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Optimization of the extraction process of *Phyllanthus niruri L.*

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Abstract

Phyllanthus niruri L., known as stone breaker, is a plant in the Phyllanthaceae family, belonging to the genus Phyllanthus. In this study, the factorial design was evaluated to study the importance of factors (concentration of ethanol and extraction process) on the concentration of flavonoids and phenolic compounds present in *P. niruri* extracts. Different extracts of *P. niruri* was prepared using 50 and 96% ethanol, using three different extraction methods: maceration, percolation and ultrasound. The quantification of flavonoids was performed by the spectrophotometric method with 10% $AlCl_3$ (v/v) in water. The Folin-Ciocalteu method were employed for the quantification of phenolic compounds. The analyses were performed in triplicate and the results were analyzed using the Minitab18® and Prisma GraphPad® software. Significant differences ($p \leq 0.05$) were observed in the values of flavonoids and total phenolic compounds in the extracts produced from *P. niruri*, when different extraction processes were used, as well as different concentrations of ethanol. Analyzing the Pareto graphs, it was possible to verify that the solvent concentration was the effect that most contributed to the extraction of both bioactive compounds. Checking the interaction graphs, it was possible to identify that 96% ethanol positively influenced the extraction of flavonoids and 50% ethanol influenced the extraction of phenolic compounds. Thus, the factorial design applied to the extraction process of *P. niruri* showed that the extraction of flavonoids can be carried out efficiently by traditional extraction methods, maceration or percolation, using 96% ethanol as a solvent. The optimized conditions for the extraction of phenolic compounds are by percolation or ultrasound with 50% ethanol.

Keywords: *Phyllanthus niruri.* Extraction Process. Flavonoids. Phenolic compounds.

INTRODUCTION

Phyllanthus niruri L. (Phyllanthaceae), popularly called stone breaker, is a species that occurs in almost the entire tropical region, even including the south of North America. In folk medicine the whole plant (aerial and root parts) is used to eliminate kidney stones and to increase urinary excretion, since it promotes relaxation of the ureters. In addition, there are reports of its use for gastrointestinal discomfort, diabetes, respiratory and liver diseases[1,2]. This species is part of the National List of Medicinal Plants of Interest to SUS, published in 2009 by the Ministry of Health. This list consists of plant species with the potential to generate products of interest to SUS[3].

Phytochemical analysis of this species revealed the presence of several groups of active ingredients, such as: flavonoids, alkaloids,

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triterpenes, tannins, lignans, polyphenols and sterols. Thus, the presence of these compounds in *P. niruri* is of great interest to researchers who are interested in determining their pharmacological effects[4].

Recently, there has been an increase in interest in the herbal medicine market. The global market for this class of drugs is estimated to reach US$ 20 million per year[5]. Therefore, there is a need to obtain standardized extracts[6]. The production of standardized extracts is conditioned to the monitoring of the quantity of chemical markers present, in addition to a correct identification of the plant species, information on the conditions of cultivation, extraction and purification processes. The development of standardized plant extracts results in obtaining pharmaceutical products with a high quality standard and with certain parameters of safety and therapeutic efficacy[7,8]. Among the aforementioned parameters, extraction is a crucial step in the preparation of herbal medicines and the method choice must be based on the efficiency, stability of the extracted substances and the availability of the means and cost of the process[9,10].

P. niruri is a species widely used by the population in the form of tea. However, this common form of use, and even the plant itself, are not standardized and may lead to adverse effects or lack of effectiveness[11]. The objective of this work was to study the influence of two factors (concentration of ethanol and extraction process) on the concentration of flavonoids and phenolic compounds present in the extracts of *P. niruri*.

MATERIALS AND METHODS

Plant material

The dry plant material was obtained from the São Camilo University Pharmacy - FUSC. This plant material was purchased from the company SantosFlora Comércio de Ervas Ltda., corresponding to lot 1608175268 with an authenticity report provided by the company.

Preparation of *Phyllanthus niruri* extracts

The entire plant was ground in the knife mill and subjected to the screening process, using a 20-mesh sieve (850 µm). The extraction processes used were maceration, ultrasound and percolation, obtaining 3 hydroethanolic extracts with 50% ethanol and another 3 extracts with 96% ethanol. All extracts were prepared in a 1:10 ratio (mass of the plant drug versus liquid extractor). The extraction processes were carried out in triplicate.

In the maceration process, the powdered leaves were in contact with the extracting liquid for 7 days. The percolation process was performed at an average speed of 50 drops/minute[12]. In the ultrasound process, the plant material was in contact with the extracting liquid for 30 minutes at room temperature[13]. The extracts produced were concentrated on a rotary evaporator at 40°C.

Experimental design

In this study, a 2x3 factorial design was proposed, with the ethanol and the extraction process as factors. The ethanol in concentrations used were 50% and 96% as well as the following extraction processes: maceration, ultrasound and percolation (table 1).

Table 1 – Experimental planning carried out to optimize the *Phyllanthus niruri* extraction process. Having X_{ij} where i is the type of extraction and j is the concentration of ethanol.

Extraction Process Factor	Ethanol Factor	
	50%	96%
Maceration	X11	X12
Ultrasound	X21	X22
Percolation	X31	X32

Quantification of flavonoids

The determination of the total flavonoid content in the different extracts was performed by UV/Vis spectrophotometry (Shimadzu® UV-1280) using aluminum chloride ($AlCl_3$) as a complexing agent[14]. To perform the technique, quercetin (Sigma Aldrich®) in 96% ethanol was used as CRS. 10 mg of each sample were weighed and diluted in 10 mL of 96% ethanol for extracts with 96% ethanol, and for extracts with 50% ethanol a water:ethanol (1:1 v/v) ratio was used obtaining a final concentration of 1 mg/mL . In test tubes, 0.5 mL of the sample, 0.1 mL of aqueous sodium acetate solution and 0.1 mL of 10% $AlCl_3$ in water were added. The volume was completed with 96% ethanol, reaching 5.0 mL. After 30 minutes of the addition of the complexing agent, the absorbances were determined in a spectrophotometer at a wavelength of 415 nm. The blank was prepared following the same methodology, only 0.5 mL of 50% ethanol (for extracts with 50% ethanol) or 96% ethanol (for extracts with 96% ethanol) were added instead of the sample. The analyses were performed in triplicates[15].

Quantification of phenolic substances

The Folin-Ciocalteu method was used for the quantification of phenolic compounds. To determine the content of phenolic substances, 10 mg of each 50% ethanol extract and 30 mg of each 96% ethanol extract were weighed, diluted in 10 ml of a 1:1 water:ethanol (v/v) ratio for the 50% ethanol extracts and ethanol 96% for the 96% ethanol extracts, obtaining a final concentration of 1 mg/mL and 3 mg/mL, respectively. In test tubes, 0.1 mL of the sample, 6.0 mL of water and 0.5 mL of the Folin-Ciocalteu reagent were added. The tubes were shaken and left to stand for 8 minutes. In each tube, 1.5 mL of a solution of sodium carbonate (Na_2CO_3) added to 20% water and 1.9 mL of water were added.

The blank was prepared by the same method, but 0.1 mL of 50% ethanol (50% ethanol extracts) or 96% ethanol (for 96% ethanol extracts) was added instead of the sample.

After two hours of reacting in the absence of light, absorbance was determined at a wavelength of 760 nm in a spectrophotometer (Shimadzu® UV-1280). Gallic acid in 50% ethanol was used as CRS. The readings were performed in triplicate[16].

Chromatographic Profile Determination

The crude extracts of *P. niruri* made with 96% ethanol and 50% ethanol by different extraction methods were subjected to thin layer chromatography (TLC) in order to indicate substances of interest through comparison with standard. The chromatographic system used in this research was described by Wagner and Bladt[17], aimed at flavonoids. The stationary phase employed was silica gel 60 GF254 Merck on aluminum plate 0.2 mm thick. The mobile phase was composed of ethyl acetate, glacial acetic acid, formic acid and distilled water (100:11:11:26). The developer was composed of 1% diphenylboryloxyethylamine (NP) in methanol. The standard used was rutin solubilized in ethanol. The chromatoplate was visualized under UV light (366 nm).

Statistical analysis

The results were submitted to statistical treatment, through analysis of variance (ANOVA) followed by the Tukey test. The normality of the data was tested since the values obtained were greater than 0.05. The normality test used was the D'Agostino-Pearson omnibus test. This test is the one recommended by the Prisma GraphPad statistical program, which was the software used to perform the calculations. In addition,

the homoscedasticity of the data (response - flavonoids versus ethanol concentration and response - flavonoids versus extraction process) was evaluated. The same was performed for the data verifying the presence of phenolic compounds. As the presence of these assumptions was verified, ANOVA was applied. To assess the influence of the effects (extraction process and solvent) among the concentration of flavonoids and phenolic compounds, in addition to checking whether there was any interaction between them, Minitab18® software was used. The results were considered statistically different when the p-value was less than or equal to 0.05 ($p \leq 0.05$).

RESULTS

The analyses concerning the quantification of flavonoids in the different extracts were performed using quercetin as an external standard for the construction of the standard curve (figure 1). After calculating the linear equation, total flavonoid values were obtained for each extract. Below are the results (table 2).

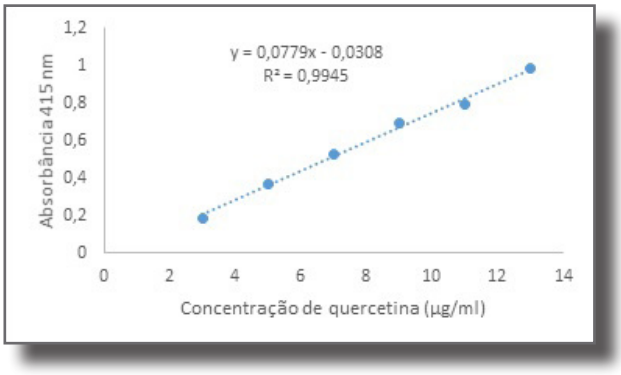


Figure 1 – Standard curve used for the quantification of total flavonoids in the different crude extracts of *Phyllanthus niruri* (São Paulo – SP, may/june, 2019).

Table 2 – Quantification of total flavonoids in the different crude extracts of *Phyllanthus niruri* using $AlCl_3$ as a complexing agent. The results for total flavonoids are expressed as means followed by their standard deviations. ANOVA followed by the Tukey test ($p \leq 0.05$). (São Paulo – SP, may/june, 2019).

Extract	Total flavonoids (mg/g crude extract) ± SD
Maceration 50% ethanol	35.67 ± 6.139[a f]
Maceration 96% ethanol	55.72 ± 3.137[b]
Percolation 50% ethanol	29.65 ± 4.813[a d]
Percolation 96% ethanol	50.09 ± 7.287 [b]
Ultrasound 50% ethanol	25.30 ± 2.087[d]
Ultrasound 96% ethanol	37.72 ± 3.970[e f]

Extracts that do not share the same letter indicate a statistical difference ($p \leq 0.05$).

Table 2 shows the amount of flavonoids in the different crude extracts of P. niruri. With the aid of the Pareto chart, it was possible to verify the factors influence (extraction process and solvent) on the concentration of flavonoids (figure 2).

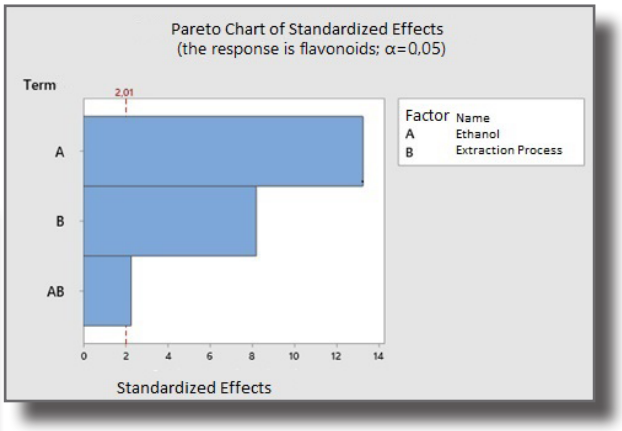


Figure 2 – Influence of the solvent concentration and extraction process parameters on the extraction of flavonoids from *Phyllanthus niruri* (São Paulo – SP, may/june, 2019.

As the interaction between the factors evaluated was verified, a graph of interaction between those factors was created (figure 3).

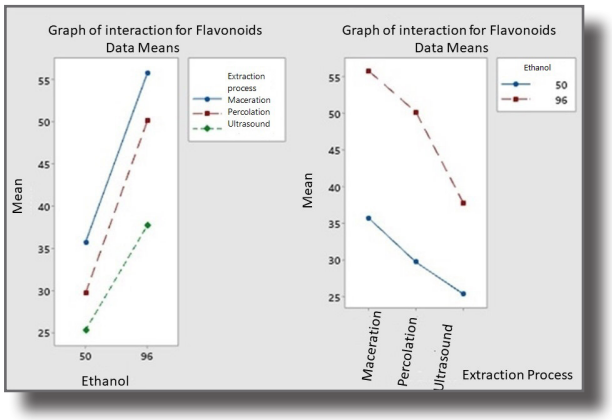


Figure 3 – Graph of interaction between factors (solvent concentration and extraction process) in the extraction of flavonoids from *Phyllanthus niruri* (São Paulo – SP, may/june, 2019)

The analyses referring to the quantification of phenolic compounds in the different extracts of *P. niruri* were performed using gallic acid as an external standard for the construction of the standard curve (figure 4). After the calculation performed using the linear equation, the values of total phenolic compounds in gallic acid equivalents per gram of extract were obtained. Below are the results (table 3).

Table 3 – Quantification of total phenolic compounds in the different crude extracts of *P. niruri* using the Folin-Ciocalteu reagent. The results are expressed as means followed by their standard deviations. ANOVA followed by the Tukey test, ($p \leq 0.05$). (São Paulo - SP, may/june, 2019).

Extract	Total phenolic compounds (mg/g crude extract) ± SD
Maceration 50% ethanol	281.0 ± 12.33[a]
Maceration 96% ethanol	70.49 ± 32.79[b]
Percolation 50% ethanol	355.4 ± 58.75[c]
Percolation 96% ethanol	43.70 ± 6.712[b]
Ultrasound 50% ethanol	327.5 ± 17.36[c]
Ultrasound 96% ethanol	117.5 ± 32.63[d]

Extracts that do not share the same letter indicate a statistical difference ($p \leq 0.05$).

Table 3 shows the amount of total phenolic compounds in the different crude extracts of *P. niruri*.

Analyzing the Pareto Diagram, represented in figure 5, it was verified the importance of each factor and the interaction between them in the value of total phenolic compounds present in the different extracts. As there was an interaction between the evaluated factors, an interaction graph was constructed (figure 6).

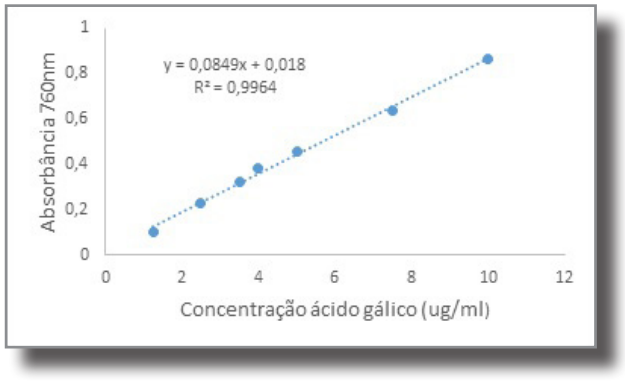


Figure 4 – Standard curve used for the quantification of total phenolic compounds in the different crude extracts of *Phyllanthus niruri* (São Paulo – SP, may, 2019).

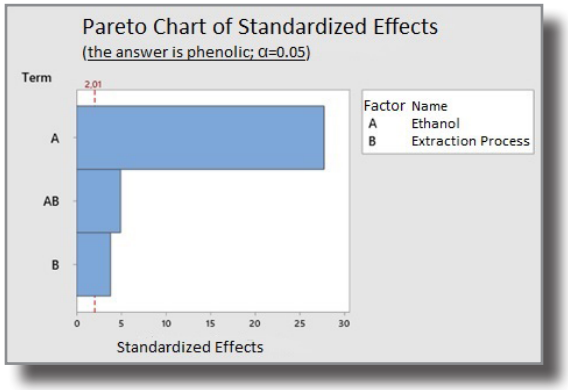


Figure 5 – Influence of the solvent concentration and extraction process parameters on the extraction of phenolic compounds from *Phyllanthus niruri*. (São Paulo - SP, may/june, 2019).

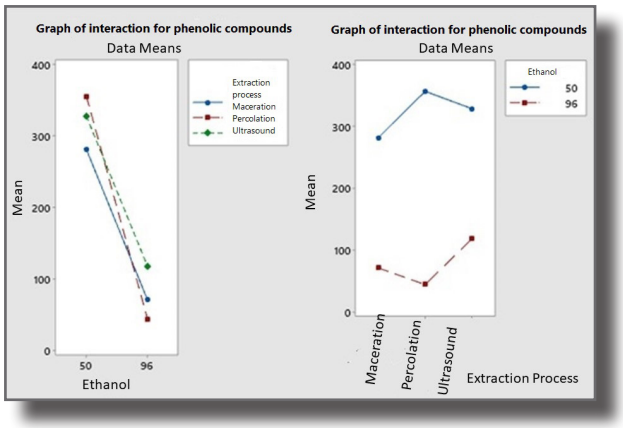
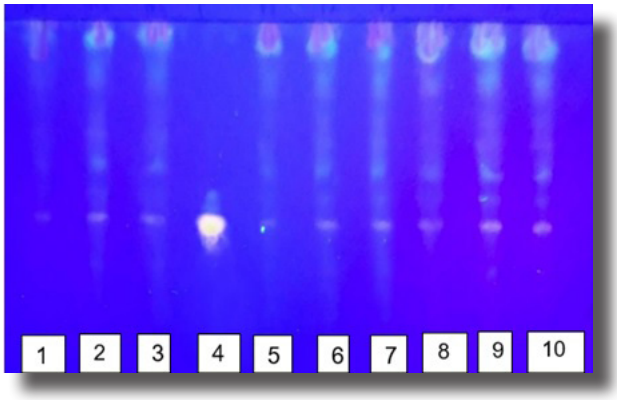


Figure 6 – Graph of interaction between factors (solvent concentration and extraction process) in the extraction of phenolic compounds from *Phyllanthus niruri*. (São Paulo - SP, may/june, 2019).

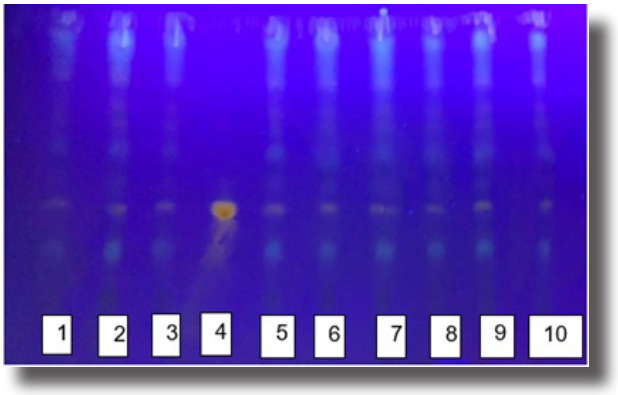
Chromatographic Profile Determination

The chromatographic profile for the different extracts produced with 96% and 50% ethanol is shown below (figure 7 and figure 8).



Legend: 1. Crude extract prepared by maceration (M1); 2. Crude extract prepared by maceration (M2); 3. Crude extract prepared by maceration (M3); 4. 0.5% rutin in methanol; 5. Crude extract prepared by percolation (P1); 6. Crude extract prepared by percolation (P2); 7. Crude extract prepared by percolation (P3); 8. Crude extract prepared by ultrasound (U1); 9. Crude extract prepared by ultrasound (U2); 10. Crude extract prepared by ultrasound (U3). Mobile Phase (MP): Ethyl acetate:Formic acid:Glacial acetic acid:H_2O (100:11:11:26). Detection with diphenylboryloxyehtylamine (NP) 1% in methanol. Distance traveled in the mobile phase: 10 cm. Visualization under 366 nm UV light.

Figure 7 – Comparative chromatoplate of crude extracts of *Phyllanthus niruri* elaborated by different extraction processes with 96% ethanol. (São Paulo – SP, november/december, 2018).



Legend: 1. Crude extract prepared by maceration (M1); 2. Crude extract prepared by maceration (M2); 3. Crude extract prepared by maceration (M3); 4. 0.5% rutin in methanol; 5. Crude extract prepared by percolation (P1); 6. Crude extract prepared by percolation (P2); 7. Crude extract prepared by percolation (P3); 8. Crude extract prepared by ultrasound (U1); 9. Crude extract prepared by ultrasound (U2); 10. Crude extract prepared by ultrasound (U3). Mobile Phase (MP): Ethyl acetate:Formic acid:Glacial acetic acid:H2O (100:11:11:26). Development with 1% diphenylboryloxyethylamine (NP) in methanol. Distance traveled in the mobile phase: 10 cm. Visualization under 366 nm UV light.

Figure 8 – Comparative chromatoplate of crude *Phyllanthus niruri* extracts prepared by different extraction processes with 50% ethanol. (São Paulo – SP, november/december, 2018).

DISCUSSION

The method of extracting active metabolites has a critical influence on pharmacological studies. Thus, the optimization and development of a new protocol is relevant[18]. Several processes can be used in the extraction of plant drugs such as conventional ones like decoction, infusion, maceration, percolation and Soxhlet extraction, and the methods considered modern, such as extraction assisted by ultrasound, microwaves, turbo-extraction, among others[19,20]. Another factor that influences an extraction process is the type of solvent used, because the solvent will diffuse into the plant material and solubilize compounds with similar polarity[21].

Some published works report the extraction of *P. niruri* with methanol and water[22,23]. Although methanol facilitates the extraction of phenolic compounds from *P. niruri*, its use is not recommended for the production of pharmaceutical products[24]. Therefore, ethanol and a hydroalcoholic mixture were used in this work.

During the development of a standardized plant extract, several decisions must be taken to obtain a product with an adequate amount of bioactive compounds, as well as to economize materials and solvents, which makes it valid to know the best conditions for extracting active ingredients[25].

In order to optimize the extraction process of flavonoids and phenolic compounds, the objective of the study was to evaluate two extraction processes, of which two were traditional (maceration and percolation) and one was unconventional (ultrasound), together with variations in the concentration of solvent (ethanol).

Several authors have studied the influence of the extraction process of medicinal plants in order to optimize the extraction of active ingredients[25,6,26]. Migliato *et al.*[27] evaluated the influence of the extraction process and the solvent on the production of crude extract of jambolan fruits (*Syzygium cumini* (L.) Skeels) on the dry residue content and on the determination of antimicrobial activity. Oliveira *et al.*[26] also optimized the extraction of rosmarinic acid, carnosol and carnosic acid, substances that have antioxidant activity, found in *Rosmarinus officinalis* L. The efficiency of the tested variables (extraction time, ethanol concentration and solid-liquid rate) was determined by the concentrations of rosmarinic acid, carnosol and carnosic acid, and also by the yield of the extraction process.

Regarding *P. niruri,* there are few studies in the literature reporting the effect of the solvent and the extraction method on the extraction of active components[28,24], although several studies have been found reporting pharmacological studies, as well as the identification, isolation and phytochemical analysis of compounds present in extracts of P. niruri[4,29].

There were significant differences ($p \leq 0.05$) in the values of total flavonoids in the extracts produced when different extraction processes were used, as well as different concentrations of ethanol. The highest concentration of flavonoids was observed in the crude extracts produced by maceration and percolation with 96% ethanol. This is because there was no statistical difference in the concentration of this secondary metabolite when the said extraction processes were used with 96% ethanol ($p \geq 0.05$). The lowest concentration of flavonoids in the crude extracts was seen in the ultrasound extraction process using 50% ethanol as the extraction liquid.

Amin *et al.*[30] also observed a high concentration of flavonoids when using 96% ethanol to produce extracts of *P. niruri* when compared to the aqueous extract. It is considered that most of these compounds are antioxidants, an important characteristic for prevention of several chronic diseases[31].

The Pareto graph (figure 2) showed that the concentration of the solvent and the extraction process are statistically significant, and the effect that most contributes to the extraction of flavonoids is the solvent ($p \leq 0.05$). In addition, there was a statistical difference in the interaction ($p \leq 0.05$) between the solvent (50% ethanol and 96% ethanol) and the extraction process (maceration, percolation and ultrasound) in the extraction of this secondary metabolite. It was observed, through the interaction graph, that the interactive effect indicates that the relationship between extraction process

and the concentration of flavonoids in the crude extracts of *P. niruri* depends on the concentration of ethanol; that is, it was found that 96% ethanol positively influenced the extraction of flavonoids from plant material.

With the phenolic compound quantification experiment, it was found that 50% ethanol had a positive influence on the extraction of total phenolic compounds.

Since there was no statistical difference in the concentration of this secondary metabolite, when the referred extraction processes with 50% ethanol were used, it was determined that the crude extracts produced by percolation and ultrasound with 50% ethanol were more efficient for extracting phenolic compounds ($p \geq 0.05$). The lowest concentration of total phenolic compounds in the crude extracts was verified when the extraction process was used for maceration and percolation with 96% ethanol as the extraction liquid (Table 3).

Nguang, *et al.*[24] found that the greater extraction of phenolic compounds present in the species was demonstrated when a 40% hydroethanolic mixture was used, a coherent fact since the species has hydrolyzable tannins as major a compound. Markom *et al.*[28] also found a more efficient yield in the extraction of tannins in hydroethanolic extracts and that some bioactive compounds, such as gallic acid, ellagic acid and corilagin present in *P. niruri*, were more efficiently extracted when using polar solvents (water and hydroethanolic mixture); suggesting that the solvent has greater interference in the extraction of active substances.

The Pareto graph (figure 5) indicated that the solvent was the factor that most influenced the efficiency of extractions ($p \leq 0.05$) of phenolic compounds. In addition, it was observed that the extraction process factor, as well as the interaction between both factors, were significant ($p \leq 0.05$). The interaction graph (figure 6) suggests that using the appropriate solvent (50% ethanol) and selecting the percolation or ultrasound extraction process results in the most efficient method for the extraction of phenolic compounds present in *P. niruri*.

Finally, the analysis of the chemical profiles of the different crude extracts, produced with 50% and 96% ethanol by TLC were similar. In addition, it was possible to see several intense spots on the chromatoplates suggesting a high concentration of flavonoids. The presence of a band of intense color was seen in all samples tested. This band has a retention factor ($Rf = 0.4$) and a rutin-like color, which was the standard used. This flavonoid was identified in the species using ^{1}H-Nuclear Magnetic Resonance techniques and correlated with antioxidant activity (DPPH radical sequestration)[32]. Other flavonoids have also been identified in the species under study, such as quercetin and apigenin[33].

CONCLUSION

The results presented in the present work suggest the importance of choosing the extraction method and the extracting solvent to obtain the best efficiency in the extraction of bioactive compounds. Thus, the factorial design applied to the extraction process of *P. niruri* showed that the extraction of flavonoids from *P. niruri* can be carried

out efficiently by the traditional extraction methods, maceration or percolation, using 96% ethanol as a solvent. The optimized conditions for the extraction of phenolic compounds from *P. niruri* is percolation or ultrasound using 50% ethanol.

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