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Development of Validated HPTLC Method for the Standardization of *Euphorbia Hirta* using Gallic Acid, Rutin and Quercetin as Phytochemical Markers

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ABSTRACT

Euphorbia Hirta belonging to the Euphorbiaceae family contains more amounts of phenolic compounds and flavonoids which are responsible for the main pharmacological actions like anti-oxidant, anti-inflammatory, anti-dengue and anti-cancer. Considering the current importance of these ingredients, an attempt has been made for the simultaneous estimation of gallic acid, rutin and quercetin from *euphorbia hirta* by successive extraction involves the use of solvents in an order of increasing polarity in soxhlet extractor at 30-*45 °C* using 800 ml solvents for 5 hours in increasing polarity to isolate the active constituents without other interferences. Hence we proposed to develop easy, rapid, accurate, precise and reliable analytical HPTLC method for the standardization of Euphorbia hirta(L) using gallic acid, rutin and quercetin as phytochemical markers from its methanolic extract and herbal capsule formulation. The separation was performed on TLC aluminum Plates precoated with silica gel $60F_{254}$, good separation was achieved in the mobile phase of butyl acetate: 1,4-dioxane (5:5% v/v) and densitometric determination of gallic acid, rutin and quercetin was carried out at 266nm.The linear regression data showed a good linearity in the concentration range of 136-748ng/spot of gallic acid, rutin and quercetin with a good correlation coefficient of 0.9989. Limit of detection was found to be 102 ng/spot of gallic acid,17ng/spot of rutin and 68ng/spot of quercetin. The limit of quantification for the estimation of gallic acid, rutin and quercetin was found to be 136ng/spot.

Keywords: *Euphorbia Hirta,* gallic acid, rutin, quercetin, methanol, phytochemical markers.

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INTRODUCTION

Euphorbia hirta contains more amounts of phenolic compounds and flavonoids[1] which are responsible for the main pharmacological actions like anti-oxidant, anti-inflammatory, anti-dengue, and anti-cancer[2-5]. Literature survey revealed that there are no analytical methods reported for the standardization of Euphorbia hirta (L) using gallic acid, rutin and quercetin as phytochemical markers. Hence we proposed to develop easy, rapid, accurate, precise and reliable analytical methods for the standardization of Euphorbia hirta (L) using gallic acid, rutin and quercetin simultaneously as phytochemical markers from its methanolic extract and herbal capsule formulation. *The International Conference on Harmonization (ICH) guideline entitled Validation of Analytical procedure*[6,7]. The importance of validation is producing reliable and repeatable results for routine analysis and stability analysis.The main objective of the study is to develop new analytical methods for assurance of quality, safety and efficacy of herbal drugs and pharmaceuticals which is quite important because of their use not only as health care products but also life-saving substances.

MATERIALS AND METHOD

Materials:

The Euphorbia hirta L fresh plant was procured from the SOUTH INDIA AGRO HERBAL IMPEX, Tuticorin, TN, India. Pure drug samples of gallic acid, rutin and quercetin (phyto-chemical reference standards) were procured from Sigma-Aldrich, Bangalore and S.D. Fine Chemicals Ltd., India. The Euphorbia hirta L formulation of TAWA-TAWA capsules was procured from the Ruby gold company, Green meadows, Davao city, Philippines. Silica gel G60 F_{254}, 20x10cm TLC plate was procured from Merck Germany. Butyl acetate, 1,4-dioxane, methanol were supplied by S.D. Fine Chemicals Ltd., India, Qualigens Fine Chemicals Ltd., and Mumbai, India and Ranbaxy chemicals Ltd., New Delhi, India.

Instruments used:

Millipore Milli-Q water purifier, Elico LI 127 pH meter, Leela Sonic Ultrasonicator, Shimadzu digital electronic balance, Camag HPTLC system (with TLC scanner, Wincats software and Linomat -V as application device), hot air oven.

Selection and prewashing of the plate

The selection of the plate for HPTLC separation is very important as the stationary phase plays a vital role in the separation of the components. Silica gel G60 F_{254} on aluminum sheet with100-250

mm thickness was selected as the stationary phase for the separation. The pre-activation of the plate involves washing it with methanol and activated in an oven at 115^0C for 10 minutes.

Selection of solvent

The solubility of gallic acid, rutin and quercetin was tried in a series of solvents like methanol, ethanol, acetonitrile, chloroform, ethyl acetate, butyl acetate, toluene, and water. Among these solvents gallic acid, rutin and quercetin were freely soluble in methanol and showed good stability. So methanol was selected as the solvent for the study.

Selection of detection wavelength

The drugs were dissolved in methanol and the solution was scanned in the UV- region between 200-400nm and detection wavelength of 266nm was selected for the simultaneous determination of gallic acid, rutin and quercetin.

Selection of mobile phase

Based on the solubility and polarity the separation of gallic acid, rutin and quercetin was carried out using different solvent systems like Ethyl acetate: methanol: toluene, Butyl acetate : methanol : toluene, Toluene : n-propanol, Toluene : methanol, Butyl acetate: Toluene : propanol, Butyl acetate: Toluene : methanol, Ethyl acetate : methanol : toluene, Butyl acetate : methanol: 1,4-dioxane, Butyl acetate: Toluene : 1,4 – dioxane, Butyl acetate : 1,4-dioxane. Among all the solvent system tried butyl acetate: 1, 4- dioxane gave good separation of gallic acid, rutin and quercetin with good peak shape.

Optimization of mobile phase ratio:

To obtain good compact spots different ratios of butyl acetate : 1,4-dioxane (8:2, 6:4, 7:3, 2:8, 4:6, 3:7, 5:5)were tried it was found that the 5:5% v/v gave good separation form the solvent front as well as from spots.

Fixed chromatographic conditions:

Silica gel G $60F_{254}$ on aluminum sheet was selected as the stationary phase for the separation. Linear ascending developments of the plates to a distance of 90 mm was performed with butyl acetate: 1, 4- dioxane (5:5) as mobile phase in a twin trough glass chamber previously saturated with mobile phase vapour for 20 min at room temperature. After development the plate was scanned at 266 nm by means of a Camag TLC scanner in absorbance mode, using the deuterium lamp. The slit dimensions were 5 × 0.45 mm and the scanning speed was 20 mm/s.

Preparation of standard stock solution:

10mg of gallic acid, rutin and quercetin were weighed and transferred into a 10ml standard flask, dissolved using methanol and the volume was made up to get a concentration 1000µg/ml.

Preparation of working standard solution:

From the stock solution about 3.4ml was pipetted out, transferred into a 10ml standard flask and the volume was made up with methanol to get a concentration of 340µg/ml for each solution.

Preparation of standard graph:

From the working standard solution about 0.4 to 2.2 µl was spotted on the TLC plate, followed by the spots were developed using mobile phase, dried scanned and the absorption overlay spectrum of gallic acid, rutin and quercetin was recorded. The peak areas were recorded. Calibration graph was obtained by plotting the peak areas against the corresponding concentration of the standard solutions. The linearity was observed in the range of 136-680ng/spot of gallic acid, rutin and quercetin.

Extraction procedure[8]:

Plant was dried at room temperature and ground well. To isolate the active constituents without other interferences, 100 g of whole plant was extracted successively in soxlet extractor at 30-*45 °C* using 800 ml solvents for 5 hours in increasing polarity. The collected extracts were dried separately and used for further analysis. Preliminary phytochemical screening of successive extracts of Euphorbia hirta showed that the methanolic extract contains more flavonoids and poly phenolic acids.

Analysis of the extracts:

Accurately weighed 10mg of methanolic extract and transferred to 10 ml standard flask. It was dissolved in methanol to get concentration, 1000µg/ml. Then the solution is applied onto the plate, developed and scanned at 266nm.

Analysis of formulation[9]:

20 capsules (TAWA-TAWA, ruby gold company) were weighed and the average weight was calculated. A quantity equivalent to 10mg of tawa-tawa formulation was weighed, transferred to 10 ml volumetric flask, dissolved in methanol and made upto the volume to get concentration of 1000µg/ml. Then the solution was diluted to the linearity range, applied onto the plate, developed and scanned at 266nm. The concentration of a gallic acid, rutin and quercetin found in the tawa-tawa formulation was determined from the peak areas obtained.

Validation of the method

The developed HPTLC method was validated in terms of linearity, range, limit of detection (LOD), limit of quantification (LOQ), inter and intraday precision, repeatability and recovery studies.

Linearity:

The linearity was assessed using different volume of stock solution, were spotted on TLC plate to obtain the concentration of 136-680ng/spot. The spots were developed and evaluated densitometrically using CAMAG HPTLC system. Peak area were noted for each spot and plotted against concentration to get a linear graph.

Limit of detection and limit of quantification:

The limit of detection and limit of quantification was determined by applying the least concentration of the standard in the plate.

Precision:

The precision of the developed method was determined by intra and interday studies.

Intraday studies:

Intraday precision was determined by the analysis of the standard drug solution of two different concentrations 0.6 and 0.8µl which are within the linearity range for six times on a day and the %RSD was calculated.

Inter-day studies:

Inter-day precision was found out by the analysis of two different concentrations 0.6 and 0.8µl of the standard solution which are within the linearity range for two different days and the %RSD was calculated.

Repeatability

Repeatability of sample application:

Repeatability of sample application was assessed by the analysis of a concentration of a standard solution within the linearity range which is applied six times on the precoated TLC-plate, developed and the spots were scanned. The %RSD was calculated from the peak areas.

Repeatability of measurement:

Repeatability of measurement was determined by measuring the peak area of a concentration of standard solution within the linearity range for five times on the pre-coated TLC plate and the plate was scanned without changing the position of the plate and the %RSD was calculated.

Accuracy:

Accuracy of the developed method was determined by recovery study which is done by the addition of a quantity of the pure drug into the preanalyzed formulation at 100% level. The percentage recovery and %RSD were calculated.

Stability studies:

When the developed chromatographic plate was exposed to the atmosphere, the analytes are likely to decompose. The stability of the individual components in the plate was studied at different time intervals and the peak areas were compared with the peak area of the standard solution.

RESULTS AND DISCUSSION

Precoated silica gel $60F_{254}$ in aluminum sheet was selected as stationary phase. Methanol was chosen as a best solvent for performing the complete study since it solubilizes the drug and shows good stability in its solution form. Several different runs using mobile phase of varying polarity was performed to choose the appropriate mobile phase. Butyl acetate: 1, 4- dioxane gave acceptable separation of gallic acid, rutin and quercetin with good peak shape and dense compact spot. 266nm was an ideal wavelength chosen which gives good response and maximum absorbance for the drug to be detected. The spots were applied and developed with butyl acetate: 1, 4- dioxane, dried and the absorption spectrum was recorded and it resulted in good symmetric peak with R_f value 0.64, 0.15, 0.78. The overlay spectrum was shown in the figure 1. The linearity was observed in the range of 136-680ng/spot of gallic acid, rutin and quercetin and the standard representative chromatogram of 544 ng/spot of gallic acid, rutin and quercetin was shown in figure 2.

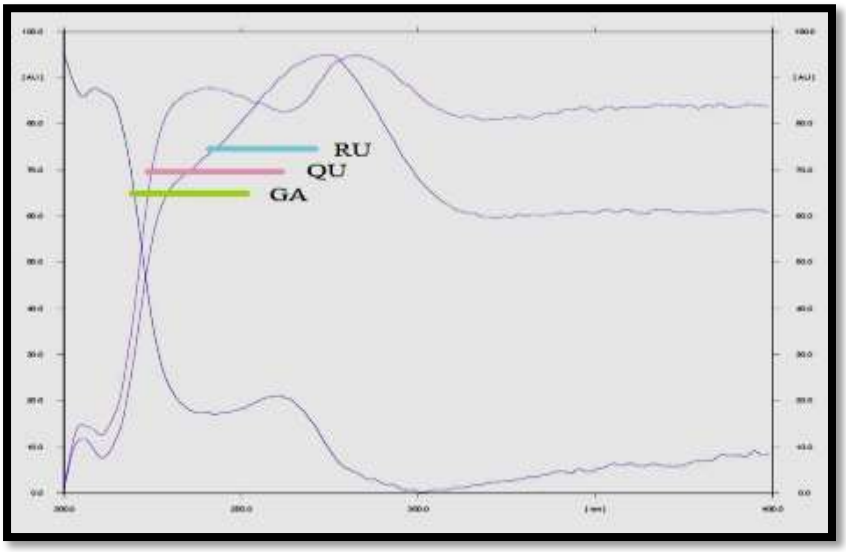

Figure 1: Overlay Spectrum of gallic acid, rutin and quercetin

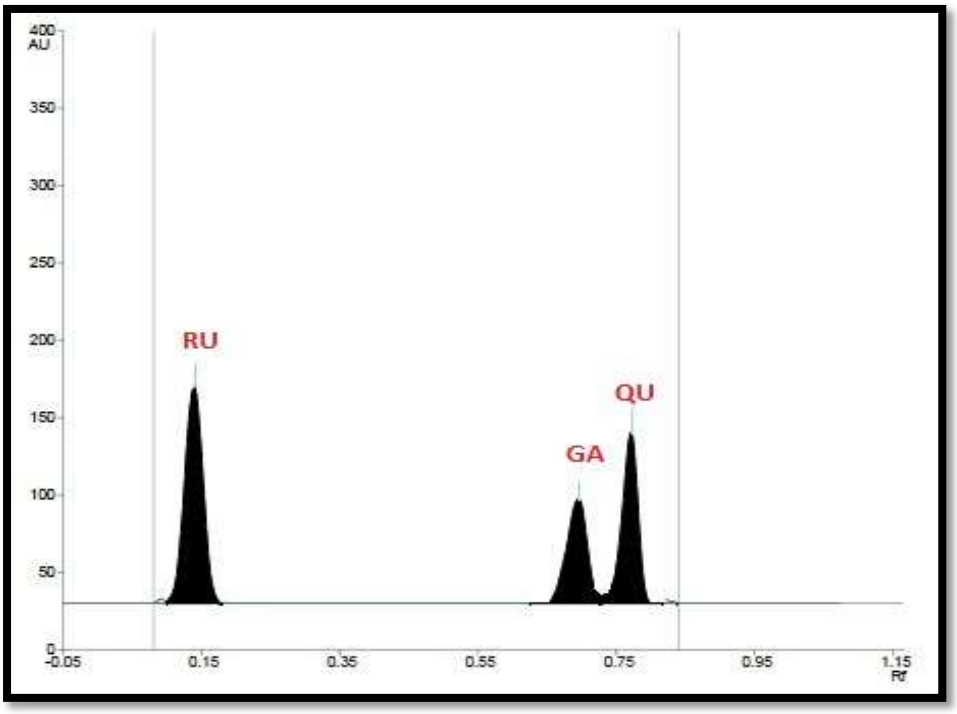

Figure 2: 544 ng/spot of gallic acid, rutin and quercetin

Analysis of extracts:

The chromatogram representing the separation of gallic acid, rutin and quercetin using methanolic extract was shown in the figure 3 and the concentration obtained was calculated and tabulated in table 1.

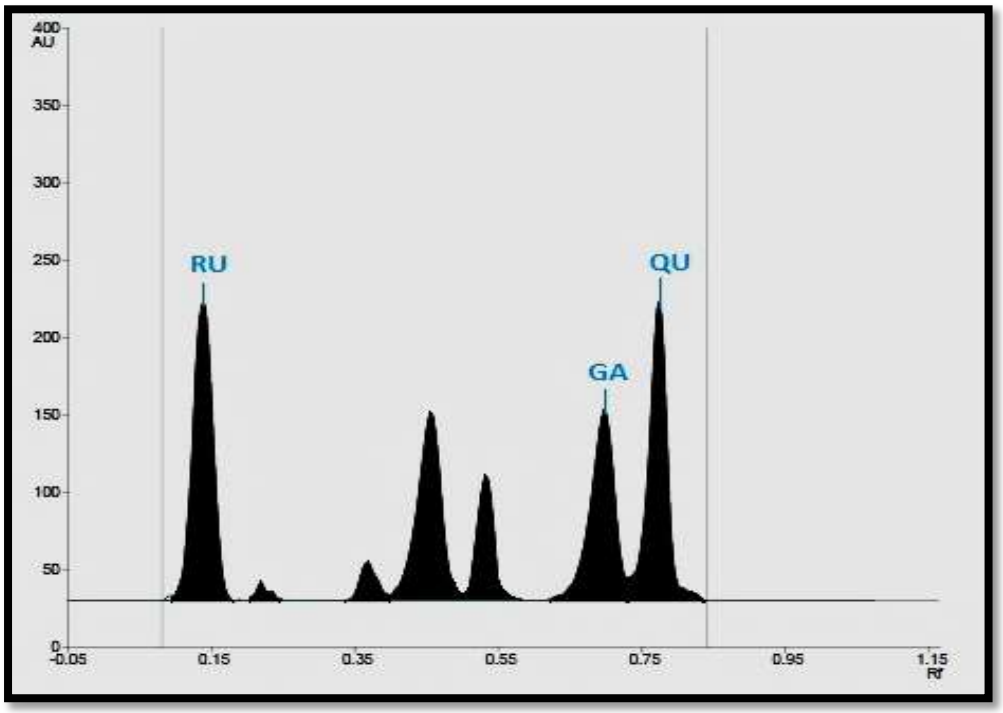

Figure 3: Methanolic extract

Table 1: Analysis of extract:

Successive extract	Concentration(ng/spot)*		
	Gallic acid	**Rutin**	**Quercetin**
Methanol	291.3ng/spot	363.1ng/spot	311.6ng/spot

*Mean of six determination

Analysis of formulation:

The concentration of a gallic acid, rutin and quercetin found in the tawa-tawa formulation was determined from the peak areas and the corresponding chromatogram was shown in figure 4. Concentration of desired active constituents found from formulation was given in table 2.

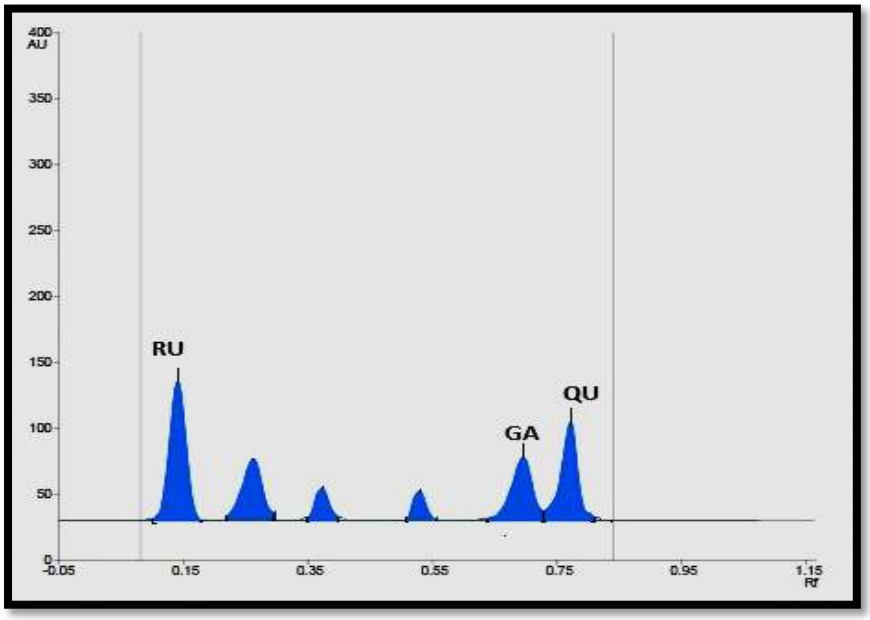

Figure 4: Chromatogram of formulation

Table 2: Analysis of formulation

	Concentration(ng/spot)*	
Gallic acid	**Rutin**	**Quercetin**
577.5 ng/spot	457.4ng/spot	508.6ng/spot

*Mean of six determination

Validation of the method:

Linearity and range:

The linear regression data showed a good linearity in the concentration range of 136-748ng/spot and the calibration graphs of gallic acid, rutin and quercetin was shown in figure 5,6 and 7. The slope, intercept and correlation coefficient values for gallic acid, rutin and quercetin was found to be 501.40, 4.62, 0.99948, 264.83, 2.063, 0.99910 and 184.765, 4.456, 0.99899 respectively.

Calibration graphs

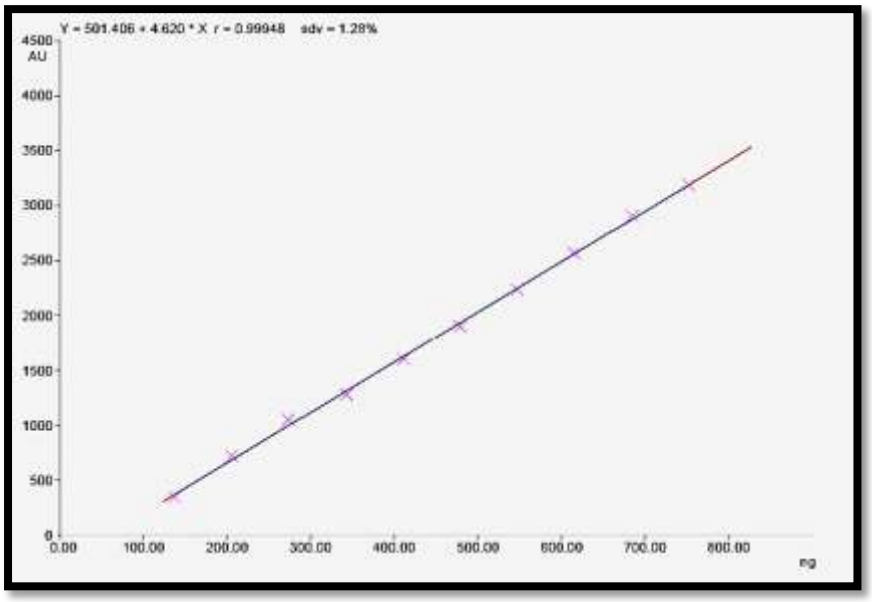

Figure 5: Gallic acid (136-748ng/spot)

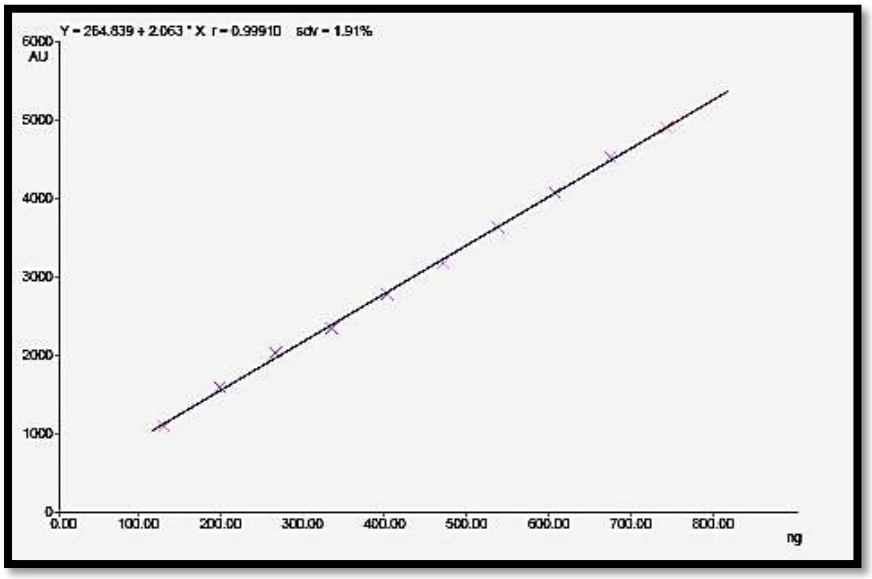

Figure 6: Rutin (136-748ng/spot)

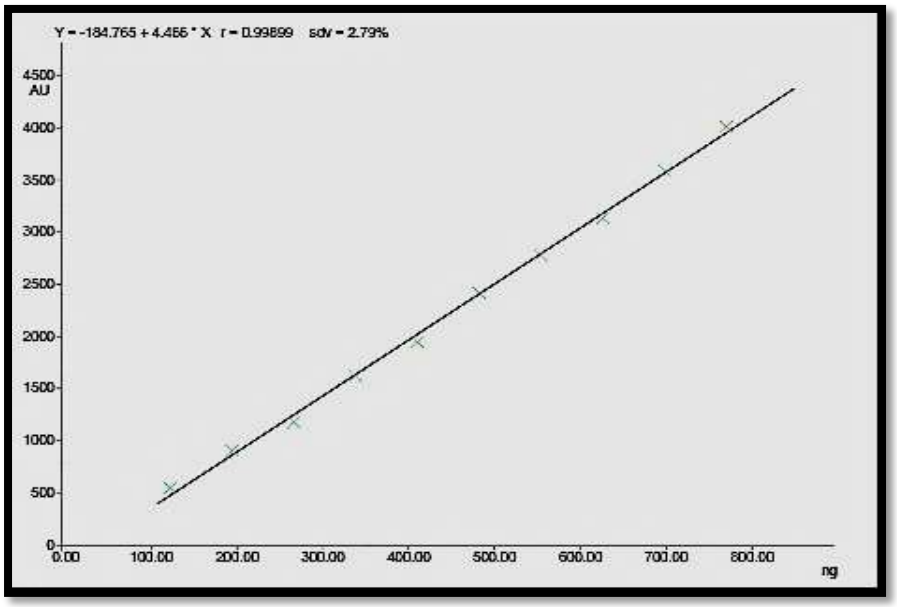

Figure 7: Quercetin (136-748ng/spot)

Limit of detection and limit of quantification:

Limit of detection was found to be 102 ng/spot of gallic acid, 17ng/spot of rutin and 68ng/spot of quercetin. The limit of quantification for the estimation of gallic acid, rutin and quercetin was found to be 136ng/spot.

Precision:

The intraday, interday and repeatability studies were carried out and the corresponding %RSD was calculated and tabulated in table 3,4,5, and 6. % RSD values were recorded to be < 2 and this proves that the method developed is more precise.

Table 3: Intraday studies

Volume applied in µl	Peak area			%RSD*		
	Gallic acid	Rutin	Quercetin	Gallic acid	Rutin	Quercetin
0.6	592.1	1520.7	782.1	0.189	0.069	0.153
	593.3	1521.8	783.4			
	592.9	1522.8	782.7			
	594.7	1523.7	780.9			
	592.3	1522.6	784.3			
	591.7	1521.5	783.5			
0.8	822.9	1830.8	1030.3	0.293	0.052	0.085
	820.1	1828.9	1029.4			
	821.5	1829.1	1030.8			
	823.8	1830.6	1028.3			
	823.2	1829.5	1029.1			
	822.8	1828.4	1029.7			

*mean of six determinations

Table 4: Inter-day studies

Volume applied in µl	Days	Peak area			%RSD*		
		Gallic acid	rutin	Quercetin	Gallic acid	rutin	Quercetin
0.6	1st	593.3	1522.6	782.1	0.1283	0.0902	0.0833
		594.6	1520.4	783.4			
		593.7	1522.4	782.7			
		592.8	1523.7	780.9			
	2nd	592.7	1523.6	783.4	0.2331	0.0414	0.0851
		594.6	1523.2	784.6			
		594.9	1524.7	783.5			
		592.1	1523.7	783.9			
0.8	1st	826.9	1828.6	1029.2	0.1065	0.0518	0.0858
		825.7	1830.7	1027.5			
		827.7	1830.5	1028.6			
		827.4	1829.8	1029.5			
	2nd	828.5	1830.5	1032.7	0.0912	0.0483	0.1867
		827.7	1829.4	1031.8			
		826.7	1828.7	1030.5			
		827.3	1830.5	1029.5			

*mean of four determinations

Table 5: Repeatability of sample application

Volume applied in µl	Peak area			%RSD*		
	Gallic acid	rutin	Quercetin	Gallic acid	rutin	Quercetin
0.8	831.3	1829.7	1033.5	0.0607	0.0322	0.0769
	830.7	1828.9	1032.8			
	831.5	1829.5	1033.4			
	832.1	1829.7	1033.8			
	831.6	1830.1	1032.6			
	830.9	1828.5	1031.6			

*mean of six determinations

Table 6: Repeatability of sample Measurement

Volume applied in µl	Peak area			%RSD*		
	Gallic acid	rutin	Quercetin	Gallic acid	rutin	Quercetin
0.8	831.3	1829.7	1033.5	0.0617	0.0332	0.0759
	831.7	1828.9	1032.8			
	831.5	1829.5	1033.4			
	832.1	1829.7	1033.8			
	831.6	1830.1	1032.6			
	830.9	1829.5	1032.6			

*mean of six determinations

Recovery studies:

Recovery values were observed to be close to 100 which were shown in table 7, and this proved that the method is accurate.

Table 7: Recovery studies

Level	% Recovery			%RSD*		
	Gallic acid	rutin	Quercetin	Gallic acid	rutin	Quercetin
100%	99.5	99.4	99.8	0.2151	0.3121	0.2039

*mean of six determinations

Stability studies:

The analytes were found be to stable on the plate for about 6 hours, which was shown in table 8.

Table 8: Stability studies

Volume applied µl	Time in hours	Peak area		
		Gallic acid	Rutin	Quercetin
0.6	1	595.7	1522.5	792.5
	2	593.3	1521.6	790.6
	3	593.0	1520.5	787.5
	4	591.4	1520.0	780.0
	5	590.2	1519.8	779.8
	6	**588.3**	**1518.5**	**771.5**
	7	550.7	1501.7	765.7
	8	545.8	1498.3	761.3

CONCLUSION

Euphorbia hirta (L) is one of the commonly used herbal capsule formulation for the treatment of dengue fever. At present, gallic acid, rutin and quercetin are considered to be appropriate chemical compounds for the quality control of herbal medicines, since they are convenient in terms of method development and marker availability. In the present study standardization of Euphorbia hirta(L) methanolic extract and herbal capsule formulation using gallic acid, rutin and quercetin as phytochemical markers was established by HPTLC method and was validated according to *ICH* guidelines. It was found to be easy, rapid, accurate, precise and reliable. Hence this proposed method can be adopted for fingerprint analysis and routine quality control of Euphorbia hirta (L) in marketed herbal capsule formulation.

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euphorbiaceae family and heartfelt and sincere thanks to SNR Sons Charitable trust, Coimbatore for providing the necessary infrastructural facilities to perform this study.

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