

Quantification of total alkaloid, tannin, flavonoid, phenolic, and chlorogenic acid contents of *Leea macrophylla* Roxb. ex Hornem

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Abstract

Objective: The aim of the present work was to assess the total alkaloid content (TAC), total tannin content (TTC), total phenolic content (TPC), and total flavonoid content (TFC) of crude methanolic extract of root, stem, and leaf of *Leea macrophylla* Roxb. ex Hornem. which is a medicinally important plant belonging to the family Vitaceae and used extensively in ethnomedicine with its significant therapeutic significance. This article also aims to assess chlorogenic acid content in root, stem, and leaf of plant using high profile thin-layer chromatography (HPTLC). **Materials and Methods:** Root, stem, and leaf powder of *L. macrophylla* were used for extract preparation and quantification. The amount of total alkaloids analyzed by modern method of analysis, total tannin by titrimetric method, the amount of total flavonoids by aluminum chloride assay, and total phenols by Folin–Ciocalteu assay. HPTLC method has been used for quantification of chlorogenic acid. **Results:** The TAC of the methanolic extract of root, stem, and leaf was 0.37%, 0.50%, and 0.52% w/w, respectively, TTC was 2.01%, 1.23%, and 3.67% w/w, respectively. The TFC and TPC of the methanolic extract of root, stem, and leaf were 361.67 ± 14.43 , 233.33 ± 5.77 , and 395 ± 25 mg chrysin equivalent per gram and 243.33 ± 18.01 , 232.33 ± 2.31 , and 376 ± 8.54 mg gallic acid equivalent per gram, respectively. Chlorogenic acid contents of stem and leaf are 27.24 and 12.21 $\mu\text{g}/\text{mg}$, while root does not show its presence using this method. **Conclusion:** The results of the study highlighted a potent alkaloid, tannins, flavonoid, phenol, and chlorogenic acid contents in the methanolic extract of *L. macrophylla* and thus can be used for medicinal purposes as components present in the plant possesses varied biological activities.

Key words: Chlorogenic acid, high profile thin-layer chromatography, *Leea macrophylla*, total alkaloid content, total flavonoid content, total phenolic content

INTRODUCTION

Therapeutic potential of medicinal plants is based on bioactive compounds present in them. These compounds may elicit a long range of different effects in man and animal seating the plants dependent on plant species and amount eaten.^[1] Secondary metabolites are produced within the plants beside the primary biosynthetic and metabolic routes of compounds aimed at plant growth and development such as carbohydrates, amino acids, proteins, and lipids.^[1]

The most important of these biologically active ingredients is alkaloids, flavonoids, steroids,

glycosides, terpenes, phenolic compounds, and tannins. These active chemical components can be extracted and used in the preparation of useful drugs. The importance of biological, chemical, and pharmacological evaluation of plant-derived bioactive compounds used to cure numerous human ailments has been increasingly recognized in the last few decades,

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but still, there are innumerable potentially useful medicinal plants and herbs waiting to be evaluated and exploited for their effective therapeutic application.^[2-5]

Identifying the chemical nature and their quantity in a given medicinal plant becomes more valuable so as to use these plants in the treatment of various diseases or identifying the lead compound for future new drug development. As natural products from medicinal plants, either as pure compounds or as standardized extracts, provide unlimited opportunities for new drug leads because of the unmatched availability of chemical diversity.^[6]

Leea macrophylla, family Vitaceae, known as *Hastikarna*^[7,8]/*Hastikarna palasha*^[9] in Ayurveda, locally known as *Hanshia dabar* in, Gandhamardan hill ranges, Odisha, is a herb or herbaceous shrub.^[10] The main phytochemicals of the plant are alkaloids, tannins, flavonoids, phenols, and steroids.^[11] Antioxidant,^[12] antiurolithiatic,^[13] anti-inflammatory,^[14] antithrombotic,^[15] hepatoprotective,^[12] neuroprotective,^[16] wound healing,^[17] etc., are the reported pharmacological activities of the plant.^[18] Only root part of unifoliate variety of plant has been studied and phytoconstituents present in them have been quantified.^[11] But until date, no study has been done on compound leaf variety of *L. macrophylla* regarding the presence of phytoconstituents in them and their quantification.

Based on the strong evidence of the biological and pharmacological activities of these bioactive components, the present study was conducted to determine the total alkaloid, tannins, flavonoid, phenolic, and chlorogenic acid contents of a root, stem, and leaf samples and methanolic extracts of these parts of *L. macrophylla*.

MATERIALS AND METHODS

Chemicals and Reagents

All the chemicals and reagents used in the present study were of analytical grade and were obtained from Sigma, Merck, and SD Fine.

Plant Collection and Authentication

Whole plant of *Hastikarna* was collected by the first author with the help of traditional practitioner from its natural habitat Gandhamardan hills, Paikmal, Odisha and identified by the local taxonomist during the month of October 2016. Herbarium was prepared from the collected plant material and was authenticated from BSI, Kolkata (Specimen No. CNH/2016/Tech. II/68 dated 31/01/2017) as *L. macrophylla* Roxb. ex. Hornem., family – Vitaceae. A specimen of the sample herbarium has been deposited in Pharmacognosy Laboratory, I. P. G. T and R. A, Jamnagar (Specimen No. Ph. M: 6234) for further references.

Extract Preparation

Five grams of powder extracted with methanol (100 ml), keeping it for overnight with initial occasional shaking up to 6 h and then set aside. After 24 h, it was filtered and alcoholic extract was collected.

Spectrophotometric Measurements

Ultraviolet/visible double beam spectrophotometer (Systronics, Model 2202) and standard quartz cuvette were used for all the absorbance measurements. Instrumental conditions (measuring mode-absorbance; slit width (nm)-1.0 fixed; wavelength type-point; wave length (nm)-765/510; calibration-fixed wavelength).

Preparation of Standard Solution

About 10 mg each of chrysin and gallic acid were accurately weighed into clean and dry volumetric flasks, dissolved in methanol, and the volume was made up to 10 ml using the same solvent so as to make the concentration of the solution as 1 mg/ml.

Preparation of Test Sample

A stock solution of the test substance (*L. macrophylla* root, stem, and leaf) was prepared by dissolving 10 mg of dried methanol extract in 10 ml same solvent to give a concentration of 1 mg/ml.

Determination of Total Alkaloid Content (TAC)

Accurately weighed 2.5 g of samples (*L. macrophylla* root, stem, and leaf powder) was transferred to clean conical flasks separately. The sample was moistened with ammonia and kept overnight. The next day 50 ml ethyl acetate was added. The flask was shaken and kept aside for 5–6 h. On completion, the sample was filtered through simple filter paper and filtrate was taken in a previously dried and weighed, porcelain-evaporating dish and evaporated on a hot water bath. It was dried until constant weight in an oven and weighed.^[19]

Determination of Total Tannin Content (TTC)

Quantitative estimation of tannin was performed by titrating the extract with standard potassium permanganate solution following the method of AOAC.^[20,21]

Briefly, 5 ml aliquot of the extract was mixed with 12.5 ml of indigo carmine solution and 375 ml of distilled water. This mixture was titrated against KMnO₄ solution (“Y” mL). As titration preceded, the blue color of the indigo carmine passes through many shades to a final yellow with a faint pink tint at

the rim. It was taken as the end-point. This volume of KMnO_4 was used to titrate total tannin plus all other related compounds. To determine the volume of KMnO_4 ("X" ml.) used to titrate nontannin (related) compound, another aliquot of 50 ml extract was mixed with 25 ml of gelatin solution (25 g. gelatin was soaked for 1 h in saturated NaCl solution. The mixture was then warmed until the gelatin has dissolved and after cooling the solution was made up to 1 l with saturated NaCl), 50 ml of the acidic NaCl solution (25 ml of concentrated H_2SO_4 was added to 975 ml of saturated NaCl solution.), and 5 g powdered kaolin. The mixture was shaken for 15 min and filtered through Whatman No. 1 filter paper. 12.5 ml of the filtrate was mixed with same volume of indigo carmine solution and 375 ml of distilled water. This mixture was again titrated against KMnO_4 solution until color changed to faint pink as earlier. The volume of KMnO_4 used to titrate true tannin was calculated by the values of Y and X. The concentration of tannin was estimated using the following relationship:

1 ml of standard KMnO_4 solution = 0.595 ml of 0.1 N Oxalic acid

1 ml of 0.1 N Oxalic acid = 0.0042 g of tannin.

Determination of Total Flavonoid Content (TFC)

The flavonoids content was determined by aluminum trichloride method.^[22]

Reagents: 5% NaNO_2 w/v, 10% AlCl_3 w/v, 4% NaOH w/v, Chrysin (Standard).

Procedure: The TFC of the root, stem, and leaf extract was determined by aluminum chloride colorimetric assay. Briefly, 0.5 ml aliquots of the extract and standard solution (0.01–1.0 mg/ml) of chrysin were added with 2 ml of distilled water and subsequently with 0.15 ml of sodium nitrite (5% NaNO_2 w/v) solution and mixed. After 6 min, 0.15 ml of (10% AlCl_3 w/v) solution was added. The solutions were allowed to stand for further 6 min and after that 2 ml of sodium hydroxide (4% NaOH w/v) solution was added to the mixture. The final volume was adjusted to 5 ml with immediate addition of distilled water, mixed thoroughly and allowed to stand for another 15 min. The absorbance of each mixture was determined at 510 nm against the same mixture but without extract as a blank. TFC was determined as mg chrysin equivalent per gram of sample with the help of calibration curve of chrysin. All determinations were performed in triplicate ($n = 3$).

Determination of Total Phenolic Content (TPC)^[23,24]

The phenolic is all those compounds, which phenolic group not hydroxyl. It may be monophenol, di-phenol, tri-phenol, or polyphenol. The TPC of the extracts was estimated by the method of Singleton *et al.* (1999).

Reagents: 10% Folin–Ciocalteu's reagent, 7.5% of NaHCO_3 , gallic acid (Standard).

Procedure: Estimation of total phenol content in the selected plant extract was measured spectrophotometrically by Folin–Ciocalteu colorimetric method, using gallic acid as the standard and expressing results as gallic acid equivalent (GAE) per gram of sample. Different concentrations (0.01–1 mg/ml) of gallic acid were prepared in methanol. Aliquots of 0.5 ml of the test sample and each sample of the standard solution were taken, mixed with 2 ml of Folin–Ciocalteu reagent (10% v/v) and 5 min later 4 ml of a saturated solution of sodium carbonate (7.5% w/v). The tubes were covered with silver foils and incubate at room temperature for 30 min with intermittent shaking. The absorbance was taken at 765 nm using methanol as blank. All the samples were analyzed in three replications. The total phenol was determined with the help of a standard curve prepared from the pure phenolic standard (gallic acid).

Folin–Ciocalteu is a very sensitive reagent containing phosphomolybdate and phosphotungstate that form blue-complex in alkaline solution by the reduction of phenols. This blue color was measured spectrophotometrically.

Quantification of Chlorogenic Acid Contents Using High Profile Thin-layer Chromatography (HPTLC)

Equipment of HPTLC

A CAMAG (Switzerland) HPTLC system equipped with a sample applicator Linomat V was used for application of samples. CAMAG TLC scanner III., ReproSTAR, and WinCATS 1.3.4 were used for scanning the plates. CAMAG twin through glass chamber was used for developing the plates.

HPTLC

HPTLC was performed on 10×20 cm HPTLC silica gel 60 F254 plates (Merck, Germany). Ethyl acetate-formic acid-acetic acid-water in volume ratio 6.7:0.7:0.7:1.7 (v/v) was used as a mobile phase.^[25,26] After development plates were air dried and recorded at 366 nm, identification and quantification were performed by TLC densitometry using CAMAG TLC scanner 3 and winCATS software version 1.3.4 (Switzerland). Quantification was performed using calibration curves (peak area of chromatogram versus mass of standard applied in the form of band) for individual standard in triplicate. Application volume–track 1:4 μl (chlorogenic acid); track 2:5 μl (chlorogenic acid); track 3:6 μl (chlorogenic acid); track 4:5 μl (methanol extract of root of *L. macrophylla*); track 5:5 μl (methanol extract of stem of *L. macrophylla*); and track 6:5 μl (methanol extract of leaf of *L. macrophylla*).

Preparation of Standard Chlorogenic Acid Solution

Accurately weighed 0.3 mg of chlorogenic acid was taken and to it 3 ml of methanol was added and dissolved to get standard solution with concentration of 0.1 mg/ml.

Sample Preparation

To the dried extract of sample, methanol was added in a proportion so as to make sample solution with concentration of 1 mg/ml

Statistical Analysis

All the determinations were replicated in three independent assays and the results were reported as a mean \pm standard deviation.

RESULTS AND DISCUSSION

Medicinal plants contain many secondary metabolites and other chemical compounds having biochemical effects in human beings and animals. The huge amounts of such compounds have a broad range of effects, from being acute deadly to being healthy or curative. According to the World Health Organization, more than 80% of the world's population relies on traditional medicine for their primary healthcare needs. That's why identifying the chemical nature and their quantity in a given medicinal plant becomes more valuable so as to use these plants in the treatment of various diseases or identifying the lead compound for future new drug development. As natural products from medicinal plants, either as pure compounds or as standardized extracts, provide unlimited opportunities for new drug leads because of the unmatched availability of chemical diversity.^[6]

The present study has been carried out for quantification of the total alkaloid, tannins, flavonoid, phenolic, and chlorogenic acid contents of methanolic extract of root, stem, and leaf of *L. macrophylla*. The TAC of root, stem, and leaf in the crude extract was 0.37%, 0.50%, and 0.52% w/w, respectively. The TTC of root, stem, and leaf in the crude extract was 2.01%, 1.23%, and 3.67% w/w, respectively. The concentration of flavonoids in MeOH extract of root, stem, and leaf, (mg/g) in chrysin equivalent determined from regression equation of calibration curve ($y = 0.002x - 0.0044$ $R^2 = 0.9911$) was 361.67 ± 14.43 , 233.33 ± 5.77 , and 395 ± 25 , respectively, and the content of the phenolic compounds in the MeOH extract of root, stem, and leaf determined from regression equation of calibration curve ($y = 0.0209x - 0.0472$ $R^2 = 0.9996$) and expressed in GAE was 243.33 ± 18.01 , 232.33 ± 2.31 , and 376 ± 8.54 , respectively. The standard calibration curves of chrysin and gallic acid are shown in Figures 1 and 2, respectively. The results are shown in Tables 1 and 2.

Quantification of Chlorogenic Acid Using HPTLC

Chlorogenic acid is one of the chemicals present in the *L. macrophylla* which have a significant role in the biological action of plant thus having its medicinal importance. That's why it was quantified using the modern HPTLC method.

In HPTLC study, standard chlorogenic acid showed R_f values as 0.57, 0.55, and 0.54. While stem and leaf samples of the

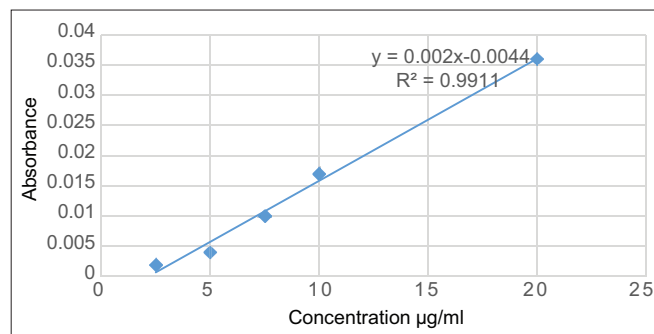


Figure 1: Standard calibration curve of chrysin for total flavonoid content

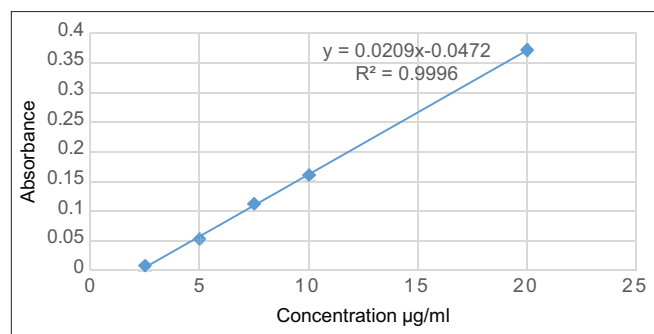


Figure 2: Standard calibration curve of gallic acid for total phenolic content

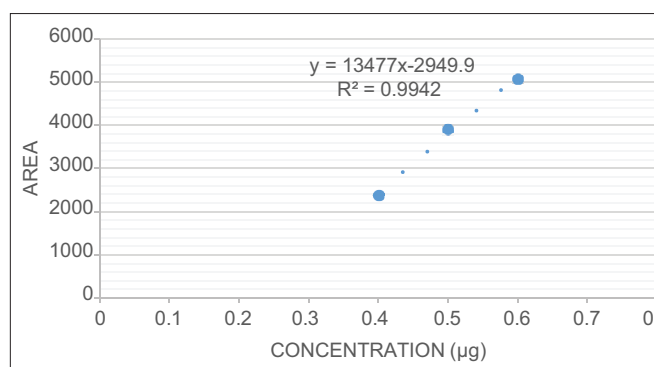


Figure 3: Standard calibration curve of chlorogenic acid

Table 1: Total alkaloid and tannin content of *Leea macrophylla*

parameters	Root	Stem	Leaf
Total alkaloid (% w/w)	0.37	0.50	0.52
Total tannin (% w/w)	2.01	1.23	3.67

plant showed R_f value 0.53, which shows that chlorogenic acid moieties are present in stem and leaf plant samples. One spot is observed in the case of root sample, three spots are observed in the case of stem sample, and four spots are observed in

the case of leaf sample. The number of spots at different R_f of the root, stem, and leaf samples are depicted in Table 3. Graphs, densitogram and spectral comparison obtained from the HPTLC study are depicted in Figures 4 and 5.

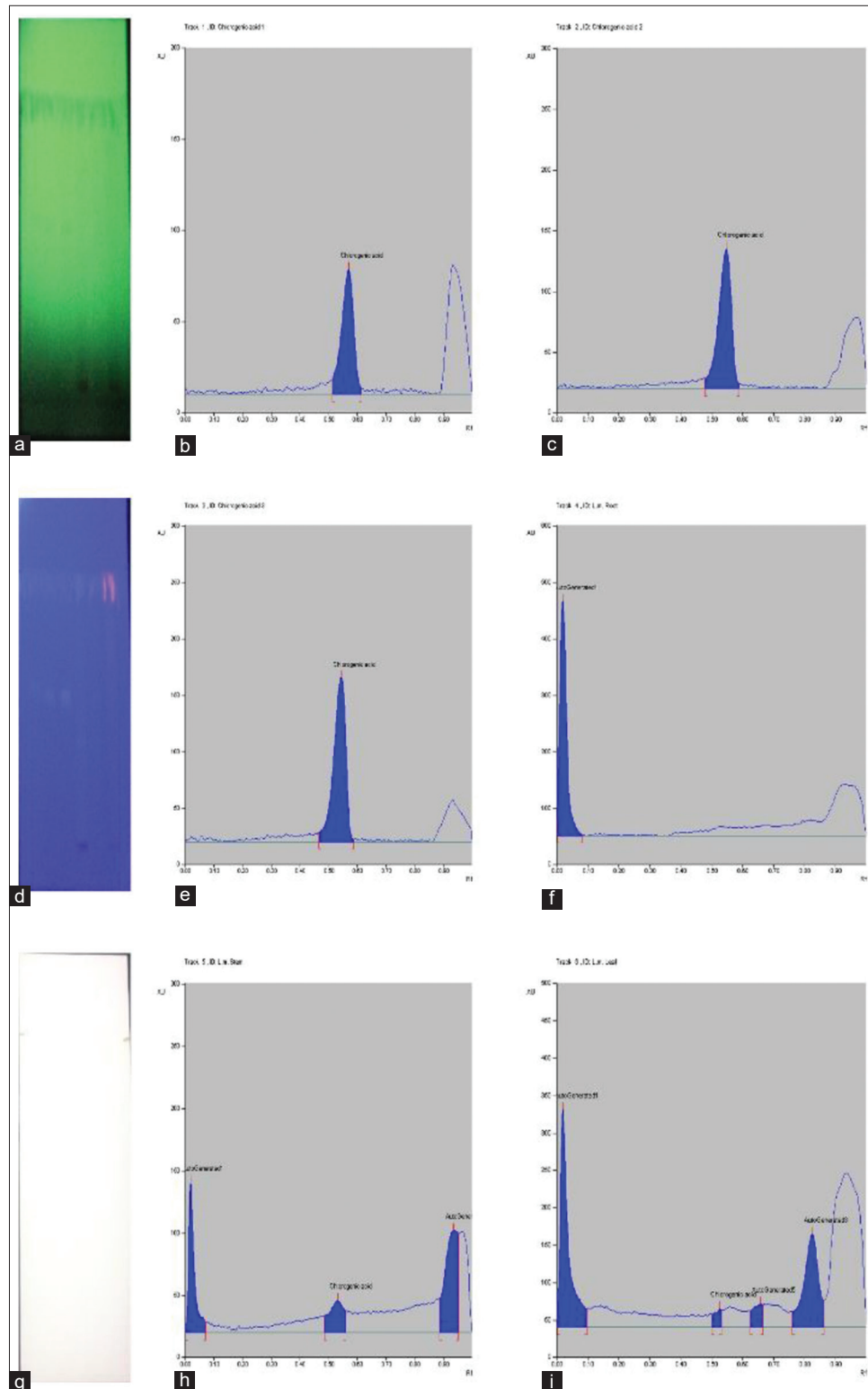


Figure 4: High profile thin-layer chromatography (HPTLC) study showing plates and tracks, a: HPTLC plate at 254 nm– ultraviolet (UV) short, b: Track 1-peak display of standard chlorogenic acid at 366 nm, c: Track 2-peak display of standard chlorogenic acid at 366 nm, d: HPTLC plate at 366 nm– UV long, e: Track 3-peak display of standard chlorogenic acid at 366 nm, f: Track 4-peak display of *L. macrophylla* root sample at 366 nm, g: HPTLC plate in visible daylight, h: Track 5-peak display of *L. macrophylla* stem sample at 366 nm, i: Track 6-peak display of *L. macrophylla* leaf sample at 366 nm

Preparation of Standard Curve

The calibration curve was plotted of concentration versus area; the linearity graph is depicted in Figure 3. The three concentrations taken for linearity are 0.4, 0.5, and 0.6 μg and are obtained 2381.4, 3907.7, and 5076.8, respectively, R^2 is 0.9942.

The quantity of chlorogenic acid was calculated by common cross multiplication formula as below and depicted in Table 4.

Concentration of sample = Area of sample/Factor.

The results revealed that the test MeOH extract contains potent amounts of alkaloids, tannins, flavonoid, and phenolic compounds. Many of the curative properties of this plant may

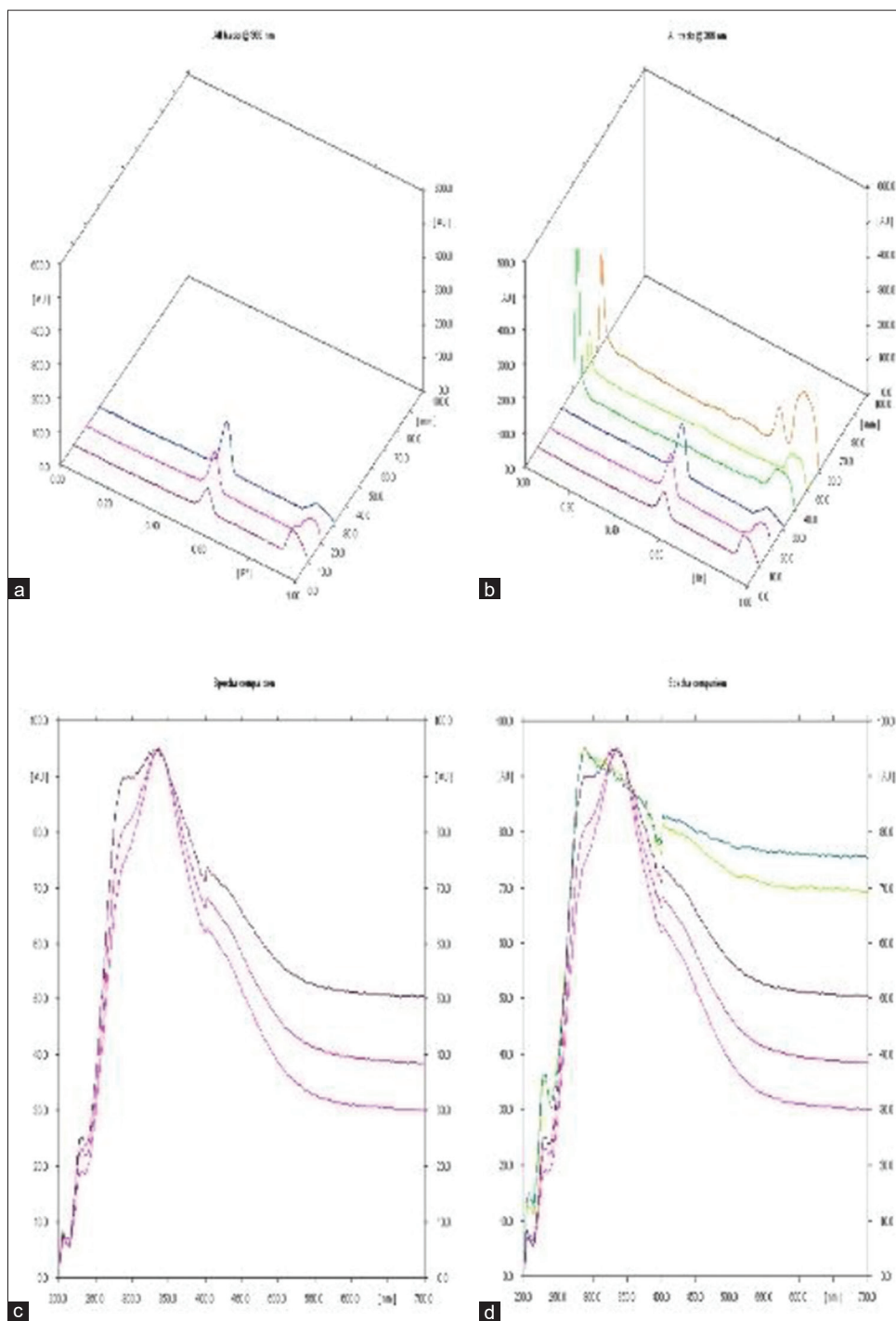


Figure 5: High profile thin-layer chromatography study showing densitogram and spectral comparison. a: Densitogram showing peaks of different concentrations of standard chlorogenic acid at 366 nm, b: Densitogram showing peak comparison of standard chlorogenic acid with samples at 366 nm, c: Spectral comparison of different concentrations of standard chlorogenic acid at 366 nm, d: Spectral comparison of standard chlorogenic acid with samples at 366 nm

Table 2: Flavonoid and phenol contents of *Leea macrophylla*

Parameters	MeOH extract of root (content mg/g)	MeOH extract of stem (content mg/g)	MeOH extract of leaf (content mg/g)
Total flavonoid (CE)	361.67±14.43	233.33±5.77	395±25
Total phenol, (GAE)	243.33±18.01	232.33±2.31	376±8.54

Values are represented as mean±SD (n=3), CE: Chrysin equivalent, GAE: Gallic acid equivalent, MeOH: Methyl alcohol

Table 3: R_f s obtained at 366 nm in different samples of chlorogenic acid and plant samples

Track no. /mode	366 nm	
	Number of spots	R _f value
Track 1 (chlorogenic acid)	1	0.57
Track 2 (chlorogenic acid)	1	0.55
Track 3 (chlorogenic acid)	1	0.54
Track 4 (root)	1	0.02
Track 5 (stem)	3	0.02, 0.53, 0.94
Track 6 (leaf)	4	0.02, 0.53, 0.66, 0.83

Table 4: Quantitative estimation of chlorogenic acid moieties in stem and leaf samples

Track no./mode	Quantity of chlorogenic acid moieties (µg/mg)
Track 5 (Stem)	27.24
Track 6 (Leaf)	12.21

depend on these bioactive components.^[11-18] These findings supported the uses of *L. macrophylla* as anti-microbial, anti-inflammatory, anti-diabetic, antitumor, antioxidant, free radical scavenging agent, etc. Further, more progress in the detailed examination of the composition of these bioactive chemicals in plant extract is required for the complete evaluation of the individual compounds exhibiting the different biochemical properties.

HPTLC method showed the presence of chlorogenic acid moieties in the stem and leaf samples of the *L. macrophylla*. It is taken as a dietary supplement, chlorogenic acid slightly reduces blood pressure,^[27,28] It has been investigated for possible anti-inflammatory effects.^[29] Chlorogenic acid demonstrated the effective promotion of wound closure and capillary tube formation and also enhanced keratinocyte wound closure.^[30] These findings supports the plants use in various ailments.

CONCLUSION

In the present study, we have found that the plant is rich in alkaloid, tannins, flavonoid, and phenolic compounds, also plant exhibited the significant presence of chlorogenic acid,

and therefore, has provided some biochemical basis for the ethnomedicinal use of plant *L. macrophylla*. As a promising source of bioactive compounds, it can be an excellent source of useful drug leads which are concerned. Moreover, it can also be concluded that the methanolic extract of root, stem, and leaf of *L. macrophylla* can also serve as a much potent antioxidant agent. It will obviously be due to the high contents of the phytochemicals in the methanolic extract, thus supports its use in various ethnomedicinal claims such as wound healing.

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