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Original Article

Total phytosterol content in drug materials and extracts from roots of *Acanthospermum hispidum* by UV-VIS spectrophotometry

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ABSTRACT

Acanthospermum hispidum DC., Asteraceae, is widely used in folk medicine in Brazil to treat respiratory diseases; this biological property has been attributed to its phytosterol content. This study evaluated the spectrophotometric assay method to quantify the total phytosterol content in raw materials and extracts from roots of *A. hispidum*. The procedure was based on the quantification at 625 nm after the Liebermann-Burchard reaction. The method was evaluated for linearity, repeatability, intermediate precision, accuracy and robustness. The date indicated that the procedure is a valid analytical tool for materials and herbal derivatives from *A. hispidum*.

Introduction

The plant *Acanthospermum hispidum* DC., Asteraceae, has aroused interest in studies of their biological activity, based on its popular use in Brazil and around the world (Fleischer et al., 2003; Maciel et al., 1997; Noumi and Dibakto, 2000). In Northeastern Brazil, the species has been traditionally used to treat asthma and bronchitis, and the an expectorant (Agra et al., 2008; Agra et al., 2007). Trials on the use of a syrup made from *A. hispidum* in herbal medicine in the public-health service have shown promising results in treating patients with bronchial asthma (Araujo et al., 2008; Maciel et al., 1997). Significant considering the content of phytosterols in this herbal drug and the published reports on the biological properties of these substances, it is believed that the phytosterols contained in *A. hispidum* are related to its therapeutic potential (Araujo et al., 2008). In spite of the pharmacological potential and strong interest in the scientific community, the species does not appear in official monographs and the specifications and/or analytical procedures have yet been developed for quality

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control of substances derived from this plant. Therefore, the development and validation of an analytical method applied to the herbal drug extracted from *A. hispidum* are important prerequisites for the establishment of quality parameters to assure its quality, therapeutic efficacy and safety (Anvisa, 2010; Araujo et al., 2008).

Several analytical methods have been reported for the quantification of phytosterols in herbal material, mainly by HPLC (coupled to a UV and/or MS detector) and/or GC (coupled to an FID and/or MS detector) (Abidi, 2001; Lagarda et al., 2006; Lembcke et al., 2005; Liu and Ruan, 2013). In spite of the selectivity and sensitivity of these methods, the quantification of phytosterols by HPLC or CG shows some limitations, such as detection (either by a UV and/or DAD detector), high operational cost (MS detector), or the need for several steps in the sample preparation or derivatization (Burkard et al., 2004; Kuksis, 2001).

Additionally, the quantification of a set of isolated compounds or compounds in biological matrices such as herbal materials by the use of selective procedures is a controversial point in analysis and quality control. The pharmacological activity of herbal drugs or phytopharmaceuticals is a result of the combined effect of a group of substances, unlike synthetic or purified drugs (Marques et al., 2012).

On the other hand, the analytical methodologies using spectrophotometry UV/VIS are the most commonly used tools for the control of plant based materials in chemical quality due to its simplicity andlow implementation cost reproducibility (Fernandes et al., 2012; Komarova et al., 2009; Marques et al., 2013; Silva et al., 2009).

Several colorimetric assays have been proposed for the identification and quantification of the derivates of sterols (Bartos and Pesez, 1976; Kumar et al., 2011). The reaction described in 1885 by Liebermann and then applied to the analysis of cholesterol by Burchard colorimetric procedure is the most widely foranalysis of sterol (Artiss and Zak, 2000; Burke et al., 1974; Liebermann, 1885). Originally proposed for clinical determination of cholesterols, the Liebermann-Burchard reaction has also been used with satisfactory results for qualitative-quantitative analysis of sterols from matrices of animal and plant origin (Xiong et al., 2002).

In view of the importance of phytosterols to several biological properties of drug materials, this study evaluated the performance of the spectrophotometric assay after application of the Liebermann-Buchard reaction, to quantify the total phytosterol content in the herbal drug and hydroethanol extract from roots of *A. hispidum*.

Material and methods

Plant material

The roots of *Acanthospermum hispidum* DC., Asteraceae, were collected in Olinda, Pernambuco, Brazil. The raw materials was identified by Dr. Evani L. Araújo, and a voucher specimen was deposited at the Herbarium Dárdano de Andrade Lima at the Agronomic Institute of Pernambuco, Recife, Penambuco, under registration number 73350.

Reagents and reference substance

All solvents used were analytical grade: chloroform (FMaia® and Quimex®), acetic anhydride PA (FMaia®), sulfuric acid (FMaia®), and ethanol (Cinética®). β-sitosterol (Sigma-Aldrich®) was used to the standard.

Liebermann-Burchard Reagent (LB)

A volume of 50 ml of acetic anhydride was transferred to an amber glass vial and kept in an ice bath. After 30 min, 5 ml of sulfuric acid was added carefully to acetic anhydride (Kenny, 1952).

Hydroethanolic extract

The hydroethanolic extract was prepared by maceration for seven days, using ethanol 70% (v/v) solvent and the drug to:solvent proportion of 2:10 (w/v).

Method development

The specifications for sample preparation, analysis of the experimental conditions, as well as the spectrophotometric adjustments are described in the following articles.

Preparation of samples for quantitative analysis

General procedure for phytosterol content in herbal drugs

The herbal drug was prepared by reflux, using chloroform as solvent. The extraction was performed in the round-bottomed flask containing 6.25 g of drug equipment and 30 ml of chloroform for 30 min. The extract was cooled to room temperature (25°C), filtered on cotton, and the residue (cotton and plant materials) re-extracted twice, using 30 ml of chloroform for 15 min. The filtered fractions were collected in the extract solution and dried under reduced pressure at 40°C. The residue was resuspended in 20 ml of chloroform, and the volume adjusted to 50 ml with the same solvent (SM). Aliquots from SM were transferred to 10 ml volumetric flasks and 2 ml of LB reagent was added. The volume was adjusted with chloroform. The absorptions were measured in the UV/Vis spectrophotometer (Evolution 60S, Thermo Scientific®, Germany), 5 min after addition of the LB reagent. Chloroform was used as the blank.

General procedure for phytosterol content in hydroethanolic extract

Hydroethanolic extract (100 ml) was dried under reduced pressure at 40°C. The residue was resuspended in 20 ml of chloroform and the volume adjusted to 50 ml with the same solvent (SM). SM aliquots were transferred to 10 ml volumetric flasks and 2 ml of LB reagent was added. The volume was adjusted with chloroform. The absorptions were measured in a spectrophotometer UV/Vis (Evolution 60S, Thermo Scientific®, Germany), 5 min after the addition of the reagent LB. The chloroform was used as a blank.

Wavelength selection and concentration of the sample

Aliquots of the samples were analyzed in a scanning spectrophotometer in the region 400-900 nm, 5 min after addition of the reagent, and the chloroform measured using the blank. The spectra were used to identify the most appropriate dilutions and determine the wavelength of maximum absorption.

Reaction time

The influence of the reaction time on the responses of the method is both the samples and for the standard (β-sitosterol) was studied by performing a kinetic reaction. For this purpose, after addition of the reagent, the absorbance was measured every 5 min for 1 h.

Liebermann-Burchard reagent optimization

The samples were prepared as described above, and transferred to 10 ml volumetric flasks. Different aliquots of reagent LB (1, 2 and 3 ml) were added and the volume was adjusted with chloroform. After 5 min, the absorbances were measured in the UV-Vis spectrophotometer, using chloroform to the blank.

Total phytosterol content

The phytosterol content (TPC) was calculated as β-sitosterol (g%) using the photometric standard equation to calculate steroids proposed by Kim and Goldberg (1969):

$$TPC = C_s \cdot \frac{A_a}{A_s}$$

Where: C_s= Standard Concentration; A_a = Absorbance of the sample; A_s =Absorbance of the standard.

Method validation

The method was validated using parameters prescribed by ICH guidelines and the Brazilian validation guide, including linearity, limit of sensitivities, selectivity, precision, accuracy, recovery and robustness (Anvisa, 2003; ICH, 2005). Statistical analyzes were performed using linear regression, ANOVA and Student's t-test, for using the software Excel (Microsoft Corporation, USA).

Specificity

The specificity of the method was demonstrated by the overlapping of the standard solution (β-sitosterol) and the samples containing roots of *A. hispidum* obtained in the range of 400-900 nm.

Standard calibration curve

The calibration curves were evaluated by analyzing three authentic curves, constructed with the β-sitosterol standard solution at five concentration levels (0.02-0.10 mg/ml). The results were analyzed by linear regression using the least squares method, in order to define the coefficient of determination (R2).

Linearity

The extract from roots of *A. hispidum* was filtered through filter paper and diluted with chloroform. The calibration curve was calculated by linear regression, and the results represented the mean of three curves obtained by three measurements of each concentration. The date were calculated by linear regression using the least squares method, in order to define the coefficient of determination (R2).

Limits of detection (LOD) and quantification (LOQ)

The limits of detection (Lembcke, 2005) and quantification (LOQ) were calculated in mg/ml according to the following equations: DL = DPa × 3/IC and QL = DPa × 10/IC, where DPa and the standard deviation of the y-intercept, obtained from three linear curves; and IC is the mean angular coefficient (slope of the line) of the respective curves.

Precision

Precision was evaluated by repeatability. Six individual determinations for the samples at a concentration of 100% for analysis were examined for repeatability. Intermediate precision was estimated from measurements made by two analysts on two consecutive days, using samples prepared under the same conditions.

Accuracy

The recovery was determined by addition of known increasing amounts of the standard solution of β-sitosterol (0.02 to 0.06 mg/ml) to samples at 100% of the concentration analysis. The recovery values were expressed the percentages for the ratio of the total phytosterol contents experimentally determined and their theoretical concentrations. Each sample was tested three times, and the amount recovered was calculated.

Robustness

The robustness was preliminarily estimated by using different solvent from suppliers, and several wavelengths for the analysis.

Results and discussion

Method development

Wavelength selection

In the presence of the Liebermann-Burchard reagent ($HOAc/H_2SO_4$), the phytosterols are protonated, followed by dehydration with loss of H_2O, which forms the carbonium ion of 3,5-cholestadiene. This process is the first step in the colorimetric reaction of LB. Next, the blue color is formed by

an oxidation reaction, from pentacyclic cations (Brieskorn and Hofmann, 1964; Sorensen, 1965; Xiong et al., 2007; Yoder and Thomas, 1954). The maximum observed for the blue oxidation product occurs at 625 nm. Two additional maxima can be observed at 389 nm, due to a tetracyclic cation with the benzene ring (Deno et al., 1964); and at 410 nm from aromatic sulfonic acids, after being rearranged (Burke et al., 1974; Velapoldi et al., 1974). For the samples and standard (Fig. 1), the appropriate wavelength for the analysis was 625 nm. This wavelength accords with the literature, and is more specific for the reaction product from LB-phytosterols.

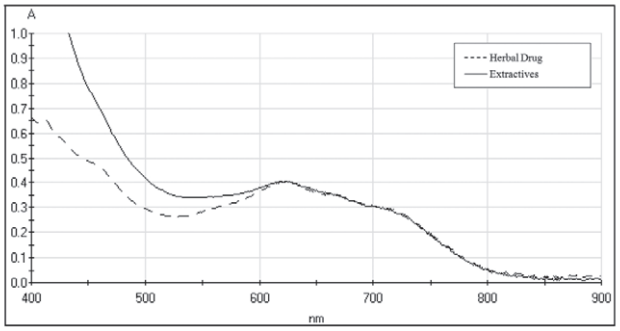

Fig. 1 - Spectrum for the Liebermann-Burchard reaction product (400-900 nm): herbal materials and extracts from roots of *Acanthospermum hispidum*.

Reaction time

According to the reaction-time kinetics for the samples and the standard, maximum absorbance occurs 5 min after the addition of the reagent (Fig. 2). The data also confirm the accelerated behavior of the reaction as described by (Moore and Baumann, 1952), possibly due to conversion of acetate derivatives of the steroids after the reaction with LB reagent (Xiong et al., 2007); as well as the instability of the reaction product, resulting in a decrease in the absorbance read after the initial 5 min. On the other hand, the high reproducibility of the method ensured that if the determination was made exactly 5 min after addition of the reagent, the response of the method was not significantly affected.

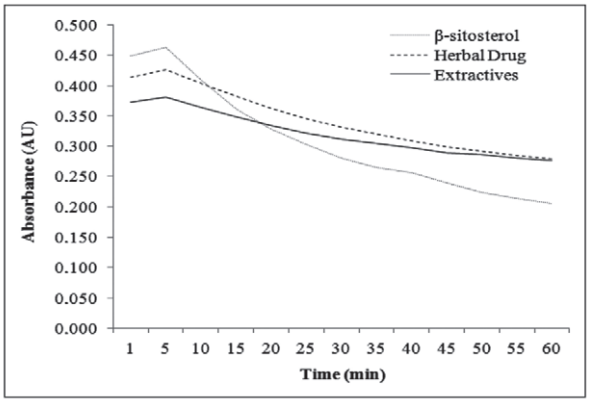

Fig. 2 - Influence of reaction time on the response of the method (absorbance) for the kinetics of the reaction time for samples and standards.

Liebermann-Burchard reagent optimization

The addition of excess acid by increasing the amount of Liebermann-Burchard reagent resulted in decreased overall phytosterol content. This was observed after 2 ml of the Liebermann-Burchard reagent was added to the samples (Fig. 3)

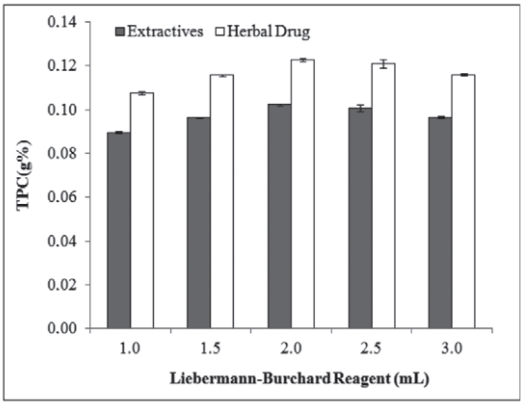

Fig. 3 - Influence of the Liebermann-Burchard reagent on total phytosterol content (TPC g%) calculated the standard (β-sitosterol) for both the drug and herbal extracts from roots of *Acanthospermum hispidum*.

Validation

Specificity

The specificity of spectrophotometric methods plays an important role in the analyses of complex matrices such as drugs and herbal derivatives. Interference is the main difficulty in developing reliable procedures. Comparative evaluation of the experimental spectra provides the necessary information about similarities between the standard solutions and the samples. Fig. 4 shows the spectra for the standard β-sitosterol solution and the drug and herbal extracts from *A. hispidum*. The spectrum was obtained after the reaction with the reagent Liebermann-Buchard , and all solutions showed very similar behavior, with a maximum at 625 nm.

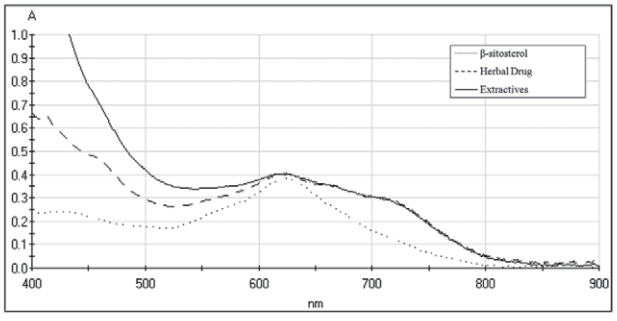

Fig. 4 - Spectrum for the Liebermann-Burchard reaction product (400-900 nm): reference solution (β-sitosterol), herbal materials and extracts from roots of *Acanthospermum hispidum*.

Calibration curves, linearity, detection and quantification limits
Calibration curves were evaluated after regression analysis, and linearity was estimated by the coefficients of determination (R2) for concentrations ranging from 80 to 120% of the working concentration. The date presented in Table 1 suggest that for the standard curves and both samples (herbal materials and extracts from *A. hispidum*) were linear; the R2 values were 0.99 higher than those prescribed by the Brazilian Agency (Anvisa, 2003). Thus, more than 99% of the experimental variability could be explained by the linear models, which confirms the satisfactory relationship between the analyte concentrations and the spectrophotometric responses. Regarding the detection and quantification limits (LOD and LOQ), the spectrophotometric procedure was sensitive for the detection and quantification of phytosterols in full both the herbal materials and the extract, with no interference from the important instrumental technique.

Table 1
Calibration data for the standard (β-sitosterol) and the drug and herbal extracts from roots of *Acanthospermum hispidum*.

Sample	Model Coefficients	R2	Range (mg/ml)	LOD (g%)	LOQ (g%)
β-sitosterol	a = 6.3117 b = 0.0124	0.9981	0.02-0.10	-	-
Herbal drug	a = 0.0078 b = 0.0022	0.9975	37.0-87.5	0,0086	0,0285
Extractives	a = 0.0817 b = 0.0199	0.9918	3.08-9.24	0,0069	0,0232

Precision
The precision of the analytical procedures was assayed at two levels: repeatability and intermediate precision. The method showed low relative standard deviations (RSD%) at the two levels for both samples (drug and herbal extracts). Thus, in the first level (repeatability) the maximum relative standard deviation was 2.99% (Table 2). There was no statistical difference to the second level (intermediate precision), although the method has been assessed at different days (Table 3). Therefore, the method was considered precise according to the national statutory requirements (Anvisa, 2003).

Table 2
Repeatability test: total phytosterol content (TPC g%) for the drug and herbal extracts from roots of *Acanthospermum hispidum*.

Sample	TPC (g%) (mean ± sd; RSD%)
Herbal drug	0.146 ± 0,014 (2.99)
Extractives	0.096 ± 0.001 (1.41)

Table 3
Intermediate precision test: total phytosterol content (TPC g%) for the drug and herbal extracts from roots of *Acanthospermum hispidum*.

	Day 1		Day 2	
	Drug material	Extractives	Drug material	Extractives
Analyst 1	0.146 ± 0.014 (2.98)	0.099 ± 0.001 (1.08)	0.145 ± 0.018 (3.85)	0.099 ± 0.001 (1.48)
Analyst 2	0.142 ± 0.017 (3.75)	0.098 ± 0.001 (1.36)	0.143 ± 0.009 (2.04)	0.102 ± 0.001 (1.14)

Accuracy
The accuracy and recovery of an analytical method expresses the nature of a complex matrix such as plant-based materials. Several sources of interference or variations, such as extraction conditions and extraction (partial degradation) chromophores similar reagents etc. can improve or decrease the analytical response, leading to incorrect results. Accurate procedures must be free from interference, and the method responses should be due to the analyte concentration. In order to determine the accuracy for both samples (drug and herbal extracts), they were spiked with the standard (0.02 to 0.06 mg/ml) and the parameter was calculated from the recovery of the total phytosterol content. The data showed recoveries from 98.5 to 100.38% for the herbal drug, and from 97.51 to 98.99% for the extracts from roots of *A. hispidum* (Table 4). In both cases, the recovery levels higher than 95% that confirm the proposed quantitative procedures are in accordance with statutory requirements, and provide reliable results.

Table 4
Accuracy test: recovery (%) for the drug and herbal extracts from roots of *Acanthospermum hispidum*.

Sample	Herbal Drug	Extractives
	Accuracy (%)	Accuracy (%)
Sample 1	100.39 ± 1.87 (1.87)	97.52 ± 1.26 (1.29)
Sample 2	100.18 ± 3.41 (3.40)	99.74 ± 0.83 (0.83)
Sample 3	98.85 ± 3.99 (4.04)	99.00 ± 1.55 (1.56)

Robustness
To assess the robustness, we used small and deliberate variations in analytical methodology because the objective of this study was to develop and standardize an analytical method, its validity was tested with different sources of variation, including the solvent manufacturer (Quimex® and Fmaia®) and the wavelength (623 to 627 nm). Solvent

obtained from different manufacturers (Quimex and Fmaia) did not significantly affect the phytosterol content, nor did the wavelength, indicating that also the proposed method is also resistant to changes in standard conditions. The coefficients of variation ranged from 0.66 to 2.37 for the herbal drugs, and from 0.71 to 1.85 for the hydroethanol extract (Table 5), that indicating the sources of variation in the study remained below the prescribed maximum of 5% (Anvisa, 2003), confirming the robustness of the method.

Table 5
Robustness test: total phytosterol content (TPC %) for the drug and herbal extracts from roots of *Acanthospermum hispidum*.

Source or Variation	Herbal Drug	Extractives
	TPC (g%)	TPC (g%)
Solvent supplier		
Quimex ®	0.114 ± 0.003 (2.37)	0.096 ± 0.001 (1.08)
Fmaia ®	0.111 ± 0.001 (0.88)	0.099 ± 0.002 (1.86)
λ nm		
623nm	0.123 ± 0.003 (0.66)	0.103 ± 0.001 (0.71)
624nm	0.124 ± 0.003 (0.74)	0.104 ± 0.001 (0.79)
625nm	0.124 ± 0.004 (1.00)	0.103 ± 0.001 (0.87)
626nm	0.124 ± 0.005 (1.37)	0.103 ± 0.001 (0.75)
627nm	0.124 ± 0.005 (1.14)	0.103 ± 0.001 (0.76)

Conclusion

In order to achieve the quality of an herbal medicine, the qualitative and quantitative phytochemical analyses play an important role. In this way, an analytical methodology by UV-Vis spectrophotometry was developed and validated to quantify phytosterols in the roots of *A. hispidum* and their extracts. The analytical procedure was validated at 625 nm showing linearity and could be considered robust, precise, and accurate under the conditions studieds. The analytical method using UV-Vis spectrophotometry therefore constitutes an alternative tool that is useful, low in cost and easy to perform, for employment in full quality control of total phytosterols in roots of *A. hispidum* and their extracts.

Authorship

LBDCA (MSc student), SLS (undergraduate student) and MAMG (MSc student) contributed in plant sample identification, confection of herbarium, running the laboratory work, analysis of the date and drafted the paper. MRAF contributed to date analysis and discussion. ELA contributed to plant collection and identification. All laboratorial activities were supervised by KPR and LALS. All authors read and approved the final manuscript submission.

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