Spectrophotometric Method for Polyphenols Analysis
Validation and application on *Trapa Natans* L. *Species*

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The objective of this work is to validate the spectrophotometric method to detect and quantify the total polyphenols from different sources. The validation procedure was applied to evaluate the total polyphenols of Trapa natans L. species by the UV-VIS spectrophotometric method with Folin - Ciocalteu reagent, as a chromogenic agent. The method was validated as per ICH rules for linearity, detection limit, quantification limit, precision, and accuracy. Calibration curves show a linear relationship between the absorbance and the concentration. The linearity concentration range was 1.0 to 6.0μg/mL with the correlation coefficient of 0.9994. The limit of detection and limit of quantitation values were found to be 0.2096 μg/mL and 0.6987 μg/mL. We verified the performance parameters after experimental studies and we established that the spectrophotometric method can be applied for the determination of total polyphenols in water caltrop roots, leaves, hulls and pulp hidroalcoolic extracts.

Keywords: spectrophotometric, validation, total polyphenol

Plants are established sources of pharmaceutical compounds, aromatic compounds and industrial compounds; the civilization is linked to their world and they have constituted for millennia the major sources of production of bio-products for the survival of the entire world [1, 2].

Recent statistics show that over 1500 novel compounds are identified in different plants species annually and that about a quarter of drugs prescriptions contain substances of plant origin [3].

In Romania the predominant species in the Danube Delta is *Trapa natans*. Water caltrop belongs to the family *Trapaceae* and is a plant that belongs to the annual aquatic plants with floating leaves. The plant grows in shallow water fields, lakes and pools [4].

Trapa natans has been traditionally used for many purposes such as inflammation, swelling, sore mouth [5], diarrhoea, dysentery, haemorrhages [6], urinary discharges, fractures, bronchitis [7].

There is little information on the polyphenolic content of water caltrop pericarps [6, 7]. The polyphenols are secondary metabolites in plants, most of them are responsible for their antioxidant properties [8].

From the pharmaceutical point of view, the polyphenols are an important group of substances found in the *Trapa natans* [9-11]. They represent an important source of natural antioxidants compounds investigated to combat oxidative stress by reactive oxygen species, which in moderate quantities have a better shaped physiological role, but they have also harmful and destructive potential to cells if they are used in inadequate quantities [12].

The objectives of the study are to validate the spectrophotometric method for the quantification of total phenol content and to determine the total phenol content in water caltrop hulls, pulp, leaves and roots.

Many plants have been identified as containing polyphenols and their consumption is recommended.

In the last years the number of publications on the potential health benefits of polyphenols has increased [13]. Today it is of interest to investigate the polyphenols in plants extract, especially those traditionally used in traditional medicine and there are recommendations to increase the intake of foods rich in polyphenols, which are antioxidant compounds.

The principle of the method is based on determining the intensity of blue coloration of molybdenum oxides formed through the reaction of reducing the Folin-Ciocalteu reactive (fosfomolibdowolframic acid) by polyphenols. Measurements were made at the maximum absorbance, λ = 725 nm. The spectrophotometric method of determining the visible area was developed to allow the calculation of the amount of total polyphenols in the components of the species *Trapa natans* [14].

Experimental part

Apparatus

We used the UV-VIS spectrophotometer model V 630 Jasco (Jasco, United Kingdom), which is operative in the 190 – 1100 nm spectral domain, with PC-HP XXX UV-VIS and 1 cm quartz cells were used for all absorbance measurements.

Reagent and solutions

We used for plant extraction the 99% ethanol (Merck, Germany) solution. For samples preparation the sodium carbonate (Merck, Germany) was used; the solution of 20% sodium carbonate decahydrate was prepared by dissolving 20.0 g in 80.0 g double distilled water. The distilled water was used throughout and it was prepared using a Millipore system.

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As chromogenic agent we used the Folin-Ciocalteu reagent (Merck, Germany). The filtration of prepared sample was performed with 0.45 μm Minisart-plus membrane filter (Sartorius AG, Germany).

For calibration curves we used gallic acid from Chromadex, Germany.

Plant material

The fresh water caltrops were collected in august 2009 from the Sinoe Lake, Romania. The taxonomic identities of these plants were confirmed in the Department of General, Vegetal and Animal Biology, Faculty of Pharmacy, Ovidius University of Constanta, Romania.

The fresh water caltrops were manually removed and separated into four samples: roots, leaves, pulp and hulls. The samples were air-dried; each sample was powdered and stored at 4°C until further analysis.

Preparation of samples for validation

The primary analyte stock standard solution was prepared by dissolving 10.0 mg gallic acid in water and diluting to 100.0 mL with the same solvent. In 10 mL volumetric flask the secondary stock solution was prepared, by mixing the adequate volume of primary stock solution of gallic acid (0.1, 0.2, 0.3, 0.4, 0.5, 0.6 mL) with 1 mL of Folin-Ciocalteu reagent and made up to 10 mL with solution of 20% sodium carbonate. After 40 min incubation at room temperature and filtration, the absorbance at 725 nm was measured. Blank solution was prepared and measured in the same condition, but without analyte.

Execution of validation

The development of a simple, rapid, sensitive and accurate analytical method for the routine quantitative determination of the total polyphenols in samples, leads to the reduction of unnecessary sample preparations and of materials and labour costs.

The validation of the spectrophotometric method consists in the determination of the following parameters: the response function linearity, the results linearity, precision, accuracy, limit of detection and limit of quantification [15-18].

For the response function linearity, standards and blanks six concentrations were prepared (1.0, 2.0, 3.0, 4.0, 5.0, 6.0 μg/mL gallic acid), and measured in triplicate to establish the regression equation. The linearity was evaluated by the least-squares regression method and was analyzed and confirmed by statistical analysis.

Through the calibration equation there are calculated the values of the found concentrations, in order to also evaluate the linearity of the results. The calibration equation was used to calculate the retrieved concentrations, to determine the linearity of the results and to evaluate the statistical parameters of retrieved concentrations.

The performance and accuracy of analytical methods are essential to the quality control of plant material.

In order to evaluate the detection's repeatability, there were performed a number of 10 determinations for each sample concentration (4 μg/mL of gallic acid), in the same day and respectively the same experimental conditions.

In order to determine the repeatability of the presented spectrophotometric method, it was chosen the option of working with a minimum of 9 determinations covering the specific domain of concentration: 3 determinations at 3 values of concentration (2 μg/mL, 4 μg/mL and 5 μg/mL).

To determine the intermediary precision, the entire experiment was performed on the second day, with newly prepared reagents, the procedure being identical as in the case of determining the method's repeatability. It was chosen the option of working with a minimum of 9 determinations covering the specific domain of concentration: 3 determinations at 3 values of concentration 2 μg/mL, 4 μg/mL and 5 μg/mL). We wanted to demonstrate the determination method of the gallic acid, repeated at intervals of at least one day between 2 successive analytical measurements, over some identical samples, which must generate similar results.

The admissibility conditions are fulfilled subject to the fact that the average retrieval degree is found in the interval of 95 – 105%, and the relative standard deviation must not exceed 5% [15].

In order to confirm the accuracy of the proposed method, there were analyzed 9 samples using 3 levels of concentration which should cover the work interval.

The limit of detection (LOD) was calculated using the following equation according to definition LOD= 3s/k, where s is standard error of the calibration curve and k is the sensitivity, namely the slope of the calibration curve [15].

The limit of quantitation (LOQ) was calculated using the following equation: LOQ=10s/k [18].

The recovery, which indicates the accuracy of the experimental procedure, was appreciated by adding 1mg/mL of gallic acid to ethanolic extracts.

Preparation of extracts for determination of total polyphenols

Each powdered plant material (10 g of each sample) was extracted with 100 mL aqueous ethanol 50% (2 x 50 mL) at 40°C in hot continuous extraction using a Soxhlet apparatus for 6 h . After cooling, the extracts were filtered through a 0.45 μm membrane. They were brought to 100 mL in a volumetric flask, by washing the residue with the same solvent. All the extracts were stored at -4°C.

The total polyphenols of the samples were determined according to the method described by Bucur L., with minor modification.

1 mL of each sample (10^2 mg/mL) was mixed with 1 mL of diluted Folin-Ciocalteu reagent (diluted 1:1) and double distilled water up to 25 mL solution (solution A). After 5 min incubation at room temperature, there were prepared the sample solutions by diluting solution A with the solution of 20% sodium carbonate (w/v).

The blank solution was prepared in the same conditions with double distilled water, but without analyt. After 40 min incubation at room temperature, the absorbance was measured and converted to total polyphenols contents according to the calibration curves of gallic acid (mg GAE/g samples dry plant material). The absorbance was measured at 725 nm at 20°C. The estimation of the total polyphenolic content was carried out for five times.

Statistics

For all analyses, the data were subject to an analysis of variance (ANOVA). The regression analyses were performed with the Microsoft Excel software.

Results and discussions

Analysis of validation results of the spectrophotometric method

A linear relationship was found between the absorbance at 725 nmand the concentration of gallic acid in the range of 1.0 to 6.0μg/mL. The representative linear equation was $y = 0.1383 x + 0.0225$ where: y is the absorbance, x is gallic acid concentration (μg/mL), calculated by the least squares method. The regression coefficient (r) of the

standard curve was 0.9994 (fig. 1) indicating good linearity ($r > 0.999$). The performance parameters of linear regression equation are presented in table 1.

Table 1
VALIDATION PARAMETERS FOR THE RESPONSE FUNCTION LINEARITY

Parameter	Value
Observations	6
Linear range (µg/mL)	1.0– 6.0
Slope	0.1383
Intercept	0.0225
Regression coefficient r	0.9994
Coefficient of determination r^2	0.9989
Standard error of the regression line (SE)	0.0096
Mean	0.5063
Standard deviation (SD)	0.2588

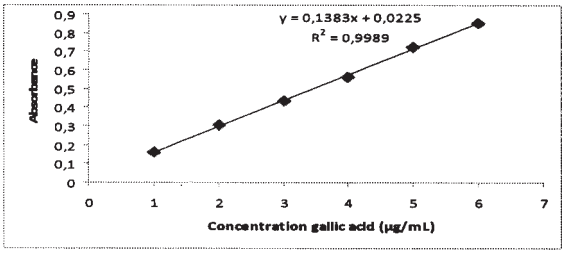

Fig.1. The linear regression curve for the determination of gallic acid etalon curve

To evaluate the linearity of the results through the calibration equation, there were calculated the values of the found concentrations and the validation parameters for the results linearity (table 2). Using the calibration line equation between the theoretically introduced concentration and the calculated one, there is a linear correlation.

Table 2
VALIDATION PARAMETERS FOR THE RESULTS LINEARITY

Parameter	Value
Observations	6
Linear range (µg/mL)	1.0– 6.0
Slope	0.9997
Intercept	-0.0002
Regression coefficient r	0.9994
Coefficient of determination r^2	0.9989
Standard error of the results linearity (SE)	0.0698
Mean ($\bar{x}$)	3.4987
Standard deviation (SD)	1.8713

Table 3
STATISTICAL PARAMETERS WHICH CHARACTERIZE THE PERCENT RETRIEVAL DEPENDING ON THE THEORETICAL CONCENTRATION OF GALLIC ACID

Parameter	Value
Observations n	6
Work domain	97.50 – 101.80 %
Mean of retrieval ($\bar{x}$)	100.14%
Standard deviation of retrieval (SD)	1.69

There are calculated the absolute error, the relative error and the retrieval in order to validate the results linearity. The statistical parameters which characterize the percent retrieval depending on the theoretical concentration of gallic acid are presented in table 3.

It can be appreciated that the retrieval percent is practically constant within the studied linearity interval, the retrieval's deviation being 1.69, lower than 2%.

Quantitative measurements of accuracy and precision depend on the chosen conditions: detection repeatability, repeatability of the method and intermediate precision. The statistical parameters which characterize the precision and accuracy of the sprectrophotometric method of determination of gallic acid are presented in table 4.

According to the equations presented, LOD was found to be 0.2096 µg/mL and LOQ 0.6987 µg/mL.

Evaluation of total polyphenols content and recovery studies in Trapa natans extracts

Total polyphenols were estimated as gallic acid equivalents (GAE) and they are expressed as milligrams of gallic acid per gram (dry weight) of ethanolic extract (table 5).

The recovery was appreciable, indicating the accuracy of this experimental procedure and was determined by adding 1.00 mg/mL amounts of gallic acid to plant extracts (t6able 5). Differences of estimated concentrations to actual concentrations varied from 2.55 to 4.01%. The differences may be due to the presence of some traces of unreacted reagent.

Ethanolic extract of roots has the highest content on polyphenols, followed by ethanolic extracts of leaves, hulls and of pulp (fig.2).

Parameter	detection precision	method precision	intermediary precision	accuracy
Observations n	10	9	9	9
Mean of retrieval ($\bar{x}$)	0.51	97.84	99.01	99.03
Standard deviation of accuracy (SD)	0.001	1.57	1.01	1.53
RSD	0.34	0.53	1.02	1.55

Table 4
STATISTICAL PARAMETERS WHICH CHARACTERIZE THE PRECISION AND ACCURACY OF THE GALLIC ACID DETERMINATION METHOD

Extracts	$\bar{X} \pm SD$ (mg GAE/g of extract)	Recovery
Roots	2.76± 0.18	96.81
Leaves	1.19± 0.21	96.42
Pulp	0.47± 0.08	97.45
Hulls	1.12± 0.039	95.99

Table 5
QUANTIFICATION OF TOTAL POLYPHENOLS AND THE RECOVERY STUDIES IN *TRAPA NATANS* EXTRACTS

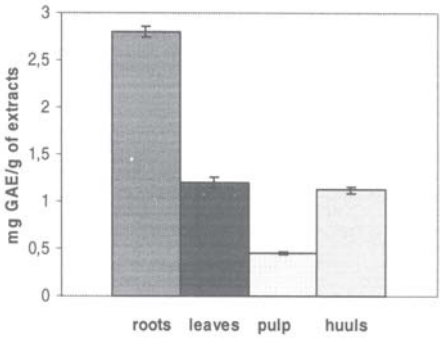

Fig. 2. Comparision of total polyphenolic content in *Trapa natans* extracts

Conclusions

In this paper we study a spectrophotometric method for quantifying polyphenols in plant extracts and we validate this assay. The method is selective, precise, reproducible, repeatable, accurate and linear over the concentration range studied.

In all analyzed extracts obtained from the aquatic part, the aerial part, the fruit pulp and fruit hulls of the *Trapa natans* L., extracts obtained by solid-liquid extraction in Soxhlet extractor, the polyphenols were outlined.

This method can be used for routine analysis for the determination of total polyphenols content in the plants issues.

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