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Research article

Determination and validation assay for total phenolic content in two *Euphorbiaceous* plants by Folin Ciocalteu Method

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ABSTRACT

The *Euphorbia hirta* L. (Euphorbiaceae) commonly known as *dudheli* in vernacular language (Gujarati) commonly grows, widely in most parts of India and in other tropical countries, especially on roadsides and on wasteland. The *Euphorbia thymifolia* L. (Euphorbiaceae) commonly known as *nani dudheli* in vernacular language (Gujarati). The *E. thymifolia* is a prostrate, annual herb; stems many, spreading on the ground. The quantitative analysis of phenols manly done through Folin-Ciocalteu (FC) method to determine total phenolic content as gallic acid equivalent (GAE) of plant extracts. Quantitation and validation assay of an analytical method implicated to the extracts from *E. hirta* and *E. thymifolia* are fundamental factors for quality control parameters. The linearity of GA is found to be ranging from 10-100 mg/L (R^2 = 0.9998). The highest total phenolic content of *E. hirta* is found 21.216 ± 6.03 % W/W and 2.7 % W/W of dry extract and dry wet of herb respectively. Hence, the proposed method was found to be specific, accurate, precise, linear and reproducible.

Keywords: *Euphorbia hirta* L, *Euphorbia thymifolia* L, Folin-Ciocalteu (FC),Total Phenolic content

INTRODUCTION

The *Euphorbia hirta* L. (Euphorbiaceae) commonly known as *dudheli* in vernacular language (Gujarati) commonly grows, widely in most parts of India and in other tropical countries, especially on roadsides and on wasteland [1]. Traditionally *E. hirta* is used in a respiratory disease like asthma, bronchitis, hay fever analgesic and anti-inflammatory activity [2-4]. The literature survey reported that *E. hirta* contains flavonoids, sterols, tannins and triterpenoids [5-7]. In recent study methanolic extract of *E. hirta* showed cytotoxicity on HCT15 and Vero cell lines [8]. Sharma et al. reported that aqueous and methanol extracts of *E. hirta* showed in vitro α-glucosidase inhibitory activity of aqueous, hydro alcoholic and methanol extract respectively [9]. The *Euphorbia thymifolia* L. (Euphorbiaceae) commonly known as *nani dudheli* in vernacular language (Gujarati). The *E. thymifolia* is a prostrate, annual herb; stems many, spreading on the ground. The *E. thymifolia* is traditionally used as an antiviral in bronchial asthma bronchitis and hay fever [10]. Charaka prescribed *E. thymifolia* in diarrhea and piles. *E. thymifolia* is believed to uses as antidiarrheal, antimalarial and antidysentery [11]. The *E. thymifolia* contains hydrolysable tannins , flavonoids, triterpenoids and essential oils [10]. In recent study found that *E. thymifolia* treatment suppress iNOS protein expression, also inhibited generation of tumor necrosis factor (TNF), interleukin-6 (IL-6), IL-1 and CCL2 in LPS stimulated BV2 Microglia [12].

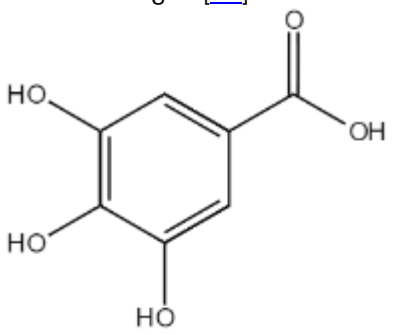

Figure 1: Chemical structure of gallic acid

Gallic acid (3,4,5-trihydroxybenzoic acid: Mol. Wt. 170.12 g/mol, Figure 1) is a phenolic acid, which is amply found in plants. Gallic acid (GA) mainly found as free form or conjugated with sugar in plants. GA and its alkyl esters serve as antioxidants via their effect on CYP450 and inhibition of ATP synthesis. GA also has variety of applications in cosmetics and pharmaceutical industry. Additionally, it is also employed as raw material for inks and paints manufacturing [13]. Several literature surveys reported that this compound have numerous therapeutic potentials including as anticancer [14], antifungal [15], antiviral [16], and bacteriostatic properties [17].

Although, several analytical techniques have been proposed for quantification of polyphenols in plant extracts due to their structural complexity and diversity [18]. The estimation of specific polyphenols is not enough to state the amount of procyanidins, UV-visible spectrophotometry is adequate to determine total polyphenol content (TPC) [19]. The quantitative analysis of phenols mainly done through Folin-Ciocalteu (FC) method to determine TPC as gallic acid equivalent (GAE) of plant extracts. Follin and Denis reported general technique of FC method for quantification of phenols is based on the spectrophotometric determination of phosphotungustic-phosphomolybdic complex [20], In this oxidation-reduction reaction, phenolate ion is oxidized while phosphotungustic-phosphomolybdic complex is reduced and form blue chromophore. The maximum absorption of this chromophore is depend upon pH, amount of The FC reagent, concentration of Na_2CO_3 and amount of phenolic acid present in extract [20].

Table 1 Total Phenol content of *E. hirta* and *E. thymifolia* determined by Folin-Ciocalteu method.

Marker compound	Total Phenolic (GAE)Content [a] (% w/w dry extract and dry powder)			
	ETM	ETW	EHM	EHW
GA	8.106 ± 5.46 (1.04*)	9.780 ± 0.85 (2.38*)	21.216 ± 6.03 (2.7*)	10.728 ± 4.44 (2.5*)

[a]Mean ± SD (n = 3), *calculated on the basis of dry weight of herb

Table 2 Quantifying and validation assay GA by UV spectrophotometric method

Sr. No.	Parameter	GA
1	Selectivity	Selective
2	Specificity	Specific
3	λ_{max}	760 nm
4	Molar Absorptivity (L mol-1 cm-1)	0.485×10^3
5	Beer's law limit (µg/mL)	5-100
6	Regression Equation Y=mX+c	y = 0.0033x + 0.0005
7	Correlation coefficient (r^2)	0.9994
8	LOD (µg/mL)	5.03
9	LOQ (µg/mL)	15.24
10	Repeatability (n=10, %RSD)	0.33
11	Average recovery (%)	102.36 ± 3.31
12	Precision (%RSD)	
	Inter day	1.31-1.84
	Intra day	0.52-0.63

Several authors have been reported TPC from *E. hirta* and *E. thymifolia* plant extracts by FC method via colorimetric assay using UV-visible spectrophotometer. Analytical method validation involving UV-visible spectrophotometric quantitation for TPC, as GAE is poorly described in literature [21, 22]. The therapeutic potential and intense attention in the field of traditional medicine, the specification and/or analytical procedure have not been developed yet.

Therefore, the quantitation and validation assay of an analytical method implicated to the extracts from *E. hirta* and *E. thymifolia* are fundamental factors for quality control parameters. The present method has been validated as per the ICH guidelines [23] and similar to the methods reported by laboratory on UV-Visible spectrophotometer method development and validation for herbal drugs [20]. However, this research work is intended to perform validation assay via a simplified UV-visible spectrophotometric method for quantification of TPC, as GAE per gm of *E. hirta and E. thymifolia* aqueous and methanol extracts.

MATERIALS AND METHODS

Reagents and standards

Standard gallic acid (purity, 98%) was purchased from Sigma Aldrich, India. All solvents and chemicals such as; methanol, Na_2CO_3 and Folin-

Ciocalteu used in experiment were of analytical grade and bought from SD Fine Chemicals, India.

Plant material and preparation of extracts

Fresh plants of *E. hirta* and *E. thymifolia* were collected from Amargadh village, Rajkot, Gujarat, India. The plants were identified and authenticated by CSIR-NISCAIR, New Delhi, India. Voucher specimens (DP/SVU/PHCOG/Herb/02 & DP/SVU/PHCOG/Herb/03) of same have been deposited in Sumandeep Vidyapeeth University, Vadodara for future.

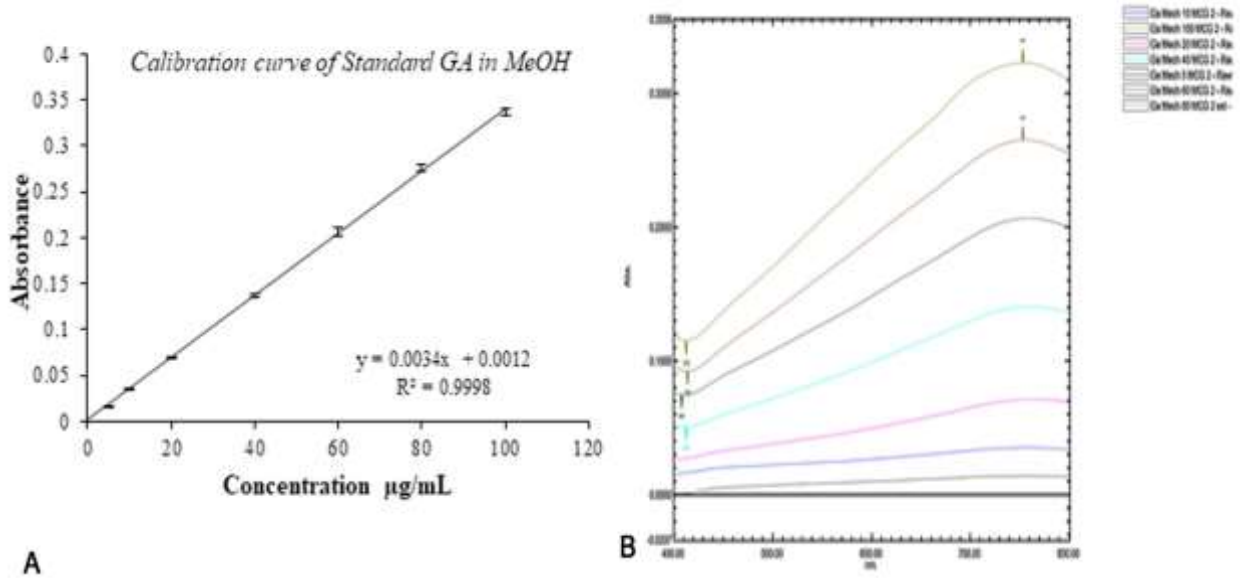

Figure 2. (A) Calibration curve of GA (B) Overlay spectra of GA in MeOH

Sample preparation

The collected aerial parts were sun dried, pulverized and sieved through mesh size 40. The cold maceration method was utilized to prepare aqueous and methanolic extracts of *E. hirta* and *E. thymifolia* (EHW, ETW EHM and ETM respectively) for 72 h. Extracts were concentrated using a water bath (Mack, Ahmedabad, India) at a temperature of 70°C and subsequently lyophilized and stored in a vacuum desiccator and used for further studies. The % yield of EHM, ETM, EHW and ETW extracts was 16.34% and 6.25% (w/w) respectively.

Method for TP analysis of extracts from ET and EH

The quantitative analysis of phenols was mainly done through Folin-Ciocalteu (FC) method to determine TPC as gallic acid equivalent (GAE) of plant extracts. Follin and Denis reported general technique FC method for quantification of phenols is based on the spectrophotometric determination of phosphotungustic-phosphomolybdic complex [20].

Standard stock solution of gallic acid and extracts (1 mg/mL) was prepared in methanol. From the stock solution 400 µL of aliquot was transferred to 10 mL volumetric flask and 4 mL DW was added to make, to this 0.6 mL of FCR was added and mixed well. After 5 min, 1.6 mL of Na_2CO_3 (20% w/v) solution was added to this solution and mixed well and the final volume 10 mL was made up with double distilled water. Keep the mixture for 30 min and absorbance was measured at 760 nm using in a UV-visible spectrophotometer (Shimadzu UV-1800, Japan) against blank. Total Phenolic (GAE) Content was expressed as % w/w dry extract and dry powder.

Method validation

1.1.1 Standard curve and linearity

To establish linearity, various working standards were prepared in different concentration ranging from the gallic acid standard stock (1 mg/mL) in six replicates of six working standard solutions to follow the Lambert-Beer law. Each working standard solutions was prepared according to the procedure describe above for quantification of gallic acid. Each sample was scanned at absorbance maxima (760 nm) against blank. The slope and standard error of intercept of calibration curve were calculated by linear regression with the Microsoft Excel software.

1.1.2 Specificity

Specificity was determined by adding 1 ml of 40 µg/mL of gallic acid solution to the linearity solutions of extract and compared with the linearity of the same extract. The specificity of method was considered if the slope of linear

equation in the test for linearity and specificity are equal or very similar.

1.1.3 Limit of detection (LOD) and limit of quantification (LOQ)

The LOD and LOQ were determined from the standard deviation (SD) of rutin hydrate linearity and the slope of the calibration curve with suitable multiplier. Different dilutions of standard gallic acid along with blank, and LOD and LOQ were determined from using formula given below:

$$LOD = \frac{3.3\ \sigma}{S}$$

$$LOQ = \frac{10\ \sigma}{S}$$

Where;

σ = the SD of the analysis

S = the slope from linearity equation

1.1.4 Precision

System precision (repeatability) was confirmed according to ICH guideline by repeated (n=10) scanning of corresponding reference standard (40 µg/mL) and expressed as relative standard deviation (RSD). The precision of method was assessed by analyzing aliquots of gallic acid containing 50%, 100% and 150% (n=3 for each set) of mid-point from linearity range, on the same day (inter day) and on different days (intraday). The results were expressed as %RSD, ≤ 2 was acceptable.

1.1.5 Accuracy

The accuracy of method was determined by calculating recoveries of respective reference standard by standard addition method. Known amount of standard solution was added to a pre-quantified sample solution of extract and results were calculated as recoveries and average recoveries.

1.1.6 Specificity

Specificity was determined by adding 1 mL of 40 µg/mL of reference standard solution to the linearity solutions of extract and compared with the linearity of the same extract.

STATISTICAL ANALYSIS

The results were expressed as mean ± SD (%RSD). Test for linearity was performed by simple regression analysis and the r^2 was set as equal to or greater than 0.99 using Microsoft EXCEL 2016. The statistical analysis ANOVA followed by post hoc Dunnett's test, with 95% confidence interval as significance level was performed using GraphPad Prism 7 (GraphPad Software Inc.).

RESULTS AND DISCUSSION

The calibration curve and overlay spectra of GA is represented in Fig. 2A and 2B respectively. The calibration curve is linear when the concentration According to the results are shown in of GA in the ranging from 10-100 mg/L (R^2 = 0.9998).

Quantification of total phenolic content in methanol and aqueous extracts of aerial parts of *E. hirta* and *E. thymifolia* were calculated using linearity equation of calibration curve of GA and results are shown in. TP content was found higher in methanol extract of *E. hirta* than aqueous extract. In contrary, higher amount of TP were present in aqueous extract of *E. thymifolia* than methanol extract. However, both methanol and water extracts of *E. hirta* showed comparatively higher amount of total phenols than those of *E. thymifolia*. There is not much difference in the amounts of total phenols in the methanol and water extracts of *E. thymifolia*. This is not so in the case of the extracts of *E. hirta*. Methanol extract had higher amounts of total phenols than water extracts. However, when calculated in terms of dry weight of the herbs, the values are more or less similar. This is because of the higher extractive values of *E. hirta* in water than in methanol.

Validation parameters viz. linearity, selectivity, specificity, molar absorptivity, repeatability, LOQ and LOD and range has summarized in Table

The results of the specificity test showed that the method is specific. The precision and accuracy of the proposed UV-Visible spectrophotometric method was determined by estimating the GA concentration at three different concentration levels (50, 100 and 150%). The inter day and intra precision of the proposed method was performed three set of experiment within same day and different day (Table). The RSD values of precision study were found to be less than 2% indicates the precision of the method.

The recovery study was established using the standard addition method. A known amount of standard GA was added to pre-analyzed extract and per cent recovery and average recovery were determined. The results of the study are shown in Table . The average recovery was found to be 102.36% showed that method has good accuracy for estimation of total phenolic in extracts.

Repeatability was performed by repeated scanning (n= 10) of the same concentration of GA and values are expressed as %RSD (0.33%). The linearity equation of calibration curve was Y = 0.0033X + 0.0005 and the correlation coefficient was 0.998 shown that method was linear. The result of the proposed method was found specific, accurate, precise, linear and reproducible.

CONCLUSION

The total phenolic content was found in methanol extract of *E. hirta* than aqueous extract. In contrary, higher amount of TP were present in aqueous extract of *E. thymifolia* than methanol extract. However, both methanol and water extracts of *E. hirta* showed comparatively higher amount of total phenols than those of *E. thymifolia*. There is not much difference in the amounts of total phenols in the methanol and water extracts of *E. thymifolia*. However, the result of the proposed method was found specific, accurate, precise, linear and reproducible.

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