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Technical aspects on production of fluid extract from *Brosimum gaudichaudii* Trécul roots

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ABSTRACT

Instruction: Despite the increased use of Brosimum gaudichaudii roots as raw material on medicine to treatment of vitiligo, there are not studies that showing the impact of unit operations on the quality and standardized of the extract of B. gaudichaudii. The quality of the herbal extract is essential to ensure the safety and efficacy of pharmaceutical product. Due the medical and commercial importance, this study aimed to evaluate the impact of the extraction method (ultrasound or percolation) on the quality of herbal extract and optimize the extraction of psoralen and 8-methoxypsoralen (8-MOP) from B. gaudichaudii. **Materials and Methods:** The extraction recovery was evaluate by high-performance liquid chromatography (C8 reverse phase column and acetonitrile: Water 45:55 and flow rate 0.6 mL/min). The extraction was performed by ultrasound-assisted extraction (UEA) or percolation using a Box-Behnken design. **Results:** From both chemical markers (psoralen and bergapten), the optimal conditions for the UEA were an extraction time of 25 min, the mean particle size of 100 μm, and an ethanol: Water ratio of 55:45 (v/v). **Conclusion:** The extraction by percolation revealed that ethanol 55% was more efficient than ethanol 80% to extract psoralen and bergapten.

Key words: Box-Behnken design, optimization, percolation, ultrasound-assisted extraction

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INTRODUCTION

The extraction method of bioactive compounds is an important step in the manufacturing of herbal medicines, because secondary metabolites with therapeutic potential are usually found in small quantities in plant materials. New extraction methods must be developed or optimized to increase yields and selectivity of the process. Several extraction methods exist, including those employing (i) heating maceration, (ii) refluxing, (iii) Soxhlet extraction, (iv) supercritical fluids.[1-5] Same this techniques generally require long extraction time, organic solvents toxics that may have potential negative on human health and environment.[6-8] Among these technics, the ultrasound-assisted extraction (UEA) and percolations associate with chemometrics technics as response surface methodology (RSM) are commonly used in pharmaceutical industries. Due to their advantages over other extraction technologies, including operational flexibility, low cost, high yield, increase energy efficient and they applicability for heat-sensitive materials.[9-12]

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Now novel medicines for the treatment of vitiligo and psoriasis have been developed with *Brosimum gaudichaudii* Trécul roots. The roots of this species has a high amount of psoralen and bergapten 8-methoxypsoralen (8-MOP) that are photosensitizing agents in PUVA therapy. Moreover, *B. gaudichaudii* has antifungal, antiviral, and antimicrobial properties and is also used atopic dermatitis.[13,14]

Psoralen and bergapten may be obtained by extraction from herbal species or chemical synthesis. However, recovery in the extraction is low, and the synthesis is expensive and generates toxics waste. Accentuating the importance of developing high-performance extraction processes for *B. gaudichaudii*.[13,15]

Despite the increased use of *B. gaudichaudii* roots as raw material on medicine, there are not studies that showing the impact of unit operations on the quality and standardized of the *B. gaudichaudii* extract. The quality of the herbal extract is essential to ensure the safety and efficacy of pharmaceutical product.

Due to the medical and commercial importance of psoralen and bergapten, this study aimed to evaluate the impact of the extraction method on the quality of herbal extract

and optimize the extraction of psoralen and 8-MOP from *B. gaudichaudii*.

MATERIALS AND METHODS

Herbal material

The roots of *B. gaudichaudii* Trécul were collected in the city of Jussára, Goiás, Brazil. The species was identified, and a voucher was stored at the UFG Herbarium (UFG-45.517).[1-3] The herbal material was dried at 40°C in forced ventilation and ground in a knife mill.

Reagents

The ethanol used in the extraction procedure was of analytical-reagent grade (95%.v/v). High-performance liquid chromatography (HPLC)-grade acetonitrile was purchased from Merck.

High-performance liquid chromatography-photodiode array detector psoralen and bergapten analysis

High-performance liquid chromatography analyses of herbal extracts and powered roots were performed using a Waters HPLC system (Alience), e2695 separation module, e2998 photodiode array detector, and Empower 3 data processing system.

The separations were performed according to the methodology developed by Martins *et al.* 2011.[16] The following analysis conditions were used: A C8 reverse phase column (Luna 250 × 4.6 mm. Phenomenex®), acetonitrile: Water (45:55) mobile phase, the flow rate of 0.6 mL/min, and detection wavelengths of 244 nm for psoralen and 220 nm for bergapten. The analytical method was validated according to guideline Q2 (R1) from the International Conference on Harmonization.[17]

Evaluation of degradation of psoralen and bergapten by ultrasound

A previous study of stability was done with markers, psoralen and bergapten. A solution hydroethanolic of psoralen and bergapten (2 mg/mL) was kept for 50 min in the ultrasound bath (37°) (UNIQUE® USC 4800, 40 KHz). A control solution in the same contraction was made, and the areas of chemical makers were compared by HPLC.

EXPERIMENTAL DESIGN

Extraction by ultrasound-assisted extraction

The UEA was performed in an ultrasonic bath (UNIQUE® USC 4800, 40 KHz). The extractions were performed in 25 mL volumetric flask with 50 mg of powered roots and 25 mL of the hydroethanolic mixture. The volumetric flask was partially immersed in the ultrasonic bath (37°C) and ultrasonicated for a predetermined time [Table 1], and the extracts were analyzed by HPLC.

The influence of ultrasonication on the psoralen and bergapten yield was evaluated using a 3^3 factorial drawing (Box-Behnken) with 17 experimental runs, including five replicates at the center point. The effects of variability in the observed response due to extraneous factors were minimized by randomizing the order of the experiments. The factorial design matrix was comprised of extraction time (X_1), particle size (X_2) and ethanol: Water ratio (X_3), as shown in Table 1.

The experimental data were fitted to a second-order polynomial model, and the regression coefficients were obtained by analyses of variance (ANOVA). The generalized second-order polynomial model used in the RSM analysis was as follows (Equation 1): Where *y* is the dependent variable, β_0 is a constant term, *k* is the number of variables, β_i represents the coefficients of the linear parameters, β_{ii} represents the coefficients of the quadratic terms, and β_{ij} represents the coefficients of the interaction parameters. Matlab R2009b software was used to generate response surfaces (RS). To verify the model's predictive capability, the optimal conditions were established by RSM and the predicted, and experimental values were compared by experimental verification using the presumed optimal conditions.

$$y = \beta_0 + \sum_{i=1}^{k} \beta_i x_i + \sum_{i=1}^{k} \beta_{ii} x_i^2 + \sum\sum \beta_{ij} x_i x_j \quad (1)$$

Extraction by percolator

Stainless steel percolator (5 L) was used to obtain the percolated extract. The percolation lasted 16-25 days with a flow of 0.2 mL/min of hydroethanolic mixture (55% v/v) or (80% v/v). The test was evaluated in triplicate, and used 1 kg powder roots (355 μm) per replicate.

RESULTS AND DISCUSSION

It can be observed that all the system suitability parameters were in accordance with the literature

Table 1: Coded factors and respective levels in the factorial design

Factors	Level		
	−1	0	+1
(X_{E1}) Extraction time (min)	5	20	35
(X_{E2}) Particle size (μm)	50	150	250
(X_{E3}) Ethanol: Water ratio (% v/v)	35	65	95

specifications [Table 2]. Thus, the HPLC system and procedure showed to be capable of providing data of acceptable quality. Performing the selectivity test, it was found for all sample that there was no compound interfering with the retention time of psoralen and 8-MOP. Furthermore, well-resolved peaks indicate the specificity of the method [Figure 1].

Table 3 resumes the parameters values obtained from method validation. The calibration curves showed a linear response obtaining correlation coefficients (*r*) 0.999 to psoralen e0.998-8-MOP. The LOD (0.044 μg/mL to psoralen and 2.25-8-MOP) and LOQ (0.14 to psoralen and 14.12 to bergapten) showed that the present method has adequate sensitivity to detect and quantification of psoralen and bergapten in the prepared sample.

The stability study showed that content of psoralen and 8-MOP ware not altered by the action of ultrasound, there was a range of <1% between the sample area and the control.

Table 2: System suitability parameters values to psoralen and bergapten from *B. gaudichaudii*

Parameter	Psoralen	8-MOP	Recommendations
Repeatability	<0.1%	<0.3%	RSD<1% to *n*>5
Tailing factor (*T*)	1.0	1.1	<2.0
Theoretical plates (*N*)	13,523	14,023	>2000

B. gaudichaudii: Brosimum gaudichaudii; MOP: Methoxypsoralen; RSD: Relative standard deviation

Table 3: Validation parameters values obtained from HPLC-PDA method for the determination of psoralen and bergapten (8-MOP) from *B. gaudichaudii*

Parameter	Psoralen	8-MOP
RT		
Minute	12.5	16.07
Linearity		
Linearity range (μg/mL)	1.0-16.0	80-160
Sensitivity		
LOD, μg/mL	0.044	2.25
LOQ, μg/mL	0.14	14.12
Precision		
RSD %	0.1	0.15
Accuracy (%)		
Recovery 80	100.54%±0.08[a]	100.3%±0.19[a]
Recovery 100	100.45%±0.44[a]	99,62%±0.10[a]
Recovery 120	100.88%±0.7[a]	100.4%±0.25[a]
Robustness (%)		
Changing column mark/RSD	<0.1	
Temperature of column/RSD		
Ratio of solvent/RSD		

[a]Data expressed as mean±SD. RT: Retention time; SD: Standard deviation; RSD: Relative standard deviation; HPLC: High performance liquid chromatography; PDA: Photodiode array detector; MOP: Methoxypsoralen; LOD: Limit of deteccion; LOQ: Limit of quantification; *B. gaudichaudii: Brosimum gaudichaudii*

The UEA yields (EY %) and ANOVA are summarized in Tables 4 and 5, respectively. This analysis showed that X_{E3}, X_{E2}^2 and X_{E3}^2 significantly affected the EY % of psoralen. The EY % of bergapten was influenced by the interaction between X_{E2}, X_{E3} and X_{E2}^2.

Figures 1 and 2 show the surface response plot for the EY % of psoralen and bergapten. The factors that most influenced the psoralen and bergapten extractions were the quadratic interaction of particle size (X_{E2}^2) and the linear interaction between X_{E2} and X_{E3}, respectively [Table 5]. The F-test for the psoralen extraction revealed a 2.2% probability for F to be interpreted as noise and a 1.55% probability for the bergapten value to be interpreted as noise.

Table 4: 3^3 Box-Behnken factorial design matrices and result of UAE

X_{E1}	X_{E2}	X_{E3}	Psoralen (%w/w)	Bergapten (%w/w)
25	250	75	0.100	0.478
15	50	55	0.101	0.472
5	50	75	0.093	0.428
15	250	95	0.081	0.444
15	150	75	0.099	0.461
15	250	55	0.091	0.400
25	50	75	0.093	0.433
15	150	75	0.102	0.475
25	150	55	0.110	0.487
5	150	55	0.098	0.460
25	150	95	0.096	0.458
5	150	95	0.098	0.451
15	50	95	0.072	0.393
15	150	75	0.094	0.449
15	150	75	0.094	0.436
15	150	75	0.097	0.457
5	250	75	0.096	0.413

UAE: Ultrasound-assisted extraction

Table 5: Summary of factor effects and significances (*P*) ANOVA

Factors	Coefficients (% (w/w))	
	Psoralen	Bergapten
Intercept	+0.097	+0.46
X_{E1}×extraction time	$+1.65\times10^{-3}$	+0.012[a]
X_{E2}×particle size	$+1.05\times10^{-3}$	$+2.0\times10^{-3}$
X_{E3}×ethanol water ratio	-6.60×10^{-3}[b]	-9.53×10^{-3}
$X_{E1}\times X_{E2}$	$+7.26\times10^{-3}$	+0.015
$X_{E1}\times X_{E3}$	-3.656×10^{-3}	-6.16×10^{-3}
$X_{E2}\times X_{E3}$	$+5.03\times10^{-3}$	+0.029[b]
X_{E1}^2	$+6.14\times10^{-3}$[c]	$+9.11\times10^{-3}$
X_{E2}^2	-7.65×10^{-3}[c]	−0.027[b]
X_{E3}^2	-2.87×10^{-3}	$+4.02\times10^{-4}$
Lack of fit	2.49	0.75

Significant: [a]0.1%; [b]1%; [c]5%. ANOVA: Analysis of variance

The lack-of-fit values were 2.49 for psoralen and 0.75 for bergapten; neither of these values was significant ($P < 0.05$) in the models, indicating that the model was well fit by Equations 2 and 3. The lack of fit of the model indicates whether the estimated RS represents the actual shape of the surface. An r^2 value (multiple correlation coefficient) closer to one denotes better correlation between the observed and predicted values. In this case, the high values of r (0.87 and 0.88) indicate good correlation between the experimental and predicted values.

$$Tp = 0.097 - 6.60 \times 10^{-3} X_E{}^3 + 6.14 \times 10^{-3} X_E 1^2 - 7.65 \times 10^{-3} X_E 2^2 \quad (2)$$
$$r = 0.87$$

$$Tb = +0.46 + 0.012.X_E{}^1 + 0.029.X_E{}^2.X_E{}^3 - 0.027.X_E 2^2 \quad (3)$$
$$r = 0.88$$

The optimal theoretical extraction parameters for psoralen (0.111% w/w) and bergapten (0.491% w/w) from *B. gaudichaudii* roots were a 25 min extraction, 100 μm particle size, and ethanol: Water ratio of 55%. The verification test showed that the psoralen and bergapten contents obtained from extraction under optimal conditions were 0.113 ± 0.001% w/w (n = 3) and 0.497 ± 0.002% w/w (n = 3), respectively. The good correlation between the theoretical results and the rechecked values confirmed that the response model represented the expected optimization well.

These results collaborate those of Celeghini *et al.,* (2007), who evaluated the influence of the ethanol: Water (v/v) ratio on the extraction of psoralen and bergapten from *Dorstenia brasiliensis* L. roots. The optimal value of the ethanol/water mixture obtained in the previous work was 1:1. The same author also evaluated the influence of extraction time on process efficiency, reporting an optimal extraction time of 30 min.[18]

The percolation extraction was run for 15 days. In the first day the content of psoralen and 8-MOP were 20.39 μg/mL and 73.43 μg/mL when using ethanol 80% and 25.44 μg/mL e80.22 μg/mL to ethanol 55%. After 15 days of extraction the contents of psoralen reduced 99.2% and 97% to 8-MOP, however the content of psoralen and bergapten extracted by ethanol 55% was higher (1.1% to psoralen 2.9% and to 8-MOP) than extracted by 80% ethanol (0.7% to 2.3% and to psoralen 8-MOP) Figure 3.

The percolation method extracted 10-fold and 6-fold more psoralen and bergapten, respectively, than the ultrasonic extraction method. However, the extraction of 1.1% psoralen and 2.9% bergapten was 98 times faster using the ultrasound-assisted method [Figure 4].

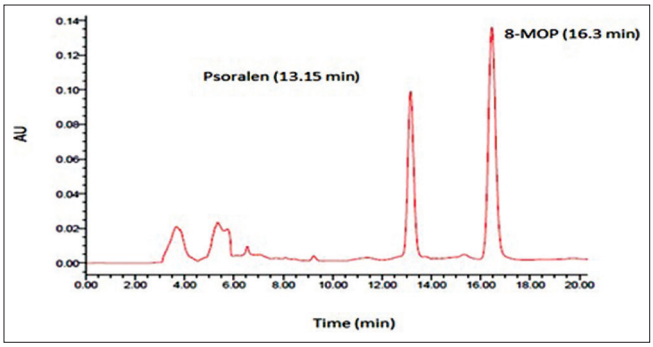

Figure 1: High-performance liquid chromatography-photodiode array detector chromatograms of *Brosimum gaudichaudii* extract obtained at 222 and 244 nm. The first peak is psoralen (13.15 min), and the second is bergapten (16.3 min). Chromatographic conditions: Column C8 (Luna 250 × 4.6 mm. Phenomenex®), mobile phase (acetonitrile: Water 45:55), a flow rate of 0.6 mL/min

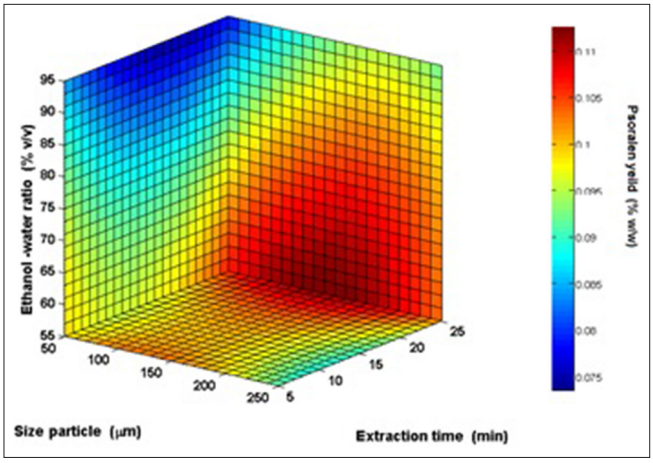

Figure 2: Surface plot of psoralen content as a function of ethanol water ratio, particle size and extraction time

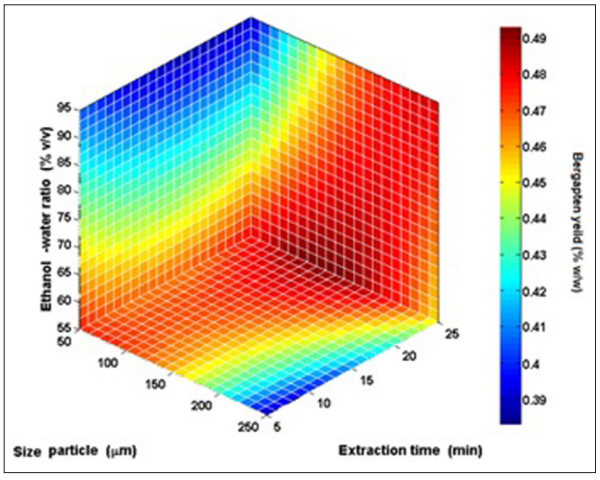

Figure 3: Surface plot of the bergapten content as a function of ethanol water ratio, particle size and extraction time

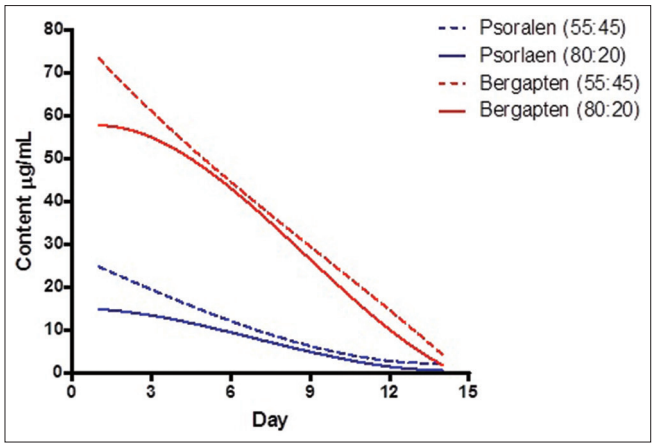

Figure 4: Decrease contents of psoralen and bergapten in function of time. Extraction condition: (- - -) extract with ethanol: Water (55:45), (___) ethanol: Water (80:20), and flux of 0.2 mL/min

The higher extraction yields obtained by the ultrasound-assisted method may be attributed to the effects of acoustic cavitations produced in the solvent. The ultrasonic wave also exerts a mechanical effect, allowing greater penetration of the solvent into the herbal matrix, which increases the contact surface between the solid and liquid phases and encourages the solute to diffuse from the solid phase into the solvent.[19-21] Several authors have reported high efficiencies for the UEA of foods and bioactive compounds.[22-24]

Optimization studies are important for predicting the extraction behavior of herbal compounds of interest in terms of controllable factors, such as extraction time, alcohol content, and particle size, to predict and minimize the costs involved in the production of herbal extracts.

CONCLUSION

The results of this study indicate that UEA and percolation were effective approaches to psoralen and bergapten extraction from *B. gaudichaudii* Trécul roots. However, UEA leads to a more rapid extraction than the percolation method. These results justify the use of ultrasonic extraction in industrial and laboratory extraction processes.

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