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Development of RP-HPLC method for Qualitative Analysis of Active Ingredient (Gallic acid) from Stem Bark of *Dendrophthoe falcate* Linn.

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ABSTRACT
A simple, precise and sensitive, RP-HPLC method with UV detection at 271nm was developed and validated for qualitative determination of active ingredient Gallic acid from stem bark of *Dendrophthoe falcate* Linn. Separation was performed on a ThermoMOS 2 HYPERSIL C_{18} column (250 cm × 4.6 mm, 5µm ODS 3) using mobile phase comprising of 0.1% Orthophosphoric acid : Acetonitrile (400 cm^3 : 600 cm^3) with a flow rate of 1 ml/minute with a short run time of 13 minute. The method was validated according to the regulatory guidelines with respect to linearity, system suitability, precision, solution stability, accuracy, robustness, assay and recovery. Detector response was linear for HPLC in the range of 0.04 to 0.16 mg/cm^3. The system suitability, precision, solution stability, accuracy, robustness, assay and recovery was assessed by calculating % COV for all these parameters which is less than two as expected. The recovery of the method for Gallic acid was found 98.94% which shows that method is accurate. The described method has the advantage of being rapid and easy hence it can be applied for routine quality control analysis of Gallic acid from *Dendrophthoe falcate* Linn.

Keywords: Gallic acid, RP-HPLC, *Dendrophthoe falcate* Linn.

INTRODUCTION

Dendrophthoe falcate Linn. is genus of evergreen, shrubby, parasite that is distributed in the tropical and sub-tropical regions of the old world. *Dendrophthoe falcate* Linn. (Loranthaceae) is an important plant in Indian system of medicine. It is a parasitic shrub that grows on a variety of host plants namely mango, jack and other trees. The plant has been used as an aphrodiasic, astringent, narcotic and diuretic. [1] Chemically the plant has been found to be rich in phenols and flavonoids; catechins, gallic acid, ellagic acid, chebulinic acid, quercetin [2], kaempferol, rutin and quercetrin. [3]

Dendrophthoe falcate Linn has been used traditionally in the treatment of pulmonary tuberculosis, asthma, menstrual disorders, swelling wounds, ulcers, renal and vesical calculi and vitiated conditions of kapha and pitta. [1] In Nepal, the leaf along with Urtica doica is made into a paste and used to treat bone fractures.Members of genus Dendrophthoe are reported to have anti-oxidant, anti-microbial, anticancer, anti-diabetic [4], anti-lithiatic and antihypertensive [5] properties. Study was undertaken for identification of Gallic acid in *Dendrophthoe falcate* Linn. stem-bark.

EXPERIMENTAL

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Fig.1 (a): *Dendrophthoe Falcate* Linn Parasite on *Magnifera indica* (Mango), Mahad

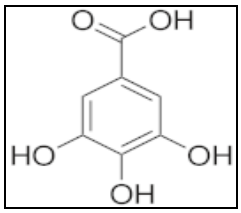

Fig. 1 (b): Gallic acid

Chemical and reagents

The Gallic acid working standards were obtained as a gift sample from J B Pharmaceutical Ltd., Mumbai with purity of 99.98%. All the reagents which were used were of chromatographic grade. All dilutions were performed in standard volumetric flask.

Instrumentation and chromatographic conditions

Jasco, High performance liquid chromatograph equipped with PU 980 isocratic pump, fitted with an autosampler, a Rheodyne injector fitted with 20µl sample loop and Jasco UV- Visible detector (UV-970) was employed. The instrument was connected to Borwin chromatographic software (Version 1.21) for data acquisition and integration. The chromatographic conditions had previously been optimized to achieve the best resolution and peak shape. Detection was performed at λ=271nm having flow rate 1ml/min. The typical chromatogram for standard and sample is shown in Fig. 2.

Preparation of standard solution of Gallic acid (solution 1)

In a series of 10 cm^3 standard volumetric flasks varying amounts of Gallic acid were taken. To each of these flasks methanol was added and diluted up to the mark. These solutions were use for the linearity study.

Preparation of sample solution for assay experiment (solution 2)

Three grams of the plant powder was accurately weighed and taken in a 100 cm^3 of standard volumetric flask to this methanol was added and diluted up to the mark, sonicated for five minutes and then flask was kept aside overnight for extraction. This solution was filtered through the Whatmann filter paper No. 41 in another dry stopperred conical flask. This solution (Solution 2) was used for assay experiment.

Procedure of sample preparation for recovery experiment (solution 3)

Set I

Three grams of the plant powder was accurately weighed and taken in a 100 cm^3 of standard volumetric flask, to this methanol was added and diluted up to the mark, sonicated for five minutes and then flask was kept aside overnight for extraction. This solution was filtered through the Whatmann filter paper No. 41 in another dry stopperred conical flask (Solution 3). This served as the zero level.

Set II, III and IV

A fixed volume of the pre-analysed sample (Solution 3) was taken in a three different standard volumetric flasks and to it different quantities (12.5 mg, 13.1 mg and 16.2 mg) of standard Gallic acid was added and diluted up to the mark with methanol.

VALIDATION PROCEDURE

Linear working range

The linearity experiment was carried out in triplicate. Into a series of 10 cm^3 standard volumetric flasks varying amounts of Gallic acid were taken. To each of these flasks methanol was added and diluted up to the mark to obtain desired linearity range. 10µl of each solution was injected and the detector response for the different concentration was measured. The peak area was calculated for each concentration level and a graph was plotted of concentration of Gallic acid against the peak area. The plot was linear in the range of 0.04 mg/cm^3 to 0.16 mg/cm^3. The mean peak area response was used for calculations. The data was analyzed by linear regression least square fitting. The statistical data obtained are given in Table 1.

System suitability test

A system suitability experiment was performed before determination of Gallic acid in samples. The coefficient of variation for peak area, retention time, theoretical plate and asymmetry factor value for Gallic acid was less than 2.00% for five replicate measurements of the same sample having 0.1 mg/cm^3 concentration as expected.

Robustness of the method

In the present work effect of deliberate change in wavelength {± 2nm i.e. (271nm, 269nm, and 273nm)}, mobile phase composition {± 5% change in organic solvent i.e. (40: 60, 37: 63, 43: 57) volumes ratio of 0.1% Ortho-Phosphoric Acid: Acetonitrile and + 10% change in organic solvent i.e. (40: 60, 46: 54) and volume ratio of 0.05M KH_2PO_4}, and temperature (±5°C) was studied for the robustness of the method.

Peak asymmetry and peak tailing

The result shows that within the concentration ranges mentioned above there was an excellent correlation between peak area and concentration of the Gallic Acid.

In the present experimental work the peak asymmetry factor and peak tailing factor calculated for Gallic acid are given in Table 6.

Solution stability

In the present study mobile phase is considered for the study of the solution stability of Gallic acid from *Dendrophthoe falcate* Linn.

Assay experiment

10µl of solution 2 were injected and chromatogram were developed and evaluated. The procedure was repeated twice, individually weighing the plant powder (3 g) each time. The responses from the standard and sample were used to calculate the amounts of the Gallic acid in the plant powder. In the present assay experiment the standard deviation was found to be 0.0141; while percentage coefficient of variation was found to be 0.1186 for Gallic acid. These values of low percentage coefficient of variation indicate high precision of the method.

Recovery experiment

The accuracy of the experiment was established by using solution 3. The mean recovery is within acceptable limits which indicate that the method is accurate.

Determination of reliability of the assay method

Statistical parameters namely standard deviation (S.D) and percentage coefficient of variation (C.O.V. %) were calculated to study the reliability of the method.

Table 1: Linear regression data for calibration curve

Regression Output	Gallic Acid
Constant (C)	250013.14
Standard error of Y estimate	$5.767857488\times 10^{-7}$
R squared	0.993
No. observations	7
Degree of freedom	5
X Coefficient (S) (m)	117489370
Standard error of coefficient	2.64×10^{-3}

RESULTS AND DISCUSSION

The actual photograph of *Dendrophthoe falcate* Linn parasite grown on Mango tree (*Magnifera indica*) in Mahad taluka and structure of Gallic acid are shown in the Fig. 1 (a) and Fig. 1 (b) respectively.

Use of 0.1% Ortho-phosphoric Acid: Acetonitrile in the volume ratio of 400cm^3: 600cm^3 resulted in good separation of Gallic acid. The chromatogram obtained using mobile phase 0.1% Ortho-phosphoric Acid: Acetonitrile at flow rate 1ml/ min, showed no interfering peak at the retention time of the Gallic acid viz. 12.81 minute as shown in Fig. 2. Fig. 2 (a) shows HPLC chromatogram for Gallic acid in sample.

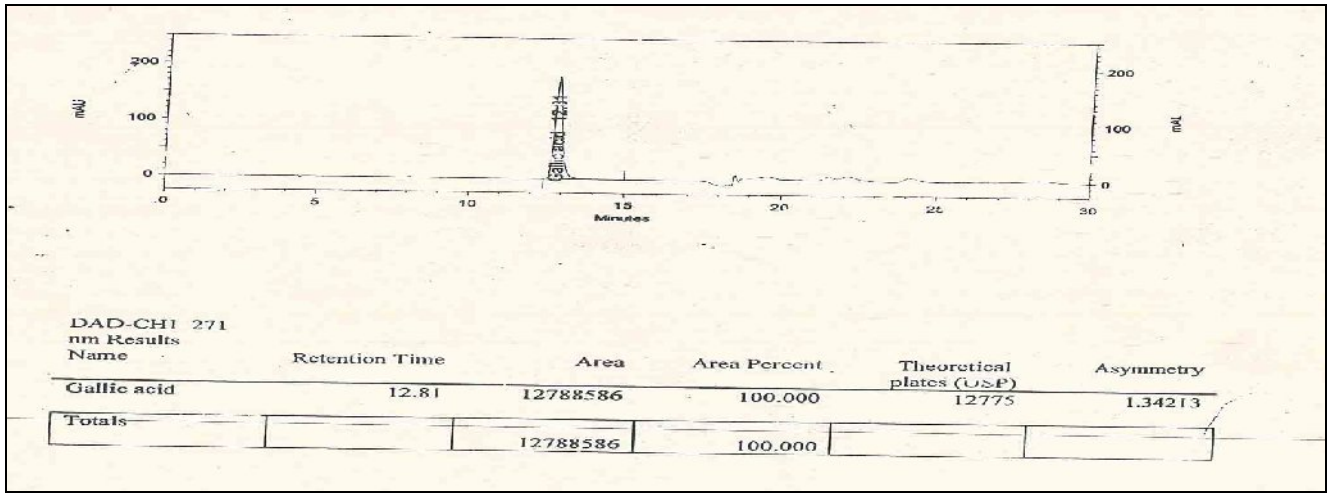

Fig. 2: HPLC Chromatogram showing Standard Gallic acid

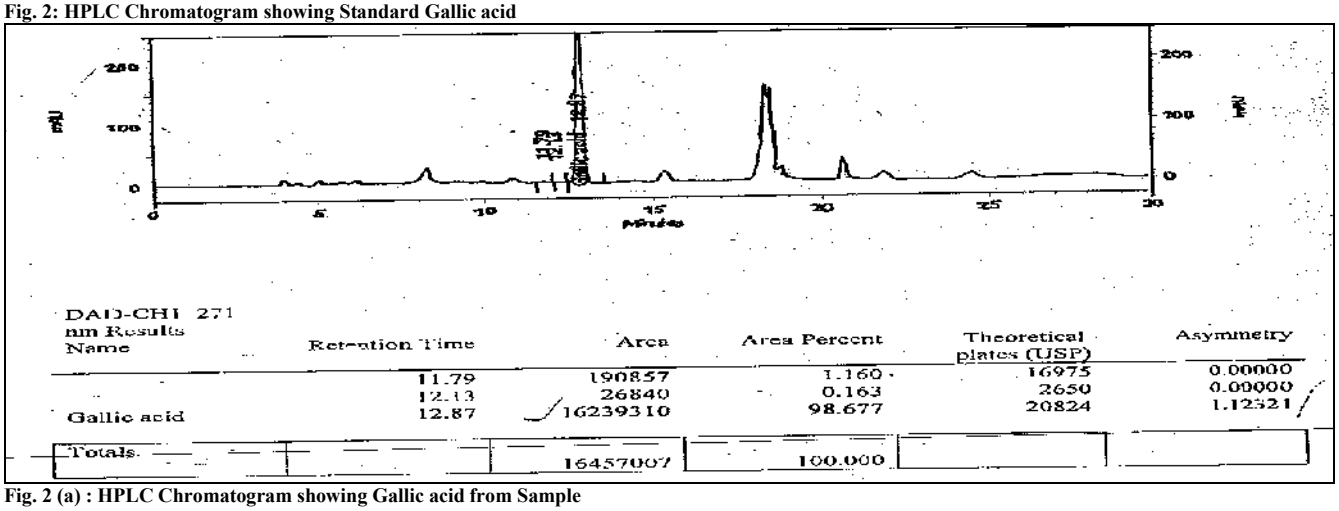

Fig. 2 (a) : HPLC Chromatogram showing Gallic acid from Sample

Regression analysis for the calibration data for Gallic acid showed that the dependent variable (peak area) and independent variable (concentration) were represented by the equation Y=117489370X + 250013.14 for Gallic acid (Table 1).

The correlation coefficient obtained was 0.993 for Gallic acid. That means the good linear relationship between the concentration range 0.04 mg to 0.16 mg.

A system suitability experiment was performed before determination of Gallic acid in samples. The percent coefficient of variation for peak area, retention time, theoretical plate and asymmetry factor value for Gallic acid was less than 2% for five replicate measurements of the same sample having 0.1mg/cm^3 concentration. This showed that the method and the system are suitable for determination of Gallic acid. Results are given in Table 2.

Table 2: Mean results of system suitability for Gallic acid

S. No.	Parameters	Mean Observations	C.O.V%
1	Peak Area For Gallic acid	12162969.4	0.6896
2	Retention Time For Gallic acid	13.05	0.5264
3	Theoretical Plate For Gallic acid	12268	1.06820
4	Asymmetry Factor For Gallic acid	1.33	0.00

The results observed for the robustness are summarized in table 3, 4, and 5 respectively for Gallic acid which shows that method is not affected by deliberate change in the wavelength, mobile phase composition and temperature.

Table 3: Robustness of the method- effect of wavelength (Gallic acid)

Wavelength	Retention Time	Theoretical Plate	Asymmetry factor
271	13.05	12278	1.66
269	13.35	11406.24	0.00
273	13.31	11337.9904	1.66

Table 4: Robustness of the method- effect of mobile phase composition (Gallic acid)

Mobile Phase Composition	Retention Time	Theoretical Plate	Asymmetry factor
40: 60	13.07	12295	1.2
37: 63	14.67	7032.0112	1.33
43: 57	13.9	8593.2536	0.84
46: 54	12.17	2928	0.29

Table 5: Robustness of the method- effect of temperature (Gallic acid)

Temperature	Retention Time	Theoretical Plates	Asymmetry Factor
Original temperature 25°C	12.49	17215.5	1.2
30°C	11.998	15806.8	1.21396

The result shows that within the concentration ranges mentioned above there was an excellent correlation between peak area and concentration of the Gallic Acid. The results are given in Table 6.

Table 6: Asymmetry factor and peak tailing factor calculation

Components	Asymmetry Factor	Peak Tailing Factor
Gallic acid	1.2	1.2

The low values of percent coefficient of variation for retention time, theoretical plate and asymmetry factor shows that solution is suitable for quality control analysis of Gallic acid in *Dendrophthoe falcate* Linn. Results are given in the Table 7 and 8.

Table 7: Results of solution stability- effect of pH of mobile phase (0.1% OPA: ACN 400:600) pH 2.44

S. No	Retention Time	Peak Area	HETP	Asymmetry
1	12.76	13260476	21087	1.1139
2	12.71	13230312	21008	1.1127
3	12.91	13137755	21547	1.1099
4	13.05	12791321	21680	1.0847
5	13.17	12894323	21835	1.1066
Mean	12.92	13062837.4	21431.4	1.1056
SD	0.1727	209069.3665	366.0386	0.0119
C.O.V %	1.3367	1.6005	1.7079	1.0763

Table 8: **Results of solution stability- effect of pH of mobile phase (0.05M KH_2PO_4: Methanol 400:600) pH 3.5**

S. No	Retention Time	Peak Area	HETP	Asymmetry
1	13.26	15364616	21358	1.29
2	13.37	15070079	21480	1.27
Mean	13.315	15217347.5	21419	1.28
SD	0.0778	30287786.32	86.2670	0.0141
C.O.V %	0.5843	199.0346	0.4027	1.1015

The low values of percent coefficient of variation and standard deviation from assay indicates method is highly precise. Results obtained are shown in Table 9.

Table 9: Results of assay experiment from plant

S. No.	Weight of Sample taken in gm	Peak Area of sample	Amount of Gallic acid found in mg
1	3.00	14656980	11.89
2	3.00	14639431	11.87
		Mean	11.88
		SD	0.0141
		C.O.V%	0.1186

The recovery of Gallic acid was found to be 98.94% which indicates high accuracy of the method. The results of recovery analysis are given in Table 10.

Table 10: Results of recovery experiment from plant

Amount of Standard Added in mg	Total Amount of Gallic acid Found in mg (Sample + Standard Added) Added in mg			Mean	Expected Amount in mg
	1	2	3		
0.00	11.89	11.87	11.89	11.88	
12.5	13.1	13.1	13.1	13.1	13.1
13.1	13.7	13.8	13.8	13.8	13.7
16.2	16.7	16.8	16.8	16.8	16.8

From this the recovery was found to be 98.94 %.

Low values of statistical parameters namely standard deviation and percent coefficient of variation for recovery indicates high reliability of the method, these values are given in Table 11.

Table 11: Statistical evaluation of recovery experiment for Gallic acid from plant

Level	Total Amount of Gallic acid Found in mg (Sample + Standard Added)	SD	C.O.V%
0	11.88	0.0122	0.1026
1	13.1	0.00	0.00
2	13.8	0.0070	0.0507
3	16.8	0.0070	0.0416

The validated RP-HPLC method was used for determination of Gallic acid from the stem bark of *Dendrophthoe falcate* Linn. The mean assay result, expressed as a percentage in the stem bark is shown in Table 9. The results indicate that the method is highly precise.

As the proposed method is highly accurate, selective and precise hence can be used for a routine quality control analysis of Gallic acid from stem bark of the *Dendrophthoe falcate* Linn. The method is also fast and requires approximately 13 minute for analysis.

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