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Research Article

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Selective extraction of betulinic acid from *Zizyphus joazeiro* Mart. bark: A preliminary study

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ABSTRACT

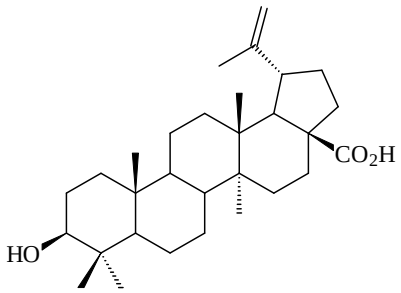
Betulinic acid is a pentacyclic lupane-type triterpenoid with a variety of biological applications; for example, its chemical derivative has antineoplastic action. The static maceration, heat reflux and focused microwave-assisted extraction of betulinic acid from Zizyphus joazeiro bark was investigated. Ethyl acetate was used as a solvent extractor due to its higher selectivity when compared to other solvents. Thus, the yield of the extracted betulinic acid ranged from 0.41 to 2.1 %. The conditions that produced the highest yield of betulinic acid (2.1 %) were a temperature of 77 ºC and an extraction time of 45 min by focused microwave-assisted extraction.

Keywords: betulinic acid, *Zizyphus joazeiro*, Rhamnaceae, focused microwave-assisted extraction.

INTRODUCTION

Zizyphus joazeiro Mart. (Rhamnaceae), which is a tree that grows in northeastern Brazil, is widely used in traditional Brazilian medicine for the treatment of fever, mycosis, chronic bronchitis and gastric ulcers [1, 2]. The chemical study of this species has led to was possibly isolated saponins and triterpenoids [2, 3, 4]. In the triterpene class, betulinic acid [3β-hydroxy-lup-20(29)-en-28-oic acid] (Figure 1) is found in *Z. joazeiro* bark at a considerable concentration and deserves great interest due to its several biological activities.

Figure 1: The chemical structure of betulinic acid



Betulinic acid (BA) shows several biological actions and medicinal properties such as inhibition of human immunodeficiency virus (HIV) and protein tyrosine phosphatase (treatment of type II diabetes and obesity) [5, 6]. BA is also a traditional molecule that is used in the treatment of several cancers [7]. Due to its selective cytotoxicity against melanoma and tumor cells [8], the action of BA can be compared to that of doxorubicin in human cell lines and toward neoplastic and non-neoplastic proliferation of normal lymphocytes. BA-increased inhibition was evident in all cell lines regardless of the position of the p53 protein, which is responsible for the proliferation of tumor cell [7]. Modification of the membrane potential of the mitochondria that produces reactive oxygen species and the

opening of transition channels can be responsible for apoptotic action that liberates apogenic mitochondrial factors, activates caspases and fragments DNA [9, 10].

However, BA has received more attention recently due to its use as a raw material for the synthesis of molecules that are more active against several cancers. It is known that pharmacological treatment of several cancer types have not shown the desired efficiency; therefore, new molecules should be investigated [11]. Structural modifications to BA have been made with the objective of achieving the highest action against cancer. Its nitrogenous derivatives showed excellent results as anti-proliferative drugs and have been used as pro-drugs because they are not toxic, not hemolytic and are more hydro-soluble that betulinic acid [12].

BA is undoubtedly an important natural compound that is used as a raw material for the synthesis of anticancer substances, and a few studies have shown conditions of its selective extraction from natural sources. However, this does not occur when using its biogenetic precursor betulin [13]. In this study, the extraction of betulinic acid from *Z. joazeiro* was investigated by conventional (static maceration and heat reflux) and focused microwave-assistance methods.

EXPERIMENTAL SECTION

Reagents and standard

Ethyl acetate and ethanol (95 %) were analytical grade (Synth-Brazil®). The betulinic acid that was used as a standard was purchased from Sigma-Aldrich®. TLC Silica Gel 60 (Merck®, Darmstadt, Germany) was used for the controlled analysis of the extractions. Hexane:ethyl acetate (1/1) was used as the mobile phase for TLC analysis, and a sulfuric acid solution (20 %) was used for revelation.

Plant material

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

Equipment

FMAE was performed using a Discover microwave system (CEM), which operated at a maximum power of 300 W. The system provided constant feedback control of the extraction temperature through the continuous monitoring of the solvent temperature in the control vessel. NMR spectra were obtained using a Bruker AC-500 (^{1}H: 500 MHz).

Effect of the solvent on the extraction of BA

One gram of powdered bark of *Z. joazeiro* was placed in 100-mL vials, 30 mL of each solvent was added, and extraction was achieved (ethyl acetate and ethanol 95 %) by focused microwave-assistance. The temperature (70 ºC) and extraction time (two hours) parameters were fixed to check the selectivity of the solvents for BA. After extraction, the samples were filtered and dried under reduced pressure. The efficiency of the extraction was calculated as follows: percentage extraction (w/w) = mass of extracts/ mass of dried material (bark) x 100.

Statistical analysis

All analytical determinations were performed in triplicate and the results are presented as average values ± standard deviations. Data were analysed using one-way analysis of variance (ANOVA) and the Tukey multiple comparison test was used to determine significant differences ($p < 0.05$) between mean values. SISVAR 5.3 (UFLA, Brazil) was the software used for data analyses.

RESULTS AND DISCUSSION

The selectivity of the extraction of natural products from plant material depends on various factors; and solvent choice constitutes one critical parameter for successfully obtaining a pure compound [14]. In the specific case of extraction of BA, the ethyl acetate and hydroethanolic solutions (95 %) were interesting solvent extractors that resulted in the highest recovery (1.75 and 1.86 %, respectively) in white birch (*Betula platyphylla* suk.) bark when compared to other solvents (dichloromethane, acetone, chloroform and methanol) [15, 16, 17]. Based on these studies, we first investigated the selective extraction of BA from *Z. joazeiro* bark using an ethyl acetate and hydroethanolic solution (95 %).

Figure 2. ^{1}H NMR spectra of ethyl acetate and ethanolic extracts

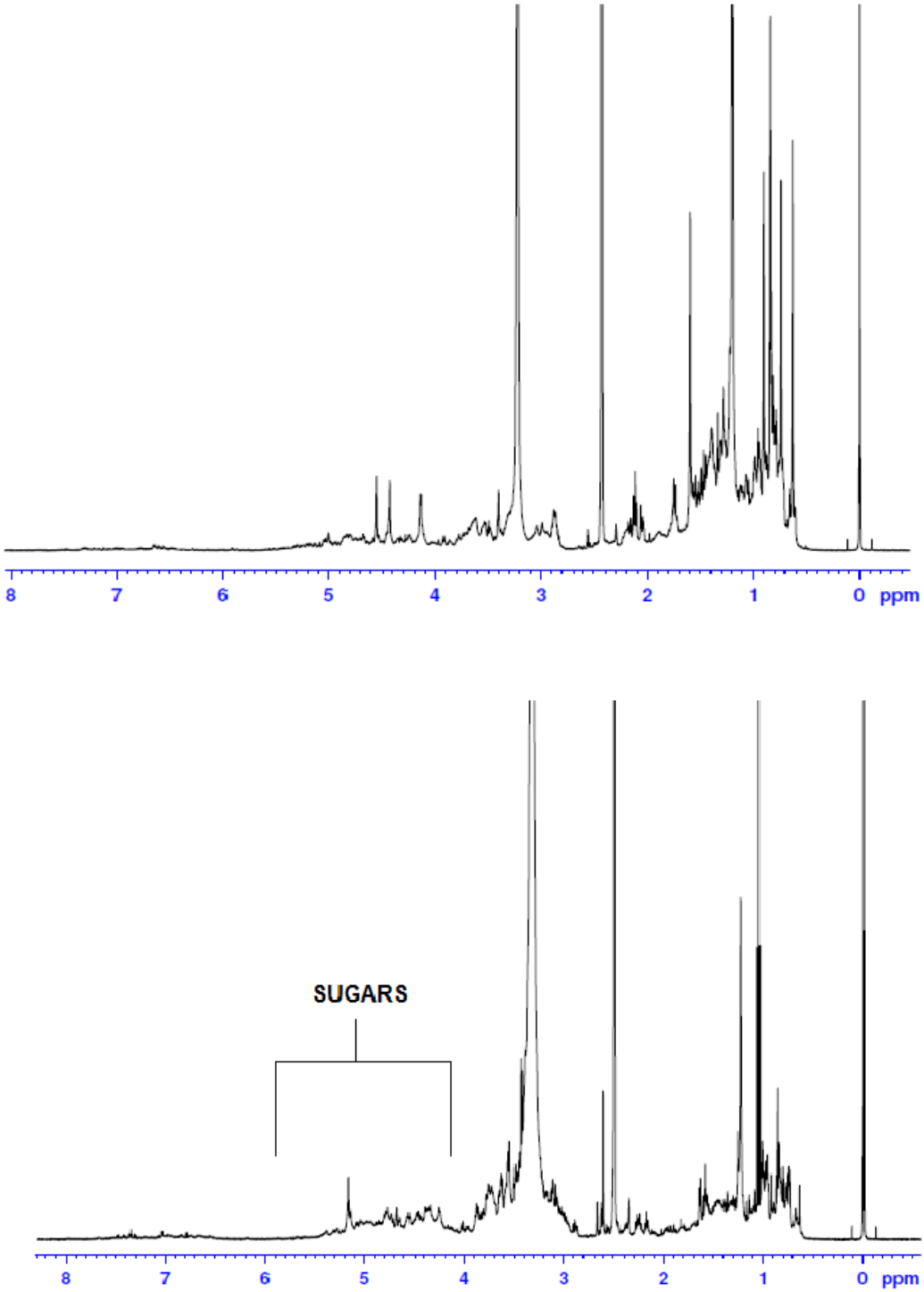
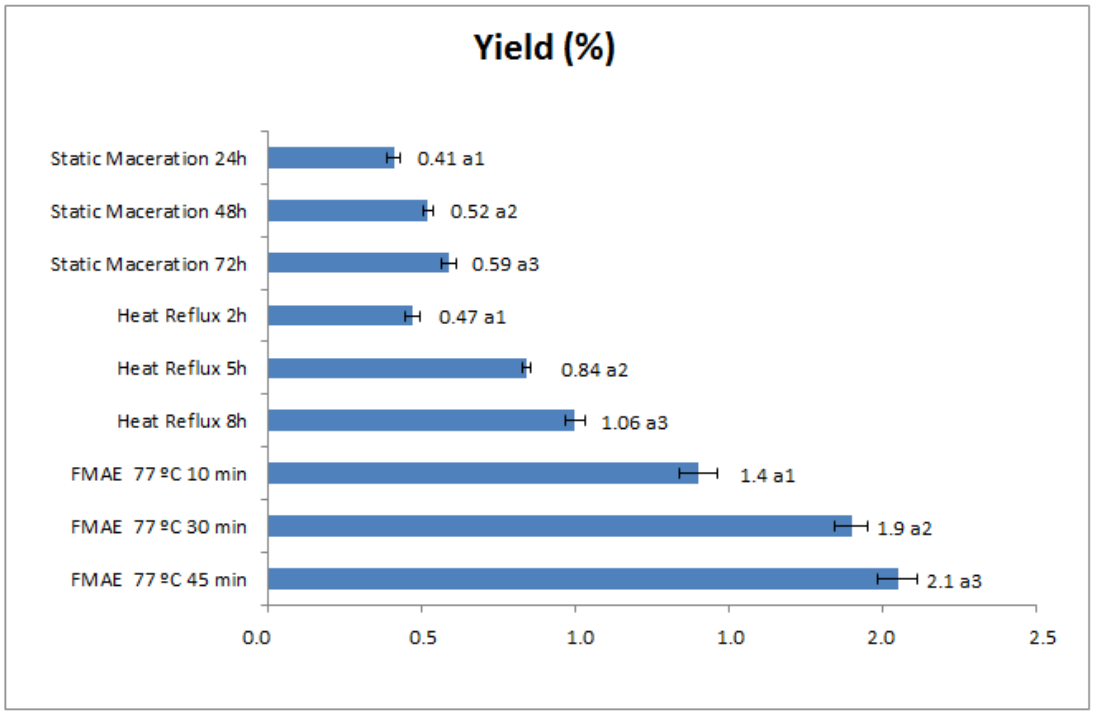


Figure 2 shows the ^{1}H NMR spectra of the extract that was obtained using the solvent extractors ethyl acetate and hydroethanolic solution (95 %). Its analysis confirms the high selectivity of the ethyl acetate solvent for BA extraction through the comparative intensities of the determinate signals. The ethyl acetate extract showed a majority of signals that were characteristic of a pentacyclic triterpene with seven methyl groups, two olefinic hydrogens and one carbinolic hydrogen. However, the ^{1}H NMR spectra of the hydroethanolic solution (95 %) showed low intensity of betulinic acid and additional signals in the sugars region between 4-5 ppm. Due to the high selectivity of the extraction of BA from *Z. joazeiro* bark, ethyl acetate was used as a solvent to compare the yields from different methods.

The BA extraction was performed using conventional (static maceration and heat reflux) and focused microwave-assisted methods. The times used for maceration and heat-reflux extraction were 24, 48, 72 and 2, 5, 8 hours, respectively. The temperature that was used for focused microwave-assisted extraction was the boiling point of the extracting solvent, ethyl acetate (77 °C), for the following times: 15, 30 and 45 minutes. Figure 3 shows the yields of the extractions.

The difference (0.47 %) between the yields that were obtained by the maceration (at 8 hours) and heat reflux methods (at 72 h) demonstrates the influence of temperature on the yield of BA when using these extraction techniques. The extraction of bioactive compounds by microwave-assisted extraction (MAE) has been shown to have many advantages over other conventional extraction methods, principally in yield versus time of operation [13, 18]. The application of microwave irradiation of the BA showed high yields at 45 minutes (2.1 %). This result was in agreement with other extractions using the same method. Thus, the extraction time that was used in the experiment (72 hours, 8 hours and 45 minutes) using FMAE was approximately eleven times faster than using heat reflux and 96 times faster than when using maceration.

Figure 3: The yield of extractives (%) obtained from the bark of *Zizyphus joazeiro* Mart. using different extraction techniques
For each method, columns with the same letter denote no statistically significant differences between samples (Tukey's p<0.05)



All extractions were monitored by TLC, which showed only one spot with the same time retention pattern of betulinic acid that confirmed the selective extraction of ethyl acetate as the solvent extractor in the conventional and microwave methods.

The selectivity of the extraction of the betulinic acid has not been cited in the literature. MeOH 95 % used in different techniques (continuous shaking, Soxhlet, ultra sonication and microwave-assisted) for extracting BA from *Ancistrocladus heyneanus* leaves with good yield [19]. The evaluation of the content of betulin and betulinic acids in Birch (*Betula pendula* Roth.) barks using 95 % ethanol and a sonication method and obtained the highest content [20]. However, both of the above-mentioned examples did not show selectivity for BA extraction. The conventional extraction methods used here showed low yields (w/w) when compared to the focused microwave method. It was indicated that, in addition to heat, the effects of microwave energy influenced the enhancement of secondary metabolites. Several works have described this fact, showing that microwave is an efficient method of extraction [21, 22].

The extraction of betulinic acid by ethyl acetate from *Z. joazeiro* using focused microwave energy was compared with conventional extraction methods (maceration and reflux). The solvent extractor that was used showed selectivity to betulinic acid, and the focused microwave extraction was a better method when using a time of 45 minutes and a temperature of 77 ºC (2.1 %).

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