Pharmacogn. Mag.
A multifaceted peer reviewed journal in the field of Pharmacognosy and Natural Products
www.phcog.com | www.phcog.net

ORIGINAL ARTICLE

Optimization of Ultrasound-assisted Extraction of Phenolic Compounds from *Myrcia amazonica* DC. (*Myrtaceae*) Leaves

Mariana Cristina de Morais Rodrigues, Leonardo Luiz Borges, Frederico Severino Martins[1], Rosa Helena V. Mourão[2], Edemilson Cardoso da Conceição

Laboratory of PD&I of Bioproducts, School of Pharmacy, Federal University of Goiás, Jataí, [1]Department of Pharmaceutical Sciences, Sciences of Ribeirão Preto, University of São Paulo, Ribeirão Preto, [2]Laboratory of Bioprospecting and experimental Biology, Department of Natural Resources of Amazônia, Federal University of Oeste do Pará, Santarém - PA, Brazil

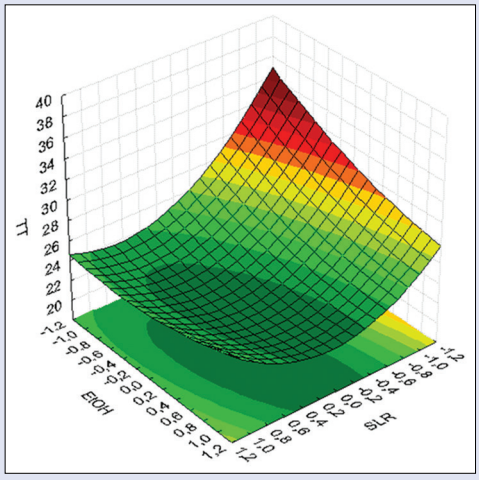
ABSTRACT

Background: *Myrcia amazonica*. DC is a species predominantly found in northern Brazil, and belongs to the *Myrtaceae* family, which possess various species used in folk medicine to treat gastrointestinal disorders, infectious diseases, and hemorrhagic conditions and are known for their essential oil contents. **Materials and Methods:** This study aimed applied the Box–Behnken design combined with response surface methodology to optimize ultrasound-assisted extraction of total polyphenols, total tannins (TT), and total flavonoids (TF) from *M. amazonica* DC. **Results:** The results indicated that the best conditions to obtain highest yields of TT were in lower levels of alcohol degree (65%), time (15 min), and also solid: Liquid ratio (solid to liquid ratio; 20 mg: 5 mL). The TF could be extracted with high amounts with higher extraction times (45 min), lower values of solid: Liquid ratio (20 mg: mL), and intermediate alcohol degree level. **Conclusion:** The exploitation of the natural plant resources present very important impact for the economic development, and also the valorization of great Brazilian biodiversity. The knowledge obtained from this work should be useful to further exploit and apply this raw material.

Key words: Box–Behnken, flavonoids, *Myrcia amazônica,* polyphenols, tannins

SUMMARY

- *Myrcia amazonica* leaves possess phenolic compounds with biological applications;
- Lower levels of ethanolic strength are more suitable to obtain a igher levels of phenolic compouds such as tannins;
- Box-Behnken design indicates to be useful to explore the best conditions of ultrasound assisted extraction.



Abbreviation used: Nomenclature ES: Ethanolic strength, ET: Extraction time, SLR: Solid to liquid ratio, TFc: Total flavonoid contents, TPc: Total polyphenol contents, TTc: Total tannin contents.

Correspondence:

Mariana Cristina de Morais Rodrigues,
Laboratório de PD and de Bioprodutos,
Faculdade de Farmácia,
Universidade Federal de Goiás,
CP 131, 74001-970 Praça Universitária,
N° 1166 Setor Universitário Goiânia, GO, Brazil.
E-mail: marianacmfarma@gmail.com

DOI : 10.4103/0973-1296.175997

Access this article online
Website: www.phcog.com
Quick Response Code:

INTRODUCTION

The *Myrtaceae* is a family constituted by 130 genera and 5700 species, it is very common in Brazil, and several of its species can be found in the Amazon region. The *Myrcia* genus, with 374 species occurs mainly in the Central America to Northern Argentina.[1] The species of the *Myrtaceae* family are popularly known for having woody aspect, however, the Brazilian generally do not produce valuable timber, restricting themselves to providing firewood for use in small parts or objects and other forms of local use.[2] In Brazil, the largest use of plants in this family is related to their fruits, they are usually marketed and known to possess very characteristic aroma and flavor (e.g., guava, *Psidium guajava* L., *Jabuticabeira*, *Myrciaria cauliflora* (Mart.) O. Berg, *Surinam cherry*, *Eugenia uniflora* L.).[3,4] This family has a diversity of species with medicinal properties already confirmed such as the works diuretic action, anti-hemorrhagic, astringent, hypoglycemic, and antihypertensive among others.[5]

Secondary metabolites are directly related to the development, protection, and dissemination of the plant, as a result, these compounds play a series of therapeutic activities to the human body. Among the secondary metabolites, phenolic compounds are substances that present

Cite this article as: de Morais Rodrigues MC, Borges LL, Martins FS, Mourão RV, da Conceição EC. Optimization of ultrasound-assisted extraction of phenolic compounds from *Myrcia amazonica* DC. (*Myrtaceae*) leaves. Phcog Mag 2016;12:9-12.

an important role in protection against pathogens and predators, these compounds are very diverse and can be divided into several classes (including the flavonoids [Fv], and condensed and hydrolysable tannins).[6] Several Fv has antioxidant activity and has a high redox potential, thus they can reduce the free radicals that can be harmful and also has a great potential for chelating metals.[7] The main class of tannins are hydrolysable and condensed being that the former are derived from a gallic acid,[8] they precipitate with proteins, anti-oxidants are also its antimicrobial power has been much studies in recent times and agents.[9]

The *Myrtaceae* family is characterized by the presence of secondary metabolites such as phenolic compounds (mainly Fv and tannins) and essential oils.[4,10]

The evaluation of the influence of several factors simultaneously must be necessary to find optimum conditions in several processes. Thus, currently several statistical models have been employed to solve this problem, among them the Box–Behnken design and response surface methodology (RSM), allow for the studies on factors and its multiple interactions.[11,12]

Myrcia amazonica DC. is a species predominantly found in northern Brazil, and there are few studies in the literature. Thus, this study aimed to perform an optimization of extraction of phenolic compounds, Fv, and tannins in leaves of *Myrcia amazoncia* DC. employing ultrasound-assisted extraction.

MATERIALS AND METHODS

Plant material

Leaves of native specimens of *M. amazonica* DC., *Myrtaceae*, were collected in Santarém, Pará, Brazil (02°30.464' S; 054°50.931' W). The plants were identified by Dra. Rosa Helena Veras Mourão and a voucher #250.3639 of the specimen were deposited at Herbarium of the National Institute of Amazon Research. The plant material was dried at 40°C, and pulverized by knife mill, and stored sheltered from light and moisture for subsequent use in the extraction procedure.

Chemicals

Rutin (95%) and tannic acid (98%) were purchased from Sigma–Aldrich® (Sigma–Aldrich Brasil Ltd., a, São Paulo, Brazil), and ultrapure water from a Milli-Q system (Millipore®, Bedford, MA, USA) were used. All reagents were of analytical grade.

Total polyphenol contents, total tannin contents, and total flavonoids contents

For calculated total polyphenol contents (TPc) was employed Hagerman and Butler method, adapted by Waterman and Mole,[13,14] the absorbance was read at 510 nm. The calibration curve was prepared with tannic acid at the dilutions: 0.1, 0.15, 0.2, 0.25, 0.3, and 0.35 mg/mL. The correlation coefficient calculated for this curve was 0.9983.

For total tannin contents (TTc) was employed Hagerman and Butler method, adapted by Waterman and Mole,[13,15] the absorbance was read at 510 nm. The calibration curve was prepared with tannic acid at the dilutions: 0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 0.7, and 0.8 mg/mL. The correlation coefficient calculated for this curve was 0.9985.

For total flavonoids content (TFc), the ethanolic extract was directly read at 361 nm.[16] The calibration curve was prepared with rutin at the dilutions: 0.01, 0.02, 0.03, 0.04, 0.05, 0.06, 0.07, 0.08, and 0.1 mg/mL. The correlation coefficient calculated for this curve was 0.9939.

Experimental design

Box–Behnken experimental design (3^3) with three factors and three levels was used to optimize and evaluate main effects, interaction effects, and quadratic effects of the process variables.[17] The factors studied were: (i) Extraction times (ETs) of 5, 10, and 15 min, (ii) solid: Liquid ratio of 100, 150, and 200 mg/mL, and (iii) ethanolic strength (ES) 65, 80, and 95% (v/v), show in Table 1. Experiments were performed using a 40 kHz ultrasonic UNIQUE® (USC 4800). The experimental runs were randomized to satisfy the statistical requirement of independence of observations, show in Table 2. A second-order polynomial regression model was used to express the yield as a function of the independent variables as follows:

$$y = \beta_0 + \sum_{i=1}^{k} \beta_i x_i + \sum_{i=1}^{k} \beta_{ii} x_i^2 + \sum\sum \beta_{ij} x_i x_j \quad (1)$$

Where y represents the response variables, β_0 is a constant, β_i, β_{ii}, and β_{ij} are the linear, quadratic and interactive coefficients, respectively. x_i and x_j are the levels of the independent variables. All statistical analysis was conducted using the software Design Expert® version 7.0.[18] The factors with a significance higher than 5% ($P < 0.05$) was considered.

RESULTS AND DISCUSSION

Table 2 shows the results with the corresponding responses data for the *M. amazonica* leaves, regarding TPc, TTc, and TFc. The highest value obtained of TPc under the experimental design was 13.66% (Run 2). Regarding total tannins (TT) content, experiment 3 showed the highest value, which was 39.12%, and in this extraction was employed the same alcohol degree that the experiment described above.

The highest content of TF found was 2.60% (Run 4), and this value was also obtained with the 80% alcohol degree, the same of the runs early. The similarity of these experiments regarding the highest values of the responses suggests the influence of alcohol in the extraction process, and this fact will be discussed later.

The second-order polynomial model was fitted to each of the response variables (Equation 1). The ANOVA provides the summary of main effects and their significance in Table 3, which the level of significance

Table 1: Code and level of factors chosen for the experiments

Factor	Low	Center	High
ET (min)	−1 (15)	0 (30)	+1 (45)
SLR (mg/mL)	−1 (100)	0 (150)	+1 (200)
ES (v/v %)	−1 (65)	0 (80)	+1 (95)

ET: Extraction time; SLR: Solid to liquid ratio; ES: Ethanolic strength

Table 2: Box-Behnken design and observed responses of ultrasound-assisted extraction of *Myrcia amazonica* leaves

Run	ET	SLR	ES	TPc	TTc	TFc
1	−1	−1	0	12.70	30.92	2.42
2	1	−1	0	13.66	33.98	2.43
3	−1	1	0	10.87	39.12	2.29
4	1	1	0	10.28	18.83	2.60
5	−1	0	−1	11.58	35.98	2.25
6	1	0	−1	9.17	23.39	2.12
7	−1	0	1	7.89	25.85	1.75
8	1	0	1	10.32	27.82	2.11
9	0	−1	−1	7.56	32.12	1.95
10	0	1	−1	7.21	22.85	1.84
11	0	−1	1	6.73	27.38	1.74
12	0	1	1	7.82	24.43	1.60
13	0	0	0	10.03	19.22	2.22
14	0	0	0	7.78	23.98	2.34
15	0	0	0	6.38	24.97	2.15

TPc: Total polyphenol contents; TFc: Total flavoid contentes; TTc: Total tannin contents; ET: Extraction time; SLR: Solid to liquid ratio; ES: Ethanolic strength

are represented as percentages. This analysis showed that the ES had a significant effect on the total phenols, TT, and TF content at the 5% level.

The ANOVA revealed that only ET^2 exerted an influence on the TPc at a significance level of 5%. None of the others effects and the interactive terms were significant. Thus, in the levels investigated, this squared term had a negative influence over the total phenols content. The coefficient of this independent variable is shown in the following fitted equation:

$$TPc = 7.64 + 0.049\ ET + 3.11\ ET^2\ (R = 0.7327) \quad (2)$$

Figure 1a and b show the surfaces plots of TTc as a function of ET, solid to liquid ratio (SLR), and ES. These graphs showed that ET and SLR exerted a nonlinear effect on TTc. In general, higher and lower levels of SLR provided better yields of the tannins by ultrasound-assisted extraction, thus its possible make the extraction in these two regions of the surface. The fact of the obtainment of great amounts of tannins with lower SLR could present an economy of this raw material. Sousa *et al*. found similar results with the stem barks of the species *Stryphnodendron adstringens* (Mar.) Coville, which lower levels of SLR results in great yields of tannins.[19] Other studies investigated the influence of various parameters on the tannins obtained by ultrasound-assisted extraction, such the article of Martins *et al*., where was studied the parameters ET, ES, and particle size of raw material on the tannins amount of *Dipteryx alata* Vogel fruits.[20]

The application of experimental design to increase the yield of extraction markers has been very employed in early years. The ultrasound-assisted extraction of the phenolic compound 4-nerolidylcatechol was investigated in roots of species *Pothomorphe umbellata*, and the main factors that exerted influence in this extraction process were ethanol to water ratio and ET.[21]

According to Figure 1b, it can observed that lower amounts of ethanol in extraction solvent had a positive influence in yields of TT, which could be explained by the higher affinity to these polar compounds with polar solvents (with more water).[19,22]

The fitted equation of TT is given by:

$$TTc = 23.19 - 3.48\ ET + 4.72\ ET^2 - 2.40\ SLR + 3.16\ SLR^2 - 1.11\ ES - 5.84\ ET \times SLR + 3.64\ ET \times ES\ (R = 0.9579) \quad (3)$$

Figure 2 presents the surface responses of TFc as a function of the terms ET, ES, and SLR. The graphs show that lowest amounts of SLR result in better yields of Fv extraction. Moreover, intermediate values of ES (80%, v/v) associated superior ET (45 min) provided improve yields of Fv extraction. Figure 2 shows the interaction of *ES* and *SLR*, and from this response surface, it can be observed that the better condition to obtainment higher amounts of Fv. The second-order behavior in the optimization of Fv extraction by ultrasound was also found in a study conducted by Zhang *et al.*, which SLR and ES were the quadratic terms.[23] The coefficients of independent variables to this model are shown in the following equation:

$$TFc = 2.21 + 0.068\ ET + 0.24\ ET^2 - 0.12\ ES - 0.41\ ES^2 + 0.12\ ET \times ES\ (R = 0.9722) \quad (4)$$

The dried extract obtained presented a yield of 68.40% (w/w). The levels of total polyphenols, TT, and total FV were, respectively 18.875%, 18.33%, and 7.95% (w/w). These contents of phenolic compounds found were relevant because the values were greater than other species of the same genus.[24]

In this work, the ultrasound-assisted extraction of total phenols, TT, and TF from *M. amazonica* leaves was investigated with a three-variable experiment Box–Behnken design based on an RSM in enhancing the yields of these marker classes. The best extraction parameters in the levels studied were obtained. The knowledge gained in this study has a great importance for further investigations and application of this vegetal species.

Table 3: Summary of factor effects on *Myrcia amazonica* powder properties

Factor	TPc	TTc	TFc
ET	0.09722	−6.9582*	2.091365
ET^2	−3.11446*	−4.7802*	0.136811
SLR	−1.11729	−4.7959*	−0.235664*
SLR^2	−0.70095	−3.2149*	−0.053010
ES	−0.68944	−2.2153	0.037269
ES^2	1.43641	−0.7620	−0.237385*
ET x SLR	−0.77336	−11.6775*	0.413907*
ET x ES	2.41700	7.2822*	0.150955
SLR x ES	0.72396	3.1580	0.239325*

*Significant at 5%; TPc: Total polyphenol contents (% w/w); TFc: Total flavonoid contentes (% w/w); TTc: Total tannin contents (% w/w); ET: Extraction time; SLR: Solid to liquid ratio; ES: Ethanolic strength

Acknowledgment

The authors gratefully acknowledge the financial support obtained from CNPq and CAPES.

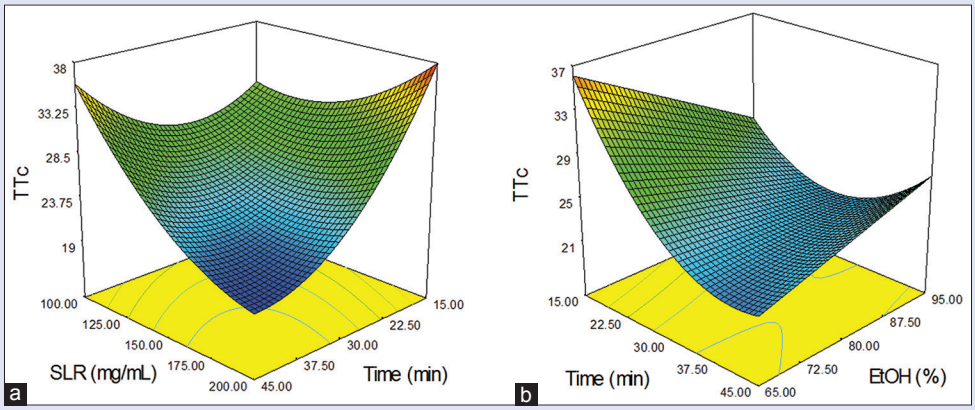


Figure 1: (a) Surface plot of total tannin content as a function of solid to liquid ratio and extraction time (b) surface plot of total tannin content as a function of ethanolic strength and extraction time

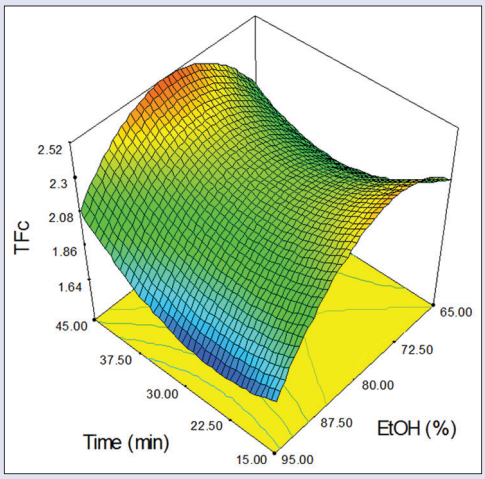


Figure 2: Surface plot of total flavonoid content as a function of ethanolic strength and extraction time

Financial support and sponsorship

The financial support obtained from CNPq and CAPES.

Conflicts of interest

There are no conflicts of interest.

REFERENCES

1. Govaerts R, Sobral M, Ashton P, Barrie F, Holst BK, Landrum LR, *et al.* World Checklist of Myrtaceae. Ver. 1. Kew: Royal Botanic Garden; 2008. p. 57-9.
2. Marchiori JN, Sobral M, editors. Dendrologia das Angiospermas-Myrtales. Santa Maria: Universidade Federal de Santa Maria; 1997. p. 304.
3. Borges LL, Conceição EC, Silveira D. Active compounds and medicinal properties of *Myrciaria* genus. Food Chem 2014;153:224-33.
4. Sá FA, Borges LL, Paula JA, Sampaio BL, Ferri PH, Paula JR. Essential oils in aerial parts of *Myrcia tomentosa*: Composition and variability. Rev Bras Farmacogn 2012;22:1233-40.
5. Russo EM, Reichelt AA, De-Sá JR, Furlanetto RP, Moisés RC, Kasamatsu TS, *et al.* Clinical trial of *Myrcia uniflora* and *Bauhinia forficata* leaf extracts in normal and diabetic patients. Braz J Med Biol Res 1990;23:11-20.
6. Hurtado-Fernández E, Gómez-Romero M, Carrasco-Pancorbo A, Fernández-Gutiérrez A. Application and potential of capillary electroseparation methods to determine antioxidant phenolic compounds from plant food material. J Pharm Biomed Anal 2010;53:1130-60.
7. Tsao R, Yang R. Optimization of a new mobile phase to know the complex and real polyphenolic composition: Towards a total phenolic index using high-performance liquid chromatography. J Chromatogr A 2003;1018:29-40.
8. Porter LJ. Tannins. In: Harborne JB, editor. Methods in Plant Biochemistry. London: Academic Press; 1989.
9. Hangerman AE. Tannin Handbook. USA: Departament of Chemistry and Biochemistry Miami University; 2002.
10. Borges LL, Alves SF, Bara MT, Conceição EC, Ferri PH, Paula JR. Influence of environmental factors on the composition of essential oils from leaves of *Myrcia tomentosa* (Aubl.) DC. Bol Latinoam Caribe Plant Med Aromat 2013;6:572-80.
11. Zafar M, Kumar S, Kumar S. Optimization of naphthalene biodegradation by a genetic algorithm based response surface methodology. Braz J Chem Eng 2010;27:89-99.
12. Weuster-Botz D. Experimental design for fermentation media development: Statistical design or global random search? J Biosci Bioeng 2000;90:473-83.
13. Hagerman AE, Butler LG. Protein precipitation method for the quantitative determination of tannins. J Agric Food Chem 1978;26:809-12.
14. Waterman PG, Mole S. A critical analysis of techniques for measuring tannins in ecological studies I: Techniques for chemically defining tannins. Oecologia 1987;72:137-47.
15. Waterman PG, Mole S. A critical analysis of techniques for measuring tannins in ecological studies II: Techniques for chemically defining tannins. Oecologia 1987;72:148-56.
16. Rolim A, Maciel CP, Kaneko TM, Consiglieri VO, Salgado-Santos IM, Velasco MV. Validation assay for total flavonoids, as rutin equivalents, from *Trichilia catigua* Adr. Juss (*Meliaceae*) and *Ptychopetalum olacoides* Bentham (*Olacaceae*) commercial extract. J AOAC Int 2005;88:1015-9.
17. Box GE, Hunter WG, Hunter JS. Statistics for Experimenters. New York: John Wiley and Sons; 1978.
18. Stat-Ease, Inc. 2021 East Hennepin Ave., Suite 480, Minneapolis, MN 55413,USA: Design-Expert version 7.0.0, 2005.
19. Sousa JN, Pedroso NB, Borges LL, Oliveira GA, Paula JR, Conceição EC. Optimization of Ultrasound-assisted extraction of polyphenols, tannins and epigallocatechin gallate from barks of *Stryphnodendron adstringens* (Mart.) Coville bark extracts. Pharmacogn Mag 2014;10 Suppl 2:S318-23.
20. Martins FS, Borges LL, Paula JR, Conceição EC. Impact of different extraction methods on the quality of *Dipteryx alata* extracts. Rev Bras Farmacogn 2013;23:521-6.
21. Costa FS, de Araújo Júnior CA, Silva Ede J, Bara MT, Lima EM, Valadares MC, *et al.* Impact of ultrasound-assisted extraction on quality and photostability of the *Pothomorphe umbellata* extracts. Ultrason Sonochem 2011;18:1002-7.
22. Rusak G, Komes D, Likic S, Horžic D, Kovac M. Phenolic content and antioxidative capacity of green and white tea extracts depending on extraction conditions and the solvent used. Food Chem 2008;110:852-8.
23. Zhang G, He L, Hu M. Optimized ultrasonic-assisted extraction of flavonoids from *Prunella vulgaris* L. and evaluation of antioxidant activities *in vitro*. Innov Food Sci Emerg Technol 2011;12:18-25.
24. Borges LL, Alves SF, Sampaio BL, Conceição EC, Bara MT, Paula JR. Environmental factors affecting the concentration of phenolic compounds in *Myrcia tomentosa* leaves. Rev Bras Farmacogn 2013;23:230-8.

Mariana Cristina de Morais Rodrigues

ABOUT AUTHOR

Mariana Cristina de Morais Rodrigues, is pharmacist graduated in the State University of Goiás, Brazil, and currently is a Master degree student in the Federal University of Goiás, under guidance of Prof. Dr. Edemilson Cardoso da Conceição. Her research it's concentrated mainly with pharmaceutical technology applied to natural products and development of intermediate and final products from medicinal plants.