Lat. Am. J. Pharm. **22** (3): 203-7 (2003)
Recibido el 11 de diciembre de 2002
Aceptado el 17 de abril de 2003

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

Luiz Alberto Lira SOARES [1]*, Valquíria Link BASSANI [2],
George GONZÁLEZ ORTEGA [2] & Pedro Ros PETROVICK [2]

[1] *PPGCF - Departamento de Farmácia - Universidade Federal do Rio Grande do Norte, Avenida General Cordeiro de Farias, s/n, 59010-180, Natal, RN, Brasil.*
[2] *PPGCF - Faculdade de Farmácia - Universidade Federal do Rio Grande do Sul, Avenida Ipiranga 2752, 90610-000, Porto Alegre, RS, Brasil.*

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 $AlCl_3$ 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.

RESUMEN "Determinación de flavonoides totales para el control de calidad de extractivos acuosos de *Phyllantus niruri* L." El objetivo de este estudio ha sido el desarrollo de una metodología de control de calidad tecnológico para la determinación de flavonoides en extractos acuosos de *Phyllanthus niruri.* El método de análisis se evaluó, con varias modificaciones, para determinar su adecuabilidad para la determinación cuantitativa de flavonoides totales en la materia prima vegetal y en sus extractos acuosos. El método de análisis para este grupo químico se basa en una evaluación espectrofotométrica de un complejo formado con cloruro de aluminio tras la hidrólisis ácida de los glucósidos. Los análisis estadísticos han demostrado que el método es adecuado para el análisis cuantitativo de la materia prima vegetal, pero presenta limitaciones para la evaluación de las soluciones extractivas acuosas. Dichas limitaciones pueden ser solucionadas utilizando un rango definido de cantidad de materia prima vegetal y una alícuota estándar de la solución extractiva obtenida.

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 $AlCl_3$ 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,

* Author to whom correspondence should be addressed. E-mail: phtech@uol.com.br

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 [6]

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 $AlCl_3$ 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), calculated as quercetin, using the equation 1, represents the average of six determinations.

$$TFC = \frac{A \cdot DF}{E^{\,1cm}_{1\%} \cdot (w - t)} \qquad (1)$$

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

Evaluation of drug:solvent 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 $AlCl_3$ 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.

$$TFC = \frac{A \cdot DF}{E^{\,1cm}_{1\%} \cdot w} \qquad (2)$$

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 $AlCl_3$-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

total flavonoid content calculated using equation 2. Results are the means of three determinations.

A regression curve was estimated by the last squares method and the confidence limits were calculated. The confidence limits for intercept and slope were calculated using equations 3 and 4 [12]:

$$L_a = a \pm (t_{\alpha\,[df]}\, s_y) \quad (3)$$

$$L_b = b \pm (t_{\alpha\,[df]}\, s_y) \quad (4)$$

Where: L_a = confidence limits for intercept value; L_b = confidence limits for slope value; a = intercept for the regression curve; b = slope for the regression curve; s_y = standard deviation; $t_{\alpha\,[df]}$ = table value for "t", $\alpha = 0.05$ and df = n-1

RESULTS AND DISCUSSION

The spectra of hydrolyzed rutin, treated aqueous extract and raw plant material after reaction with $AlCl_3$ all have the absorption maximum between 420 and 430 nm, which agrees with the published data for the rutin aglycone-$AlCl_3$ (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 + $AlCl_3$) [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 ± 0.0305 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 reveled relative standard deviation maximal of 3.74%. These data were considered very satisfactory considering the multiple step technique and the matrix complexity.

Amount (g)	Δ Conc.	TFC (g%) experimental	TFC (g%) calculated	TFC_{exp} - TFC_{calc}	$\Delta\%_{TFCcalc}$
0.400	-	0.819	0.819	-	-
0.750	1.8750	0.826	0.820	0.006	0.67
1.125	2.8125	0.790	0.820	0.030	3.75
1.500	3.7500	0.706	0.819	0.114	16.14
1.850	4.6250	0.677	0.820	0.143	21.06

Table 1. Total flavonoid content (TFC) as a function of the drug sample amount. DF = 625 (Equation 1); TFC_{exp} = experimental total flavonoid content; TFC_{calc} = calculated total flavonoid content; (%TFC_{calc} = difference between experimental and estimated TFT (%).

	Day 1			Day 2		
	Sample 1	Sample 2	Sample 3	Sample 4	Sample 5	Sample 6
1	0,8188	0,8340	0,7862	0,8015	0,7828	0,7702
2	0,8223	0,8685	0,7862	0,7981	0,7828	0,7685
3	0,8188	0,8340	0,7689	0,7946	0,7724	0,7479
Mean	0,8200	0,8455	0,7805	0,7981	0,7793	0,7628
sd	0,002	0,0199	0,0100	0,0035	0,0060	0,0130
RSD %	0,24	2,35	1,28	0,43	0,77	1,71
Repeatability	0,8153 ± 0,0305 (3,742%)			0,7801 ± 0,0107 (2,177%)		

Table 2. Repeatability and intermediate precision data for the total flavonoid assay in *P. niruri* raw material.

The F-test for inter-day precision analysis showed no statistical significant difference (α = 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 [6], 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-Al-Cl_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-

Statistical Data	Aliquot volume (ml) 20	30	40
a ± sd	0.0268 ± 0.0040	0.037 ± 0.0111	0.0265 ± 0.0029
Conf. Limt. (a)	0.0332 to - 0.0204	0.057 to 0.0166	0.032 to 0.0211
b ± sd	0.0196 ± 0.0006	0.0312 ± 0.00162	0.0485 ± 0.00043
Conf. Limt. (b)	0.0206 to 0.0187	0.034 to 0.028	0.049 to 0.047
R^2	0.9982	0.9946	0.9998

Table 3. Statistical analysis for response curves of the flavonoid-$AlCl_3$ complex after the use various volumes of SS, assuming a first order equation (y = a + bx).

X	y (calculated)	y' (experimental)	y" without error (a = 0)	difference (%)
2.5	0.148	0.148	0.121	18.24
5.0	0.269	0.266	0.243	9.85
7.5	0.390	0.391	0.364	6.7
10.0	0.511	0.504	0.485	5.3

Table 4. Constant systematic errors for the curve with 40 ml SS. Where: x = drug conc. (%, w/v); y = absorbance; a = 0,0265; b = 0,0485.

Drug conc. (w/v)	Δ Conc.	TFC (g%) experimental	TFC (g%) calculated	TFC_{exp}-TFC_{calc}	$\Delta\ \%_{TFCcalc}$
2.5	1	0.0032	0.0030	0.0002	- 6.66
5.0	2	0.0059	0.0059	0	0
7.5	3	0.0083	0.0089	0.0007	+ 7.95
10.0	4	0.0123	0.0119	0.0004	- 3.39

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

man 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% (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).

Acknowledgement. This work was supported by CEME/Ministry of Health/Brazil and CNPq.

REFERENCES

1. Santos, D.R. (1990) *Chá de quebra-pedra (Phyllanthus niruri) na litíase urinária em humanos e em ratos.* Escola Paulista de Medicina, São Paulo. M.Sc. Dissertation.
2. Venkateswaran P.S., I. Millman & B.S. Blumberg (1987) *Proc. Natl. Acad. Sci. USA* **84**: 274-8.
3. Simões C.M.O., L.A. Mentz, E.P. Schenkel, B.E. Irgang, & J.R. Stehmann (1998) *Plantas da medicina popular do Rio Grande do Sul.* 5th Ed. Porto Alegre: Editora da Universidade/ UFRGS.
4. Brasil, Ministério da Saúde, Secretaria de Vigilância Sanitária. (2000) Resolução n. 17 de 2000, *Diário Oficial da União.*
5. Hartke K., & E. Mutschler (1986) *Kommentar des deutschen Arzneibuches* - 9. Ausgabe. Stuttgart, Wissenschaftliche, Bd.**3**: 2944-7.
6. Bundesvereinigung Deutscher Apothekerverbände (Hrsg.) (1986) Deutscher Arzneimittel - Codex. Govi, Frankfurt, Deutscher Apotheker, Stuttgart, Bd. **2**: 4-5, Lief. 88.
7. Hartke K, H. Hartke, E. Mutschler, E. Rücke & M. Wichtl (1991) Deutsches Arzneibuch 10. Aufgabe - Kommentar, Wissenschaftliche, Stuttgart, GOVI, Frankfurt. Bd. II/1, Monography B39 (Birkenblätter).
8. Schmidt P.C. & G. González Ortega (1993) *Dtsch. Apoth. Ztg.* **133**: 4457-66.
9. Soares, L.A.L., K.C.B. De Souza, V.L. Bassani, G. González Ortega & P.R. Petrovick (1997) *"Quantitative evaluation of glycosilated flavonoids in the crude drugs Phyllanthus niruiri L. (Euphorbiaceae) and Passiflora edulis (Passifloraceae)"*. In: II World Congress on medicinal and aromatic Plants for Human Welfare, 2, 1997, Mendoza, *Abstracts*... P;460, Mendoza: Argentina.
10. Petry, R.D., K.C.B. De Souza, V.L. Bassani, P.R. Petrovick & G. González Ortega (1998) *Rev. Bras. Farm*. **79**: 7-10.
11. Petry R.D., G. González Ortega & W.B. Silva (2001) *Pharmazie* **56**: 465-70.
12. Funck W., V. Dammann & G. Donnevert (1992) *Qualitätssicherung in der analytischen Chemie.* Weinheim: VHC.
13. Nara T.K., E. Gleye, E. Lavergne de Cerval & E. Stanislas (1977) *Plantes Méd. Phytothér.* **2**: 82-6.
14. Chauan J.S., S. Mohamad & S.K. Srivastava (1977) *Planta Med.* **32**:217-22.
15. Mabry T.J., K.R. Markham & M.B. Thomas (1970) Reagents and Procedures for the Ultraviolet Spectral Analysis of Flavonoids. In: *The Systematic Identification of Flavonoids.* Berlin: Springer.