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RESEARCH ARTICLE

Quantitative Determination of Gallic Acid from Methanolic Extract of *Cocculus hirsutus* using HPTLC

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ABSTRACT

Cocculus hirsutus is antipyretic, tonic, lessens thirst, good for fractures and useful in tubercular glands related problems. It is a well-known herb used as first aid remedy in minor injuries. During the present study a rapid, simple, accurate and specific HPTLC method for quantitative estimation of Gallic acid present in the dried leaf powder and callus of *C. hirsutus* has been developed. The method used in this work resulted in good peak shape and enabled good resolution of Gallic acid from *C. hirsutus* samples. Gallic acid was identified in *in vivo* (leaf) and *in vitro* (six weeks old callus) tissues. Presence of isolated Gallic acid was further confirmed by super imposable IR spectra of isolated and authentic samples of Gallic acid. Variation in Gallic acid content in *in-vivo* and *in-vitro* samples in *C. hirsutus* was observed. *In vivo* leaf had maximum amount of Gallic acid (0.44%) while minimum amount was found in *in vitro* callus (0.10 %). High content of Gallic acid in leaf shows its potential of synthesizing Gallic acid. This study is also of practical importance because Gallic acid has quite interest of in food, cosmetic and pharmaceutical industries, as substitutes for synthetic antioxidants. The Gallic acid exhibit a wide range of biological effects including antibacterial, anti-inflammatory, antiallergic, hepatoprotective, antithrombotic, antiviral, anticarcinogenic and vasodilatory actions.

Key words: *Cocculus hirsutus*, HPTLC, IR, Gallic acid, leaf, callus.

INTRODUCTION

Tannins are natural compounds widely distributed in the plant kingdom. The role of tannins is in the defense system of plants against microbial and animal attacks due to their astringent capacity and the ability to form complexes with proteins and polysaccharides[1]. Tannins are secondary metabolites of plants. It is generally accepted that considering their sugar content, polymerization and esterification degrees they are divided into three groups: condensed tannins, hydrolysable tannins and complex tannins.

Gallic acid (GA) is a phenolic compound in which the phenolic groups that serve as a source of readily available hydrogen atoms such that the subsequent radicals produced can be delocalized over the phenolic structure[2,3]. The interest in these compounds is due to their pharmacological activity as radical scavengers [4]. It has been proved that they have potential preventive and therapeutic effects in many diseases, where the oxidative stress has been implicated, including cardiovascular diseases,cancer, neurodegenerative disorders and in aging. The phenolics attract great interest in food, cosmetic and pharmaceutical industries, as substitutes for synthetic antioxidants [5]. Several chromatographic methods have been documented for determination of Gallic acid in plant extracts [6] but due to the complex nature and inherent variability of the chemical constituents of the plant based drugs, it is difficult to establish quality control parameters and hence modern analytical techniques are expected to help in circumvention of this problem.

Cocculus hirsutus is antipyretic, tonic, lessens thirst, good for fractures, and useful in tubercular glands related problems. It is a well-known herb used as first aid remedy in minor injuries. It alleviates *kapha* and *vata* doshas. It is used as *deepanee*, *pachanee* and *raktdoshagni*. It possesses light, oily and slimy attributes. It has a special potency as a detoxifier. It is an aphrodisiac and tonic in properties [7]. Due to the presence of phenolic compounds in the plant, it is used as antimicrobial, preventive in infection and it enhances healing. Tannins and flavanoids are present in glycosidic combination. Tannins have

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stringent and healing properties. Hence it is used in the treatment of wounds, burns and ulcers. Gallic acid has been reported in many plants by several workers[8-10].The objective of the present investigation was to establish and validate the fast and sensitive high performance thin layer liquid chromatography (HPTLC) method for determination of gallic acid in methanolic extract of *Cocculus hirsutus*.

MATERIALS AND METHODS

Chemicals

All the chemicals used in the experiments were of analytical grade procured from Merck. Reference standard Gallic acid was purchased from Sigma Chemicals.

Plant material

The leaves of *C. hirsutus* used in the present investigation were collected fresh from their natural habitats and cut into small pieces, dried, powdered and used for the estimation of Gallic acid contents. Unorganized cultures were raised and established. These cultures were harvested at their maximum growth. Tissue samples were dried at 100^0C for 15 min. to inactivate enzymes followed by 60^0C till constant weight was achieved. The dried leaves were then powdered and analyzed separately for their Gallic acid contents. Five replicates were taken in each case.

Extraction procedure

Each of the powdered tissue samples viz. *in vivo* (leaf) and *in vitro* (callus) was taken in a soxhlet apparatus and extracted with methanol for 24 hrs. The solvent was recovered by distillation. The residue was concentrated, dried and stored in the desiccators for further experiment and analysis. The dried residue was taken up in methanol for further analysis. Isolation, identification and quantification of gallic acid were carried out by TLC, HPTLC and IR spectral studies in the plant species.

HPTLC Chromatographic conditions

Stationary Phase:
Precoated silica gel plates Merck 60 F_{254} (10 x 10, 0.2 mm thickness).
Mobile Phase:
Toluene: ethyl acetate: formic acid: methanol (3:3:0.8:0.2 v/v)
Spotting Device:
Linomat V Automatic sample spotter, CAMAG (Switzerland).
Development Mode:
CAMAG twin trough chamber, CAMAG Densitometer: TLC Scanner III, CATS software, CAMAG.

Preparation of standard solution

The stock solution of gallic acid (20µg/ml) was prepared by transferring 2 mg of gallic acid, accurately weighed, into a 100 ml volumetric flask, dissolving in 50 ml methanol. It was then sonicated for 10 minutes and the final volume of the solution was made up to100 ml with methanol to get a solution of appropriate range of Gallic acid.

Instrumentation and chromatographic conditions

HPTLC was performed on 20 cm×10 cm aluminium packed plates coated with silica gel 60 F254 (Merck, Mumbai, India). Standard solution of Gallic acidand sample solution were applied to the plates as bands 8.0 mm wide, 30.0 mm apart and 10.0 mm from the bottom edge of the same chromatographic plate by use of a Camag (Muttenz, Switzerland) Linomat V sample applicator equipped with a 100µL Hamilton (USA) syringe. Ascending development to a distance of 80 mm was performed at room temperature (28 ± 2^0C), with Toluene: ethyl acetate: formic acid: methanol (3:3:0.8:0.2 v/v) as mobile phase in a Camag glass twin-trough chamber previously saturated with mobile phase vapour for 20 minutes. After development, the plates were dried in air and were sprayed with 5% methanolic $FeCl_3$and subsequently heated at 120^0C for derivatization. These plates were scanned and visualized under visible light at 525 nm and UV light at 254 nm and 366 nm absorbance/reflection mode using reflection mode by CAMAG Scanner III and CATS software and deuterium lamp was used to analyze the plates.

Calibration curve of the standard

A stock solution of standard gallic acid (20µg/ml) was prepared in methanol. Different volumes of stock solution 2,4,6,8 and 10 µl were spotted on to TLC plate to obtain concentration 20, 40, 80, 120, 160, 180 and 200 µg/spot of gallic acid respectively. The working standard was applied on precoated silica gel F_{254} HPTLC plates and the plates were developed as described earlier. The peak areas were recorded. The calibration curve of the standard concentration (X-axis) over the average peak height/area (Y-axis) was prepared to

get a regression equation by Win Cats software, which was used for the estimation of Gallic acid.

HPTLC Quantification of the extracts

The gallic acid content of various extracts was determined by comparing the area of chromatogram with the calibration curve of concentration of standards. The R_f value of standard gallic acid (0.41) was compared with the R_f value of the extracts. Quantitative estimation of the plate was performed in the remission/absorption mode at 254 nm; with the following conditions slit width 6.00x0.30 mm, micro scanning speed 20 mm/s and data resolution 100 µm step. Calibration parameters were as follows: calibration mode- single level, statistics mode-cv, evolution mode- peak height. The average content of gallic acid in different extracts was expressed in percentage.

IR Spectral studies

Each of the fluorescent spots coinciding with those of standard reference compound of Gallic acid was marked, scrapped and collected separately with the adsorbent from plates. The bands were then eluted with methanol, elutes dried in vacuo and crystallized separately with acetone and methanol. Each of the crystallized isolates from all the samples tested were subjected to Infra-red spectrophotometric (Perkin-Elmer 337 Grating, Infra-red spectrophotometer using nujol or potassium bromide pellets) studies along with respective standard compound of Gallic acid.

RESULTS AND DISSCUSION

In the present study, the HPTLC procedure was optimized with a view to quantify the samples extract. The conditions used to extract samples have an important effect on the efficiency of extraction and subsequent isolation of marker compounds present in the samples. Different extraction solvents (diethyl ether, methanol, 95% ethanol, petroleum ether, and ethyl acetate) were tested and methanol was finally adopted because the efficiency of extraction was high and fewer interfering compounds were extracted. Initially toluene: ethyl acetate: formic acid: methanol in varying ratio was tried. The mobile phase toluene: ethyl acetate: formic acid: methanol (3:3:0.8:0.2 v/v) gave good resolution with R_f= 0.41. Well defined spots were obtained when the chamber was saturated with mobile phase for 20 min. at room temperature. The TLC plates were visualized under UV light at 254 nm after derivatization. A photograph of a TLC plate after chromatography of Gallic acid standard and a methanolic extract of the samples of *C. hirsutus* was shown in (**Plate-1, Figs. A&B**).

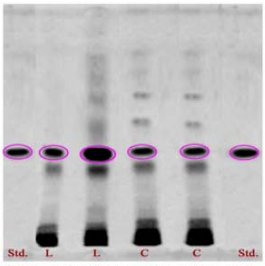


Fig. A: TLC of gallic acid in visible light.

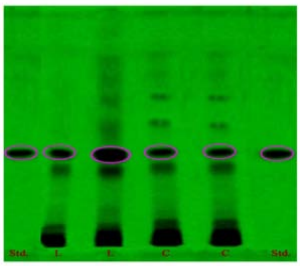


Fig. B: TLC of gallic acid at 254 nm.

PLATE-1

The identity of the Gallic acid bands in the sample chromatogram was confirmed by the chromatogram obtained from the sample with that obtained from the reference standard solution. The R_f's obtained for the said plant extracts closely replicate the R_f's found for standard gallic acid, thus making it a significant fingerprint parameter. The chromatogram of standard gallic acid is shown in (**Plate-2, Fig. A**)

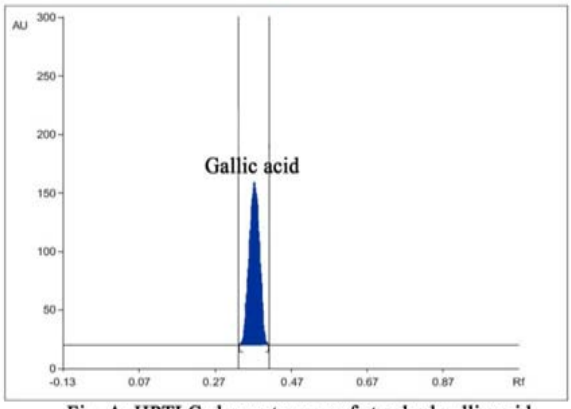


Fig. A: HPTLC chromatogram of standard gallic acid.

PLATE-2

and the gallic acid identified in *C. hirsutus* leaf and callus samples are shown in (**Plate-2, Figs. B&C.**)

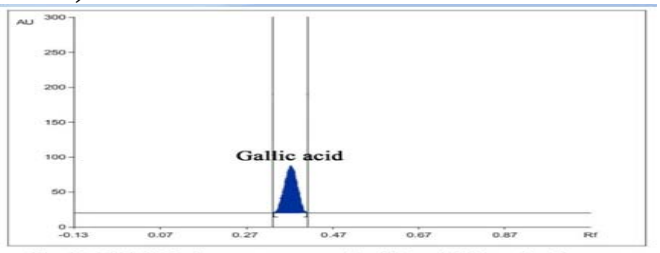


Fig. B: HPTLC chromatogram of gallic acid from leaf extract.

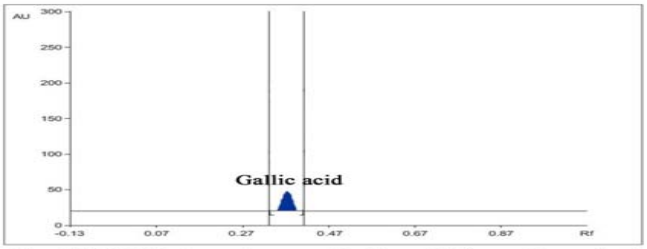


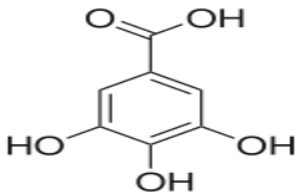
Fig. C: HPTLC chromatogram of gallic acid from callus extract.

PLATE-2

The respective R_f's obtained for each sample are shown in (**Table 1**).

Table 1: Chromatographic data for HPTLC of Gallic acid

Sample	Rf	Maximum height	Area	Content (%)
Standard	0.41	170.6	9701.8	100%
Leaf	0.40	95.5	2750.8	0.44%
Callus	0.42	48.3	642.4	0.10%



Structure of Gallic acid

The peak corresponding to gallic acid (0.40, 0.42) from the sample solution had same retention factor as that of standard gallic acid (0.41). The characteristics IR spectral peaks were found to be superimposable with those of their respective standard reference of gallic acid (**Plate3, Fig A**).

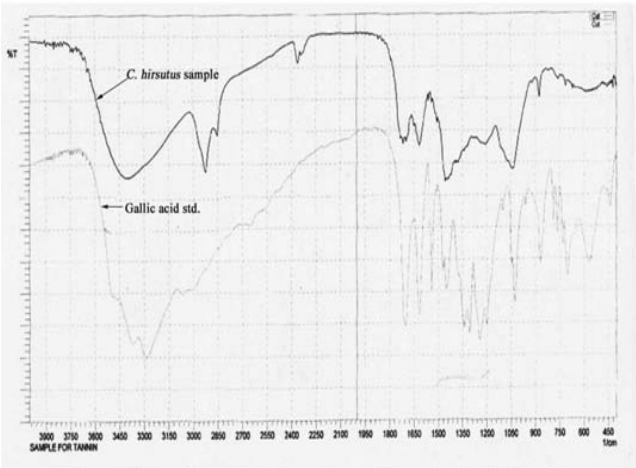


Fig. A: Superimposed IR spectra of standard gallic acid and isolated gallic acid.

PLATE-3

The 3D spectra of all tracks scanned at 580 nm are shown in (**Plate 3, Fig B**).

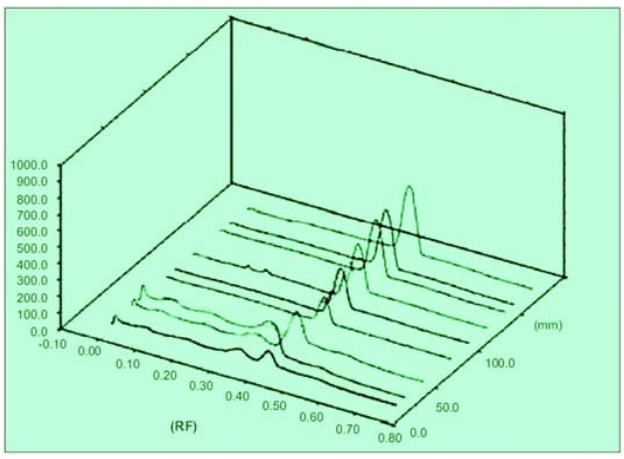


Fig. B: 3-D spectra of gallic acid.

PLATE-3

A three dimensional view enhances the visible similarities amongst all spectral tracks at a selected wavelength which in the present study was 580 nm at different vertices thus bringing out the desired fingerprints. The 3D spectra obtained from the present study has brought out the spectra's for all tracks viewed together and are suggestive of similarities between the test tracks and the standard tracks also elucidating strong presence of the biomarker in the plant extracts. The linearity regression for the calibration showed correlation coefficient of 0.99 with respect to height and area in the range of 2.0-10.0 µl and the content of gallic acid in methanolic extract of leaf and callus was found to be 0.44 % and 0.10% respectively.

In the present study, gallic acid was identified in *in vivo* (leaf) and *in vitro* (six weeks old) callus tissues. Presence of isolated gallic acid was further confirmed by super imposable IR spectra of isolated and authentic samples of Gallic acid. Variation in Gallic acid content in *in vivo* and *in vitro* samples in *C. hirsutus* was observed. *In vivo* leaf had maximum amount of gallic acid (0.44%) while minimum amount was found in *in vitro* callus (0.10 %). This study found that methanol was the best solvent for the extraction of *C. hirsutus* since it produced good resolution of gallic acid from *C. hirsutus* samples. There are several reports, which depict the presence of gallic acid from various plant species *in vivo* and *in vitro* viz. *Terminaliachebula*[11],*Droseraindica*[12],*Polyalthi alongifolia*[13],*Cassia alata* and *Andrographis paniculata*[14], *Schinopsis brasiliensis*[15]and *Zanthoxylum acanthopodium*[16]. This suggests that *Cocculus hirsutus* can be considered as a potential Gallic acid source. From this point of view, our first study in gallic acid isolation and identification from *in vivo* and *in vitro* plant parts of *C.hirsutus* can fulfill an important deficiency in the published research findings; can be a startingpoint for the study of the potential pharmacological properties of this plant species.

CONCLUSION

On the basis of the results of the present study, it was concluded that the gallic acid was successfully isolated from *Cocculus hirsutus*. A rapid, simple, accurate and specific HPTLC method for quantitative estimation of gallic acid present in the dried leaf and callus of *C. hirsutus* has been developed. The method used in this work

resulted in good peak shape and enabled good resolution of gallic acid from *C. hirsutus* samples. The newly established method for isolation and purification enables ready access to large amounts of highly pure samples of the reference standards. The simple, practical, and low cost HPTLC method described for assay of the important Gallic acid chosen as marker can be successfully used to evaluate the quality of *C. hirsutus* with good sensitivity and linearity .The more research on the purification and structure elucidation of Gallic acid in the *Cocculus hirsutus* leaves may be focused and carried out in future.

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