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Determination and validation of spectrophotometric analytical method for quantification of total flavonoids in the leaves of *Azadirachta indica* A. Juss. (Meliacease) and optimization of the ultrasound-assisted extraction conditions

Determinação e validação de método analítico espectrofotométrico para quantificação de flavonoides totais nas folhas de *Azadirachta indica* A. Juss. (Meliaceae) e otimização das condições de extração assistida por ultrassom

Determinación y validación del método analítico espectrofotométrico para la cuantificación de los flavonoides totales en las hojas de *Azadirachta indica* A. Juss. (Meliaceae) y optimización de las condiciones de extracción asistida por ultrasonidos

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Leiza Fagundes Soares
ORCID: https://orcid.org/0000-0003-3866-1472
Universidade Estadual de Goiás, Brazil
E-mail: leizafagundes@hotmail.com
Luana Cristina Machado
ORCID: https://orcid.org/0000-0001-7991-3221
Universidade Estadual de Goiás, Brazil
E-mail: luanac689@gmail.com
Eduardo Júnio Pelegrini dos Santos
ORCID: https://orcid.org/0000-0002-1725-9858
Universidade Estadual de Goiás, Brazil
E-mail: eduardo.junio2015@outlook.com
Debborah Gonçalves Bezerra
ORCID: https://orcid.org/0000-0002-5450-9940
Universidade Estadual de Goiás, Brazil
E-mail: debborah.b@gmail.com
Leonardo Luiz Borges
ORCID: https://orcid.org/0000-0003-2183-3944
Universidade Estadual de Goiás, Brazil
E-mail: leonardo.borges@ueg.br
Vanessa Cristiane Santana Amaral
ORCID: https://orcid.org/0000-0001-5964-4888
Universidade Estadual de Goiás, Brazil
E-mail: vanessa.cristiane@ueg.br
José Realino de Paula
ORCID: https://orcid.org/0000-0002-4424-7692
Universidade Federal de Goiás, Brazil
E-mail: pjrpaula@gmail.com
Joelma Abadia Marciano de Paula
ORCID: https://orcid.org/0000-0003-0737-2600
Universidade Estadual de Goiás, Brazil
E-mail: joelma.paula@ueg.br

Abstract

Azadirachta indica A. Juss. (Meliaceae) is a plant whose leaves present several pharmacological activities, among them: antioxidant, antimicrobial and hypoglycemic. The present work aims to determine and validate a spectrophotometric analytical method for quantification of total flavonoids, expressed as rutin, in *A. indica* leaves and to optimize the extraction conditions of these flavonoids. For the selection of the spectrophotometric method, four analytical methods were investigated. The method selected as the most efficient, simple, fast and sustainable has been validated and optimized. The validation was performed according to criteria of national and international regulatory agencies. Box Behnken design (3^3) and the response surface methodology (RSM) were used to estimate the best extraction conditions. The analytical method that met the study criteria consisted of ultrasound-assisted extraction (UAE) of the plant material in hydroethanolic solution, at a temperature of 60 ºC, for 30 minutes, followed by filtration, dilution of the extract in ethanol/0.02 M acetic acid solution (99:1) and absorbance reading in a spectrophotometer at

364nm. In the validation stages, this method was shown to be linear (linear interval - 20 to 80 µg/mL), precise (intraday and interday), accurate (mean recovery of 120.8%), robust and selective (absence of matrix effect). The best conditions for the UAE process were: Ethanol content 30% (p/p), plant material/solvent ratio 0.2 g/mL and extraction time of 33 minutes. The spectrophotometric method and the UAE conditions determined in this study are suitable for use in the quality control of plant drugs and ethanol extracts from the leaves of *Azadirachta indica*.
Keywords: Neem; Quality control; Herbal medicine; Rutin.

Resumo

Azadirachta indica A. Juss. (Meliaceae) é uma planta cujas folhas apresentam diversas atividades farmacológicas, entre elas: antioxidante, antimicrobiana e hipoglicemiante. O presente trabalho visa determinar e validar um método analítico espectrofotométrico para quantificação de flavonoides totais, expressos como rutina, nas folhas de *A. indica* e otimizar as condições de extração desses flavonoides. Para a seleção do método espectrofotométrico foram investigados quatro métodos analíticos. O método selecionado como o mais eficiente, simples, rápido e sustentável foi validado e otimizado. A validação foi realizada segundo critérios de agências reguladoras nacionais e internacionais. Planejamentos fatoriais Box Behnken (3^3) e a metodologia de superfície de resposta (MSR) foram utilizados para estimar as melhores condições de extração. O método analítico que atendeu aos critérios do estudo consistiu em extração assistida por ultrassom (EAU) do material vegetal em solução hidroetanólica, à temperatura de 60 ºC, por 30 minutos, seguida de filtração, diluição do extrato em solução de etanol/ácido acético 0,02 M (99:1) e leitura das absorvâncias em espectrofotômetro a 364nm. Nas etapas da validação esse método mostrou ser linear (intervalo linear - 20 a 80 µg/mL), preciso (intradia e interdia), exato (recuperação média de 120,8%), robusto e seletivo (ausência de efeito matriz). As melhores condições para o processo de EAU foram: Teor etanólico 30%(p/p), proporção material vegetal/solvente 0,2 g/mL e tempo de extração 33 minutos. O método espectrofotométrico e as condições de EAU determinados nesse estudo estão aptos a serem utilizados no controle de qualidade da droga vegetal e extratos etanólicos das folhas de *Azadirachta indica*.
Palavras-chave: Neem; Controle de qualidade; Fitoterápico; Rutina.

Resumen

Azadirachta indica A. Juss. (Meliaceae) es una planta cuyas hojas presentan diversas actividades farmacológicas, entre ellas: antioxidante, antimicrobiano e hipoglicemiante. El presente trabajo tiene como objetivo determinar y validar un método analítico espectrofotométrico para la cuantificación de flavonoides totales, expresados como rutina, en las hojas de *A. indica* y optimizar las condiciones de extracción de esos flavonoides. Para la selección del método espectrofotométrico se investigaron cuatro métodos analíticos. El método seleccionado como el más eficiente, simple, rápido y sostenible ha sido validado y optimizado. La validación se realizó según criterios de agencias reguladoras nacionales e internacionales. La planificación factorial Box Behnken (3^3) y la metodología de superficie de respuesta (MSR) se utilizaron para estimar las mejores condiciones de extracción. El método analítico que cumplió los criterios del estudio consistió en la extracción asistida por ultrasonido (EAU) del material vegetal en solución hidroetanólica, a una temperatura de 60 ºC, durante 30 minutos, seguida de filtración, dilución del extracto en solución de etanol/ácido acético 0,02 M (99:1) y lectura de las absorbancias en espectrofotómetro a 364 nm. En las etapas de la validación este método demostró ser lineal (intervalo lineal - 20 a 80 µg/mL), preciso (intradía e interdia), exacto (recuperación media de 120,8%), robusto y selectivo (ausencia de efecto matriz). Las mejores condiciones para el proceso de EAU fueron: Contenido etanólico 30% (p/p), proporción material vegetal/solvente 0,2 g/ml y tiempo de extracción 33 minutos. El método espectrofotométrico y las condiciones de EAU determinados en ese estudio están aptos para ser utilizados en el control de calidad de la droga vegetal y extractos etanólicos de las hojas de *Azadirachta indica*.
Palabras clave: Neem; Control de calidad; Fitoterápico; Rutina.

1. Introduction

Azadirachta indica A. Juss. is a plant species from the Meliaceae family, used to treat several acute crises and chronic diseases in different parts of Asia and Africa, known worldwide as neem or "nim" (Gupta et al., 2017). Extracts of its leaves have demonstrated antioxidant, antibacterial (Manandhar et al., 2019), antitumor (Supriyanto et al., 2020), antiviral (Gupta et al., 2014) and hypoglycemic activities (Mostofa et al., 2007; Akinola et al., 2010; Akinola et al., 2011).

In the leaves of this species were identified compounds such as: limonoids, flavonoids, tannins, alkaloids and coumarins (Silva Neto et al., 2020), with possible correlation with their biological activities described in the literature. Phenolic compounds, such as flavonoids, can play an important role in antioxidant and hypoglycemic activities. Flavonoids have the ability to nullify reactive oxygen species, such as the superoxide anion (O^{2-}) (Simões et al., 2016). This action may also play a role in the treatment of diabetes mellitus, since the onset of insulin resistance is associated with oxidative stress (Sarian et al., 2017).

In Brazil, for herbal medicine to be registered and sold, it is necessary to have proven quality, safety and efficacy. With regard to quality assurance, Good Manufacturing and Control Practices (GMCP) and the performance of physicochemical tests are essential, both in vegetable raw material and in derived and finished products (Brazil, 2014). Methods and criteria recognised by regulatory agencies should be followed in order to carry out quality control tests on plant and phytotherapeutic raw materials and, in the absence of a pharmacopoeic method, the analytical method should be validated (Brazil, 2014; Brazil, 2017a; FDA, 1996; ICH, 2005; USP, 2008). Currently, we are looking for analytical methods that are more efficient, simple, fast, economically viable and mainly sustainable. The validation of the analytical methods must comply with the parameters recommended by regulatory agencies, such as linearity, matrix effect, precision, accuracy, robustness, limit of detection and quantification, in order to seek evidence that the method is suitable for the intended use (Brazil, 2017a; FDA, 1996; ICH, 2005; USP, 2008).

The present study aimed to determine and validate a spectrophotometric analytical method for quantification of total flavonoids, expressed as rutin, in *A. indica* leaves, choosing an efficient, simple, fast, cheap and environmentally sustainable method. Concomitantly, ultrasound-assisted extraction of total flavonoids present in *A. indica* leaves was investigated in order to optimize the parameters of this process, to be used in the execution of the validated spectrophotometric analytical method.

2. Methodology

2.1 Plant material

The samples were composed of leaves, collected in July 2018 from *A. indica* trees grown in woods located in front of the Empresa Brasileira de Pesquisa Agropecuária (EMBRAPA Arroz e Feijão), in the city of Santo Antônio, Goiás, Brazil (S 16º 30' 26,0994"; W 49º 16' 58,8720"; 821m). Vouchers of flowering branches were deposited in the Herbarium of the Universidade Federal de Goiás (UFG), registration number UFG-48590.

The leaves were cleaned in potable water and purified water, subjected to drying in an oven with air-forced circulation (Solab, model SL-102) at 37 ±5 ºC until reaching desiccation loss values between 8 and 14%. Then the material was powdered in a knife mill (Marconi, model MA-580) and packed in plastic bags with protection from light and moisture.

2.2 Spectrophotometric analytical methods investigated for the determination of total flavonoids in *A. Indica* leaves

For the determination of total flavonoids in *A. indica* leaves, four spectrophotometric analytical methods were investigated. All trials were performed in triplicate. The choice of the most promising method was based on sustainability and extraction efficiency criteria (Barba et al., 2016; Chemat et al., 2017). For this purpose, we considered the practicality of the flavonoid extraction process, the lower energy consumption of the method and the low toxicity of the reagents used in the execution of the steps, as well as the extraction of higher flavonoid contents from *A. indica* leaves. The method that best met these criteria was validated and optimized. The investigated methods are described below:

Method 1 - Initially we tested the method of determination of total flavonoids described in the "sabugueiro" monograph (*Sambucus nigra flos*) according to the Brazilian Pharmacopoeia 5th edition (Brazil, 2010).

Method 2 - The method of total flavonoid determination of "sabugueiro" (*Sambucus nigra flos*) was tested, with the following adaptation: the extraction stage was performed in an ultrasound bath (UNIQUE mod. USC - 2800A, frequency 40kHz and power 154W) at 60 ºC for 30 minutes, replacing reflux extraction.

Method 3 - The method of Rolim et al. (2005) was evaluated with adaptations. For this, 1g of the plant drug was transferred to a 10 mL volumetric balloon, the volume was completed with 70% ethanol (w/w), remained in an ultrasound bath for 30 minutes at room temperature, filtered in qualitative filter paper. Then, from this stock solution, the samples were prepared, using 100 µl of the stock solution and sufficient quantity for 2 mL of 95% P.A. ethanol/0.02 M acetic acid (99:1). The analytical curve of the rutin standard was prepared in triplicate from a stock solution consisting of 0.010g rutin (Sigma-Aldrich) in 100 mL

of 95% P.A. ethanol/0.02 M acetic acid (99:1). Seven serial concentrations were prepared from this stock solution: 5, 10, 15, 20, 25, 30 and 35 µg/mL, all in 95% P.A. ethanol/0.02 M acetic acid (99:1). Then, the absorbance readings of the solutions in the Perkinelmer UV-VIS spectrophotometer, model Lambda 25, were performed at a wavelength of 364 nm, using 95% P.A. ethanol/0.02 M acetic acid (99:1) as blank. The total flavonoid concentration, expressed as rutin, was calculated using the straight-line equation, obtained by linear regression analysis of the rutin standard calibration curve. The total flavonoid content (%) expressed as rutin was calculated according to Equation 1.

Content (%) = C/Cs × 100 (Eq. 1)

Where:

C = total flavonoid concentration expressed as rutin in the sample (µg/mL)

Cs = Sample concentration (vegetable raw material) (µg/mL)

Method 4 - New adaptations to the method of Rolim et al. (2005) were tested. For this, 1g of the plant drug was transferred to a 10 mL volumetric balloon, the volume was supplemented with 50% ethanol (w/w) instead of 70% ethanol (w/w), and remained in an ultrasound bath at 60 ºC (instead of room temperature) for 30 minutes. The other stages were performed in the same way as Method 3.

2.3 Validation of the spectrophotometric analytical method

The analytical method that best met the pre-established criteria was validated (Brazil, 2017a; FDA, 1996; ICH, 2005; USP, 2008). For this, the linearity, precision, accuracy and robustness of the method were determined. Limits of detection and quantification and the matrix effect were also determined.

Linearity: The linearity of the method was determined by the calibration curve generated of 7 concentration levels of the rutin standard (20, 30, 40, 50, 60, 70 and 80 µg/mL) prepared independently, in triplicate, in 95% P.A. ethanol/acetic acid 0.02 M (99:1).

Precision: The precision was determined through 6 spectrophotometric readings of the sample (at 100% of the total flavonoid concentration), prepared individually and performed interday (intermediate precision) and intraday (repeatability).

Accuracy: The accuracy was verified by the recovery method, by the absorbance readings of samples prepared in triplicate, in three concentration levels relative to the linear interval (low (80%), mean (100%) and high (120%) concentrations), and samples with the standard added (50µg/mL) under these same conditions, in triplicate. The accuracy was calculated for each concentration level according to Equation 2 (Brazil, 2017a).

$$\text{Recovery} = \frac{AC\ (\text{sample added}) - CA\ (\text{sample})}{TAC} x\ 100 \quad \text{(Eq.2)}$$

Where:

AC - analyte experimental concentration (total flavonoids expressed as rutin, in µg/mL)

TAC - theoretical analyte concentration (rutin standard, in µg/mL)

Robustness: The robustness of the method was determined by obtaining the concentration of flavonoids, expressed as rutin, in the sample analyzed under the conditions of the original method and after small variations in wavelength (363 and 365 nm) and type of solvent (methanol and ethanol) used in sample dilution.

Limits of detection and quantification: The limits of detection and quantification were estimated according to the parameters of the analytical curve, from Equations 3 and 4 (Brazil, 2017a), respectively.

$$LD = \frac{3{,}3*\sigma}{S} \qquad LQ = \frac{10*\sigma}{S} \qquad \text{(Eq. 3 e 4)}$$

Where:

LD - limit of detection

LQ - limit of quantification

σ - Standard deviation (SD) calculated in two ways, SD of intercept with Y axis and residual SD of regression line

S - average of slopes of calibration curves

Matrix effect: The determination of the matrix effect was performed by constructing two analytical curves (analyte concentration x absorbance): one obtained from extract samples prepared without addition of rutin standard and another from extract samples strengthened with rutin. Both curves were prepared to obtain 5 levels of total flavonoid concentrations, expressed as rutin, within the linear range (40 to 80 µg/mL). The linear regression analysis of the two curves aimed to demonstrate the parallelism between the lines and the coincidence of the angular coefficients. All samples were prepared in triplicate and independently.

All statistical analyses were performed with the aid of Action Stat software (version 3.5) coupled to Excel 2016, at a significance level of 5% (Action, 2014; Brazil, 2017a).

2.4 Optimisation of ultrasound-assisted extraction conditions of total flavonoids, expressed as rutin, in *A. indica* leaves

Box-Behnken design (3^3) and the Response Surface Methodology (RSM) were used to optimize ultrasound-assisted extraction (UAE) of total flavonoids, expressed as rutin, in *A. indica* leaves. The choice of variables investigated in the UAE took into account the parameters of extractions performed in methods 3 and 4, previously described, in which were used: liquid extractor – 70% ethanol (w/w) and 50% ethanol (w/w); temperature - environment (25 °C) and 60 °C and plant material/solvent ratio - 0.1 g/mL. The results obtained in these preliminary trials led to the decision to set the extraction temperature at 60 °C and to vary the parameters in the optimization experiments: extractor liquid, extraction time, and plant material/solvent ratio. For this, two batteries of experiments were carried out. For the first battery (Table 1), 15 experiments were generated using the software Statistica® version 12.0 (Statsoft, 2010) and their execution was performed randomly. Three experiments were repetitions of the central point, used in the evaluation of pure error. The second battery also had 15 random experiments, in which the same factors were investigated at different levels (Table 2) to better investigate the results of the first battery.

Table 1 - Independent variables and their levels, evaluated by RSM in a Box-Behnken 3^3 model, for the optimization of the UAE of total flavonoids, expressed as rutin, in *A. indica* leaves, in the 1st factorial planning.

Independent variables (factors)	**Levels**		
	-1	**0**	**+1**
Extraction time (minutes)	10	30	50
Plant material/Solvent Ratio (g/mL)	0.10	0.15	0.20
Ethanol content (% w/w)	30	50	70

Legend: RSM: Response Surface Methodology; UAE: Ultrasound-Assisted Extraction. Source: Authors (2020).

Table 2 - Independent variables and their levels, evaluated by RSM, in a Box-Behnken 3^3 model, for the optimization of the UAE of total flavonoids, expressed as rutin, in *A. indica* leaves, in the 2nd factorial planning.

Independent variables (factors)	Levels		
	-1	**0**	**+1**
Extraction time (minutes)	20	30	40
Drug/Solvent Ratio (g/mL)	0.06	0.1	0.2
Ethanolic content (% w/w)	10	30	50

Legend: RSM: Response Surface Methodology; UAE: Ultrasound-Assisted Extraction. Source: Authors (2020).

The response variable of all experiments was the total flavonoid concentrations (μg/mL), determined by the validated spectrophotometric method, considering the dilution factors used.

All statistical analyses and response surfaces were generated with Statistica® 12.0 software (STATSOFT, 2010). Multiple linear regression analysis was performed using the quadratic equation of the polynomial model (Eq. 5).

$$Y = B_0 + B_1X_1 + B_2X_2 + B_3X_3 + B_{11}X_1^2 + B_{22}X_2^2 + B_{33}X_3^2 + B_{12}X_1X_2 + B_{13}X_1X_3 + B_{23}X_2X_3 \quad \text{(Eq.5)}$$

Where:

Y - predicted response;

B_0 - model constant;

X_1, X_2 and X_3 - independent variables;

B_1, B_2 and B_3 - linear coefficients;

B_{12}, B_{13} and B_{23} - interaction coefficients between the independent variables;

B_{11}, B_{22} and B_{33} - quadratic coefficients.

The F test was used to obtain the coefficients. In the factorial planning, p values < 0.05 were considered significant. Data analysis was performed through regression analysis, variance analysis (ANOVA) and response surface plotting.

The conditions considered optimal were conducted experimentally, in triplicate, in order to validate whether the results of the total flavonoid concentrations, expressed as rutin, correspond to the values predicted by the model.

3. Results and Discussion

3.1 Spectrophotometric analytical methods investigated

Method 1, based on the pharmacopoeic method of "sabugueiro" (*Sambucus nigra flos*) (Brazil, 2010), expresses the total flavonoid content as quercetin equivalents. For this method, the total flavonoid content of *A. indica* leaves was 0.45319% (±0.0070). Toxic and environmentally harmful compounds such as methenamine, hydrochloric acid, acetone, ethyl acetate, aluminum chloride and acetic acid were used in this method, as well as requiring higher energy, water and time costs for its execution, in a large number of stages.

In Method 2, at the extraction stage, the reflux used in Method 1 was replaced by an ultrasound bath at 60 ºC for 30 minutes. The flavonoid content expressed as quercetin obtained was 0.8431% (±0.0312). In this method, there was a reduction in time and water wastage in relation to Method 1, but compounds such as acetone, methenamine and hydrochloric acid were still used. In addition to decreasing the time spent on the experiment, the total flavonoid content almost doubled in relation to Method 1, demonstrating the efficiency of ultrasonic waves in the extraction of flavonoids.

In Method 3, based on Rolim et al. (2005), the total flavonoid content is expressed as rutin equivalent. In this case, in which 70% ethanol (w/w) was used as a liquid extractor and ultrasound extraction lasted for 30 minutes at room temperature, total flavonoid content, expressed as rutin, was 0.10803% (±0.01159) in the leaves of *A. indica*. In this method, in addition to decreasing the time spent, the use of solvents was reduced, since only ethanol and acetic acid are used.

Finally, in Method 4, in which 50% ethanol (w/w) was used as a liquid extractor and extraction was performed in an ultrasound bath at 60 ºC for 30 minutes, the total flavonoid content, expressed as rutin, was 0.33664% (±0.3143). The results indicate that by increasing the temperature and decreasing the ethanol content, the extraction of flavonoids tends to improve, since the content tripled in relation to Method 3. The results also indicate that doing the reflux replacement by ultrasound bath (as in Method 2) and by changing the ethanol content (as in Method 4) it is possible to extract higher levels of flavonoids in plant material, reducing expenses, time and consumption of solvents. It is worth noting that the results of the first two methods are not comparable with the last two, since the flavonoid contents in Methods 1 and 2 are expressed as quercetin and in Methods 3 and 4 they are expressed as rutin.

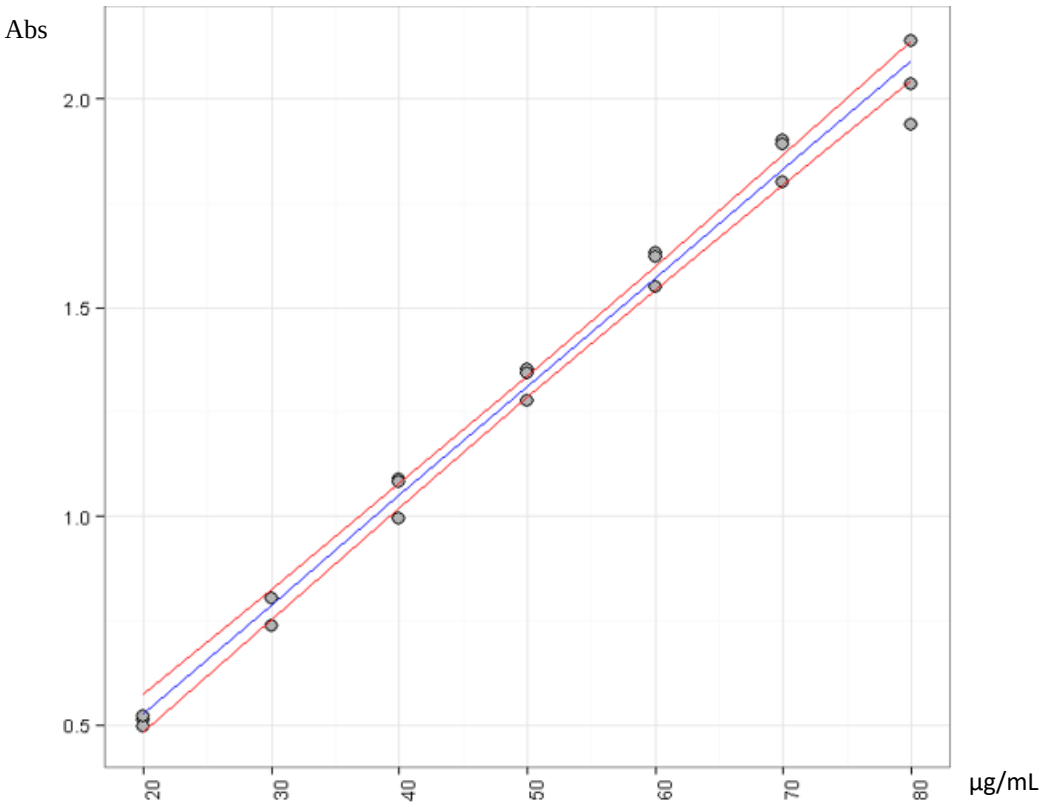
The literature is scarce on methods for quantitative analysis of flavonoids from the leaves of *A. indica*. The quality control of herbal medicine inputs from the species, especially from seeds, is mostly based on the content of azadirachtin and/or related compounds, which are very frequent in this species. However, after High Performance Liquid Chromatography (HPLC) analysis in a sample of the *A. indica* leaf extract from this study, carried out at the Natural Products Research Laboratory of the Federal University of Goiás (UFG), according to the methodology of Paula et al. (2016), the presence of azadirachtin in the sample was not detected (data not shown). In studies by Deshpandes et al. (2014), the presence of flavonoids among other compounds in the *A. indica* leaves was observed. Due to the high pharmacological potential of flavonoids, this group of compounds was chosen as possible markers of the plant raw material from *A. indica* leaves in this study.

After verification of the conditions for extraction and quantification of total flavonoids in the plant material of *A. indica*, Method 4 was considered as the most appropriate, compared to the others. It is a simple method, with lower energy consumption and low toxicity, besides providing the extraction of higher levels of flavonoids, expressed as rutin, in the leaves of *A. indica*. Briefly, the method consisted of ultrasound-assisted extraction of 1g of the plant material in sufficient quantity to complete 10 mL of 50% ethanol (w/w), at a temperature of 60 ºC, for 30 minutes, followed by filtration and reading of the absorbances in spectrophotometer at 364nm. For the absorbance readings the extract was diluted (100 µl) in sufficient quantity for 2 mL of 95% P.A. ethanol/0.02 M acetic acid solution (99:1), using 95% P.A. ethanol/0.02 M acetic acid solution (99:1) as blank. This method was validated, the UAE conditions were optimized and the results are demonstrated below.

3.2 Validation of the spectrophotometric analytical method

Linearity: The linearity of the rutin calibration curve was demonstrated in the interval of 20 to 80 µg/mL (Figure 1), obtaining the following representative equation: y=0.0261+0.0071 (R=0.9948), which was estimated by the Ordinary Minimum Square Method (OMSM) after proving the homocedasticity of the data (Cochran test, p=0.138) and establishing the variances of the responses (absorbances) for each rutin concentration used in the interval (C), considering a significance level of 5%.

Figure 1 - Mean calibration curve of the rutin standard in the concentration range of 20 to 80 µg/mL for the spectrophotometric method.

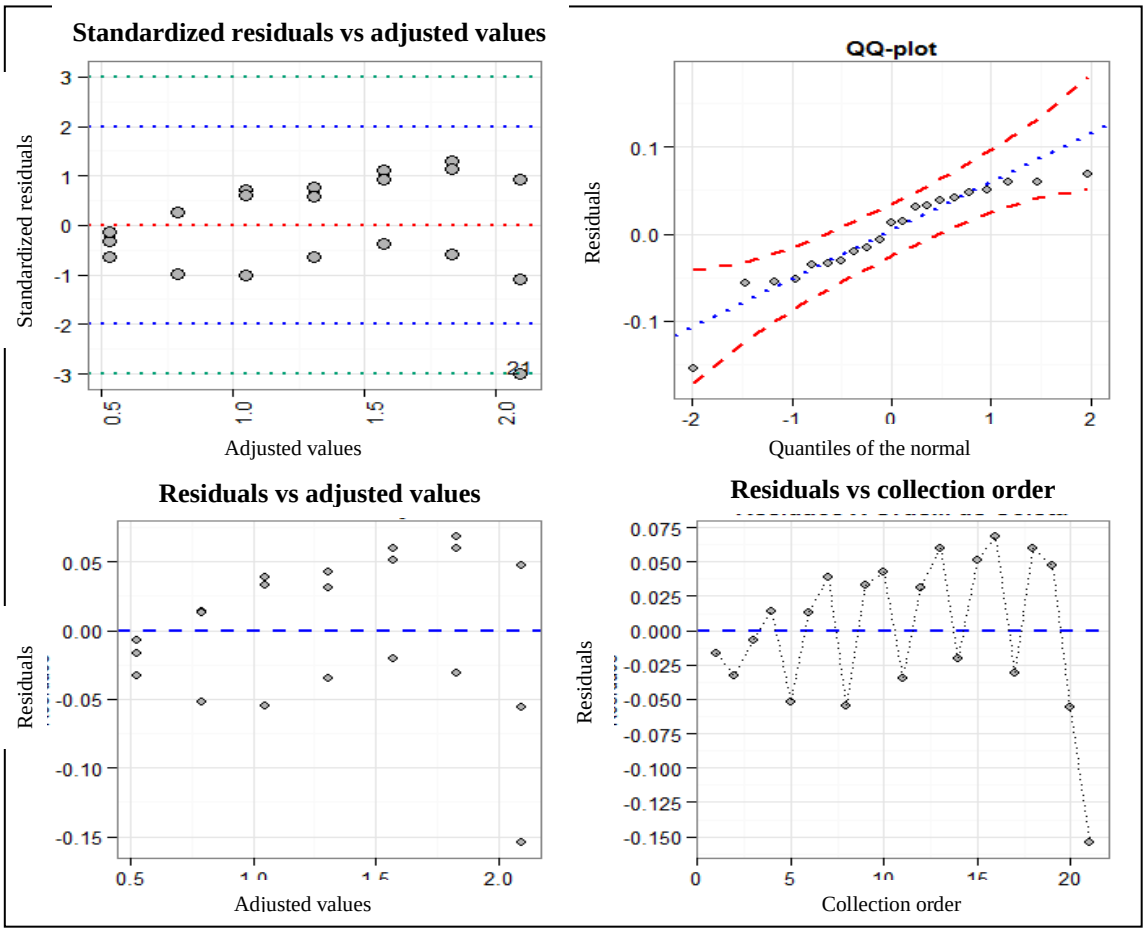


Source: Authors (2020).

The angular coefficient, evaluated by the F test, was statistically different from zero (p=0.828104968) and the linear coefficient, evaluated by the ANOVA T-test, was not statistically different from zero (p=2.32002x10^{-20}); this demonstrates the linearity of the method.

Residue analysis shows how predictive the model is from a minimum distance between the actual value and the estimated value. The visual analysis of the graphs (Figure 2) shows the randomness of the distribution of the points within the limits established by the blue line in the first graph. When analyzing the QQ-Plot graph, the highest number of points was within the red line and within the blue line, the residues are minimal and adequate, as recommended. In order to prove homocedasticity, the null hypothesis should be confirmed by means of the normality and residue independence test, at a significance level of 5%. The normality test was performed according to Anderson-Darling, with a p value of 0.1523, and the independence test was performed according to Durbin-Watson, with a p value of 0.0759. Therefore, the residues are independent and have a normal distribution. For confirmation purposes, the lack of fit test was performed, which represents the error embedded in the analysis and whether this error is compromising the linearity of the method. The value of p= 0.4069 shows to be non-significant, thus proving the H_0 and showing that the method had no lack of fit. Therefore, through the analysis of the statistical parameters, it was verified that the method has the ability to generate analytical responses directly proportional to the flavonoid concentration expressed as rutin (Brazil, 2017b).

Figure 2 - Graphs of the standardized residues for the linearity of the spectrophotometric method.



Source: Authors (2020).

Limits of Detection and Quantification: The limit of detection refers to the smallest amount of analyte present in the sample that can be detected, not necessarily quantified, under the experimental conditions established in the method. The limit of quantification is the smallest amount of the analyte present in the sample that can be determined with acceptable precision and accuracy under the experimental conditions established in the method (Brazil, 2017a). For the present study, the limits of detection and quantification, estimated based on the standard deviation of the intercept with the y-axis, were 3.59 µg/mL and 10.88 µg/mL, respectively. The same limits estimated based on the standard deviation of residues were 7.06 µg/mL and 21.4 µg/mL, respectively.

Precision: In the determination of the method's precision (Table 3), Relative Standard Deviation (RSD) obtained on day 1 (Analyst 1) was 3.349% for total flavonoid concentrations expressed as rutin in the six samples of plant material of *A. indica* prepared at 100% of the test concentration. On day 2 (analyst 2), the RSD was 4.360% and the inter-day RSD was 6.702%.

Table 3 - Precision data for the quantification of total flavonoids, expressed as rutin equivalents, by spectrophotometric method.

Extract	**Absorbance (364 nm)**	**Flavonoid concentration expressed as rutin (µg/mL)**
Intra-day (Analyst 1, day 1)		
1	1.292	49.229
2	1.267	48.272
3	1.239	47.199
4	1.325	50.494
5	1.253	47.735
6	1.204	45.859
Mean	1.263	48.131
RSD (%)	3.331	3.349
Intra-day (Analyst 2, day 2)		
1	1.181	44.977
2	1.183	45.053
3	1.099	41.835
4	1.069	40.685
5	1.108	42.180
6	1.171	44.593
Mean	1.135	43.220
RSD (%)	3.955	4.360
Inter-day		
Mean	1.199	45.676
RSD (%)	6.379	6.702

RSD: Relative standard deviation. Source: Authors (2020).

Accuracy: The results of the accuracy obtained by the recovery method are shown in Table 4. The samples strengthened with the rutin standard (50µg/mL) presented an average recovery of 120.11% ±17.8469 and RSD of 14.85%, i.e., within the acceptable limits for accuracy (Possas et al., 2012).

Table 4 - Accuracy data for the quantification of total flavonoids expressed as rutin equivalents, by spectrophotometric method.

Sample concentration level relative to linear range (%)	Total flavonoid concentration in the sample (µg/mL)	Total flavonoid concentration in sample strengthened with rutin pattern (µg/mL)	Recovery (%)
Low	15.31	50.99	142.68
	21.60	42.90	85.21
	15.89	50.87	139.92
Medium	24.67	54.28	118.46
	23.90	51.18	109.11
	19.76	49.61	119.38
High	32.67	60.30	110.49
	25.89	59.53	134.55
	31.83	62,14	121.22
Theoretical concentration of the rutin standard		50 µg/ml	
		Mean	120.11
		RSD (%)	14.85

Legend: RSD: Relative standard deviation. Source: Authors (2020).

Robustness: The results of the method's robustness evaluations are shown in Table 5. The RSD for the total flavonoid concentrations obtained at 363nm was 4.828%, at 365nm of 5.334%, and the RSD between the two wavelengths was 6.951%. In relation to the type of solvent, the RSD obtained with the use of methanol was 3.205%, with ethanol it was 3.331%, and the RSD among the solvents was 5.210%.

The maximum acceptable RSD value for the validation of analytical methods should be defined according to the method employed, the type of matrix, the analyte concentration in the sample and the purpose of the method. According to the Brazilian guidance for registration of herbal medicine and registration and notification of a traditional herbal product (Brazil, 2014), it is recommended that RSD results in the validation of analytical methods should not be higher than 15%. Therefore, the method has been shown to be suitable for the quantification of total flavonoids, expressed as rutin, in *A. indica* leaves. In addition, the robustness tests of the method have shown that the use of ethanol in the preparation of solutions used to dilute extracts for spectrophotometric readings do not cause changes in absorbance as compared to methanol; hence the choice of ethanol, a less toxic solvent, proved to be suitable for the method.

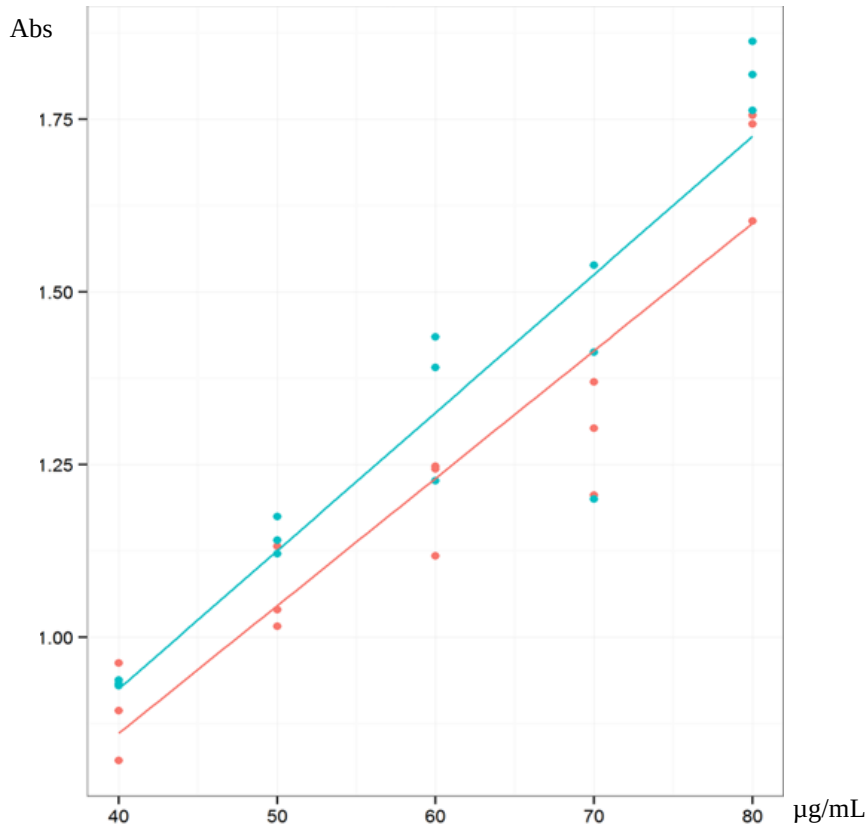
Table 5 - Robustness data for the quantification of total flavonoids, expressed as rutin equivalents, by spectrophotometric method.

	Absorbances (nm)	Mean	RSD (%)
363nm	1.313 1.408 1.370 1.251 1.354 1.431	1.35	4.82
365nm	1.282 1.327 1.216 1.187 1.229 1.144	1.23	5.33
363 and 365nm		1.29	6.95
Methanol	1.181 1.181 1.147 1.129 1.133 1.227	1.16	3.20
Ethanol	1.292 1.267 1.239 1.325 1.253 1.204	1.26	3.33
Methanol and Ethanol		1.21	5.21

Legend: RSD: Relative standard deviation. Source: Authors (2020).

Matrix Effect: Plant extracts are composed of complex matrices; therefore, the effect of matrix components on the analytical response must be determined (Brazil, 2017a). This determination is made by checking the parallelism of the lines, comparing the angular coefficients of the calibration curves constituted with the fortified sample with the analytical standard and the sample without analytical standard at the same linearity concentration levels. The presence of this parallelism indicates the absence of interference from the matrix constituents (Brazil, 2017a). The two calibration curves generated a dispersion diagram shown in Figure 3. The comparison test (F test) of the curves showed no significant difference, at the significance level of 5%, for the equality of the intercept (p=0.98433341), parallelism of the lines (p=0.59499914) and coincidence (p=0.069867601), confirming the absence of interference of matrix compounds in the analytical response.

Figure 3 - Absorbance dispersion diagram (364nm) as a function of flavonoid concentrations (µg/mL), constructed from the sample without rutin standard and from the sample fortified with rutin standard, at the same linearity concentration levels (40 - 80 µg/mL). Blue line represents the curve of the sample WITHOUT rutin standard and red line represents the curve of the sample WITH rutin standard.



Source: Authors (2020).

3.3 Optimization of UAE conditions of total flavonoids, expressed as rutin, for the validated method

After finding that Method 4 was capable of generating linear analytical responses, and that it was precise, accurate, robust for the legal parameters and was satisfactorily selective by proving the absence of matrix effect, the extractive conditions used in the method were investigated in order to optimize them. The results are presented below.

Multiple linear/quadratic regression analysis for the first battery of 15 experiments for UAE optimization was performed based on data from Table 6.

According to ANOVA and multiple linear/quadratic regression using RSM, it was observed that in the first set of experiments, the model used was significant (p=0.000190), not significant for lack of fit (p=0.564186), the coefficient of determination (R^2) was 0.99768 and R^2 adjusted for total flavonoids expressed as rutin was 0.99351. A significant linear effect (p<0.05) of ethanol content (X_1), plant material/solvent ratio (X_2) and time (X_3) was observed. A significant quadratic effect for the variables: ethanol content (X_{12}) and plant material/solvent ratio (X_{22}), and interaction of ethanol content (X_1) and time (X_3) as shown in Table 7.

Table 6 - 1st Experimental Box Behnken (3^3) design and total flavonoid concentrations, expressed as rutin (µg/mL), for the UAE of *A. indica* leaves

Sample	Ethanol content (%p/p)	PMS (g/mL)	Time (min)	Total flavonoid concentrations (µg/mL)
1	30	0.1	30	2519.38
2	70	0.1	30	1670.15
3	30	0.2	30	5051.69
4	70	0.2	30	3624.00
5	30	0.15	10	3531.69
6	30	0.15	50	1805.53
7	70	0.15	10	3633.23
8	70	0.15	50	3122.46
9	50	0.1	10	1907.07
10	50	0.2	10	3984.00
11	50	0.1	50	2217.84
12	50	0.2	50	4710.15
13	50	0.15	30	3390.15
14	50	0.15	30	3537.84
15	50	0.15	30	3534.76

Caption: PMS - Plant material/solvent ratio. UAE - Ultrasound assisted extraction. Source: Authors (2020).

Table 7 - Variance analysis for the first battery of experiments of the polynomial quadratic regression model for ultrasound-assisted extraction of total flavonoids, expressed as rutin equivalents, in *A. indica* leaves.

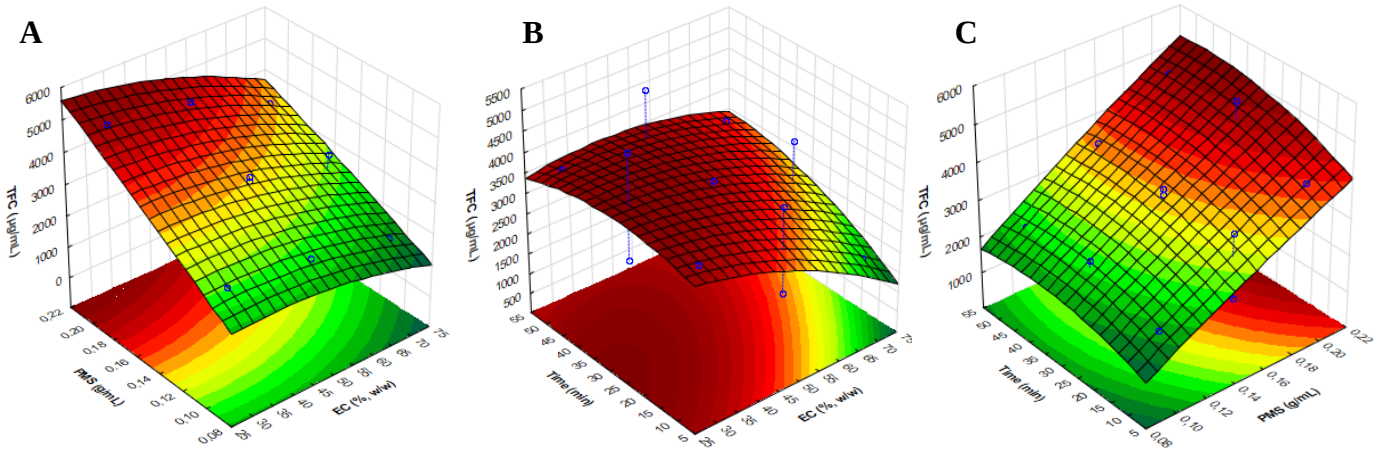
Factor	Sum of squares	degree of freedom	Square average	F	*p*
x_1	2546851	1	2546851	357.5691	0.002785*
x_2	2985298	1	2985298	419.1255	0.002377*
x_3	753614	1	753614	105.8048	0.009319*
x_1^2	189274	1	189274	26.5734	0.035632*
x_2^2	7434	1	7434	1.0438	0.414408
x_3^2	209057	1	209057	29.3509	0.032423*
x_1x_2	83654	1	82654	11.7448	0.075614
x_1x_3	369290	1	369290	51.8470	0.018747*
x_2x_3	43136	1	43136	6.0562	0.132969
Lack of fit	19259	3	6420	0.9013	0.564186
Pure error	14245	2	7123		
Total square sum	14450477	14			

Legend: *x1* – ethanol content (% p/p) linear; *x2* – plant material/solvent ratio (g/mL) linear; *x3*- time (minutes) linear; $x1^2$– ethanol content (% p/p) quadratic; $x2^2$ – plant material/solvent ratio (g/mL) quadratic; $x3^2$ - time (minutes) quadratic; *$p \leq 0.05$. Source: Authors (2020).

The response surface graphs (Figure 4) show the effects of the independent variables on the flavonoid concentrations, expressed as rutin, of the first battery of experiments. Figure 4A shows that the maximum extraction of flavonoids was obtained between the ethanolic graduations of 25 to 40 % (w/w) and the drug/solvent ratio between 0.22 and 0.18 g/mL, and the ethanolic

content variable had a negative linear effect, that is, increasing the ethanolic content the extraction of flavonoids tends to decrease and the variable drug/solvent ratio presented a positive linear effect. The quadratic effect of the parameters was also demonstrated. In Figure 4B, it was observed that the maximum extraction of flavonoids was achieved at an ethanolic content between 25 and 30% (w/w) and extraction time between 20 and 35 minutes. Figure 4C shows that the highest flavonoid concentrations were reached in drug/solvent ratios between 0.2 g/mL and 0.22 g/mL and time around 30 minutes.

Figure 4 - Response surface graphs (A - C) of the first battery of ultrasound-assisted extraction experiments (UAE) of flavonoids, expressed as rutin equivalents, from *A. indica* leaves.



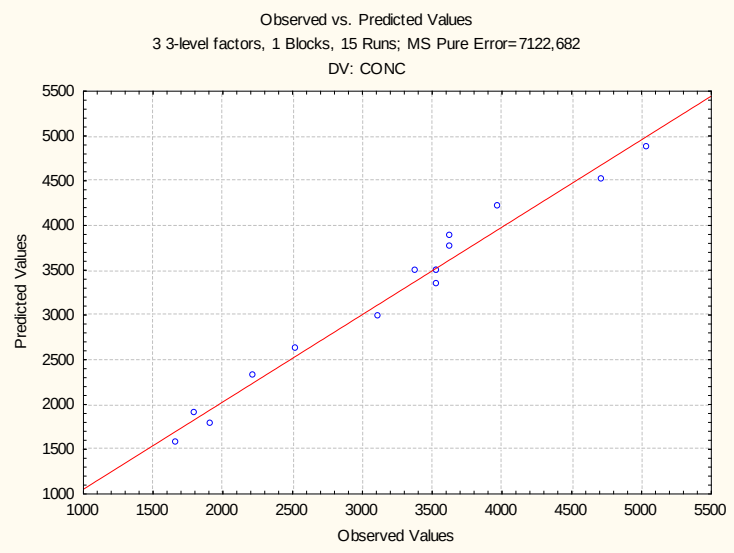
Legend: TFC - total flavonoid concentration, expressed as rutin (µg/mL); PMS – Plant material/solvent ratio (g/mL); EC - ethanol content (%, w/w). Source: Authors (2020).

The results observed in the model of the first battery of experiments are close to the values predicted by the model, as shown in Figure 5, pointing out that the model had a good predictive capacity.

According to response surface data analysis and the general optimization function, the UAE conditions for the optimization of responses were determined. Therefore, the model suggested the following optimal extraction parameters: Ethanol content of 31.37% (p/p), plant material/solvent ratio of 0.199 g/mL and extraction time of 33 minutes. Under these conditions the predicted value for the concentration of total flavonoids, expressed as rutin, is 5052.622 µg/mL.

A second model was carried out to better investigate the results obtained in the first battery of experiments, modifying some parameters according to the results of the first model. Thus, ethanol contents were 10, 30 and 50% (w/w), plant material/solvent ratio of 0.06, 0.1 and 0.2 (g/mL) and time of 20, 30 and 40 minutes. The results of the 15 experiments of the second battery are shown in Table 8.

Figure 5 - Values predicted by the model and values observed for the first set of experiments.



Source: Authors (2020).

Table 8 - 2nd Experimental Box Behnken (3^3) design and total flavonoid concentrations, expressed as rutin, for the UAE of *A. indica* leaves.

Sample	Ethanol content (%w/w)	PMS (g/mL)	Time (min)	Total flavonoid concentrations (µg/mL)
1	10	0.20	30	3821.54
2	50	0.20	30	3683.07
3	10	0.06	30	1529.23
4	50	0.06	30	1636.92
5	10	0.10	20	2275.38
6	50	0.10	20	2029.23
7	10	0.10	40	1852.31
8	50	0.10	40	1975.38
9	30	0.20	20	3913.84
10	30	0.06	20	1690.77
11	30	0.20	40	3321.54
12	30	0.06	40	1852.31
13	30	0.10	30	2290.77
14	30	0.10	30	2267.69
15	30	0.10	30	2690.77

Legend: PMS – Plant material/solvent ratio. Source: Authors (2020).

ANOVA and multiple linear/quadratic regression using the RSM of the second battery of experiments demonstrated that the model employed was significant (p = 0.003211), without lack of fit (p=0.756696), the coefficient of determination (R^2) was 0.98079 and the R^2 adjusted for total flavonoids was 0.94621. However, only one significant linear effect (p 0.05) was observed, which was the plant material/solvent ratio (X_1) on the total flavonoid concentration. No quadratic or interaction effects of the variables was significant, as shown in Table 9.

Table 9 - Variance analysis for the second battery of 15 experiments, for the polynomial quadratic regression model for total flavonoids, expressed as rutin equivalents, in *A. indica* leaves.

Factor	Sum of squares	degree of freedom	Square average	F	*p*
x_1	6792	1	6791.8	0.12002	0.762063
x_2	757102	1	757101.6	13.37924	0.0067286*
x_3	165955	1	165955	2.93270	0.228934
x_1^2	155363	1	155363.4	2.74553	0.239373
x_2^2	2335	1	2334.8	0.04126	0.857828
x_3^2	117257	1	117256.9	2.93270	0.286660
x_1x_2	11734	1	11733.7	0.020735	0.693508
x_1x_3	34083	1	34083	0.60230	0.518908
x_2x_3	126729	1	126729.2	2.23952	0.273193
Lack of fit	72277	3	24092.2	0.42575	0.756696
Pure error	113176	2	56587.8		
Total square sum	9652757	14			

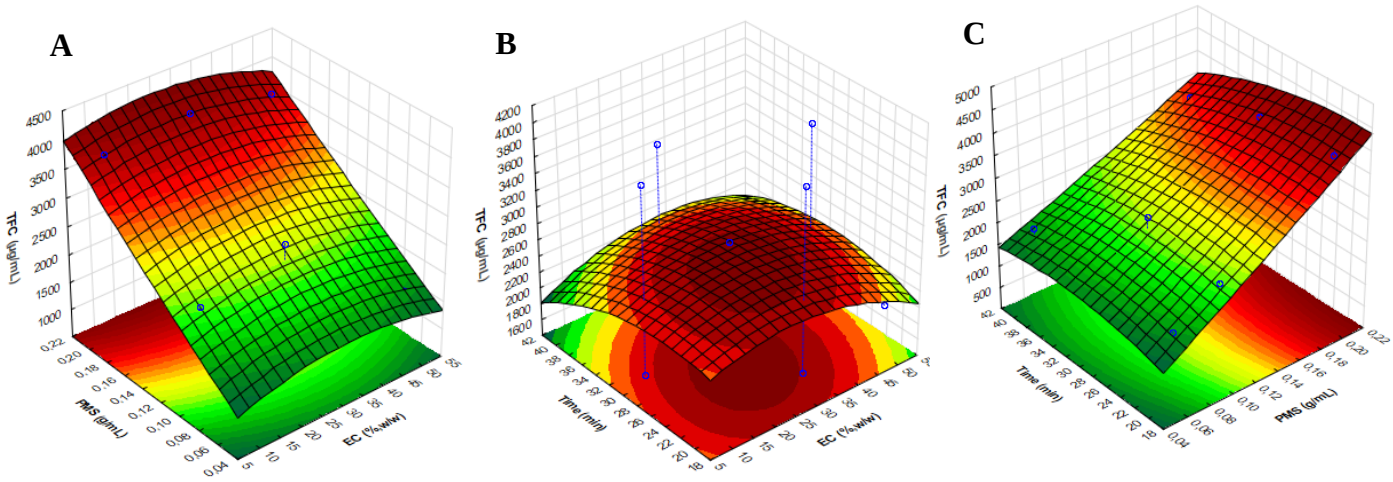
Legend: x_1 – ethanol content (% w/w) linear; x_2 – plant material/solvent ratio (g/mL) linear; x_3- time (minutes) linear; x_1^2– ethanol content (% w/w) quadratic; x_2^2 – plant material/solvent ratio (g/mL) quadratic; x_3^2 - time (minutes) quadratic; *$p \leq 0.05$. Source: Authors (2020).

Although the interactions between the parameters (factors) evaluated were not significant, the analysis of the response surface graphs (Figures 6A, B and C) confirmed that the optimal values predicted in the first set of experiments were adequate. Figure 6A showed that the highest flavonoid concentrations were obtained with a drug/solvent ratio between 0.2 and 0.22. Figure 6B showed that the highest flavonoid concentrations were obtained in ethanolic grades between 15 and 35% and times of 18 to 34 minutes. Figure 6C showed that the variable drug/solvent ratio led to maximum flavonoid concentration between 0.2 and 0.22 g/mL.

The significant effects of UAE parameters on total flavonoid concentrations, expressed as rutin, were best evidenced in the first battery of experiments and confirmed in the second battery. Therefore, the optimal extraction conditions suggested by the first battery were rounded to the following values: ethanol content of 30% (w/w), plant material/solvent ratio of 0.2 g/mL and extraction time of 30 minutes. Under these conditions, the concentration of total flavonoids, expressed as rutin, in *A. indica* leaves and predicted by the model is 5052.622 µg/mL.

In order to validate the conditions suggested in the statistical model, UAE was performed in triplicate in the proposed parameters. The values found for the total flavonoid concentrations were: 4077.94 µg/mL; 5547.17 µg/mL and 5281.66 µg/mL, an average of 4968.92 µg/mL. This value corresponds to 98.34% of the value predicted by the model, confirming that these are the best UAE conditions for this material.

Figure 6 - Response surface graphs (A - C) of the second battery of ultrasound-assisted extraction experiments (UAE) of flavonoids, expressed as rutin equivalents, from *A. indica* leaves.



Legend: TFC - total flavonoid concentration, expressed as rutin (µg/mL); PMS – plant material solvent ratio (g/mL); EC - ethanol content (%, w/w). Source: Authors (2020).

The ethanolic content, which in the present study ranged from 70% (w/w) to 10% (w/w), was significant in the extraction of flavonoids, similar to the results of other studies, such as Zhang et al. (2011) and Huang et al. (2009), who found ethanol levels close to 40% as an ideal condition in flavonoid extraction. For the variable time, we also observed similar values in the literature. Wang et al. (2012) concluded that an approximate time of 30 minutes in ultrasound is required for maximum utilization in flavonoid extraction.

The UAE method is seen as a simple and effective extraction alternative; it is a modern, fast and green technique, suitable for improving the extraction efficiency of bioactive compounds, as well as producing considerable environmental benefits (Wen et al., 2018). In studies by Özgür and Çimen (2018), four techniques for extracting anthocyanins from red rose petals were compared: UAE, reflux extraction, Soxhlet extraction and marinated extraction. The UAE method proved to be the most suitable due to its high efficiency and short extraction time.

Currently the statistical tools of multivariate analysis are used in the optimization of analytical methods, mainly by reducing the number of experiments. Recent studies have successfully demonstrated that RSM can be used to optimize the extraction of bioactive compounds (Lu et al., 2015; Paula et al., 2016; Jang et al., 2017) and, among the most used models associated with RSM, is Box-Behnken design. Research conducted by Zaizhi et al. (2019) tested a combined homogenate and ultrasonic cavitation system (HUCS) for the extraction of flavonoids from the leaves of *Cinnamomum camphora* J. Presi (Lauraceae). Response Surface Methodology and Box-Behnken design were used to optimize the extraction process, and the results suggested that HUCS is a green and efficient method for the extraction of flavonoids from *C. camphora*, as well as other natural plant products.

4. Conclusion

The spectrophotometric analytical method for quantification of total flavonoids, expressed as rutin, in *A. indica* leaves indicates that it best met the criteria of efficiency, simplicity, rapidity and sustainability in this study. It consisted of the following steps, determined after validation and UAE optimisation studies: ultrasound-assisted extraction of 2g of plant material in sufficient quantity to complete 10 mL of 30% ethanol (w/w) at a temperature of 60 ºC, for 30 minutes followed by filtration and reading of the absorbances in a spectrophotometer at 364nm. For the absorbance readings, the extract should be diluted (100 µl) in sufficient quantity to 2 ml of 95% P.A. ethanol/0.02 M acetic acid solution (99:1) using this same solution as blank. Total

flavonoid concentrations (μg/mL) should be estimated using the straight-line equation, generated by linear regression analysis of at least 5 rutin concentration levels, prepared in 95% P.A. ethanol/0.02 M acetic acid solution (99:1) in triplicate. This method was shown to be linear (linear interval - 20 to 80 μg/mL), precise, accurate, robust and selective (absence of matrix effect), and can be used for the quality control of plant drugs and ethanol extracts of *Azadirachta indica* leaves.

Future studies about the adequacy of this method in the quantification of flavonoids in final products, e.g., tablets and capsules of extracts from the *A. indica* leaves are recommended. Adjuvants are often used in the formulation of these preparations, thus, the effects of the adjuvants on the method parameters would be evaluated.

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