Betulinic Acid from *Zizyphus Joazeiro* Bark Using Focused Microwave-Assisted Extraction and Response Surface Methodology

Fernanda Carolina Sousa Fonseca, Letícia Caribé Batista Reis, Jener David Gonçalves dos Santos, Carla Rodrigues Cardoso Branco, Sergio Luis da Costa Ferreira[1], Jorge Mauricio David[1], Alexsandro Branco

Department of Health, State University of Feira de Santana, Feira de Santana, [1]Department of Organic Chemistry, Federal University of Bahia, Salvador, Bahia, Brazil
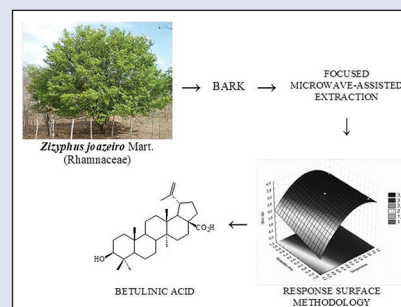
ABSTRACT

Background: The effect of the extraction time (min) and temperature (ºC) on the yield of betulinic acid (BA) from *Zizyphus joazeiro* barks using focused microwave-assisted extraction was investigated. **Materials and Methods:** The ethyl acetate was used as extractor solvent because it was shown to provide a betulinic acid-clean extract. A full two-level statistical factorial design was applied to determine the important effects and interactions of these independent variables upon the yield of BA. **Results:** The conditions that produced the highest yield of BA were at temperature of 70 ºC and an extraction time of 15 min (3.33 mg per gram of plant). **Conclusion:** The BA has drawn attention due to its use as a raw material in the synthesis of active compounds against the Human Immunodeficiency Virus (HIV).

Key words: Betulinic acid, experimental design, focused microwave-assisted extraction, *Zizyphus joazeiro*

SUMMARY

- The extraction of betulinic acid (BA) from *Zizyphus joazeiro* barks using focused microwave was investigated
- A full two-level statistical factorial design was applied to determine the effects and interactions of the independent variables (time and temperature) upon the yield of BA
- The reversed-phase high-performance liquid chromatography with diode-array detector was used for quantification.

Access this article online
Website: www.phcog.com
Quick Response Code:

INTRODUCTION

Zizyphus joazeiro Mart., (Rhamnaceae), a tree growing in Northeastern Brazil, is widely used in traditional Brazilian medicine for the treatment of fever, chronic bronchitis, gastric ulcers, blood diseases, and headaches,[1] and its barks bioproduce betulinic acid (BA).[2-4] BA has a range of biological activities that can be used in the treatment of Type-II diabetes, obesity,[5] and cancer.[6] Recently, this triterpene pentacyclic has drawn attention due to its use as a raw material in the synthesis of numerous potentially active compounds, mainly against human immunodeficiency virus.[7-10]

In the extraction process, the selective solubility in an organic solvent coefficient is a significant effect in the design and optimization of the separation of natural products. Chebil *et al.*[11] showed that the solubility of flavonoids (quercetin, isoquercitrin, rutin, chrysin, naringenin, and hesperetin) was strongly affected by both the nature of the solvent and the flavonoid structure. The conventional extraction with organic solvents, such as static maceration or reflux, is widely used to obtain triterpenes. However, there has recently been an increasing demand for new, more efficient, economical, and eco-friendly extraction techniques. Examples of these techniques include the use of microwave-assisted extraction (MAE),[12] ultrasonic extraction,[13] supercritical fluid extraction,[14] and pressurized solvent extraction.[15]

MAE offers a rapid delivery of energy to the total volume of a solvent and causes rapid heating due to collisions with surrounding molecules.[12] In a previous study on the extraction of BA from *Z. joazeiro* bark by maceration, reflux, and focused MAE (FMAE) were compared.[16] This paper describes the optimization of FMAE of BA [Figure 1] from the same plant using the response surface methodology.
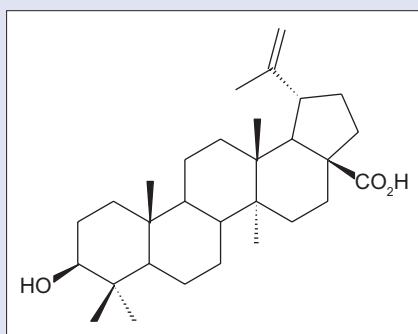
MATERIALS AND METHODS

Reagents and equipment

The ethyl acetate and ethanol used were analytical grade (Synth-Brazil®). The BA that was used as a standard was purchased from Sigma-Aldrich®.

Figure 1: The chemical structure of betulinic acid

Table 1: Yield (mg/g) of betulinic acid using focused microwave-assisted extraction

Assay	Variables		Yield
	T (°C) (X_1)	Extraction time (min) (X_2)	Betulinic acid (mg)
1	75 (1)	20 (1)	3.25
2	75 (1)	10 (−1)	3.09
3	65 (−1)	20 (1)	2.29
4	65 (−1)	10 (−1)	1.33
5c	70 (0)	15 (0)	3.33
6c	70 (0)	15 (0)	3.32
7c	70 (0)	15 (0)	3.33

High-performance liquid chromatography (HPLC)-grade methanol and acetonitrile were purchased from Vetec®. A Milli-Q System® (Bedford, MA, USA) was used to purify the water. FMAE was performed using a discover microwave system (CEM), which operates at a maximum power of 300 W. The system provides constant feedback control of the extraction temperature through the continuous monitoring of the solvent temperature in the control vessel. The quantification of BA from the samples treated with FMAE was carried out by means of HPLC. The HPLC apparatus was equipped with a VRW HITACHI L-2130 pump, a VRW HITACHI L-2300 diode array detector and an auto sampler with a 100 μL loop. The data acquired were processed using the Ezchrom Elite software (Agilent Technologies Inc., California, USA).

Plant material

Barks of *Z. joazeiro* Mart., Rhamnaceae, were collected in Feira de Santana, state of Bahia, Brazil, in 2008. A voucher specimen (HUEFS 61790) was deposited at the Herbarium of the State University of Feira de Santana, Brazil.

Experimental design

The results obtained in our laboratories prior to the preliminary extraction of the BA from *Z. juazeiro* were used here for the decision on the choice of the variables.[16] Thus, the two variables (temperature and extraction time) were monitored using a full two-level factorial design [Table 1] for the BA extraction. Three repetitions of the central point (c) to a total of seven experiments were performed to estimate the possible pure error, and the experimental treatments were varied randomly to detect the presence of possible systematic errors. The regression analysis allowed the obtaining of the mathematical model:

$$Y = \beta_0 + \beta_1 X_1 + \beta_2 X_2 + \beta_{11} X_1^2 + \beta_{22} X_2^2 + \beta_{12} X_1 X_2 \tag{1}$$

The coded (−1; +1) levels were used for each independent variable: The −1 level corresponds to the lower value of each variable and + 1 to the upper one. These limits were selected on the basis of previous studies[16] and the optimization procedure was carried out within these domains. Concerning the Eq. 1: Y is the predicted response, X_1 and X_2 are the independent variables, b_0 is the offset term, β_1 and β_2 are the linear effects, β_{11} and β_{22} are the squared effects, and β_{12} is the interaction term. All the calculations and graphics in this work were performed using the Statistica® software, including the analysis of variance for the responses of this study. The efficiency of the extraction was calculated as follows: Percentage of extraction (w/w) = mass of extracts/mass of dried material (bark) ×100. Each sample (1 g) was placed in a 100 mL flat-bottomed narrow-neck flask topped by a vapor condenser and suspended in ethyl acetate (30 mL).

Reversed-phase high-performance liquid chromatography-diode array detector quantification

An aliquot of 20 μL of each sample was injected into an HPLC column (Purospher STAR® RP8e column, 250 mm × 4.6 mm, i.d., 5 μm particle size), and elution was carried out using an 80:20 acetonitrile:acidified water (with 0.05% phosphoric acid) solution at a flow rate of 1 mL/min in a 35°C oven. The eluate was monitored at a detection wavelength of 205 nm. The chromatographic conditions were based on those used in previous studies; however, some modifications were made to enhance HPLC separation.[17-19] The procedure was repeated three times for each sample. Samples precisely weighed obtained using the FMAE technique were dissolved in methanol in an ultra-sonic bath for 10 min and filtered through a 0.45 μm filter before injection. The method was validated by an external calibration curve using standard BA solutions prepared in methanol in ten different concentrations, ranging from 0.01 to 0.1 mg/mL. Each solution was injected three times, and the curve was constructed on Microsoft Office Excel 2007 using the average of the area.

Linearity

The linearity was checked by preparing standard solutions of BA at 10 concentrations ranging from 0.01 to 0.1 mg/mL. A calibration curve was plotted using the standard BA data, which resulted in the equation $y = 41307171.5152x + 88910.6667$, which was found to be linear ($r^2 = 0.9979$) in the specified concentration range. The detection and quantification limits were also calculated on the basis of signal-to-noise ratios of 3 and 10, respectively; the detection limit was 0.002 mg/mL, and the quantification limit was 0.013 mg/mL.

Precisions and accuracy

Intra- and inter-day measurements were utilized to determine the precision of the assay method developed; these measurements were conducted using three different working solutions prepared with standard BA (0.02, 0.04, and 0.08 mg/mL). Each solution was injected into the HPLC apparatus in triplicate, and variations were expressed by the relative standard deviations (RSD). The accuracy was expressed as the agreement between the set reference value and the measured value (96.84, 92.88, and 93.54%). The calculated RSD and accuracy values were well within the accepted limits.

The chromatograms of the extractions obtained though FMAE and the standard BA standards show a separate distinct peak for the BA. The retention time for BA was the same for all samples analyzed, which emphasizes the specificity of the HPLC method.

Specificity

The specificity was determined through the analysis of all chromatograms, where the chromatographic peaks of BA were confirmed by comparing their retention time, ultraviolet (UV) spectra, and purity. The specificity of the method was also determined by comparing the UV spectra of the standards and the FMAE samples. All of the samples analyzed showed the same profile of absorption at 205 nm. To further improve the specificity of the method, the purity of the peak was calculated by overlaying the UV spectra in three different regions, which showed similarity values above 0.99.

RESULTS AND DISCUSSION

Figure 2 shows the chromatography profile by means of HPLC-diode-array detection (DAD), and it was possible to confirm that the ethyl acetate solvent showed a clean extraction to BA using FMAE when compared with ethanol. Thus, to evaluate the optimum experimental conditions for the BA extraction by means of FMAE, we employed a factorial design to assess the influencing factors that are involved in the yield: Time and temperature. The calculated $F = 21.01$ obtained from the ratio 1.1210/0.05356 was greater than the tabulated $F = 9.27$ for 3° of freedom at the 95% confidence level (F 0.95; 3; 3), indicating that treatments are significantly different. The highest BA yield (3.33 mg/g of plant) was obtained when barks of *Z. joazeiro* were extracted at 75°C for 15 min [Table 1]. The second-order polynomial

equation that represents the behavior of the yield of BA was obtained, describing the response surface contour lines (Eq. 2).

$$Y = 3.32 + 0.682X_1 - 0.8358X_1{}^2 + 0.282X_2 - 0.2025X_1X_2 \qquad (2)$$

The contour lines on the top surface [Figure 3], corresponding to the experiment 1 [Table 1], show that the BA values, predicted by the model, increase substantially with the temperature increase and that is consistent with the positive value of the coefficient X_1. The absolute difference between the coefficients X_1 and X_2 indicates that temperature contributes positively, about two times more regarding the extraction time. The negative interaction X_1X_2 indicates that there is no synergism between the extraction time and temperature and that the linear model (Eq. 2) is superior to the quadratic model.

As for the temperatures, elevated temperatures should result in higher extraction efficiencies because they give the solvents a greater capacity to dissolve the analytes. Surface tension and solvent viscosity also decrease with temperature, improving matrix penetration.[20] The BA yields are changed as temperature increases. The results reveal that under 65°C, the yield of the 20 min extraction is almost double that of the 10 min extraction. As the temperature is increased, the percentage yields of BA become more similar, regardless of the extraction time. The results obtained confirm that the ideal parameters in this study are those with intermediate values.

In accordance with the information described previously in the experimental design, for a 10 min extraction time, when the temperature is below 65°C, the yield is about 2.3 times lower than when a temperature of 75°C is applied. If the irradiation time is extended with additional 10 min, the extraction yield maintains the same average, suggesting that the time of extraction can be reduced. That was also observed in the extraction of triterpenoid saponins from *Ganoderma atrum,* where the recovery of saponins decreased when extraction time was increased beyond 20 min.[20] Based on the results so far, 15 min was found to be an optimum operating time for the FMAE of BA from *Z. Joazeiro*.

The microwave energy reaches and exceeds the boiling point of the solvent in a matter of seconds, negatively affecting the reaction. For this reason, the maximum temperatures were previously defined. In a focused microwave apparatus, as the temperature reaches the value set, the power is reduced so that the extraction reaction does not exceed the maximum temperature point.[21] The power then remains at a lower level to maintain the temperature set for the entire reaction. Figure 4 shows the power utilized in all experiments, and one can note that a minimum
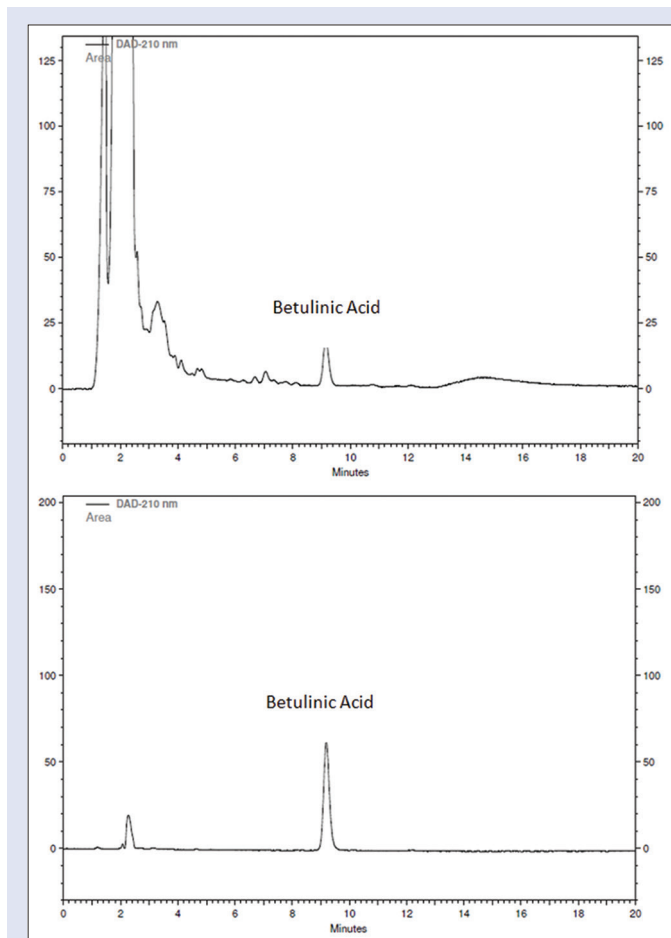
Figure 2: High-performance liquid chromatography-diode array detector chromatogram of betulinic acid extraction employing ethanol (upper) and ethyl acetate (down)
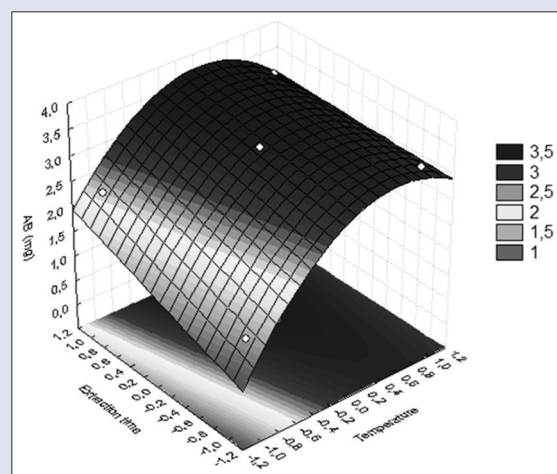
Figure 3: The yield of the betulinic acid from *Zizyphus joazeiro* Mart., Bark (mg) by response surface methodology
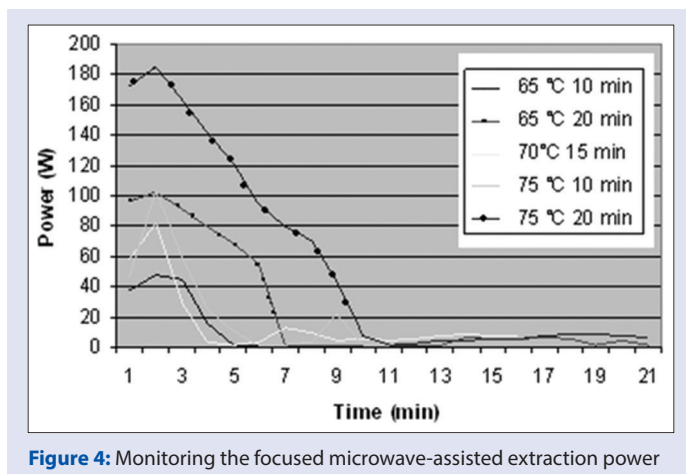
Figure 4: Monitoring the focused microwave-assisted extraction power

of a 2 min reaction time is necessary to achieve the temperature set. That is a long time for a typical microwave reaction; however, ethyl acetate is a low microwave-absorbing solvent, which means it takes much longer to be heated.

The response of the extract yield is an important parameter in industrial processes because the extraction and subsequent separation are the steps that involve higher costs, and the higher the yield of the process, the greater the concentration of the product of interest.

CONCLUSION

The extraction of betulinic acid from *Z. joazeiro* using focused microwave was optimized by the central composite experimental design. This study proves the suitability of the focused microwave-assisted extraction as a rapid, clean and efficient extraction procedure. Although these facts can be rationalized, the best temperature and time of extraction are 70°C and 15 minutes, respectively.

Conflicts of interest

There are no conflicts of interest.

REFERENCES

1. Schühly W, Heilmann J, Calis I, Sticher O. New triterpenoids with antibacterial activity from *Zizyphus joazeiro*. Planta Med 1999;65:740-3.

2. Higuchi R, Kubota S, Komori T, Kawasaki T, Pandey VB, Singh JP, *et al*. Triterpenoid saponins from the bark of *Zizyphus joazeiro*. Phytochemistry 2004;23:2597-600.

3. Barbosa-Filho JM, Trigueiro JA, Cheriyan UO, Bhattacharyya J. Constituents of the stem-bark of *Zizyphus joazeiro*. J Nat Prod 1985;48:152-3.

4. Schuhly W, Heilmann J, Çalis I, Sticher O. Novel triterpene saponins from *Zizyphus joazeiro*. Helv Chim Acta 2000;83:1509-15.

5. Choi JY, Na M, Hyun Hwang I, Ho Lee S, Young Bae E, Yeon Kim B, *et al*. Isolation of betulinic acid, its methyl ester and guaiane sesquiterpenoids with protein tyrosine phosphatase 1B inhibitory activity from the roots of *Saussurea lappa* C.B.Clarke. Molecules 2009;14:266-72.

6. Fulda S. Betulinic acid for cancer treatment and prevention. Int J Mol Sci 2008;9:1096-107.

7. Huang L, Ho P, Lee KH, Chen CH. Synthesis and anti-HIV activity of bi-functional betulinic acid derivatives. Bioorg Med Chem 2006;14:2279-89.

8. Qian K, Nakagawa-Goto K, Yu D, Morris-Natschke SL, Nitz TJ, Kilgore N, *et al*. Anti-AIDS agents 73: Structure-activity relationship study and asymmetric synthesis of 3-O-monomethylsuccinyl-betulinic acid derivatives. Bioorg Med Chem Lett 2007;17:6553-7.

9. Ganser-Pornillos BK, Yeager M, Sundquist WI. The structural biology of HIV assembly. Curr Opin Struct Biol 2008;18:203-17.

10. Dafonseca S, Coric P, Gay B, Hong SS, Bouaziz S, Boulanger P. The inhibition of assembly of HIV-1 virus-like particles by 3-O-(3',3'-dimethylsuccinyl) betulinic acid (DSB) is counteracted by Vif and requires its Zinc-binding domain. Virol J 2008;5:162.

11. Chebil L, Humeau C, Anthoni J, Dehez F, Engasser JM, Ghoul M. Solubility of flavonoids in organic solvents. J Chem Eng Data 2007;52:1552-6. [2013].

12. Beejmohun V, Fliniaux O, Grand E, Lamblin F, Bensaddek L, Christen P, *et al*. Microwave-assisted extraction of the main phenolic compounds in flaxseed. Phytochem Anal 2007;18:275-82.

13. Lavoie JM, Stevanovic T. Selective ultrasound-assisted extractions of lipophilic constituents from *Betula alleghaniensis* and *B. papyrifera* wood at low temperatures. Phytochem Anal 2007;18:291-9.

14. Khundker S, Dean JR, Jones P. A comparison between solid phase extraction and supercritical fluid extraction for the determination of fluconazole from animal feed. J Pharm Biomed Anal 1995;13:1441-7.

15. Lang Q, Wai CM. Supercritical fluid extraction in herbal and natural product studies – A practical review. Talanta 2001;53:771-82.

16. Fonseca FC, Santos JD, Branco A. Selective extraction of betulinic acid from *Zizyphus joazeiro* Mart. bark: A preliminary study. J Chem Pharm Res 2013;5:417-21.

17. Zhao G, Yan W, Cao D. Simultaneous determination of betulin and betulinic acid in white birch bark using RP-HPLC. J Pharm Biomed Anal 2007;43:959-62.

18. Guo S, Duan JA, Tang Y, Su S, Shang E, Ni S, *et al*. High-performance liquid chromatography – Two wavelength detection of triterpenoid acids from the fruits of *Ziziphus jujuba* containing various cultivars in different regions and classification using chemometric analysis. J Pharm Biomed Anal 2009;49:1296-302.

19. Kumar D, Mallick S, Vedasiromoni JR, Pal BC. Anti-leukemic activity of *Dillenia indica* L. fruit extract and quantification of betulinic acid by HPLC. Phytomedicine 2010;17:431-5.

20. Chen Y, Xie MY, Gong XF. Microwave-assisted extraction used for the isolation of total triterpenoid saponins from *Ganoderma atrum*. J Food Eng 2007;81:162-70.

21. Hayes BL. Microwave Synthesis: Chemistry at the Speed of Light. Matthews, NC: CEM; 2002.