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Phyllanthin and hypophyllanthin determination by gas chromatography-mass spectrometry of six stonebreaker species from different regions of Brazil

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Stonebreaker is a medicinal plant traditionally used for the treatment of urolithiasis among other illnesses. However, several species of the genus *Phyllanthus* L. are called "quebra-pedra" (stonebreaker) by the general population in Brazil, due to taxonomic similarities, although the use of a species with a low content of active compounds may lead to a less effective treatment. *P. niruri* and *P. tenellus* are recognized as stonebreaker in the Brazilian Pharmacopoeia, and the Brazilian Ministry of Health included *Phyllanthus* spp in a list of medicinal plants recommended for research and development, indicating the need for further studies. Quantitative determination of the active compounds of different *Phyllanthus* species from Brazil is important because it allows to assess the impact of their role in public health and so contribute to the goal of RENISUS, the official list of species of interest to the national health service. Lignans comprise a group of compounds found in *Phyllanthus* species that have diverse pharmacological properties. Some of the most studied lignans are phyllanthin and hypophyllanthin, which have antihyperuricemic activity. The aim of this study was to develop an analytical method for the quantitative determination of phyllanthin and hypophyllanthin in *Phyllanthus* species by GC-MS and to assess the content of these lignans in six species of the genus from four different regions of Brazil. The phyllanthin and hypophyllanthin contents were found to be very variable in the analyzed species, ranging from not detectable or a mere trace to 0.6% (m/m), the highest value, found in *P. amarus.*

Key words: Traditional medicine, urolithiasis, stonebreaker, quebra-pedra, *Phyllanthus*, lignans, gas chromatography-mass spectrometry (GC-MS)

INTRODUCTION

Stonebreaker is a medicinal plant traditionally used for the treatment of urolithiasis, diabetes and hepatitis.

Several species of the genus *Phyllanthus* L. (Phyllanthaceae) are commonly recognized as “quebra-pedra” (stonebreaker) and due to their taxonomic similarities, all of them are collected as such by a population unable to differentiate them scientifically, which is liable to result in a less effective treatment when a species with low content of the active compounds is used (Inglis et al., 2018). Officially in Brazil, *Phyllanthus niruri* and *Phyllanthus tenellus* both have monographs in the Brazilian Pharmacopoeia (Brazilian Health Surveillance Agency, 2019) and both the infusion and the tincture from *P. niruri* are indicated for the treatment of urolithiasis, as specified in the National Phytotherapy Formulary of the Brazilian Pharmacopoeia (Brazilian Health Surveillance Agency, 2011). The Brazilian Ministry of Health also included *Phyllanthus* spp in a list of species recommended for research and development of herbal medicines for public health indicating the need for further studies. The list included four species of the genus found in Brazil, to be compared in order to define scientifically which is best suited for production and therapeutic use (Brazilian Ministry of Health, 2009).

Several pharmacological properties are described for species of the genus *Phyllanthus*, justifying the interest on these species. They include the activities: anti-inflammatory and analgesic (Santos et al., 1994; Calixto et al., 1998; Zhang et al., 2014; Chen and Chen, 2011; Chopade and Sayyad, 2013), antihyperuricemic (Murugaiyah and Chan, 2006), urolithiasis (Boim et al., 2010; Woottisin et al., 2011; Barros et al., 2003, 2006), hepatoprotective (Huang et al., 2003; Srirama et al., 2012; Lee et al., 2006; Jain and Singhai, 2011), hypoglycemic (Hnatyszyn et al., 2002) and antibacterial (Oliveira et al., 2007; Silva et al., 2010; Windayani et al., 2015; Cesari et al., 2015). Different classes of compounds present in *Phyllanthus* extracts are believed to be responsible for the pharmacological properties, among them tannins, alkaloids, lignans and flavonoids (Calixto et al., 1998). Variability in the content of these active compounds among different species of the genus *Phyllanthus* was already known in India (Ravikanth et al., 2012) and poses a significant challenge also in Brazil because of the diversity of environments the country has. Therefore, the quantitative determination of these compounds in different species of this genus, and of their differences in content, would help assess the impact of their use in health (Nahar et al., 2012) and so contribute to the goal of RENISUS, the official list of species of interest to the national health service (Brazilian Ministry of Health, 2009).

Lignans are among the most important active in *Phyllanthus* species, as they have many described pharmacological properties such as antihyperuricemic (Murugaiyah and Chan, 2006) and hepatoprotective activities (Huang et al., 2003; Srirama et al., 2012; Lee et al., 2006; Jain and Singhai, 2011). These are phenylpropanoid dimers with a broad range of structural diversity widely distributed in higher plants and identified in species of some 70 families. Many of these compounds have been used in traditional medicine and isolated from different plant parts such as bark, wood, resin, roots, leaves, flowers, fruits and seeds (Konuklugil, 1995). Antinociceptive studies using a murine model by Chopade and Sayyad (2013) showed that the tannin corilagin and the lignans phyllanthin and hypophyllanthin were responsible for the analgesic effect of *Phyllanthus amarus* and *Phyllanthus fraternus* extracts. Murugaiyah and Chan (2006) demonstrated, also in a murine model, that the methanolic extract obtained from *P. niruri* and its lignans were able to reverse the high plasmatic uric acid levels in hyperuricemic animals. The antihyperuricemic effect was attributed to the lignans phyllanthin, hypophyllanthin and phyltetralin.

Tripathi et al. (2006) have quantitatively determined phyllanthin and hypophyllanthin (Figure 1) in several *Phyllanthus* species from India and found significant variation in the contents of these two lignans, which raises even more concern given the taxonomic similarities among these species. However, the monographs of *P. niruri* and *P. tenellus* in Brazilian Pharmacopoeia do not include the analysis of the lignan content, as though these species do not have lignans. Therefore, the aim of this study is to determine the contents of phyllanthin and hypophyllanthin in the four species of the genus *Phyllanthus* of the RENISUS list plus two other species commonly found in Brazil. To achieve this goal, Gas Chromatography-Mass Spectrometry (GC-MS) was used, three different GC columns were tested and a central composite design was performed to develop a method in the selected column.

MATERIALS AND METHODS

Plant sample collection

Samples were obtained from collection sites in South, Southeast, Central-West, Northeast and North regions of Brazil. *Phyllanthus niruri* L. and *Phyllanthus stipulatus* (Raf.) G.L. Webster were obtained from the Collection of Bioactive Plants of the Agricultural Research and Rural Extension Company of Santa Catarina Epagri, in Itajaí, Santa Catarina, Brazil (GPS 26º57'10"S; 48º45'47"W, South region); *P. amarus* Schum. & Thonn. was collected in the Multidisciplinary Center for Chemical, Biological and Agricultural Research in Campinas, São Paulo, Brazil (GPS22º47'43"S;

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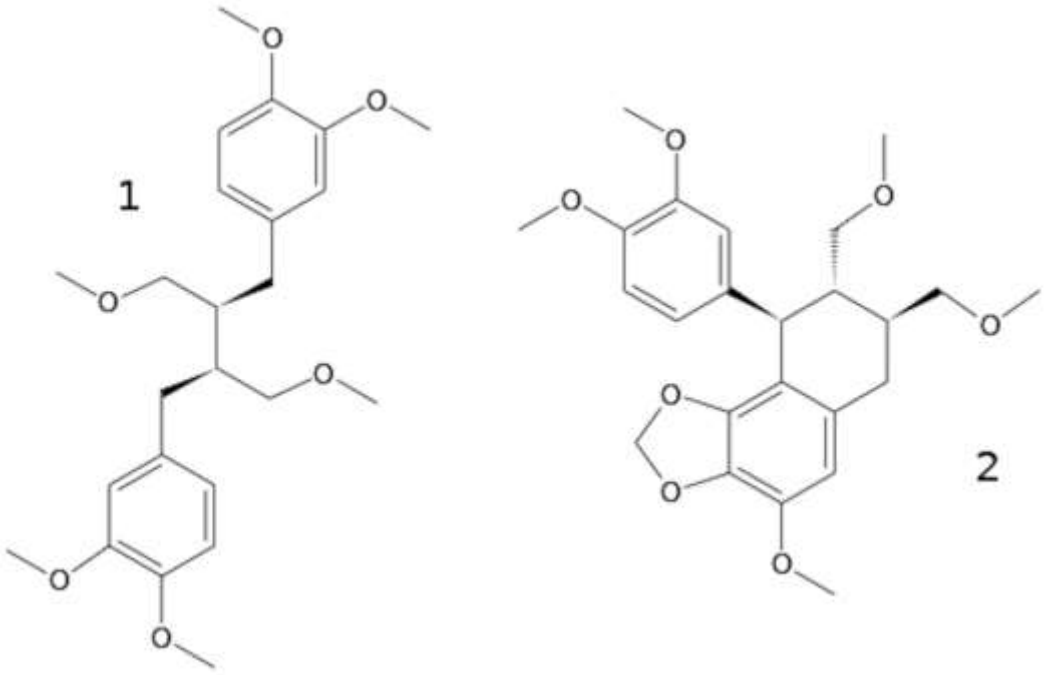

Figure 1. Chemical structures of (1) Phyllanthin and (2) Hypophyllanthin.

47º06'46''W, Southeast region); *P. tenellus* Roxb. was collected in the State University of Santa Cruz in Ilhéus, Bahia, Brazil (GPS 14º10'26''S; 47º04'28''W, Northeast region); *Phyllanthus caroliniensis* Walter was collected from the Brazilian Agricultural Research Corporation, Embrapa, Genetic Resources and Biotechnology, Brasília, Brazil (GPS 15º43'50.1''S; 47º54'06.2''W, Central-West region) and *Phyllanthus urinaria* L. was collected in the Embrapa Eastern Amazon Research Institute in Belém, Pará, Brazil (GPS 01º27'21''S; 48º30'14''W, North region). The vouchers were deposited and identified at the Embrapa CEN Herbarium.

Plant sample preparation

Samples were processed and storage according to the Good Practices for medicinal plants (Brazilian Health Surveillance Agency, 2013). Aerial parts were dried in a Marconi laboratory stove model MA 035/5 (Piracicaba, Brazil) and ground in a Tecnal mill model TE-650 (Piracicaba, Brazil). The resulting material was sieved to particle size between 32-60 mesh. Approximately 100 mg of each sample was weighed on an analytical balance (Sartorius model CP225D) in a glass tube. To each sample was added 1 ml of *n*-hexane 95% (HPLC grade/ Spectro, Tedia, Brazil) and then sonicated (USC 1850 A, UNIQUE) for 45 min at 25°C, 25 KHZ frequency and power of 154W. The samples were filtered with hydrophobic polytetrafluoroethylene filters (FilterPro) with 0.22 µm pore size and 4 mm diameter into pore size and 4 mm diameter into 1 ml volumetric flasks. Samples were transferred to vials with an insert and analyzed.

Development of the analytical method

The chromatographic method was developed using a sample of *P. niruri* L. The development of the method for the lignans analysis was carried out in an Agilent GC 6890N gas chromatograph (GC) equipped with a GC Sampler 120 automatic liquid sampler and coupled with a mass spectrometer (MS) model 5973N. Helium was used as carrier gas. Initially three capillary columns were tested, all had polymethylphenylsiloxane stationary phase with different proportions of phenyl and methyl groups: DB-5ms (5% phenyl), DB-35 (35% phenyl) and DB-17HT (50% phenyl). All columns were from Agilent Technologies and had the following dimensions: 30 m length, 0.25 mm inner diameter and 0.250 µm film thickness. For the comparison between columns, a method published by Moslka et al.(2014) was used. The chromatographic method was further developed with the DB-17HT column. The development was performed by central composite design, with the following parameters: Initial oven temperature, oven heating rate and carrier gas flow. Table 1 shows the performed experiments. All calculations were performed in JMP Statistical Discovery Software version 8.0 (SAS, Cary, USA). The other chromatographic parameters were set to: Injection volume of 1 µm pore size and 4 mm diameter, injector temperature at 300°C in splitless mode, transfer line temperature at 300°C, ion source temperature at 300°C and quadrupole analyzer at 200°C. These parameters were maintained in all analysis. Injection of the samples in the different experiments was randomly performed. The responses used were four resolutions between major fragments of different peaks of lignans and their contaminants. The resolutions with the respective fragments and the substances involved are presented in Table 2.

Quantitative determination of phyllanthin and hypophyllanthin

Identification of phyllanthin and hypophyllanthin

Phyllanthin and hypophyllanthin were identified by comparison with standard mass spectra and retention times (Rt). The standards

Table 1. Central composite design.

Coded design	Initial oven temperature (°C)	Heating rate (°C/min)	Flow rate (ml/min)
−−−	60	6	1
−−+	60	6	2
−+−	60	18	1
−++	60	18	2
+−−	120	6	1
+−+	120	6	2
++−	120	18	1
+++	120	18	2
a00	39.55	12	1.5
A00	140.45	12	1.5
0a0	90	1.90	1.5
0A0	90	22.09	1.5
00a	90	12	0.6
00A	90	12	2.4
000	90	12	1.5
000	90	12	1.5

(A) Upper axial level; (a) lower axial level; (-) lower level: (0) center level: (+) upper level.

Table 2. Central composite design responses.

Responses	Fragments (*m/z*)	Substances
R1	339 - 57	Phyltetralin - fatty acid
R2	414 - 430	Steroid - Nirtetralin
R3	430 - 43	Nirtetralin - Steroid
R4	323 - 339	Lintetralin - Phyltetralin

were purchased from Chromadex (Irvine, USA) and both have 98.9% purity.

Preparation of internal standard solution

Alpha-humulene (Sigma-Aldrich, Brazil) was weighed (37.90 mg) into a 5 ml volumetric flask to prepare the internal standard stock solution. This compound was dissolved in 95% *n*-hexane (HPLC grade/Spectro, Tedia, Brazil), in an ultrasonic bath, and after dissolution the flask volume was completed with *n*-hexane. The internal standard working solution was prepared by diluting 187.5 µm pore size and 4 mm diameter into the stock solution in a 5 ml volumetric flask, with a final concentration of 284.25 µm pore size and 4 mm diameter in g/ml.

Method validation

Validation was performed based on the International Council for Harmonisation (ICH) guidelines (2005). Standard calibration curves of phyllanthin and hypophyllanthin lignans were prepared. The stock solution of each lignan was prepared with 1 mg of each standard, weighed in a 2 ml volumetric flask. Subsequently, the compounds were dissolved with *n*-hexane (HPLC grade/ Spectro, Tedia, Brazil) using an ultrasonic bath, after dissolution the flask volume was completed with *n*-hexane. Solutions of different concentrations of the calibration curve were prepared from this solution (ranging from about 10 to 500 µg/ml) with the addition of 50 µm pore size and 4 mm diameter into the internal standard working solution. For quantitative determination of the samples, the fragments *m/z* 151 of phyllanthin and hypophyllanthin were divided by the area of fragments *m/z* 93 of the internal standard. With this value, the linear regression was calculated. Precision was calculated as the coefficient of variation between different preparations of the *P. niruri* sample. Limit of quantification (LOQ) was considered the lowest concentration of the calibration curve and limit of detection (LOD) was calculated from the analytical noise of the chromatographic method.

Sample preparation method for the quantitative determination of lignans

Sample preparation followed the same method used for the standard stock solutions with the addition of 50 µl of internal standard working solution before completing the volume.

Lignans content

The phyllanthin and hypophyllanthin contents (% m/m) were calculated using the following expression:

$$\frac{\left(\left(\frac{A_{Sa}}{A_{IS}}\right)-a\right)\cdot\left(\frac{1}{b}\right)\cdot 100}{M_{Sa}\cdot 1000}$$

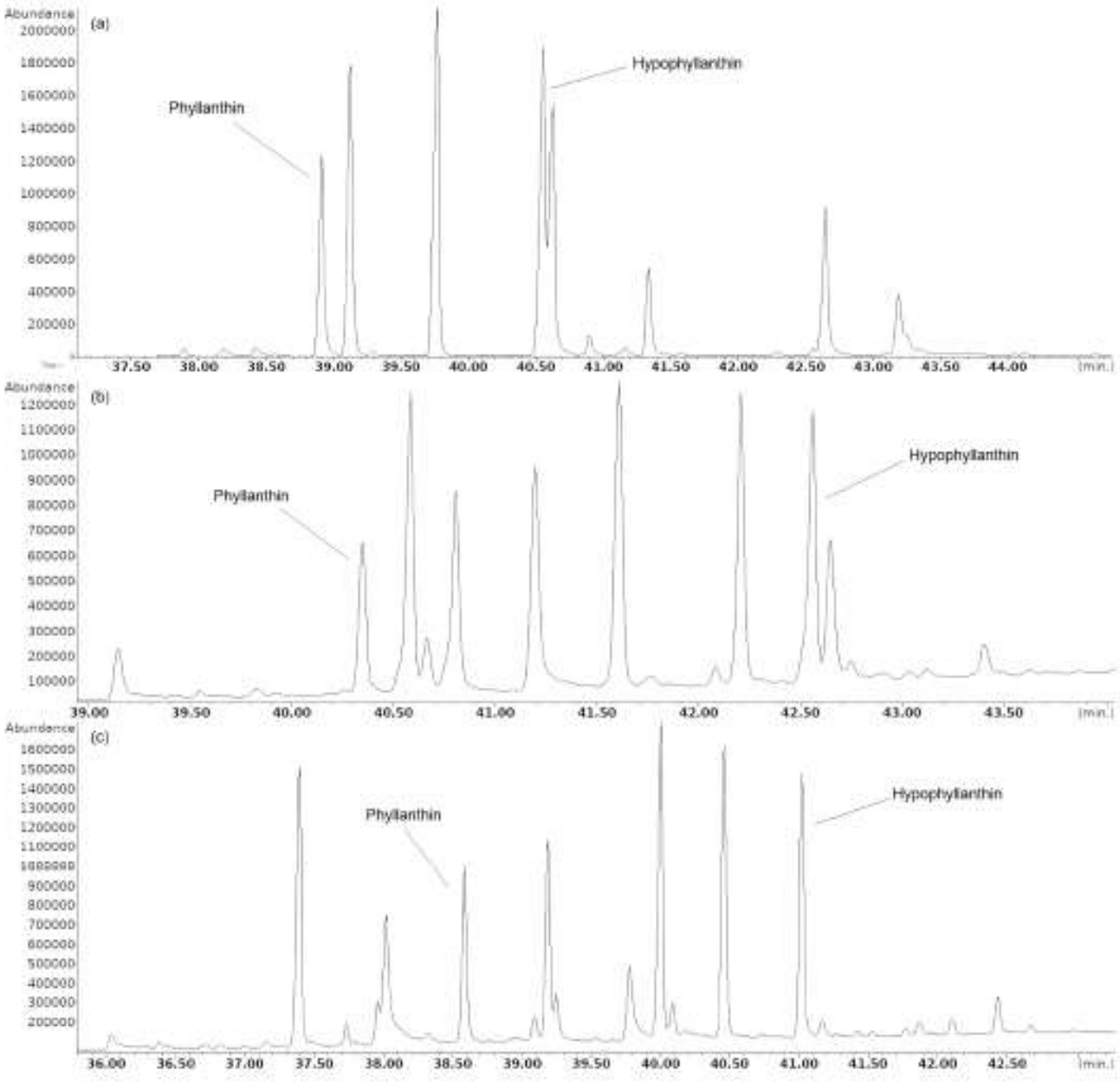

Figure 2. Total ion chromatograms of P. *niruri* in different columns (a) DB-5ms (b) DB-35 and (c) DB-17HT in the same method.

Where *ASa* is the area of fragment *m/z* 151 of each lignan, *AIS* is the area of fragment *m/z* 93 of the internal standard, *a* is the linear coefficient of the curve, *b* is the angular coefficient of the curve and *MSa* is the sample weight.

RESULTS AND DISCUSSION

Chromatographic method development

The choice of Gas Chromatography-Mass Spectrometry as analytical technique rather than the more frequently used liquid chromatography for the analysis of phyllanthin and hypophyllanthin has the advantage of GC-MS being less expensive to acquire and to maintain, using minimum amounts of organic solvents and able to identify several lignans as demonstrated by Molska et al. (2014).

Analysis of the *P. niruri* sample in different columns showed that the DB-35 and DB-17HT columns have a greater discrimination capacity for lignans than the DB-5ms column, similar to that used by Molska et al. (2014) (Figure 2). The higher content of phenyl groups in these columns allows for a better interaction of the lignans with

Table 3. Lignans detected in *Phyllanthus* species.

Phyllanthus species	5-Demetoxiniranthin	Phyllanthin	Urinatetralin	Lintetralin	Phyltetralin	Urinaligran	Nirthetralin	Niranthin	Hypophyllanthin	Hinokinin	Dextrobursehernin
P. niruri	-	+	-	+	+	-	+	+	+	-	+
P. stipulatus	-	-	-	-	-	-	-	-	-	-	-
P. amarus	+	+	+	+	+	+	+	+	+	+	+
P. tenellus	-	-	-	-	-	-	-	-	-	-	-
P. caroliniensis	-	-	-	-	-	-	-	-	-	-	-
P. urinaria	-	+	-	-	+	-	+	+	+	-	-

(+) detected: (-) not detected.

the stationary phase, due to the higher availability of groups able to perform dipole interactions, which results in better separations. Between the columns DB-35 and DB-17HT, the latter showed a slightly better separation and therefore was chosen for optimization of the analytical method. Before the optimization, several lignans present in the *P. niruri* sample were identified by CG-MS and are presented in Table 3.

As can be observed in Figure 2, although phyllanthin and hypophyllanthin peaks showed good chromatographic resolution, the chromatogram of the sample *P. niruri* in DB-17HT column shows some peaks coeluting with other lignans, indicating the need for further optimization of the chromatographic method. An experimental design was therefore, performed to optimize the analytical method. The three factors studied, initial oven temperature, oven heating rate and carrier gas flow rate were chosen because they were the most significant factors in previous studies. The responses used were four resolutions, indicated as R1, R2, R3 and R4, between two lignans or a lignan and another substance. These are detailed in Table 2 which presents the two substances involved in each resolution, along with their characteristic fragments used for resolution calculation. The study intervals of each factor were based on the method of Molska et al. (2014), using the author's initial oven temperature (60°C) as the lowest value (-1), their oven heating rate (6°C/min) used as the lowest value (-1) and their carrier gas flow rate (1.5 ml/min) used as the central value (0). The three factors were significant for the responses studied as shown in Figure 3. The best chromatographic condition was established by contour analysis. Figure 4 presents a contour graph between carrier gas flow and oven heating rate as the two most significant factors. For this contour graph, initial oven temperature is fixed at 120°C. In the graph, the regions where the resolutions were below 1.5 were shaded. Only a small region was not shaded, with high carrier gas flow and low heating rate. Within this region, the best chromatographic condition comprises a 120°C initial oven temperature, 6°C/min heating rate and 2 ml/min carrier gas flow rate. A chromatogram of *P. niruri* sample in this condition is presented in Figure 5.

Method validation

The calibration curves of phyllanthin and hypophyllanthin were considered adequate with a coefficient of correlation r^2= 0.996. A duplicate analysis of this sample showed a coefficient of variation below 4% and was therefore considered adequate. The LOQ of both the lignans was considered the lowest concentration of the calibration curve, at 12.2 µg/ml for phyllanthin and at 11.1 µg/ml for hypophyllanthin. The LOD was obtained for phyllanthin at 1.4 µg/ml and for hypophyllanthin at 0.8 µg/ml.

Phyllanthin and hypophyllanthin quantitative determination

The contents of phyllanthin and hypophyllanthin determined are shown in Table 4. Phyllanthin and hypophyllanthin were detected in three of six samples: *P. niruri*, *P. amarus* and *P. urinaria*. Nevertheless, only *P. amarus* had minimal quantifiable contents of both lignans. *P. amarus* had about 0.6% (m/m) of phyllanthin, very similar to the contents found by Tripathi et al. (2006) although the content of hypophyllanthin was about ten times smaller. The same authors did not detect these lignans in *P. urinaria* although this study found them in small amounts in this species. Nahar et al. (2012) claimed that *P. niruri* and *P. urinaria* are the major lignan-producing species, but the results here obtained indicate that although they do produce lignans, *P. amarus* is the major producing species among the samples analyzed.

The results also contradict the *P. niruri* monograph in the Brazilian Pharmacopoeia which

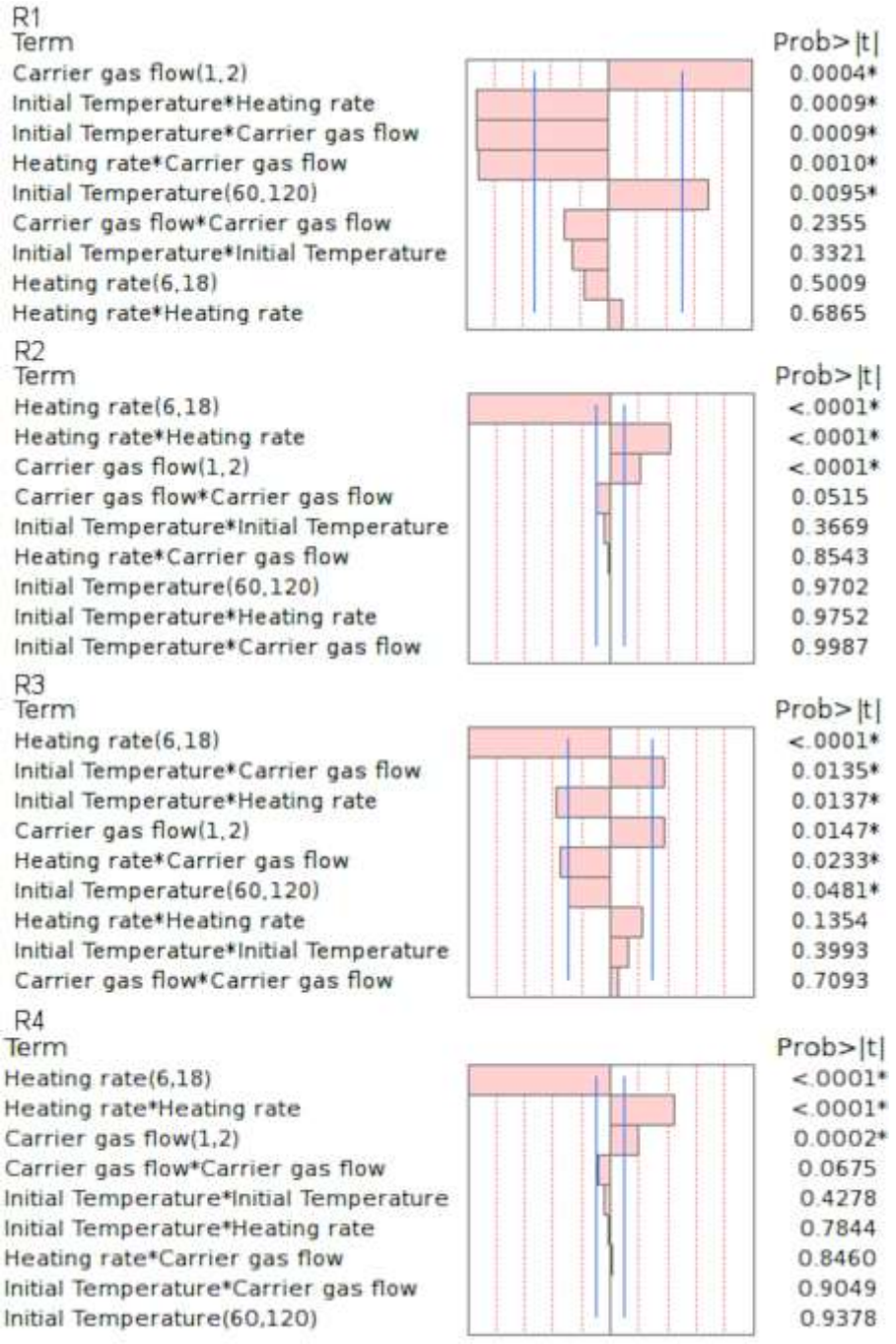

Figure 3. Significance charts of the factors for the different responses.

states that this species does not have phyllanthin. At the same time, *P. amarus* was the only species that produced substantial amounts of phyllanthin and hypophyllanthin. The overall results demonstrate the high variability of phyllanthin and hypophyllanthin production among different species of the genus *Phyllanthus* and even the variability in the production of these lignans by the same species from different countries, implying that a broader study encompassing several species from different regions of Brazil is necessary, in order to establish the best conditions for cultivation and to confirm *P. amarus* as the most suitable. The RENISUS list, as already stated, includes *Phyllanthus* spp, without defining which ones but indicating four promising species to be studied (*P. amarus, P. niruri, P. tenellus* and *P. urinaria*). This study contributes to this effort, at least in relation to

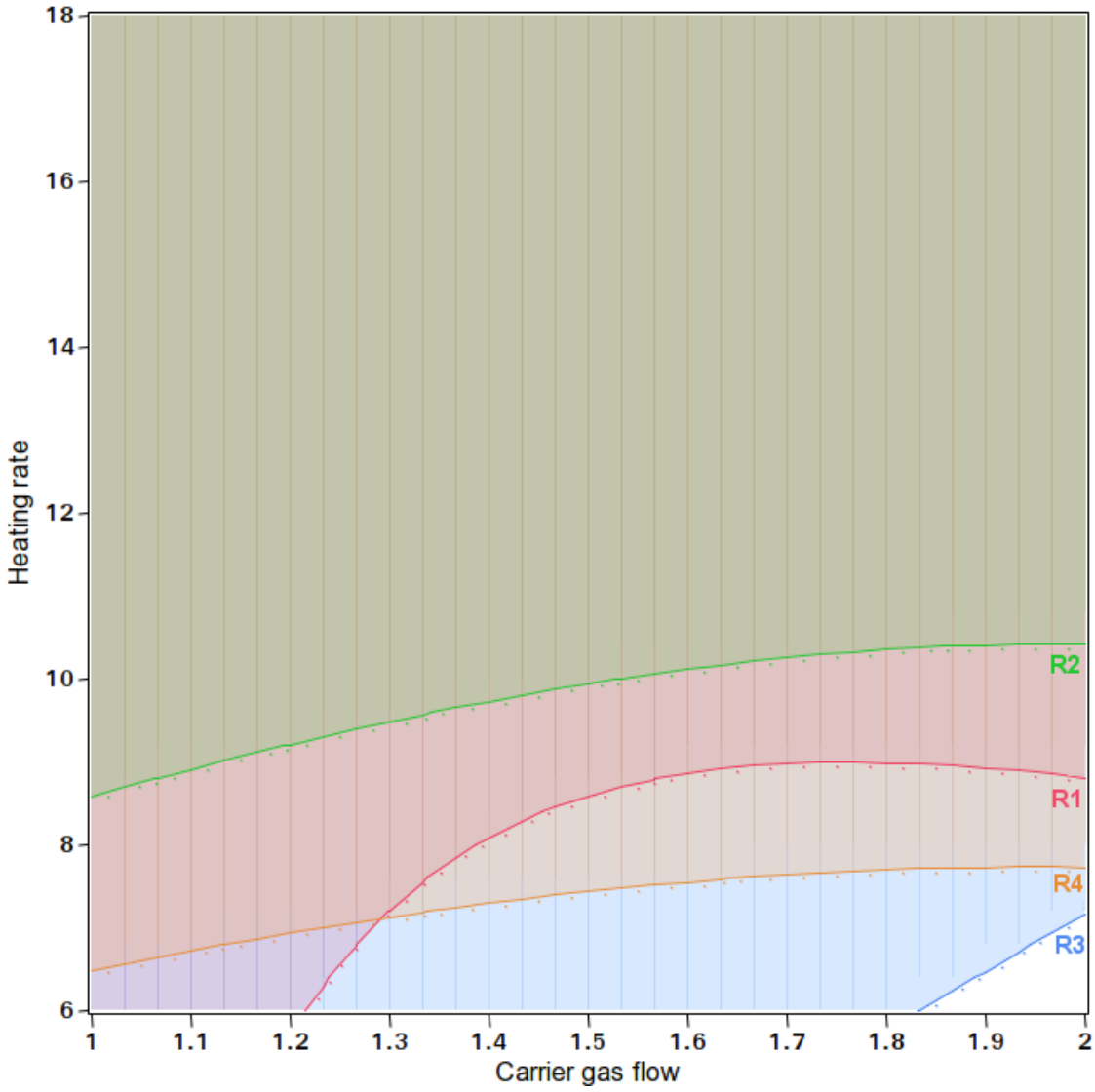

Figure 4. Contour chart of the four responses.

phyllanthin and hypophyllanthin content, by revealing *P. amarus* as the most promising species.

Lignan content in the different *Phyllanthus* species

Eleven lignans were identified in the samples analyzed (Table 3), with *P. amarus* being the only sample in which all eleven were detected and had been all previously detected in other studies (Nahar et al., 2012; Molska et al., 2014; Qi et al., 2014), confirming that it was the major producer of lignans among the studied species. Seven lignans were detected in *P. niruri* but, only niranthin, hypophyllanthin and dextrobursehernin were detected for the first time (Nahar et al., 2012; Qi et al., 2014). Five lignans were detected in *P. urinaria,* and of these only hypophyllanthin was detected for the first time (Nahar et al., 2012; Molska et al., 2014). No lignans were detected in the remaining three species analyzed in agreement with previously published literature. These results demonstrate again the high variability in the occurrence of lignans among *Phyllanthus* species and in the same species from different locations. The authors reaffirm the necessity of a broader study in Brazil of the occurrence of lignans in *Phyllanthus* species.

Conclusion

These results confirmed the high variability of lignan content among species of *Phyllanthus.* Furthermore, they demonstrate that *P. amarus* species is the most promising species to be studied in Brazil due to its high lignan content. The results also contribute to the RENISUS

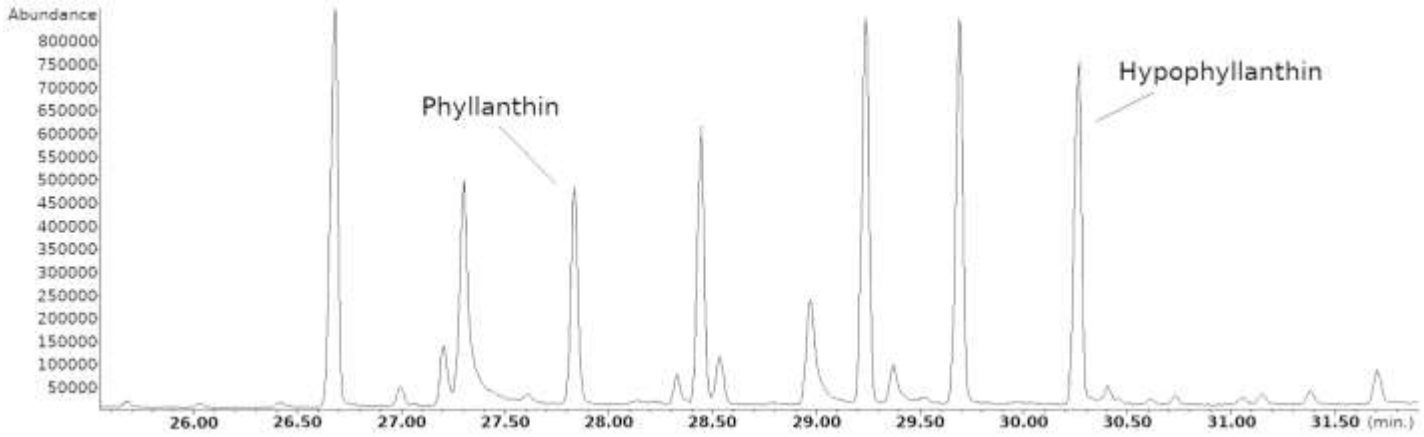

Figure 5. Total ion chromatogram of *P. niruri* using the optimized method.

Table 4. Content of phyllanthin and hypophyllanthin in the analyzed species.

***Phyllanthus* species**	**Phyllanthin % (m/m)**	**Hypophyllanthin % (m/m)**
P. niruri	Below LOQ	Below LOQ
P. urinaria	Below LOQ	Below LOQ
P. amarus	0.600	0.027
P. tenellus	n.d.	n.d.
P. stipulatus	n.d.	n.d.
P. caroliniensis	n.d.	n.d.

(LOQ) limit of quantitation; (n.d.) not detected.

goal for developing a herbal medicine from *Phyllanthus* spp. Additionally, the method developed by GC-MS offers a precise identification and quantitation of lignans found in *Phyllanthus* spp is less expensive and complies with the principles of green chemistry.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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