GSC Biological and Pharmaceutical Sciences, 2020, 13(01), 054-061

Available online at GSC Online Press Directory

GSC Biological and Pharmaceutical Sciences

e-ISSN: 2581-3250, CODEN (USA): GBPSC2

Journal homepage: https://www.gsconlinepress.com/journals/gscbps

(RESEARCH ARTICLE)

Simultaneous quantitative estimation of ellagic acid and gallic acid in Sudanese *Solanum dubium* seed by high performance thin layer chromatography (HPTLC)

Iman Tagelsir Abdalla Mohamed [1,*], Wasim Khan [2], Karishma Chester [2], Abdelwahab Hassan Mohamed [3] Sayeed Ahmad [2] and Saad Mohamed Hussien Ayoub [4]

[1]*Department of Chemistry, Preparatory College, University of Medical Sciences and Technology, Khartoum Sudan.*
[2] *Natural Product Laboratory, Faculty of Pharmacy, Hamdard University, Hamdard Nagar, New Delhi 110062,*
[3] *Department of Pharmacology, Faculty of Pharmacy, National Ribat, University, P.O. Box 2404 Khartoum Sudan.*
[4]*Department of Pharmacognosy, Faculty of Pharmacy, University of Medical Sciences and Technology, P.O. Box 12810, Khartoum, Sudan.*

Publication history**:** Received on 25 September 2020; revised on 07 October 2020; accepted on 12 October 2020

Article DOI: https://doi.org/10.30574/gscbps.2020.13.1.0311

Abstract

Background: *Solanum dubium* is a plant believed to have several therapeutic effects including anti-asthmatic properties. The objective of this study was to investigate the quantitative estimation of Gallic acid and Ellagic acid from the seed extract of Sudanese *Solanum dubium*.

Methods: A simple and rapid high-performance thin-layer chromatographic method was developed and validated for quantitative estimation of Gallic acid and Ellagic acid from the seed extract of Sudanese *Solanum dubium*.

Results: Ellagic acid and Gallic acid were quantified by using HPTLC. The seeds were found to contain 1.1% w/w of Ellagic acid and 2.1% w/w of Gallic acid in extract. Gallic acid and Ellagic acid were chromatographed on silica gel 60 F254 TLC plate using Toluene: Ethyl acetate – Methanol – Formic acid (3:3:1:0.4 v/v/v/v) as mobile phase and quantified by densitometric scanning at 280 nm. The method was found to give compact spots for Gallic acid (Rf 0.35)

and Ellagic acid (Rf 0.21). The linear regression analysis data for standard Gallic and Ellagic acids spots showed good linear relationship with r2 = 0.988 and 0.994 respectively, in the concentration range 100-3000 ng/spot, accurate (99.23-102.7%), (98.7-100.2%); precise (% RSD ≤ 2); robust (% RSD ≤ 2) and specific. The LOD and LOQ of the method were found as 653 and 396.8ng/spot and 215.5and 130 ng/spot, of Gallic acid and Ellagic acid respectively.

Conclusion: The method was validated for precision, recovery and repeatability as per the International Conference on Harmonization Guidelines for Gallic acid and Ellagic acid. Statistical analysis of the data showed that the method is precise, accurate, reproducible and selective for the analysis of Gallic acid and Ellagic acid.

Keywords: Sudanese *Solanum dubium* seed; Gallic acid; Ellagic acid; HPTLC.

1. Introduction

Solanum dubium Fresen is a medicinal plant member of the *Solanaceae* family of plants, which comprises many genera, well known in Sudan for their therapeutic properties. *S. dubium* Fresen, in Sudan, is a well known wild plant that grows

*Corresponding author: Iman Tagelsir Abdalla Mohamed
Department of Chemistry, Preparatory College, University of Medical Sciences and Technology, Khartoum Sudan.

widely in Khartoum during the rainy season. It also grows in the west and east shores of the White Nile, South of the Blue Nile, Gezira, Kordfan and Darfur regions. [Salih,1979].

High performance thin layer chromatography (HPTLC) can be used qualitatively and quantitatively for the estimation of chemical constituents present in the plant materials. [Ravishankara, et, al. 2000]. Phenolic acids have been considered as potential therapeutic agents against a wide range of ailments including neurodegenerative diseases, cancer, diabetes, cardiovascular dysfunction, inflammatory diseases and in ageing [Rebecca, 2003]. They are widely distributed in the plant kingdom. The importance of antioxidant activities of phenolic acids and their possible usage in processed foods as a natural antioxidants have reached a milestone both in food biotechnology and in the health care sector. Several phenolic acids [Gupta, et, al. 2012] were reported (i.e Ferulic acid, ellagic acid, caffeic acid etc.,) from plants. One such prominent phenolic acid is gallic acid. It is found in a wide variety of foods including vegetables, fruits, tea, coffee, and wine. Gallic acid elicits several interesting and various biological responses, such as antibacterial, anti-fungal, anti-inflammatory, antiviral, anticancer, antioxidant [Weerasak, and Suwanna, 2007], antimutagenic, and anti-diabetic activities. Gallic acid is trihydroxybenzoic acid, a type of phenolic acid. It is found both free and as a part of tannins which are astringent in nature. As the importance of these antioxidants to human health becomes clearer there is a rapidly expanding search for rich plant sources of these compounds with much attention focusing on the antioxidant potential of Ellagic acid (EA). Ellagic acid has been found to be Hepatoprotective, Antioxidant, Antimutagenic and Antimicrobial.[Sheng, et,al. 2011]. All these properties make Gallic acid and Ellagic acid very important molecules with medicinal values.

The present work reports the results of quantitative estimation of gallic and ellagic acids in *S. dubium* seed extracts by HPTLC, which proved to be rapid, simple and accurate method for quality control of the said two acids in plant extracts.

2. Material andmethods

2.1. Plant Material

The samples of *S. dubium* were collected in the area Al-Halfaya in Khartoum, Sudan and authenticated in Bioactive Natural Product Laboratory, Hamdard University, and Voucher specimen was deposited as (JH/FP/BNPL/2014/IMAN/S1).

2.2. Chemicals and Reference Compounds

Ellagic acid and gallic acid from sigma were used as reference compounds (purity >98%) and other chemicals and reagents used were of analytical grade (AR) and procured from Merck Ltd. India.

2.3. Preparation of standard mixed Ellagic acid and Gallic acid standards

The solutions of the ellagic acid and gallic acid standards 1mg/ml each were prepared in HPLC grade methanol. A mixture of the ellagic acid and gallic acid was prepared by mixing 100 µl of each compound so that the concentration of each compound in the mixture was 1 mg/ml (1000 µg/ml). The mixed solution was used for application on HPTLC plates to prepare the standard plot.

2.4. Preparation of sample extracts

Ten gm of dried and powdered *S. dubium* seeds were extracted using 300 ml of methanol by sonication at 40°C for one hour. The mixture was filtered using Whatman No. 1 filter paper and washed with methanol. To the filtrate dilute HCl was added, heated and concentrated to half and extracted with ethyl acetate. The ethyl acetate fraction was evaporated to dryness in a water bath at 40°C. The dried extract was further dissolved in methanol and filtered using a 0.2-µm syringe filter. The final volume was brought to 5.0 ml with methanol and stored at 4 °C prior application to HPTLC plates for quantification.

2.5. Sample application

The samples were applied as bands (4 mm width), with a CAMAG microlitre syringe on precoated HPTLC silica gel glass plates (60F254; 20 × 10 cm, Merck KGaA, Germany) using a CAMAG Linomat V (Muttenz, Switzerland) and were controlled by WinCATS software (CAMAG). A constant application rate of 100 nl/s was employed and the space between two bands was 7.2 mm. The slit dimension was maintained at 5.0 × 0.30 mm, and a 20 mm/s scanning speed was

employed. The mobile phase consisted of toluene: ethyl acetate: methanol: formic acid: (3:3:1:0.4 v/v/v/v). Linear ascending development was carried out in a 20 × 10 cm twin trough glass chamber, saturated with the mobile phase. The optimized chamber saturation time for the mobile phase was 15 min at room temperature. The length of chromatogram run was 80 mm. Subsequent to development; HPTLC plates were dried in an oven at 60°C for five min. Densitometric scanning was performed on a CAMAG TLC scanner IV (absorbance mode 280 nm) with WinCATS software.

2.6. Calibration curve of Ellagic acid and Gallic acid

Different volumes of the SGE mixture standard solution (0.1, 0.2, 0.4, 0.6, 0.8, 1.0, 2.0, 3.0 µl mg/ml) were spotted in triplicate on the HPTLC plate to obtain 100, 200, 400, 600, 800, 1000, 2000, 3000 ng/spot of each standard, respectively. The data for the peak area Vs ellagic acid and gallic acid concentrations were treated with the linear-least square regression, and the regression equation obtained from the standard curve was used to estimate gallic and ellagic acids in the extract.

2.7. Quantification of Ellagic acid and Gallic acid in extracts

The method was applied to analyze ellagic acid and gallic acid content in *S. dubium* seed extract. Samples of 5.0 µl each were applied in quadruplicate on TLC plates. The *S. dubium* seed yield was quantified using the regression equation from the calibration curve.

2.8. Method validation

The developed method was validated as per the ICH guidelines [Branch, et, al., 2005] similar to the other chromatographic HPTLC methods reported by laboratory [Kamal, et, al., 2012 and Singh, et, al., 2011] which are in use for the quality control of herbal drugs.

2.9. Accuracy as recovery

The accuracy of the present method was assessed in samples through recovery studies, in which pre-analyzed samples were spiked with an extra 50%, 100% and 150% of the standard mixture and analyzed by the proposed method. The experiments were conducted six times, the average recovered ellagic acid and gallic acid content was quantified using the regression equation, and the % recovery was calculated accordingly.

2.10. Precision

Inter-day and intra-day precision analyses were performed by spotting three different concentrations of the ellagic acid and gallic acid stock solution 100, 600 and 2000 ng/spot ng/spot of each for ellagic acid and gallic acid in the same day and in three different days, respectively. Inter-analyst precision was carried out by repeating same procedure with a different analyst. Intermediate precisions were determined in terms of % RSD of the area.

2.11. Specificity

The specificity of the method was assessed by comparing the Rf and absorption spectra of ellagic acid and gallic acid in sample and standard tracks. The peak purity of ellagic acid and gallic acid was assessed by comparing the spectra at three levels, i.e., peak start, peak apex, and peak end position of the band.

2.12. Robustness

The robustness of the proposed method was determined at three concentrations; (100, 600 and 2000 ng/spot of each for ellagic acid and gallic acid) in two different ways, i.e., by changing the composition of mobile phase and changing the detecting wavelength. The % RSD of the peak area was calculated to assess the robustness of the method.

2.13. Sensitivity

The sensitivity of the method was determined as the limits of detection (LOD) and quantification (LOQ). Decreasing amounts of the ellagic acid and gallic acid standard solution were applied to a plate and the chromatographic–densitometric analysis was performed as described above. The concentration of sample giving a signal to noise ratio of three was fixed as the LOD, whereas the concentration of the sample giving a signal to noise ratio of 10 was fixed as LOQ.

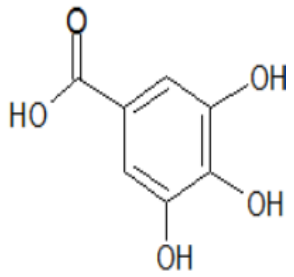
3. Results and discussion

3.1. Optimization of Mobile Phase

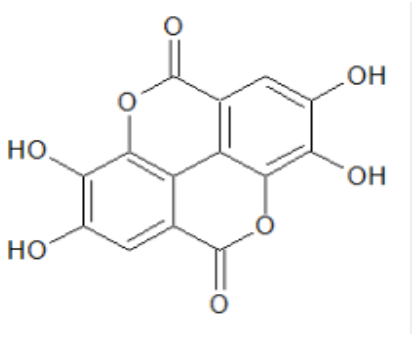
The composition of the mobile phase was optimized by testing different solvent compositions of varying polarities. From the different compositions of the mobile phase tried the desired resolution of compounds together with symmetrical and reproducible peaks, was achieved using toluene: ethyl acetate: methanol: formic acid: (3:3:1:0.4 v/v/v/v) as the mobile phase. Well-separated and compact bands of gallic and ellagic acid were visualized at the R_f value 0.21 and 0.35 respectively, Chromatograms were scanned at 280 nm (Fig. 3 & 4).

3.2. Calibration Curve for Ellagic acid and Gallic acid

The linear regression data for the calibration plot are indicative of a good linear relationship between the peak area and a wide range of concentrations. The linear regression calibration curves plotted the peak area against the concentration and were linear from 100-3000 ng/ml for all standard namely ellagic acid and gallic acid with good linear relationships of r2 = 0.994 and 0.988, respectively, Table1.



Gallic acid



Ellagic acid

Table 1: Linearity data of chromatographic HPTLC method for mixed standards (n=3)

NO.	Biomarkers	Solvent system (v/v/v)	Linearity (ng/spot)	Equation	Regression ± SD	slope ± SD	intercept ± SD	LOD (ng/spot)	LOQ (ng/spot)
1	Ellagic acid	Toluene: ethyl acetate: methanol formic acid(3:3:0.4:1 v/v/v/v)	100-3000	Y=7929± 0.002x	0.988± 0.001	-0.0024	7929±	215	653
2	Gallic acid		100-3000	Y=5734+ 11.97x	0.994± 0.0005	11.97	5734±	130	396.8

LOD: limit of detection; LOQ: Limit of quantification; SD: standard deviation.

3.3. Validation of the method

3.3.1. Accuracy

The accuracy of the method was analysed by the standard addition method at the following levels (0, 50, 100, and 150), which showed good recovery within the range of 98.7-100.2%, 99.23-102.7% and for ellagic acid and gallic acid, respectively Table 2.

Table 2: Accuracy of HPTLC methods for the estimation of mixed standards (n=2)

% of standard spiked to the sample	Theoretical content (µg/ml)	Amount of drug recovered (µg/ml)	% of drug recovered	%RSD
Ellagic acid				
0	2.17	2.15	99.08	1.50
50	3.25	3.20	98.46	0.24
100	6.51	6.40	100.21	0.12
150	9.76	9.50	98.87	0.34
Gallic acid				
0	1.05	1.08	99.23	0.06
50	1.57	1.65	102.34	0.08
100	3.15	3.20	101.58	0.09
150	4.72	4.85	102.34	0.06

3.4. Precision

Intermediate precisions were determined and reported in terms of % RSD. Intermediate precision includes data from inter-day, intra-day and inter-analyst precision measurements. The satisfactory result (% RSD≤ 2) of the precision measurements indicates that the method can be adopted in any laboratory and by any qualified person for the routine analysis of ellagic acid and gallic acid. The results of the analysis are depicted in Table3.

Table 3: Precision of the method for estimation of mixed standards (n=2)

Conc (ng/spot)	Inter-day precision		Intra-day precision		Inter-analyst precision	
	Mean peak area ± SD	% RSD	Mean peak area ± SD	% RSD	Mean peak area ± SD	% RSD
Ellagic acid						
100	3332.96±51	1.5	3367.66±69	2.0	3405.03±55	1.6
600	8486.58±11	0.1	8596.04±13	0.2	8428.81±37	0.4
2000	7751.58±12	0.2	7841.62±25	0.3	7904.31±23	0.3
Gallic acid						
100	3448.5±52	1.5	3267.11±84	2.5	3231.29±94	2.9
600	11198.19±34	0.3	11752.85±170	0.1	12141.80±23	0.2
2000	20858.67±100	0.5	21418.38±56	0.3	22602.29±43	0.2

Conc.: concentration; SD: Standard deviation; RSD: Relative standard deviation.

3.5. Specificity

The spectra of gallic and ellagic acid in both the standard and sample tracks were highly correlated and confirm that the scanning mode is effective and sensitive.

3.6. Robustness

The robustness of the method was assessed by introducing small changes in the composition of the mobile phase and detection wavelength, and the effect on the desired result was reported as % RSD. Mobile phases with compositions of toluene: ethyl acetate: methanol: formic acid (3:3:0.8:0.4, v/v/v/v), (3:3:0.5:0.2, v/v/v/v and (3:3:0.5:0.8 v/v/v/v) were used and a detection wavelength of 280 ± 2 nm varied to identify any variation. The results of the robustness measurements are shown in Table 4 (a and b).

Table 4: Robustness of the HPTLC method for estimation of mixed standards by changing a) detecting wavelengths (n=2) and (b) mobile phase composition (n=2)

Detection wavelength				
Components	**Conc (ng/spot)**	**Wave length used**	**Mean area ± SD**	**%RSD of area**
Ellagic acid	100	270	3540.41±74	2.1
		290	3425.08±37	1.1
	600	270	8719.85±39	0.4
		290	8460.19±43	0.5
	2000	270	7555.97±11	0.1
		290	7908.97±50	0.6
Gallic acid	100	270	3272.77±73	2.2
		290	3415.85±28	0.8
	600	270	12630.05±32	0.3
		290	11491.79±83	0.7
	2000	270	22178.70±97	0.4
		290	25036.49±57	0.2

Mobile phase composition Toulene: ethyl acetate: formic acid: methanol (3:3:1:0.4 v/v/v/v)							
		3:3:0.8:0.4		3:3:0.5:0.2		3:3:0.5:0.8	
Components	Conc (ng/spot)	Mean peak area ± SD	% RSD	Mean peak area ± SD	% RSD	Mean peak area ± SD	% RSD
Ellagic acid	100	3251.58±28	0.9	3275.07±50	1.5	3140.74±34	1.1
	600	8829.35±32	0.4	8730.25±35	0.4	8319.52±33	0.4
	2000	7813.24±74	0.9	7800.31±82	1.5	7526.97±61	0.8
Gallic acid	100	3580±32	0.9	3124.09±67	0.3	3450.23±20	0.6
	600	12415.12±23	0.2	11982.34±11	1.3	13667.23±45	0.3
	2000	25334.16±47	0.2	23445.45±40	1.5	22897.67±49	0.2

3.7. Sensitivity

The limit of detection (LOD) and limit of quantification (LOQ) represent the concentration of the analyte that would yield signal to noise ratio of 3 and 10, respectively. For the proposed HPTLC method, the LOD and LOQ of ellagic acid and gallic acid were 215.5 and 130, 653 and 396.8 ng/spot respectively.

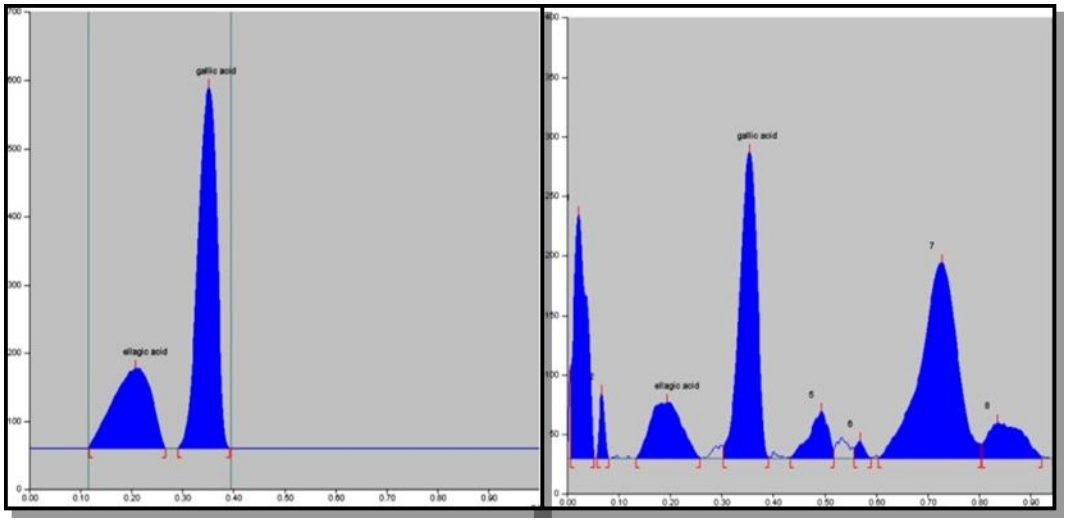


Figure 1: HPTLC chromatogram of standards ellagic acid (R_f 0.21) and gallic acid (R_f 0.35) at 280 nm.

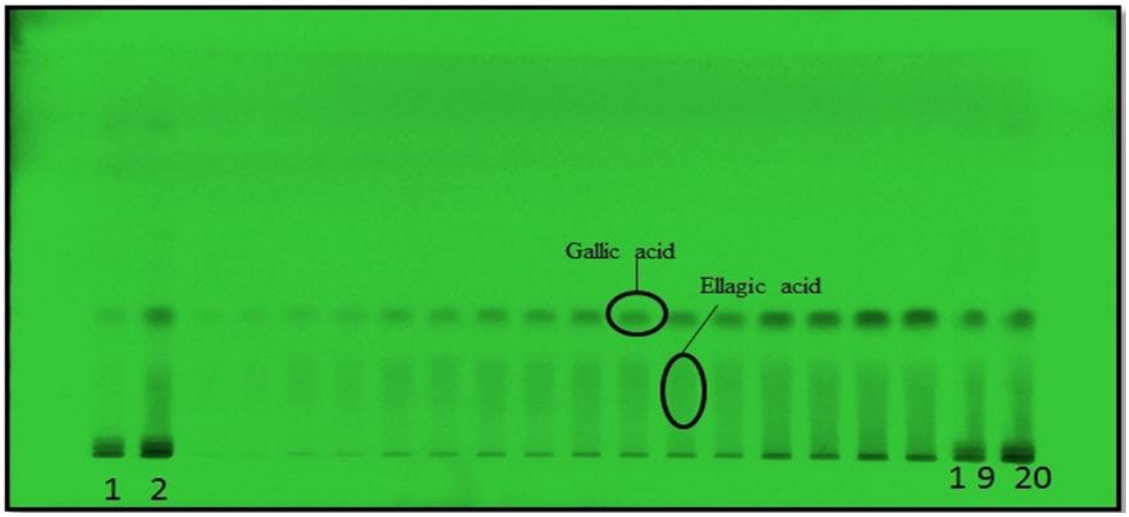


Figure 2: HPTLC Chromatogram of the sample at 280 nm using the solvent system toluene: ethyl acetate: methanol: formic acid (3:3:0.4:1, v/v/v/v) showed the presence of ellagic acid and gallic acid

Track number: 1, 2, 19 and 20 correspond to *S. dubium* seed extract

3.8. Quantitative Evaluation of Gallic acid and Ellagic acid in *S. dubium* seed

Gallic acid and ellagic acid concentrations in sample were analysed from the regression equation using values of area obtained from WinCATS software. The mean values of gallic acid and ellagic acid in sample are shown in Fig. 4. The study revealed that *S. dubium* seeds contained gallic acid 2.1% and ellagic acid 1.4% Table 5. gallic acid and ellagic acid could be good lead compounds for new drugs development from *S. dubium* seed.

Table 5: The content of ellagic acid and gallic acid present in sample of *S. dubium* seed

Sample	**Content %**	
	Ellagic acid	Gallic acid
S.dubium seed extract	1.4	2.1

4. Conclusion

A specific, precise, accurate, sensitive, rapid and reliable HPTLC method has been developed and validated. This method can be used in the Quality Control Department for estimation of gallic acid and ellagic acid in *S. dubium* seed. The proposed HPTLC method was found to be rapid, simple and accurate for quantitative estimation of gallic acid and ellagic acid in extracts. The recovery values of gallic acid were found to be 99.23- 102.7 % and 98.7- 100.2%, for ellagic acid which shows the reliability and suitability of the method. The seeds were found to contain 1.4% w/w of ellagic acid and 2.1% w/w of gallic acid in extract.

Compliance with ethical standards

Acknowledgments

We are grateful to the Department of Bioactive Natural Product Laboratory, Faculty of Pharmacy, Hamdard University, Hamdard Nagar, New Delhi, India-110062, especially Dr. Sayeed Ahmad for providing laboratory space and facilities to complete this work.

Disclosure of conflict of interest

There is no any conflict of interest exist.

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