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SIMULTANEOUS ESTIMATION OF GALLIC ACID, QUERCETIN AND KAEMPFEROL FROM EUPHORBIA PROSTRATA W. AIT BY HIGH PERFORMANCE THIN LAYER CHROMATOGRAPHY

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ABSTRACT

A sensitive and reliable quantitative High Performance Thin Layer Chromatographic (HPTLC) method have been developed for the quantitation of Gallic acid, Quercetin and Kaempferol from whole plant powder of *Euphorbia prostrata* W. Ait. which has antidiabetic activity. Methanolic extract of the whole plant powder of *Euphorbia prostrata* W. Ait. used for carrying out thin layer chromatography (TLC) on silica gel 60 F254 plates. Detection and quantitation was performed by using deuterium lamp at λ = 254 nm. Accuracy of the developed HPTLC method was checked by conducting the recovery study. The average percent content of Gallic acid, Quercetin and Kaempferol in whole plant powder of the used plant as estimated by the proposed method was found to be 0.3939mg/g, 0.1197 mg/g, and 0.2421mg/g respectively. The HPTLC method developed for the quantitative determination of Gallic acid Quercetin and Kaempferol from the whole plant powder of *Euphorbia prostrata* W. Ait. is rapid, simple and precise.

KEYWORDS: **Gallic acid, Quercetin, Kaempferol, *Euphorbia prostrata* W. Ait. and High Performance Thin Layer Chromatography (HPTLC)**

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INTRODUCTION

Euphorbia prtostrata W. Ait. is commonly called as laghudugdhika, a medicinal plant belonging to Euphorbiaceae family. A branched, prostate, fleshy, spreading milky weed growing throughout India in plains. It has various medicinal properties. *Euphorbia prostrata* W. Ait. is reported to have antidiabetic activity[1-2] anti bacterial activity[3-4], anti-inflammatory activity[156] and anthelmintic activity[7]. Plant contains many phytoconstituents such as Gallic acid, Kaempferol, Quercetin, Quercetin-3-rhamnoside, apigenin-7-glucoside, β- sitosterol and stigmasterol[18]. Gallic acid is a phenolic acid. Quercetin and Kaempferol are flavonoids. Gallic acid is reported to have several biological activities such as anti-cancer activity[9], anti-inflammatory[10], anti-angiogenic[11], anti-atherosclerosis[12], anti-bacterial[13], anti-carcinogenic[14], antioxidant[15]. Quercetin is reported to show down activity of antidiabetic[16-17], antioxidant [18], anti- inflammatory[19] and anti-cancer activity[20]. Kaempferol is known for its strong antioxidant and anti-inflammatory properties[21]. Considering medicinal properties of Gallic acid, Quercetin and Kaempferol in the present research work, HPTLC method was developed for the separation and quantification of these phytoconstituents. Very few researchers have attempted to use HPTLC method for quantitation of Gallic acid, Quercetin and Kaempferol. Simultaneous estimation of Gallic acid, Curcumin and Quercetin by HPTLC on silica gel 60F254 percolated TLC plate using toluene: ethyl acetate: formic acid (4.5:3.0:0.2 *v/v/v*) as solvent system have been reported[22]. HPTLC method have been developed for quantitation of Quercetin in the dried flowers of *Nymphaea stellata* willd using toluene: ethyl acetate: formic acid, (5: 4: 0.2 *v/v/v*), as mobile phase[23]. HPTLC method have developed for simultaneous estimation of Quercetin, Kaempferol and asiatic acid in the methanolic extract of leaves of two chemotypes of *Centella asiatica* using a suitable mobile phase toluene: ethyl acetate: chloroform: formic acid (6:6:4:1 *v/v/v*)[24]. However, no HPTLC method was reported for simultaneous quantitation of Gallic acid, Quercetin and Kaempferol from *Euphorbia prostrata* W.Ait. Thus, precise and accurate HPTLC method has been developed and validated using International Conference on Harmonization (ICH) guidelines for simultaneous quantitation of Gallic acid Quercetin and Kaempferol from *Euphorbia prostrata* W.Ait.

MATERIALS AND METHODS

Reagents and Standards

Toluene, ethyl acetate, methanol and formic acid were of AR grade with 99.5%, 99.5%, 99.8% and 100.0 % purities respectively and were obtained from Qualigens Fine Chemicals, Mumbai, India. The reference standards Gallic acid (purity 97.5%), Quercetin (purity 95.0%) and kempferol (purity 97.0%) were purchased from Sigma-Aldrich Chemie GmbH (Aldrich Division, Steinbeim, Germany).

Plant material

Euphorbia prostrata W. Ait. was collected from Satara district from Maharashtra, India and its herbarium was prepared and authenticated from Botonical Servey of India (BSI), Pune, India. A duplicate herbarium was prepared and preserved in Ramnarain Ruia College. The whole plant of *Euphorbia prostrata* W. Ait., was washed with water to remove soil particles, dried at 45±2ºC.[25] A dried plant was powdered and then sieved through BSS mesh size 85 and stored in an air tight container at room temperature (25 ± 2°)

Preparation of solutions

Preparation of stock solution of Gallic acid (50.0µg/mL)

10.30 mg of Gallic acid was accurately weighed and transferred to 10.0 mL volumetric flask. 5.0 mL of methanol was added and the mixture was sonicated in an ultrasonic bath (Model:TRANS-O-SONIC, Frequency: 50 Hz) for 5 minutes for complete dissolution of Gallic acid. The mixture was then diluted up to the mark with methanol to obtain a solution of Gallic acid with concentration of 1000.0µg/mL. A 500 µL of above stock solution of Gallic acid was then transferred to 10.0 mL volumetric flask and diluted up to mark by methanol to obtain working solution of acid with concentration of 50.0µg/mL

Preparation of stock solution of Quercetin (5.0µg/mL)

10.00 mg of Quercetin was accurately weighed and transferred to 10.0 mL volumetric flask. 5.0 mL of methanol was added and the mixture was sonicated for 5 minutes for complete dissolution Quercetin. The mixture was then diluted up to the mark with methanol to obtain a solution of Quercetin with concentration of 1000.0µg/mL. A 50 µL of above stock solution of Quercetin was then transferred to 10.0mL volumetric flask and diluted up to mark by methanol to obtain working solution of Quercetin with concentration of 5.0µg/mL.

Preparation of stock solution of Kaempferol (25.0µg/mL)

About 10.00 mg of Kaempferol was accurately weighed and transferred to 10.0mL volumetric flask. 5.0 mL of methanol was added and the mixture was sonicated in an ultrasonic bath for 5 minutes for complete dissolution Kaempferol. The mixture was then diluted up to the mark with methanol to obtain a solution of Kaempferol with concentration of 1000.0µg/mL. A 250µL of above stock solution of Kaempferol was then transferred to 10.0mL volumetric flask and diluted up to mark by methanol to obtain working solution of Kaempferol with concentration of 25.0µg/mL

Preparation of sample solution

Dried whole plant powder (about 1000 mg) of *Euphorbia prostrata* W. Ait. was accurately weighed and added to 20 mL standard volumetric flask. To this, 10 mL of methanol was added. The content of the standard volumetric flask was shaken on a mechanical shaker for 10 min, followed by sonication in an ultra-sonic bath for 15 min and were allowed to stand overnight, at room temperature (25º±2ºC). This solution was filtered through Whatmann filter paper No. 41 (Merck, Mumbai, India). The clear filtrate was collected in a dry 10mL standard volumetric flask. This solution was used for the assay experiment.

Chromatographic conditions

Chromatography was performed on 5.0 cm x 10.0 cm TLC plates which were cut from 20.0 cm x 20.0 cm TLC aluminum plates pre-coated with 200µm layers of silica gel 60F254 (E. Merck, Mumbai, India). The plates were prewashed with methanol and activated at 105-110ºC for 15 minutes before analysis. 10 µL of each of the standard solutions of Gallic acid, Quercetin, Kaempferol and sample solution were applied as bands with the help of CAMAG Linomat IV sample applicator with a 100 µL syringe (Hamilton, Bonaduz, Switzerland), at a distance of 10.0 mm from the bottom edge of the chromatographic plate as bands of 6.0 mm width at a distance of 8.8 mm from each other. Linear ascending development was carried out in a twin-trough glass chamber (Camag, Muttenz, Switzerland) saturated with mobile phase comprising of toluene : ethyl acetate : formic acid : methanol (3.0:3.0:0.8:0.2 *v/v/v/v*). The optimized chamber saturation time for the mobile phase was 20 minutes at room temperature (25º±2ºC). The plates were developed to a distance of 80 mm from the bottom edge of the plate. After development, a plate was air dried and the response of standards Gallic acid, Quercetin, Kaempferol was monitored using CAMAG III TLC Scanner with Win CATS software version 2.01.2., set at a wavelength, λ= 254 nm, in Deuterium lig

Method validation

Linearity

Prepartion of calibration curve for Gallic acid

A stock solution of standard Gallic acid (50.0µg/mL) was prepared in methanol. Applying 1 µL, 2 µL, 4 µL, 6 µL 8 µL, 10 µL, 12 µL, 14 µL, 16 µL, 18 µL, 20 µL, 22 µL from stock solution of 50.00 µg/mL of standard Gallic acid on TLC plate to obtain concentrations of 0.05 µg/band to 1.0 µg/band. The chromatograms were recorded and peak areas for each applied concentration of Gallic acid were noted.

Prepartion of calibration curve for Quercetin

A stock solution of standard Quercetin(5.0µg/mL) was prepared in methanol. Applying 5 µL, 10 µL, 15 µL, 20 µL, 30 µL, 40 µL, 60 µL, 70 µL, 80 µL, 90 µL 100 µL from stock solution of 5.00 µg/mL of standard Quercetin on TLC plate to obtain concentrations of 0.025 µg/band to 0.500 µg/band. The chromatograms were recorded and peak areas for each applied concentration of Quercetin were noted.

Prepartion of calibration curve for Kaempferol

A stock solution of standard Kaempferol (25.0 µg/mL) was prepared in methanol. Applying 2 µL, 4 µL, 6 µL, 8 µL, 10 µL, 12 µL, 14 µL, 16 µL, 18 µL, 20 µL from stock solution of 5.00 µg/mL of standard Kaempferol on TLC plate to obtain concentrations of 0.050 µg/band to 0.500 µg/band.The chromatograms were recorded and peak areas for each applied concentration of Kaempferol were noted. Each concentration of Gallic acid, Quercetin and Kaempferol was applied in triplicate under 6.0 mm bands on the TLC plate by means of CAMAG Linomat IV automatic sample applicator. The plates were developed under specified chromatographic conditions and scanned and the peak areas were recorded for each concentration. The calibration curves of Gallic acid, Quercetin and Kaempferol were obtained by plotting graphs of mean peak areas vs. corresponding concentrations. The results listed in Table 1. For all the standards, within the concentration range indicated, there was a good correlation between mean peak area and concentration of standards.

Limit of Detection (LOD) and Limit of Quantitation (LOQ)

The limit of detection (LOD) and limit of quantitation (LOQ) were determined at signal to noise ratios of 3:1 and 10:1, respectively. The LOD and LOQ values obtained for both the components are listed in Table 1.

System Suitability

System suitability was carried out to verify that resolution and reproducibility of the system were acceptable for the analysis. System suitability test was carried out by applying standard solution of Gallic acid with concentration of 0.5 µg/band, Quercetin with concentration 0.150 µg/band and Kaempferol with concentration of 0.2 µg/band on same TLC plate in six replicates under specified chromatographic conditions. The chromatograms for all standards were recorded. The values of mean peak area, standard deviation (S.D.) and the percent relative standard deviations (%R.S.D) of Gallic acid, Quercetin and Kaempferol were calculated.

Precision

Intra-assay precision and intermediate precision of the method were determined. Intra assay precision was evaluated by analysis of three replicate applications of freshly prepared standard solutions of same concentration, on the same day. Intermediate precision was evaluated by analysis of three replicate applications of standard solution of same concentration on three different days.

Robustness

Robustness tests examine the effect of the operational parameters on the analysis results. By introducing small changes in the mobile phase composition, the effects on the results were examined. The mobile phase in the developed method was toluene : ethyl acetate : formic acid : methanol (3.0:3.0:0.8:0.2 *v/v/v/v*). The composition of the mobile phase was altered as toluene : ethyl acetate : formic acid : methanol (3.1: 2.9 : 0.8 : 0.2 *v/v/v/v*), toluene : ethyl acetate : formic acid : methanol (2.9: 3.1: 0.8 : 0.2 *v/v/v/v*) and toluene : ethyl acetate : formic acid : methanol (3.0 : 3.0 : 0.7 : 0.3 *v/v/v/v*). The amounts of Gallic acid, Quercetin and Kaempferol from dried whole plant powder of *Euphorbia prostrata* W.Ait. obtained by normal method and that by altered method was found to be similar. The modifications did not affect the system suitability criteria. From observations, it was concluded that the method is robust as the changes in operational parameter did not affect the results.

Assay Procedure

The developed and validated HPTLC method was used for quantification of Gallic acid, Quercetin and Kaempferol from the methanolic extract of dried whole plant powder of *Euphorbia prostrata* W.Ait. 10µL of this methanolic extract was applied as a band on the same TLC plate (n=7). The plate was developed and scanned under the optimized chromatographic conditions. The

chromatograms were recorded. Amount of Gallic acid, Quercetin and Kaempferol present in the sample solution were determined from the calibration curve by using the peak area of Gallic acid, Quercetin and Kaempferol in the sample. The amount of Gallic acid, Quercetin and Kaempferol were found to be 0.3939 mg/g, 0.1197 mg/g and 0.2421 mg/g respectively.

Accuracy

The accuracy of the method was established by performing recovery experiment using standard addition method at three different levels. To accurately weighed about 1.0 g of dried whole plant powder of *Euphorbia prostrata* W.Ait., known amount of standard Gallic acid, Quercetin and Kaempferol i.e. 0.1 mg, 0.2 mg, 0.3 mg were added, and extracted using methanol. Gallic acid, Quercetin and Kaempferol contents were quantified by the proposed method and the percentage recovery was calculated. The values of percent recoveries obtained were 98.16%, 98.54 %and 95.60% for Gallic acid, Quercetin and Kaempferol respectively. The results of accuracy are listed in Table 2.

RESULTS

HPTLC separation of Gallic acid, Quercetin and Kaempferol from dried whole plant powder of *Euphorbia prostrata* W. Ait. and good separation was achieved by using toluene : ethyl acetate : formic acid : methanol (3.0:3.0:0.8:0.2 v/v) as mobile phase with double development of the plate. Detection was carried out densitometrically using a CAMAG TLC Scanner at λ = Different mobile phases were tried for simultaneous 254 nm as Gallic acid, Quercetin and Kaempferol showed maximum response at this wavelength. The identity of the bands of Gallic acid, Quercetin and Kaempferol in the sample solutions was confirmed by comparing their retention factor (Rf) values of reference standards. Figure 1 represents a typical TLC plate showing separation of Gallic acid, Quercetin and Kaempferol. The Rf values for Gallic acid, Quercetin and Kaempferol were 0.31, 0.48 and 0.58 respectively. Figure 2 shows typical HPTLC chromatograms of standard Gallic acid, Quercetin and Kaempferol and methanolic extract of dried whole plant powder of *Euphorbia prostrata* W.Ait. A good linear relationship was observed for Gallic acid, Quercetin and Kaempferol in the concentration range of 0.2 µg/band to 0.8 µg/band (Figure 3), 0.075 µg/band to 0.350 µg/band (Figure 4)and 0.2 µg/band to 0.5 µg/band(Figure 5) respectively with correlation coefficient of 0.999 for all the components (Table 1). When the method was validated for repeatability and intermediate precision, the values of percentage relative standard deviations were less than 2, indicating the proposed method is precise and repeatable (Table 1). The mean amounts of Gallic acid, Quercetin and Kaempferol from the methanolic extract of dried whole plant powder of *Euphorbia prostrata* W.Ait. were found to be 0.3939 mg/g, 0.1197 mg/g, and 0.2421 mg/g respectively. The values of percent recoveries of Gallic acid, Quercetin and Kaempferol at three levels were 98.16%, 98.54% and 95.60% respectively indicating accuracy of the method (Table 2).

Table 1
Method validation data for simultaneous quantification of Gallic acid, Quercetin and Kaempferol

Parameters	Results		
	Gallic acid	Quercetin	Kaempferol
Linear range (n=3) (µg/mL)	0.05-1.00	0.025-0.500	0.05-0.500
Correlation coefficient (r)	0.999	0.999	0.999
LOD (µg/mL)	0.05	0.025	0.05
LOQ (µg/mL)	0.2	0.075	0.2
Repeatability (% R.S.D.) (n=3) (On the same day)	0.61	0.491	0.787
Intermediate precision (% R.S.D.) (n=9)(For three successive days)	0.646	0.411	0.648

Table 2
Results of recovery study for simultaneous HPTLC quantification of Gallic acid, Quercetin and Kaempferol from methanolic extract of dried whole plant powder of Euphorbia prostrata W.Ait.

Level	Weight of sample taken(g)	Weight of Std added to sample (mg)	Mean Weight of std found in sample (mg)± SD*	Percentage recovery(%)
Gallic acid				
0	1.003	0.000	0.3939	98.16
1	1.002	0.100	0.4871	
2	1.001	0.200	0.5886	
3	1.003	0.300	0.6874	
Quercetin				
0	1.003	0.000	0.1195	98.54
1	1.002	0.100	0.2176	
2	1.001	0.200	0.3180	
3	1.003	0.300	0.4186	
Kaempferol				
0	1.003	0.000	0.2421	95.60
1	1.002	0.100	0.3366	
2	1.001	0.200	0.4352	
3	1.003	0.300	0.5279	

Mean ±S.D. (n=7)

Figure 1
HPTLC plate showing separation of methanolic extract of dried whole plant powder of Euphorbia prostrata W.Ait.. (A), standard Gallic acid (B) and standard Quercetin (C) standard Kaempferol (D)

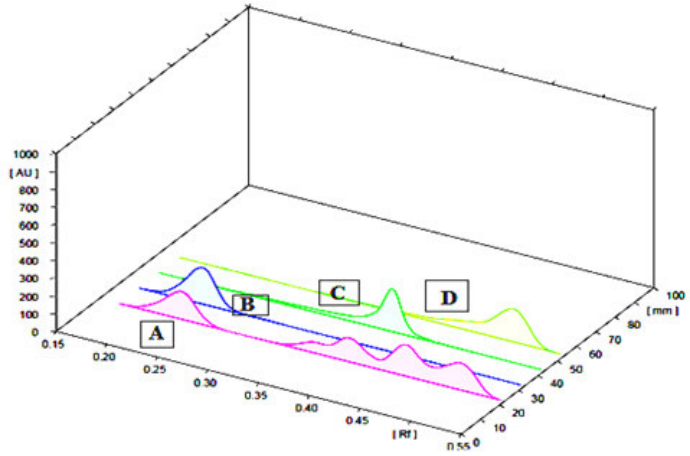

Figure 2
HPTLC chromatograms obtained for methanolic extract of dried whole plant powder of Euphorbia prostrata W.Ait. (A), standard Gallic acid (B) and standard Quercetin (C) standard Kaempferol

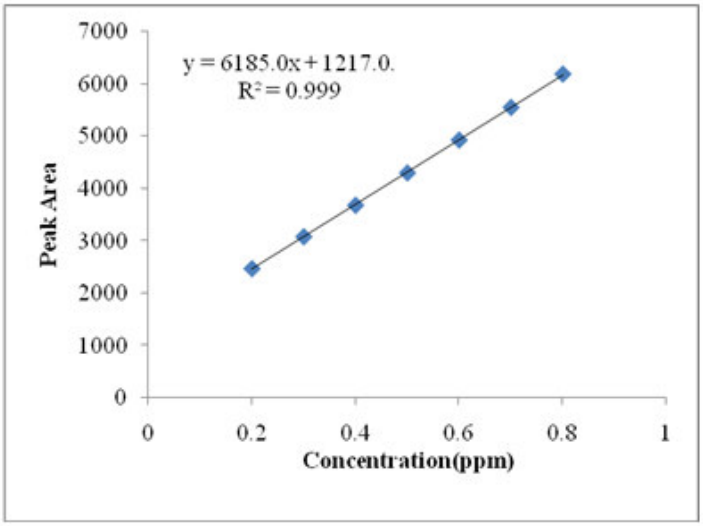

Figure 3
Graph of peak area vs. concentration of Gallic acid Linear working range of Gallic acid

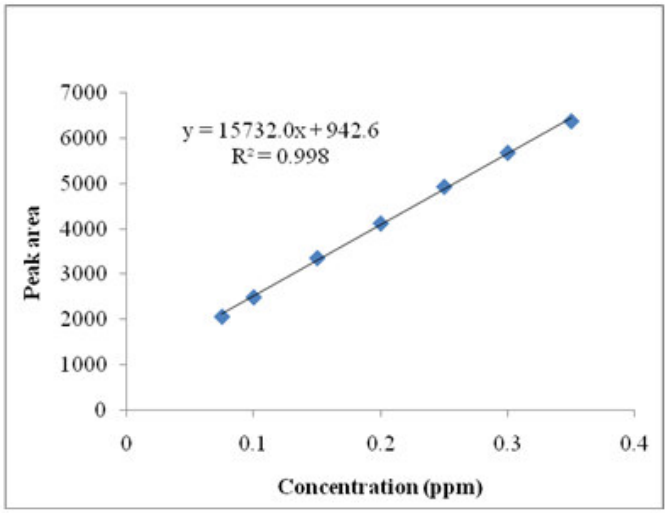

Figure 4
Graph of peak area vs. concentration of Quercetin Linear working range of Quercetin

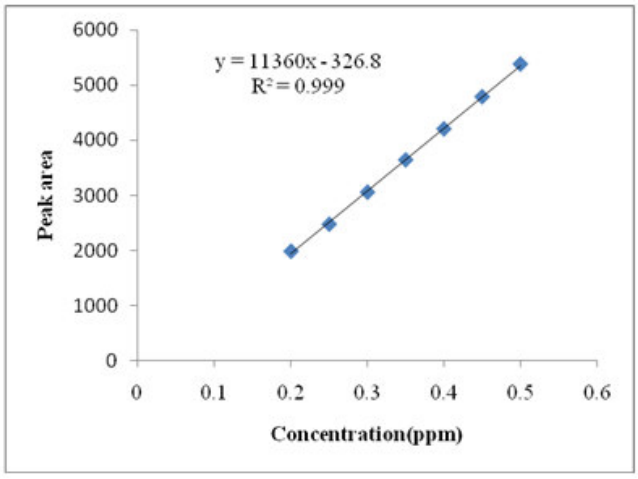

Figure 5
Graph of peak area vs. concentration of Kaempferol Linear working range of Kaempferol

DISCUSSION

In literature survey((Vinitkumar,*et al*, 2011), polyherbal formulation was standardized by using Gallic acid, Curcumin and Quercetin as a biomarker by HPTLC analysis, using toluene: ethyl acetate: formic acid (4.5:3.0:0.2,*v/v/v*) as solvent system. Densitometry scanning was performed under reflectance absorbance mode at 366 nm to quantify the spots. The Rf values of Gallic acid, curcumin and Quercetin were 0.40, 0.73 and 0.55 respectively. Quantitation of Quercetin in the dried flowers of *Nymphaea stellata* willd. The hydroalcoholic extract of *N. stellata* (Rakesh, *et al*, 2009) was chromatographed with toluene: ethyl acetate: formic acid, (5: 4: 0.2 *v/v/v*), as mobile phase. Detection and quantitation were performed by densitrometric scanning at λ= 380 nm, by using deuterium lamp. The Rf value found to be 0.29. Simultaneous estimation of Quercetin, Kaempferol and Asiatic acid in the methanolic extract of leaves of two chemotypes of *Centella asiatica* (Joshi C, *et al*, 2012)was achieved by mobile phase toluene: ethyl acetate : chloroform : formic acid (6:6:4:1 *v/v/v*). The plates were derivatized with anisaldehyde reagent and densitometric determination was performed at wavelength 530nm. The Rf values for asiatic acid, Quercetin, and Kaempferol were 0.33, 0.44 and 0.55 respectively. In present research work, mobile phase selected was toluene: ethyl acetate: formic acid: methanol (3.0:3.0:0.8:0.2 *v/v/v/v*). The mobile phase helped to separate Gallic acid, Quercetin and Kaempferol with a very little resolution. The Rf values for Gallic acid, Quercetin and Kaempferol were 0.31, 0.48 and 0.58 respectively The developed method was successfully used for separation of Gallic acid, Quercetin and Kaempferol from *Euphorbia prostrata* W. Ait.

CONCLUSION

The developed HPTLC technique is precise, specific and accurate. It can be used for the routine quality control analysis and simultaneous quantitative determination Gallic acid, Quercetin and Kaempferol standards.

CONFLICT OF INTEREST

Conflict of interest declared none.

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