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Comparison of betulinic acid extraction techniques from *Eugenia florida* DC (*Myrtaceae*) leaves

Abstract

Eugenia florida DC belongs to the Myrtaceae family. Previous phytochemical studies with leaves extracts of E. florida revealed the occurrence of triterpenes, such as betulinic acid (BA) which presents a vast literature with different biological activities: anti-inflammatory, antimalarial, antimicrobial, antiviral and anticancer. The objective of the present study was to evaluate the efficiency of different extraction methods (static maceration, dynamic maceration, percolation, soxhlet and ultrasonic waves) to extract BA from the leaves of *Eugenia florida*. The solvents and particle sizes were also evaluated. The results showed that the better solvents to extract BA were ethyl acetate and chloroform. The highest percentages of BA were obtained by percolation and maceration, probably due to the swelling of the plant material and the renewal of the solvent process. In the assays using different particle sizes of leaves of *E. florida*, relating to BA recovery and extraction yield, different extraction profiles were observed among the various solvents used. Ethyl acetate and chloroform showed no significant differences in both yields, however 50% of decreasing on extraction efficiency was observed when plant smaller particles were used with methanol and ethanol.

Keywords: betulinic acid, *Eugenia florida*, myrtaceae, extraction, HSP

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Abbreviations: BA, Betulinic Acid; HSP, Hansen Solubility Parameter; RED, relative energy difference; δD, dispersion bonds; δP, polar bonds; δH, hydrogen bonds; HEX, hexane; $CHCl_3$, chloroform; EtOAc, ethyl acetate; EtOH, ethanol; MeOH, methanol

Introduction

Eugenia florida DC. belongs to the Myrtaceae family.[1,2] Previous phytochemical studies with in leaves extracts of *E. florida* revealed the occurrence of triterpenes, and considered as an important source of betulinic acid (BA) (Figure 1), belonging to the lupanic group and presents many biological activities already described in literature, such as anti-inflamatory,[3,4] antimalarial, antiviral,[5–7] anti HIV[8,9] and antitumoral.[10–13]

E. florida, like other plants, presents in its composition a wide variety of substances forming a complex matrix. When a target molecule is desired, an important step is related to its extraction and recovery. The extraction process to obtain these compounds can be directly influenced by the time the material is in contact with the solvent, the degree of grinding of the plant, the extraction temperature, the polarity of the solvent, the solvent reactivity to the product to be extracted and the amount of solvent.[14] This study aimed to evaluate the efficiency of different extraction techniques to obtain BA from the leaves of *E. florida*.

Material and methods

Solvents and reagents

The following analytical grade chemicals were used: ethanol 99.8%, methanol 99.9%, ethyl acetate, chloroform and n-hexane 95%. All solvents were purchased from Tedia. Standard of Betulinic Acid 90% and Diazald were obtained from Sigma-Aldrich.

Plant material

Leaves of *E. florida* were collected at Oswaldo Cruz Foundation, Rio de Janeiro, Brazil during August 2011. A voucher was deposited in Herbarium of Jardim Botânico of Rio de Janeiro, Brazil (RB 328.061). The leaves were dried in oven with air circulation at 25-30ºC and grinded in industrial mills.

Diazomethane preparation

For diazomethane synthesis, a joint-type distillation apparatus Clear seal (T 19/22) was applied. 5g of potassium hydroxide was weighed and solubilized in 30mL of ethyl alcohol and 8mL of distilled water. This solution was transferred to the distillation system flask and heated to 55-65°C with stirring. The mixture of 11g Diazald reagent solubilized in 75mL of ethyl ether was transferred to the addition funnel. With stable bath temperature, it was started the addition of Diazald's solution into the reaction medium. After complete transfer of the Diazald solution, the addition funnel was washed with 25mL of ethyl ether, which was also added to the reaction flask. The reaction was completed when the reaction medium was very light yellow.

Extract preparation

Five different extraction methods were carried out to prepare extracts from leaves of *Eugenia florida*. At the end of each extraction procedure, the extract was filtered in a 0,45µm filter paper and then evaporated under reduced pressure in order to obtain the crude extract. The samples were preserved at 4°C until analysis. Each procedure was performed in triplicate and the medium values were used to construct the graphics.

All the extraction procedures were performed with 1:10 plant: solvent ratio.

Static maceration extraction

Dried and ground plant material (10g) was taken into flasks and received 100 mL of solvent. With the flasks capped and sealed, extraction lasted 72 h, at room temperature. After 24h the extraction has begun, the extraction solvent was renewed and this procedure was repeated after 24h to ensure better depletion of plant material.

Dynamic maceration extraction

Powdered leaves (10g) were placed in 250 mL flasks and extracted with 100 mL of solvent. With the flasks capped and sealed, the plant was in contact with the solvent in shaker table for 72h (8 h / day with agitation and 16 h/ day with no agitation), at room temperature. The solvent was renewed three times to ensure better depletion of plant material. Once the percolation has ended, the plant used for extraction, still soaked in the solvent, is pressed to recover the residual liquid absorbed by the plant matter, which is then added to the macerated.

Percolation extraction

The swelling step was performed outside the percolator. The dried plant matter (40g) was placed in a beaker in contact with the solvent (100 mL) for 2 h, so that the forces resulting from expansion do not affected the experiment. With the moistened plant matter in percolator, the solvent was slowly passed through the plant (1 to 2mL /min.), and pushed away by another pure solvent that was added from above. The total amount of solvent used in the percolation was 400 mL. Once the percolation has ended, the plant used for extraction, still soaked in the solvent, was pressed to recover the residual liquid absorbed by the plant matter, which was then added to the leach ate.

Soxhlet extraction

In reflux extraction, 32g of the dried and ground plant material was placed in a Soxhlet apparatus with 300 mL of solvent. This method of extraction was performed until the solvent stayed limpid, which lasted two days with 8h of reflux.

Ultrasound extraction

In ultrasonic extraction, a Bransonic Model ultrasound equipment, power of 135 W at a frequency of 42kHz and with a radiation intensity of 0.19W / cm^2 was employed Bath dimensions were 29.2cm x 24.13 cm x 15.2 cm. Extraction temperature was kept constant at 25°C using water bath.. In this experiment, 4g of dried and grinded leaves were placed in a centrifuge tube with 40mL of extraction solvent. The capped tube was placed on ultrasound and extraction was performed for 20 minutes. The extraction time was determined in previous experiments.[15]

Particle size effect

After performing the tests to evaluate the efficiencies of extraction solvents and techniques, a trial to assess the effect of particle size on extraction was performed, since this is an important factor in pre-formulation studies. The same batch of *E. florida* leaves was grinded into different particle sizes as shown in Table 1.

Table 1 Parameters used to obtain different particle sizes of the leaves of *Eugenia florida*

Mesh (#)	Equipment	Dried Plant (g)	Ground Plant (g)	Grinding Time (min)
Sprayed	Ball Mill Marconi MA 021	100	96.95	3
0.5 (mm)	Knife Mill Marconi MA 680	100	99.3	18
0.85 (mm)	Knife Mill Marconi MA 680	100	98.05	17
1.7 (mm)	Knife Mill Marconi MA 680	100	98.05	17

The extraction technique chosen was extraction by ultrasound for 20 minutes since it was the fastest procedure with the least amount of solvent studied in this work. Five experiments were performed simultaneously (in triplicate) with different solvents: ethanol, methanol, ethyl acetate, chloroform and hexane.

Extract yield percentage

The extraction yield was presented in percentage (%) and was calculated by the ratio between the mass of extract recovered and the initial amount of dried and powdered plant material used for extraction. The extract yield was determined for each extraction technique evaluated.

Chromatographic conditions

Instrumentation consisted of a Gas Chromatography with Flame Ionization Detector System (GC-FID), AGILENT 6890N equipped with an automatic injector model 7683. Capillary column DB-5 (30m X 320 μm X 1.5 μm).

Oven temperature was programmed from 200°C (hold for 2 min) to 250°C at a rate of 10°C/min, then 15°C/min to 320°C, ending with a 28.34 min isothermal at 320°C. The particle-free derivatized crude extracts (1 μL) were taken in a syringe and injected into injector with a split ratio 5:1. Helium (99.999%) was used as carrier gas at a constant flow of 1ml/min, detector at 350 ºC, hydrogen and air synthetic flow of 35ml/min and 350 ml/min, respectively. The procedure was repeated in triplicate for each sample.

Sample preparation

Precisely weighed samples (1g each) were derivatized with diazomethane solution (1 mL). After complete evaporation, the samples were solubilized in methanol (1 mL), and 1μL injected into GC-FID.

Standard solutions

The standard calibration curve for BA was performed using standard solutions prepared with methanol in volumetric flasks in the concentration ranging from 10 to100 μg/ml, and an 1.0 ml aliquot of each calibration solution were derivatized with diazomethane (1 mL). After complete evaporation, they were solubilized with MeOH (1 mL), and 1μL was injected into a GC-FID. The average value of triplicates of each calibration level was used to build the calibration curve in Microsoft Office Excel 2007.

Statistical analysis

All data were analyzed by Student's t-test and F-test and values with $p < 0.05$ were considered significantly different. Microsoft Office Excel 2007 was used to provide the statistical calculation.

Hansen solubility parameters (HSP)

The HSP distance between two molecules (1 and 2), conventionally called Ra, is measure by the formula:

$$Ra^2 = 4(\delta D_1 - \delta D_2)^2 + (\delta P_1 - \delta P_2)^2 + (\delta H_1 - \delta H_2)^2$$

where:

δD - dispersion bonds

δP -polar bonds

δH- hydrogen bonds

The relative energy difference between a solute and a solvent is

called RED number and is calculated by the ratio Ra/Ro. The Ro value was estimated based on the solubility (extraction) experimental results.

The formulas used in this work to calculate Ra, Ro and RED parameters were taken from Hansen solubility parameters: a user's handbook.[16]

Results

The choice of the extraction solvent is certainly one of the main challenges to be considered, since the chemicals are extracted in different solvents with various levels of intensity, which can be affected by many factors.[14] High temperatures, for example, can facilitate the extraction of substances, but it should be controlled in order to prevent the degradation of thermo sensitive substances. Bearing in mind a large scale production and the chemical structure of BA, the preliminary choice of solvents were selected based on its polarity, cost, and environmental effect (Figure 1).

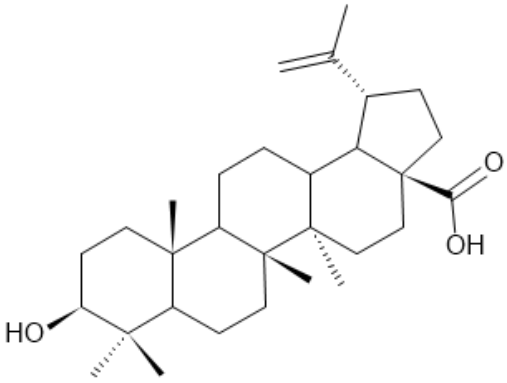

Figure 1 Chemical structure of betulinic acid.

The yield of the crude extracts of *E. florida* leaves was evaluated by different techniques and solvents (Figure 2), as well as the extraction efficiency of BA (Figure 3).

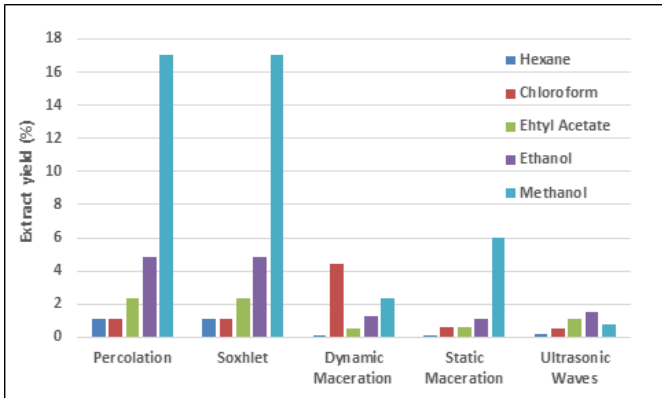

Figure 2 Extract yields (%) of the crude extracts from leaves of *Eugenia florida* DC. obtained by different extraction techniques.

EtOAc, ethyl acetate; $CHCl_3$, chloroform; EtOH, ethanol; MeOH, methanol; Hex, hexane

In order to evaluate the effect of particle size on extraction efficiency, the ultrasonic waves technique was chosen due to be a fast analysis and have a lower expense of solvent. The same five solvents were tested in this experiment and the results can be seen in Figures 4&5.

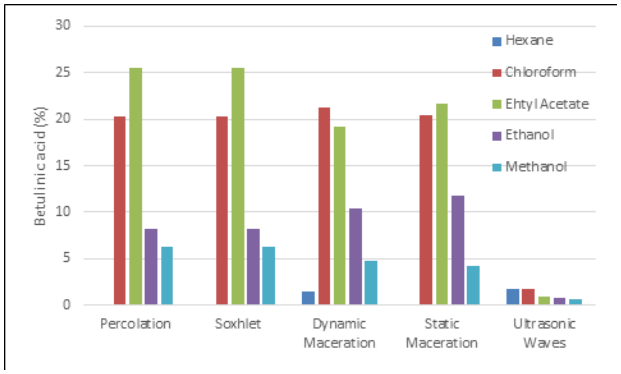

Figure 3 Betulinic acid (%) in the crude extracts from leaves of *Eugenia florida* DC. obtained by different extraction techniques.

EtOAc, ethyl acetate; $CHCl_3$, chloroform; EtOH, ethanol; MeOH, methanol; Hex, hexane

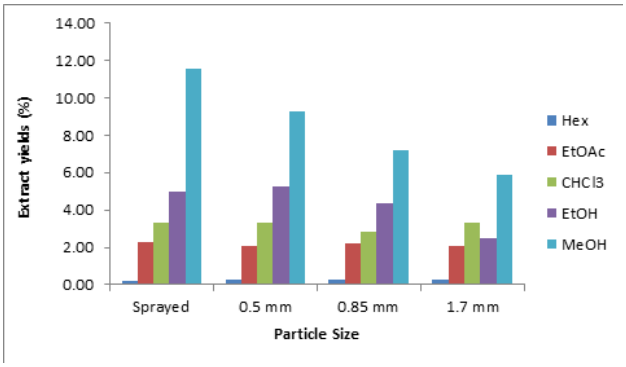

Figure 4 Extract yields of the *Eugenia florida* leaves extracts obtained by different particle size (Ultrasound extraction).

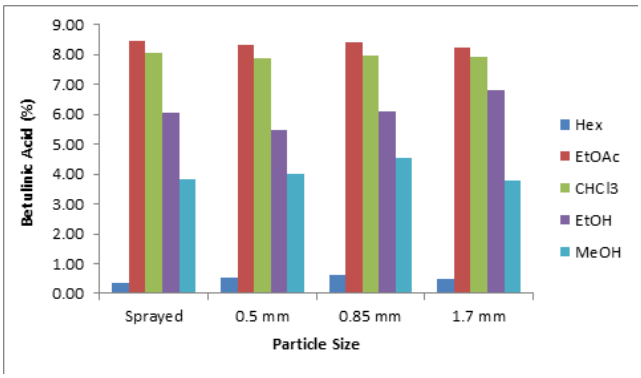

Figure 5 Betulinic acid yields of the *Eugenia florida* leaves extracts obtained by different particle size (Ultrasound extraction).

Discussion

Although methanol presented the highest extraction yields (Figure 2), ethyl acetate and chloroform exhibited the best results of extracted BA (Figure 3) showing 14-25% in the different extraction processes applied (except for ultrasonic waves).

Considering Hansen Solubility Parameters (HSP) is possible to explain the chemical behavior of ethyl acetate and chloroform as the best solvents for BA extraction. Hansen solubility parameter values are based on dispersion bonds (δD), polar bonds (δP) and hydrogen bonds (δH), which contain information on inter-molecular interactions among solvents and also with solutes.[16] This rational evaluation allows the exchange of solvents to be more effective in

dissolving solutes and with low environmental impact. The HSP distance between two molecules (1 and 2), conventionally called Ra, is the measure of how alike they are. The smaller the Ra, higher the probability to be compatible.

Conveniently, it makes HSP plots into nice spheres. Inside the sphere radius (Ro) are located all the good solvents. Solubility, or high affinity, requires that Ra be less than Ro. The ratio Ra/Ro is called RED number, reflecting the relative energy difference. RED numbers below 1.0 indicate high affinity; RED equal to or close to 1.0 is a boundary condition; and higher RED numbers indicate progressively lower affinities. Based on literature,[16,17] HSP values for the extraction solvents and for BA were taken. Ra and RED numbers were calculated to evaluate the theoretical solubility of BA (Table 2). Ro value was estimated regarding the Ra numbers for the two best solvents (score 1). According to Table 2, ethyl acetate and chloroform demonstrated RED numbers bellow 1 (0.49 and 0.86, respectively), confirming their efficiency in BA extraction from the plant material.

Table 2 RED evaluation for betulinic acid solubility using different extraction solvents

Solvents	δD (Mpa1/2)	δP(Mpa1/2)	δH(Mpa1/2)	Score	Ra (BA-solvent)(Mpa1/2)	Ro	RED
Ethyl Acetate	15.8	5.3	7.2	1	2.95	6	0.49
Chloroform	17.8	3.1	5.7	1	5.13		0.86
Hexane	14.9	0	0	0	11.33		1.89
Ethanol	15.8	8.8	19.4	0	11.96		1.99
Methanol	14.7	12.3	22.3	0	15.98		2.66
Solute	dD	dP	dH				
Betulinic acid (BA)	16.8	7.4	7.7				

δD, dispersion bonds; δP, polar bonds; δH, hydrogen bonds; Ra, Hansen solubility parameter distance; Ro, sphere radius

For the solvents applied in this work, the dispersion value did not imply significant effect on BA solubility, as can be observed when chloroform is used (Figure 5). Ethanol presents polar forces (δP) close to betulinic acid (8.8 and 7.4 for ethanol and BA, respectively) which could promote a reasonable BA yields (8.2-12.1%) in different extractions techniques (percolation, maceration and soxhlet). Despite hexane presented a RED number close to ethanol, the results indicated a poor extraction power developed by this solvent, showing that dispersion forces (mostly Van der Waals force) are the HSP most important in its weak interaction with BA (Figure 6).

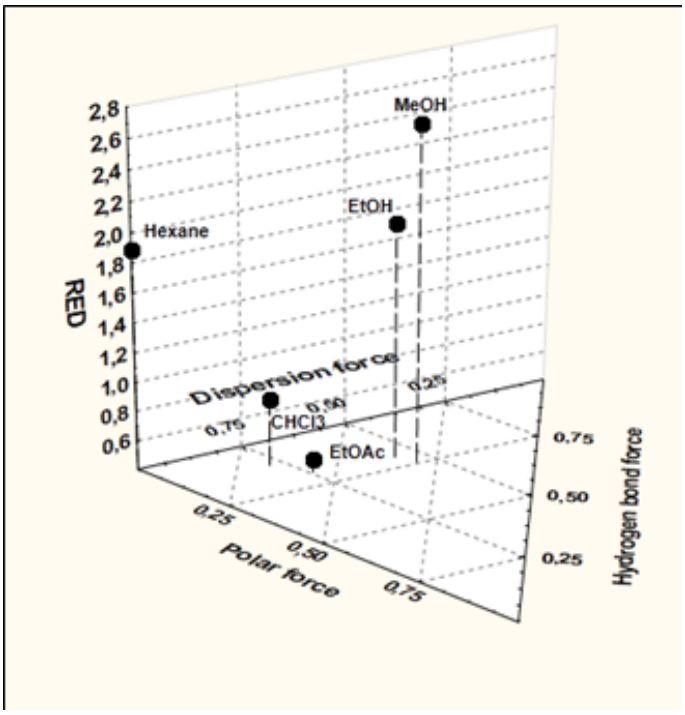

Figure 6 Evaluation of RED number against Hansen solubility parameters of extraction solvents.

Figure 4 clearly depicts how Hansen Solubility Parameters classified solvents in three different groups: polar aprotics (ethyl acetate and chloroform), polar protics (ethanol and methanol) and apolar (hexane).

Hildebrand solubility parameter theory also points to smaller molar volume solvents as being better than those with larger molar volumes, even though they may have identical solubility parameters.[16] Likewise, the low content of betulinic acid found after n-hexane extraction may be related to its low permeability into the cells and better affinity to cell surface substances, such as waxes and lipids or other substances of nonpolar nature. Consequently, extraction efficiency of the substances present in plants depends on not only the solvent used, but also its location in the plant, which is determined by chemical nature of substance.

The crude extract yield showed the highest percentage result with methanol (17.1% w/w percolation, 14.9% w/w soxhlet, 2.3% w/w dynamic maceration, 6.0% w/w static maceration and 0.8% w/w ultrasonic waves). Molar volume can be an important parameter that may explain higher extractive yields (%) of the crude methanol extracts when compared to other solvents. The size of solvent and solute molecules is important for solubility, permeation, diffusion, and chemical resistance phenomena, as depicted in Figure 7 (Soxhlet extraction was elected as example since this technique showed the best results for crude extract yield).

The spheres diameter presented in Figure 7 are proportional to each solvent molar volume value (Methanol: 41 cm^3/mol, Ethanol: 59 cm^3/mol, Chloroform: 81 cm^3/mol, Ethyl acetate: 99 cm^3/mol, Hexane: 131 cm^3/mol). The outlier position of methanol demonstrates how extraction yield (%) is impacted positively by the facilitated diffusion promoted from the small size of this solvent, however this molecular characteristic did not promote the necessary selectivity for BA extraction. Large molar volumes (hexane) seems impair BA solubility, besides its low cell permeability.

As previously commented, the techniques selected for this study were continuous hot extraction (soxhlet), static and dynamic

maceration, percolation and ultrasonic waves. As depicted in Figure 3, the best extraction techniques for obtaining BA were maceration (dynamic and static) and percolation.

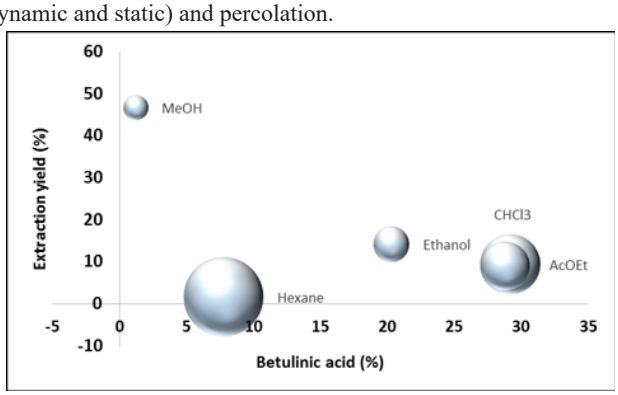

Figure 7 Molar Volume effect in extraction efficiency (Soxhlet extraction) of betulinic acid from leaves of *Eugenia florida.*

Maceration exploits the phenomenon of diffusion of the solvent through the plant tissue. In dynamic extraction, the contact of the plant material with the solvent is facilitated due to constant stirring.[18] In the extraction by percolation, plant goes through the initial process of swelling and after this period is continuously in contact with the new solvent for its downward movement. This process improves the balance time between the solvent located inside and outside the plant cell.[19]

Soxhlet extraction is a continuous method widely used in the extraction of organic substances. The advantages of this extraction process are continuous extraction with renewed and reduced solvent volume. The main disadvantage is that the extract remains in contact with the solvent at its boiling point for the entire process. In some cases, this condition may promote degradation of the extracted components,[20] which may explain the lower results for betulinic acid extraction compared to both types of maceration extraction.

The principle of ultrasonic extraction process consists in the fact that a stream of a liquid or liquid mixture is pushed on pressure for a metallic blade that vibrates at are sonant cavity. The phenomenon consists in the conversion of electrical energy of high frequency, via vibrations in mechanical pressure waves in solution. These waves create a huge number of microscopic bubbles that implode violently causing the intense agitation of the molecules.[21] Using this technique, plant tissue cells can have their membranes lysed, allowing the intracellular molecular components to spill into the solution, facilitating the extraction process.

Percolation showed the low yield of crude extract (Figure 2) but demonstrated the high yield of extracted BA (Figure 3). The high selectivity of this technique for BA extraction may be due to the higher osmotic force in the plant cells promoted by the constant renewal of the solvent during the process. In a different case, solvents can show a high extract yield but it does not necessarily imply extraction efficiency of the substance of interest, as seen with methanol in Soxhlet extraction. Higher temperature extractions (Soxhlet) can facilitate solvent diffusion and, consequently, better extract yield (methanol). Nevertheless, methanol is not selective for BA, although BA has polar moieties in its structure, the non polar moiety is also very relevant (Figure 1).

It can be observed that ultrasonic waves technique although faster compared to the other techniques did not present good results for BA extraction in *E. florida*. Despite sound waves break cells and facilitating the release of substances, degradation may occur, depending on the compound and the time of extraction. Besides, the great variety and amount of substances present in plant cell can promote lower extraction power of BA by solvent saturation, like observed in maceration extraction process.

Regarding the environmental risks involved with the solvents used in this work, Tobiszewski et al.,[22] developed a study to assess of risk based on detailed hazard and exposure investigations. The assessment procedure holds promise for solvent selection during process design as well as in finding alternatives to hazardous solvents used in existing processes. The authors[22] applied the multi-criteria decision analysis (MCDA) as an approach that supports the decision making process when complex problems need to be solved. The study results show that alcohols and esters can be considered as low environmental risk solvents (green solvent), whereas chlorinated solvents or aromatic hydrocarbons are the most problematic. In Tobiszewski et al.,[22] ranking, ethanol and ethyl acetate presented the highest scores (2º and 12º places) as green solvents (high rank means low environmental risk) among those chosen for this work.

The particle size evaluation of plant material is an important step because it may represent a direct influence on the efficiency of the extraction process.[23] It is evident from Figure 4 that the statement applies only to the yields of the crude extract obtained using ethanol and methanol, as particle size decreases the yield also reduces (50%). Differently than expected, the reduction in particle size did not imply significantly different results for BA yield

Conclusion

The highest percentages of BA were obtained by percolation and maceration. BA molecule has polar and non-polar characteristics and the solvents that presented greater percentage of this compound were ethyl acetate and chloroform, as demonstrated by RED numbers below 1 for those solvents. HSP assessment showed to be a useful tool to predict efficient solvents for betulinic acid extraction. Although ethanol achieved results inferior to chloroform in BA extraction (%), it is recognized as a green solvent, as is ethyl acetate.

Acknowledgments

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Conflicts of interest

There is no conflict of interest in this study.

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