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Development and Validation of UV-Visible Method to Determine Gallic Acid in Hydroalcoholic Extract of *Erythrina fusca* Leaves

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ABSTRACT

Gallic acid (GA) inhibitory potential against oxidative stress and associated diseases, creates the importance for GA standardization in plant medicines. The present investigation was intended to quantify the amount of GA in *Erythrina fusca* leaves hydro-alcoholic extract (EFLHE) by the development of UV/Visible-spectrophotometric method and its validation. The study involved of EFLHE preparation via ethanolic maceration, followed by estimation of total phenolic content (TPC) or GA through the development of Folin-Ciocalteu reagent-based UV/Visible-spectrometric method and standard GA calibration curve. Developed method to estimate GA was validated using linearity, accuracy, precision, repeatability and ruggedness studies. The TPC analysis of EFLHE in concentration of 0.5, 1.0, 2.5, 3.0, 5.0, 10.0 μg/ml exhibited amount of GA as 98.0±8.71, 117.0±1.73, 217.1±3.45, 276.0±0.80, 289.1±1.11 and 295.2±1.19 GA equivalent (mg/g, dry weight) respectively. The linearity study revealed the range of GA from 0.5-10 μg/ml. The correlation coefficient for GA was found as 0.997 at 212 nm. Recovery analysis (accuracy) showed that little change in drug concentration could be accurately estimated. The precision study revealed low %RSD with the highest value of 0.43%, indicating substantial precision. The present study concludes that advanced method to estimate GA in EFLHE is rapid, simple, accurate, precise, and economic; and validated for linearity, accuracy, precision, repeatability and ruggedness. The study recommends that this method can be used for GA estimation in *Erythrina fusca* leaves extract.

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INTRODUCTION

The expense of health care is rising at an alarming rate across the globe. The world market for phytopharmaceutical is also growing steadily. An estimation of the World Bank shows that trade in medicinal plants, raw materials and botanical drug products are growing at an annual rate of 5-15% (Patwardhan *et al.*, 2005). Around 75-80% of the world population use herbal medicines (Pan *et al.*, 2014). Standardization is considered as the critical factor to assure herbal drug quality. Access to herbal drug quality is attributed to active constituent concen-

tration. Phytochemical constituents are crucial for the pharmacological action of herbal formulation. Thus, it is essential to set up a system of standardization for every plant medicine in the market (Kumar *et al.*, 2011; Sitapara *et al.*, 2011). It is very challenging for investigators to develop various authentic and accurate analytical protocols that could screen the phyto composition, including quantitative analysis of marker/bio active compounds is a significant challenge for scientists (Rasheed *et al.*, 2012). Studies revealed that *Erythrina fusca* contains gallic acid (GA) that possess significant antioxidant, antidiabetic, antimicrobial property and high efficacy against periodontitis (Fuloria *et al.*, 2019).

Facts suggest many methods for the determination of GA as total phenolic content (TPC) individually and in combination with other drugs (Fernandes and Salgado, 2016). Various studies reported quantification and validation of GA in different plant extracts using Folin-Ciocalteu reagent based UV spectrophotometric method. This is because GA shows maximum absorption near 272 nm (Blainski *et al.*, 2013; Kamboj *et al.*, 2015). The study suggests *Erythrina fusca* plant possess high antioxidant potential (Subal *et al.*, 2010; Innok *et al.*, 2009). But till date, none of the studies performed the validation and development of estimation of GA in *Erythrina fusca* hydro-alcoholic extract (EFLHE). Hence, the present study was intended to quantify the amount of gallic acid (GA) in EFLHE using UV-Visible spectrophotometric method (Folin-Ciocalteu reagent method).

MATERIALS AND METHODS

The solvents, reagents and consumable were procured from Sigma Aldrich, SD Fine, Merck, and R&M chemicals. The glasswares were cleaned using deionised H_2O and dried at 160 °C for 2 hours before the experiment. The spectrophotometric analysis was done using Shimadzu double beam UV-Visible spectrophotometer, model U-2800 (200-800 nm).

Preparation of EFLHE

The *E. fusca* leaves were collected (from the campus of AIMST University, Malaysia), washed (with H_2O to remove dust), air-dried (until crispy) and powdered (coarse). The preparation of EFLHE was based on established maceration protocol with slight modification (Anjum and Chandra, 2015). Briefly, in a 500 ml conical flask 100 g of EFL and mixture of ethanol and distilled H2O (1:1) were added with 1:10 w/v sample to solvent ratio. The obtained mixture was swirled for seven days on mechanical shaker maintained at 100 rpm. After seven days, the supernatant liquid was filtered, and the filtrate was dried using rotavapor at 70 °C. Next, the EFLHE was air-dried (for completely drying), kept in a desiccator (to remove moisture) and finally stored in a refrigerator.

Development of UV-Visible spectrometric method

Solvent Selection

The procedure for the selection of solvents for UV-Visible analytical method development was based on standard protocol with slight modification (Bhardwaj *et al.*, 2017). In the protocol, various solvents were tested for the UV-Visible analytical method development, out of which Methanol and H2O (1:9) was selected based on solubility, peak quality, and non-interference at a specified wavelength.

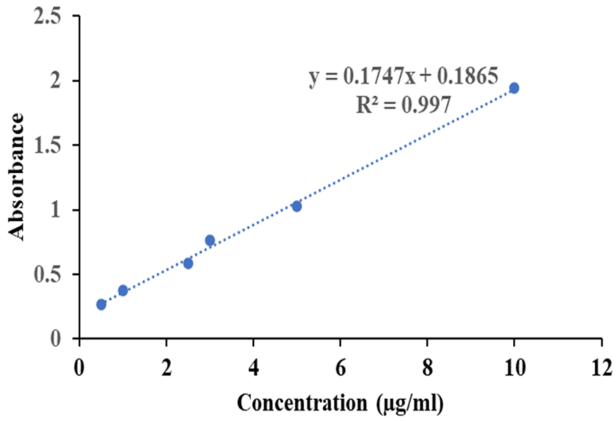

Figure 1: Calibration curve for GA

Wavelength Selection

A representative spectrum of GA solution (10 μg/ml) in Methanol and deionised H_2O (1:9) was obtained by scanning them in the UV range (200-400 nm) in 10 mm cell against blank solvent. Current protocol was founded on the established procedure with small variation (Bhardwaj *et al.*, 2017).

Preparation of Stock Solution and Standard Curve

Accurately weighed quantity of GA (10 mg) was transferred into the 10 ml of volumetric flask and dissolved by diluting up to mark using Methanol and deionised H_2O (1:9) to get a concentration of 1000 μg/ml. From this stock solution, the aliquots of working standard solution of GA were formulated using methanol and deionised H_2O (1:9) solution to get a concentration in a range of 0.5-10 μg/ml for GA (Bueno *et al.*, 2012). The absorbance value for each concentration of the standard solution of GA was measured at λ_{max} of 212 nm. A calibration curve between concentrations versus absorbance was built to study the Beer-Lambert's Law and

Table 1: TPC (mg GAE/g) of EFLHE.

Conc (μg/ml)	Abs	Amount of GA as TPC, mg GAE/g	Mean±SD
0.5	0.194	92.0	98.0±8.71
	0.196	94.0	
	0.195	108.0	
1.0	0.203	118.0	117.0±1.73
	0.208	115.0	
	0.210	118.0	
2.5	0.280	214.0	217.1±3.45
	0.281	216.4	
	0.283	220.8	
3.0	0.305	276.0	276.0±0.80
	0.306	276.8	
	0.310	275.2	
5.0	0.439	289.0	289.1±1.11
	0.440	290.2	
	0.438	288.0	
10.0	0.703	295.6	295.2±1.19
	0.700	293.9	
	0.704	296.2	

regression equation.

Total Phenolic Content

Accurately 10 mg of EFLHE was dissolved ad diluted with Methanol and deionised H_2O (1:9) to get a concentration of 1000 μg/ml. From this stock solution, aliquots of EFLHE solution were prepared with Methanol and deionised H_2O (1:9) to get a concentration in the range of 0.5-10 μg/m (International Conference on Harmonization, 2005). The reaction mixtures were prepared by mixing 0.5 ml of each EFLHE solution with 2.5 ml of 1% Folin-Ciocalteu reagent dissolved in distilled H_2O and 2.5 ml of sodium bicarbonate. Next, the reaction mixtures were incubated at 45 °C for 15 minutes. The prepared solutions absorbances were measured at 212 nm against a blank. The quantification of TPC of EFLHE was founded on a standard curve of GA that acts as a standard phenolic compound and expressed as mg of GA equivalent (GAE) per gram of EFLHE. The TPC was estimated using the following expression:

$$\mathbf{T = \frac{C \times V}{M}}$$

Where: T = Total phenolic content in mg/g as GAE of EFLHE, C = Concentration of GA in mg/ml (established from calibration curve), V = Volume in ml of EFLHE solution, and M = Weight of EFLHE in gram.

Method Validation

The developed analytical protocol validation was based on linearity, accuracy, precision, repeatability and ruggedness, using ICH guidelines Q2 (R1) (International Conference on Harmonization, 2005).

Linearity

The 0.5 ml of each GA standard solution (0.5-10 μg/mL) was mixed with 2.5 ml of 1% Folin-Ciocalteu reagent (dissolved in distilled H_2O) and 2.5 ml of sodium bicarbonate. Each reaction mixture was incubated at 45 °C for 15 minutes and scanned at 212 nm against Methanol and H_2O (1:9) as blank in triplicate. A calibration curve was constructed by plotting concentration against absorbance (Figure 1). The regression equation and correlation coefficient were determined using GA standard concentrations (0.5-10 μg/ml). The relation between drug and absorbance is expressed as y = MX + c. Where, m = slope, c = intercept and x = concentration. Based on GA standard curve, TPC of EFLHE was estimated and expressed as mg of GAE per gram of plant extracts.

Accuracy

Analytical method accuracy was estimated by carrying out a recovery analysis of 80%, 100% and 120% of EFLHE concentration as per ICH guidelines in triplicate. Percentage recovery was estimated using the following expression:

$$\mathbf{\%\ Recovery = \frac{A.100}{A^T}}$$

Where, 'A' is EFLHE absorbance after addition of standard, 'A^T' is theoretical absorbance (sum of absorbance of GA standard and expected absorbance of GA in sample extract based on calibration curve).

Precision

The developed analytical protocol was tested for precision founded on intraday and interday variations. Intraday precision was established by analyzing the 10, 15 and 20 ug/ml of EFLHE for three times on the same day.

Interday precision was calculated by analyzing the 10, 15, and 20 ug/ml of EFLHE daily for three days.

Repeatability

The proposed protocol was validated for repeatability by analyzing 10 μg/ml of sample extract solution for six times.

Ruggedness

The analytical protocol ruggedness was determined by spiking the standard six times with different analyst using the same instrument.

RESULTS AND DISCUSSION

Facts suggest GA possess a significant antioxidant potential and protects the human body from free radicals harmful actions (Rasool *et al.*, 2010).

Investigation suggests GA extracted from grape seeds induced the programmed death of prostate cancer cells (Kaur *et al.*, 2009). Besides, GA is beneficial for diabetes patients as they can trigger the release of insulin by the pancreatic cells (Sameermahmood *et al.*, 2010). These biological activities indicate the potential use of GA (Masoud *et al.*, 2012; Phiriyawirut and Phachamud, 2011). Based on the facts over GA to exhibit its absorption in UV region, various researchers performed GA estimation and validation studies over plant extracts involving Folin-Ciocalteu reagent based spectrophotometric analysis (Singh and Avupati, 2017; Purohit *et al.*, 2014).

However, to date, none of the studies suggested GA estimation and validation in *Erythrina fusca ethanolic extract (EFLHE)*. Based on these facts present study was intended to quantify the amount of GA in EFLHE using UV-Visible spectrophotometry (Folin-Ciocalteu reagent method). Application of given formula estimated associated with *E. fusca* leaves was found to be 32%. The per cent yield of EFLHE was estimated based on the dry weight of EFLHE (X) and EFLHE soaked (Y) using the given formula:

$$\%\text{Yield} = \frac{X}{Y}$$

Development of UV-Visible spectrometric method

The earlier study suggests that solvents substantially affects the quality of the spectrophotometric signals (Bhardwaj *et al.*, 2017). Hence, in the present study, the selection of solvents for UV-Visible method development was made by testing different solvents based on solubility, peak quality, and non-interference at a specified wavelength. Solvent optimization study revealed Methanol and H_2O (1:9) as the most suitable solvent for the current protocol. For wavelength optimisation, a representative spectrum of GA solution (10 μg/ml) in Methanol and deionised H_2O (1:9) was scanned from 200 to 400 nm. The UV-Visible spectrum revealed well-defined λ_{max} at 212 nm for GA. The analysis of EFLHE for GA as TPC was carried out as per the protocol given in the experimental part of the present study. The resultant data for the same is given in Table 1.

Method Validation

Linearity

The analytical protocol for linearity is protocol ability to deliver results in a specified range directly or through mathematical expression, proportional to analyze concentration (Jain *et al.*, 2011). The linearity results for EFLHE were derived from the calibration curve of GA (0.5-10 μg/ml).

The correlation coefficient (r^2) from the calibration curve was found to be 0.997 (Figure 1) and expressed in GAE per gram dry EFLHE weight. The content of phenolic compounds in EFLHE extracts ranged from 98 to 295.2 mg GAE/g, representing an approximate four-fold variation (Table 1). The linearity results are shown in Table 2. The linear regression data for calibration curves showed good linear relationship over the concentration range of 0.5-10 ug/ml. A linear regression equation was found to be y=0.0547x+0.1547. It has a slope of 0.0547 and y-intercept of 0.1545. The coefficient of correlation or value for GA in EFLHE at 212 nm is 0.997. As per the study done by Kaur et al. when the value is greater than 0.99, then the regression line from linearity studies exhibits accuracy (Kaur *et al.*, 2009).

Accuracy

Generally, the accuracy of the analytical protocol is the closeness of practical result to theoretical value (Bhardwaj *et al.*, 2017). Accuracy study was conducted as per the experimental protocol and resulted in data for the same is given in Table 3. The results of the accuracy study revealed percentage recovery of 86.80±0.49, 87.60±0.36 and

Table 2: Linearity data of standard gallic acid and gallic acid in EFLHE.

Parameters	Gallic Acid	Gallic acid in EFLHE
Detection wavelength	212 nm	212 nm
Linearity range	0.5-10 μg/ml	0.5 -10
Slope	0.1747	0.0547
Intercept	0.1865	0.1545
Correlation Coefficient	0.997	0.997
Regression Equation	y=0.1747x+0.1865	y = 0.0547x + 0.1545

Table 3: Accuracy data.

Pre-analysed sample solution μg/ml	Amount of GA Added, %	Amount recovered (mg GAE/g)	Mean$\pm$SD	%Recovery	%RSD
10	80	405	404.3$\pm$3.05	86.80$\pm$0.49	0.56
		401			
		407			
10	100	424	426$\pm$2.00	87.60$\pm$0.36	0.41
		428			
		426			
10	120	452	453$\pm$1.73	89.07$\pm$0.24	0.27
		455			
		452			

Table 4: Intraday precision data.

Conc. of extract (μg/ml)	Intraday precision		
	Absorbance	Amount of GA as TPC, mg GAE/g	Mean $\pm$ SD
10	0.705	297.0	296.3 $\pm$ 1.2
	0.701	295.0	
	0.706	297.0	
15	0.981	303.3	303.1 $\pm$ 0.3
	0.980	302.7	
	0.981	303.3	
20	1.331	327.5	326.7 $\pm$ 0.8
	1.328	326.5	
	1.325	326.0	

89.07$\pm$0.24 respectively, for the 80 %, 100 % and 120 % of the test concentration. As per the report of Andressa Blainski *et al.* these percentages were within the range of 85%-115% which indicate that the method has good accuracy for quantification of GA from EFLHE (Blainski *et al.*, 2013).

Precision

The precision of an analytical protocol is a degree of repeatability under the normal operation conditions. Precision studies of the developed method were conducted as per the intraday and interday experimental protocol of the present study. The results for precision study are reported in Tables 4 and 5. Both intra and the inter-day precision study revealed quiet low % RSD with the highest value of 0.43%. A study claimed that %RSD less than 2% indicates good precision (Pawar and Salunkhe, 2013).

Repeatability

Repeatability study of the developed method was conducted as per the experimental protocol of the present study and resulted in data given in Table 6. The 10 μg/ml of EFLHE were analyzed for six times, and amount of GA as TPC found was of little differ-

Table 5: Interday precision data.

Conc. of extract (μg/ml)	Interday precision		
	Absorbance	Amount of GA as TPC, mg GAE/g	Mean $\pm$ SD
10	0.703	295.0	294.0 $\pm$ 1.4
	0.698	292.0	
	0.701	295.0	
15	0.975	300.7	301.1 $\pm$ 0.3
	0.977	301.3	
	0.977	301.3	
20	1.335	328.5	328.8 $\pm$ 1.5
	1.330	327.5	
	1.3341	330.5	

Table 6: Repeatability data.

Conc. of extract (μg/ml)	Absorbance	Amount of GA as TPC, mg GAE/g	Mean $\pm$SD
10	0.703	296.0	295.0 $\pm$ 1.6
	0.703	296.0	
	0.705	297.0	
	0.700	294.0	
	0.698	293.0	
	0.700	294.0	

Table 7: Ruggedness data

Conc. of extract (μg/ml)	Analyst 1		Analyst 2	
	Amount of GA as TPC, mg GAE/g	Mean $\pm$ SD	Amount of GA as TPC, mg GAE/g	Mean $\pm$ SD
20	326.0	327.1 $\pm$ 0.74	318.0	324.5
	327.5		321.5	
	326.5		322.0	
	328.0		322.5	
	327.0		318.5	
	327.5		324.5	

ence with a standard deviation of 1.6, which indicates good repeatability. Amount of GA as TPC found in 10 μg/ml of sample extract (n=6) found was 295$\pm$1.6 GAE (mg/g).

Ruggedness

Ruggedness study over-developed analytical method was performed according to the present study experimental protocol, and results are given in data given in Table 7. The ruggedness of the method was assessed by spiking the standard six times with different analyst by using the same equipment.

The results showed that for 20 μg/ml of sample extract, both analyst 1 and analyst 2 obtain an amount of Gallic Acid of 327.1$\pm$0.74 GAE (mg/g) and 321.2$\pm$2.48 GAE (mg/g) respectively.

Both analysts had %RSD less than 2 %, which are 0.22 % and 0.77 % respectively. As the percentage RSD value is less than 2 %, so the variation of analysts will not affect the UV method in the quantification of GA in EFLHE (Pawar and Salunkhe, 2013). Based on resultant data, the present study reveals that quantification of GA in EFLHE through UV-Spectrophotometer is an economical and straight forward method. The validation of the analytical protocol developed in this study has been proven to be linear, specific, precise, accurate, reproducible, rugged, and easy.

CONCLUSIONS

This is the first-time study to develop and validate the method for quantification the amount of gallic acid in *Erythrina fusca* hydro-alcoholic extract using UV-Visible spectrophotometric method (Folin-Ciocalteu reagent method). Based on the experimental results of the present study, it can be concluded that quantification of gallic acid in a hydro-alcoholic extract of *Erythrina fusca* leaves can be carried out by UV-spectro photometric technique. The developed method is quite simple and less time-consuming. Besides, this method requires less labor cost and less sophisticated and less expensive equipment. Apart from it, this method has been validated as required by ICH guidelines. The current study recommends that in the future estimation of gallic acid in other parts of *Erythrina fusca* plant such as bark or stem can also be done. Moreover, variant extraction methods may also be used to replace simple maceration to compare the amount of gallic acid.

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Conflict of Interest

The authors declare that they have no conflict of interest for this study.

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