

Quantitative Estimation of Gallic Acid From Hydroalcoholic Extract of Dried Flowers of *Nymphaea stellata*, Willd by RP-HPLC

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Several polyphenols have been tested in *Nymphaea stellata*, Willd. The flavonoid content is important because of the pharmacological properties of these compounds, whereas quercetin has been proved to be an antioxidant, antiinflammatory and hepatoprotective compound. A reversed-phase HPLC method has been developed and applied to determine quercetin content in hydroalcoholic extract of dried flowers of *Nymphaea stellata* in a single analysis. The gallic acid was analyzed with a HiQ Sil C-18 column by isocratic elution using 0.01 % (v/v) orthophosphoric acid:acetonitrile (95:5 v/v) as the mobile phase. The flow rate was 1.2 mL min⁻¹ and detection was set at 265 nm. The recovery of the method was in the range of 98.50-99.40 % and all the compounds showed good linearity (r = 0.98332) in a relatively wide concentration range.

Key Words: *N. stellata* Willd, Gallic acid, RP-HPLC, Extract.

INTRODUCTION

Nymphaea stellata Willd. (Nymphaeaceae), a medicinal plant has been reported traditionally for the treatment of liver disorders in Ayurveda. The leaves, roots and flowers have a wide range of pharmacological activities and are used for diabetes, eruptive fevers and as cardiogenic, emollient, diuretic, narcotic and aphrodisiac^{1,2}. The plant also has antihepatotoxic³, antidiabetic⁴ and antihyperlipidaemic⁵ activities. The flowers of plant contains⁶ flavanoids, gallic acid, astragalgin, quercetin and kaempferol. Structurally they have phenolic groups which serve as a source of readily available hydrogen atoms such that the subsequent radicals produced can be delocalized over the phenolic structure^{7,8}. The interest in these compounds is due to their pharmacological activity as radical scavengers^{9,10}. They have been proved to have potential preventive and therapeutic effects in many diseases, where the oxidative stress has been implicated, including cardiovascular diseases, cancer, neurodegenerative disorders and in aging¹¹⁻¹⁵. The phenolics are also of interest in food, cosmetic and pharmaceutical industries, as substitutes for synthetic antioxidants.

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These phenolic compounds widely distribute in the plant kingdom¹⁶⁻¹⁸. Several high-performance liquid chromatographic (HPLC) methods and capillary electrophoretic methods have been developed for the determination of these constituents in herbs and Chinese medicinal preparations¹⁹⁻³¹, but none on estimation of quercetin in flowers of *N. stellata* by HPLC method. In present study, HPLC with UV detector was developed for quercetin in hydroalcoholic extract of dried flowers of *N. stellata*. The assay was validated to determine linearity, precision, recovery, selectivity and stability.

EXPERIMENTAL

Quercetin (99.0 % pure) was purchased from SISCO Research Laboratories, India. Other reagents were analytical grade and purchased from Loba chemie Pvt. Ltd., Mumbai, India. The deionized water was prepared from Millipore water purification system (Bharti Vidhypeeth, Pune, India) and was filtered with 0.45 μm membranes.

The herbarium of *Nymphaea stellata* Willd. was identified and authenticated from Satara Ayurvedic Arkshala, Satara having voucher No. 648/A dated 30-06-2008 and from Botanist, Prof. B.D. Patil, Department of Botany, Sant Gadge Maharaj College of Science, Tal. Karad, Dist. Satara, India, having voucher No. SGM/BDP/03/2008. About 300 g of the powder was taken in a Soxhlet extractor, defatted with petroleum ether and then extracted with hydroalcohol (70 %). The solvent was recovered by glass distillation. The residue was concentrated, dried and stored in the dessicator for subsequent use of experiments.

An isocratic HPLC PU 2080 Plus (JASCO) with UV-2075 Plus detector and RP-C₁₈ column was used. A rheodyne injector with a 20 μL loop was used for the injection of sample. The HPLC system was equipped with borwin software for data processing. Separations were carried out with a HiQ Sil C₁₈ reversed-phase column (250 mm \times 4.6 mm) (KYA TECH Corporation, Japan). The mobile phase was isocratic of aqueous 0.01 % (v/v) orthophosphoric acid-acetonitrile (95:5 % v/v). The flow rate was 1 mL min⁻¹. The detection was set at UV 265 nm and absorption spectra of compounds were recorded between 200 and 400 nm. The column temperature was 25 °C and the sample injection volume was 20 μL . The gallic acid was identified from hydroalcoholic extract by comparing retention time value with those of the standard. All solvents were of HPLC-grade and were filtered and degassed before their use.

Calibration curve: Marker compound, gallic acid was accurately weighed and dissolved in 50 % methanol to give serial concentrations within the range of 0.5-50 $\mu\text{g mL}^{-1}$. The calibration curve was obtained from peak areas of the standard solutions over the concentrations. Concentration of gallic acid in sample was calculated from the regression analysis.

Sample preparation: The hydroalcoholic extract was dissolved with 50 % methanol into a volumetric flask. The final volume of the extracting solution was

set to 50 mL. For determination of gallic acid, the solutions were filtered through a membrane (0.45 µm) and then injected into HPLC directly.

Recovery study: An appropriate amount of extract was taken as control and spiked with 50, 100 and 150 % of standard gallic acid with that of sample extract. Samples were filtered through a 0.45 µm membrane filter and assayed by HPLC to calculate recoveries.

RESULTS AND DISCUSSION

Optimization of separation conditions: Absorption maxima of gallic acid was observed to be in the range of 200-400 nm on the UV spectra with dimensional chromatograms and a monitoring wavelength for quantitative determination at 265 nm was altered to obtain the baseline separation of marker compound. Isocratic elution was carried out to successfully separate the compounds in hydroalcoholic extract of *N. Stellata* using 0.01 % (v/v) orthophosphoric acid:acetonitrile (95:5, v/v) as mobile phase in less than 10 min. There were no interfered peaks within the time frame in which gallic acid in the extract was detected.

Regression equation: Linear regression analysis for gallic acid was performed by the external standard method. The calculated results were given in Table-1, where a, b and r were the coefficients of the regression equation $Y = ax + b$, x referred to the concentration of the marker compound ($\mu\text{g mL}^{-1}$), Y the peak area and r the correlation coefficient of the equation. The marker substance showed good linearity ($r = 0.98332$) in a relatively wide concentration range. The limits of detection (LOD) for gallic acid were 18.35 ng mL^{-1} , detected at 265 nm.

TABLE-1
LINEAR RELATION BETWEEN PEAK AREA AND CONCENTRATION (n = 6)

Marker compounds	Regression equation	R	Linear range ($\mu\text{g mL}^{-1}$)	LOD (ng mL^{-1})
Gallic acid	$Y = 649079.7597x + 39615.9610$	0.98332	0.5-2.5	18.35

Y = peak area, x = concentration ($\mu\text{g mL}^{-1}$). Triplicate assay about the different concentration (n = 6).

Precision and accuracy: The within-day and the day-to-day accuracy data for marker substance are listed in Table-2, relative standard deviations (RSD) of the within-day and day-to-day were 0.029-0.9 % and 0.0085-0.21 %, respectively.

TABLE-2
WITHIN-DAY AND DAY-TO-DAY RELATIVE STANDARD DEVIATION (RSD) FOR THE RP-HPLC METHOD FOR THE DETERMINATION OF GALLIC ACID

Conc. ($\mu\text{g mL}^{-1}$)	Observed concentration ($\mu\text{g mL}^{-1}$) \pm SD		RSD (%)		Accuracy (%)	
	Within-day ^a	Day-to-day ^b	Within-day ^a	Day-to-day ^b	Within-day ^a	Day-to-day ^b
2.5	2.46 \pm 0.900	2.51 \pm 0.210	0.900	0.2100	98.40	100.40
2.0	1.99 \pm 0.029	1.98 \pm 0.029	0.029	0.0290	99.50	99.50
1.5	1.52 \pm 1.250	1.49 \pm 0.085	0.120	0.0085	101.33	99.33

^aWithin-day precision test at six times in 1 day; ^bDay- to- day precision on four different days.

Recovery: The average recovery of standard spiked into extract was 98.78 % for gallic acid (average of three), as shown in Table-3.

TABLE-3
RECOVERY OF GALLIC ACID (n = 3)

Initial amount ($\mu\text{g mL}^{-1}$)	Added amount ($\mu\text{g mL}^{-1}$)	Detected amount ($\mu\text{g mL}^{-1}$)	Recovery (%)	RSD (%)
1	0.5	1.48	98.66	0.0081
1	1.0	1.97	98.50	0.0410
1	1.5	2.48	99.20	0.0080

Determination of gallic acid in hydroalcoholic extract: Figs. 1-3 shows the chromatogram obtained from RP-HPLC separation of standard gallic acid, typical chromatogram of hydroalcoholic extract and comparative chromatogram of gallic acid with hydroalcoholic extract, respectively. The retention time of the standard gallic acid was 6.5 min. The content of gallic acid was calculated from the corresponding calibration curve. Gallic acid content in hydroalcoholic extract of *Nymphaea stellata*, Willd was found to be 15.43 mg/g.

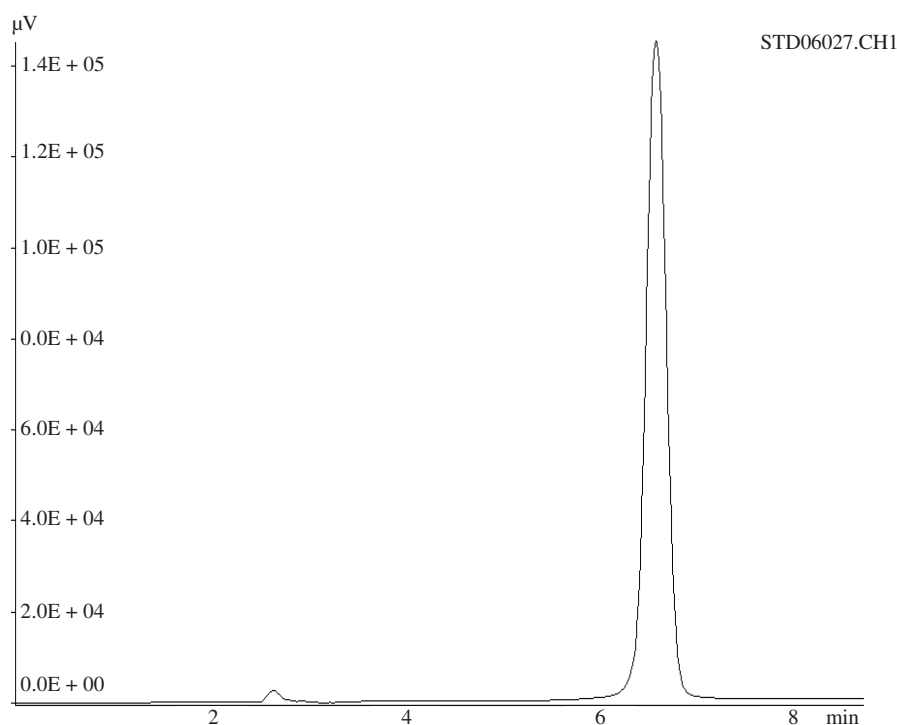


Fig. 1. Typical chromatogram of gallic acid, $3 \mu\text{g mL}^{-1}$

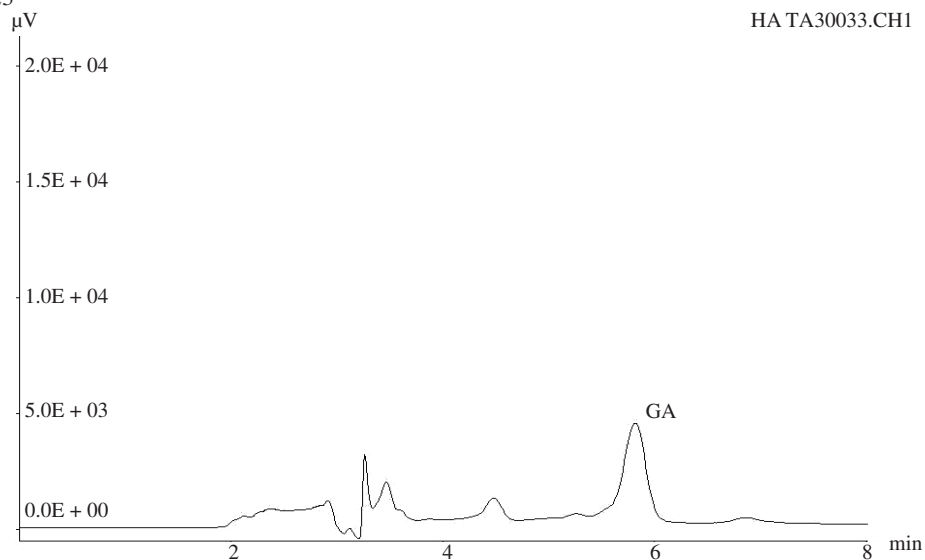


Fig. 2. Typical chromatogram of hydroalcoholic extract of *Nymphaea stellata*, Willd, $100 \mu\text{g mL}^{-1}$. Conditions: mobile phase, 0.01 % orthophosphoric acid:acetonitrile; flow rate, 1 mL min^{-1} ; detection wavelength, 265 nm; column temperature, $25 \text{ }^\circ\text{C}$ injection volume, $20 \mu\text{L}$

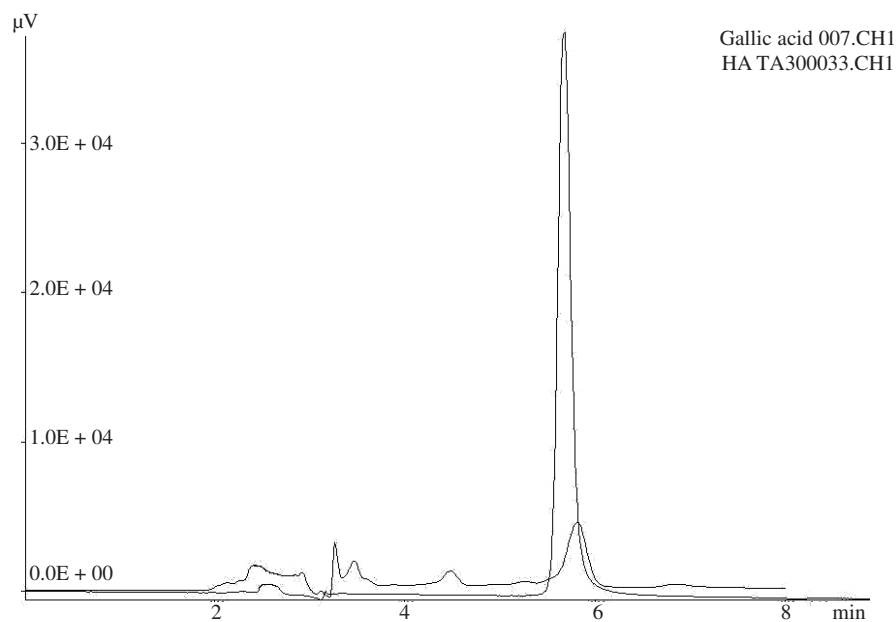


Fig. 3. Comparative chromatogram of gallic acid and hydroalcoholic extract of *Nymphaea stellata*

Conclusion

This was the first report of identification and quantification of the gallic acid in hydroalcoholic extract of dried flowers of *Nymphaea stellata*, Willd with a short analysis time. A simple, rapid and accurate assay approach was presented. The experimental results indicated that hydroalcoholic extract of dried flowers of *N. stellata* contained high concentration of gallic acid. Since the phenolic compounds have been of interest of health benefits, the present analytical study could be a potential application to identify and quantify the phenolic compound in other flower extracts.

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