MODIFICATION AND VALIDITATION OF HPLC METHOD FOR ISOLATION OF ELLAGIC ACID FROM PUNICA GRANTUM

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ABSTRACT

A simple isolation method and rapid, specific high-performance liquid chromatographic method was developed and validated for Ellagic acid from Punica grantum extract. HPLC analysis was performed on C_{18} column using a 90:10 (v/v) mixtures of acetonitrile and methanol as isocratic mobile phase at flow rate 1.0 ml/min. UV detection was at 254 nm for filixic acid PBP. Filixic acid shown retention time at 4.02 min. Method was validated for accuracy, precision, linearity, specificity and sensitivity in accordance with International Conference on Harmonisation guidelines. Validation studies revealed that the method is specific, accurate, precise, reliable and reproducible. Good linear correlation coefficient (r2 > 0.993) was obtained for calibration plots in the range tested. The limit of detection was 2.25 µg/ml and limit of quantification was 7.53 µg/ml for ellagic acid. Intra and inter-day relative standard deviation of precision was less than 1.50 %. Recovery was between 94.57 and 101.05 % ellagic acid. The method can be successfully used for quantitative analysis of ellagic acid in Punica grantum for day-to-day studies.

Keyword : - *Punicagrantum, ellagic acid, HPLC, isolation.*

1. INTRODUCTION

Plants have created in interest among the people by its clinically proven effects. Also the over use of synthetic drugs, which results in higher incidence of adverse drug reactions, have motivated the humans to go back to nature for safer remedies. Medicinal plants as potential source of therapeutic aids has attained a significant role in health system all over the world for both humans and animals not only in the diseased condition but also as potential material for maintaining proper health (nutraceuticals).

Among them Ellagic acid (EA) is a phenolic compound that exhibits both antimutagenic and anticarcinogenic activity in a wide range of assays *invitro* and *in vivo* (1, 2). It can act as an antioxidant, and has been found to cause apoptosis (cell death) in cancer cells (3). Ellagic acid has also been said toreduce heart disease, birth defects, liver problems, and to promote wound healing (4). There are alsoreports that it may help the liver to break down or remove some cancer-causing substances from theblood (5). Several studies have found that ellagic acid can inhibit the growth of skin, esophagus, lung, and other tumors caused by carcinogens (6,7). Recently Italian researchers found that ellagic acid seemed to reduce the side effects of chemotherapy in men with advanced prostate cancer, although it did not help to slow disease progression or improve survival (8). However, further studies would be needed to confirm these results and to determine if other results apply to humans.

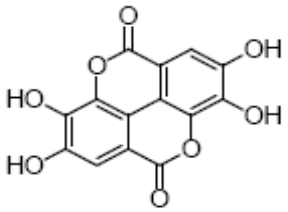

Fig- 1: Structure of Ellagic Acid

Ellagic acid has been found to have antioxidant, anticarcinogenic, antifibrosis, antiplasmodial activity and chemo preventive activity (3, 6-8).

In the first method ellagic acid was precipitated by adding water to concentrated methanolic extract. Precipitate obtained was further purified and characterized.

In the other method rind powder was extracted with water and water extract was subjected to acid hydrolysis, which precipitated ellagic acid. Product obtained by precipitation of methanolic extract was better in appearance than hydrolysis method.Ellagic acid showed positive ferric chloride test and Reichel test. Melting point was above 300^0C. UV spectroscopy study showed a characteristic spectrum of phenolic compounds with two maxima at 254 and 363 nm. Broad band at 3150 cm^{-1} due to O-H stretching and a sharp band at 1724 cm^{-1} due to C=O stretching in IR shows the presence of hydroxyl and carbonyl of lactone group respectively. Molecular ion peak at 303 m/e gives the molecular weight of ellagic acid. HPLC chromatogram of standard ellagic acid and isolated ellagic acid were also matching.

In present study attempt was made to isolate the ellagic acid from *Punicagranatum*. The specific, reliable and reproducible HPLC method was developed and validated according to International Conference on Harmonization (ICH) guidelines10.

2 EXPERIMENTAL

2.1 Plant Material and Chemical

Air dried rhizomes and fond bases of *D. filx-mas* were collected in January (Three samples) from Mumbai, authenticated and voucher specimen was deposited in Institute of Chemical Technology, Mumbai. Analytical grade acetonitrile, methanol and chloroform were purchased from Merck, India for HPLC study. All chemicals were of analytical grade purchased from Sigma-Aldrich. All solvents for HPLC study were filtered through 0.45 μm pore size filter (Milipore Bedford).

2.2 Isolation of Ellagic Acid

Dry fruit powder of pomegranate was purchased from the local market in Mumbai. The extraction of ellagic acid was carried out in two steps.In the first set of experiments, dry fruit powder of pomegranatewas first refluxed with hexane and chloroform for 1 hr. This leads to removal of non-polar impurities. Followed by extraction,vacuum filtration was carried out extraction was performed. After separation, the obtained residue was subjected to vacuum drying. The obtained residue was used for further studies.In the second set of experiments, the residue which was obtained in the first method was again extracted methanol for 3 hrs. The solvent was removed and the process was repeated for one more time to remove the final traces of ellagic acidfrom ground powder of pomegranate.The extracts were then combined and concentrated on rotary evaporator under reduced pressure. Subsequently, the water was added to the concentrated methanol extract. This leads to the precipitation of ellagic acid, which was separated by filtration. This residue was again washed by water in order to remove water soluble impurities. The obtained compound was subjected for characterization study for confirmation of ellagic acid.

For TLC, Toluene: ethyl acetate (9:1) was used as a solvent system and vanillin-sulphuric acid as a derivatising agent. Isolated compound was confirmed as filixic acid PBP (1.3 g) by melting point, UV, IR, NMR and Mass spectroscopic studies. The NMR spectral analysis was done in CDCl3, using TMS as internal reference.

2.2.1 Determination of Ellagic Acid using HPLC

The Agilent (Germany) HPLC system, consisting of a model G1329A standard auto-sampler, model G1316A thermostat column, model G1322 A vacuum degasser, quaternary pump, model G1314B variable wavelength detector, was used.The separation was achieved on a stainless steel silica based Zorbax Eclipse XDB–C18 column (ϕ4.6 mm×150 mm, 5 µm). The column temperature was maintained at 30ºC. Ellagic Acid was eluted using mobile phase consisting of methanol and 0.1% H_3PO_4v/v (70:30) at the flow rate of 1 ml/min. The eluent was monitored at 223 nm. The standard curve was obtained by analyzing known concentration of Ellagic Acid. The standard curve was plotted between the concentration of Ellagic Acid and the area under the curve. This plot was used for the determination of concentration of the Ellagic Acid in the unknown solution. All the samples were prepared in the methanol of 10 mg/l concentration and filtered through 0.22 µm filter remove any suspended particles. The amount of sample injected in the column was kept constant at 10 µl. All the solvents used in the HPLC analysis were first filtered through 0.22 µm filter and then sonicated for 10 min to remove any dissolved gases.

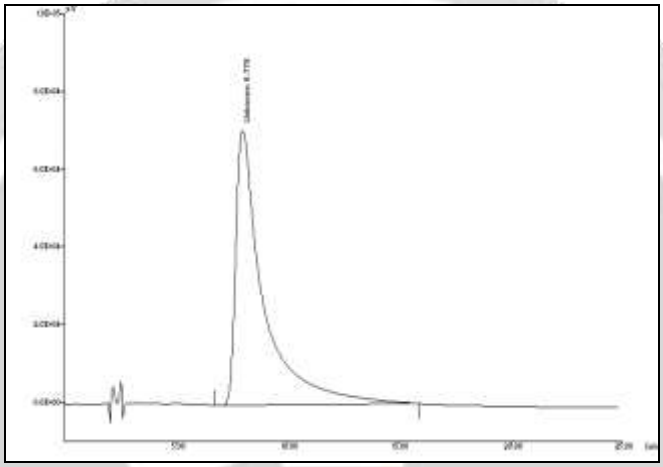

Fig-2: Chromatogram of Standard Ellagic Acid

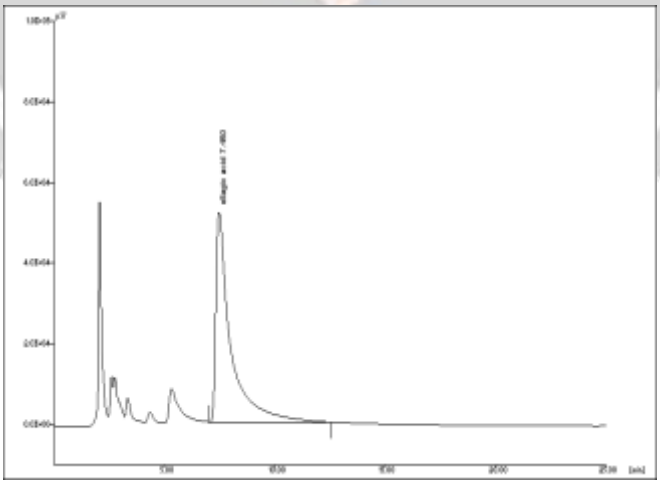

Fig-3 : Chromatogram of Alcoholic Extract

2.2.2 Chromatography

HPLC analysis was performed with a JASCO (Hachioji, Tokyo, Japan) system consisting of an intelligent pump (PU-1580, PU-2080), a high-pressure mixer (MX-2080-31), a manual sample injection valve (Rheodyne 7725i)

equipped with a 20 µL loop, and a UV–visible detector (UV- 1575). RP-18 endcapped column (250 mm × 4.6 mm i.d., 5 µm particle, HibarLichrocart Purospher Star, Merck, Darmstadt, Germany).

2.3 Validation of chromatographic method

HPLC method development and validation studies carried out using Acetonitrile: Methanol (90:10) as isocratic mobile phase at flow rate of 1 ml/min, at UV at 254 nm. The analysis was performed at ambient temperature and data were analyzed on a computer equipped with Borwinsoftware.5 g drug sample was extracted with 100 ml methanol by Soxhlet apparatus for 24 h. Extract was filtered and transferred to 100 ml volumetric flask and volume was made with methanol (Sample stock solution). Standard stock solution (1 mg/ml) of filixic acid PBP was freshly prepared in methanol. Calibration curve was prepared by using standard solutions of different concentrations (10, 20, 30, 40 and 50 µg/ml). In validation study calibration plots were constructed for standard filixic acid, after triplicate analysis of each solution.

LOD and LOQ were experimentally verified by diluting known concentration of filixic acid until the average response were approximately three to ten times the standard deviation of Response for six replicate determinations. Precision was determined as the intra-day and inter-day variation of results from analysis of three different standard solutions. Intra-day precision was determined by triplicate analysis of each solution on a single day. Inter-day precision was determined by triplicate analysis of the solutions on two successive days.

The accuracy of the method was determined by application of the standard addition method. Known amounts of the standard (1ml of 10, 50, and 100 µg/ml standard solutions) were added to the 1 ml of extract (sample stock solution) and analyzed in triplicate as described above. The total amount of filixic acid was calculated from the corresponding calibration plot and the recovery of filixic acid was calculated by using following equation:
Recovery (%) = (amount found - amount contained) / amount added x 100

3. RESULT AND DISCUSSION

3.1 Characterization of Isolated Compound

Brown powder UV λ max 253, 364 nm (methanol); ESI-MS (negative mode) *m/z* 301.1 [M - H]; 1H NMR (CDCl3) as tetra acetate δ 7.87 (2H, s, aromatic); δ 2.35 (6H, s, CH3CO2); δ 2.30 (6H, s, CH3CO2).

3.2 Validation of chromatographic method

In HPLC study filixic acid PBP was resolved with retention time (Rt) at 4.03. Reproducible results were obtained for each of three samples. Validation of HPLC method was done for filixic acid PBP. Chromatogram obtained from standard solution shown good resolution.

3.2.1 Linearity

The linearity of the calibration curve for filixic acid PBP (10, 20, 30, 40 and 50 µg/ml Concentration) was good (r = 0.993) over the concentration range investigated. Calibration curvewere prepared by plotting peak area *vs* concentration of filixic acid PBP. 20 µl standard solutionswere injected and respective peak areas were recorded by following regression equation.

$$Y = 6871X + 28428.$$

Where, Y is peak area and X is concentration.

3.2.2 Sensitivity

LOD and LOQ were 2.25µg /mland 7.52 µg/ml respectively, which were calculated by using the following formulae.LOD =3.3 σ/ S and LOQ = 10 σ/ S.
Where σ is the standard deviation of the response and S is the slope of the calibration plot.

3.2.3 Precision

Analysis of filixic acid PBP was performed at different solution of 10, 20 and 30 µg/mlconcentrations. No significant intra-day and inter-day variation was observed. Relative standard eviation (RSD) was always less than 1.5 % (Table I).

Table-1: Method of validation parameters for the quantification of Ellagic Acid by the proposed method

Parameters	Results
Linear range (µg/ml)	5.0-75 µg/ml
Regression equation	y = 4.913x
Correlation coefficient (r^2)	0.9999
LOQ (µg/ml)	0.02
LOD (µg/ml)	0.05

Where,
x is the concentration of stevioside in µg/ml
y is the peak area at 219 nm

3.2.4 Recovery

For all drug samples after spiking with standard solution of 10, 50 and 100 µg/ml concentration, the method shown total recovery of filixic acid PBP, between 94.57 to 101.05 % (Table II).

Table-2: Precision and Stability of Ellagic Acid

Analyte		**Precision (RSD, %)**				**Stability**
		Intra-day (n=3)		**Inter-day (n=3)**		**RSD of P_a (%)**
		R_t	**P_a**	**R_t**	**P_a**	
	25µg/ml	2.6	0.33	2.64	0.35	0.31
	50 µg/ml	2.67	0.37	2.68	0.38	0.37
Stevioside	75 µg/ml	2.69	0.39	2.71	0.41	0.40

3.3 Sample analysis

Isolated filixic acid PBP shown purity above 98 %. The retention time of filixic acid PBP was4.07 min (Figure 1). All samples of *D. filix-mas* were analyzed under the same chromatographiccondition. The analysis, including sample preparation, was performed in triplicate (Table III).

Table- 3: Recovery of Ellagic Acid

Analyte	**Contained (µg/ml)**	**Added (µg/ml)**	**Found (µg/ml)**	**Recovery (%)**	**Mean (%)**	**RSD (%)**
	78.7	100	164.87	95.76		
	78.7	100	167.69	95.81		
Stevioside	78.7	100	165.58	95.49		
	78.7	50	125.62	97.29		
	78.7	50	124.76	96.81	97.30%	2.25

	78.7	50	125.12	97.89		
	78.7	10	88.67	98.56		
	78.7	10	88.52	99.11		
	78.7	10	88.59	98.92		

4. CONCLUSIONS

In conclusion, appreciable amount of ellagic acid from Punica granatum has been isolated by above method and its structure was confirmed by UV, IR, NMR and Mass spectroscopic studies. A simple, rapid, reproducible, and sensitive HPLC method has been developed for analysis of filixic acid PBP first time. The method was validated for linearity, LOD, LOQ, precision, inter-day and intra-day variation, and recovery study. Method is suitable for processing of many samples in limited time for day to day analysis, pharmacokinetic and bioequivalence studies.

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