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Research Article

HPLC standardization of the methanolic extract of *Acrocarpus fraxinifolius* leaves based on Gallic acid Content

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ABSTRACT

High-Performance Liquid Chromatography (HPLC) was developed for the standardization and quantification of gallic acid in the methanolic extract of *Acrocarpus fraxinifolius* Weight & Arn leaves. Linearity was observed in the range of 0.4–2 mg/mL with a correlation coefficient (r^2) of 0.9978. The limit of detection (LOD) and limit of quantification (LOQ) were 0.0054 mg/mL and 0.0163 mg/mL, respectively indicating the sensitivity of the applied method. Recovery values of 100.377% indicate the best accuracy of the method. Gallic acid content was quantified as 168.75 ± 1.05 mg/g methanolic extract. This developed method was simple, accurate and precise to be used as a reference standard method for determination of gallic acid content in the plant extract.

Keywords: HPLC; Standardization; Quantification; gallic acid; *Acrocarpus fraxinifolius*; linearity; accuracy.

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1. INTRODUCTION

Standardization of herbal extracts is considered as a great important tool for quality control in production and manufacturing process of herbal drugs to define the chemical profile of the product and to assess consistent biological activity **[1]**. The validation parameters of analytical separation method are accuracy, precision, linearity, range, detection limit and quantification limit **[2-4]**. Two ambiguous parameters are detection limit and quantification limit, The notion of detection limits was first encountered during the golden era of quantitative trace environment analysis, during the mid to late 1980s **[5]**. The limit of detection (LOD) of an analyte is described as that concentration which gives an instrumental signal significantly different from the blank or background signal **[6]**. The importance of calibration linearity in the context of the quantification limit is considered, since the estimated concentrations of this limit, are deduced from the regression line. In the chromatographic analysis, it was proven that the best method is based on the use of the parameters obtained from the analytical curve, which are statistically reliable **[4]**. *Acrocarpus fraxinifolius* Weight & Arn is a member of the tree family Fabaceae belonging to subfamily Caesalpinioideae **[7]**. The literature showed that many galloylated compounds such as 2,3 digalloyl-α,β-glucoside, quercetin-(2"-galloyl-)3-O-β-glucopyranoside, myrecetin-(2"-galloyl-)3-O-β -glucopyranoside as well as gallic acid were isolated from the *Acrocarpus fraxinifolius* Weight & Arn extract **[8]**. This study was

conducted to obtain a valid method for standardization and quantification of gallic acid in the methanolic extract of *Acrocarpus fraxinifolius* leaves using HPLC.

2. MATERIALS AND METHODS

2.1. Plant Material

Fresh leaves of *Acrocarpus Fraxinifolius* (Fabaceae) were collected from plants grown in ZOO Botanical Garden, Ministry of Agriculture, Giza, on March (2013). They were kindly authenticated by Mrs. Tereize Labib, Agricultural Engineer, El-Orman Botanical Garden, Giza, Egypt. Voucher specimens of the authenticated plant PHG-P-AF130 were deposited at the Department of Pharmacognosy, Faculty of Pharmacy, Ain Shams University.

2.2. Plant Extract

The intact air-dried plant material (3 kg) was comminuted to powder then boiled in distilled water for 2 h then filtered while hot. The filtrate was completely evaporated in vacuo at ≈ 55 - 60 ºC till dryness. The solid residue was then extracted with methanol at 40 ºC till exhaustion. The combined methanolic extracts were evaporated in vacuo till dryness. The process yielded finally 90 g of a sticky dark brown material.

2.3. Chemical Reagents

All used reagents such as acetic acid, water, and methanol were of HPLC grade. The active compound gallic acid, used as a reference standard, was purchased from (Sigma-Aldrich).

2.4. Chromatographic Conditions and Procedures

The methanolic leaf extract of *Acrocarpus fraxinifolius* was standardized using an Agilent 1200 series HPLC equipped with an Agilent quaternary pump connected to a photodiode array detector with variable wavelengths, the separation was carried out on RP C18 column with dimensions (150mm, 4.6mm, 5 µm) according to the previously described method **[9]**. Gradient elution preceded using 4% acetic acid in water (solvent A) and methanol (solvent B) according to the gradient program of **table 1**. The mobile phase was filtered through a 0.45 µm millipore filter and degassed by sonication for 30 min. The flow rate was adjusted at 0.6 mL/min and injection volume 20µL. The detection was done at wavelength 280 nm. The sample was repeated in triplicates.

Table 1 Time Table for HPLC Gradient Elution Process using 4% Acetic Acid and Methanol as Solvents.

Time (min)	Solvent A (4% acetic acid) (%)	Solvent B (Methanol) (%)
0.00-4.00	100.0	0.0
4.01-10.00	50.0	50.0
10.01-20.00	20.0	80.0
20.01-22.00	50.0	50.0
22.01- 26.00	100.0	0.0

2.5. Preparation of the Standard Solution

Standard solution of pure gallic acid was prepared by dissolving 40 mg in 10 ml of methanol in a volumetric flask (stock solution). For linearity study and construction of calibration curve, five different concentration of stock gallic acid solution after dilution with HPLC grade methanol (0.4, 0.8, 1.2, 1.6 and 0.3 mg/mL) was injected in triplicates.

2.6. Preparation of Sample Solution

0.1 gm of methanolic extract of Acrocarpus fraxinifolius leaves was dissolved in 25 ml HPLC grade methanol. The sample was sonicated for 20 min. After sonication, the sample was filtered through a 0.45 µm membrane.

2.7. Validation of the HPLC Method

2.7.1. Limits of Detection (LOD) and Quantification (LOQ)

For determination of LOD and LOQ, different dilutions of the standard gallic solution were analyzed 6 times using the mobile phase as a blank (**Fig. 1).**

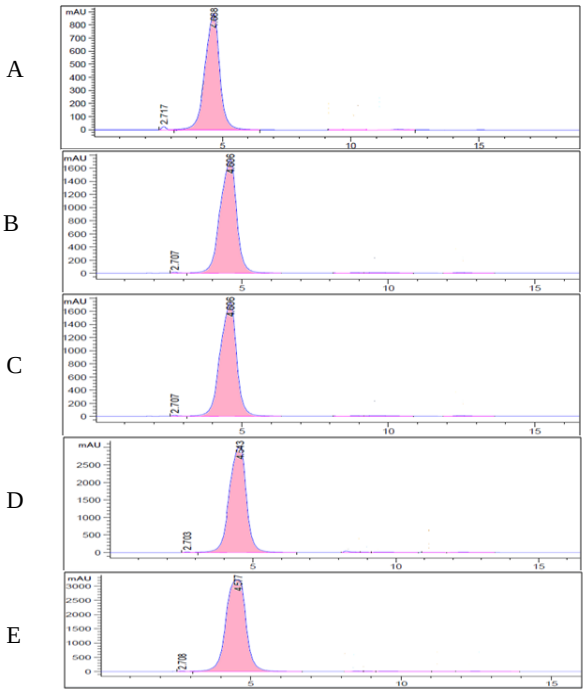

Fig. 1 HPLC Chromatogram of Reference Standard (Gallic acid), at Concentrations (A) 0.4; (B) 0.8; (C) 1.2; (D) 1.6 and (E) 2 mg/mL

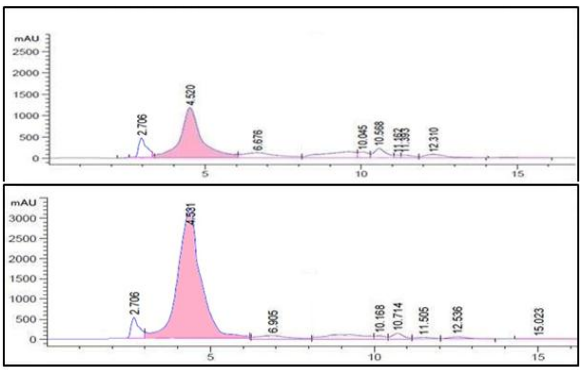

Fig. 2 HPLC Chromatogram of Spiked *Acrocarpus fraxinifolius* Extract with Known Concentration of the Standard (Gallic Acid).

The LOD and LOQ were determined on the basis of signal-to-noise ratio until the average responses were approximately three and ten times the responses of the blank respectively.

2.7.2. Accuracy (Percentage of Recovery)

The accuracy of the applied method was ascertained by spiking the plant extract sample with a known amount of standard solution as shown in **Fig. 2**. The accuracy was estimated by applying values of peak area to the regression equations of the calibration graph. Three replicate samples of each concentration level were prepared.

2.7.3. Method precision (Repeatability)

The precision of the instruments was checked by repeatedly injecting (n=6) and analyzing standard solution. The results are reported in terms of relative standard deviation (RSD).

2.7.4. Intermediate Precision (Reproducibility)

The interday and intraday precision of the proposed method were determined by analyzing standard solution at different concentrations (0.4, 0.8, 1.2, 1.6 and 0.3 mg/mL) three times on the same day and on three different days. The results are reported in terms of RSD.

3. RESULTS AND DISCUSSION

3.1. HPLC Method Validation

The calibration curve was constructed by plotting the peak areas against five standard gallic acid concentrations; regression equation and correlation coefficient (r^2) were derived and illustrated in fig3. Linearity was obtained in the range of 0.4–2 mg/mL. The regression equation was found as y = 70827x + 8139.6 with a correlation coefficient (r^2) of 0.9978, indicating best linearity as indicated in **Fig. 3**. The correlation coefficient was larger than 0.995 indicating a good relationship between peak areas and concentrations **[10]**. The method was validated in terms of accuracy, precision, repeatability, reproducibility, LOD, and LOQ and illustrated in **table 2**. The LOD value was found to be 0.0054 mg/mL which is the concentration that yields a signal-to-noise (S/N) ratio of 3:1. The LOQ value was 0.0163 mg/mL under the described conditions with an S/N ratio of 10:1. This confirmed the sensitivity for quantification of gallic acid in the plant extract. A recovery value of 100.377% (Average recovery percent of

different concentrations of the standard) indicated the best accuracy of the method.

Table 2 Validation Parameters of Applied HPLC Method

Validation Parameter	Result
Accuracy (mean ± %RSD)	100.37 ± 1.036
Precision (%RSD)	
✓ Repeatability	1.036
Intermediate Precision	
✓ Intraday	1.106
✓ Interday	0.918
Regression Equation	y = 70827x + 8139.6
Linearity range (mg/ mL)	0.4-2
Linearity	
✓ Intercept	8139.6
✓ Slope	70827
✓ Correlation Coefficient (r^2)	0.9972
LOD (mg/mL)	0.0054
LOQ (mg/mL)	0.0163

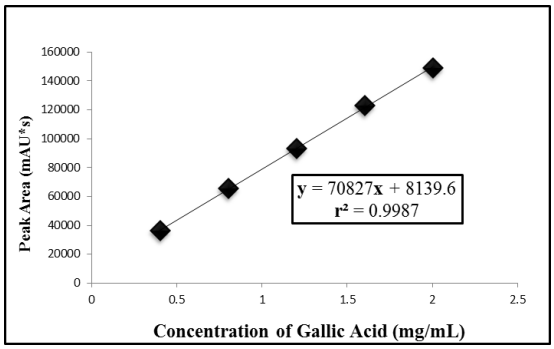

Fig. 3 Calibration Curve of Standard Gallic Acid.

3.2. HPLC Quantification of Gallic Acid in the Methanolic Extract of *Acrocarpus fraxinifolius* Weight & Arn Leaves

Quantification of gallic acid content in Acrocarpus fraxinifolius was estimated from the regression equation as 168.75 ± 1.05 mg/g methanolic extract. The result is expressed in term of mean ± SD of three determinations.

4. CONCLUSION

The HPLC applied method was simple, accurate, linear, repeatable, reproducible and sensitive. The gallic acid content was found to be 168.75 ± 1.05 mg/g methanolic extract of *Acrocarpus fraxinifolius* Weight & Arn Leaves. This result can be used for routine quality control analysis.

Conflict of Interest

The authors report that they have no conflicts of interest.

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