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CONNIE CAMPANO CABRAL

COMPARATIVE EXTRACTION OF PHENOLIC AND OTHER MINOR COMPOUNDS FROM *PHYLLANTHUS AMARUS* AND *PHYLLANTHUS NIRURI* BY UPLC-QTOF-MS/MS AND CHEMOMETRIC ANALYSIS

FORTALEZA

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Undergraduate thesis presented to the Chemistry program in the Exact Sciences Department at the Federal University of Ceará as a partial requirement for the acquisition of a bachelor's degree in Chemistry with Industrial Licence.

Educational Mentor: Prof. Dr. Dávila de Souza Zampieri

Professional Mentor: Dr. Guilherme Julião Zocolo

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Approved in: ___/___/_____.

EXAMINING BOARD

_____

Prof. Dr. Dávila de Souza Zampieri

Universidade Federal do Ceará – UFC

_____

Dr. Guilherme Julião Zocolo

Empresa Brasileira de Pesquisa Agropecuária-EMBRAPA

_____

Dr. Gisele Silvestre da Silva

Empresa Brasileira de Pesquisa Agropecuária-EMBRAPA

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ABSTRACT

Phyllanthus amarus and *Phyllanthus niruri* are species of medicinal plants, popularly known as “quebra-pedra”, commonly used in folk medicine in tropical and subtropical countries, such as India, Brazil and Malaysia. They are used to cure pathologies such as hepatitis B, kidney stones, diabetes and other infectious diseases. Their medicinal properties are due to the presence of phenolic compounds in their composition. This study aimed to achieve the best extraction method, whether it was by ultrasound or maceration, and solvent proportion for a better extraction of phenolic compounds. The extracts were prepared with pure water and different methanol/water and ethanol/water ratios to be analysed by ultra performance liquid chromatography coupled to mass spectrometry (UPLC-QTOF-MS/MS) system and by chemometrics for a more detailed information.

Keywords: *Phyllathus amarus*, *Phyllanthus niruri*, Phenolic compounds, Extraction method.

RESUMO

Phyllanthus amarus e *Phyllanthus niruri* são espécies de plantas medicinais, popularmente conhecidas como “quebra-pedra”, geralmente usadas na medicina popular em países tropicais e sub-tropicais, como Índia, Brasil e Malásia. São usadas para curar patologias como hepatite B, pedras renais, diabetes e outras doenças infecciosas. Suas propriedades medicinais são justificadas pela presença de compostos fenólicos nas respectivas composições. Este trabalho teve como objetivo encontrar o melhor método de extração, seja por ultrassom ou maceração, e a melhor proporção de solventes para melhorar a extração de compostos fenólicos. Os extratos foram preparados com água pura e misturas de metanol/água e etanol/água em diferentes proporções para serem analisados por um sistema de cromatografia líquida de ultra eficiência acoplada a Espectrometria de massas (CLUE-EM) e por quimiometria para uma informação mais detalhada.

Palavras-chave: *Phyllanthus amarus*, *Phyllanthus niruri*, Compostos fenólicos, método de extração.

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LIST OF ABBREVIATIONS AND ACRONYMS

ASCII	American Standard Code for Information Interchange
EAC	Erhlich Ascites Carcinoma
ESI MS	Electrospray ionization mass spectrometers
HIV – RT	Human Immunodeficiency virus reverse transcriptase
HPLC	High Performance Liquid Chromatography
LC – MS	Liquid Chromatography coupled to Mass Spectrometry
MeOH	Methanol
m/z	mass to charge ratio
MS	Mass Spectrometry
PCA	Principal Component Analysis
ppm	parts per million
PTFE	Polytetrafluoroethylene
Q-TOF-MS	Quadrupole Time-of-Flight Mass Spectrometry
SPE	Solid Phase Extraction
SVD	Singular Value Decomposition
TIC	Total Ion Chromatogram
T_R	Retention time
UPLC	Ultra Performance Liquid Chromatography
US	Ultrasound
^{13}C NMR	Carbon-13 Nuclear Magnetic Resonance
^{1}H NMR	Hydrogen-1 Nuclear Magnetic Resonance

CONTENT

1. INTRODUCTION

Phyllanthus amarus Schum & Thonn and *Phyllanthus niruri* Linn (Euphorbiaceae), are small herbs, commonly used in folk medicine to treat several diseases such as intestinal infections, diabetes, urinary, sexual disorders, hepatitis (SARIN *et al.*, 2014). The plants are a rich source of bioactive compounds including lignans, alkaloids, triterpenes, and polyphenols as quercetin, rutin, corilagin, and gallic acid (PATEL *et al.*, 2011; SOUSA *et al.*, 2016) .

Indeed, the specific metabolic composition determines biological functions and ecological interactions between plants, their enemies and beneficial organisms (SCHWEIGER *et al*., 2014). Previous studies on *Phyllanthus* species have reported differences among the extractive processes, enhancing the amount of the targeted molecules in qualitative and quantitative composition (KUMAR *et al.*, 2015)

To date, pressurized liquid, conventional and ultrasound extraction are common to the genus *Phyllanthus* (PATEL *et al.*, 2011). Each parameter method influences on the chemical composition and in the best conditions predicted in the experiment. Specifically, pressurized liquid extraction exhibited greater solubility of phenolic content compared to conventional and ultrasound extraction.

Additionally, ultrasound extraction proved to be inefficient to extract ellagitannins. However, evaluations of extractions in different proportions of solvent yielded an increase in extracting phenolic compounds (SOUSA *et al.*, 2016).It has been known that the use of ultrasonic means for the extraction of secondary metabolites in various raw materials is considered an economical alternative over traditional extraction processes, which is an emerging industry demand for sustainable development (GALVAN D'ALESSANDRO *et al.*, 2012; GARCIA-SALAS *et al.*, 2010).

Targeted and untargeted approaches have found successful applications with mass spectrometry (MS) and nuclear magnetic resonance (MEDIANI *et al.*, 2017; ZHOU *et al.*, 2012). Untargeted approach is a screening tool used to identify all metabolites in a sample. Due to the variability of compounds in different samples, targeted approach summarizes the chemical information at a region leading to the specific metabolites in different profile samples. Combined with chemometric analysis,

the study shows relevant molecular markers related to sample discrimination (GOODACRE *et al.*, 2003).

Hence, the aim of this work was to evaluate the effect of the application of ultrasound waves on the kinetics of extraction of organic-water solvent extraction systems. More specifically, it is intended to delineate the potential of ultrasonic assisted extraction in the preparation of extracts rich in polyphenols and other minor compounds contained in *P. amarus* and *P. niruri* leaves.

Therefore, screening of chemical composition of leaves was also evaluated considering changes in the extraction model by ultra performance liquid chromatography–quadrupole time-of-flight mass spectrometry (UPLC–QTOF-MS/MS) system combined with multivariate data by the targeted and untargeted approach.

2. LITERATURE REVIEW

2.1 *Phyllanthus amarus and Phyllanthus niruri*

The genus *Phyllanthus* belongs to the Euphorbiaceae family and is well known for containing species that are commonly used as medicinal plants. The studies on these types of plants have increased due to pharmacological properties with great potential. Two species are considered the richest among the *Phyllanthus* genus: *Phyllanthus amarus* and *Phyllanthus niruri*, both popularly known as “stone breakers” (“quebra-pedra” in Brazil), they are mostly found in tropical and subtropical countries, especially in Brazil and India (CALIXTO *et al.*, 1998). For a long time, they have been confused due to their similar physical and phytochemical properties, and chemical composition. Both are small annual herbs growing up to 30 – 50 cm and with small elliptical leaves.

Previous studies show that aqueous, methanolic and ethanolic extracts of *P. amarus* and *P. niruri* contain a set of secondary metabolites: alkaloids, ellagitannins, polyphenols, lignans, triterpenes, flavonoids, sterols and volatile oil in *P. amarus* (PATEL *et al.*, 2011)and lignans, tannins, alkaloids, flavonoids, terpenes, coumarins, saponins, phenylpropanoids and sterols in *P. niruri* (BAGALKOTKAR *et al.*, 2006).

The classes of compounds found in both species are the main reason they are regularly used to treat several diseases, including hepatitis-B, kidney stones (the reason

for being popularly known as “stone breakers”), asthma, diabetes, tumours, urinary problems, liver problems, viral and bacterial infections (BAGALKOTKAR *et al.*, 2006; PATEL *et al.*, 2011) and exclusively for *P. niruri*, human immunodeficiency virus reverse transcriptase (HIV-RT) (OGATA *et al.*, 1992).

Therefore, in view of the significant bioactive content of *Phyllanthus* species that justifies the extensive use of *P. amarus* and *P. niruri* in folk medicine, it is extremely important to develop extraction methods and techniques that lead to improved yield of the most relevant compounds.

2.2 Ultrasound-assisted Extraction

The method of ultrasound assisted extraction consists of subjecting the sample to an ultrasonic bath with certain pre-established parameters (KULKARNI; RATHOD, 2014) in order to extract substances with better yields than the conventional method (KHAN *et al.*, 2010).

The mechanism of this method is based on ultrasonic pressure waves through a fluid inducing cavitation phenomenon (CHEMAT *et al.*, 2017), which is a formation of liquid-free cavities (bubbles) in the fluid (WU *et al.*, 2013). This causes disruption of cell walls, turbulence and further implosion of the bubbles, that allows access of the solvent to the targeted compounds. Thus, the compounds dissolve with a better yield in a shorter period of time (SHIRSATH; SONAWANE; GOGATE, 2012).

Compared to other extraction methods, there are mainly two advantages of using ultrasound assisted extraction: it is economical, for it requires less amount of solvent, and, according to previous reports, the time of the extraction can be three times shorter than what would have been if the conventional method, such as maceration, were to be applied (SHIRSATH; SONAWANE; GOGATE, 2012).

The uses of ultrasound-assisted extraction have been widely used for the extraction of phenolic compounds from different types of plant materials due to their shorter extraction times, higher yield, simplicity, lower cost, and eco-friendly properties (ISMAIL *et al.*, 2018; ŽIVKOVIĆ *et al.*, 2018).

2.3 Phenolic Compounds

Phenolic compounds are secondary metabolites and belong to one of the largest groups of substances with over 8000 known structures (BRAVO, 1998). The basic structure consists in an aromatic ring with one or more hydroxyl substituents. Some of the main classes of phenolic compounds are flavonoids, tannins, lignans, coumarins and phenolic acids (CAI *et al.*, 2004; NACZK; SHAHIDI, 2004; PANCHE; DIWAN; CHANDRA, 2016).

The phenolic compounds present in *Phyllanthus amarus* and in *Phyllanthus niruri* made these species the focus of the genus *Phyllanthus*. Among the numerous species in this genus, *P. amarus* and *P. niruri* have been tested and their extracts are well-tolerated in human beings and presented no side effects (CALIXTO *et al.*, 1998), which supports further phytochemical investigation and studies to explore the medicinal potential of these species. Repandusinic acid A, Phyllanthin and Hypophyllanthin are some examples of phenolic compounds with significant therapeutic importance.

Repandusinic acid A (Figure 1) is one of the many phenolic compounds identified in *P. niruri* and is an example of medicinal potential when treating viral diseases. It has been identified as an inhibitor of HIV-RT (OGATA *et al.*, 1992).

A) B)

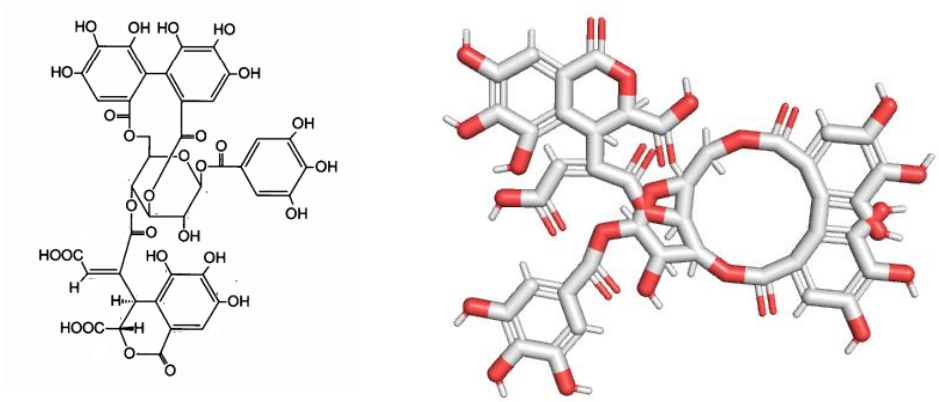


Figure 1. 2D and 3D structure of Repandusinic acid A (1-A and 2-B, respectively).

Phyllanthin and hypophyllanthin (Figure 2) are lignans isolated from *P. amarus* that presented antitumor activity as the mixture (1:1) of the lignans decreased the

growth of solid tumour mass induced by Ehrlich Ascites Carcinoma cells in swiss albino mice (ISLAM *et al.*, 2008).

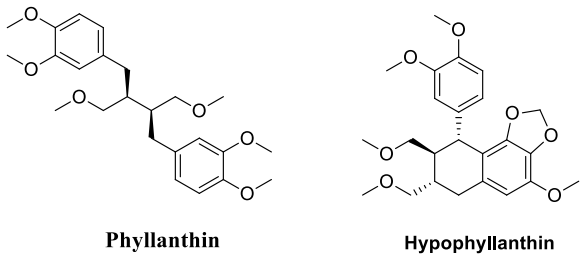


Figure 2. Phyllanthin and hypophyllanthin

3. OBJECTIVES

3.1 General Objectives:

Compare extraction processes of phenolic and other minor compounds from *P. amarus* and *P. niruri* by UPLC-QTOF-MS/MS and chemometric analysis.

3.2 Specific Objectives:

·Prepare ethanolic, methanolic and aqueous extracts of both species by ultrasound and maceration;

·Analyse the samples of each extract in a UPLC-QTOF-MS/MS system to characterize the samples;

·Use the data from the chromatographic analysis to perform chemometric analysis to obtain detailed information of the differences in each extract.

4. MATERIALS AND METHODS

4.1 Chemicals and reagents

Acetonitrile and formic acid (LC–MS grade) were obtained from Merck (Darmstadt, Germany). Water was obtained using a Milli-Q water purifier system from Millipore (Billerica, MA, USA). Standard for thymoquinone, magnoflorine, hederagenin, and kaempferol were purchased from Chromadex (Wesel, Germany). All other chemicals and standards were provided by Sigma Aldrich (St. Louis, MO, USA)

4.2 Ultrasound assisted preparation of *P. amarus* and *P. niruri*

Ultrasound assisted extraction was carried out in an ultrasonic bath (Model ECO-SONICS Q 3.0/40A) with a working ultrasonic frequency of 40 kHz at constant temperature (25 ° C). All the experiments were carried out in glass tubes of known dimension placed in an ultrasonic bath (Total dimensions (cm) : 24 x 13,7 x 10) at known height and position.

50mg of dried leaves, from both species, were initially used and each sample was submitted to extraction with 4 mL of hexane for 15 minutes, to eliminate nonpolar substances (which are interfering substances). After that, the samples were extracted with 4 mL of MeOH/water, EtOH/water, in different proportions, and in pure water. The tested proportions were: 3:7, 1:1, 7:3 (v:v). Each of the systems was submitted to extractions by different methods: (1) ultrasound (US) for 20 minutes and (2) maceration for 24 hours. After the extractions, the samples were analysed with UPLC-QTOF-MS/MS, to evaluate the relative efficiency of the extraction process. All extraction attempts were performed in triplicate (n = 3).

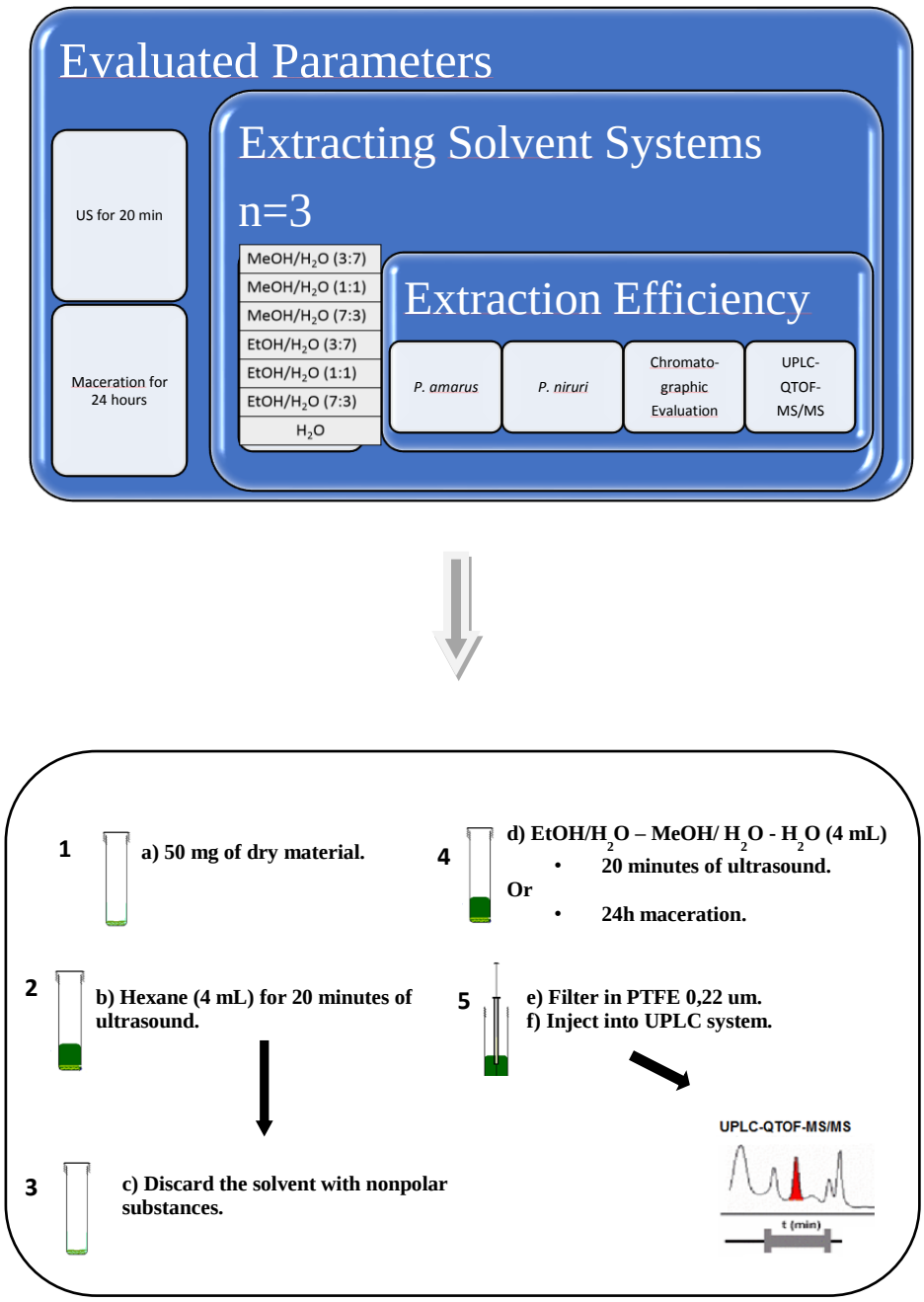


Figure 3. Optimization of the extraction experiment using stone breaker as model plant material.

4.3 UPLC–QTOF-MS/MS

The samples were filtered with Syringe filters (PTFE, 0.2 µm pore and 13 mm diameter, Millipore Millex) and 5 µL of each sample was injected into the UPLC system (Waters Co., Milford, MA, USA). The instrumental UPLC analysis was performed in an ACQUITY UPLC BEH column (150 × 2.1 mm, 1.7 um; Waters Co.)

on a Waters Acquity UPLC system. The column temperature was set at 40 °C. The binary gradient elution system consisted of 0.1 % formic acid in water (A) and 0.1 % formic acid in acetonitrile (B), with linear gradient from 2 to 95 % B (0-15 min), with a flow rate of 0.4 $mL.min^{-1}$.

The profiling was performed by coupling the Waters ACQUITY UPLC system to the Q-TOF Premier mass spectrometer (Waters MS Technologies, Manchester, UK) with electrospray ionization (ESI) interface in the negative ionizations mode. The desolvation gas used was nitrogen and set at 350 °C with flow rate of 500 L/h. The capillary and cone voltages were adjusted to 2.6 kV and 0.5 V, respectively. The mass accuracy and reproducibility were maintained by infusing lock mass (leucine-enkephalin, 0.2 ng/µL; $[M-H]^-$ ion at *m/z* 556.2771). MS data were collected for *m/z* values in the range of 110-1180 Da with a scan time of 0.1 over an analysis time of 19 min. The accurate mass and molecular formula assignments were obtained with the MassLynx 4.1 software (Waters MS Technologies).

4.4 Chemometric analysis

The UPLC-MS chromatogram files were converted to American Standard Code for Information Interchange (ASCII) files for both numeric matrices construction (*P. amarus* e *P. niruri*), which were exported to The Unscrambler X™ program 10.4 (CAMO software, Woodbridge, NJ, USA)for chemometric analysis. The chromatogram region between 0.5 and 7.3 min was selected for both *Phyllanthus* species. The Principal Component Analysis (PCA) analysis was developed using the Singular Value Decomposition (SVD) algorithm to decompose the matrices, which was performed after baseline correction and normalization over the chromatograms (samples) and mean-centered processing over the variables (compounds).

5. RESULTS AND DISCUSSION

5.1 Ultrasound assisted extraction

In order to analyse the metabolic profile of the *Phyllanthus* species in the water/organic solvent system, it was first necessary to verify the best extraction method to be used.

Due to the uses and advantages previously studied in phenolic compounds, this study aims the extraction method that has the best yield in extracting as many phenolic compounds as possible.

The method of ultrasound-assisted extraction was chosen because it has been considered an advanced extraction technique, leading to high recovery yields of bioactive compounds due to cavitation (CHEMAT; ZILL-E-HUMA; KHAN, 2011; PAPOUTSIS *et al.*, 2018). Besides that, it is simple to apply in different types of samples, economical and still to be considered an eco-friendly technique (TENG *et al.*, 2016).

However, it is known that the quantity and quality of polyphenol compounds may vary depending on the conditions of the ultrasound-assisted extraction process (PAPOUTSIS *et al.*, 2018). Therefore, optimizing the extraction conditions in such process is relevant.

Thus, the ultrasonic method was used in the investigation of the extraction of phenolic and other minor compounds from *P. amarus* e *P. niruri*. Initially, two main variables were considered in this study of extraction yield. Firstly, the use of ultrasound or simple maceration, and secondly, the organic/water solvent ratio.

Table 1 lists the polar solvent conditions investigated in this ultrasound-assisted extraction study. The extraction time and mass of plant material were set at 20 minutes and 50 mg, respectively for all the extraction attempts performed in this study.

Table 1. Organic-water solvent extraction systems ultrasound-assisted

Experiment	Condition	Solvent (4 mL)	v/v
1	(1)	MeOH:H_2O	3:7
2	(1)	MeOH:H_2O	1:1
3	(1)	MeOH:H_2O	7:3
4	(1)	EtOH:H_2O	3:7
5	(1)	EtOH:H_2O	1:1
6	(1)	MeOH:H_2O	7:3
1	(2)	EtOH:H_2O	3:7
2	(2)	EtOH:H_2O	1:1
3	(2)	EtOH:H_2O	7:3
4	(2)	EtOH:H_2O	3:7
5	(2)	EtOH:H_2O	1:1
6	(2)	EtOH:H_2O	7:3
7	(1)	H_2O	-
7	(2)	H_2O	-

(1) ultrasound (US) for 20 minutes, Ultrasonic frequency - 40 kHz, T: 25° C; (2) maceration for 24 hours;

As expected, for both *P. amarus* and *P niruri* evaluation, the differences in the extraction processes were clearly evidenced using only water as solvent compared to ethanolic and methanolic extractions. The ultrasound processing did not present relevant effect for all the extraction processes (water, ethanol, or methanol), but it was the chosen method due to its economic and environmental-friendly properties.

However, the detailed investigations applied for ethanolic and methanolic extracts for both *P. amarus* and *P. niruri* show that the separations of the samples using ethanol as solvent were more evident than methanol and that there was a better extraction of phenolic compounds, when comparing in chemometric analysis, which extract contains the most phenolic compounds, seen furthermore.

For an accurate response to which extraction system yields the best extraction of phenolic compounds, further investigations must be made, such as total phenolic content in each extract. This will enhance the comparison among the different extraction

systems. At this moment, the number of identified phenolic compounds in each extract is being compared with chemometric analysis.

The methodology development in this study reduced amounts of sample, solvents, in addition to a shorter extraction time.The development of this extraction methodology gives us an overview of extraction methods when subjected to ultrasonic conditions.

Additionally, it has shown to be economically and environmentally feasible, since it allows the application of the method to many samples, using a minimum amount of solvent, with low environmental impact, because it is a mixture of ethanol or methanol/ water that used a small amount of plant material.

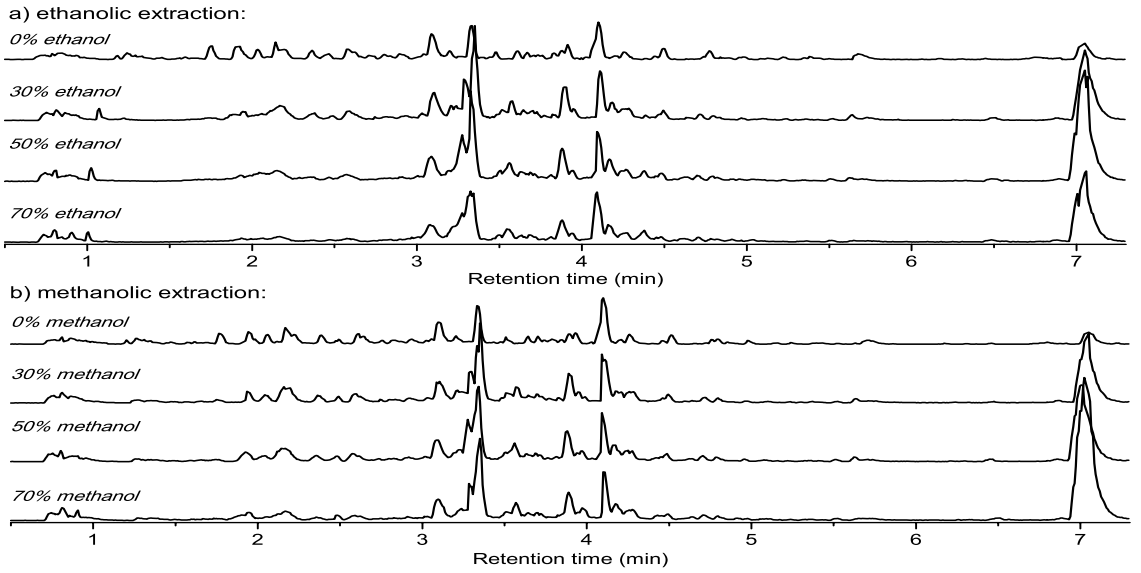
5.2 UPLC–QTOF–MS/MS-ESI

UPLC–QTOF–MS/MS-ESI was used to detect major and minor secondary metabolites. The ultra-high performance liquid chromatography-quadrupole time-of-flight mass spectrometry with electrospray ionization technique is an approach that offers high sensitivity, excellent resolution, efficient separation of metabolites, and allows a reduction of the analysis time (NOVÁKOVÁ; SVOBODA; PAVLÍK, 2017).

In agreement with previous reports (SOUSA *et al.*, 2016), it was possible to characterize the compounds according to their *m/z* values and fragmentation profiles. The tentative identification of many minor and major unknown compounds was performed by the accurate mass (mass error <11 ppm) and fragmentation patterns.

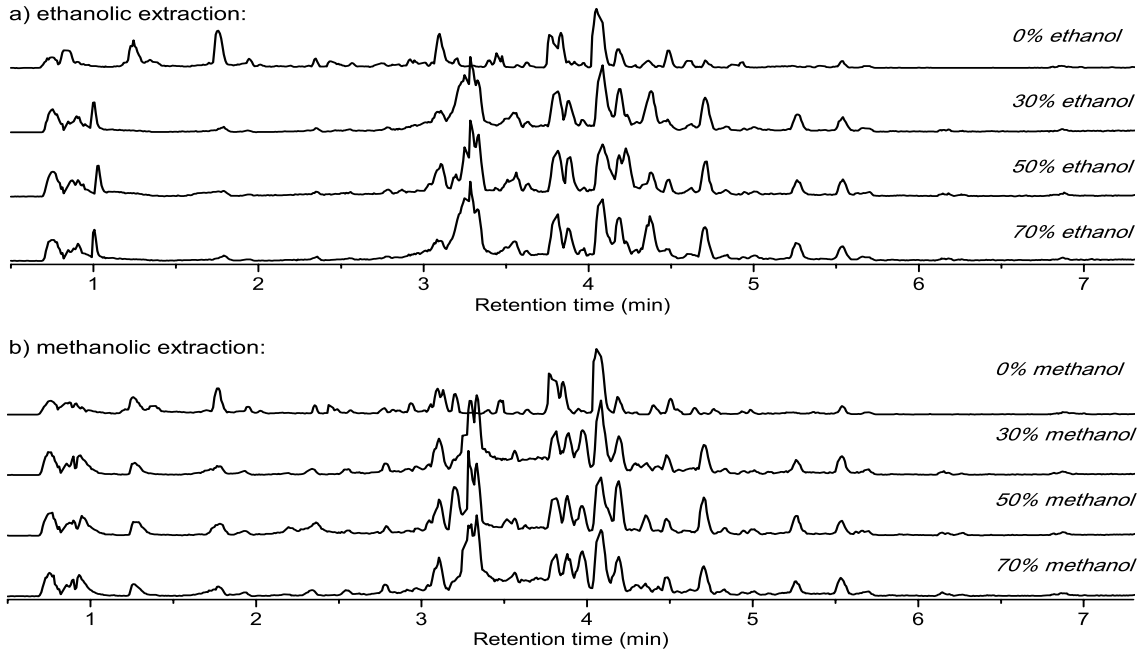
As described below, the phenolic content was the main chemical group of substances observed in the polar extractions of both species of *Phyllanthus* (*P. amarus* e *P. niruri*). Typical chromatographic profiles (in the same intensity) for *P. amarus* samples from the ethanolic (a) and methanolic (b) extractions obtained through ultrasound, are illustrated in Figures 4a and 4b, respectively. In the same approach, Figures 5a and 5b present the chromatographic profiles for *P. niruri* extracts obtained through ultrasound.

The extractions obtained through maceration showed no relevant differences in the analyses. Table 2 summarizes the characterized compounds for both *Phyllanthus* species.



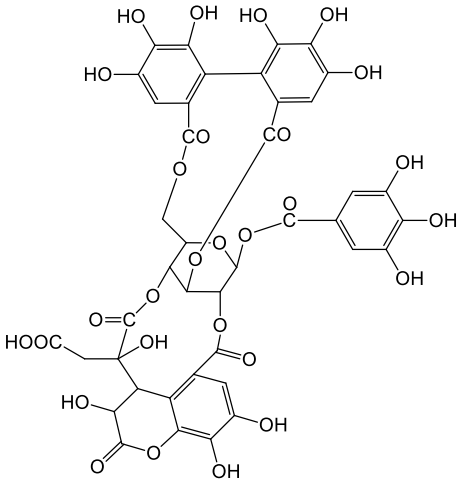
Source: The authors.

Figure 4. Chromatograms (TIC, negative mode) of *P. amarus*' ethanolic (a) and methanolic (b) extracts obtained through US.

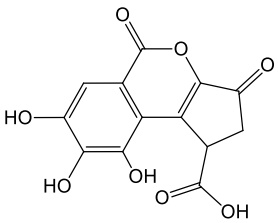


Source: The authors.

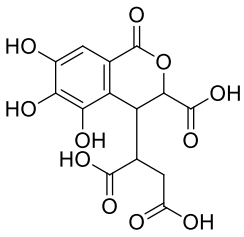
Figure 5. Chromatograms (TIC, negative mode) of *P. niruri*'s ethanolic (a) and methanolic (b) extracts obtained through US.



Amariinic acid



Brevifolin Carboxylic Acid



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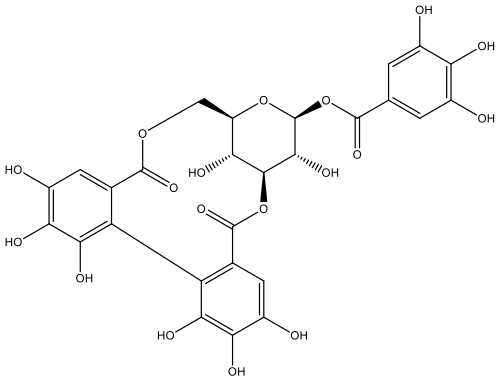

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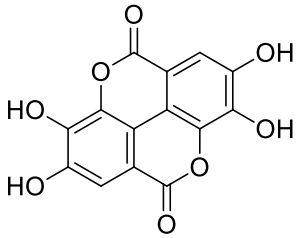
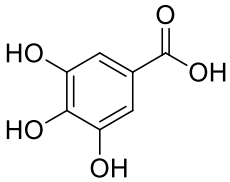


Ellagic acid



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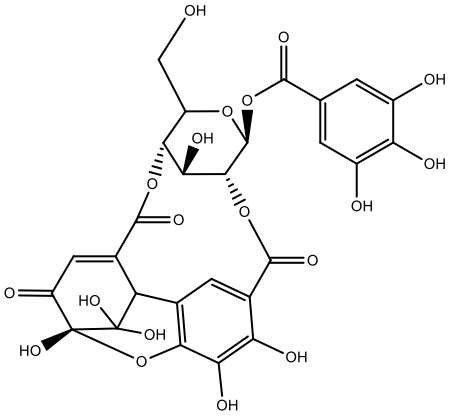
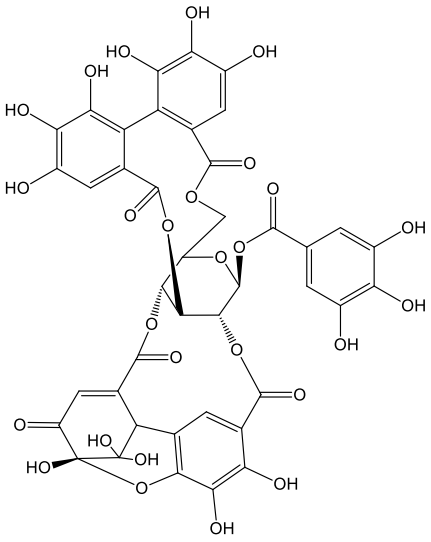
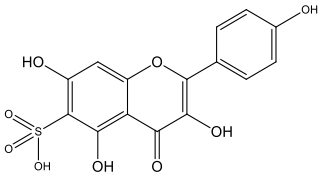


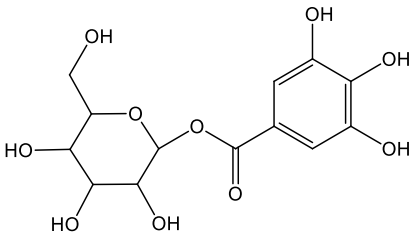
Furosin



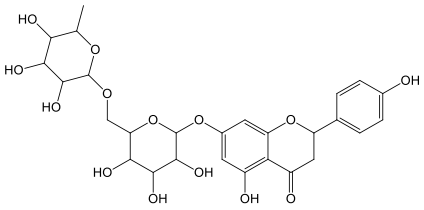
Geraniin



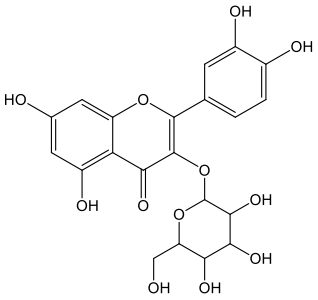
Niruriflavone



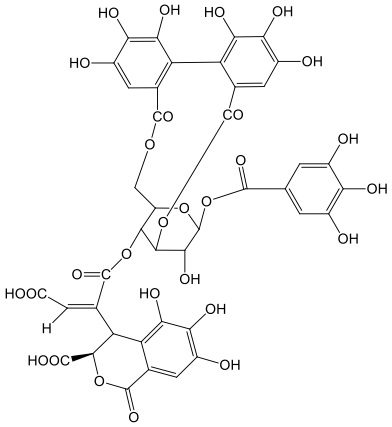
Monogalloylhexoside



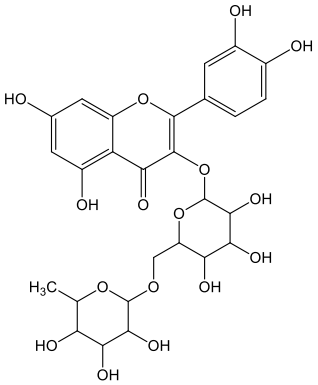
Narirutin



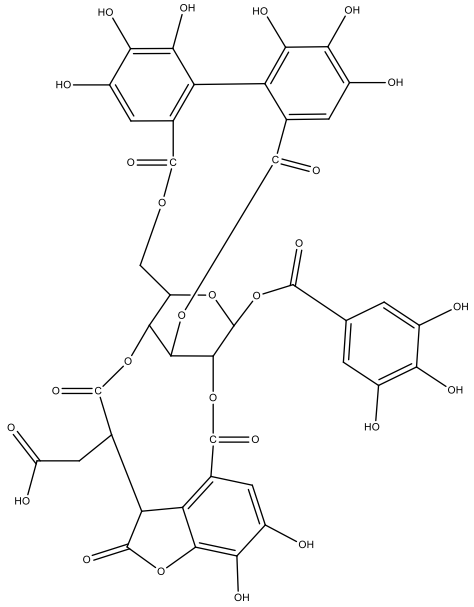
Quercetin-3-*O*-hexoside



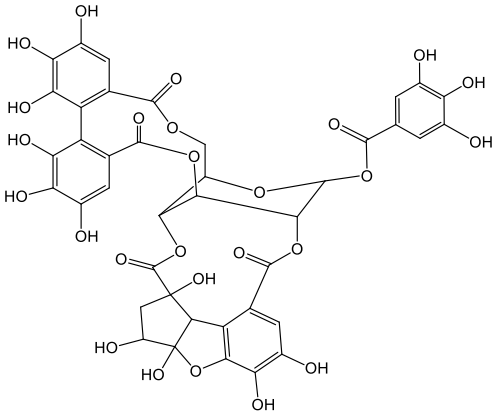
Repandusinic acid



Rutin



Phyllanthusiin U



Phyllanthusiin C

Figure 6. Structures of the phenolic compounds detected in the composition of *P. amarus* and *P. niruri.*

Table 2. Compounds tentatively determined by UPLC-QTOF-MS/MS in the *P. amarus* and *P. niruri* extracts.

Nº Peaks	**R_t (min)**	**$[M-H]^-$ *m/z***	***MS/MS* Fragments**	**Molecular Formula**	**Error (ppm)**	**Proposed compound**	*P. amarus*	*P. niruri*	**Reference**
1	0.86	209.0269	191.0524	$C_6H_{10}O_8$	-2.8	Mucic acid	X	X	(YANG *et al.*, 2012)
2	0.91	191.0529	-	$C_7H_{12}O_7$	-2.7	Quinic acid	-	X	(KUMAR *et al.*, 2015)
3	0.94	515.0638	335, 226	-	-	Unknown	-	-	-
4	1.06	355.0310	337.0197	$C_{14}H_{12}O_{11}$	2.5	Chebulic acid	-	X	(YANG *et al.*, 2012)
5	1.28	191.0188	-	$C_6H_8O_7$	2.1	Mucic acid lactone	X	-	(YANG *et al.*, 2012)
6	1.3	-	-	$C_{12}H_{15}NO_3$	0	Niruroidine	X	X	(GUO *et al.*, 2015)
7	1.41	-	-	$C_{12}H_{15}NO_3$	5.4	Niruroidine (isomer)	X	X	(GUO *et al.*, 2015)
8	1.61	331.0665	271.0484 211.0218 169.0114	$C_{13}H_{16}O_{10}$	-3.9	Monogalloyl-hexoside	X	X	(SENTANDREU; CERDÁN-CALERO; SENDRA, 2013)
9	1.62	-	-	$C_{12}H_{15}NO_3$	5.4	Niruroidine (isomer)	X	X	(GUO *et al.*, 2015)
10	1.73	169.0126	125.0237	$C_7H_6O_5$	-6.5	Gallic acid	X	X	(YANG *et al.*, 2012)

Table 2

Nº Peaks	**R_t (min)**	**$[M-H]^-$ *m/z***	***MS/MS* Fragments**	**Molecular Formula**	**Error (ppm)**	**Proposed compound**	*P. amarus*	*P. niruri*	**Reference**
11	2.17	-	120	$C_9H_{11}NO_2$	4.2	Phenylalanine	-	X	(HANHINEVA *et al.*, 2008)
12	2.39	447.1145	-	$C_{18}H_{24}O_{13}$	1.3	Unknown	-	X	
13	2.53	669.0915	337.0197	$C_{27}H_{26}O_{20}$	-3.6	Neochebuloylgalloylglucose	-	X	(YANG *et al.*, 2012)
14	2.64	649.0670	169.0135 435.0543	$C_{27}H_{22}O_{19}$	-1.1	Furosin	-	X	(DU *et al.*, 2018)
15	2.87	649.0709	169.0134 435.0540	$C_{27}H_{22}O_{19}$	4.9	Furosin (isomer)	-	X	(DU *et al.*, 2018)
16	2.88	463.0515	-	$C_{20}H_{16}O_{13}$	0.4	Ellagic acid hexose	-	X	(YANG *et al.*, 2012)
17	3.07	969.0904	169.0121 247.0229 300.9979	$C_{41}H_{30}O_{28}$	6.1	Repandusinic acid A	-	X	(OGATA *et al.*, 1992)
18	3.13	291.0133	247.0228	$C_{13}H_8O_8$	-2.1	Brevifolin carboxylic acid	X	X	(KUMAR *et al.*, 2015)

Table 2

Nº Peaks	**R_t (min)**	**$[M-H]^-$ *m/z***	***MS/MS* Fragments**	**Molecular Formula**	**Error (ppm)**	**Proposed compound**	*P. amarus*	*P. niruri*	**Reference**
19	3.23	969.0867	169.0134 247.0228 300.9966	$C_{41}H_{30}O_{28}$	2.3	Repandusinic acid A (isomer)	-	X	(OGATA *et al.*, 1992)
20	3.24	969.0858	247.0228 300.9971	$C_{41}H_{30}O_{28}$	1.3	Amariinic acid	X	-	(YEAP FOO, 1995)
21	3.24	633.0714	247.0226 300.9967 463.0463	$C_{27}H_{22}O_{18}$	-2.2	Corilagin	X	X	(KUMAR *et al.*, 2015)
22	3.26	-	-	$C_{34}H_{22}O_{22}$	1.8	Emblicanin A	X	X	(GUO *et al.*, 2015)
23	3.32	-	-	$C_{34}H_{22}O_{22}$	1.4	Emblicanin A(isomer)	X	X	(GUO *et al.*, 2015)
24	3.32	951.0797	169.0144 300.9981 933.0655	$C_{41}H_{28}O_{27}$	6.0	Geraniin	X	X	(KUMAR *et al.*, 2015)
25	3.51	305.0685	225	$C_{15}H_{14}O_7$	7.9	Gallocatechin	X	X	(HOSSAIN *et al.*, 2010)

Table 2

Nº Peaks	**R_t (min)**	**$[M-H]^-$ *m/z***	***MS/MS* Fragments**	**Molecular Formula**	**Error (ppm)**	**Proposed compound**	*P. amarus*	*P. niruri*	**Reference**
26	3.52	387.1638	169.0123 207.1026 329.0899	$C_{18}H_{30}O_9$	-4.4	Tuberonic acid hexoside	-	X	(KUMAR *et al.*, 2015)
27	3.58	925.0967	300.9972	$C_{40}H_{30}O_{26}$	2.2	Phyllanthusiin C	X	X	(LATTÉ; KOLODZIEJ, 2000)
28	3.65	969.0998	169.0126 247.0231 300.9975	$C_{41}H_{30}O_{28}$	5.8	Repandusinic acid A (isomer)	-	X	(OGATA *et al.*, 1992)
29	3.81	593.1506	327.0494 357.0640 429.0833 473.1087	$C_{27}H_{30}O_{15}$	-4.0	Orientin-2″-*O*’-rhamnoside	-	X	(FILHO *et al.*, 2018)
30	3.84	247.0232	-	$C_{12}H_8O_6$	4.5	Brevifolin	X	X	(SENTANDREU; CERDÁN-CALERO; SENDRA, 2013)
31	3.89	951.0757	169.0134 300.9977 363.0745	$C_{41}H_{28}O_{27}$	1.8	Geraniin	X	X	(KUMAR *et al.*, 2015)

Table 2

Nº Peaks	**R_t (min)**	**$[M-H]^-$ *m/z***	***MS/MS* Fragments**	**Molecular Formula**	**Error (ppm)**	**Proposed compound**	*P. amarus*	*P. niruri*	**Reference**
32	3.96	395.1538	-	-	-	Unknown	X	-	
33	4.09	577.1548	293.0421 300.9949	$C_{27}H_{30}O_{14}$	-1.6	Vitexin-2″-*O*-rhamnoside	-	X	(SPRENGER; CASS, 2013)
34	4.12	300.9952	206.0800	$C_{14}H_6O_8$	-10.6	Ellagic acid	X	-	(KUMAR *et al.*, 2015)
35	4.13	609.1458	-	$C_{27}H_{30}O_{16}$	0.3	Rutin	X	-	(HOSSAIN *et al.*, 2010)
36	4.20	463.0877	197.0414 300.9974	$C_{21}H_{20}O_{12}$	0.0	Quercetin-3-*O*-hexoside	X	X	(HOSSAIN *et al.*, 2010)
37	4.32	-	-	$C_{15}H_{10}O_7$	0.7	Quercetin	X	X	(GUO *et al.*, 2015)
38	4.45	363.0169	169.0134 300.9981	$C_{21}H_{12}O_8S$	-3.3	Niruriflavone	-	X	(GUO *et al.*, 2015)
39	4.51	579.1713	245.0903	$C_{27}H_{32}O_{14}$	-0.1	Narirutin	X	-	(FILHO *et al.*, 2018)
40	4.56	-	-	-	-	Unknown	X	X	
41	4.73	-	-	-	-	Unknown	-	X	
42	4.80	237.1178	-	-	-	Unknown	X	-	

Table 2

Nº Peaks	**R_t (min)**	**$[M-H]^-$ *m/z***	***MS/MS* Fragments**	**Molecular Formula**	**Error (ppm)**	**Proposed compound**	*P. amarus*	*P. niruri*	**Reference**
43	4.9	923.0833	-	$C_{40}H_{28}O_{26}$	4.5	Phyllanthusiin U	-	X	Chen et al., 2011
44	5.28	583.1088	169.0136 395.0781 413.0871 431.0963	$C_{28}H_{24}O_{14}$	0.0	Kaempferol-*O*-galloyl-deoxyhexoside	-	X	Gu et al., 2013
45	5.40	629.2107	-	-	-	Unknown	X	-	
46	5.56	559.1463	395.0782 483.1870 545.2019	$C_{27}H_{28}O_{13}$	2.0	3-*O*-Sinapoyl-5-*O*-caffeoylquinic acid	-	X	Kuhnert et al. 2011
47	5.62	497.0748	-	-	-	Unknown	X	X	
48	6.65	343.0438	328.0220	$C_{17}H_{12}O_8$	-4.7	Tri-*O*-methylellagic acid	X	-	(KUMAR *et al.*, 2015)
49	7.22	363.0138	-	$C_{16}H_{12}O_8S$	-10.2	Niruriflavone	X	-	(GUO *et al.*, 2015)

According to Table 2, it was observed in the chromatograms the presence of 34 phenolic compounds, of which 14 were detected in the *P. amarus*' sample, shown in Figure 6 and 22 in the *P. niruri* sample, also shown in Figure 6. They are: Mono galloyl-hexoside (peak 8), Gallic acid (peak 10), Brevifolin Carboxylic acid (peak 18), Amariinic acid (peak 20), Corilagin (peak 21), Geraniin (peak 24), Phyllanthusiin C (peak 27), Ellagic acid (peak 34), Rutin (peak 35), Quercetin-3-*O*-hexoside (peak 36), Narirutin (peak 39), Tri-methyl ellagic acid (peak 48) in *P. amarus*, and Chebulic acid (peak 4), Mono galloyl-hexoside (peak 8), Gallic acid (peak 10), Neochebuloylgalloylglucose (peak 13), Furosin (peak 14), Ellagic acid hexose (peak 16), Repandusinic acid A (peak 17), Brevifolin Carboxylic acid (peak 18), Corilagin (peak 21), Geraniin (peak 24), Phyllanthusiin C (peak 27), Orientin-2''-*O*-rhamnoside (peak 29), Vitexin-2''-*O*-rhamnoside (peak 33), Quercetin-3-*O*-hexoside (peak 36), Niruriflavone (peak 38), Phyllanthusiin U (peak 43), Kaempferol-*O*-galloyl-deoxyhexoside (peak 44), 3-*O*-Sinapoyl-5-*O*-caffeoylquinic acid (peak 46) in *P. niruri*.

Other 5 minor compounds were detected in the chromatograms, of which three are carboxylic acids, one is an alkaloid and another one is an amino acid.

5.3 Chemometric Analysis

5.3.1 *P. amarus*

The chromatograms present a complex set of organic compounds and a visual distinction is challenging. Therefore, chemometric analysis was performed to investigate precisely the differences of the content of organic profile associated with different extraction processes and solvents for both *Phyllanthus* species. Figure 7a shows the PC1 *vs* PC2 scores plot from the evaluation of all the extracts from *P. amarus*. In addition, a detailed investigation of the differences among the extracts composition according to ethanol/water and methanol/water ratios as extraction solvent, a PCA was developed only for ethanolic (green) and methanolic (red) extracts, which is presented in Figure 7b.

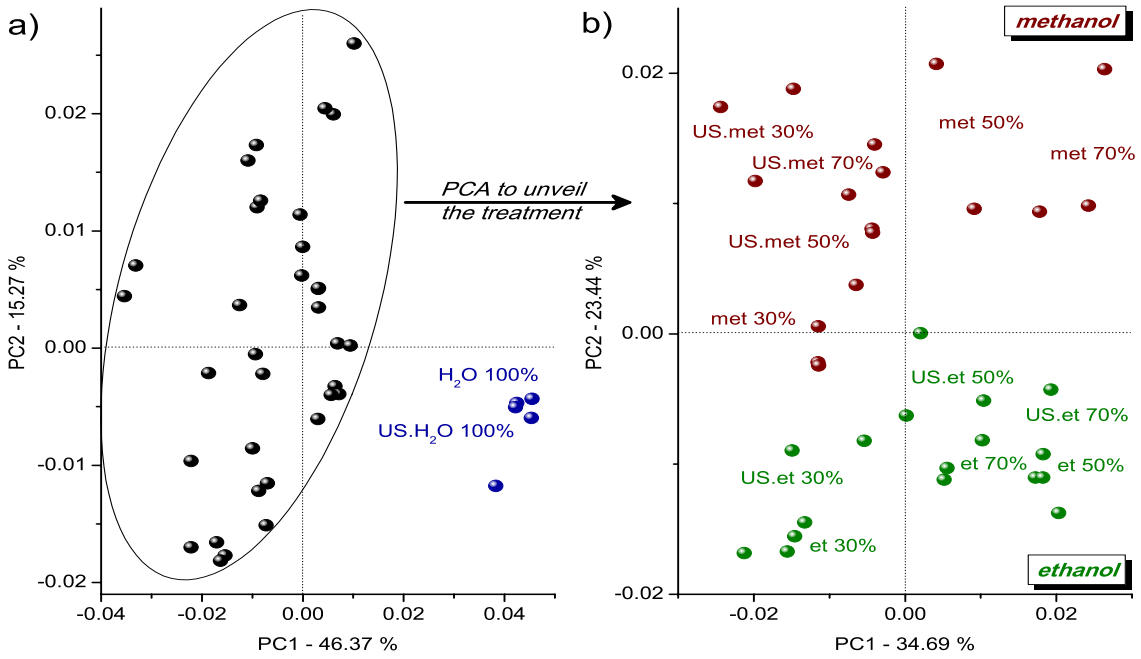


Figure 7. a) PC1 *vs*. PC2 scores plot from the evaluation of all the *P. amarus* extracts composition; b) PC1 *vs*. PC2 scores plot only for ethanolic (green) and methanolic (red) extracts.

Figure 7a presents a clear separation of the resultant extracts using only water (H_2O in blue) as solvent from the ethanolic and methanolic extracts (in black) only according to PC1 axis. The detailed investigation applied for the ethanolic and methanolic extracts (Figure 7b) evidenced the separation tendency of the ethanolic extracts (in green) and methanolic extracts (in red) according to PC2 axis. In addition, Figure 7b presented a separation tendency of the samples related to ethanol/water and methanol/water ratios according to PC1 axis. Basically, the positive scores of PC1 are the extracts using higher amounts of ethanol and methanol (70 % and 50 %), and in the negative scores are the extracts mainly with lower amount of ethanol (30 %). The ultrasound (US) processing did not present relevant effect in the extract's composition using water, ethanol, or methanol as solvent.

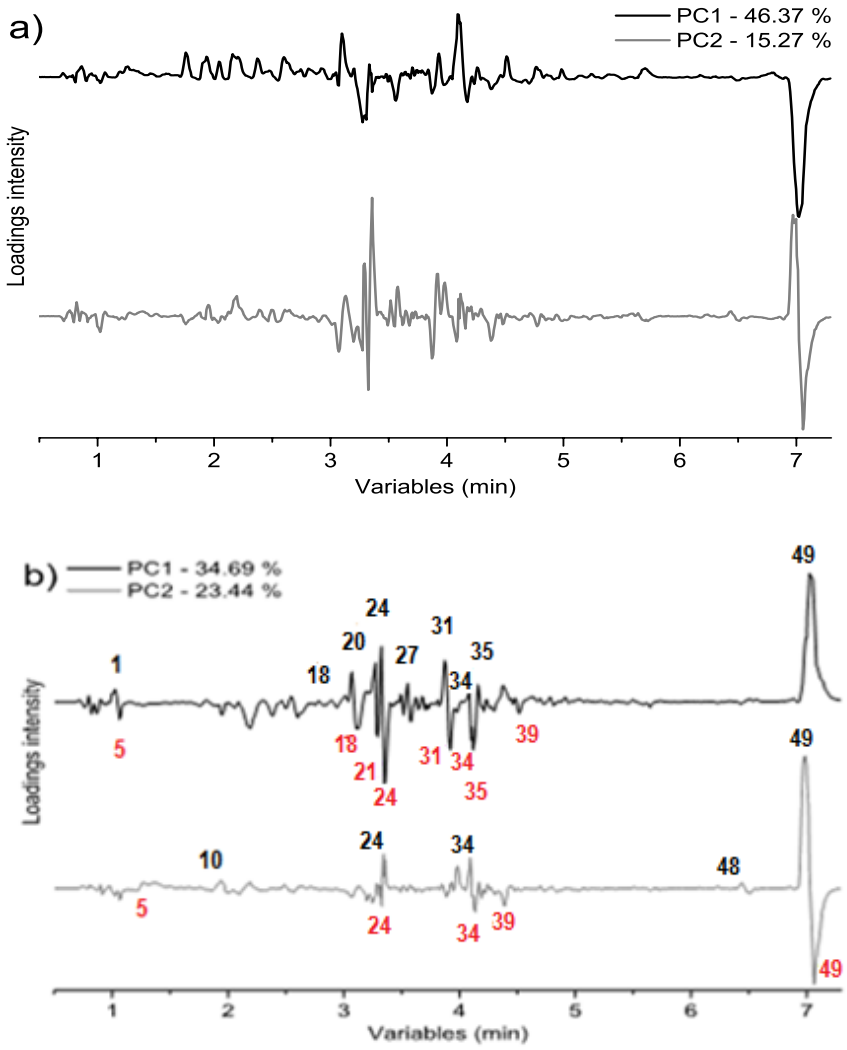


Figure 8. Loadings from *P.amarus* extracts plotted in lines: a) all the extracts; b) only for ethanolic and methanolic extracts.

In a detailed view, the differences in each extract concerning the methanol/water and ethanol/water ratio, according to PC1 axis, are summarized in Table 3 after analyzing 9 major loading intensities of the positive scores, and 8 of the negative scores in Figure 8b.

Table 3. Main loading intensities of PC1 axis and their correspondent proposed compounds.

Positive	Retention time (min)	Proposed compounds	Negative	Retention time (min)	Proposed compounds
1	0,86	Mucic Acid	5	1,28	Mucic Acid Lactone
18	3,13	Brevifolin Carboxylic acid	17	3,13	Brevifolin Carboxylic acid
20	3,24	Amariinic Acid	21	3,24	Corilagin
24	3,32	Geraniin	24	3,32	Geraniin
27	3,58	Phyllanthusin C	31	3,89	Geraniin
31	3,89	Geraniin	34	4,12	Ellagic Acid
34	4,12	Ellagic Acid	35	4,13	Rutin
35	4,13	Rutin	39	4,51	Narirutin
49	7,07	Niruriflavone			

Therefore, the difference between the methanol/water and ethanol/water ratio concerning which one yielded a better extraction of phenolic compounds and other minor compounds is very slim.

The differences in each extract regarding the solvent that was used, according to PC2 axis, are summarized in Table 4 after analysing 5 major loading intensities of the positive scores and 5 of the negative scores in Figure 8b.

Table 4. Main loading intensities of PC2 axis and their correspondent proposed compounds.

Positive	Retention time(min)	Proposed compounds	Negative	Retention time (min)	Proposed compounds
10	1,73	Gallic Acid	5	1,28	Mucic Acid Lactone
24	3,32	Geraniin	24	3,32	Geraniin
34	4,12	Ellagic Acid	34	4,12	Ellagic Acid
48	6,55	Tri-*O*-methyl ellagic acid	39	4,51	Narirutin
49	7,07	Niruriflavone	49	7,07	Niruriflavone

According to Table 4, the ethanolic extract (green) in negative values of PC2 yielded a better extraction of phenolic compounds.

5.3.2 *P. niruri*

As presented for *P. amarus* extracts, Figure 9a shows the PC1 *vs* PC2 scores plot for the evaluation of all the extracts from *P. niruri*, and the detailed analysis performed only for ethanolic (green) and methanolic (red) extracts is shown in Figure 9b.

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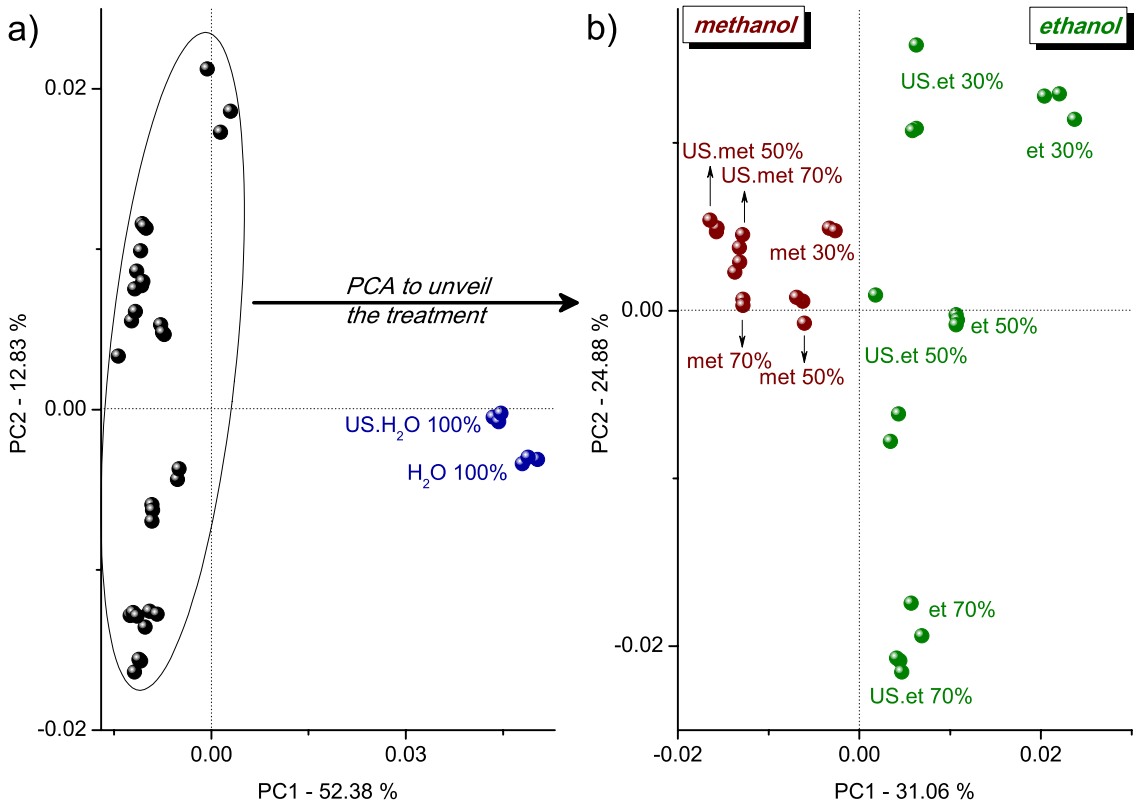

Figure 9. a) PC1 vs. PC2 scores plot from the evaluation of all the *P. niruri* extracts composition; b) PC1 vs. PC2 scores plot only for ethanolic (green) and methanolic (red) extracts

As presented in the PCA analysis for *P. amarus* extracts, PC1 axis presented in Figure 9a shows the separation of the resultant extracts using only water (H_2O in blue) as solvent from the others ethanolic and methanolic extracts (in black). The detailed investigation only for ethanolic and methanolic extracts presented in Figure 9b demonstrated the separation tendency of the ethanolic extracts (in green) and methanolic extracts (in red) according to PC1 axis. In addition, the separation of the extracts related to the ethanol/water and methanol/water ratio was more evident in the ethanolic extraction, with a higher percentage of ethanol (70 % and 50 %) in negative values of PC1 and a lower percentage in positive values of the same PC. As presented for *P. amarus* evaluation, the ultrasound (US) processing did not present significant effect for extractions using water, ethanol, and methanol.

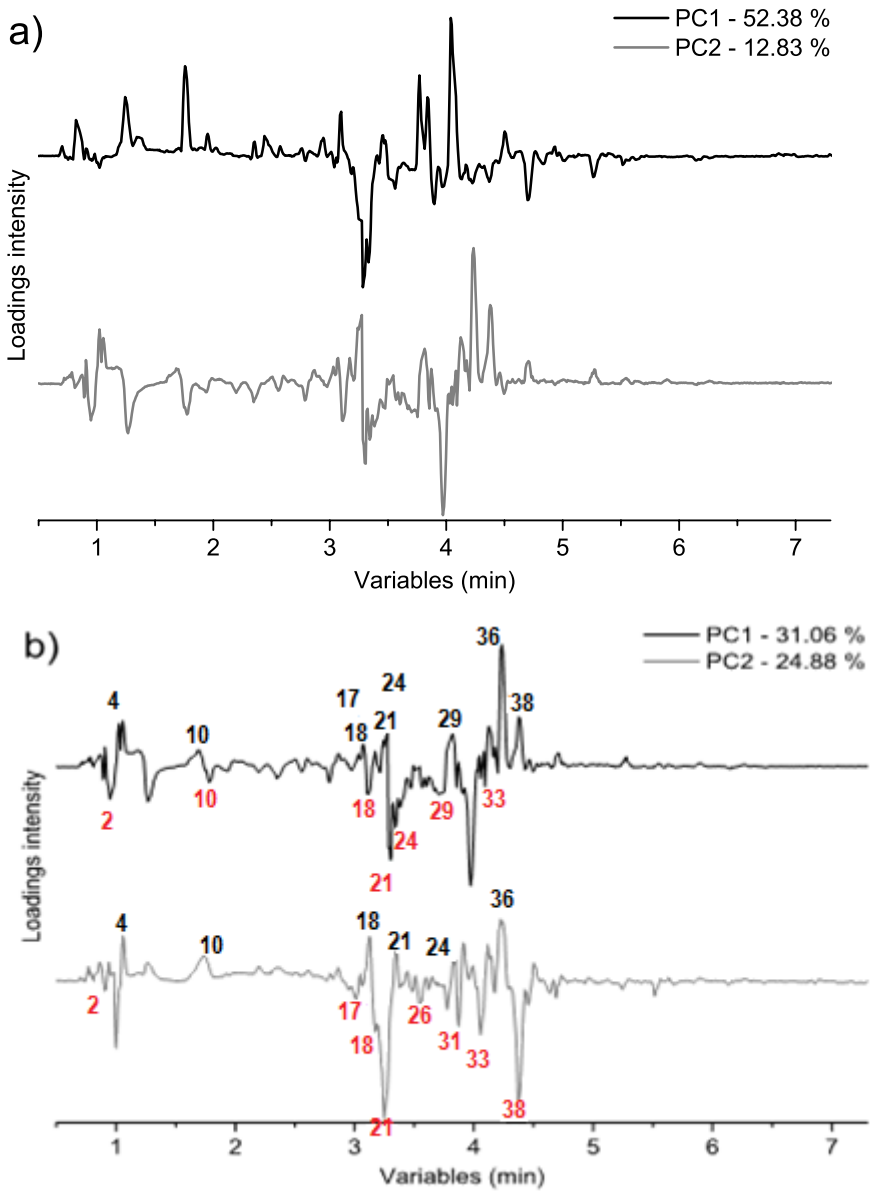

Figure 10. Loadings from *P. niruri* extracts plotted in lines: a) all the extracts; b) only for ethanolic and methanolic extracts.

In a detailed view, the differences in each extract concerning the extraction solvent (methanol and ethanol), according to PC1 axis, are summarized in Table 5 after analyzing 9 major loading intensities of the positive scores, and 7 of the negative scores in Figure 10b.

Table 5. Main loading intensities of PC1 axis and their correspondent proposed compounds.

Positive	**Retention time (min)**	**Proposed compounds**	**Negative**	**Retention time (min)**	**Proposed compounds**
4	1,06	Chebulic Acid	2	0,91	Quinic Acid
10	1,73	Gallic Acid	10	1,73	Gallic Acid
17	3,07	Repandusinic Acid A	18	3,13	Brevifolin Carboxylic Acid
18	3,13	Brevifolin Carboxylic Acid	21	3,24	Corilagin
21	3,24	Corilagin	24	3,32	Geraniin
24	3,32	Geraniin	29	3,81	Orientin-2”-*O*-rhamnoside
29	3,81	Orientin-2”-*O*-rhamnoside	33	4,09	Vitexin-2”-*O*-rhamnoside
36	4,20	Quercetin-3-*O*-hexoside			
38	4,45	Niruriflavone			

The positive values of PC1 present a higher number of phenolic compounds. Therefore, the ethanolic extract yielded a better extraction of the latter compared to the methanolic extract.

The differences in each extract concerning the methanol/water and ethanol/water ratio, according to PC2 axis, are summarized in Table 6 after analyzing 6 major loading intensities of the positive scores, and 8 of the negative scores in Figure 10b.

Table 6. Main loading intensities of PC2 axis and their correspondent proposed compounds.

Positive	**Retention time (min)**	**Proposed compounds**	**Negative**	**Retention time (min)**	**Proposed compounds**
4	1,06	Chebulic Acid	2	0,91	Quinic Acid
10	1,73	Gallic Acid	17	3,07	Repandusinic Acid A
18	3,13	Brevifolin Carboxylic Acid	18	3,13	Brevifolin Carboxylic Acid
24	3,32	Geraniin	21	3,24	Corilagin
29	3,81	Orientin-2”-*O*-rhamnoside	26	3,52	Tuberonic Acid hexoside
36	4,20	Quercetin-3-*O*-hexoside	31	3,89	Geraniin
			33	4,09	Vitexin-2”-*O*-rhamnoside
			38	4,45	Niruriflavone

6. CONCLUSIONS

Metabolites of *P. niruri and P. amarus* were extracted under optimized conditions. After evaluation of the ultrasound-assisted extraction method by varying the solvent and the use of ultrasonic technique, it was observed that the best extraction system was the 3: 7 (v/v) ethanol/water mixture, with US (1) for 20 min. This is observed based on chemometric analysis that gives us the identified phenolic compounds in each extraction system, and on the benefits this extraction system provides, regarding the environment and the lower use of resources. The 3:7 (v/v) ethanol/water mixture was proven to be more selective in extracting phenolic compounds.

Thus, we have developed a promising ecologically correct alternative, providing good chromatographic separation and protection against the degradation of unstable polyphenols in the presence of light.

Methanol and ethanol/water extracts of *P. niruri and P. amarus* were analyzed by UPLC–QTOF-MS/MS with the objectives to establish a model for quality prediction and to identify potential marker metabolites. Chromatographic fingerprinting demonstrated the similarities and differences between samples analyzed.

A total of 41 metabolites were evaluated via a UPLC-QTOF-MS/MS-based metabolomic study. Among them, 14 and 22 phenolic compounds were detected in the *P. amarus* and *P. niruri*, respectively. Therefore, in total 34 phenolic compounds were tentatively identified.

Additionally, due to the complexity of the UPLC-QTOF-MS/MS data, the chemometric analysis was applied for evaluation of samples. Thus, a chemometric technique was used to discriminate between methanolic and ethanolic sample of *P. niruri and P. amarus.*

The PCA data showed that the ethanolic extract provided a better extraction in relation to the methanolic extract, in view of the greater quantity of phenolic compounds obtained, seen in tables 3, 4, 5 and 6.

However, for a more accurate response to which extraction system provides a better extraction of phenolic compounds, a further investigation must be made, such as total phenolic content.

The chemometrics evaluation of the UPLC-QTOF-MS/MS dataset was suitable to follow changes in *P. niruri* and *P. amarus* under different polar solvents.

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