Development and validation of analytical methodology for quantification of total flavonoids of *Morus nigra* by ultraviolet-visible absorption spectrophotometry

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*Morus nigra* L. is known in the region of Brazilian São Francisco Valley as "amora-miúra". It is widely used in traditional medicine, mainly in treatment of diabetes, hypercholesterolemia, cardiovascular problems, obesity and gout. This study proposed to develop and validate a fast, simple, efficient and low cost analytical method that could quantify total flavonoid content present in crude ethanolic extract of leaves of *M. nigra* (Mn EtOH). For this, ultraviolet-visible (UV-Vis) absorption spectrophotometry was used as the analytical tool and the complexation with aluminium chloride (AlCl₃). The quantification of flavonoids by this method was based on the complexation of AlCl₃ with the flavonoid nucleus as a selectivity tool made by two procedures, without complexation (NCP) and with complexation (WCP), in order to evaluate the effects of the complexing agent in quantification, proving the efficiency of this technique. In this way, it became necessary to validate the method used to ensure its efficiency. The validation of the method of quantification of total flavonoid content by UV-Vis demonstrated that the method was selective, linear, precise, accurate and robust.

**Key words:** *Morus nigra*, ultraviolet-visible (UV-Vis), Moraceae, total flavonoids, natural products.

**INTRODUCTION**

The use of plants in the treatment of diseases often symbolizes the only therapeutic resource of several people around the world (Maciel et al., 2002). However, many medicinal plants still require more detailed studies.

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In 2009, the Brazilian Ministry of Health published the National List of Medicinal Plants of Interest to the Unified Health System, with the purpose of guiding research on medicinal plants. Among the plants mentioned are those of the genus Morus, which belongs to the family Moraceae and comprises around 24 species and approximately 100 varieties (Oliveira et al., 2013).

*Morus nigra* L. originates in far East, being brought to the region of the São Francisco Valley (Brazil) by Japanese immigrants, adapting well to the climate and soil conditions of the region. In folk medicine, the population uses this species as leaf tea (decoction) for the treatment of diabetes, cholesterol, cardiovascular problems, obesity, and gout. It is popularly known as “amora-miúra”, however, in other regions of this country, it is called as “amora-preta” or "blackberry" (Souza et al., 2015).

Although widely used by traditional communities, there are still few chemical and pharmacological studies. However, some important pharmacological activities have already been proven. Naderi et al. (2004) demonstrated that the compounds present in three different types of extracts obtained from *M. nigra* fruit presented protective activity against the peroxidative damage of biomembranes and biomolecules. The results show that all the three extracts inhibited haemoglobin glycosylation induced by glucose to differing degrees. Anti-inflammatory and antinociceptive activities have also been demonstrated for its fruit extract and were related to the presence of flavonoids such as rutin (RUT), the major compound in the extracts (Chen et al., 2016). Other studies revealed its antinociceptive and anti-inflammatory activities using different parts of this plant species (Souza et al., 2000; Padilha et al., 2009, 2010).

The main bioactive substances present in *M. nigra* are phenolic compounds and flavonoids are the most studied because they stand out due to their wide range of pharmacological activities that have already been demonstrated experimentally in the literature, such as antitumor, antioxidant, antiviral, photoprotective and anti-inflammatory, which gives it significant pharmacological importance (Nestel, 2003; Alencar Filho et al., 2016).

The standardization of the vegetal raw material is essential to guarantee its quality, effectiveness and safety, and to prove the therapeutic effects of the vegetal drug used and consequently of the final product (Fonseca, 2007). In order for an herbal remedy to be effective, the chemical integrity of the active ingredients must be preserved, guaranteeing the pharmacological action. In this way, the plant used requires necessarily previous studies related to the botanical, phytochemical and development of analytical methodologies, highlighting the profile of the chemical constituents of interest (Toledo et al., 2003). Analysis of the content of the main bioactive compounds in raw material of plant origin is an essential step for safety and efficacy.

Several techniques can be used to quantify flavonoids in plant materials. Ultraviolet-visible absorption spectrophotometry (UV-Vis) is used in the analysis of these compounds as it is a simple, fast, low cost, easy to perform and with high reliability of results. This technique is recognized by the advantages of its use, being used mainly in quality control in pharmaceutical industry, which demands speed and reliability of the results (Alves et al., 2010).

Thus, for the method to be recognized, the analytical procedures require an evaluation that estimates its efficiency in the laboratory routine and its capacity to detect and quantify a particular analyte denominated validation (Brito et al., 2003). In Brazil, the National Health Surveillance Agency (ANVISA), through specific resolution (ER 899, 2003), defines that “validation must guarantee, through experimental studies, that the method meets the requirements of the applications analytically, assuring the reliability of the results” (BRASIL, 2003). Thus, it is necessary to carry out tests for the determination of the specificity, linearity, limit of detection, limit of quantification, accuracy, precision and robustness for the analysis.

Considering the medicinal interest in *M. nigra*, the aim of this study was to develop and validate a UV-Vis region absorption spectrophotometry methodology capable of quantifying the total flavonoids present in the leaves of this species.

**MATERIALS AND METHODS**

**Chemicals, glassware and solvents**

All solvents used were of analytical grade: Aluminium chloride (Vetec®), methanol (MeOH, Synth®), and ethanol (EtOH, Synth®). Phox® glassware was used. As standard for flavonoids, hydrated rutin (Sigma-aldrich®); purity ≥94% was used.

**Equipment**

The equipment used were EVEN® analytical balance (model FA-2204B), Cristofoli® ultrasonic bath, ETHIK TECHNOLOGY® stove with air circulation (model 420-STD), SOLAB® knife mill (model SL-31), EVEN® UV-Vis spectrophotometer (model IL-592), and Nova Instruments® UV-Vis Spectrophotometer (Model NI-1600 UV).

**Plant**

The harvest of *M. nigra* leaves were performed at Fazenda Ouro Verde, located in the municipality of Casa Nova-BA (S 9°16′15″; W 40°51′44″). The samples were identified by the botanist José Alves de Siqueira Filho from Centro de Recuperação de Áreas Degradadas da Caatinga-CRAD. A voucher specimen was deposited in the HVASF Federal University of San Francisco Valley Herbarium (voucher number 1764).

**Experimental procedures**

The plant material was subjected to a drying process in an oven with circulating air at 40°C for five days. Subsequently, it was
pulverized in a knife mill, obtaining a dry and pulverized vegetable material (500 g of dry powder). Exhaustive maceration was used to prepare the crude ethanolic extract, using ethanol 95%, for 12 days, performing successive extractions every 72 h. The extractive solution was concentrated in a rotary evaporator under reduced pressure at 50°C, obtaining 57.24 g of the crude ethanolic extract of *M. nigra* (Mn-EtOH).

The stock solution was prepared from Mn-EtOH (10 mg/ml) in MeOH (99.8%) with sonication for 10 min. This solution was diluted for 1.0 mg/ml in methanol concentration for further analysis. The AlCl₃ solution was prepared at 5% (w/v) with EtOH (99.5%). Triplicate tests were performed with 9, 10 and 11 ml of AlCl₃ added to the test solution and in the time of 0, 10, 20 and 30 min to verify the complexation reaction (Marques et al., 2012). The volume of 10 ml was chosen for the next analyses.

The procedures were performed without complexation (NCP) and with complexation (WCP), in order to evaluate the effect of the complexing agent (AlCl₃) on increasing the selectivity for the quantification of the flavonoids. Aliquots were withdrawn in 0.5, 1 and 1.5 ml of the stock solution and completed to a volume of 100 ml with distilled water (NCP) and adding 10 ml of 5% AlCl₃, adjusting the volume to 100 ml with distilled water (WCP), obtaining the test solutions.

**Analysis with NCP**

Initially, the analysis of the three levels of the samples in NCP was performed in triplicate, with the 1.0 mg/ml solution of Mn-EtOH. The analysis was carried out by scanning the UV-Vis spectrophotometer (EVEN®, model IL -592) with glass cuvettes, varying the wavelength from 300 to 500 nm (5 in 5 nm), using distilled water as the blank of the experiment, and the absorbance measured and recorded.

**Analysis with WCP**

The samples were then analyzed in triplicate with 1.0 mg/ml solution of Mn-EtOH containing 10 ml of AlCl₃ (WCP), first to verify the complexation reaction with the flavonoids, at 0, 10, 20 and 30 min. With this, it was possible to observe that there was no significant difference in relation to time, noting that the complexation reaction was immediate. Then, the same analysis of the three levels of the samples was carried out, in triplicate, in a spectrophotometer scan, varying the wavelength at 300 to 500 nm (5 in 5 nm), using 10 ml of AlCl₃ as the blank of the experiment adjusted for the final volume of 100 mL.

**Determination of sample concentration and wavelength for reading**

After the UV scan analysis, the means of each triplicate of the test solution (0.5, 1 and 1.5 ml stock solution in the NCP and WCP), were used to obtain a scan curve of each sample. Then, the wavelength where the highest absorption of the analytes occurred (major peak) was determined (Marques et al., 2012).

**Validation of the analytical method**

The procedures were evaluated according to the norms established by ANVISA, through specific resolution (ER 899, 2003), which defines what should be considered during the validation of analytical methods. The parameters specificity, linearity, precision (repeatability, intermediate precision and reproducibility), limits of detection and quantification, accuracy and robustness were evaluated (Brazil, 2003).

**Linearity**

To measure linearity, three different calibration curves were prepared from seven concentration levels (0.05 to 0.15 mg/ml) at a volume adjusted to 100 ml, determining the equation of the line and the coefficient of determination for NCP and WCP. The calibration curves were obtained from the mean absorbance as a function of concentration.

**Limits of detection (LOD) and quantification (LOQ)**

LOD and LOQ were calculated from the calibration curves for the NCP and WCP, according to the following equations:

\[
\text{LOD} = \text{SDa} \times 3/\text{IC} \\
\text{LOQ} = \text{SDa} \times 10/\text{IC}
\]

Where, SDa is the standard deviation of the intercept with the Y axis, obtained from the average of the three linearity curves and IC is the slope of the line of the respective calibration curves.

**Specificity**

This parameter was determined for the overlap of UV spectra of RUT (200 µg/mL) and Mn-EtOH sample (1 mg/mL) in the treatment with and without complexing with 5% (m/v) AlCl₃ (WCP and NCP) in the range of 300 to 500 nm.

**Precision**

Precision was evaluated by three subparameters, repeatability, intermediate precision and reproducibility. Repeatability was performed by measuring the absorbance in six-fold of each triplicate of the stock solution (10 mg/ml) by a single-day analyst, resulting in a total of 18 determinations. Intermediate precision was performed in the same way, in six replicates of each of the three test solutions and the analyses were done by two analysts on two distinct days, with a total of 18 determinations each. The reproducibility analysis of the method was carried out in another laboratory, by varying the UV-Vis spectrophotometer model (Nova Instruments®, model NI-1600 UV).

**Accuracy**

Accuracy was analyzed by the recovery test, from the addition of a known amount of RUT (200 µg/ml). For the NCP samples, 2 ml of the test solution was added to each cuvette, with which the reading was read and then 100 µl of the rutin solution was added. In the WCP samples, the same procedures were performed and the reading was taken for 5 min, a time considered satisfactory for RUT complexation. The result of the recovery was obtained by Equation 3:

\[
R (%) = \frac{\text{TFC} \times \text{CFE} / \text{CFP} \times 100}{\text{CFP}}
\]

Where, R is the percent recovery, TFC corresponds to the total flavonoid concentration (RUT) added to the Mn-EtOH solution, CFE corresponds to the concentration of the RUT in Mn-EtOH and CFP concentration of the RUT.
Table 1. Linear equation, linearity, limit of detection (LOD) and quantification (LOQ) of rutin and Mn-EtOH (WCP and NCP).

<table>
<thead>
<tr>
<th>Sample</th>
<th>Type</th>
<th>Linear equation</th>
<th>$R^2$</th>
<th>LOD (μg/mL)</th>
<th>LOQ (μg/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rutin</td>
<td>NCP</td>
<td>$y = (-0.02) + 2.14x$</td>
<td>0.983</td>
<td>18.1</td>
<td>60.3</td>
</tr>
<tr>
<td></td>
<td>WCP</td>
<td>$y = (-0.002) + 3.37x$</td>
<td>0.997</td>
<td>2.2</td>
<td>7.4</td>
</tr>
<tr>
<td>Mn-EtOH</td>
<td>NCP</td>
<td>$y = 0.02 + 2.19x$</td>
<td>0.997</td>
<td>3.6</td>
<td>12.1</td>
</tr>
<tr>
<td></td>
<td>WCP</td>
<td>$y = (-0.0328) + 8.162x$</td>
<td>0.997</td>
<td>$12.2 \times 10^3$</td>
<td>$40.8 \times 10^3$</td>
</tr>
</tbody>
</table>

The total flavonoids calculated for Mn-EtOH was 228 ± 0.0037 μg of rutin equivalents/mg of the extract.

**Robustness**

The robustness of the method was performed by varying the manufacturer of the MeOH used, Synth® by Vetec®. The procedure was performed with three stock solutions analyzed in triplicate.

**Statistical analysis**

All analyzes were performed in triplicate and the reliability of the parameters was verified by the relative standard deviation (RSD%). The results were analyzed statistically by analysis of variance (ANOVA); One-Way or Two-Way, when applicable, being considered statistically significant $F$ calculated less than tabulated $F$ ($p > 0.05$). The statistical treatment was obtained by the software OriginPro 8®.

**RESULTS AND DISCUSSION**

Triplicate tests were performed with 9, 10, and 11 ml AlCl$_3$ added to the test solution. After that, statistical treatment of the absorbances was done at 0, 10, 20, and 30 min, and it was verified that there was no significant difference between the results, that is, the complexation reaction happened immediately. The 10 ml volume was chosen to continue the next analyzes, due to the critical $F$ value (6.9427) that was lower than the calculated $F$ (3078.587).

**Linearity**

This parameter was determined by constructing calibration curves of the extracts (RUT and Mn-EtOH) in seven concentration levels (0.05 to 0.15 mg/ml), of determination for NCP and WCP. The results are shown in Table 1. The correlation coefficients ($R^2$) were obtained from linear regression analysis and were higher than 0.98 (WCP) and 0.99 (NCP) for RUT and 0.99 (WCP and NCP) for Mn-EtOH (Table 1).

The Brazilian legislation (Brazil, 2003) allows the methods to develop complex mixtures that are validated with correlation coefficient value, since there is more difficulty in obtaining accurate results when working with this type of sample.

**LOD and LOQ**

The LOD and LOQ results were obtained from three different calibration curves for each standard (RUT) and Mn-EtOH, respectively. The LOD results found were 18.1 and 2.2 μg/mL for NCP and WCP, respectively. The LOQ results found were 60.3 and 7.4 μg/mL for NCP and WCP, respectively. The LOQ results found were 12.1 and 40.8 $\times 10^3$ μg/mL for NCP and WCP, respectively (Table 1).

From these results, it is possible to observe that the method provides spectrophotometric responses with high sensitivity to detect and quantify RUT in extract of *M. nigra*, with expected reliability and without changes of intrinsic factors.

**Specificity**

It was possible to observe in the overlap of UV spectra, RUT that presents maximum absorption in 355 nm, while Mn-EtOH presents maximum absorption band near this wavelength, confirming that in this wavelength it is possible specifically to quantify the standard for flavonoids contained in the sample, even in the presence of other components of the extract. In the spectra of RUT + AlCl$_3$ and Mn-EtOH + AlCl$_3$ samples, a shift of the absorption band was observed for a bigger wavelength and a hyperchromic effect due to the complexation of the flavonoid with Al$^{3+}$ leading to a wavelength energy absorption higher than other phenolic compounds, avoiding interference in absorbance measurements (Fonseca et al., 2007).

**Precision**

Precision was evaluated by three sub parameters: repeatability, intermediate precision and reproducibility both performed by two different analysts using the same equipment on two consecutive days. Repeatability and intermediate precision were assessed. The results are
Table 2. Repeatability parameter.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Mean (µg/mL)</th>
<th>DP</th>
<th>RSD%</th>
</tr>
</thead>
<tbody>
<tr>
<td>NCP</td>
<td>0.276</td>
<td>0.01</td>
<td>3.64</td>
</tr>
<tr>
<td>WCP</td>
<td>0.808</td>
<td>0.008</td>
<td>0.99</td>
</tr>
</tbody>
</table>

Table 3. Intermediate precision analysis.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Analyst</th>
<th>Day 1</th>
<th>Day 2</th>
<th>F cal</th>
<th>F tab</th>
</tr>
</thead>
<tbody>
<tr>
<td>NCP</td>
<td>1</td>
<td>0.276</td>
<td>0.288</td>
<td>F cal 0.00437</td>
<td>F tab 0.99564</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>0.279</td>
<td>0.295</td>
<td></td>
<td></td>
</tr>
<tr>
<td>WCP</td>
<td>1</td>
<td>0.807</td>
<td>0.823</td>
<td>F cal 0.15206</td>
<td></td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>0.819</td>
<td>0.840</td>
<td>F tab 0.71263</td>
<td></td>
</tr>
</tbody>
</table>

Table 4. Reproducibility analysis.

<table>
<thead>
<tr>
<th>Spectrophotometers UV-Vis (model)</th>
<th>Mean (µg/mL) ± RSD%</th>
<th>F cal</th>
<th>F tab</th>
</tr>
</thead>
<tbody>
<tr>
<td>EVEN® (model IL-592)</td>
<td>NCP 0.2802 ± 0.00838</td>
<td>0.3333</td>
<td>0.6667</td>
</tr>
<tr>
<td></td>
<td>WCP 0.825 ± 0.00933</td>
<td>0.3333</td>
<td>0.6667</td>
</tr>
<tr>
<td>Nova Instruments® (model NI-1600 UV)</td>
<td>NCP 0.2916 ± 0.0147</td>
<td>0.3333</td>
<td>0.6667</td>
</tr>
<tr>
<td></td>
<td>WCP 0.804 ± 0.0183</td>
<td>0.3333</td>
<td>0.6667</td>
</tr>
</tbody>
</table>

Table 5. Robustness analysis.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Variable</th>
<th>Mean (µg/mL) ± RSD%</th>
<th>F cal</th>
<th>F tab</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Synth®</td>
<td>NCP 0.2802 ± 0.00838</td>
<td>0.3333</td>
<td>0.6667</td>
</tr>
<tr>
<td></td>
<td></td>
<td>WCP 0.825 ± 0.00933</td>
<td>0.3333</td>
<td>0.6667</td>
</tr>
<tr>
<td></td>
<td>Solvent acquisition</td>
<td>NCP 0.2458 ± 0.0151</td>
<td>0.3333</td>
<td>0.6667</td>
</tr>
<tr>
<td></td>
<td></td>
<td>WCP 0.7504 ± 0.00168</td>
<td>0.3333</td>
<td>0.6667</td>
</tr>
</tbody>
</table>

Accuracy

This parameter was evaluated by the analyte recovery method, adding a known amount of the RUT standard (200 µg/mL) in test solution. The result of the recovery was obtained in percentage by the equation described on validation procedures. The experimental data obtained revealed the average standard recovery of 118.32 ± 1.436%, coefficient of variance 1.21%, attesting that this value is acceptable for natural products. These values show that the analytical method developed is sufficiently accurate.

Robustness

For robustness of the solvent-proof procedures of the solvent acquisition MeOH (99.8%), the obtained data shown in Tables 2 to 4.

The results for repeatability (intra-day) and intermediate precision (inter-day) showed values for RSD% lower than 15%, which is the maximum value pre-acquired by ANVISA for plant material. For the repeatability parameter, RSD% values were 3.64% for NCP and 0.99% for WCP (Table 2).

For intermediate precision, calculated F was lower than the tabulated F (p>0.05), so, no significant statistical difference was observed when the same analyst evaluated the method on different days and when different analysts evaluated on different days (Table 3). For reproducibility parameter, the calculated F was also lower than the tabulated F, inferring that no statistical difference was observed, and therefore the reproducible method. Therefore, the results are reliable and confirm an accuracy in accordance with what is recommended by the Brazilian legislation (Table 4).
show that all the procedures (NCP and WCP) were robust regarding the parameter analyzed, since the calculated F values were lower than the tabulated F (Table 5).

**Conclusion**

In this work, a spectrophotometric method was developed to be used for routine analysis of flavonoids present in ethanolic extract of leaves of *M. nigra*. The reported developed protocol is simple, fast, specific, precise, accurate, robust and inexpensive and thus recommended for quantification and quality analysis of flavonoids in plant leaves.

**CONFLICT OF INTERESTS**

The authors have not declared any conflict of interests.

**REFERENCES**


