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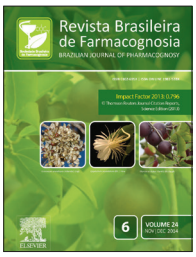

Original article

Validation of a spectrophotometric methodology for the quantification of polysaccharides from roots of *Operculina macrocarpa* (jalapa)

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ABSTRACT

The roots from *Operculina macrocarpa* (L.) Urb., Convolvulaceae, are widely used in Brazilian traditional medicine as a laxative and purgative. The biological properties of this drug material have been attributed to its polysaccharides content. Thus, the aim of this study was to evaluate the polysaccharide content in drug material from *O. macrocarpa* by spectrophotometric quantitative analysis. The root was used as plant material and the botanical identification was performed by macro and microscopic analysis. The plant material was used to validate the spectrophotometric procedures at 490 nm for the quantification of the reaction product from drug polysaccharides and phenol-sulfuric acid solution. The analytical procedure was evaluated in order to comply with the necessary legal requirements by the determination of the following parameters: specificity, linearity, selectivity, precision, accuracy and robustness. This study provides with a simple and valid analytical procedure (linear, precise, accurate and reproducible), which can be satisfactorily used for quality control and standardization of herbal drug from *O. macrocarpa*.

Introduction

Operculina macrocarpa (L.) Urb., Convolvulaceae, popularly known in Brazil as "batata-de-purga" or "jalapa" (Matos, 1994), is widely used in traditional medicine as a laxative and purgative (Matos, 1982; Martins et al., 2000; Michelin and Salgado, 2004; Gomes et al., 2008; Michelin et al., 2010). Moreover, traditional products from roots of this species are used to treat circulatory impairments (Matos et al., 1982), such as thrombosis, and recently its antiplatelet and anticoagulant activities were experimentally studied (Pierdoná et al., 2014). Regarding its laxative activity, several studies attribute this property to the glycosidic resins present in the flowers of *Ipomoea murucoides* and the roots of *Ipomoea purga* ("Mexican Jalap") (Chérigo and Pereda-Miranda, 2006; Pereda-Miranda et al., 2006).

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The resin glycosides are found throughout the species from the Convolvulaceae family, and they are the active ingredients related to purgative or laxative activities in several medicinal plants traditionally used as laxative; including *O. macrocarpa* (Lima et al., 2006). The chemical composition of the resin can be divided in two groups: the ether-soluble resin glycosides, known as jalapin; and, the ether-insoluble resin glycosides, called convolvulin or jalapurgin (Gomes et al., 2008, Akiyama et al., 2014). Although there are several reports on the isolation and elucidation of jalapin, convolvulin remains poorly understood (Akiyama et al., 2014).

Considering the relevance of *O. macrocarpa* as a traditional herbal drug and also as a herbal product in the Brazilian market, the quantitative determination of total polysaccharides content (TPC) from this species is essential as a criteria for quality control analysis of the raw materials, extractive process control and/or the evaluation of phytopharmaceuticals. There are some modern analytical procedures for the quantification of polysaccharides in drug materials reported in the literature, such as HPLC-RID (Kakita et al., 2002; Liang et al., 2006) and GC-MS (Guadalupe et al., 2012). However, due to the chemical compounds diversity and method limitations (high operational cost, experimental complexity and absence of appropriate markers), the chromatographic procedures are often unable to properly quantify the TPC. Additionally, the pharmacological properties of most herbal products are the result of the synergic effect of several compounds, and the quantification of isolated compounds can overestimate the efficiency or safety of a herbal product (Wagner, 2011).

Regarding the quantitative determination of polysaccharides from the roots of *O. macrocarpa*, a gravimetric method was previously reported (Lima et al., 2006). However, the procedure includes several steps such as partition, dry residue and reagents addition, resulting in a long analysis time that could increase the method's variability. In this context, the spectrophotometric methods are appropriate tools for total quantification, as well as having operational advantages such as: low cost, simplicity and experimental agility (Fernandes et al., 2012; Marques et al., 2012; Araújo et al., 2013).

In order to quantify the total polysaccharide content in herbal drugs, the colorimetric procedure proposed by Dubois et al. in 1956 plays a key role still. The TPC is determined after a reaction with phenol-sulfuric acid and some studies have shown its applicability to drug materials (Cuesta et al., 2003; Masuko et al., 2005; Mecozzi, 2005). Recently, the procedure was successful in testing complex polysaccharide mixtures from *Camellia sinensis*. However, several modifications were necessary to improve the method in order to achieve the accuracy and reproducibility required (Xi et al., 2010).

Therefore, in view of the importance of the polysaccharides from the root of *O. macrocarpa*, and the chemical diversity of the polysaccharides, the aim of this study was to evaluate and validate the analytical procedures for quantification of the total polysaccharides Content by means of the phenol-sulfuric acid procedure.

Material and methods

Plant material

The roots of *Operculina macrocarpa* (L.) Urb., Convolvulaceae, were purchased from the company Santos Flora Comércio de Ervas Ltda (São Paulo, SP; batch: JALA01/0411; manufacturing date: April/2011; expiry date April/2014). The drug material was botanically identified in our laboratory by using macroscopic and microscopic data, and used as reference (JLP1) for comparative studies (Fig. 1). Three commercial samples of the drug material were also obtained from the local market (Recife, Pernambuco).

Chromatographic profile of polysaccharides by Thin Layer Chromatography

The sample was obtained by decoction using 2 g of herbal drug (reference or commercial samples) in 20 ml of methanol and water (1:1) for 10 min at 100°C and the solution was then filtered in cotton. The standard used was D-maltose (Ref. ASB-00013040-001, Chromadex, USA) at a concentration of 1 mg/ml in methanol. Plates of silica gel were used as support (Ref. 818333, Alugram® SIL G/UV254, Macherey-Nagel, Germany), and ethyl acetate, formic acid, glacial acetic acid and water (100:11:11:27, v/v/v/v) as the mobile phase. Aliquots of 10 µl of each sample or standard solution were separately applied to the chromatoplate. After elution, the plate was developed using thymol solution (0.5 g thymol in 95 ml of ethanol GL 96, kept in an ice bath for 15 min and slowly added to 5 ml of homogenizing sulfuric acid PA). Finally, it was dried under laminar flow and heated to 100 °C for 2 min.

Method development

Specifications for sample preparation, analysis of the experimental conditions, as well as the spectrophotometric adjustments are described further below.

Preparation of samples for quantitative analysis

General procedure for polysaccharide content in herbal drugs

The herbal drug was prepared by reflux, using water as solvent. The extraction was performed in a round-bottom flask containing 2 g of drug and 100 ml of water for 30 min (85°C). The extract was cooled to room temperature (25°C), filtered using filter paper and transferred to a 100 ml volumetric flask. The volume was adjusted with the same solvent, yielding the *Stock Solution (SS)*. An aliquot of 3 ml from SS was transferred to a 250 ml round-bottomed flask and 1 ml of phenol solution 5% (w/v) and 5 ml of sulfuric acid was added. The mixture was then heated up in a water bath, under reflux for 30 min (85°C). After cooling to room temperature, 0.550 ml from this solution was transferred to 10 ml volumetric flasks, and the volume was completed with distilled water (probe solution, PS). The absorbance of PS was determined immediately after preparation, at 490 nm (A_1) and using water as the blank (spectrophotometer mod. Evolution 60S, Thermo Scientific®, Germany).

Figure 1 – Herbal material (roots) of *Operculina macrocarpa* (L.) Urb., Convolvulaceae.

Standard Solution

Before use, 50 mg of D-maltose SQR was immediately dissolved in a volumetric flask with 25 ml of distilled water. An aliquot of 3 ml was transferred to a 250 ml round-bottom flask and 1 ml of phenol solution 5% (w/v) and 5 ml of sulfuric acid was added. The mixture was heated in a water bath, under reflux for 30 min (85°C). After cooling to room temperature, an aliquot of 0.500 ml from this solution was transferred to a 10 ml volumetric flask and the volume was completed with distilled water. The absorbance was determined immediately after dilution, at 490 nm (A_2) and using water as the blank.

Wavelength selection and concentration of the sample

Aliquots ranging from 0.400 to 0.700 ml of the sample solution were analyzed in a scanning spectrophotometer in the region 300-650 nm and water was used as the blank. The spectra were used to identify the wavelength of maximum absorption and the analytical range.

Evaluation of herbal material concentration

Samples of the herbal material ranging from 1 to 5 g were submitted to the general procedure of polysaccharide content quantification, using water as solvent. The absorbance was determined in a spectrophotometer at 490 nm. The results express the average of three determinations.

Total polysaccharide content

The polysaccharide content was calculated as D-maltose (g%) using the equation:

$$TPT = \frac{A_1 \text{ x } m_2 \text{ x } 363{,}64}{(m_1 - p) \text{ x } A_2}$$

Where: TPC = Total Polysaccharide Content (g%); A_1 = Absorbance of the Probe Solution; m_1= weight of drug; m_2= weight of standard; p = loss on drying; A_2= absorbance of standard solution.

Method validation

The method was validated in compliance with ICH guidelines and the Brazilian legislation, including linearity, limit of sensitivities, selectivity, precision, accuracy and robustness (Anvisa, 2003; ICH, 2005). Statistical analyses were performed using linear regression, ANOVA and Student's t-test, with Excel (Microsoft Corporation, USA).

Standard calibration curve

The calibration curves were evaluated by analyzing three authentic curves, constructed with the D-maltose standard solution at six concentration levels (20-46.7 µg/ml). The results were analyzed by linear regression using the least squares method, in order to define the coefficient of determination (R^2).

Linearity

The extract from roots of *O. macrocarpa* was prepared as previously described, and the dilutions were performed at six concentration levels (166.67-666.67 µg/ml). 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 data was calculated by linear regression using the least squares method, in order to define the coefficient of determination (R^2).

Limits of detection (LOD) and quantification (LOQ)

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

Specificity

The specificity of the method was demonstrated by overlapping the standard solution (D-maltose) to the sample containing root of *O. macrocarpa*, obtained in the range of 300-650 nm.

Selectivity

The selectivity was evaluated by comparing the regression data (coefficient of determination and slopes) of the sample calibration curve and as spiked sample calibration curve. Thus, 0.300 ml of the reference solution (containing 200 µg of D-maltose) was added to the sample solution and three authentic standard curves with six different concentrations were prepared. The regression analysis was performed and the curves (sample and spiked) were compared using the slopes in order to assess the specificity of the method (Bueno et al., 2012).

Precision

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

Accuracy

The sample was prepared according to the steps to obtain the Stock Solution and the Sample Solution, as well as the procedure for obtaining the *Standard Solution*. The recovery was determined by the addition of known increasing amounts of the *Standard Solution* of D-maltose to Probe Solution at 100% of the concentration analysis. The recovery values were expressed as the ratio percentages of the total polysaccharide content experimentally determined and their theoretical concentrations. Each sample was tested three times, and the recovered amount was calculated.

Robustness

The robustness of the method was evaluated through the changes in concentration of phenol employed; different reading times after sample preparation; variation in the time of extraction of extractive solutions and changes in the wavelength of the reading samples. To compare the results obtained from the averages found for three authentic determinations of each parameter calculation, RSD% was performed.

Results and discussion

Chromatographic profile of polysaccharides by thin layer chromatography

The chromatographic profile was established for the authentic sample (JLP1) and commercial samples (JLP2, JLP3 and JLP4), using D-maltose as standard, for evidencing the presence of polysaccharide in the materials. The chromatogram obtained for the samples of "Jalapa", showed pink bands after heating. One of the bands corresponds in color and *Rf* value (0.65) to that obtained for the band of standard D-maltose (Fig. 2).

Method development

According to Masuko et al. (2005), the sequence of addition of reagents (sample-phenol-sulfuric acid; phenol-sulfuric acid-sample and sample-sulfuric acid-phenol) has an influence in the absorbance value obtained. In this study, no difference was observed in the absorbance value obtained, therefore, for this experiment the sample-phenol-sulfuric acid sequence was chosen due to its greater safety, because the order prioritizes the addition of the acid in aqueous solution and is also in accordance to the method originally proposed by Dubois et al. (1956).

Influence of drug: solvent ratio

By comparing the amounts of drug used, the proportion of 2 g drug to 100 ml of liquid extract was chosen to standardize the extraction process, since it showed a higher spectrophotometric response compared to other ratios evaluated with the method in question (Fig. 3).

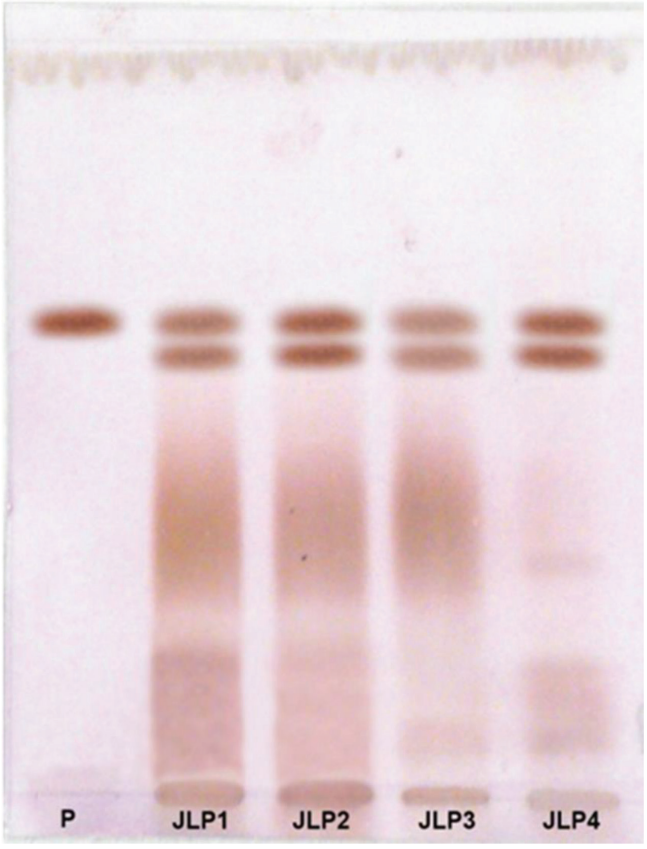

Figure 2 – Chromatographic profile obtained for standard D-maltose (P), reference sample (JLP1) and commercial samples (JPL2, JPL3 and JPL4) of *Operculina macrocarpa*.

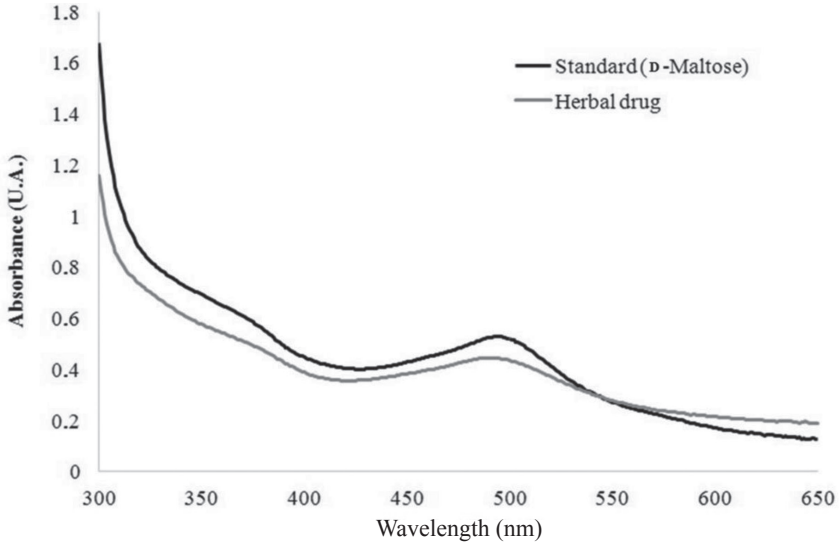

Figure 3 – Spectra (300-650 nm) for the phenol-sulfuric reaction products for herbal drug and standard (D-maltose).

Method validation

Calibration curves, linearity, detection and quantification limits
Calibration curves were evaluated after regression analysis, and linearity was estimated by the coefficients of determination (R^2) for concentrations ranging from 80 to 120% of the working concentration. The data presented in Table 1 suggest that the curves for the standard and herbal material from the root of *O. macrocarpa* were linear; the R^2 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. The *F* value found for the models was greater than the critical *F* value, and is the reverse of the lack of fit for adjustment. Regarding the detection and quantification limits (LOD and LOQ), the spectrophotometric procedure was sensitive to the detection and quantification of polysaccharides in the herbal material, with no interference from the important instrumental technique.

Specificity
Simple sugars, polysaccharides and their derivatives, give a yellow-orange color when treated with aqueous solution of phenols and concentrated sulfuric acid, and the intensity of the color produced is related to the concentration of added phenol, thus, proportional to the amount of sugar present in the sample (Dubois et al., 1956). Furthermore, the color is stable and the spectrum generated shows a maximum that is well defined for the sample and standard. Some authors report that most sugars have their maximum absorptions between 480 and 495 nm (Masuko et al., 2005). In Fig. 4, it can be observed that the spectra obtained for the standard D-maltose and the *O. macrocarpa* extract are in the range of between 300-650 nm, evidenced only by a maximum absorption at 490 nm, thus it is possible to specifically detect the standard in the solution containing root of *O. macrocarpa*, even with impurities.

Selectivity
Regarding the complex nature of biological materials such as herbal drugs, the study of selectivity of analytical procedures plays an important role. However, the absence of analyte-free matrices remains a challenge for the development and validation of several analytical methods applied to herbal drugs or herbal medicines. In order to decrease the matrix interference, several strategies for sample preparation have been used to clean up the samples such as SPE. Nevertheless, there does not exist any strategy to obtain analyte-free

Table 1
Calibration data for the standard (D-maltose) and the herbal drug from roots of *Operculina macrocarpa*.

Sample	Model Coefficients	R^2	Range (µg/ml)	LOD	LOQ
D-maltose	a = 0.0238 b = - 0.2610	0.9992	20.0-46.7	-	-
Herbal drug	a = 0.0012 b = - 0.0059	0.9996	166.67-666.67	1.8043	6.0143

matrices. Thus, the analytical method specificity can be studied by using spiked samples such as in the recovery assays. In this way, the regression data from the spiked curve is compared to the data from the sample curve. If both curves are parallel, no interference can be attributed to the matrix. As can be observed in Fig. 5, the spiking curve with a known amount of D-maltose provides a constant shifting response for all concentrations. The regression data shows that the addition of a reference solution allows a linear correlation (R^2 = 0.9981). Furthermore, no difference was observed between the slopes. These data confirm the parallelism of curves and, consequently, the dependence of method response from analyte (reaction product from polysaccharides, phenol and sulfuric acid).

Precision

The data for procedure of precision, assayed at two levels: repeatability and intermediate precision, showed that the method was precise due to the lower relative standard deviations (RSD%), for both parameters. Thus, in the repeatability the maximum relative standard deviation was 1.21%. Furthermore there was no statistical difference in the intermediate precision, although the method had been assessed on different days (Table 2). Therefore, the method could be considered precise according to the national regulatory requirement (Anvisa, 2003).

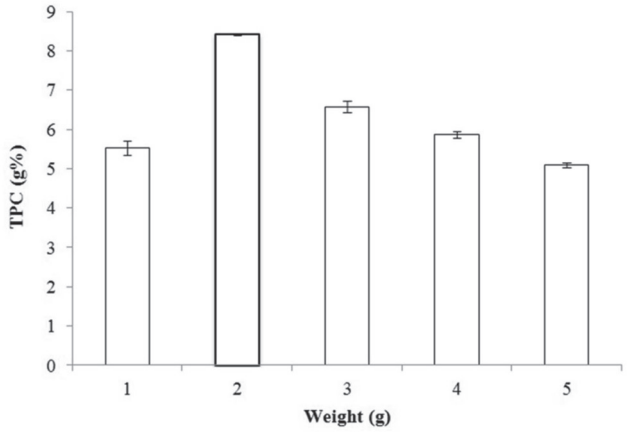

Figure 4 – Influence of drug concentration on total polysaccharide content (TPC) from the herbal material.

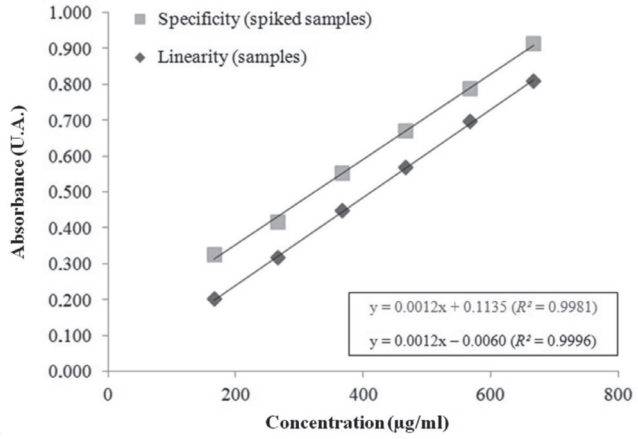

Figure 5 – Selectivity assay of the analytical method for TPC from roots of *Operculina macrocarpa*.

Accuracy

The accuracy of an analytical method expresses the nature of a complex matrix, such as those in plant-based materials. Several sources of interference or variations, such as extraction conditions and extraction (partial degradation) of chromophores and similar reagents 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 herbal drug, it was spiked with the standard (0.1 to 0.3 ml) and the parameter was calculated from the recovery of the total polysaccharide content. The data showed recoveries from 97.42 to 101.06% for the herbal drug. The recovery levels higher than 95% confirm that the proposed quantitative procedures are in accordance with statutory requirements, and provide reliable results.

Robustness

In accordance to the legislation, the assay of robustness should be performed during the development of the method and must be performed with small and deliberate variations in analytical methodology. In this step, several critical parameters should be considered as those related to sample preparation such as stability of the solutions or extraction time; as well as those derived from the analytical procedure such as pH, temperature, and solvent manufacturer (Anvisa, 2003). In our study, it was not possible to evaluate the pH of the sample solution, due to the use of concentrated sulfuric acid as reagent. In addition, the solvent supplier has no influence, since the method was based in the use of water as solvent.

Thus, the robustness of this analytical procedure was carried out by the evaluation of phenol concentration (4.75 and 5.25%; w/v); time of sample extraction (28, 30 and 32 min); reaction times after sample preparation (0, 30 and 60 min before measurement); as well the different wavelengths of measurement (488, 490 and 492 nm). The effect of each parameter was assessed by the respective relative standard deviation (RSD), which ranged from 0.70 to 1.15 for the sample absorbances (Table 3), and indicates that the variability of such error sources remained below of the international criteria of 5% (Anvisa, 2003; ICH, 2005). Thus, the data confirm the reliability and the stability of the analytical method for the laboratory routine.

Table 2
Repeatability and Intermediate precision test: total polysaccharide content (TPC) for the drug from roots of *Operculina macrocarpa*.

Test	TPC (g%) (Mean ± SD; RSD%)	
Repeatability	8.97 ± 0.109 (1.21)	
Intermediate precision	Day 1	Day 2
Analyst 1	8.88 ± 0.035 (0.40)	9.03 ± 0.060 (0.67)
Analyst 2	8.89 ± 0.081 (0.91)	9.00 ± 0.097 (1.07)

Table 3
Robustness test: total polysaccharide content (TPC) for the drug from roots of *Operculina macrocarpa*.

Source of variation	Variables	TPC g% Mean ± SD (RSD%)
Phenol concentration (%, w/v)	4.75	8.99 ± 0.060 (0.22)
	5.25	8.99 ± 0.020 (0.26)
Stability of solution (min)	30	8.90 ± 0.061 (0.68)
	60	8.84 ± 0.058 (0.65)
Extraction time (min)	28	9.00 ± 0.012 (0.13)
	32	8.98 ± 0.110 (1.22)
Wavelength (nm)	493	8.81 ± 0.020 (0.24)
	498	8.84 ± 0.029 (0.33)
Reference conditions		8.83 ± 0.060 (0.68)

Total polysaccharide content in commercial samples

The procedures were tested on commercial samples obtained in the public market (Recife, PE), after validation, to check the reproducibility of the method. The average of TPC obtained from the samples remained between 6.41 and 8.07 g%. Mean values are shown in Table 4. Data shows some variability in the polysaccharides content obtained from the different commercial samples.

Table 4
Total polysaccharide content (TPC) in commercial samples of *Operculina macrocarpa*.

Samples	TPC (g%) Mean ± SD (RSD%)
JLP2	7.30 ± 0.131 (1.80)
JLP3	6.41 ± 0.132 (2.06)
JLP4	8.07 ± 0.222 (2.75)

Conclusion

The spectrophotometric procedure for quantification of total polysaccharides content after phenol-sulfuric acid reaction previously described by Dubois et al. (1956), showed the reliability and reproducibility necessary to quantify such compounds from the root of *O. macrocarpa*. The analytical method was evaluated in accordance to the legal requirements and validated at 490 nm showing linearity, selectivity and specificity, and proves to be precise, accurate and robust. Thus, the data allowed the conclusion that this procedure combines operational and sample preparation simplicity, with feasibility and low cost, to accomplish the quality control of herbal drug (root) from *O. macrocarpa*.

Authors contributions

MAMG (MSc student) and contributed to the running of the laboratory work and analysis. MRAF contributed to date analysis and discussion. BMN (undergraduate student) and ASCOS (MSc student) contributed to identification of the herbal material. KPR and LALS designed the study and supervise the laboratory activities. All authors read and approved the final manuscript submission.

Conflicts of interest

The authors declare no conflicts of interest.

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