10 chromatograph with a UV-Vis detector SPD-10A and manual injection. The method used validated by Tacon (2012) and adapted from Nunomura et al. (2009). The chromatography was run in isocratic mode with methanol: water (20:80) as the mobile phase, column C-18 Shim-PACK CLS-(ODS 280x4.0 mm and particle size 5 µm) and the detector was set to wavelength of 272 nm. The flow rate of the mobile phase was 0.8 mL/min. The analytical curve was constructed using bergenin 95.4% (ChromaDex Ltd, USA), with bergenin concentrations ranging from 12.5 to 400 µg/mL, and a linear correlation coefficient of 0.9993. The bergenin yield was calculated based on the ratio of bergenin mass found in HPLC analysis and the weight of plant used in each of the dynamic maceration process.

Statistical analysis

The software Statistica® 9.0 (Statsoft Inc, Tulsa, USA) was used for response surface analysis of factors effects on extracts characteristics. The effects were considered statistically significant only when $p \leq 0.05$. ANOVA on the experimental data was performed using the visual general linear model (VGLM) module from Statistica®. The response functions applied were the multiple linear equations given by:

$$Y = A_0 + A_1X_1 + A_2X_2 + A_3X_3 + A_4X_1X_2 + A_5X_1X_3 + A_6X_2X_3 \qquad \text{Eq. 2}$$

Where, Y=dependent variables; X_n=factors studied and A_n=polynomial coefficients

Results and Discussion

The milling process resulted in plant material with mean particle size (d_{50}) equal to 343±14 µm. This milling step was made to reduce the particle size and increase the surface area, thus improving the extraction process (Fonseca et al., 2006; Manpong et al., 2011; Martin et al., 2011). Thus, all extraction experiments were carried out with milled bark with this size distribution. Table 3 shows the results for extract antioxidant activity, phenol and bergenin content and bergenin yield.

Table 3. Extracts characteristics.

Run	IC50 µg/mL)	Phenols content (%)	Bergenin content (%)	Bergenin yield (%)
1	4.32	40.10	36.87	1.93
2	4.05	39.68	35.79	2.26
3	4.35	47.82	34.98	2.06
4	4.73	36.09	32.37	2.16
5	4.58	37.85	34.70	2.93
6	4.65	37.05	33.66	4.00
7	5.44	31.89	38.07	0.52
8	4.02	36.12	37.01	1.47
9	4.62	38.78	31.82	3.90
10	4.71	34.65	32.06	2.71
11	4.38	36.22	34.98	0.76
12	5.87	36.63	35.56	0.39
13	4.78	39.01	35.48	2.09
14	5.09	38.85	35.41	2.03
15	5.04	38.56	33.83	2.20

Gallic acid standard: IC50 3.21 µg/mL.

In Table 3 it is observed that all the extracts showed excellent antioxidant activity, with the IC50 ranging from 4.02 to 5.87 µg/mL. The values were close to the IC50 of gallic acid (3.21 µg/mL), which is considered a reference for antioxidant activity (Lima et al., 2006; Sousa et al., 2007). These IC50 values are comparable or superior to plant extracts with recognized antioxidant activity (Araujo et al., 2010; Lima et al., 2006; Marquele et al., 2006; Souza et al., 2007).

Phenols are among the most important classes of antioxidants. The data in Table 3 demonstrate that all extracts presented high phenol contents, which ranged from 31.89 to 47.82% (Marquele et al., 2006; Politi et al., 2011). This result corroborates with the high antioxidant activity of the extracts obtained from *Endopleura uchi* (Huber) Cuatrec., Humiriaceae (Politi et al., 2011), since the phenolic compounds are able to sequester or neutralize free radicals due to their chemical structure and reducing properties. They are also able to chelate transition metals, acting both in the stage of initiation and the propagation of the oxidative process (Garrido et al., 2012; Sousa et al., 2007).

In this paper the bergenin (**1**) content refers to the concentration of this marker in each of the fifteen extracts, which was calculated from the standard curve of bergenin (HPLC). High levels of this marker are observed in all extracts, being the average 35.58%. The chromatograms of the one of extracts (run nr 1, Table 3) and of the bergenin 95.4% (ChromaDex Ltd, USA) may be observed in Figures 1a and b, respectively.

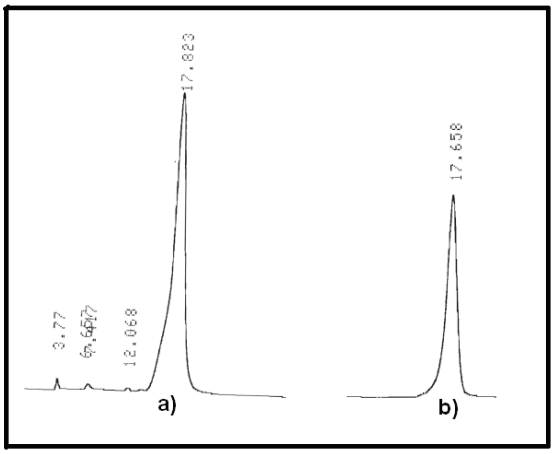

Figure 1. Chromatographic profiles of: a) extract of Uchi (Box-Behnken design-run nr1, Table 3); b) bergenin 95.4% (ChromaDex Ltd, USA).

The data on bergenin content from Table 3 can be used to calculate the weight of bergenin extracted in relation to the original weight of plant, or the yield of bergenin. The results indicate up to 4% of bergenin in the uchi bark, which is comparable to value reported in literature, 3.19% (Nunomura et al., 2009).

Statistical analysis

The Tables 4 to 7 show the results for statistical analysis, ANOVA, of the extract characteristics. Effects were considered statistically significant when $p \leq 0.05$.

Table 4 shows the results of the statistical analysis for IC50. It can be noted that the statistically significant parameters were the temperature and the quadratic term of time. These two effects can be clearly seen in Figure 2. Intermediary times were responsible for higher IC50, or lower antioxidant activity. The lowest temperature levels showed higher antioxidant activity. It is well known that higher temperatures may promote better extraction, but they may also increase the degradation of heat sensitive compounds, thereby increasing the IC50. The P/S ratio did not affect the antioxidant activity at a significant level, demonstrating that within the range of P/S ratio analyzed, IC50 is always constant.

Table 4. Summary of ANOVA analysis by response surface on IC50.

Effects	SS	MS	F	p
t	0.19158	0.19158	2.0005	0.216390
t^2	0.64013	0.64013	6.6842	0.049111
T	0.64355	0.64355	6.7199	0.048702
T^2	0.13629	0.13629	1.4231	0.286404
P/S	0.16445	0.16445	1.7172	0.247018
P/S^2	0.05044	0.05044	0.5267	0.500550
t x T	0.10498	0.10498	1.0962	0.343060
Tt x P/S	0.55652	0.55652	5.8111	0.060817
T x P/S	0.49210	0.49210	5.1385	0.072727

Where: SS-sum of squares; MS-mean square; F-f number and *p*-probability.

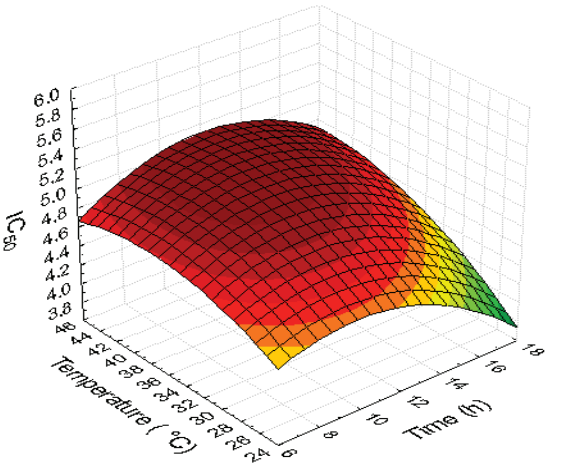

Figure 2. Surface plot of the IC50 as function of time and temperature.

Table 5 shows that none of the factors have influenced significantly the phenol content. Within the range analyzed, regardless of the operating conditions chosen, the higher levels of phenols were 31.89 to 47.82. The highest phenol content was obtained at the lower level of time (6 h), the higher level of temperature (45 ºC) and the central level of the ratio P/S (30%).

Table 5. Summary of ANOVA analysis by response surface on phenol content.

Effects	SS	MS	F	p
t	9.500	9.500	1.1015	0.341996
t^2	1.505	1.505	0.1744	0.693519
T	0.022	0.022	0.0025	0.961936
T^2	8.084	8.084	0.9373	0.377433
P/S	6.971	6.971	0.8083	0.409837
P/S^2	50.893	50.893	5.9006	0.059445
t x T	32.002	32.002	3.7104	0.112028
Tt x P/S	6.330	6.330	0.7340	0.430738
T x P/S	5.179	5.179	0.6004	0.473453

Where: SS- sum of squares; MS-mean square; F-f number and *p*-probability.

Table 6. Summary of ANOVA analysis by response surface on bergenin content.

Effects	SS	MS	F	p
t	4.186	4.186	3.107	0.138263
t^2	5.127	5.127	3.805	0.108564
T	2.515	2.515	1.867	0.230070
T^2	4.330	4.330	3.214	0.133010
P/S	22.383	22.383	16.613	0.009578
P/S^2	0.181	0.181	0.135	0.728683
t x T	0.579	0.579	0.430	0.540932
Tt x P/S	0.000	0.000	0.000	0.994249
T x P/S	0.028	0.028	0.021	0.891085

Where: SS-sum of squares; MS-mean square; F-f number and *p*-probability.

Table 7. Summary of ANOVA analysis by response surface on bergenin yield.

Effects	SS	MS	F	P
t	0.75149	0.75149	5.56230	0.064875
t^2	0.07714	0.07714	0.57093	0.483945
T	0.29102	0.29102	2.15403	0.202120
T^2	0.07805	0.07805	0.57770	0.481498
P/S	13.50562	13.50562	99.96405	0.000171
P/S^2	0.00138	0.00138	0.01023	0.923354
t x T	0.01354	0.01354	0.10023	0.764342
Tt x P/S	0.00360	0.00360	0.02668	0.876655
T x P/S	0.16889	0.16889	1.25009	0.314357

Where: SS-sum of squares; MS-mean square; F-f number and *p*-probability.

In Tables 6 and 7, it can be observed that P/S influences the bergenin content and yield both at 1% significance level, however in opposite forms. Bergenin content is directly proportional to P/S, but bergenin yield is inversely proportional. A possible explanation for this effect is that bergenin content and yield provide different information on the extractive process. As said before, bergenin contents are concentration of bergenin in fluid extracts. Bergenin yields are calculated comparing the mass of bergenin extracted to the weight of plant used in the experiment. Since bergenin contents show similar values to all P/S ratios, and bergenin yields decrease with P/S, is means that within the range of P/S studied the solvent was close to saturate with bergenin. Thus, based on bergenin yield, high P/S ratios are not recommended. These effects can be observed in Figures 3 and 4.

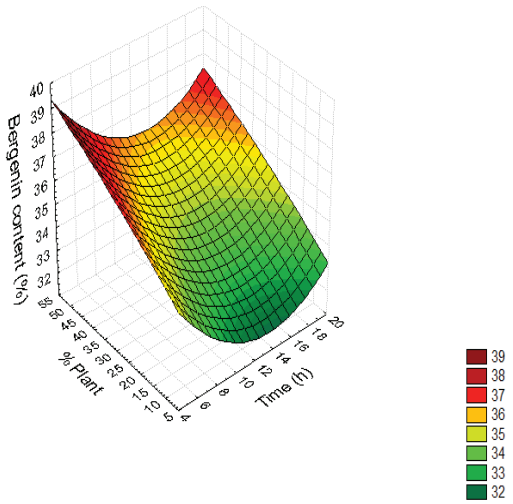

Figure 3. Response surface plot of bergenin content as function of time and P/S%.

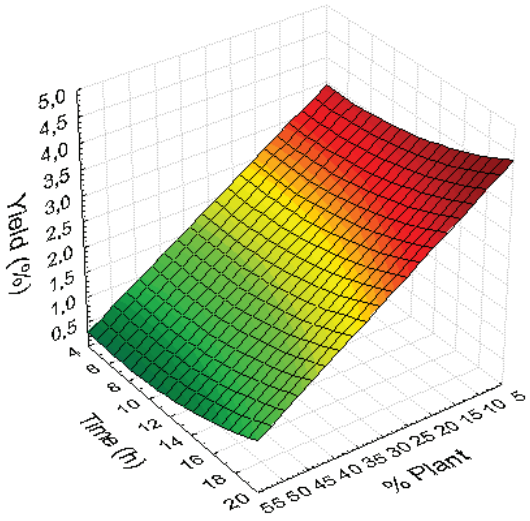

Figure 4. Response surface plot of bergenin yield as function of time and P/S%.

Furthermore, the fact that best antioxidant activities were observed for the very same extracts with higher contents of bergenin and phenols, may indicate the relationship between these compounds and the *Endopleura uchi* activities reported in folk medicine (Garrido et al., 2012; Nunomura et al., 2009; Politi et al., 2011).

Besides, the use of the statistical tool DOE, has enabled a better analysis of results and further exploration of the same, like analyzing possible interactions and indicating the appropriate levels for the process. (Peralta-Zamora et al., 2005; Couto et al., 2011).

Conclusion

The dynamic maceration parameters chosen for the extractive processes exerted a strong influence on the characteristics of the extracts, and could be altered to allow best extraction. The optimized uchi extract showed high bergenin and phenol content and antioxidant activity. The use of the statistical tool DOE can be very useful in designing future experiments in phytopharmaceutical technology.

Thus, the study of extraction process is essential in the development and standardization of *Endopleura uchi* (Huber) Cuatrec., Humiriaceae, extracts.

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