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Research Article

RAPID DENSITOMETRIC METHOD FOR QUANTIFICATION OF GALLIC ACID AND COUMARIN IN THE LEAF OF MEDICINAL PLANT *POUZOLZIA BENNETTIANA* FROM THE NILGIRI HILLS USING HPTLC

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ABSTRACT

The primary aim of the study is to identify and quantify the amount of gallic acid and coumarin present in the methanolic leaf extract of *Pouzolzia bennettiana* by HPTLC technique. The plant extract was collected using Soxhlet apparatus. Standard gallic acid and coumarin were prepared separately in 1 mg/ml concentration. The methanolic leaf extract was prepared as 100 $mgmL^{-1}$ concentration. The standard and sample were loaded on a 10 x 10 Silica gel 60F254 with 250 µm TLC plate using Hamilton syringe and CAMAG LINOMAT 5 instrument. It was developed with respective mobile phase Toluene: Ethyl Acetate: Formic acid: Methanol in the ratio of 3:6:1.6:0.4 v/v/v/v up to 80 mm. It was later scanned at 500 nm. The developed method was robust. The amount of gallic acid and coumarin were found to be 0.43 (%W/W) and 0.36 (%W/W) respectively. The LOD was 1.75062307 µg/spot for gallic acid and 1.650265 µg/spot for coumarin. The LOQ was found to be 5.30491838 µg/spot for gallic acid and 5.000802 µg/spot for coumarin. The several constituents present in the extract did not interfere with the peak of gallic acid and coumarin proved the specificity. The compounds gallic acid a part of tannin a phenolic acid and coumarin a hydroxyl cinnamic acid group has several uses. The developed HPTLC method was rapid, accurate, precise, reproducible and specific for the simultaneous estimation of gallic acid and coumarin. This can be applied in routine quality control and analysis of plants and herbal medicines.

Keywords: High performance thin layer chromatography, *Pouzolzia bennettiana,* Medicinal plant, Gallic acid, Coumarin, Quantification

INTRODUCTION

Plants have always served as novel weapon against several diseases. The most specific reasons for using medicinal plants are fewer side effects and it is cost effective[1,2]. Usage of natural products is radically changing due to market outcome in the form of new supplements and mainly is on plants and its origin. The secondary metabolites of plants proved to cure human disorders. They can be used for their several pharmacological actions. Several pharmacopoeia describe the physicochemical parameters of the plant constituents. Thus, the modern methods are used to identify and quantify the active constituents in plant material can be used for proper standardization and its formulations[3]. Those methodologies can generate fingerprint in large collections that could be useful to detect the stability of the same over a period of time. It includes methods of extraction and analytical methods such as spectrophotometric analysis, High-Performance Liquid Chromatography (HPLC), Gas Chromatography-Mass Spectrometry (GC-MS) and Fluorescence Transmission–Infrared Spectroscopy (FT-IR). These methods are developed for the study of active pharma compounds or substances[4].

HPTLC could be a good alternative tool in routine drug analysis which holds off major advantages such as reduced time, minimizing exposure risks and significant reduction in toxic environmental condition which also facilitates repeated detection of chromatogram with same or different parameters by analyzing several samples simultaneously[5-7]. *Pouzolzia bennettiana* Wight (Urticaceae) is traditionally employed as a folklore remedy to treat wide spectrum of ailments such as stomach pain, to treat cuts, for bone fracture and dislocation and to heal skin burn with inflammation[8-10]. In addition, it is eaten to increase the secretion of milk in expecting mothers. *Pouzolzia bennettiana* plant extract possess unique bio molecules which are rich in phenolic and flavonoid compounds. The plant phenolics are used as valuable sources of natural drugs, antibiotics, antiviral, antitumor, biocidal and bioactive agents[11].

The phenolic acid gallic acid and coumarin detected in the extracts possess several favourable activities on human health such as lowering low density lipoprotein, anti- inflammatory, anti-allergic, antiulcer, antiviral, anti-purgative, antimicrobial and protect cardiovascular mortality[12]. Thus the present study investigated the qualitative and quantitative determination of Coumarin and Gallic acid of methanolic leaf extract of *Pouzolzia bennettiana* against standards using HPTLC fingerprinting method.

MATERIALS AND METHODS

Instrumentation

High performance thin layer chromatography analysis for detection and quantification of gallic acid and coumarin was performed on a Camag HPTLC system fitted with Linomat V sample applicator, twin trough development chamber (10x10 size) and TLC Scanner III. Wincats integration software was used.

Reagents and Chemicals

Analytical grade Toluene, Ethyl acetate, Methanol and Formic acid were purchased from Merck Ltd, Mumbai. Pure Gallic acid and Coumarin were obtained from the Natural Remedies Ltd, Bangalore. Pre coated TLC aluminium sheets silica gel 60F254 (10 x10 cm, 0.2 mm thick) was obtained from E. Merck Ltd, Mumbai.

Collection of Raw Material

Pouzolzia bennettiana was collected from Kotagiri, The Nilgiris, Tamil Nadu, India and it was taxonomically authenticated by The Scientist, Botanical Survey of India, TNAU campus, Coimbatore. Voucher number BSI/SRC/5/23/2012-13/Tech./793.

Preparation of standard Gallic acid and Coumarin Solution

10 mg of Gallic acid and Coumarin were accurately weighed and transferred into 10 mL volumetric flask, and then the solution was made up to 10 ml with methanol (1 $mgmL^{-1}$)[13].

Preparation of Extract Solution

The leaf powder was extracted by successive solvent extraction in Soxhlet apparatus using petroleum ether and then methanol. The solvent was removed under the vacuum at temperature below 50°C and the extracts were freeze-dried. All the extracts were stored in vacuum desiccator for further studies. 1000 mg of methanolic extract of leaf was weighed in 10 mL volumetric flask. The extract was made up to 10 mL with methanol (100 $mgmL^{-1}$).

Sample application

1 mg/ml of 3 µl band length of standard and extract were loaded in the 10 x 10 Silica gel 60F254 with 250 µm thickness (E-Merck, Darmstadt, Germany) TLC plate using Hamilton syringe and CAMAG LINOMAT 5 instrument.

Development

The samples loaded plate was kept in TLC twin trough developing chamber (after saturation with Solvent vapor) with respective mobile phase Toluene: Ethyl Acetate: Formic acid: Methanol in the ratio of 3:6:1.6:0.4 v/v/v/v (Wagner and Blades, 1996) up to 80 mm. The developed plate was dried by hot air to evaporate solvents from the plate. The plate was kept in Photo-documentation chamber (CAMAG REPROSTAR 3) and the images were captured at UV 254 nm and UV 366 nm. The plate was fixed in scanner stage (CAMAG TLC SCANNER 3) and scanning was done at 500 nm.

Derivatization

The developed plate was sprayed with anisaldehyde sulphuric acid and dried at 100°c in hot air oven for 3 min. The RF values and fingerprint data were recorded by WINCATS SOFTWARE.

Stability

Sample solutions of extracts were prepared and stored at room temperature for 3 days and then applied on the sample HPTLC plate. The chromatogram was evaluated for band similarity and resolved peaks at intervals.

Calibration curve for standard gallic acid

The standard was spotted on TLC plate, developed and scanned following the chromatographic conditions mentioned above. The peak areas were noted. Calibration curve was obtained by plotting peak area against concentration of gallic acid.

Calibration curve for standard coumarin

The standard coumarin was spotted on TLC plate, developed and scanned following the chromatographic conditions mentioned above. The peak areas were noted. Calibration curve was obtained by plotting peak area against concentration of coumarin.

Validation of the method

The ICH guidelines such as linearity, accuracy, correlation coefficient, LOD, LOQ, recovery and specificity were followed to validate the analytical methods used for analysis[14,15]. Results of validation can access the consistency, quality and reliability.

Linearity was observed by applying different aliquots of standard solution for gallic acid and coumarin. The calibration curve was developed by plotting peak area range 3-15 µg/ml against concentration. The limit of detection and limit of quantification was calculated using the equation

$$\text{LOD} = \frac{3.3 \times \text{Standard Deviation of the } y\text{-intercept}}{\text{Slope of the calibration curve}}$$

$$\text{LOQ} = \frac{10 \times \text{Standard Deviation of the } y\text{-intercept}}{\text{Slope of the calibration curve}}$$

The accuracy was ascertained by repeating the experiment in triplicates and determining the recovery studies at three levels. The specificity was determined by the peak purity of the component by overlaying the fluorescence spectra of gallic acid and coumarin in the sample extract with the spectra of the reference standard of gallic acid and coumarin at the start, middle and end positions of the bands. The statistical analysis was performed using Microsoft Excel.

RESULTS AND DISCUSSION

Herbal medication is gaining importance since the human body has good compatibility and it is accepted by many. The lesser side effects are another major reason for use. Plants generally produce several secondary metabolites which are utilized by humans for various applications. At present there is increased interest on such secondary metabolites[16,17]. Quality evaluation is the basic requirement of all organizations involved in herbal preparation and it should be standardized with respect to safety before use in market[18]. According to phyto equivalence concept, chromatographic fingerprinting of herbal medicines is used to address the problem of quality control of herbal medicines.

Quantification of Gallic acid and Coumarin in methanolic leaf extract of *Pouzolzia bennettiana*

There are no reports in the literature concerning the quantification of gallic acid and coumarin in methanolic leaf extract of *Pouzolzia bennettiana*. Different mobile phase system with different composition for HPTLC analysis was tested to obtain reproducible peaks and high resolution. Finally, the mobile phase used was Toluene- Ethyl acetate- Formic acid- Methanol (3:6:1.6:0.4). Standard gallic acid and coumarin showed single peaks in chromatogram. Gallic acid and Coumarin were well resolved at R_f 0.74 and 0.95 respectively. The amount of gallic

acid and coumarin were calculated from the peak area and calibration curve. The presence of Gallic acid and coumarin was identified in nettle plant *Urtica dioica* L.[19]. Few other flavonoids such as chlorogenic acid, isoorientin, orientin and isovitexin were identified and quantified in *Cecropia* (Urticaceae)[20,21]. The amount of gallic acid in 100 mg of extract was 0.43% and amount of coumarin was 0.36% (Table 1). Around 300 types of flavonoids are identified, and they are group of poly phenolic compounds. It is widely spread in the plant kingdom. Gallic acid is a phenolic acid which occurs freely or as a part of tannin molecule. It has several properties such as antifungal, antiviral, treats albuminuria and diabetes, protects the cells from oxidative damage, antioxidant, neuropsychological, urogenital, oral health, respiratory and anticancer agent[22]. Coumarin is a fragrant organic chemical compound found in many plants. Coumarin has blood-thinning property. It also possesses anti-fungal and anti-tumor activities[23].

Table 1: Quantification of Gallic acid and Coumarin in *Pouzolzia bennettiana* methanolic leaf extract

Sample	Amount Present	
	Gallic acid (%W/W)	Coumarin (%W/W)
Extract	0.43	0.36

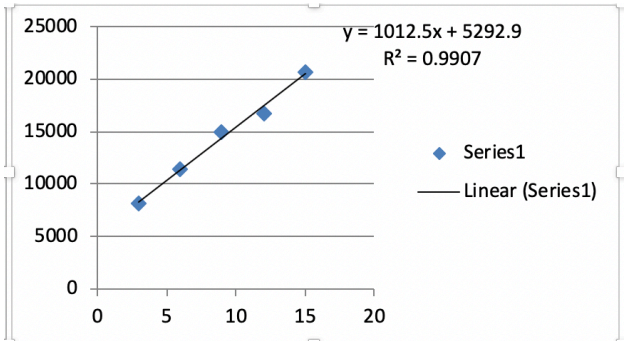

Figure 1: Calibration curve for Gallic acid

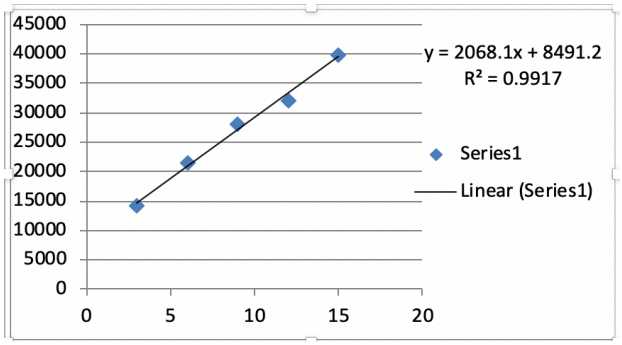

Figure 2: Calibration curve for Coumarin

Linearity

The calibration curve of gallic acid and coumarin was obtained by plotting peak area of gallic acid and coumarin against concentration of gallic acid and coumarin respectively. The linearity range was 3-15 µg/ml for both gallic acid and coumarin. The correlation coefficient was 0.990 for gallic acid and 0.991 for coumarin (Figure 1,2). Thus, it exhibits good linearity.

Limit of Detection

The minimum detection limit was found to be 1.75062307 µg/spot for gallic acid and 1.650265 µg/spot for coumarin.

Limit of Quantification

The minimum quantified limit was found to be 5.30491838 µg/spot for gallic acid and 5.000802 µg/spot for coumarin.

Table 2: Summary of Parameters

Parameters	Gallic Acid	Coumarin
Accuracy	99.6130874 ± 5.013733101 (Mean ± SD)	99.20949 ± 6.388894
Slope	1012	2068
Intercept	5292	8491
Linearity range	3-15 µg/ml	3-15 µg/ml
Correlation coefficient	0.990	0.991
SE of intercept	1084.642393	1084.642393
SD of intercept	2425.26039	2425.26039
LOD	1.75062307 µg/spot	1.650265 µg/spot
LOQ	5.30491838 µg/spot	5.000802 µg/spot

Specificity

It was found that several constituents present in the extract did not interfere with the peak of gallic acid and coumarin. The overlay spectrum was found to be similar. The peak purity was found by comparing the spectra at different levels such as peak start, peak apex and peak end positions of the spot. Thus, the method is specific.

Accuracy

The accuracy was determined by increasing amount of gallic acid and coumarin in all levels of calibration curve. The percentage recovery is tabulated (Table 3).

Table 3: **Recovery studies for Gallic acid and Coumarin**

Compound	Amount added µg	Peak area	Found Concentration	Recovery (%)
Gallic acid	3	8170	2.843873518	94.795783
	6	11427	6.062252964	101.03754
	9	14978	9.571146245	106.34606
	12	16762	11.33399209	94.44993
	15	20690	15.21541502	101.4361
Coumarin	3	14094	2.709381044	90.3127
	6	21430	6.256769826	104.2795
	9	28118	9.490812379	105.4535
	12	32098	11.41537718	95.12814
	15	39782	15.13104449	100.8736

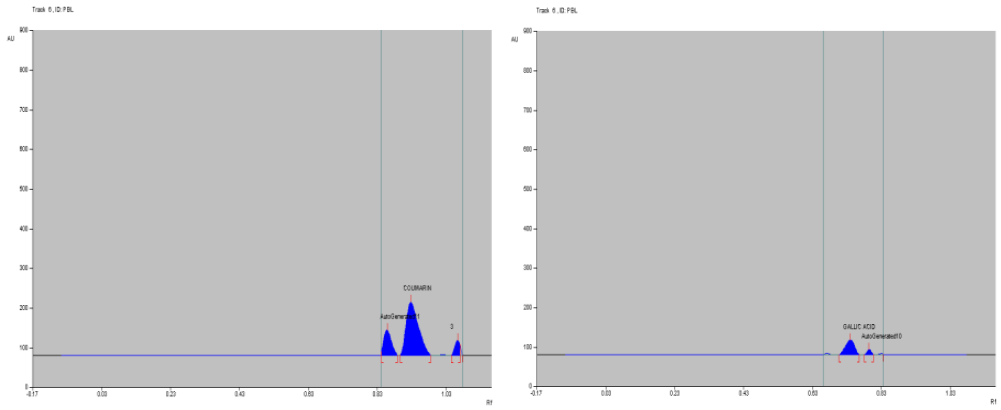

Figure 3: HPTLC Chromatogram of methanolic leaf extract of *Pouzolzia bennettianna* showing gallic acid and coumarin at 500 nm

CONCLUSION

The HPTLC method for the quantification of Gallic acid and Coumarin in the methanolic leaf extract of *Pouzolzia bennettiana* is established. The flavonoids were quantified and added merit to the scientific property of *Pouzolzia bennettiana*. Scientists in developing countries expect plants to occupy the major role in treatment. This sort of research will help in scientific analysis and drug development. This quantification method is specific, accurate, repeatable, precise and simple. It can be used in quality control of herbal medicines. The extract also possess several other compounds which are currently the subject of further investigation.

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