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Determination of Major Polyphenolic Components in *Euphoria longana* Lam. by Validated High Performance Thin-Layer Chromatography Method and Direct Analysis in Real Time Mass Spectrometry

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Abstract

In the current study, a simple, sensitive, precise, and specific method has been developed and validated for the determination of four major polyphenolic compounds in *Euphoria longana* Lam. seeds based on high performance thin-layer chromatography (HPTLC) and confirmed by direct analysis in real time mass spectrometry (DART-MS). The chromatographic separation was accomplished on Merck HPTLC aluminum plates precoated with silica gel G60 F_{254} (20 cm × 10 cm) with 250 µm thickness as stationary phase using a mobile phase composed of n-butanol: water: methanol: formic acid (7.59: 1.27: 0.13: 1.01, v/v/v/v). Densitometric measurement was performed in the absorbance mode at 280 nm. The compounds were resolved satisfactorily with R_f values of 0.40 ± 0.01, 0.57 ± 0.02, 0.69 ± 0.01, and 0.79 ± 0.01 for corilagin, ellagic acid, epicatechin, and gallic acid, respectively. The method developed was validated with acceptable linearity (r^2>0.995), sensitivity, precision (RSD ≤ 1.60%), robustness, and recovery (RSD ≤ 1.77%). The proposed method was successfully applied for the determination of active components for comprehensive quality control of *E. longana* products. Furthermore, peak identities of compounds from each band were confirmed by direct analysis in real time mass spectrometry.

Keywords: *Euphoria longana* Lam; Polyphenols; HPTLC; Validation; DART-MS

Introduction

Euphoria longana Lam. (*E. longana*) belongs to Sapindaceae family, is extensively grown in South of China, India and Southeastern Asia in which Thailand is a major exporter of *E. longana* fruit [1,2]. Dried seed extract of *E. longana* is a source of high levels of polyphenolic compounds such as corilagin, gallic acid, and ellagic acid [3]. Also, previous reports have confirmed the presence of epicatechin (Figure 1) in *E. longana* seeds as other major phenolic compound [3,4]. As *E. longana* seeds contain significant amounts of polyphenolic compounds, seed extract exhibits excellent antioxidant [1,4-7], anti-tyrosinase [1], antifungal [8], anti-inflammatory [9], antigelatinase, anti-angiogenesis, and anticancer [2,10,11] activities. Also, *E. longana* seed extract has been investigated concerning its beneficial effects on learning and memory impairment in mice [12]. As a matter of fact, these polyphenolic compounds are usually considered as the markers for quality control of *E. longana* [3,4,13]. Therefore, for the purpose, a suitable and preferred method for analysis of these polyphenolic compounds is necessary.

So far, a few methods such as high performance liquid chromatography (HPLC) [1,3,4,6] have been developed for the determination of polyphenolic compounds in *E. longana.* These liquid chromatography methods reported for quantification of only either two or three major polyphenolic compounds. Also, few other liquid chromatography tandem mass spectrometry (LC-MS) methods [4,13,14] primarily focus on isolation and characterization of polyphenolic compounds in *E. longana* seed/pericarp extracts. However, these methods suffer from longer analysis time, time-consuming sample preparation and a large amount of organic solvent consumption.

In particular, HPTLC serves as the preferred technique nowadays for the detection of analyte at a very low operating cost with advantages of the need of minimum sample cleanup, high sample throughput, and lower solvent consumption as the matter of fact several samples can be run simultaneously unlike HPLC, thus lowering analysis time and cost per analysis [15,16].

Further, a DART-MS technique was established to confirm peak identities of compounds, is a fast, reliable and non-invasive high throughput technique that involves the ionization of organic molecules in diverse samples directly from the surface. Electronically excited species such as metastable helium and nitrogen atoms ionize atmospheric water molecules that transfer protons to the sample molecules resulted in soft ionization. As the DART ion source can ionize molecules directly from the surface, compounds can be analyzed as such without sample preparation [17]. DART-MS is, therefore, an appropriate technique for the rapid confirmation of analytes from each band on HPTLC plate based on exact mass spectra acquired in negative ion mode being directly analyzed from sample bands using glass capillary without sample preparation.

Nevertheless, to the best of our knowledge, no single method has been reported for the simultaneous determination of four major polyphenols namely, gallic acid, corilagin, epicatechin, and ellagic acid in plant matrices of *E. longana.* Moreover, no method has been reported till date for quantification of these compounds using HPTLC technique. Also, this is the first ever report of HPTLC determination of corilagin in any plant matrices till date.

In this study, the authors establish cost-effective, rapid, and less time consuming analytical strategy that combines simple, precise

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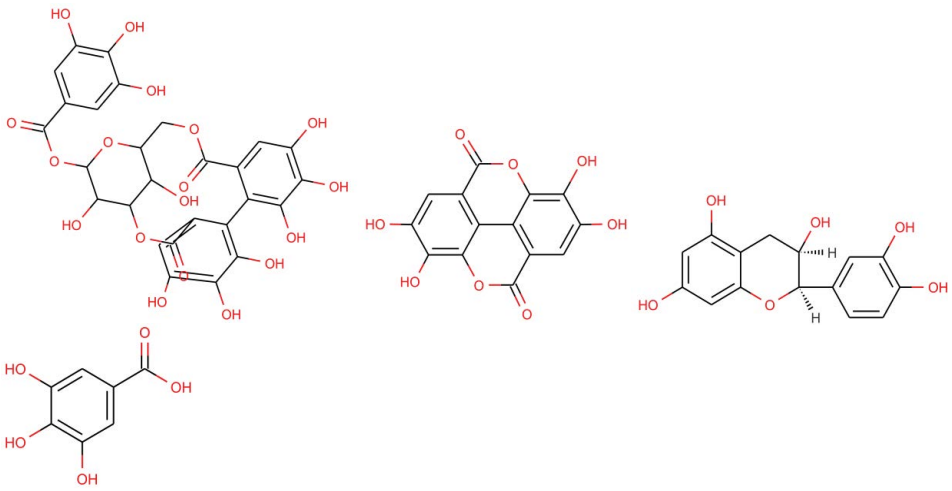

(A) (B) (C) (D)

Figure 1: Chemical structures of corilagin (A), ellagic acid (B), epicatechin (C), and gallic acid (D).

and accurate HPTLC method and DART-MS for simultaneous determination and confirmation of four major polyphenolic components in *E. longana* seeds.

Materials and Methods

Reagents and chemicals

Standard substances of gallic acid, ellagic acid and epicatechin (purity ≥ 95.5%) were purchased from Sigma-Aldrich Co. (St. Louis, MO, USA). The analytical standard of corilagin (purity ≥ 98.3%) was purchased from Chengdu Biopurify Phytochemicals Co., Ltd. (Chengdu, China). Methanol (HPLC grade), n-butanol and formic acid (analytical grade) were of from Merck (Darmstad, Germany). Ultra high purity water was prepared using a Milli-Q water purification system (Millipore Corporation, Bedford, MA, USA).

E. longana fruits (Sample 1-5) were collected from the plants grown in Muzaffarpur district, Bihar, India between the months of June to August. A voucher specimen of *E. longana* (ASR-1) was deposited in the Botanical Survey of India, Western Region Centre, Pune, India. The seeds were sundried and ground to powder using a stainless-steel grinder.

Instruments and conditions

Chromatographic analysis was performed on HPTLC plates prewashed with methanol and activated at 110°C for 5 min prior to chromatography. The samples were spotted in the form of bands 6 mm width with a Camag 100 microlitre sample syringe (Hamilton, Bonaduz, Switzerland) on silica gel precoated HPTLC aluminum plate 60 F_{254}, (20 cm × 10 cm) with 250 µm thickness; E. Merck, Darmstadt, Germany, supplied by Anchrom Technologies, Mumbai] using a Camag Linomat V applicator (Switzerland). A constant application rate of 0.1 µL/s was used and the space between two bands was 6 mm. Linear ascending development was carried out in 20 cm × 10 cm twin trough glass chamber (Camag, Muttenz, Switzerland) saturated with the mobile phase. The mobile phase was consisted of n-butanol: water: methanol: formic acid (7.59: 1.27: 0.13: 1.01, v/v/v/v) and 20 mL was used per chromatography run. The optimized chamber saturation time with mobile phase was 30 min using saturation pads at room temperature (25°C ± 2). The length of chromatogram run was 80 mm and run time was 40 min. Densitometric scanning was performed using a Camag TLC scanner III in the reflectance-absorbance mode and operated by winCATS software (V1.1.4, Camag). The slit dimension was kept at 5 mm × 0.45 mm and the scanning speed was 10 mm/s. The source of radiation used was a deuterium lamp emitting a continuous UV spectrum between 200 and 400 nm. All determinations were performed at ambient temperature with a detection wavelength of 280 nm. Concentrations of the compound chromatographed were determined from the intensity of the diffused light. Evaluation was by peak areas with linear regression.

Mass spectrometry confirmation

For analytes confirmation, all samples were analyzed using the same HPTLC conditions mentioned above and mass spectrometry detection was conducted on a JMS T100 LC (Accutof) atmospheric pressure ionization time of flight mass spectrometer (Jeol, Tokyo, Japan) fitted with a DART ion source. The mass spectrometer was operated in negative ion mode with resolving power of 6000 (full width at half maximum). The orifice 1 potential was set to 15 V, resulting in minimal fragmentation. The ring lens and orifice 2 potential were set to 13 and 5 V, respectively. Orifice 1 was set to a temperature of 100°C. The RF ion guide potential was 300 V. The DART ion source was operated with helium gas flowing at approximately 4.0 L/min. The gas heater was set to 300°C. The potential on the discharge needle electrode of the DART source was set to 3000 V, electrode 1 was 100 V and the grid was at 250 V. The samples were collected from each band of developed plate using glass capillary and positioned in the gap between the DART source and mass spectrometer for measurements. Data acquisition was from m/z 50.0 to 1000.0. Exact mass calibration was accomplished by including a mass spectrum of neat polyethylene (PEG) glycol (1:1 mixture PEG 200 and PEG 600) in the data file. m-Nitrobenzyl alcohol was also used for calibration. The mass calibration was accurate to within 0.002 u. It was found that the mass error is a linear function of the logarithm of the signal intensity adjusted to the associated lock-mass intensity. When applied to all mass data points, the correction function reduced the mass error for the majority of the tested compounds to ≤ 1 ppm over a wide range of signal intensities. The systematic error in mass measurements using TDC-based TOF-MS can only be avoided by careful and time-consuming manual analysis, limiting the exact mass

calculations to those chromatographic scans that display an analyte mass intensity similar to the lock-mass intensity. The elemental composition could be determined on selected peaks using the Mass Center software. Molecular ions of polyphenolic compounds present in the samples were found to be same with that of standard analytes at same R_f value.

Preparation of standard solutions

The appropriate amount of each standard was weighed and dissolved in methanol to make individual stock solutions. A mixed standard stock solution containing corilagin, ellagic acid, epicatechin, and gallic acid was prepared in methanol. The working standard solutions were prepared by diluting the mixed standard solution with methanol to a series of proper concentrations. Each concentration was applied six times on the HPTLC plate. The plate was then developed using the previously described mobile phase.

Preparation of sample solutions

E. longana seed powder (1 g) was weighed accurately, and the extraction process was performed as described previously [3,7] with some modification. Ultrasound-assisted extraction was implemented by an ultrasonic cleaner (Equitron, Medica Instrument Mfg. Co., Mumbai, India) using 25 mL 70% methanol for 30 min at a frequency of 53 KHz and 52°C temperature. The extracted solution was centrifuged at 12000 rpm for 10 min at 25°C, and the supernatant was transferred to the volumetric flask. The residues were then re-extracted with 25 mL 70% methanol using the same conditions. The extraction solution was collected, pooled and filtered through a 0.45 µm PTFE syringe filter. The filtrate was diluted with methanol to final working solutions and analyzed by HPTLC. All samples were stored in a refrigerator at 4°C until analysis.

Method validation

The HPTLC method was validated in terms of linearity, sensitivity, precision (intra- and inter-day variability), robustness, specificity, and accuracy in accordance with International Conference on Harmonization (ICH) guidelines on analytical method validation [18].

A series of mixed standard solutions containing appropriate concentrations of the four standards were analyzed to construct the calibration curves by plotting the peak area (y) of individual standard versus the concentration (x) of each analyte with least square linear regression of slope (m) and intercept (c) (y=mx+c). Then, the correlation coefficient (r^2) and linear range of each analyte were calculated. Limit of detection (LOD) and limit of quantification (LOQ) was determined on the basis of response and slope of each regression equation at signal-to-noise ratio (S/N) of 3:1 and 10:1, respectively for each analyte to determine the sensitivity of the method. The precision of the developed HPTLC method was analyzed by intra-day and inter-day variation. For intra-day variability test, the mixed standard solutions were analyzed for six replicates within one day, while for inter-day variability test, the solutions were examined in duplicates on three consecutive days. Variations in the precision were expressed by relative standard deviation (RSD) of the peak area of the standard. To assess the stability of the target analytes in the final extraction solution, the randomly selected *E. longana* extracted sample (S2) solution was stored at room temperature and analyzed at 0, 2, 4, 6, 8, 12 and 24 h. Robustness of the method was checked by making intentional changes in the parameters. Small change in the mobile phase composition was tried (formic acid ± 0.01 ml). The amount of mobile phase was varied in the range of ± 5%. The plates were prewashed with methanol and activated at 110°C ± 5 for 5, 10, 15 min respectively prior to chromatography. Time from spotting to chromatography and from chromatography to scanning was varied from 0, 30, 60 and 90 min. Robustness was done at three different concentration levels of each analytes. The specificity of the method was determined by analyzing standard and extract samples. The peak for polyphenols in *E. longana* extract samples was confirmed by comparing the R_f value and the spectrum of the peak with that of the standard. The peak purity of compounds was determined by comparing the spectrum at three different regions of the spot, i.e., peak start (S), peak apex (M) and the peak end (E). Moreover, peak identity and purity was confirmed by mass spectrometry. The accuracy of the method was evaluated by the standard addition method. In the accuracy experiment, known amounts of the standards at low (80% of the known amounts), medium (100% of the known amounts), and high (120% of the known amounts) levels was spiked into *E. longana* sample (S2). Three replicates were performed and the extraction recovery of each analyte was calculated using the following equation:

Recovery (%)=100 × (found amount-original)/Spiked amount

Results and Discussion

Selection of analytical wavelength

HPTLC spectrum of corilagin, ellagic acid, epicatechin, and gallic acid showed maximum absorbance at 293 nm, 278 nm, 279 nm, and 274 nm, respectively. Further, in situ HPTLC spectral overlain of corilagin, ellagic acid, epicatechin, and gallic acid was taken. Isoabsorptive point was found at 280 nm and was selected as scanning wavelength (Figure 2).

Optimization of chromatographic conditions

Several solvent systems were assessed to attain the optimum resolution of four polyphenolic compounds. Initially, the mobile phase was selected on the basis of previous reports of gallic acid and ellagic acid. The first combination of mobile phase was composed of toluene, ethyl acetate, methanol, and formic acid (3: 3: 0.2: 0.8, v/v/v/v) [19]. The mobile phase resulted in good resolution of four peaks with same R_f value of gallic acid and ellagic acid as described in reported method however corilagin peak did not migrate considerably from the spotting zone and recorded at an R_f value of 0.03. In an attempt to achieve the desired R_f value in the range (0.2-0.8), methanol in the same mobile phase was increased to 0.5, 1, and 1.5 while other components were kept constant. Despite increasing methanol, the peak of corilagin was remain constant at the starting point that indicates increasing polarity using methanol has no effect on the elution of corilagin and on the contrary this resulted in complete elution of gallic acid at the solvent front more than the desired R_f range due to high polarity difference among analytes. Any change in formic acid yielded broad peak shape of corilagin. So, it was kept constant in later trails. Further, methanol was replaced by chloroform and then acetone in different ratio, resulted in similar results with no movement of corilagin. Afterwards, trials were directed towards elution of corilagin with n-butanol-water system that had been tried in different ratio along with methanol and formic acid, which finally resulted in the movement of corilagin. Later on, fine improvements in the ratio of solvents were made to obtain optimum mobile phase composed of n-butanol: water: methanol: formic acid (7.59: 1.27: 0.13: 1.01, v/v/v/v) for required elution of corilagin and simultaneously maintaining the adequate retention of gallic acid. The optimum mobile phase resulted in well resolved peaks with good shape with R_f values of 0.40 ± 0.01, 0.57 ± 0.02, 0.69 ± 0.01, and 0.79 ± 0.01 for corilagin, ellagic acid, epicatechin, and gallic acid, respectively (Figure 3).

Method validation

The proposed HPTLC method for quantitative analysis was validated by determining the linearity, LOD, LOQ, intra-day and

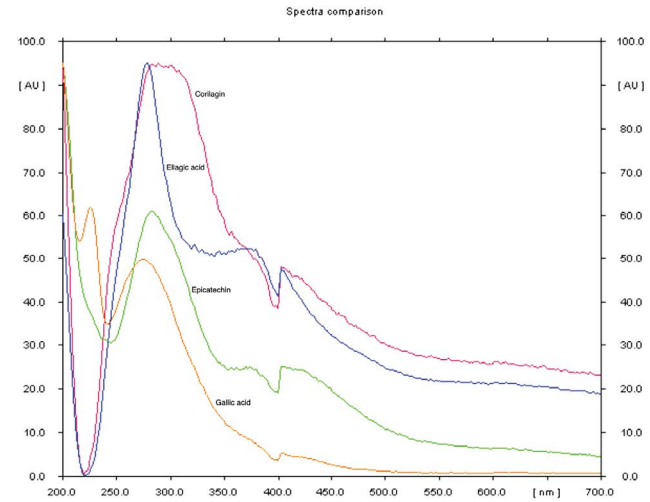

Figure 2: *In situ* HPTLC spectral overlain of corilagin, ellagic acid, epicatechin, and gallic acid in standard mixture.

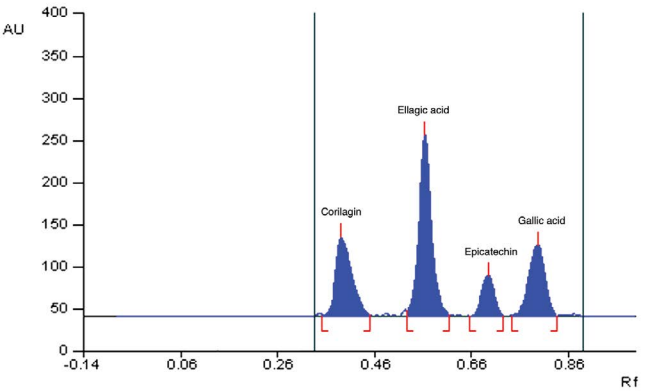

Figure 3: Densitogram of standard mixture at 600 ng/spot for corilagin (R_f 0.40), ellagic acid (R_f 0.57), epicatechin (R_f 0.69), and gallic acid (R_f 0.79) at 280 nm.

inter-day precisions, robustness, specificity, and accuracy. The linear regression equation, linearity ranges, together with the corresponding correlation coefficient (r^2) for the four analytes are listed in Table 1. All of the analytes expressed satisfactory linearity (r^2>0.995) over a relatively wide concentration range. The LOD and LOQ for the four compounds were <189.5 and 574.3 ng/spot, respectively, indicating a high sensitivity of the method with these chromatographic conditions. As shown in Table 1, the RSD values were calculated to express the intra- and inter-day precisions of the investigated compounds, ranged from 0.12 to 1.17% for the intra-day precision, and from 0.16 to 1.60% for the inter-day precision, illustrating good precision of the established method. Through analyzing the *E. longana* sample solution within 24 h, it was found to be stable as the RSD values of the peak areas were lower than 1.29%. Robustness of the proposed method was evaluated through investigating its ability to remain unaffected by small but deliberate variations. The standard deviation of the peak areas were calculated for each parameter and the RSD was found to be less than 2%. The low values of the RSD, indicated the robustness of the method. The peak purity of corilagin, ellagic acid, epicatechin, and gallic acid was assessed by comparing their respective spectra at the peak start, apex and peak end positions of the spot i.e., r (S, M)=0.999180, r (M, E)=0.998236, r (S, M)=0.999876, r (M, E)=0.999790, r (S, M)=0.999484, r (M, E)=0.998909, and r (S, M)=0.999666, r (M, E)=0.999995, respectively. A good correlation (r^2=0.998, r^2=0.999, r^2=0.998 and r^2=0.999) was obtained between the standard and *E. longana* sample spectra of corilagin, ellagic acid, epicatechin, and gallic acid, respectively (Figure 4). A further confirmation of adequate specificity and resolution was established by DART-MS. The average recoveries of the four polyphenolic compounds were in the range of 98.42-101.73% with RSD values <1.77%, indicating that the proposed method is accurate and reproducible.

Quantitative analysis of samples

The developed HPTLC method was subsequently applied for the quantitative analysis of four major polyphenolic compounds (corilagin, ellagic acid, epicatechin, and gallic acid) in *E. longana* seeds. The contents of four major components were calculated with an external standard method based on the respective calibration curves. HPTLC densitogram of *E. longana* extract sample S2 shown in Figure 5.

As per results are shown in Table 2, corilagin content ranged from 3.94 mg/g to 4.98 mg/g (sample, S5 and S1) was markedly highest among all other polyphenolic components make it considered as the most prevalent compound in *E. longana* seeds. Ellagic acid content ranged from 1.08 mg/g to 1.76 mg/g (sample, S5 and S1) was found as the other dominant polyphenolic component. Similarly, gallic acid content ranged from 1.06 mg/g to 1.61 mg/g (sample, S5 and S1). The lowest content was of epicatechin correspond to others, which ranged from 0.48 mg/g to 1.22 mg/g (sample, S5 and S3). The highest amount of total polyphenolic content was found in sample S1. The various alterations of the quality of *E. longana* samples might be due to intrinsic factors such as genetic variation, plant origin, and extrinsic factors, such as geographic location, environmental conditions, cultivation techniques, etc. [20-23]. The quantitative analysis indicated that this method has significant importance in the comprehensive evaluation of selected polyphenolic compounds, which could be used in the quality control of *E. longana* and its related preparations.

DART-MS confirmation

Representative DART-MS spectra of HPTLC bands of *E. longana* extract (60000 ng/spot) correspond to corilagin (A), ellagic acid (B), epicatechin (C), and gallic acid (D) at their respective R_f are shown in Figure 6. Elemental compositions of major peaks were calculated by a built-in software based on the exact mass numbers of the elements and deprotonated molecules $[M-H]^-$ were recorded under conditions of negative DART ionization. Based on the published literature and comparison with standards, four major polyphenolic compounds were confirmed in HPTLC bands of *E. longana* sample are shown in Table 3. According to the previously reported phytochemical studies, the mass to charge ratio of m/z 633.1488 $[M-H]^-$ was identified as deprotonated corilagin [13]. Similarly, ion peak of m/z 300.9771 $[M-H]^-$, 289.0802 $[M-H]^-$ and 169.0337 $[M-H]^-$ attributed to ellagic acid, epicatechin, and gallic acid, respectively [24-26].

To summarize, in the present study, the authors have established a suitable HPTLC method for the simultaneous determination of four major polyphenolic compounds in *E. longana*. The validated results demonstrated that the linearity, sensitivity, precision, robustness, specificity and accuracy of the proposed HPTLC method were satisfactory for the determination of polyphenolic compounds in *E. longana*. The authenticity and identification of the compounds were confirmed by DART-MS. The proposed method based on quantification and identification confirmation of bioactive components using a combination of two rapid analytical techniques was successfully applied

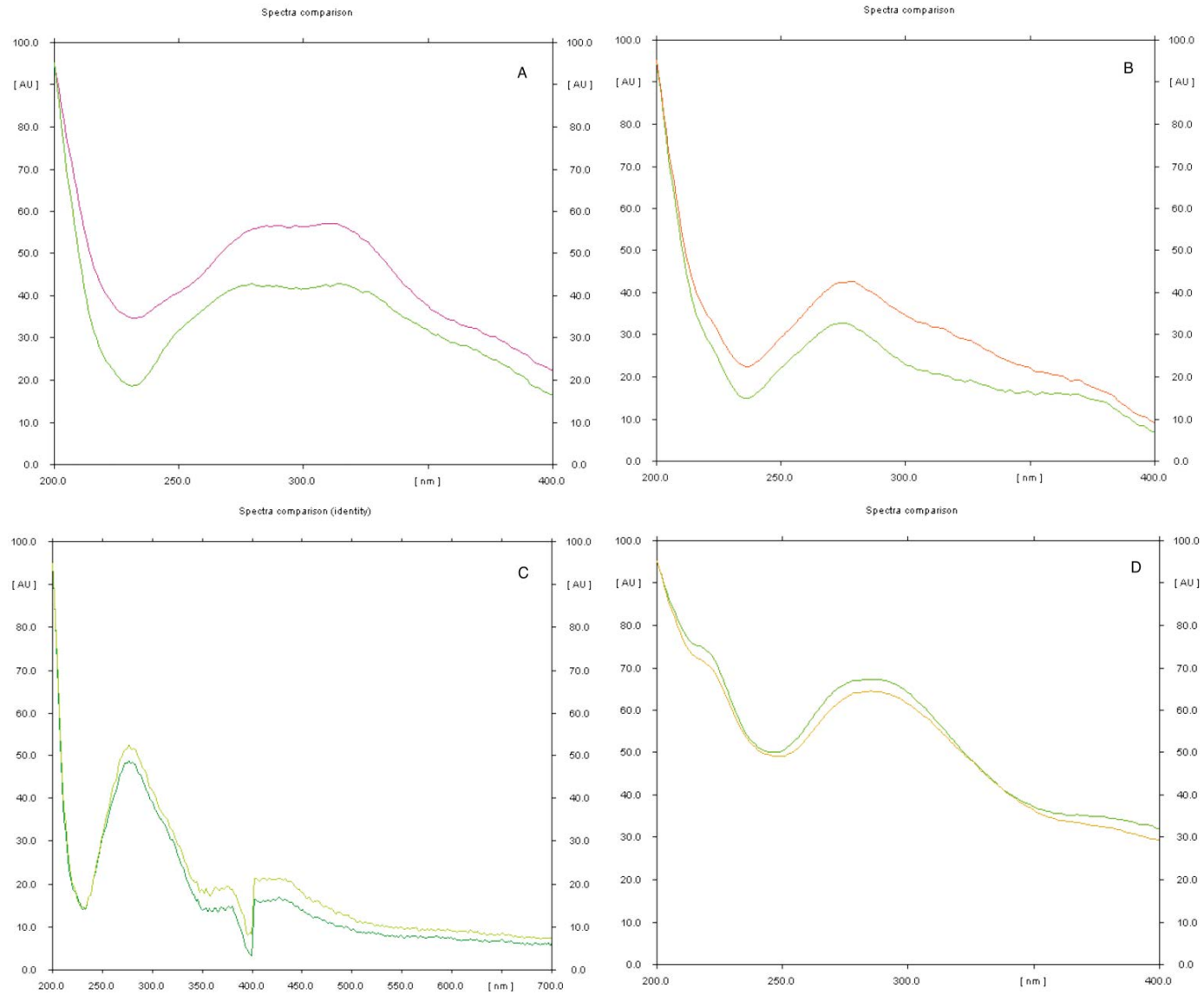

Figure 4: *In situ* HPTLC spectral overlain of corilagin (A), ellagic acid (B), epicatechin (C), and gallic acid (D) obtained from standard and *E. longana* sample.

	Corilagin	Ellagic acid	Epicatechin	Gallic acid
Regression equation	y=15.465x+187.2	y=15.987x-296.6	y=2.6583x+163.93	y=6.888x+2651.4
r^2	0.99813	0.99770	0.99941	0.99836
Linear range (ng/spot)	200-1200 ng/spot	200-1200 ng/spot	600-3600 ng/spot	200-1200 ng/spot
Sy.x*	279.6	321.1	80.9	116.8
LOD (ng/spot)	63.6	59.2	189.5	50.3
LOQ (ng/spot)	192.9	179.6	574.3	151.6
Precision RSD % (Intra-day, n=6)	0.81	0.77	1.17	0.12
Precision RSD % (Inter-day, n=6)	0.62	0.16	0.79	1.6
Stability RSD %, n=5	0.34	1.29	0.36	0.59
Recovery RSD %	1.77	0.61	1.21	0.48

*Standard deviation of residuals from line

Table 1: Method validation parameters: regression equation, correlation coefficients (r^2), limits of detection (LOD) and quantification (LOQ), precisions intra and inter-day, stability, and recovery.

for comprehensive quality control of *E. longana* products. This method could be an appropriate alternative for determination of bioactive components in plant matrices/ phytopharmaceuticals.

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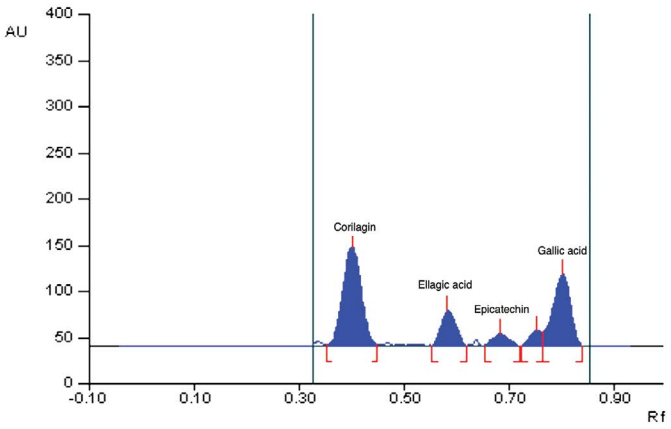

Figure 5: Densitogram of *E. longana* extract (60000 ng/spot) for corilagin (R_f 0.40), ellagic acid (R_f 0.58), epicatechin (R_f 0.68), and gallic acid (R_f 0.80) at 280 nm.

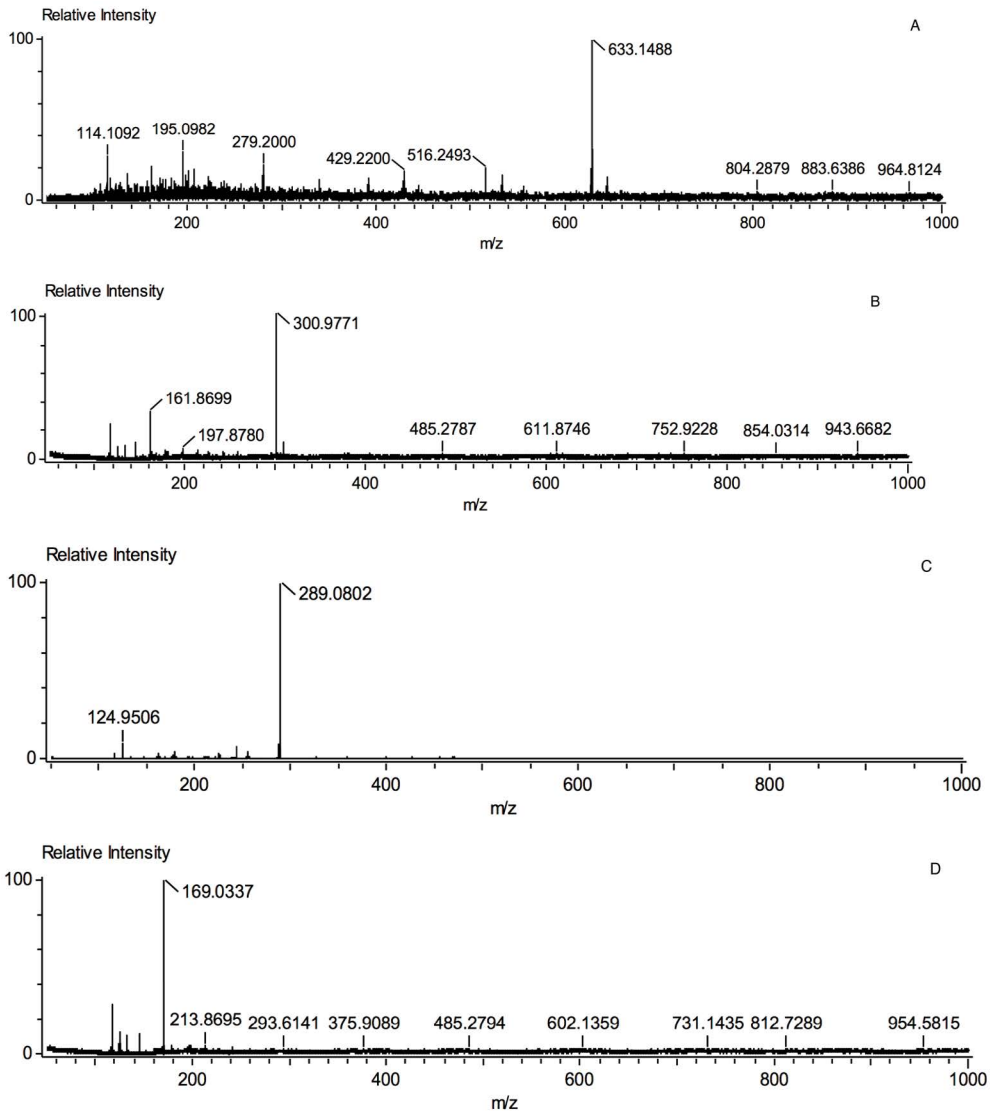

Figure 6: DART-MS spectra of HPTLC bands of *E. longana* extract (60000 ng/spot) correspond to corilagin (A), ellagic acid (B), epicatechin (C), and gallic acid (D) in negative ion mode.

Sample	Corilagin	Ellagic acid	Epicatechin	Gallic acid	Total
S1*	4.98 ± 0.02*	1.76 ± 0.05	0.92 ± 0.01	1.61 ± 0.06	9.27 ± 0.14*
S2	4.44 ± 0.15	1.61 ± 0.07	1.16 ± 0.03	1.28 ± 0.12	8.49 ± 0.37
S3	4.34 ± 0.04	1.52 ± 0.02	1.22 ± 0.16	1.57 ± 0.17	8.65 ± 0.39
S4	4.07 ± 0.10	1.13 ± 0.08	0.73 ± 0.04	1.20 ± 0.05	7.12 ± 0.27
S5	3.94 ± 0.21	1.08 ± 0.09	0.48 ± 0.10	1.06 ± 0.06	6.56 ± 0.46

*Highest content

Table 2: Content (mg/g) ± SD of polyphenolic compounds in *E. longana* seed (n=3).

Identification	[a]R_f	Molecular formula	Measured mass	Theoretical mass	Error (mmu)
Corilagin	0.40	$C_{27}H_{22}O_{18}$	633.1488 $[M-H]^-$	633.1506 $[M-H]^-$	-0.18
Ellagic acid	0.58	$C_{14}H_6O_8$	300.9771 $[M-H]^-$	300.9732 $[M-H]^-$	0.39
Epicatechin	0.68	$C_{15}H_{14}O_6$	289.0802 $[M-H]^-$	289.0790 $[M-H]^-$	0.12
Gallic acid	0.80	$C_7H_6O_5$	169.0337 $[M-H]^-$	169.0375 $[M-H]^-$	-0.38

[a]Retardation factor

Table 3: DART-MS exact mass measurement of polyphenolic compounds in HPTLC bands of *E. longana* extract.

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