The Use of qNMR for Quality Control of Coumarin-based Pharmaceuticals and Plant Medicines

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Abstract – The Coumarin (1,2-benzopyrone) is the main secondary metabolite of *Mikania laevigata* Sch. Beep ex Baker and *Mikania glomerata* Spreng., which are popularly known as guaco. These plants have been used mainly in traditional medicine in the treatment of respiratory diseases because their bronchodilator effect. However, there are around 200 species of Mikania, which are quite similar in appearance. From these, only *M. leavigata* and *M. glomerata* have high concentrations of coumarins. In this line, the falsification of products Mikania based has been frequent. In this sense, this work demonstrated the application of the easy, fast, e not destructive method based in Nuclear Magnetic Resonance in quantitative mode (qNMR) for the determination of coumarin in both commercial and homemade guaco products. Thus, in the first step the compounds were extract from guaco leaves and syrups using chloroform ($CHCl_3$), with or without ultrasound. About the method, was linear with a $R^2 = 0.9947$ for 1,2-benzopyrone, with detection and quantification limits with were 0.11 and 0.36 mg mL^{-1} respectively. In the same line, the method was safe with RSD <0.3% and with recovery ranging from 93-101%. To confirm the applicability of the method, in the last step was applied to 10 real samples (6 from leaves and 4 from syrups). The content of the coumarin in the leaf extract ranged from 0.62 to 1.30 mg mL^{-1}. For syrups I, II and IV, the content of coumarin was in accordance with the manufacturers. However, for de Syrup III, the concentration was 155% higher. In summary, the qNMR is a rapid method with minimal sample preparation that can be used to quantify coumarin in home-made plant extracts as well as in commercial samples as syrup for instance. This method is applicable for quality control of different plants-based products.

Keywords – Coumarin, Plant extracts, Syrups, qNMR.

Introduction

Herbal medicines are obtained exclusively from plants with proven therapeutic effects and safety. Since the start of human history, plants have been used for the treatment many pathologies including neoplasias,[1] infections[2] and inflammation.[3] Brazilian biodiversity makes the use of herbal medicines widespread, as it is part of the culture and popular knowledge.

The guaco is a plant of the genus *Mikania*, originating in South America, initially used by Brazilian Indians as an antiophidic, with demonstrated properties in this respect.[4-6] However, over the years, several other activities of this plant have been reported, e.g. antiallergic,[7] anti-inflammatory,[8] antitumor,[9] antibacterial[10] and larvicidal;[11]

moreover, this plant is used for the treatment of respiratory problems.[12] Coumarins are secondary metabolites derived from the chiquimic acid route, being the main component present in this species. These compounds are the main responsible the bioactivities of this plants.[5]

In view of the inherent toxicity of natural products, they require adequate quality control.[13] In this case, methodologies have been developed with this purpose to evaluate one or more markers in a complex mixture of compounds.[14-17] Chromatography is the main tool for the separation, qualification and quantification of chemical species for plant quality control.[18] In this line, extracts from various herbal products such as *Equisetum arvense L., Baccharis trimera* and *Arnica montana* have been evaluated by High Performance Liquid Chromatography (HPLC) in order to identify phenolic compounds, flavonoids and alkaloids.[19-21] However, alternative methods have been developed in order to reduce the volume of solvent used, as well as the sample preparation and analysis time. Among them, we can mention nuclear magnetic resonance

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(NMR), which provides some extra advantages: simplicity of sample preparation, rapid analysis, no sample destruction and reduced solvent usage; additionally, it provides an overview of metabolites (target and non-target). Thus, this method has been widely used for the quantification (qNMR) of markers in the analysis of medicinal plants.[14,20-23] However, some matrices are still challenging for the application of analytical quality control methods, such as syrups (due to excess sugar). Thus, this work aimed the development of a qNMR method to determine the level of coumarin in extracts of leaves (commercial and homemade), as well as in guaco syrups with minimal sample preparation. In this way, a unique easy and fast method could by apply to quality control of all products coumarin-based.

Experimental

Mikania extracts – The leaves of Mikania laevigata Sch. Bip. ex Baker were collected in Garibaldi city (29º13'32.28' S and 51º32'11.13' W), Rio Grande do Sul, Brazil and a voucher specimen deposited in the Herbarium of the University of Caxias do Sul (HUCS 38180). The extracts were obtained under sonication of dry leaves at 40% of the total amplitude of the equipment (Sonics, Vibra CellTM, Newtown, USA), at room temperature, for 20 min, using $CDCl_3$ as the solvent (1:10 m/v). The mixture was filtered, the solvent evaporated in a rotary evaporator (Büchi, Flawil, CHE) and freeze-dried. The sample was denominated 'Extract I'. This extraction procedure was the same as for commercial guaco leaves and these samples were denominate 'Extract II-Extract VI'.

For the assays, approximately 10 mg of extract were solubilized in 0.6 mL of deuterated chloroform ($CDCl_3$) (Sigma-Aldrich, Missouri, USA) and placed in 5 mm NMR tubes (WilMad-LabGlass, Vineland, NJ, USA) to be read in the NMR equipment. All analyses were performed in triplicate.

Extraction of coumarin in guaco syrup – Four samples of syrup of different commercial brands and guaco concentrations were purchased from local pharmacies. For coumarin extraction, 5.00 mL of syrup and 5.00 mL of $CDCl_3$ were added into 15.00 mL Falcon tubes. The flasks were shaken manually for 60s and centrifuged for 2 min at 2000 rpm. The supernatant was removed, and the chloroform phase was evaporated on a rotary evaporator. The obtained solid was resuspended with 0.60 mL of $CDCl_3$, which was added to 5.00 mm tubes and analyzed by ^{1}H-NMR. This samples were denominated 'Syrup I' to 'Syrup IV'. All analyses were

performed in triplicate.

NMR – All NMR measurements were obtained on a *Fourier*300 Bruker® spectrometer (Karls-ruhe, Germany) with BBI probe of 5.00 mm internal diameter, with reverse detection and field gradient coils in the coordinate. All spectra were acquired at a temperature of 298 K using $CDCl_3$ as the with 5 mm quartz tubes. The chemical shifts were reported in ppm, referenced to the $CDCl_3$ (7.26 ppm) residual. For the analysis, 64 scans (ns) were performed, with 1s of relaxation time (d1), an acquisition time of 3.14 s (aq) with 65 Kb points during (td), a pulse of zg30, and duration of 15 μs (p1). The processing of the spectra was performed with 65 kb points (si) using an exponential multiplication (lb=0.3 Hz) and manual correction of phase and baseline. The program TopSpin (Bruker Biospin®) was used for data collection and processing.

Validation – For validation, the parameters established by the International Conference on Harmonization of Technical Requirements for Registration of Pharmaceuticals for Human Use.[24]

For good selectivity, it is necessary that there are no compounds with signals with the same chemical displacement as the compounds of interest, therefore, initially, the residual solvent signal, used for calibration, and possible interference in the coumarin signal to be quantified were evaluated. Thus, for all the evaluation parameters to be evaluated, as well as for subsequent quantification, the doublet at 6.45 ppm was used. This was selected due to its selectivity in relation to the other signals in the sample, as well as its greater area of integration.

The linearity was assessed in triplicate with a coumarin standard (98% purity, Sigma Aldrich, Sigma-Aldrich, Missouri, USA) at 0.10 (P1), 0.30 (P2), 0.50 (P3), 1.00 (P4), and 3.00 (P5) mg/mL in $CDCl_3$. The limits of detection (LOD) and quantification (LOQ) were established by evaluating the signal to noise ratio (S/N). For the LOD, the value was established by multiplying the S/N ratio by 3, while for the LOQ the ratio was multiplied by 10. Both parameters were calculated using the doublet at 6.45 ppm from the smallest point on the curve (0.10 mg/mL).

The effectiveness of the method was observed through the recovery assay, where a coumarin reference standard was added to the syrup samples. For this, a stock solution of the analyte at a concentration of 10.00 mg/mL was prepared. Then it was diluted in 5 mL of syrup, in three concentration levels: 0.1, 0.5 and 1.5 mg/mL. This test was performed for all samples since there is no free matrix of the analyte and with the same type of formula-

tion. All analyzes were performed in triplicate.

Results and Discussion

Nowadays, the use of medicinal plants is increasing, especially in developing countries. In this context, Brazilians have increased their consumption of herbs, extracts and syrups from plants. Due to its wide popular use, guaco has been part of the Brazilian pharmacopoeia since its first edition in 1929. In Brazil, the National Health Surveillance Agency (ANVISA) legislates on the quality control of plants, and indicates the use of *Mikania glomerata* Spreng. as well as *Mikania laevigata* Sch. Bip. Baker in multiple forms (ethanol extract or as an infusion).[25-26]

Bolina et al. (2009) reported a comparative study of both species of *Mikania*, which highlighted the botanic similarities, as well as the chemicals detected, particularly the presence of coumarin (1,2-benzopyran), triterpenes/ steroids and flavones heterosides. In this study, the coumarin concentration in *M. glomerata* and *M. laevigata* was 0.30% and 0.45%, respectively. Quantitation was performed by HPLC-UV.[27]

The use of qNMR for the quality control of medicinal plants has been highlighted as an analysis tool due to its non-destructive nature, simple sample preparation and rapid analysis.[14] In a recent example, Bastian et al. (2018) applied qNMR for the simultaneous quantification of ellagitannins and related polyphenols in *Geranium thunbergia*.[28] However, there are no articles reporting coumarin analysis on guaco leaves or syrup using this technique. Thus, the qNMR method developed here was linear, with a correlation coefficient (R^2) higher than 0.99

(Fig. 1), and LOD and LOQ values of 0.11 and 0.36 mg/ mL, respectively.

For plant extracts and syrups, selectivity is required to verify that the extraction method used is effective in eliminating interference. The selectivity of the target compound in relation to non-target compounds present in both matrices was tested. The double at 6.45 ppm, which is referent to vinylic hydrogen, was selective and used for quantification (Fig. 2).

In the same way, the recovery values for all levels in all samples were 93% and 101%, considered satisfactory by the validation guide used in this work. Figure 3 shows that extraction was efficient for the three fortification levels (F1-F3). That can be observed by column growth as the concentration increased compared to white samples (Syrup I, II and Syrup IV). For this analysis, the RSD was less than 0.3%.

Similar results were obtained by Gasparetto and co-workers that identified and quantified coumarin and other secondary metabolites in guaco extracts utilizing chromatography methods coupled with mass spectrometry. In that work, the precision and accuracy were less than 10% and recovery values ranged from 96.3 - 103.0%.[29]

Also, utilizing ^{1}H-NMR, Yoo and co-workers (2008) developed a method to quantify the coumarin in *Angelica gigas* roots. The authors compared the qNMR method with gas chromatography coupled to mass spectrometry (GC-MS). While presenting linear values for both methods, it was shown that ^{1}H-NMR could quantify coumarin in less time than conventional GC measurements.[30]

Therewith, the real samples used for the determination of method applicability were four syrup samples, which
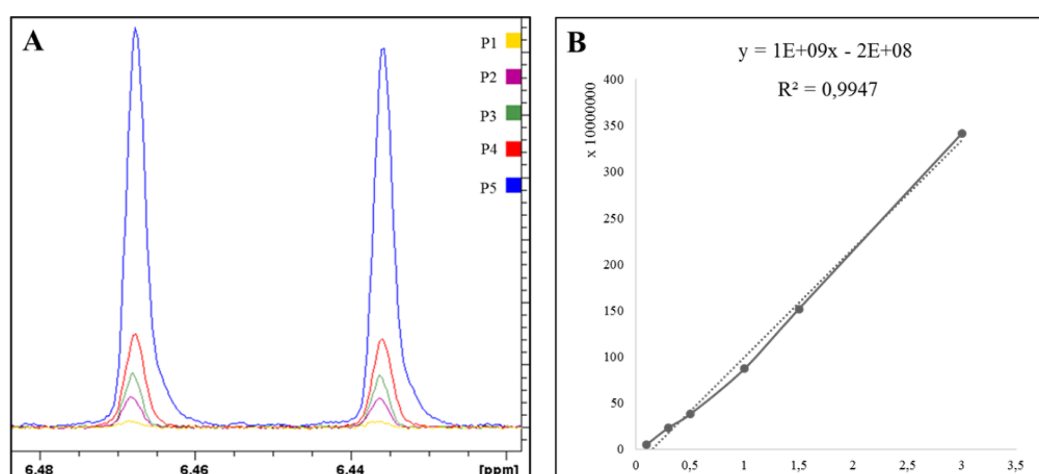

Fig. 1. Linearity results: 6.45ppm dublet in five concentrations (0.10 (P1), 0.30 (P2), 0.50 (P3), 1.00 (P4), and 3.00 (P5) mg/mL) (A), analytical curve and correlation coefficient (R2) (B).
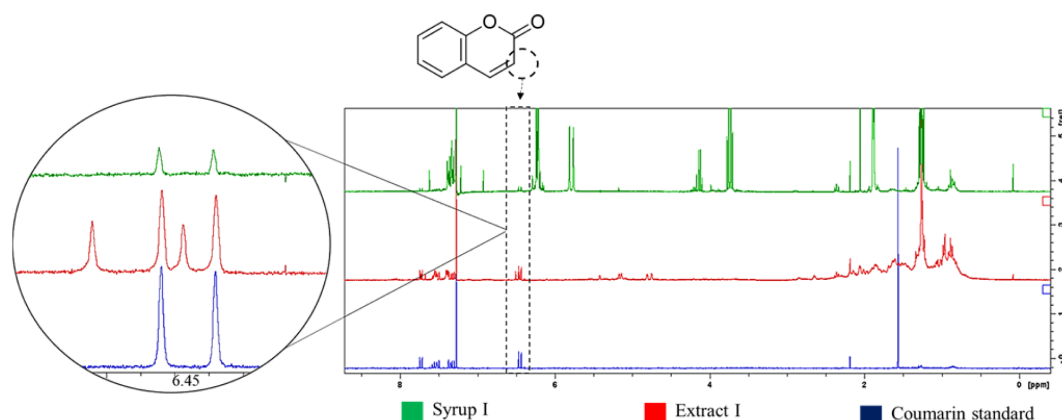
Fig. 2. Results of coumarin selectivity for standard samples, extract and syrup in relation to the other components of the sample.

Table 1. Characteristics of syrup samples, indicated coumarin concentration, other actives and excipients

Sample	Type of *Mikania*/ mg of coumarin/mL of syrup	Excipients
Syrup I	*Mikania glomerata*/0.02625 mg mL^{-1}	Sodium carmelose, potassium sorbate, sodium saccharin, sorbitol, mint flavor, honey flavor and water
Syrup II	*Mikania glomerata*/0.0882 mg mL^{-1}	Sodium carmelose, methylparaben, propylparaben, sucralose, sorbitol, mint flavor, honey flavor and water
Syrup III	*Mikania glomerata*/0.035 mg mL^{-1}	Sucrose, xanthan gum, methylparaben, citric acid, sodium citrate and water.
Syrup IV	*Mikania glomerata*/0.025 mg mL^{-1}	*Nasturtium officinale* R. brown alcohol, *Myroxylon Balsamum* (L.) Harms concentrated solute, *Aconitum napellus* L. fluid extract, sucrose, honey, ethyl alcohol, sodium benzoate, methylparaben and water

were derived from *Mikania glomerata* Spreng according to the manufacturer, as well as the tea leaves marketed. Some composition characteristics of the syrups analysed is provided in Table 1.

Regarding the results obtained for the extract samples (Table 2), it can be observed that in Extract I the coumarin concentration was 1.30 ± 0.04 mg/mL, while for the others the concentrations were between 0.62 and 1.08 mg/mL. The difference is probably because of the presence of stem in the commercial samples. Maiorano and co-workers (2005) evaluated extracts of guaco leaves, stem and roots and verified that coumarin and other non-polar compounds was present in leaves and stem extracts, but the coumarin concentration was lower in the stem extract.[6] In Brazil, only the use of leaves for phytothera-peutic purposes is established. It is possible that the branches are marketed together with the leaves, due to improper handling, cleaning and separation, or because they have a higher density, reducing the volume of material to reach the necessary weight. Although this act is doubtful, it is common to see it in herbal medicines marketed for the preparation of teas. The foreign matter index allowed by the Brazilian Pharmacopeia (2010) is, at

Table 2. Coumarin concentration found in commercial tea extracts and syrup samples

Sample	Expected concentration (mg mL^{-1})	Coumarin concentration (mg mL^{-1}) $\pm$ RSD (%)
Extract I	-	1.30 ± 0.04
Extract II	-	0.62 ± 0.02
Extract III	-	0.72 ± 0.03
Extract IV	-	1.08 ± 0.08
Extract V	-	0.73 ± 0.05
Extract VI	-	0.82 ± 0.06
Syrup I	0.22	0.22 ± 0.02
Syrup II	0.74	0.72 ± 0.05
Syrup III	0.29	0.45 ± 0.30
Syrup IV	0.21	0.20 ± 0.08

most, 2% for most vegetable medicines.[26]

The concentrations of coumarin found for the extracts made in this work are among others already presented, where the quantification was performed by chromato-graphic methods. Celeghini and co-workers (2001) found concentrations of 0.69 and 0.39 mg/mL in leaf samples of *Mikania glomerata* Spreng. using HPLC-UV and thin
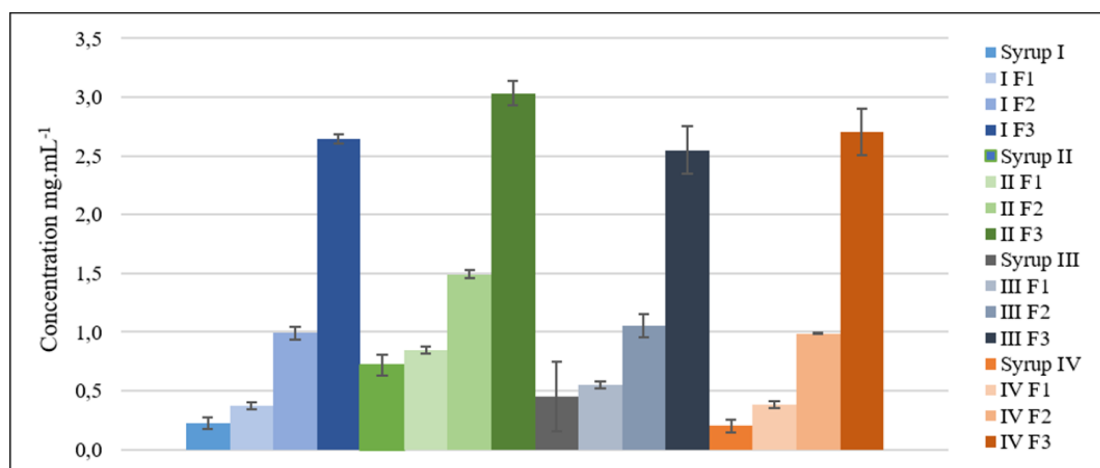
Fig. 3. Coumarin concentration levels in white syrups and their fortified samples.

layer chromatography, respectively.[31] With the same detector and matrix, Bertoldi and co-workers (2016) found values between 0.24 and 0.70 mg/mL.[32] Finally, using an aqueous infusion followed by dichloromethane extraction, Abreu and Santos (2007) found values between 1.41 and 2.99 mg/mL by analysing coumarin by gas chromatography with flame-ionization detection GC-FID.[33]

Can be observed that there is a great difference between the concentrations found (0.24-2.99 mg/mL). This may be due to different extraction methodologies, method sensitivity and analytical equipment. However, the conditions for the collection of plant material must be taken into account. The concentration of a secondary metabolite in plant samples may vary for a number of reasons: seasonality, age and plant development, temperature, water and nutrient availability, ultraviolet radiation, air pollution, pathogen attack, among others.[34]

Regarding syrup analysis, the coumarin concentration found was similar to that described on the package insert by the manufacturer, except for the Syrup III sample, as shown in Table 2. According to the Phytotherapic Form of the Brazilian Pharmacopoeia, a syrup must be composed of fluid extract, water and sugar. The indication is that 20% of extract be used, however there is no obligation to standardize it.[28] Thus, there is no information on how the extracts were obtained or how the reported coumarin concentrations were calculated.[26]

Regarding the concentration found for syrup III (0.45 ± 0.3 mg/mL), despite being almost 155% higher than indicated on the label (0.29 mg/mL), it does not present risks to the consumer health, however it characterizes a product fraudulent. This result shows that there is a need

for greater inspection in relation to herbal medicines, since they are freely sold, without the need for a medical prescription.

In the literature, few studies have been found with analysis of guaco syrup. In them, the coumarin concentration was evaluated by UV. In 2008, Da Silva et al. optimized the spectroscopic methodology developed by Celeghini et al., (2001), and found concentrations around 0.07 mg/mL, extracting the analyte with chloroform. A year later, Do Amaral and collaborators used a similar methodology to verify differences in the concentration of coumarin in syrups stored at different temperatures. In this study, concentrations from 1.19 to 1.37 mg/mL were determined, where the first refers to syrups stored at 37 ºC and the second at 10 ºC. However, the method described has not been validated, leaving room for doubtful results.[31,35,36- 37]

Thus, it can be highlighted that this work presents an unprecedented methodology for coumarin analysis and quantification in guaco syrup. Regarding the leaf extracts, the results corroborate the literature while using a rapid methodology (3.2 min); in comparison, results from traditional chromatographic methods take from 10 to 20 minutes to obtain.[38-39]

In summary, the use of qNMR proved to be a fast, efficient and safe method for the proposed work, since the results found for the extracts were similar to those reported in the literature where traditional chromatographic methods were used. Regarding the quantification of coumarin in Mikania glomerata Spreng. syrups, the concentrations found were equivalent to those described by the manufacturer, except for the syrup III sample. In addition, beyond the application of the method for

coumarin analysis on guaco leaves and syrups, it can be applied to other plants with similar metabolites.

Acknowledgments

References

(1) Fukumasu, H.; Latorre, A. O.; Bracci, N.; Górniak, S. L.; Dagli, M. L. Z. *Brazilian J. Toxicol.* **2008**, *21*, 49-59.

(2) Bains, Y.; Gugliucci, A. *Fitoterapia* **2017**, *117*, 6-10.

(3) Bulugonda, R. K.; Kumar, K. A.; Gangappa, D.; Beeda, H.; Philip, G. H.; Rao, D. M.; Faisal, S. M. *Sci. Rep.* **2017**, *7*, 42683.

(4) Collaço Rde, C.; Cogo, J. C.; Rodrigues-Simioni, L.; Rocha, T.; Oshima-Franco, Y.; Randazzo-Moura, P. *Toxicon* **2012**, *60*, 614-622.

(5) Czelusniak, K. E.; Brocco, A.; Pereira, D. F.; Freitas, G. B. L. *Rev. Bras. Plants Med.* **2012**, *14*, 400-409..

(6) Maiorano, V. A.; Marcussi, S.; Daher, M. A.; Oliveira, C. Z.; Couto, L. B.; Gomes, O. A.; França, S. C.; Soares, A. M.; Pereira, P. S. *J. Ethnopharmacol.* **2005**, *102*, 364-370.

(7) Fierro, I. M.; da Silva, A. C.; Lopes, C. da S.; de Moura, R. S.; Barja-Fidalgo, C. *J. Ethnopharmacol.* **1999**, *66*, 19-24.

(8) Alves, C. F.; Alves, V. B.; de Assis, I. P.; Clemente-Napimoga, J. T.; Uber-Bucek, E.; Dal-Secco, D.; Cunha, F. Q.; Rehder, V. L.; Napimoga, M. H. *J. Pharm. Pharmacol.* **2009**, *61*, 1097-1104.

(9) Rufatto, L. C.; Finimundy, T. C.; Roesch-Ely, M.; Moura, S. *Phytomedicine* **2013**, *20*, 883-889.

(10) Moreira, M. R.; Souza, A. B.; Soares, S.; Bianchi, T. C.; de Souza Eugênio, D.; Lemes, D. C.; Martins, C. H.; da Silva Moraes, T.; Tavares, D. C.; Ferreira, N. H.; Ambrósio, S. R.; Veneziani, R. C. *Fitoterapia* **2016**, *112*, 211-216.

(11) Araújo, A. L.; Lacerda, T.; Hiura, E.; Lopes, A. D. C. G.; Aguiar, A. R.; Tobias, F. L.; de Andrade1, M. C.; Sena, F. P.; Braga, G. M. A. M.; Ferraz, C. M.; Endringer, D. C.; Braga, F. R. *Afr. J. Pharm. Pharmacol.* **2016**, *10*, 379-384.

(12) Radünz, L. L.; Melo, E. C.; Barbosa, L. C. A.; Rocha, R. P.; Berbert, P. A. *Rev. Bras. Plants Med.* **2012**, *14*, 453-457.

(13) Turolla, M. S. dos R.; Nascimento, E. de S. *Brazilian J. Pharm. Sci.* **2006**, *42*, 289-306.

(14) Yan, K. J.; Chu, Y.; Huang, J. H.; Jiang, M. M.; Li, W.; Wang, Y. F.; Huang, H. Y.; Qin, Y. H.; Ma, X. H.; Zhou, S. P.; Sun, H.; Wang, W. *J. Pharm. Biomed. Anal.* **2016**, *131*, 183-187.

(15) Li, X.; Hu, K.; Chen, X.; Qi, J.; Yu, B. *Anal. Methods* **2017**, *9*, 5580-5585.

(16) Ribani, M.; Grespan Bottoli, C. B.; Collins, C. H.; Fontes Jardim, I. C. S.; Costa Melo, L. F. *Quim. Nova* **2004**, *27*, 771-780.

(17) Dos Reis Lívero, F. A.; da Silva, L. M.; Ferreira, D. M.; Galuppo, L. F.; Borato, D. G.; Prando, T. B.; Lourenço, E. L.; Strapasson, R. L.; Stefanello, M. É.; Werner, M. F.; Acco, A. *Naunyn Schmiedebergs Arch Pharmacol.* **2016**, *389*, 985-998.

(18) Mimica-Dukic, N.; Simin, N.; Cvejic, J.; Jovin, E.; Orcic, D.; Bozin, B. *Molecules* **2018**, *13*, 1455-1464.

(19) Wardecki, T.; Brötz, E.; De Ford C, von Loewenich F. D.; Rebets, Y.; Tokovenko, B.; Luzhetskyy, A.; Merfort, I.. *Antonie Van Leeuwenhoek* **2015**, *108*, 391-402.

(20) Jung, J. Y.; Jung, Y.; Kim, J. S.; Ryu, D. H.; Hwang, G. S. *J. Agric. Food Chem.* **2013**, *61*, 10398-10407.

(21) Li, Y.; Liu, H.; Wu, X.; Li, D.; Huang, J. *OMICS* **2013**, *17*, 252-258.

(22) Sun, L.; Wang, M.; Ren, X.; Jiang, M.; Deng, Y. *J. Pharm. Biomed. Anal.* **2018**, *160*, 323-329.

(23) Chauthe, S. K.; Sharma, R. J.; Aqil, F.; Gupta, R. C.; Singh, I. P. *Phytochem. Anal.* **2012**, *23*, 689-696.

(24) ICH. *Int. Conf. Harmon.* **1994**, 17.

(25) de Melo, L. V.; Sawaya, A. C. H. F. *Rev. Bras. Farmacogn.* **2015**, *25*, 105-110.

(26) Brazilian Pharmacopeia Comission. Brazilian Pharmacopeia. 5th. Agência Nacional De Vigilância Sanitária; Brasília, Brazil, 2010.

(27) Bolina, R. C.; Garcia, E. D. F.; Duarte, M. G. R. *Brazilian J. Pharmacogn.* **2009**, *19*, 294-298.

(28) Bastian, F.; Ito, Y.; Ogahara, E.; Ganeko, N.; Hatano, T.; Ito, H. *Molecules* **2018**, *23*, 1346.

(29) Gasparetto, J. C.; Guimarães de Francisco, T. M.; Campos, F. R.; Pontarolo, R. *J. Sep. Sci.* **2011**, *34*, 740-748.

(30) Yoo, J. S.; Ahn, E. M.; Song, M. C.; Bang, M. H.; Kim, D. H.; Han, M. W.; Kwak, H. Y.; Lee, D. Y.; Lyu, H. N.; Baek, N. I. *Food Sci. Biotechnol.* **2008**, *17*, 573-577.

(31) Celeghini, R. M. S.; Vilegas, J. H. Y.; Lanças, F. M. *J. Braz. Chem. Soc.* **2001**, *12*, 706-709.

(32) Bertoldi, F. C.; Deschamps, F. C.; Silva junior, A. A.; Correa, A. F.; Franco, M. F.; Eberlin, M. N. *Rev. Bras. Plantas Med.* **2016**, *18*, 316-325.

(33) Abreu, A. S.; Santos, A. R.; Kaiser, C. R. *Fitos* **2007**, *3*, 60-66.

(34) Veneziani, R. C. S.; Camilo, D.; de Oliveira, R. *Biochem. Syst. Ecol.* **1999**, *27*, 99-102.

(35) Soares e Silva, L.; Santos da Silva, L.; Brumano, L.; Stringheta, P. C.; Pinto, M. A. O.; Dias, L. O. M.; Muller, C. S. M.; Scio, E.; Fabri, R. L.; Castro, H. C.; Amaral, M. P. H. *Molecules.* **2012**, *17*, 10344-10354.

(36) Amaral, M. P. H.; Vieira, F. P.; Leite, M. N.; Amaral, L. H.; Pinheiro, L. C.; Fonseca, B. G.; Pereira, M. C. S.; Varejão, E. V. *Rev. Bras. Farmacogn.* **2009** *19*, 607-611.

(37) Da Silva, C. R.; Gomes, V. S.; Kulkamp, I. C.; Kanis, L. A. *Brazilian J. Pharmacogn.* **2008**, *18*, 594-599.

(38) De Melo, L. V.; Sawaya, A. C. H. F. *Rev. Bras. Farmacogn.* **2015**, *25*, 105-110.

(39) Alvarenga, F. C. R.; Garcia, E. de F.; Bastos, E. M. A. F.; Grandi, T. S. M.; Duarte, M. G. R. *Brazilian J. Pharmacogn.* **2009**, *19*, 442-448.