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Estimation of Anti-dengue Phytochemical Markers Gallic acid, Rutin and Quercetin in Methanolic Extract of *Euphorbia hirta* (L.) and Tawa-Tawa Capsule Formulation by Validated RP-HPLC Method

A. Suganthi*, T.K. Ravi

Department of Pharmaceutical Analysis, College of Pharmacy, Sri Ramakrishna Institute of Paramedical Sciences affiliated to The Tamilnadu Dr. M.G.R. Medical University, 395, Sarojini Naidu Road, Coimbatore-641 044, India

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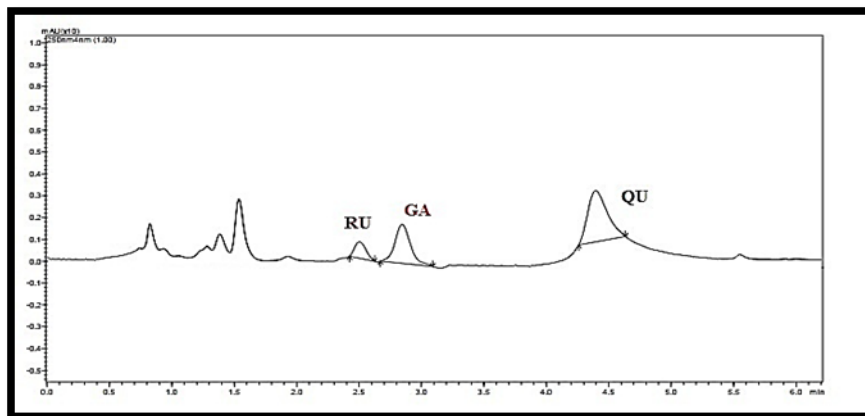
ABSTRACT

Sensitive and precise RP-HPLC method with photo diode array detector has been developed and validated for the simultaneous estimation of three commonly available anti-dengue, anti-cancer and anti-inflammatory phytochemical markers. The chromatographic separation was achieved using C18 column (250 mm × 4.0 mm, 5µm) with 0.2% v/v formic acid: acetonitrile (50:50; v/v) as mobile phase at the flow rate of 0.7 mL/min. The dual wavelength (280 and 360 nm) was selected for the identification and quantification of rutin, gallic acid and quercetin (R_t 2.56, 2.95 and 4.60 min). The method was validated as per ICH guidelines in terms of specificity, linearity, precision, and accuracy, LOD and LOQ, respectively. Linearity range for the selected markers, gallic acid, rutin and quercetin was found to be 1–10 µg/mL with the correlation coefficient value close to 1. The sensitivity of the method was demonstrated from the limit of detection which was found to be 0.4 µg/mL for gallic acid; 0.3 µg/mL for rutin and quercetin. The limit of quantification for gallic acid, rutin and quercetin was found to be 1 µg/mL, respectively. The % RSD and recovery values prove that the developed method was more precise and accurate. Hence, the proposed validated method has been successfully applied for the quality control analysis of gallic acid, rutin and quercetin in methanolic extract of *Euphorbia hirta* (L.) and Tawa-Tawa capsule formulation.

*Corresponding author: E-mail: suganlemu@gmail.com

^aDepartment of Pharmaceutical Analysis, College of Pharmacy, Sri Ramakrishna Institute of Paramedical Sciences affiliated to The Tamilnadu Dr. M.G.R. Medical University, 395, Sarojini Naidu Road, Coimbatore-641 044.
Tel: +98919 442006395

Graphical Abstract



Introduction

Dengue is a life threatening viral infection transmitted by *Aedes aegypti* mosquitoes. According to WHO, dengue is a febrile illness that affects infants, children and adults. More than 100 countries are at risk of acquiring dengue viral infection. Dengue has been an urban disease but now has spread to rural areas of India. Climate change may be a contributing factor in the global spread of dengue. It is estimated that there are approximately 500,000 cases of dengue haemorrhagic fever that must be hospitalized and 20000-25000 deaths, mainly in children [1-5].

Gallic acid is one of the main phenolic constituents found in almost all plants [6]. It is a powerful antioxidant and has wide range of therapeutic application against dengue, asthma, cancer, neural disorder, allergic rhinitis, anti-viral and anti-fungal properties [7-9]. Also, it was found to have no negative effect on healthy cells. Quercetin and rutin are flavonoids which display a variety of biological activities, including anti-dengue, anti-viral, anti-cancer and anti-inflammation [10-14]. These three active constituents are present in *Euphorbia hirta* (L.) along with the chemical constituents afzelin, quercitrin, and myricitrin, euphorbin-A, euphorbin-B, euphorbin, kaempferol, and shikmic acid, tinyatoxin, choline, camphol, and quercitol derivatives [9-15, 16]. *Euphorbia hirta* (L.) is also called as Tawa-Tawa, had been widely known to be used as a capsules and decoction for dengue fever victims due to its profound properties on increasing the platelet count which is normally low for an individual is under serious viral infection [17]. It is also used as an infusion to treat various ailments including dengue, asthma, cancer, bronchitis, hay fever, cough, cold, kidney stones, menstrual problems, sterility and venereal diseases [18-20].

Herbal drug standardization is essential to assure the quality, safety and efficacy [21, 22]. Many methods have been reported in the literature for the quantification of the selected phytochemical

markers such as gallic acid, rutin and quercetin individually or in combination with other markers using HPLC [18, 23-26] and HPTLC techniques in other herbal extracts [27, 28]. The estimation of these biomarkers in *Euphorbia hirta* (L.) by HPTLC method was already reported by the author in the year 2016 [29]. To the best of our knowledge there is no RP-HPLC method was developed for the standardization of *Euphorbia hirta* (L.) using gallic acid, rutin and quercetin as phytochemical markers. Hence our aim and objective of the present work was to develop a rapid, accurate, precise and reliable RP-HPLC method for the simultaneous standardization of gallic acid, rutin and quercetin in pure form, methanolic extract of *Euphorbia hirta* (L.) and its herbal capsule formulation. After development of the method it has been validated according to ICH guidelines [30].

Experimental

Materials and Methods

Chemicals and Instruments used

The *Euphorbia hirta* (L.) fresh plant was procured from the SOUTH INDIA AGRO HERBAL IMPEX, Tuticorin, Tamilnadu, India and it was identified (No.BSI/SRC/5/23/2012-13/Tech/1904) by Dr.G.V.S. Murthy, Scientist 'F' & Head of office, Botanical Survey of India, Southern Regional Centre, Coimbatore. Pure drug samples of gallic acid, rutin and quercetin were purchased from Sigma-Aldrich, Bangalore and S.D. Fine Chemicals Ltd., India. TAWA-TAWA capsule was obtained from the Ruby gold company, Green meadows, Davao city, Philippines. All the chemicals including methanol HPLC grade, acetonitrile HPLC grade, water purified by milli-Q water purifying system, ammonium acetate AR grade, ammonium formate AR grade, trifluoroacetic acid LR grade and triethyl amine LR grade were supplied by S.D. Fine Chemicals Ltd., India, Qualigens Fine Chemicals Ltd., and Mumbai, India and Ranbaxy chemicals Ltd., New Delhi, India.

Shimadzu HPLC class LC-10 AT VP System (Photodiode array detector), Shimadzu electronic balance, BL-220H, Millipore Milli-Q water purifier, Elico LI 127 pH meter, Leela Sonic Ultrasonicator were used for the study. Hence the selected phytochemical markers gallic acid, rutin and quercetin are polar in nature, the stationary phase selected to separate these components was RP-C18 column.

Fixed Chromatographic Conditions

The separation was done by using *LichroCART*, C18 column (250 mm × 4.0 mm, 5 µm) as stationary phase, 0.2% v/v formic acid: acetonitrile as mobile phase in the ratio of 50:50. The flow rate was

fixed as 0.7 mL/min. The detection wavelength was fixed as 280 and 360 nm for gallic acid and Quercetin; 360 nm for Rutin. The room temperature was maintained for the separation technique.

Preparation of standard solutions and mixtures

About 10 mg of gallic acid, rutin and quercetin were taken separately in 10 mL standard flask and the volume was made up with HPLC grade methanol to get a concentration of 1000 µg/mL. From the stock solution about 1 mL of gallic acid, rutin and quercetin were transferred together into a 100 mL standard flask and the volume was made up with methanol to get a concentration 10 µg/mL of gallic acid, rutin and quercetin, respectively. This concentration was used for further study.

Validation of RP-HPLC Method

The developed HPLC method was validated in terms of specificity, linearity, range, limit of detection (LOD), limit of quantification (LOQ), inter and intraday precision, repeatability and recovery studies as per International Conference on Harmonization (ICH) guidelines Q2 (R1) [30].

Specificity

The chromatogram of the blank was observed for the appearance of any additional peaks particularly at the R_t of the analytes after injecting the blank and sample solutions under optimized chromatographic condition. The peak purity index was also measured for the marker compounds and the formulation at the peak start, peak end and peak apex position.

Linearity

The linear graph was constructed at 10 concentration levels of all 3 phytochemical markers (1-10 µg/mL). From each solution, 20 µL was injected into the HPLC column (n=6) with the fixed chromatographic condition. The linearity was evaluated by the least-square regression method.

Limit of detection and Limit of quantification

For the determination of the limit of detection and limit of quantification different dilution of the standard solutions of rutin, gallic acid and quercetin were injected and analysed using the mobile phase as blank. The LOD and LOQ were determined on the basis of signal-to-noise ratio until the average responses were approximately 3 to 10 times the responses of the blank, respectively.

Repeatability of injection

The precision of the study using the instrument was checked by the repeatability of injection (n=6) and analyzing the standards (8 µg/mL) and % RSD was calculated.

Precision

The precision (reproducibility) of the developed method was determined by intra and inter-day studies. Intraday precision was determined by analyzing the standard drug solutions of 4 and 6 µg/mL for six times on the same day and inter-day precision was carried out on two different days.

Accuracy

Accuracy of the developed method was determined by calculating the recovery of each drug by standard addition method. For this known quantity of the pure drugs were added into the preanalyzed formulation at 100% level. The amount of all the drugs were estimated by applying values of peak area to the regression equation of the linear graph prepared.

Robustness

The robustness of the developed RP-HPLC method was demonstrated by making slight variations in the optimized chromatographic conditions like ± 1 in the ratio of the mobile phase, ± 0.02 in the ionic strength of formic acid and ± 0.1 in the flow rate.

Analysis of Extract

Accurately weighed 10 mg of methanolic extract was transferred to 10 mL standard flask and dissolved in methanol which was made upto the mark with mobile phase to get concentration, 1000 µg/mL. From this 10 µg/mL of the solution was prepared using the mobile phase and analyzed by fixed chromatographic condition.

Analysis of Formulation

Twenty capsules (TAWA-TAWA, ruby gold company) were weighed and the average weight was calculated. A quantity equivalent to 10 mg of formulation was weighed, transferred to 10 mL volumetric flask, dissolved in methanol and made upto the volume to get concentration of 1000 µg/mL. Then the solution was diluted to the linear range, solutions were injected into column at fixed chromatographic conditions and peak areas of these solutions were measured at selected wavelengths.

Results and discussion

Rapid and reproducible RP-HPLC method was mainly designed for the separation and quantitation of the major active constituents (gallic acid, rutin and quercetin) present in *Euphorbia hirta* (L.) methanolic extract and Tawa-Tawa capsule formulation using the corresponding phytochemical markers. The separation was achieved on C18 column by isocratic elution analysis using 0.2% v/v formic acid: acetonitrile (50:50) at the flow rate of 0.7 mL/min. All studied components were

successfully separated within 5 min. The total analysis time was 10 min per injection, which included a 3 min allowance for column equilibration prior to the next injection. In the proposed study, the UV spectra of all studied compounds were reviewed using PDA detector. The PDA detector acquisition wavelength was set in the range 200–400 nm. The results demonstrated that higher sensitivity was obtained in the range 250–380 nm with the use of 0.2% formic acid and acetonitrile instead of methanol as the eluent. Under the described conditions (HPLC apparatus and fixed chromatographic conditions section), we achieved best results at wavelength of 280 and 360 nm (Figures 1 and 2). Among the two selected wavelengths rutin showed better sensitivity at 360 nm, gallic acid at 280 nm whereas for quercetin there was not much difference in sensitivity at selected wavelengths.

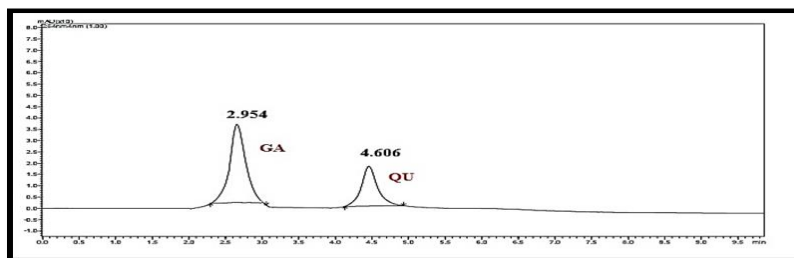


Figure 1. RP-HPLC Chromatogram of standard phytochemical markers showing the retention time for gallic acid ($R_t=2.95$ min) and quercetin ($R_t=4.60$ min) scanned at $\lambda_{\max}=280$ nm; using C18 column (250 mm \times 4.0 mm, 5 μ m) with 0.2% v/v formic acid: acetonitrile (50:50; v/v) as mobile phase at the flow rate of 0.7 mL/min.

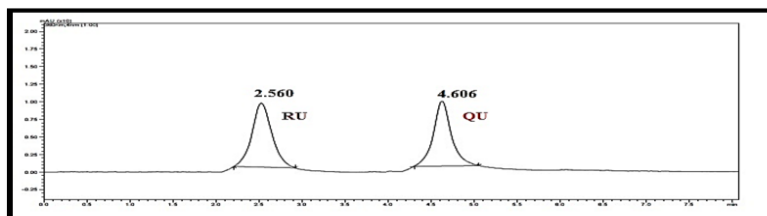


Figure 2. RP-HPLC Chromatogram of standard phytochemical markers showing the retention time for rutin ($R_t=2.56$ min) and quercetin ($R_t=4.60$ min) scanned at $\lambda_{\max}=360$ nm; using C18 column (250 mm \times 4.0 mm, 5 μ m) with 0.2% v/v formic acid: acetonitrile (50:50; v/v) as mobile phase at the flow rate of 0.7 mL/min.

Validation of RP-HPLC method

The developed HPLC method was validated under the optimized experimental conditions 0.2% formic acid: acetonitrile in the ratio of 50:50, isocratic elution system on a C18 reversed-phase column with a flow rate of 0.7 mL/min and detection at 280 and 360 nm.

Specificity

The peak purity index of gallic acid, rutin and quercetin was found to be 0.9998, 0.9999 and 0.9999, respectively. The values were close to one which revealed the specificity of gallic acid, rutin and quercetin.

Linearity and range

For quantitative applications, the response linearity was verified for all studied potential components of *Euphobia hirta* (L.) methanolic extract and Tawa-Tawa capsule formulation. The linearity of the method was investigated over a concentration range of 1–10 µg/mL for gallic acid, rutin and quercetin. The correlation coefficients of the standard curves linear regression were greater than 0.9997. The obtained linearity data are shown in Table 1.

Table 1. Statistical data for regression analysis of gallic acid, quercetin and rutin (n=3)

STANDARD ANALYTES	SLOPE±SD*	INTERCEPT±SD*	CORRELATION COEFFICIENT±SD*
Gallic acid	34596.3±297.54	360.54±0.1086	0.9999±0.00007
Quercetin	49250.1±414.51	210.56±20.108	0.9998±0.0003
Rutin	54837.0±77.78	290.65±4.16	0.9997±0.0001

* Standard deviation

Limit of detection and limit of quantification

The LOD corresponding to a S/N of approximately 3 was evaluated for the same analytes by progressive dilution. Limit of detection was found to be 0.4 µg/mL of gallic acid, 0.3 µg/mL of rutin and quercetin. The LOQ corresponding to a S/N of approximately 10 was also evaluated for the same analytes. The limit of quantification for the estimation of gallic acid, rutin and quercetin was found to be 1 µg/mL. These data support the suitability of the proposed HPLC method for its application to real samples.

Precision

In order to evaluate the precision of the method six replicate analyses of all compounds were performed on the single standard solution (8 µg/mL for each compound). The results showed that RSD value was found to be below 0.5%. Apart from this intraday precision was determined by the analysis of the standard drug solution of two different concentrations within the linearity range for six times on the same day. Inter-day precision was found out by the analysis of two different concentrations of the standard solution within the linearity range for two different days and the % RSD was calculated. In all cases, the values of the RSD were less than 1% for the peak area at the

same retention time, and less than 2.0% for the quantitation. The results of the studies are given in Table 2.

Accuracy

In order to evaluate the accuracy of the method, a known amount of the pure compounds such as gallic acid, rutin and quercetin were added to methanolic extract and Tawa-Tawa capsule formulation. Then, the fortified samples were analysed with the proposed HPLC methods. The recoveries of the added standard compounds were close to 100% with the % RSD below 1. The results proved that interferences by other matrix components are not significant, and the HPLC conditions are suitable to obtain adequate method accuracy (Table 2).

Table 2. Intra-day and Inter-day studies and accuracy data for gallic acid, rutin and quercetin

STANDARD ANALYTE	CONCENTRATION ($\mu\text{g/mL}$)	%RSD* (INTRADAY)	%RSD* (INTERDAY)	%Accuracy \pm %RSD*
Gallic acid	4	0.2091	0.2041	99.9 \pm 0.1967
	6	0.1591	0.0481	
Rutin	4	0.2171	0.2078	99.89 \pm 0.2159
	6	0.1921	0.1698	
Quercetin	4	0.0012	0.1458	100.23 \pm 0.1883
	6	0.1752	0.1687	

* mean of six determinations

Robustness

The robustness of the developed RP-HPLC method was demonstrated by making slight variations in the optimized chromatographic conditions and the changes obtained with the variation in conditions were found almost same with the standard solution of 6 $\mu\text{g/mL}$ under optimized condition. The % RSD values were below 0.5% indicates that the developed method was found to be robust (Table 3).

Table 3. Robustness data for the concentration of 6 $\mu\text{g/mL}$ by the proposed RP-HPLC method

Chromatographic condition		%RSD* OF PEAK AREA		
		Gallic acid	Quercetin	Rutin
Mobile phase ratio (50:50)	49:51	0.0974	0.1712	0.1053
	51:49	0.1072	0.1272	0.0412
Ionic strength of formic acid	0.18%	0.1421	0.2180	0.1521
	0.22%	0.1372	0.2091	0.2007
Flow rate	0.6	0.1401	0.1521	0.1672
	0.8	0.1290	0.0761	0.1495

* mean of six determinations

Analysis of Methanolic Extract and Formulation

Samples of methanolic extract and formulation were used to prepare solutions as described in the sample preparation section. The developed HPLC-PDA detector method was applied for the identification and quantitation of gallic acid, rutin and quercetin in methanolic extract of *Euphorbia hirta* (L.) and Tawa-Tawa capsule formulation. Representative chromatograms obtained from the analyzed samples of methanolic extract of *Euphorbia hirta* (L.) and Tawa-Tawa capsule formulation are reported in Figures 3 and 4, respectively. The peak identity for the identified compounds in the methanolic extract sample was confirmed by the retention time value, standard addition method and on the basis of on-line recorded UV spectra (PDA detector). Table 4 demonstrated the amount of gallic acid, rutin and quercetin found in methanolic extract of *Euphorbia hirta* (L.) and Tawa-Tawa capsule formulation.

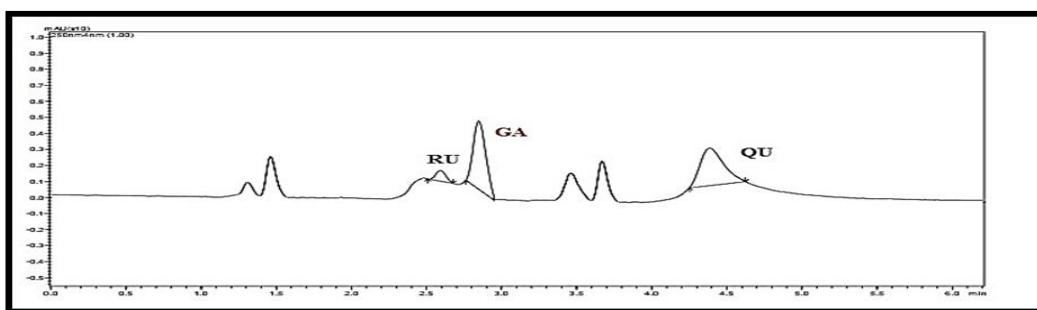


Figure 3. RP-HPLC Chromatogram of methanolic extract of *Euphorbia hirta* (L.) showing the retention time for gallic acid ($R_t=2.95$ min), rutin ($R_t=2.56$ min) and quercetin ($R_t=4.60$ min) scanned at $\lambda_{\max}=280$ nm; using C18 column (250 mm \times 4.0 mm, 5 μ m) with 0.2% v/v formic acid: acetonitrile (50:50; v/v) as mobile phase at the flow rate of 0.7 mL/min.

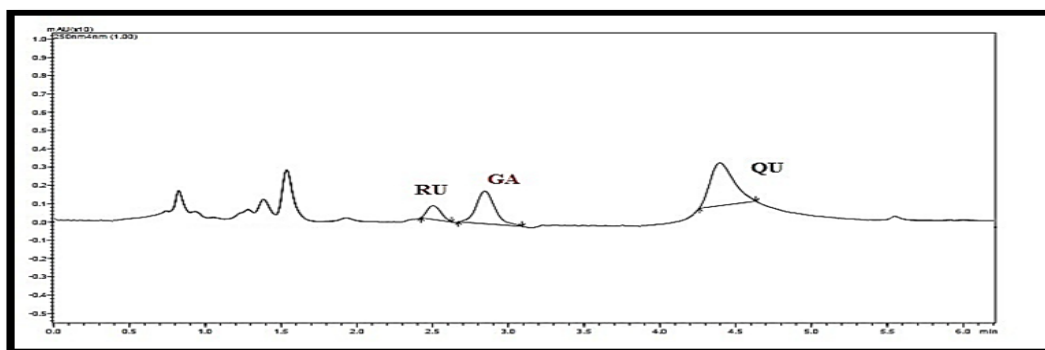


Figure 4. RP-HPLC Chromatogram of Tawa-Tawa capsule formulation showing the retention time for gallic acid ($R_t=2.95$ min), rutin ($R_t=2.56$ min) and quercetin ($R_t=4.60$ min) scanned at $\lambda_{\max}=280$ nm; using C18 column (250 mm \times 4.0 mm, 5 μ m) with 0.2% v/v formic acid: acetonitrile (50:50; v/v) as mobile phase at the flow rate of 0.7 mL/min.

Table 4. RP-HPLC analysis of gallic acid, rutin and quercetin in methanolic extract of *Euphorbia hirta* (L.) and Tawa-Tawa Capsule Formulation

Sample	Content of Gallic acid ($\mu\text{g/mL}$) $\pm\%$ RSD*	Content of Rutin ($\mu\text{g/mL}$) $\pm\%$ RSD*	Content of Quercetin ($\mu\text{g/mL}$) $\pm\%$ RSD*
Methanolic Extract	4.7 \pm 0.8694	5.5 \pm 0.5362	3.9 \pm 0.1.1251
Formulation	5.156 \pm 1.0210	4.800 \pm 0.9729	5.365 \pm 1.2411

* RSD=Relative Standard Deviation which was the average of six determination

Conclusion

In the present study, RP-HPLC based extraction and estimation of *Euphorbia hirta* (L.) plants grown in India were carried out. The research findings proved that the methanolic extract obtained from *Euphorbia hirta* (L.) plant revealed significant amount of gallic acid, rutin and quercetin, which are indicators for the treatment of dengue fever. To the best of our knowledge, no attempts have yet been made to quantify this multi phytochemicals, gallic acid, rutin and quercetin in *Euphorbia hirta* (L.) using LichroCART, C18 column (250 mm \times 4.0 mm, 5 μm) with 0.2% v/v formic acid: acetonitrile as mobile phase (50:50) in a relatively short run time (4.6 min). Reliability was guaranteed as validation experiments proved that the RP-HPLC method is linear in the proposed working range as well as specific, accurate and precise. The good recovery percentage of formulation suggests that the excipients have no interference in the determination. The proposed method was also found to be robust with respect to flow rate, ionic strength of mobile phase and detection wavelengths. Hence, the developed RP-HPLC method could be recommended for the routine quality control department for the estimation of the selected phytochemical constituents, either in the methanolic extract or in their herbal formulation. However, further studies are required to elucidate exact mechanism of action and to characterize the other phytochemical constituents responsible for the pharmacological properties of *Euphorbia hirta* (L.) as a new promising source of herbal medicine as anti-dengue agent in herbal formulations.

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