Total Flavonoid Determination for the Quality Control of Aqueous Extractives from *Phyllanthus niruri* L.

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SUMMARY. The purpose of this study has been the development of an adequate methodology for the quality control of *Phyllanthus niruri* aqueous extracts. The assay method was tested, with various modifications, to determine its suitability for the quantitative determination of total flavonoids within the crude drug raw material and aqueous extracts. The assay technique for such chemical group is based on the spectrophotometric evaluation of a complex with AlCl₃ after acid hydrolysis of the glycosides. Statistical analyses demonstrated the adequacy for the quantitative analysis of the raw material, but it had limitations for the assay of the aqueous extractive solutions. These limitations could be overcome by using a defined range of raw material amount and a standard aliquot of a stock extract solution.

INTRODUCTION

The aerial parts of *Phyllanthus niruri* L. (Euphorbiaceae) known in Brazilian folk medicine as “quebra-pedra” (stone breaker), are widely used as a tea in the treatment of genitourinary and liver disorders 1,2. Clinical trials in man and animals have demonstrated its efficacy 3. In 1983, the Brazilian Drug Agency (CEME) introduced medicinal plants into its research program policy, looking also for the development of effective pharmaceutical dosage forms with well-defined quality. The availability of suitable assay methods is an important part of the quality control of such drugs and the Brazilian health authorities have now introduced specific regulations in order to assure users of their safety and efficacy 4. Although the active substances of this medicinal plant are not yet known, flavonoids can be used as chemical markers for quality control purposes. The assay of individual flavonoid is, however, complex, time consuming and expensive and the measurement of total flavonoid content is an attractive alternative. This is an official method of the German Pharmacopoeia 5 and Drug Codex 6. It consists of acid hydrolysis of the glycosides and the spectrophotometric determination of the AlCl₃ complex formed 7. Although the method is used for many plant-derived drugs, factors as reaction time and type and concentration of flavonoids can influence the results 8-11 and hence careful standardization of the technique is necessary.

PALABRAS CLAVE: Fitoterápico, Flavonoides totales, *Phyllanthus niruri*,
KEYWORDS: Flavonoid assay, *Phyllanthus niruri*, Phytomedicine,
MATERIAL AND METHODS

Drug and reagents

Aerial parts of *Phyllanthus niruri* L., collected January 1996, were supplied by the CEME Distribution Center of the Campinas University (CPQBA/UNICAMP). The dried and ground material was used in all experiments. All reagents were pro analysis grade.

Preparation of the extractive solutions

Extracts were prepared by decocting 2.5, 5, 7.5 and 10% (w/v) of the raw material in distilled water for 15 minutes.

Total flavonoid assay for drug material

About 0.400 g of the dried and ground drug were treated in a 100 ml round bottom flask with 1.0 ml hexamethylenetetramine 0.5% (w/v), 20.0 ml acetone R and 2.0 ml hydrochloric acid R. The mixture was refluxed on a water bath for 30 min and filtered through a small cotton wool. Plant residue and filter were washed twice with 20.0 ml acetone R and the washings refluxed for 10 min. When the solutions were cool, they were filtered and made up to 100 ml with acetone. Twenty ml of this solution were transferred into a separating funnel, and extracted with 15.0 ml of ethyl acetate R. The extraction was repeated three times, using 10.0 ml of ethyl acetate each time, and the combined organic phases washed twice with 50 ml water and made up to 50 ml with ethyl acetate (Stock Solution SS). A volume of 2.0 ml AlCl₃ 2% (w/v) in ethanol were added to 10.0 ml of SS and the solution was made up to 25.0 ml with methanolic solution of acetic acid 0.5% (v/v) (Probe Solution PS). At the same time 10.0 ml of SS were made up to 25.0 ml with methanol/acetic acid solution (Contrast Solution CS). The absorbance of PS against CS was measured at 420 nm after 30 min.

The result, expressed as percentage of total flavonoids content (TFC), was calculated using the following equation:

\[
TFC = \frac{A \cdot DF}{E \cdot (w - t)}
\]  
(1)

Where: A = measured absorbance; DF = dilution factor; w = plant sample weight; t = loss on drying; \(E_{1\%1cm}\) = specific absorption of the quercetin-aluminum chloride complex (500).

Evaluation of drugsolvent influence on the total flavonoid content for the raw material

Samples of 0.400, 0.755, 1.125, 1.500 and 1.850 g of the drug raw material were treated as described previous for drug material. The total flavonoid content (TFC) was expressed as the average of three determinations.

Repeatability and intermediary precision

The repeatability was evaluated in triplicate on the same day for three samples, while the inter-day precision was assessed for two consecutive days. The data were expressed as the relative standard deviation (RSD %).

Total flavonoid assay for the extractives

About 15.0 g of the extractives, exactly weighted, were transferred to round bottom flasks and 20.0 ml acetone R, 2.0 ml hydrochloric acid R and 1.0 ml of hexamethylenetetramine 0.5% added. The mixture was refluxed on a water bath for 30 min. After cooling, the final volume was made up to 50.0 ml with acetone R (Stock Solution SS). 20.0 ml of SS and 20.0 ml water were treated once with 15.0 ml and three times with 10.0 ml ethyl acetate. The ethyl acetate phases were washed twice with 50 ml water and made up to 50.0 ml (Probe Solution PS). 10.0 ml of PS plus 2.0 ml of AlCl₃ ethanolic solution were made up to 25.0 ml with methanol/acetic acid to produce the test solution (TS). A second 10.0 ml aliquot of PS was diluted to 25.0 ml with acetic acid methanolic solution (Contrast Solution CS). After 30 min the absorbance of TS was read at 420 nm against CS. The same procedure was repeated for 30.0 and 40.0 ml of SS. Total flavonoid content was calculated using equation 2, where w represented the weight of the extractive solution, and the dilution factor DF was adjusted for each SS aliquot volume.

\[
TFC = \frac{A \cdot DF}{E \cdot (w - t)}
\]  
(2)

Wavelength selection

A rutin ethanolic solution was submitted to acid hydrolysis under the conditions described by the German Drug Codex (DAC) 6. The spectrum (200 - 500 nm) of the AlCl₃-complex solution was compared with that of an extractive solution of *Phyllanthus niruri*, prepared by decoction of 7.5% (w/v) of the drug in water for 15 min and treating as described above.

Linearity of the results for the total flavonoid assay for the extractive solution

Samples of each extractive solution were assayed according to the DAC method 6 and the
RESULTS AND DISCUSSION

The spectra of hydrolyzed rutin, treated aqueous extract and raw plant material after reaction with AlCl₃ all have the absorption maximum between 420 and 430 nm, which agrees with the published data for the rutin aglycone-AlCl₃ (quercetin) complex 7.

The similarity of the obtained spectrum could be explained by the presence of flavonoid compounds described in the literature for Phyllanthus niruri such as rutin, astragalin, quercitrin, isoquercitrin 13 and kaempferol 4′-O-α-L-rhamnopyranoside 14, whose aglycones (quercetin and kaempferol, respectively) show λ_max at 428 nm (EtOH + AlCl₃) 15.

Table 1 shows the relationship between the drug sample amount and the total flavonoid content. There is a linear relationship only between the values from 0.400 to 1.125 g of drug. The apparent decrease in flavonoid concentration at higher levels of plant material could be due to saturation of the extraction medium, or to failure of the assay method. Very low amounts of plant material gave absorbance values, which were not in accord with the Lambert-Beer Law. Considering these limitations, the total flavonoid content for the raw material was determined to be 0.80 ± 0.030 g% (w/w).

The intra-sample repeatability test demonstrated that the total flavonoid assay presented a RSD range from 0.24 to 2.35% (Table 2). The between sample repeatability revealed relative standard deviation maximal of 3.74%. These data were considered very satisfactory considering the multiple step technique and the matrix complexity.
The F-test for inter-day precision analysis showed no statistical significant difference ($\alpha = 0.05$) between the total flavonoid content peaks obtained on different days of analysis, confirming acceptable precision.

The observed assay method limitation for the plant drug material guided to the evaluation of its suitability for the quantitative analysis of the aqueous extractive solutions. Following the German Drug Code technique, 15 g of the extract, equivalent to 0.375 g raw material, was initially used. The relationship between the drug concentration in the aqueous extractive solution and the absorbance of the formed flavonoid-AlCl$_3$ complex was apparently linear. However, changing the stock solution volume from 20 to 30 and to 40 ml produced a modification in the curves. The curve parameter analysis (Table 3) showed statistical differences for the slope values of each curve indicating the existence of errors linked to the methodology.

The use of 20 ml did not, apparently, give rise to any constant systematic errors, as demonstrated by the inclusion of the zero value within the confidence limits for intercept. However, the curve for 30 ml of SS suggested a constant systematic error, because the zero value is not included within the confidence interval for the intercept. The curve for 40 ml of SS showed the higher linear correlation ($R^2 = 0.9998$), but the confidence limits analysis for intercept demonstrated the presence of a constant systematic error of 0.0265 absorbance units (Table 4).

The study of the confidence interval behavior for the intercept and slope values for all curves showed only the existence of constant systematic errors, arising from the use of different volumes of SS.

The curves plotting TFC against different amounts of the stock solution aliquots presented statistical distinct slope values, suggesting an abnormal behavior of the assay method. Considering the complexity of the sample matrix, further studies are necessary to determine the origin of these errors.

In the light of these statistical analyses, results from the method employing 20 ml SS were used to determine the total flavonoid content of the four aqueous extractive solutions from $P.$ niruri according to equation 2. The data for the total flavonoid content of the four extractive solutions (Table 5), demonstrated few and random differences from calculated values, assuming an intercept value (a) of zero and also the suitability of this assay method for the studied drug concentration range.

**CONCLUSIONS**

This study has shown that the total flavonoid content assay (TFC) recommended by the Ger-
Drug Code (DAC)\(^6\) can be used for flavonoid assay in Phyllanthus niruri raw material or aqueous extractives. The method showed selectivity, precision \((RSD < 2.5\%)\) and reproducibility \((RSD < 4.0\%)\). However, a deviation from linearity was observed when the amount of raw material was increased. Probably it occurs due to low concentration of aluminum chloride for total reaction at higher amount of sample. Therefore, the method was suitable for total flavonoid determination only in the range from 0.400 to 1.250 g of raw material. In these conditions, the total flavonoid content was 0.80 ± 0.0305 g\(\text{/(w/w)}\). As was observed to raw material, the technique could be also performed to aqueous extractives with high linearity \((R^2 = 0.9982)\) in concentrations from 2,5 to 10\%(w/v).

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### Table 5. Total flavonoid content (TFC) variation as a function of the extractive solution concentration. \(DF = 312.5\) (Equation 2); \(\text{TFC}_{\text{exp}}\) = experimental total flavonoid content; \(\text{TFC}_{\text{calc}}\) = calculated total flavonoid content; \(\Delta\%)_{\text{TFC}_{\text{calc}}} = \text{difference between estimated and experimental TFC} (\%)\.

<table>
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<th>Drug conc. ((\text{w/v}))</th>
<th>(\Delta\text{Conc.})</th>
<th>(\text{TFC (g%)}) _(experimental)</th>
<th>(\text{TFC (g%)}) _(calculated)</th>
<th>(\text{TFC}<em>{\text{exp}}-\text{TFC}</em>{\text{calc}})</th>
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<tr>
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<td>0.0089</td>
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**REFERENCES**
