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# HPTLC determination of Gallic acid in Methanol extract of *Quercus griffithii* Acorn

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**Abstract:** This paper describes HPTLC determination of gallic acid in methanol extract of *Q. griffithii* acorn. Macerated extract solution of *Q. griffithii* acorn was applied on HPTLC plate along with standard by using Camag Linomat-5. The detection of gallic acid was performed on aluminium plates pre-coated with silica gel 60  $F_{254}$  as the stationary phase. Optimized mobile phase toluene: ethyl acetate: formic acid (6:6:1) was used. Plate was developed in twin trough chamber and scanned by TLC scanner III at  $\lambda_{max}$  i.e. 297 nm. The system was found to give compact spots for gallic acid at Rf 0.41. Amount of gallic acid determined in 1 Kg dry acorn powder of *Q. griffithii* was found to be 0.19±0.01 g. The method was validated in terms of sensitivity, precision, specificity, robustness and recovery. Edibility of acorn and presence of gallic acid in *Q. griffithii* is reporting for the first time.

Key Words Quercus griffithii acorn, Edible, Gallic acid, HPTLC.

## Introduction

Quercus griffithii Hook. f. & Thomson ex Miq. (local name- Pasheng) is a deciduous tree growing up to a height of 15 m. The tree is widely distributed from East Himalayan region up to Thailand<sup>1-2</sup>. Acorn size ranges from 1.5-2 cm length to 0.8-1.2 cm diameter. The acorn matures in the month of September-October. It is an important source of fodder for wild mammals living in the temperate forest. In East Himalayan region, the plant is cultivated in large scale for fuel wood. The leaf is used for the production of humus and acorn is consumed as supplement of rice during food scarcity. Due to its bitter taste, the acorn flour is mixed with rice or barley flour (50:50 ratio), and cooked in various traditional forms. Local people believed that consumption of Q. griffithii acorn reduces one's appetite and had played important role during famine.

Gallic acid or 3,4,5-trihydroxybenzoic acid is a polyhydroxylphenolic compound (Figure 1), widely distributed in various plants<sup>3</sup>. Gallic acid has various biological activities such as anti-bacterial<sup>4</sup>, anti-inflammatory<sup>5-6</sup>, anti-melanogenic<sup>7</sup>, antioxidant<sup>8</sup>, anti-viral<sup>9</sup> anti-cancer activities in various cancer cells<sup>10-15</sup>. Gallic acid was quantitatively determined from certain plant extract such as *Arctostaphylos uva-ursi*, *Nymphaea stellata*, *Phyllanthus emblica*, *Terminalia chebula*, *Terminalia arjuna* etc.<sup>16-20</sup> by using HPTLC, however edibility of *Q. griffitiii* acorn and presence of gallic acid is reporting for the first time.

The goal of the present article is to determine the content of gallic acid in methanolic acorn extract of ethnobotanically important plant *Q. griffithii* by using the HPTLC method. For this purpose, a new, simple, sensitive, precise and robust HPTLC method was developed. The method was validated for sensitivity, precision, specificity, robustness and recovery.



Figure 1: Structure of gallic acid

#### **Materials**

Dry acorn of *Q. griffithii* collected from Tawang district of Arunachal Pradesh (India), Camag's HPTLC system comprising of a Linomat-5 applicator assisted by nitrogen gas and scanner III with *winCATS* software (Anchrome Swirtzerland), 100 mL Hamilton syringe (USA), aluminium precoated silica gel 60  $F_{254}$  (E. Merck, Germany), Camag glass twin trough chamber, Camag TLC plate heater and Camag UV chamber (254 and 366nm). All solvents used were of HPLC grade obtained from Merck India and gallic acid from SIGMA, Switzerland.

## Methods

#### **Extract preparation**

100 g of dry acorn was pulverized and extracted by cold maceration process using methanol. The final volume was concentrated to dryness by rotary evaporator at 45°C under reduced pressure<sup>21</sup>.

## Preparation of standard and sample solutions

Standard solution was prepared by dissolving gallic acid with methanol at six different concentrations (100, 200, 300, 400, 500, & 600  $\mu$ g/mL). Sample solution was prepared by mixing dried extract (acorn) in methanol at concentration of 10 mg/mL. Both solutions were sonicated for 5 minutes.

## Analytical procedure

Chromatography was performed on  $10 \times 10$  cm aluminium plates coated with 200 µm layers of silica gel 60 F<sub>254</sub>. Plate was prewashed with methanol and dried in oven at 105 °C for 30 minutes. Both standard and sample solutions were applied to the plate as bands 6.00 mm wide at a distance of 10.00 mm from the base by using Camag Linomat-5 sample applicator equipped with a 100 mL syringe. A constant rate of application of 150 nL<sup>-1</sup> was used. Plate was developed in a twin trough glass chamber of size  $10 \times 10$  cm using 13 mL optimized mobile phase. Plate was dried by hair dryer and then placed in oven for 5 minutes at 80° C for complete evaporation of mobile phase absorbed by the stationary phase. The plate was scanned at  $\lambda_{max}$  with slit dimension 4.00 x 0.30 mm micro and scanning speed of 20 mm/s with a Camag TLC scanner III in absorbance mode operated by *WinCATS* software.

#### Mobile phase optimization

Based on various reports on gallic acid separation in plant extract<sup>16-20,22</sup>, a number of mobile phase were tried in this experiment. Both standard and sample solutions were spotted on the same plate and developed. Mobile phase which gives high resolution and good peak purity of gallic acid was optimized and used for quantification of the marker.

## Determination of λ<sub>max</sub>

Both standard and sample solutions were spotted on the plate and scanned between 200-500 nm with wavelength increment of 50 nm. Gallic acid spot were identified by comparing the Rf values. Spectra of identified gallic acid spots were taken, ranging from 200-700 nm wavelengths and  $\lambda_{max}$  was determined.

#### **Calibration and quantification**

Aliquots of 1  $\mu$ L of standard solutions (100, 200, 300, 400, 500, 600 ng/spot) and 5 replicates of sample solution (100  $\mu$ g/spot) were spotted on the plates. Densitometric determination of gallic acid were performed at  $\lambda_{max}$  297 nm. The calibration of gallic acid was obtained by plotting peak areas *vs* the concentration of the compound. The amount of gallic acid present in sample was calculated using the regression of the calibration curve.

#### **Methods validation**

ICH guidelines were followed for the validation of the analytical methods developed which include sensitivity, precision, specificity, robustness and recovery<sup>23</sup>.

#### Sensitivity

The sensitivity of measurement of gallic acid was estimated as limit of detection (LOD) and limit of quantification (LOQ). Concentration of the standard solution of gallic acid was applied along with methanol as blank and determined on the basis of signal to noise ratio. The LOD and LOQ were calculated by using the equations  $LOD = 3.3 \times \sigma/S$  and  $LOQ = 10 \times \sigma/S$ , where  $\sigma$  is the standard deviation of the peak areas of the drug (n = 5), taken as a measure of noise, and S is the slope of the corresponding calibration plot.

#### Precision

Instrumental precision, intra-day precision and inter-day precision of the method were determined. Instrumental precision was measured by replicate (n=5) application of the same standard solution (500 ng/spot). Intra assay precision was evaluated by analysis of three replicate applications of standard solution on the same day. Intermediate precision was evaluated by analysis of three replicate applications of standard solution on three different days. The plate was developed under the optimized chromatographic conditions and CV % of peak area was recorded. Room temperature was maintained at 25 °C.

#### Specificity

The specificity of the method was ascertained by analyzing the standard and sample solutions. The spot for gallic acid in the sample solution was identified by comparing the Rf values and confirmed by spectra comparison. The peak purity of gallic acid was analysed by comparing the spectra at three different levels, i.e. start, middle, and end positions of the bands.

#### Robustness

Robustness of the method was determined by allowing different solvent front position (65-85 mm), tank size ( $10 \times 10$  cm,  $20 \times 10$  cm) and duration of mobile phase saturation (10-30 minutes). The effects on the results were examined.

#### Recovery

The accuracy of the method was established by performing recovery experiments at three different levels using the standard addition method. In 100  $\mu$ g/spot of sample solution, known amounts of standard solutions i.e. 0, 100 and 200 ng/spot were added. The values of percent recovery and average value of percent recovery for gallic acid were calculated, which is shown in Table 3.







Figure 4: Spectra comparison of all tracks



Figure 6: Chromatogram of sample (100 µg)

Figure 3: 3-D chromatogram of both sample and standard



Figure 5: Calibration curve of GA (peak area)



Figure 7: Developed plate captured at visible light

## **Results and Discussion**

## **Development of the optimum mobile phase**

Most of the mobile phase reported by various authors, does not give good resolution of gallic acid peak, either interfering with other peak, or gives low Rf value (Rf<0.20). Mobile containing water solvent increases the developing time. Formic acid is the most important solvent for gallic acid separation as no movement of gallic acid take place in absence of formic acid. Three solvents toluene: ethyl acetate: formic acid in the ratio of 6:6:1 gave a good separation of gallic acid with Rf = 0.41 (Figure 2). The same mobile phase was also used by Dharmender et. al. 2010<sup>24</sup> for separation of Bergenin, (+)-Catechin, Gallicin, Gallic Acid &  $\beta$ -Sitosterol. 3-Dimensional chromatogram at  $\lambda_{\text{max}}$  (297 nm) is shown in Figure 3. The same mobile phase in the ratio (7:5:1)

also gives a good resolution with Rf 0.38. Well-defined spot of gallic acid was obtained when mobile phase was sonicated for 5 minutes followed by 30 minutes saturation in TLC chamber.

## $\lambda_{max}$ of gallic acid

Maximum signal of gallic acid in the developed plate was found at wavelength 297 nm in both standard and sample solutions (Figure 4). A little absorbance was also found at wavelength 220 nm.

#### Calibration curve and its linearity

The calibration curve has been developed for gallic acid at a specific Rf value. A good linearity curve of the peak area was obtained in the concentration ranges between 100-500 ng/spot for gallic acid with linear regression equation, Y = 165.9 + 16.33 \* X; r = 0.99539 (Figure 5). The standard deviation was found to be 5.67 %.

#### Gallic acid present in Q. griffithii acorn extract

In methanol extract of acorn, 303.58 ng of gallic acid was found in 100  $\mu$ g sample extract, which means 1 Kg dry acorn powder contains 0.19 g gallic acid (Table 1). Figure 6 represents the chromatogram of sample solution (100  $\mu$ g/spot). A total of nine peaks were found in sample solution and their Rf values, peak height and peak area are given in Table 2. The peak number 4 with Rf 0.41 represents gallic acid. Figure 7 represent the developed plate captured at visible light.

#### Table 1: Quantitation of gallic acid (GA) in sample

GA quantified in 100 µg extract	: 303.58±3.30 ng
Extract yield per 1 Kg dry sample	: 60.94 g
GA present in 1 Kg dry sample	: 0.19±0.00 g

#### Table 2: No. of peaks detected in sample, their Rf values, peak height and peak area

Peak No.	Rf Values	Peak height AU	Peak area AU
1.	0.01	286.7	2219.8
2.	0.07	92.4	2831.6
3.	0.26	104.0	9022.1
4.	0.41 (GA)	167.4	4961.8
5.	0.66	69.2	1839.9
6.	0.71	125.8	3430.2
7.	0.81	45.3	1500
8.	0.89	129.1	4544.7
9.	0.96	62.3	2490

## **Methods Validated**

#### Sensitivity (LOD and LOQ)

The method was found to be very sensitive as the limits of detection and limits of quantification for gallic acid were found to be very low i.e. 0.67 ng and 2.02 ng per band, respectively.

## Precision

The developed method was very precise as the CV calculated was less than 2 % in all three precisions analyzed. CV of instrumental precision, intra-day precision and inter-day precision were calculated as 0.21 %, 0.87 % and 1.24 % respectively.

#### Specificity

The developed method was found to be specific for methanol extract as no interfering or contamination peak was detected. The spectra of marker (GA) is matching and overlapping exactly with GA spot of acorn extract with maximum absorbance at 297 nm.

## Robustness

The saturation time plays a significant role in changing the Rf value of gallic acid, however, recovery percent remains same. Saturation time was found to be inversely proportional to Rf values of gallic acid spot, which means longer is the saturation time, smaller will be Rf value. Tank size  $(10\times10, 10\times20, 20\times20\text{cm})$  and solvent front position (60-90mm) has insignificant differences in the results.

#### Recovery

The recovery percentage is given in Table 3. 100  $\mu$ g of extract was applied which contains predetermined 305.78 ng of gallic acid. There was 100 % recovery when no standard was added, while the recovery percentage goes down to 95 % in addition of 100 ng of standard gallic acid. But the recovery percent slightly increased on addition of more amount of standard gallic acid i.e. 200 ng.

Amount present in	Amount of	Amount	Recovery %
10µL sample	standard added	determined	(n=3)
303.58 ng	0 ng	306.23 ng	100.87±0.16
303.58 ng	100 ng	386.79 ng	$95.84{\pm}3.68$
303.58 ng	200 ng	486.39 ng	96.57±2.60

#### Table 3: Recovery of gallic acid

## Conclusion

Acorn of ethnobotanically important plant *Q. griffithii* contains biologically active gallic acid compound. Gallic acid determined by HPTLC was 0.19 g per Kg dry acorn. The method developed was validated in terms sensitivity, precision, specificity, robustness and recovery. The method developed gave good peak shape and enabled good resolution of gallic acid spot in methanol extract of *Q. griffithii* acorn. The acorn as edible and presence of gallic acid is reporting for the first time, and further study is going on for identification of bioactive compounds with its pharmacological activities.

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