Revista Brasileira de Farmacognosia 29 (2019) 266–270

Brazilian Journal of Pharmacognosy
REVISTA BRASILEIRA DE FARMACOGNOSIA
www.elsevier.com/locate/bjp

Short communication

An eco-friendly method for extraction and quantification of flavonoids in *Dysphania ambrosioides*

Thaís M.S. Ferreira, Júlia A. dos Santos, Leandra A. Modesto, Ludimilla S. Souza, Maria Patrícia V. dos Santos, Debborah Gonçalves Bezerra, Joelma A. M. de Paula *

Universidade Estadual de Goiás, Campus Anápolis de Ciências Exatas e Tecnológicas, Anápolis, GO, Brazil

ARTICLE INFO

Article history:
Received 31 July 2018
Accepted 11 January 2019
Available online 1 March 2019

Keywords:
Spectrophotometric method
Rutin
Validation
Ultrasound-assisted extraction
Box–Behnken design
Quality control

ABSTRACT

Dysphania ambrosioides (L.) Mosyakin and Clemants (Syn: *Chenopodium ambrosioides* L.), Amaranthaceae, is a plant with antibacterial, antifungal, antioxidant, antiparasitic and antitumor properties that is commonly used in Brazilian folk medicine. In this work we performed the optimization of ultrasound-assisted extraction of flavonoids in the aerial parts of *D. ambrosioides*. The flavonoid concentrations, as rutin equivalents, were quantified with the aid of a validated spectrophotometric method. The Box–Behnken (3^3) design with response surface methodology, for the independent variables, extraction time, temperature, and ethanol content, were used for the optimization of ultrasound-assisted extraction. The analytical method was selective, linear, without matrix interference, accurate, precise and robust. The best conditions for the ultrasound-assisted extraction of flavonoids were: time of 60 min, temperature of 57 °C and ethanol content of 57% (w/w). The methods of extracting and quantifying flavonoids developed in the present study have provided be eco-friendly, simple, and useful to determine the flavonoid content, expressed as rutin equivalents, in the aerial parts of *D. ambrosioides*.

Introduction

Dysphania ambrosioides (L.) Mosyakin and Clemants (synonym: *Chenopodium ambrosioides* L.) is a herbaceous plant that belongs to the Amaranthaceae family, which can be found in tropical regions as well as in those of temperate climate (Fank-de-Carvalho et al., 2012; Senna, 2015). Although it is considered a weed, it is one of the plants most used for therapeutic purposes in world folk practice, and it was included in the National List of Medicinal Plants of Interest of the Single Health System in Brazil (Ministério da Saúde, 2009). Innumerable biological activities are attributed to this species, such as antiparasitic, antibiotic, antifungal, antioxidant and antitumor (Barros et al., 2013; Trivellato-Grassi et al., 2013; Jesus et al., 2018).

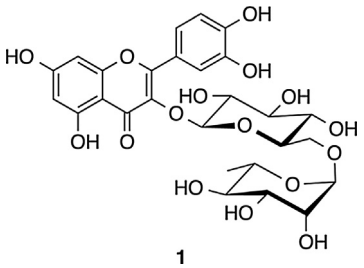
When preparing samples for quality control of plant drugs, modern extractive methods such as ultrasound-assisted extraction (UAE) are considered useful for quick and efficient small-scale extraction. They allow the use of lower temperatures, lower consumption of solvent and energy, and so they are considered green and low-cost technologies. In recent years, the ultrasound-assisted extraction method has been used to effectively extract the chemical constituents of various plant materials. This extraction method can be influenced by several factors simultaneously, such as time, temperature, and solvent (Dong et al., 2010; Wang et al., 2013; Paula et al., 2016).

Although *D. ambrosioides* is a medicinal plant of regional and international interest and despite recognition of its medicinal properties both scientifically and in folk medicine, there is a lack of research aiming to move forward in the development of phytopharmaceutical products from this species. For this, to select a chemical class or substance to be used as a reference in the quality control of herbal materials is necessary (WHO, 2011; Anvisa, 2014). Flavonoids, principally rutin (**1**), are among the phenol compounds that are most abundant in the aerial parts of this species, and the properties of flavonoids are associated with those of the *Dysphania* genus (Barros et al., 2013; Wu et al., 2016; Jesus et al., 2018). So, in the present study this class was selected as a marker, expressed as

* Corresponding author.
E-mail: joelma.paula@ueg.br (J.A. Paula).

https://doi.org/10.1016/j.bjp.2019.01.004

rutin (**1**) equivalents.



1

The aim of the present work was to determine the best conditions for the extraction of flavonoids, expressed as rutin equivalents, from the aerial parts of *D. ambrosioides* by ultrasound-assisted extraction using a validated spectrophotometric method for the quantification of flavonoids.

Materials and methods

Plant material

The aerial parts of *Dysphania ambrosioides* (L.) Mosyakin and Clemants, Amaranthaceae, were collected in the Medicinal Plant Garden of the Anápolis Campus of Exact and Technological Sciences (CCET), at the Universidade Estadual de Goiás (16°17′13.8″S; 48°57′22.7″W), in the Botanical Garden of Goiânia, Goiás, Brazil (16°19′36″S; 48°57′10″W), and in residential backyards of the cities of Anápolis (16°19′52.2″S 48°56′29.7″W, 16°22′52.9″S 48°57′17.4″W, 16°20′44.4″S 48°58′25.0″W), Goiânia (16°19′36″S 48°57′10″W), Itapuranga (15°33′49.2″S 49°57′01.6″W) and Pirenópolis (15°51′21.5″S 48°56′30.2″W). The voucher specimens were deposited in the herbarium of the Universidade Estadual de Goiás (registers HUEG11387-11392, 11873). The samples were dried at 40 °C in an oven with circulation and air renewal, pulverized in a knife mill and pooled to form a homogeneous material. The loss on drying was of 8.65% ± 0.03, determined according to WHO (2011).

Validation of a spectrophotometric method for the determination of flavonoids

For the quantification of flavonoids, expressed as rutin equivalents, the method proposed by Rolim et al. (2005) with adaptations was used. Briefly, for the extraction of the flavonoids, to 1 g of the plant drug was added enough 70% ethanol (w/w) to complete 10 ml, and the material was kept in an ultrasonic bath (Unique mod. USC – 2800 A, frequency 40 kHz and potency 154 W) for 30 min at room temperature. The extract was filtered with filter paper, and 100 μl of the filtrate was diluted in 3900 μl of a solution composed of methanol and 0.02 M acetic acid (99:1), followed by spectrophotometer reading (model SP22 Biospectro) at 364 nm, using a solution of methanol in acetic acid 0.02 M (99:1) as blank. The calculation of the flavonoid content, expressed as rutin equivalents, was performed using the equation obtained from the linear regression analysis of the calibration curve of the rutin standard Sigma Aldrich, (St. Louis, MO, USA).

The validation of the spectrophotometric method was performed according to parameters established by the International Conference on the Harmonization (ICH) of Technical Requirements for the Registration of Pharmaceuticals for Human Use (ICH, 2005) and Brazilian legislation (Anvisa, 2017). The selectivity of the method was verified through seven spectrophotometric readings of the extract at 364 nm, and compared with the reading of the rutin standard at the theoretical concentration (16 μg/ml) at the same wavelength. The percentage of recovery in the samples was calculated by the ratio between the concentration of flavonoids, as rutin equivalents, obtained in each sample and the theoretical concentration of the standard, multiplied by 100.

The linearity of the method was determined by the calibration curve obtained from seven levels of concentration of the rutin standard (1.25, 2.5, 5.0, 10.0, 20.0, 30.0, 40.0 μg/ml), prepared in triplicate, independently. The matrix effect was determined by comparing the angular coefficients from calibration curves constructed from samples strengthened with the standard and from standard only (in the same concentrations used in linearity), prepared in triplicate, independently.

The limits of detection and quantification were estimated based on parameters of the analytical curve. The precision was determined from six spectrophotometric readings of the sample (at 100% of total flavonoid concentration), individually prepared and performed intraday (repeatability) and interday (intermediate precision).

The accuracy was determined by the recovery method from the spectrophotometric reading of the samples with flavonoid concentrations, as rutin equivalents, corresponding to the low, medium, and high concentrations of the linear range, and strengthened with the standard (16 μg/ml) in these same conditions, prepared in triplicate, independently.

The robustness of the method was analyzed by obtaining the flavonoid concentration, as rutin equivalents, in the sample under the original conditions of the method and after slight variations in sample extraction time (40, 45, 60 min), solvent mark (Neon and JT Baker) and wavelength (364, 366 nm). All the statistical analyses were performed at a significance level of 5%, using the Action Stat software (version 3.0) (Action Stat (2014)) coupled to Excel 2016.

Optimization of the ultrasound-assisted extraction (UAE) of flavonoids, expressed as rutin equivalents

For UAE optimization, the response surface methodology (RSM) was used in a Box–Behnken 3^3 model. Based on previous tests, three independent variables were selected at three different levels: extraction time of 15, 30, and 45 min, extraction temperature of 30, 35, and 40 °C, and ethanol content of 60, 70, and 80% (w/w). The statistical analysis of the results guided in a second set of experiments: extraction time of 30, 45, and 60 min, temperature of 30, 45, and 60 °C and ethanol content of 30, 50, and 70% (w/w).

The response variable was expressed in flavonoid content (% w/w) as rutin equivalents, and the loss on drying the herbal drug was taken into consideration for calculation. The results were framed in a polynomial equation of the second degree by the multiple regression technique, analysis of variance (ANOVA), and response surface plotting with assistance of the Statistica® software (version 12.0) (Statistica® (2010)). The conditions considered optimal were reproduced in triplicate.

Results and discussion

Validation of the spectrophotometric analytical method

The results for the selectivity of the spectrophotometric method are shown in Table 1. The samples showed mean recovery content of 100.95% and Relative Standard Deviation (RSD) of 3.41%, demonstrating the selectivity of the method; that is its ability to quantify flavonoids, as rutin equivalents, even in the presence of matrix components (Anvisa, 2017).

The method was linear in the analyzed interval (Fig. 1), with the following representative equation: $y = 0.0218x + 0.0051$ ($R = 0.9994$), estimated by the Ordinary Least-Squares Method (OLSM). The choice of OLSM was defined after considering the variance of the responses (absorbance) for each rutin concentration

Table 1
Validation results of the spectrophotometric method for quantification of flavonoids, expressed as rutin equivalents, in the aerial parts of *Dysphania ambrosioides*.

Added standard (μg/ml)	Selectivity	Accuracy		Precision (μg/ml)		LOD (μg/ml)	LOQ (μg/ml)
	(%Recovery)	Samples extract	(%Recovery)	Intra-day	Inter-day		
16	101.75	Low	96.33	14.08	17.20	0.535	1.622
	97.45	(80%)	99.20	13.99	14.44		
	101.17		98.62	16.28	19.22		
	104.61	Medium	84.58	15.59	14.31		
	96.01	(100%)	88.88	18.11	18.94		
	100.19		86.30	15.36	18.62		
	105.47	High	88.02				
		(120%)	88.88				
			89.16				

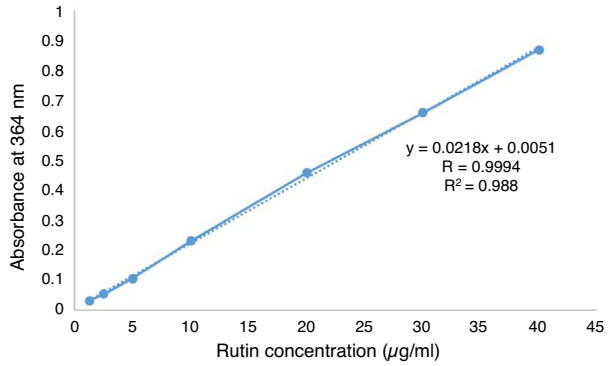


Fig. 1. Mean rutin standard calibration curve in the concentration range of 1.25 to 40 μg/ml.

employed in the interval (C), at a significance level of 5%. Thus, the calculated value of C was 0.474, lower than the critical C value of 0.561, confirming the constancy in the variance, that is, the homoscedasticity of the data.

The ANOVA showed that the angular coefficient was statistically different from zero ($p = 2.206 \times 10^{-29}$), and the linear coefficient was not statistically different from zero ($p = 0.166$), demonstrating the linearity of the method. The residue analysis is shown in Supporting Information Figure 1. This analysis quantifies the distance between the real values of the linear range and the estimated values. For this, the residues, besides the homocedastic ones, are expected to have normal distribution, which was confirmed in this study by the Anderson–Darling test ($p = 0.05$). The statistical parameters evaluated confirm that the method is able to generate analytical responses directly proportional to the concentration of flavonoids (ICH, 2005).

The detection limit indicates the lowest amount of detectable analyte present in the sample, but not necessarily quantified, under the experimental conditions established in the method. For the present study, the limits of detection and quantification, estimated based on the standard deviation of the intercept with the *y*-axis, were 0.535 and 1.622 μg/ml, respectively. These same limits, when estimated based on the standard deviation of the residues, were of 1.632 and 4.947 μg/ml, respectively.

The effect of matrix components on the analytical response should be determined, especially for samples composed of complex matrices (Anvisa, 2017), such as plant extracts. The parallelism of the lines, determined by comparing the angular coefficients of the calibration curves constructed with the analytical standard and the fortified sample with the analytical standard at the same levels of linearity concentration, is indicative of absence of interference of the matrix constituents (Anvisa, 2017). The scatter plot generated for the two calibration curves is shown in Fig. 2. The comparison tests (*F*-test) of the curves showed no significant difference at the 5% significance level for the intercept equality ($p = 0.212$), parallelism of the lines ($p = 0.588$) and coincidence ($p = 0.382$), confirming the absence of the effect of matrix components on the analytical response.

When determining the precision of the method (Table 1), on day 1 (analyst 1), a Relative Standard Deviation (RSD) of 9.85% was obtained for the flavonoid concentrations in the six samples prepared at 100% of the test concentration. On day 2 (analyst 2) the RSD was 13.07% and the inter-day RSD was 12.24%. The results of accuracy, obtained by the recovery method, are shown in Table 1. The samples, strengthened with the standard rutin, showed average recovery of 91.106% ± 5.45 and RSD of 5.99%.

In the robustness, the RSD for the flavonoid concentrations obtained in 364 nm was 8.66% and in 366 nm of 8.64%; in relation to the extraction time, the inter-solvent RSD at the wavelength of 364 nm was 5.85%, and at 366 nm it was 3.52%, and the RSD between the wavelengths was 2.69%. The guidelines for herbal medicine registration and notification and registration of traditional herbal products (Anvisa, 2014) recommend that the maximum acceptable value of RSD in the validation of analytical methods should be defined according to the method used, the concentration of analyte in the sample, the type of matrix and the purpose of the method. However, the RSD results should not exceed 15%. Therefore, the method proved to be adequate for the quantification of flavonoids, as rutin equivalents, in *D. ambrosioides*.

Optimization of the ultrasound-assisted extraction (UAE) of flavonoids, expressed as rutin equivalents

The detailed results from the UAE experiments are attached as Supporting Information (Figures 2–3, Tables 1–3). The best extraction conditions determined by the general optimization function of the model were: extraction time of 59.308 min, temperature of 57.335 °C and ethanol content of 56.968% (w/w). Under these conditions, the flavonoid content, as rutin equivalents, predicted by the model was 1.012%. Such conditions were validated in triplicate; a mean of 1.091% and a RSD of 0.17% were obtained, equivalent to 107.81% of the predicted value, which demonstrated the validity of the model.

Conclusion

The spectrophotometric analytical method chosen for the determination of flavonoids, as rutin equivalents, in the aerial parts of *D. ambrosioides* met the validation criteria established by legislation. In addition, the method is easy and simple to execute, and uses inexpensive equipment. Ultrasound-assisted extraction proved to be a fast and green technology, consumed few solvents and was efficient in the extraction process of flavonoids in the aerial parts of *D. ambrosioides*. In the future, the method here developed will be compared to other flavonoid extractive methods to consolidate the results.

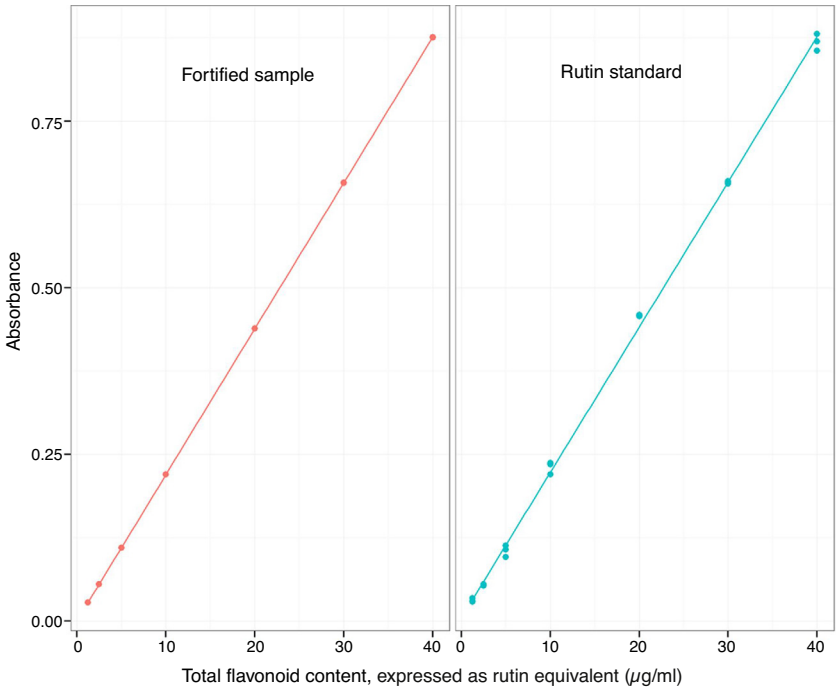


Fig. 2. Absorbance dispersion diagram (364 nm) as a function of flavonoid concentrations, constructed from rutin standard and the samples fortified with rutin standard, at the same levels of linearity concentration (1.25–40 μg/ml).

Authors' contributions

TMSF (MSc student) contributed to collecting plant samples and identification, confection of the herbarium, running the laboratory work, analysis of the data and drafting the paper. JAS contributed to collecting plant samples, confection of the herbarium, running the validation laboratory work, analysis of the data and drafting the paper. LAM and DGB contributed to collecting plant samples and identification, confection of the herbarium, running the UAE laboratory work, and analysis of the data. LSS and MPVS contributed to collecting plant samples, confection of the herbarium, running the pharmacognostic laboratory work, analysis of the data and drafting the paper. JAMP designed the study, supervised the laboratory work and contributed to critical reading of the manuscript. All the authors have read the final manuscript and approved the submission.

Conflicts of interests

The authors declare no conflicts of interest.

Acknowledgements

Many thanks to the Universidade Estadual de Goiás (UEG) for the financial support provided through a Public service contest under no. PrP 029/2016 Pro-projects/Research. The authors are grateful to the Stricto sensu scholarship program and to the Scientific and Technological Initiation program, both from UEG; to CNPq for financial support; to the Medicinal Herb Garden of the Botanic Garden of Goiânia for the supply of seedlings.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at https://doi.org/10.1016/j.bjp.2019.01.004.

References

Action Stat, 2014. Estatcamp, Consultoria em estatística e qualidade. Versão 3.5.152.34. Equipe Estatcamp, São Carlos, http://www.portalaction.com.br/ (accessed May 2018).

Anvisa, 2014. Instrução Normativa N○ 4 de 18 de junho de 2016, Guia de orientação para registro de Medicamento Fitoterápico e registro e notificação de Produto Tradicional Fitoterápico. Agência Nacional de Vigilância Sanitária, Brasília, DF.

Anvisa, 2017. Resolução da Diretoria Colegiada RDC 166 de 24 de julho de 2017. Validação de métodos analíticos. Agência Nacional de Vigilância Sanitária, Brasília, DF.

Barros, L., Pereira, E., Calhelha, R.C., Dueñas, M., Carvalho, A.M., Santos-Buelga, C., Ferreira, I.C., 2013. Bioactivity and chemical characterization in hydrophilic and lipophilic compounds of *Chenopodium ambrosioides* L. J. Funct. Foods 5, 1732–1740.

Dong, J., Liu, Y., Liang, Z., Wang, W., 2010. Investigation on ultrasound-assisted extraction of salvianolic acid B from *Salvia miltiorrhiza* root. Ultrason. Sonochem. 17, 61–65.

Fank-de-Carvalho, S.M., Báo, S.N., Marchioretto, M.S., 2012. Amaranthaceae as a bioindicator of Neotropical Savannah diversity, in: Lameed, G.A. (org.), Biodiversity Enrichment in a Diverse World. InTech, Rijeka, pp. 235–262.

ICH, 2005. Validation of analytical procedures: text and methodology Q2(R1). In: International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use (2005), http://www.ich.org/fileadmin/Public_Web_Site/ICH_Products/Guidelines/Quality/Q2_R1/Step4/Q2_R1__Guideline.pdf (accessed June 2018).

Jesus, R.S., Piana, M., Freitas, R.B., Brum, T.F., Alves, C., Belke, B.V., Mossmann, N.J., Cruz, R.C., Santos, R.C.V., Dalmolin, T., Bianchini, B.V., Campos, M., Bauermann, L.F., 2018. *In vitro* antimicrobial and antimycobacterial activity and HPLC–DAD screening of phenolics from *Chenopodium ambrosioides* L. Braz. J. Microbiol. 49, 296–302.

Ministério da Saúde, 2009. RENISUS – Relação Nacional de Plantas Medicinais de Interesse ao SUS. Espécies vegetais, Brasília, DF.

Paula, J.A.M., Brito, L.F., Caetano, K.L.F.N.C., Rodrigues, M.C.M., Borges, L.L., Conceição, E.C., 2016. Ultrasound-assisted extraction of azadirachtin from dried entire fruits of *Azadirachta indica* A Juss. (Meliaceae) and its determination by a validated HPLC-PDA method. Talanta 149, 77–84.

Rolim, A., Maciel, C.P., Kaneko, T.M., Consiglieri, V.O., Salgado-Santos, I.M., Velasco, M.V., 2005. Validation assay for total flavonoids, as rutin equivalents, from *Trichilia catigua* A Juss (Meliaceae) and *Ptychopetalum olacoides* Bentham (Olacaceae) commercial extract. J AOAC Int. 88, 1015–1019.

Senna, L., 2015. *Dysphania*. In: Lista de Espécies da Flora do Brasil. Jardim Botânico do Rio de Janeiro, http://floradobrasil.jbrj.gov.br/jabot/floradobrasil/FB139867 (accessed July 2018).

Statistica®, 2010. Data analysis software system, version 12. STATSOFT, Inc.

Trivellato-Grassi, L., Malheiros, A., Meyre-Silva, C., Buss, Z., Da, S., Monguilhott, E.D., Fröde, T.S., Silva, K.A., Souza, M.M., 2013. From popular use to pharma-

cological validation: a study of the anti-inflammatory, anti-nociceptive and healing effects of *Chenopodium ambrosioides* extract. J. Ethnopharmacol. 145, 127–138.

Wang, X., Wu, Y., Chen, G., Yue, W., Liang, Q., Wu, Q., 2013. Optimization of ultrasound assisted extraction of phenolic compounds from *Sparganii rhizoma* with response surface methodology. Ultrason. Sonochem. 20, 846–854.

WHO, 2011. Quality Control Methods for Herbal Materials. World Health Organization, Geneva.

Wu, Y., Hu, H.B., Wang, C.L., Ma, S.R., Zhang, L.L.L.L., 2016. Optimization of ultrasound-assisted extraction of flavonoids compounds from *Chenopodium hybridum* L. stem with response surface methodology IOP Conf. Ser. Earth Environ. Sci. 41, http://dx.doi.org/10.1088/1755-1315/41/1/012013.