

High-performance liquid chromatography analysis of gallic acid and kaempferol in chloroform and ethanol extract of *Cassia hirsuta* seeds

Abstract

Aim: The present study aim was to analyze the gallic acid and kaempferol in chloroform and ethanol extract of *Cassia hirsuta* seeds by high-performance liquid chromatography (HPLC) method. **Materials and Methods:** In this study, the HPLC analysis of gallic acid and kaempferol was done by selected HPLC methods and these were validated for linearity, precision, limit of detection, limit of quantitation, repeatability, and reproducibility. The corresponding concentration of gallic acid and kaempferol against respective peak area values was determined using the gallic acid and kaempferol calibration curves, respectively. **Results:** In results, it was found that the linearity was good with regression equation $y=36934x-10162$ and $y=61050x-9395.5$ for gallic acid and kaempferol, respectively, and the correlation coefficient (R_2) was 0.9982 and 0.9992, respectively. Percentage relative standard deviation of 1.6% and 0.71% for gallic acid and kaempferol, respectively, indicates the high precision. Ethanol extract of *C. hirsuta* shows high content of both gallic acid and kaempferol as compared to chloroform extract of *C. hirsuta*. **Conclusion:** In the present study, gallic acid and kaempferol content were determined in ethanol and chloroform extract of *C. hirsuta*. The developed HPLC methods have enabled rapid, linear, accurate, and reproducible analysis in these two extracts of *C. hirsuta*.

Key words: *Cassia hirsuta*, flavonoids, high-performance liquid chromatography, linearity, phenols, precision

INTRODUCTION

Traditional medicines gain wide interest in global public health, in that plant-based medicine has taken major place in extensive research on the natural origin substances. This type of interest was developed in belief that herbal medicine is safe and dependable, compared with allopathic drugs. Around \$107 billion herbal formulation market presents in India and 2.5% herbal medicines export to global markets. Most of the plants contain that phenolic flavonoids are core for plant research.^[1] Most flavonoids exhibit various pharmacological activities through antioxidant, neuroprotective effects,^[2] and immune modulator activity.^[3] Indian medicinal plants pharmacological activity is due to the presence of gallic acid, ellagic acid, catechin, kaempferol, quercetin, etc.

Cassia hirsuta plant^[4-6] belongs to the family Caesalpinaceae and commonly called as

stinking cassia and hairy senna. It is a plant from tropical America and now distributed in Indo-China, Malaysia, Thailand, African and Asian tropics, Laos, Java, Brazil, California, New Mexico, and India.^[7] In India this plant was widely distributed across the Deccan areas like Babubudaii Hills of Mysore, Rammdrug, Bellary, also in the Madras. The leaves are used to treat herpes. The seeds are a substitute for coffee. It is also used for stomach troubles, dysentery, abscesses, rheumatism, fever, and other diseases. Seeds contain phytotoxin, tannins, and 0.25% chrysarobin. Seeds also contain a water-soluble sugars extract as D-galactose and D-mannose and medicine for Parkinson's disease.^[8-11]

High-performance liquid chromatography (HPLC) with spectrophotometric detection has been widely used for identification and quantification of phenolics and flavonoids in plant extracts.^[7,12-14] Due to the presence of flavonoid chromophores, HPLC with ultraviolet (UV) detection has become the most popular detection method used in flavonoid analysis.^[13-16] Standardization of plant extract is one of the crucial parts of continuous scientific study in the herbal drug industry. With the advent of modern chromatographic systems, there is an ever-increasing intent to produce and develop easy, rapid, convenient, and cost-effective methods for standardization.^[17] For standardization of the present extracts of plant, HPLC is a sensitive and accurate tool that is widely used for the quality assessment of plant extract and its derived product.^[18] The present work describes a method for identification and quantification of gallic acid and kaempferol in chloroform and ethanol extract of *C. hirsuta* seeds using the HPLC method.

MATERIALS AND METHODS

Chemicals

The reference standard gallic acid was purchased from Hi-Media Laboratories Pvt. Ltd., Mumbai, and kaempferol purchased from Sigma-Aldrich Co., Spruce Street, St. Louis, MO, USA. HPLC grade water and methanol purchased from Merck Specialities Pvt. Ltd., Mumbai, were used in the study.

Plant Material

The plant was collected from Bagalkot, Karnataka. It was identified and authenticated by Dr. V G Jadimath, Department of Botany, Government PU College for Girls, Bagalkot - 587 101, Karnataka.

Preparation of Extract

The seeds were cleaned and air dried, then subjected to coarse powdering and passed through a sieve #44 to get uniform powder size. The collected powder was extracted with petroleum ether to defat and then by chloroform and ethanol for 24 h using Soxhlet apparatus. After the extraction, solvents were distilled off to get concentrated residue, dried in desiccator, and stored in airtight container under refrigeration.^[6]

HPLC Apparatus

The chromatography was performed on a Shimadzu LC2010CHT HPLC instrument composed of a degassing unit, low-pressure gradient unit, pump unit, mixer, ultrafast autosampler, column oven, and a UV-VIS detector with a thermostated flow cell. The LC-2010CHT is also designed for ease of use by automating the analysis process Spinchrom

LC Solution software. Enable C18G (4.6 × 250 mm, 5 μ particle size) column was used as stationary phase.

Preparation of Standard and Sample Solutions

An accurately weighed quantity of gallic acid and kaempferol (1 mg) was transferred to a 10 mL volumetric flask and dissolved in methanol. Further diluted with methanol to obtain standard stock solution of 20 μg/mL. Accurately weighed quantity of chloroform and ethanol extract of *C. hirsuta* (100 mg) was transferred to a 10 mL volumetric flask and dissolved in methanol. Further diluted to obtain solution of 1 mg/mL which was filtered through nylon filter syringe, pore size 0.45 μm.

Selection of Mobile Phase

Based on sample solubility, stability, and suitability, various mobile phase compositions were tried to get a good resolution and sharp peaks. From the various mobile phases, 0.1% v/v acetic acid in water:methanol (10:90% v/v) was chosen with detection wavelength 272 nm.^[19] As like gallic acid, the mobile phase for kaempferol was also selected from various mobile phase combinations. The buffer (pH 2):methanol (25:75% v/v) was chosen with detection wavelength 368 nm.^[20] Buffer was prepared by adding 3 ml triethanolamine in 1000 ml of HPLC water and adjusts the pH 2 with formic acid.

Chromatographic conditions

The optimized parameters which were used as a final method for the estimation of gallic acid and kaempferol represented in the below table.

Chromatographic condition for gallic acid and kaempferol

Chromatographic conditions	Gallic acid	Kaempferol
Mobile phase	0.1%v/v acetic acid in water: methanol (10:90%v/v)	Buffer (pH 2):methanol (25:75%v/v)
Stationary phase	Enable C18G (4.6×250 mm, 5 μ particle size)	Enable C18G (4.6×250 mm, 5 μ particle size)
Wavelength	272 nm	368 nm
Run time	10 min	10 min
Flow rate	1 mL/min	0.9 mL/min
Injection volume	10 μL	10 μL
Temperature	Ambient	40°C
Mode of operation	Gradient elution	Gradient elution

Validation of the Method

Validation of the analytical method was done according to the ICH guidelines. The method was validated for linearity,

precision, repeatability, reproducibility, limit of detection (LOD), and limit of quantitation (LOQ).^[21,22]

Linearity

The linearity of measurement was evaluated by analyzing different concentrations (4–24 µg/mL) of the standard solutions. Calibration curve was constructed for gallic acid and kaempferol by plotting average peak area against concentration and regression equation was found from plot. The correlation coefficient was also computed. All the samples were analyzed for repeating 6 times.

Precision

The precision of the method was tested by injecting a standard solution of gallic acid and kaempferol (20 µg/mL) 6 times. Peak areas were determined and compared. Precision was expressed as percentage relative standard deviation (% R.S.D.).

Repeatability and reproducibility

Inter- and intra-day variation was performed by injecting the standard solutions (4, 6, 8, 10, 12, and 24 µg/mL), each in six replicates, twice on the same day, and once on the next day and peak areas were determined and compared for repeatability and reproducibility of HPLC method.

Determination of LOQ and LOD

The LOD is the lowest amount of analyzing substance in a sample which can be detected but not necessarily quantified as an exact value. The LOQ is the lowest amount of analyzing substance which can be quantified with suitable precision. The LOD and LOQ of the developed method were determined by injecting progressively low concentration of the standard solution and the lowest concentrations were assayed.

Sample Analysis

The HPLC chromatograms of CECH and EECH were developed under same conditions of standard gallic acid and

kaempferol. The corresponding concentration of gallic acid and kaempferol against respective peak areas values was determined using the gallic acid and kaempferol calibration curves, respectively.

RESULTS

Linearity

A calibration curve was established for gallic acid and kaempferol by injecting 4, 8, 12, 16, 20, and 24 µg/mL of standard solution. Linearity was tested by analyzing the average peak area of gallic acid and kaempferol of different injection volume, and then, the regression equation $y = 36934x - 10162$ and $y = 61050x - 9395.5$ was obtained for gallic acid and kaempferol, respectively, and the correlation coefficient (R_2) was 0.9982 and 0.9992, respectively. Thus, a good linearity was shown when the gallic acid and kaempferol concentration ranged from 4 to 24 µg/mL. (Results are summarized in Table 1 and Figures 1-4).

Precision

The content variation in terms of percentage (RSD, %) which was found to be 1.6% and 0.71% for gallic acid and kaempferol, respectively, represents the tightly clustered data around the mean, demonstrating that the instrument used had a high precision. (Results are summarized in Table 1).

Repeatability and Reproducibility

Tables 2 and 3 reveal the intraday measurement of gallic acid and kaempferol content at 4–24 µg/mL calculated from calibration curves, which were have very near values (24.36 ± 0.0312 and 24.38 ± 0.0081 µg/mL in same day for gallic acid) (24.21 ± 0.1499 and 24.28 ± 0.1243 µg/mL in same day for kaempferol) in that same day of measurement which intern represent the HPLC methods were reproducible. Interday measurement also shows similar gallic acid and kaempferol

Table 1: Validation parameters of the HPLC method quantification of gallic acid and kaempferol

Validation parameters	Results	
	Gallic acid	Kaempferol
Retention time [Mean±S.D. (n=6)]	2.632±0.0023 min	3.185±0.0021 min
Linear range (µg/mL)	4–24	4–24
Correlation coefficient (R_2)	0.9982	0.9992
Regression equation	$y=36934x-10162$	$y=61050x-9395.5$
Precision (n=6% RSD)	1.6	0.71
LOQ (µg/mL)	1.65	0.85
LOD (µg/mL)	0.75	0.35

LOD: Limit of detection, LOQ: Limit of quantitation, HPLC: High-performance liquid chromatography, SD: Standard deviation, RSD: Relative standard deviation

Table 2: System repeatability and reproducibility of gallic acid

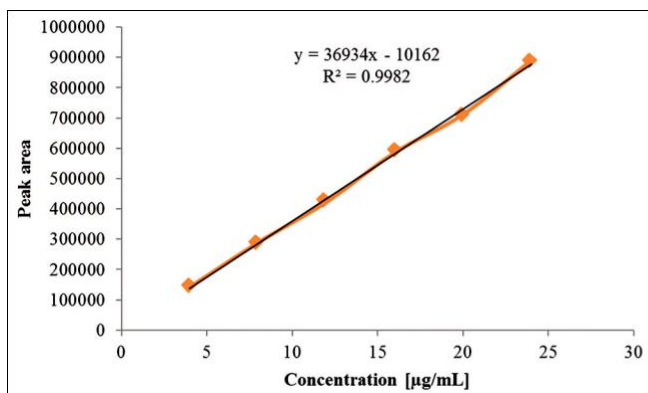
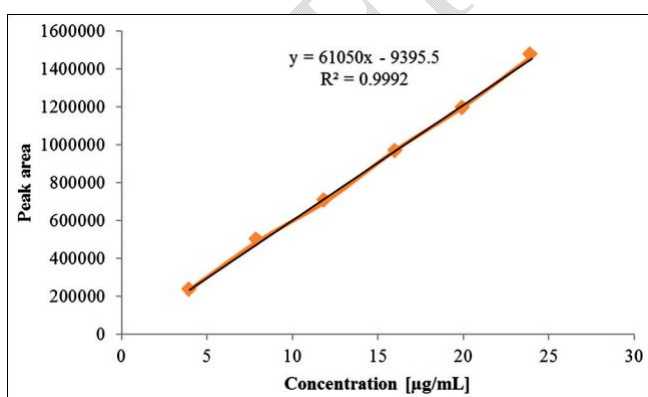
Concentration ($\mu\text{g/mL}$)	Day-1 ($\mu\text{g/mL}$)	Day-1 ($\mu\text{g/mL}$)	Day-2 ($\mu\text{g/mL}$)
4	4.122 \pm 0.0064	4.126 \pm 0.0128	4.143 \pm 0.0424
6	8.090 \pm 0.0236	8.105 \pm 0.0242	8.122 \pm 0.0495
8	11.77 \pm 0.0417	11.78 \pm 0.0338	11.79 \pm 0.0234
10	16.14 \pm 0.0121	16.15 \pm 0.0196	16.17 \pm 0.0438
12	19.59 \pm 0.0354	19.56 \pm 0.0453	19.58 \pm 0.0508
24	24.36 \pm 0.0312	24.38 \pm 0.0081	24.37 \pm 0.0432

All the values are expressed in Mean \pm S.D. ($n=6$). SD: Standard deviation

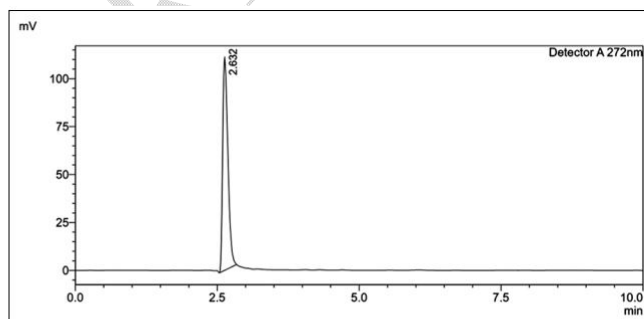
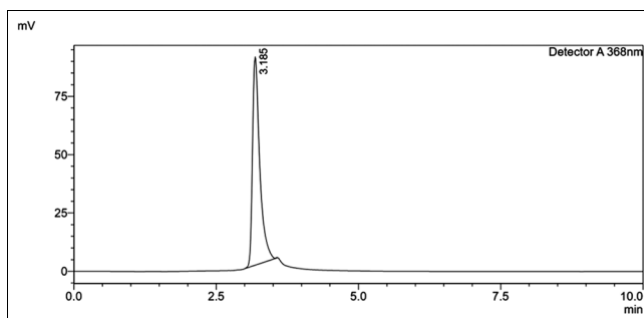
Table 3: System repeatability and reproducibility of kaempferol

Concentration ($\mu\text{g/mL}$)	Day-1 ($\mu\text{g/mL}$)	Day-1 ($\mu\text{g/mL}$)	Day-2 ($\mu\text{g/mL}$)
4	4.046 \pm 0.0125	4.060 \pm 0.0264	4.038 \pm 0.0212
6	8.437 \pm 0.2135	8.470 \pm 0.2175	8.520 \pm 0.2143
8	11.71 \pm 0.0365	11.72 \pm 0.0468	11.67 \pm 0.0924
10	16.17 \pm 0.1950	16.24 \pm 0.1475	16.25 \pm 0.3131
12	19.80 \pm 0.1628	19.86 \pm 0.1237	19.83 \pm 0.1193
24	24.21 \pm 0.1499	24.28 \pm 0.1243	24.28 \pm 0.2338

All the values are expressed in Mean \pm S.D. ($n=6$). SD: Standard deviation

**Figure 1: Standard calibration graph of gallic acid****Figure 2: Standard calibration graph of kaempferol**

contents ($24.37 \pm 0.0432 \mu\text{g/mL}$ and $24.28 \pm 0.2338 \mu\text{g/mL}$ found in next day for gallic acid and kaempferol, respectively) which indicates that the HPLC methods were reproducible.

**Figure 3: Chromatogram of standard gallic acid at 272 nm****Figure 4: Chromatogram of standard kaempferol at 368 nm**

LOD and LOQ

Signal-to-noise ratios (S/N) of 3 and 10 were considered as LOD and LOQ, respectively. The LODs for gallic acid and kaempferol were 0.75 and 0.35 $\mu\text{g/mL}$, and the LOQs for the same analytes were 1.65 and 0.85 $\mu\text{g/mL}$, respectively. This indicated that the proposed method exhibits good sensitivity

Table 4: HPLC quantification of gallic acid and kaempferol in chloroform and ethanol extracts of *Cassia hirsuta* seed

Name of extract	Gallic acid content %w/w	Kaempferol content %w/w
Chloroform extract of <i>Cassia hirsuta</i>	0.98±0.0064	0.0852±0.0039
Ethanol extract of <i>Cassia hirsuta</i>	5.954±0.0059	0.6512±0.0491

All the values are expressed in Mean±S.D. (n=6). SD: Standard deviation, HPLC: High-performance liquid chromatography

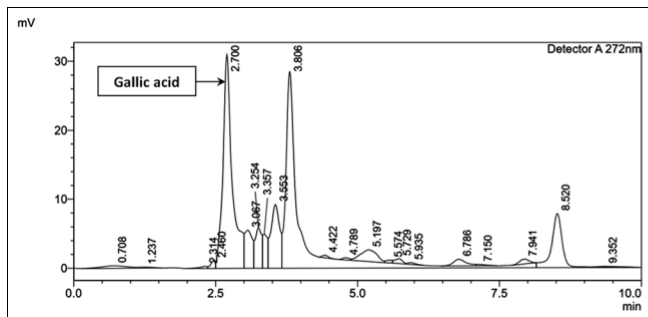


Figure 5: Chromatogram of chloroform extract of *Cassia hirsuta* at 272 nm

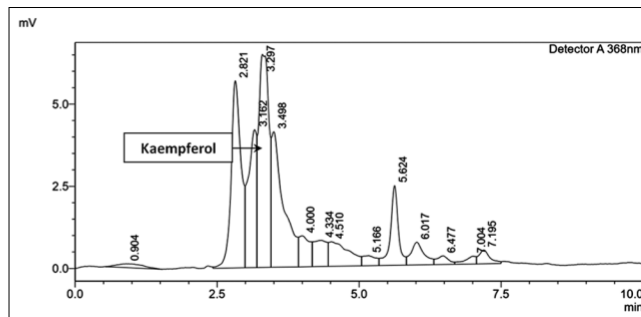


Figure 7: Chromatogram of chloroform extract of *Cassia hirsuta* at 368 nm

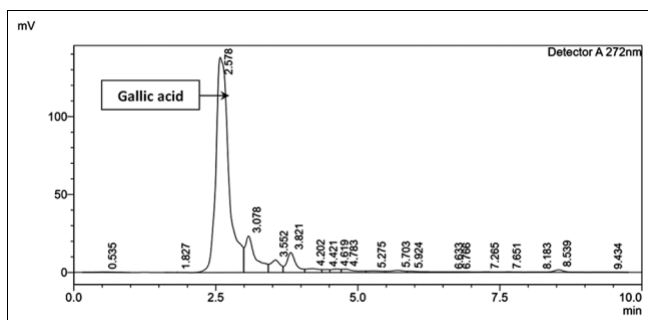


Figure 6: Chromatogram of ethanol extract of *Cassia hirsuta* at 272 nm

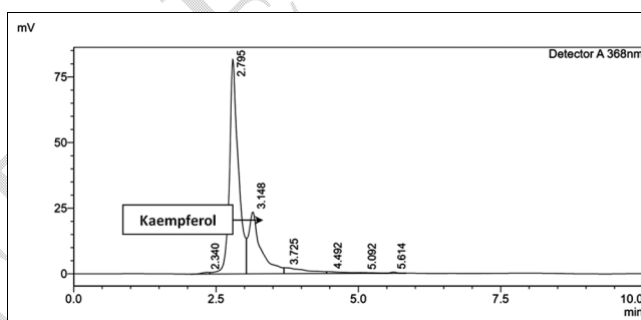


Figure 8: Chromatogram of ethanol extract of *Cassia hirsuta* at 368 nm

for the quantification of gallic acid and kaempferol. [Results are summarized in Table 1].

Sample Analysis

The results of the gallic acid and kaempferol content are given in Table 4 and Figures 5-8. Ethanol extract of *C. hirsuta* shows high content of both gallic acid and kaempferol as compared to chloroform extract of *C. hirsuta*.

DISCUSSION

Chemistry of column, type of solvent, strength of solvent (volume fraction of organic solvent(s) in the mobile phase and pH of the buffer solution), wavelength for detection, and flow rate were varied to determine the chromatographic conditions resulting in the best separation. The mobile phase conditions were optimized such that there was no interference from solvent and other compounds. Various mobile phases were tried and finally above-mentioned mobile phase and

other chromatographic conditions fixed for HPLC analysis of gallic acid and kaempferol in chloroform and ethanol extract of *C. hirsuta* seeds. Various studies were done for analyzing gallic acid^[23] as the major constituents; however, the difference between them is the type of mobile phase used. In a study of gallic acid by *Dendrophthoe falcata* L. f., Deshmukh and Prabhu^[24] found a retention time of approximately 12 min. In another work with *Schinus terebinthifolius* Raddi, gallic acid was quantified and retention times were found 5 min.^[25] Asma'a *et al.* analyzed the kaempferol and the retention time was found 4.43 min.^[22]

However, these proposed methods enable a rapid, precise, and accurate analysis of gallic acid and kaempferol using water:methanol (10:90% v/v) and buffer (pH 2):methanol (25:75% v/v) as mobile phase, respectively. The present methods offer the advantage of comparative analysis in extracts of *C. hirsuta* without any interference from other components. The result also indicates a higher gallic acid and kaempferol content in the ethanol extract of *C. hirsuta* as compared to the chloroform extract of the plant.

CONCLUSION

In the present study, gallic acid and kaempferol content were determined in ethanol and chloroform extract of *C. hirsuta*. The developed HPLC methods for the analysis of gallic acid and kaempferol have enabled rapid, linear, accurate, and reproducible analysis in these two extracts of *C. hirsuta*. The developed method can be used for quantitative analysis and quality control of extracts and commercial samples of other species containing gallic acid and kaempferol.

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