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Development and validation of novel HPLC method for the estimation of Rutin in crude hydromethanolic leaf extract of *Prosopis cineraria*

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ABSTRACT

A simple, specific, accurate and precise high performance liquid chromatography method has developed for the estimation of rutin in *Prosopis cineraria*. The chromatographic separation was achieved by using C_{18} column, 150 x 4.6mm i.d., 5µ bonded phase octadecylsilane (Thermo Labs Hypersil). Mobile phase was composed of 80 parts of methanol & 20 parts of 0.05% formic acid. The pH of the mobile phase was 3.2.The retention time of rutin was found 5.7 min with 1 mL/min flow rate at ambient temperature. The estimation was performed on PDA detector at 281 nm. In this study, an excellent linearity was obtained with r^2 0.999. Besides, the chromatographic peak was found sharp & symmetric. The proposed method was validated in terms of the analytical parameters such as accuracy, linearity, precision, robustness, limit of detection (LOD), limit of quantification (LOQ) were determined based on the International Conference on Harmonization (ICH) guidelines. The detector response was linear in the range of 2-10 µg/mL. The proposed method was successfully applied for the estimation of the constituents in crude extract of *Prosopis cineraria*. This study established a quantitative method for the determination of rutin from *Prosopis cineraria*.

Keywords: *Prosopis cineraria*, HPLC, Validation, Rutin.

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1. INTRODUCTION

The plant *Prosopis cineraria* (Khejri) is a flowering tree in the leguminous family *Fabaceae.* It is a small to medium size thorny, irregularly branched flowering tree, found widely in the Thar desert of Rajasthan, India and plays a vital role in preserving the ecosystem[1].

It is one of the most important natural resources of arid regions of India because of its economic values (fuel, fodder), ecological role in preventing soil erosion. *Prosopis cineraria* have also been used in indigenous system of medicine as a folk medicine for various ailments. The bark is dry, acrid, bitter, with sharp taste; cooling anthelmintic, tonic; cures leprosy dysentery, asthma, leucoderma, piles, tremors of the muscle, wandering of the mind. The flowers are grounded mixed with sugar and used during pregnancy as safeguard against miscarriage. The ashes of bark are rubbed over the skin to remove hair. The smoke of the leaves is good for eye troubles. Fresh Leaves juice mixed with lemon juice is used for dyspepsia; extract of crushed pods is used for earache, toothache, pain relief from fractured bones. Aqueous extract of bark and leaves applied externally to treat skin disease disinfects wounds and promotes healing[2-3].

Prosopis cineraria is mostly used as a folk medicine due to the presence numerous phytoconstituents like alkaloids (Spicigerine, Prosophylline), steroids (Campesterol, stigmasterol, sitosterol), tannins, phenolic compound (Gallic acid), flavone derivatives (Prosogerin A, B, C, D, and E), etc has been isolated. In this respect, polyphenolic compounds, like flavonoids and phenolic acids, commonly found in plants have been reported to have multiple biological effects, including antioxidant and anti inflammatory activity. Synthetic antioxidants have toxicity, Thus interest in natural antioxidant, especially of plant origin, has greatly increased in recent years[3].

Rutin (rutoside, quercetin-3-O-rutinoside) is 2-(3,4-dihydroxyphenyl)-5,7-dihydroxy-3-[_-L-rhamnopyranosyl-(1_6)-_-D-glucopyranosyloxy]-4H-chromen-4-one and is classified as a polyphenolic flavanoid (Fig. 1). It occurs in many food products of plant origin. Rutin appears as an odorless yellow crystalline powder that is practically insoluble in H_2O and poorly soluble in alcohol[4-7]. Living

organisms are incapable of synthesizing rutin. Therefore, it can only be ingested with plant products. Rutin and its aglycones were observed in various fruits, vegetables, tea leaves, coffee grains, etc[8- 9].

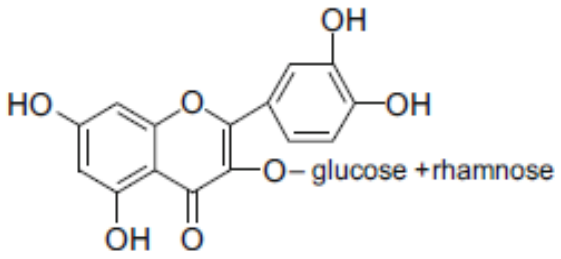

Figure 1: Structure of rutin

Identification of major and unique compounds in herbs as markers and development of analytical methodologies for monitoring them are the key steps involved in marker-based standardization[10]. High performance layer chromatography (HPLC) has recently emerged as a preferred analytical tool for fingerprints and quantification of marker compounds in herbal drugs because of its simplicity, sensitivity, accuracy, suitability for high throughput screening. HPLC method is a suitable method for estimation of chemical constituents present in plant materials[11-14]. The proposed method is optimized and validated as per the International Conference on Harmonization (ICH) guidelines[15].

The literature survey showed that there was no report about the estimation of rutin constituent in leaf extract of *Prosopis cineraria*. Quantitative estimation of this compound is important for current research and a variety of methods are required for this and in the present study the quantification of rutin was done by HPLC method. A sensitive, accurate and specific HPLC method was developed and validated for the estimation of rutin in the hydro alcoholic and methanolic leaf extract of *Prosopis cineraria*.

2. MATERIALS AND METHODS

2.1 Materials and reagents

Rutin (98%) was purchased from Sigma-Aldrich, Bangalore, India. Methanol and acetonitrile were of HPLC grade from Qualigens fine chemicals, Mumbai, India. All the reagents and chemicals used were of analytical and HPLC grade. Water (HPLC grade) was obtained from Milli Q RO system.

2.2 Plant Material

Fresh leaves of *Prosopis cineraria* were collected in month of November from Artiya Khurd village of Jodhpur Rajasthan. A voucher specimen (JJ No. 847889) was submitted in the herbarium of Botanical Survey of India Jodhpur for authentication. BSI issued a certificate of authentication with ref no. BSI/AZRC/1.12014/Tech/ 2016-17-(Pl. Id)/1062. Leaves were collected in bulk, washed, shade dried & coarsely powdered. Defated with petroleum ether & macerated 70 % methanol, the filtrated extract was then concentrated by using rotary evaporator and stored at 4^0C prior to use.

2.3 *Preparation of standard solution*

The standard stock solution of 1000 µg/mL rutin was prepared by 70% methanol. This stock solution was stored in light resistant containers. The dilute standard solutions of concentration 2-10 µg/mL of rutin were prepared from above stock solution and used for calibration curve of rutin.

2.4 *Preparation of sample solution*

About 30 g of the powdered sample was weighed and defatted with the selected solvents by Soxhlet apparatus for 72 h. The defatted residue was dried & macerated with 70 % methanol; the filtrate was dried at 50^0C under reduced pressure in a rotary evaporator (Heidolph instrument GmbH & Co.kg, Germany). The sample solution of extract of 1000 µg/mL was prepared by 70% methanol. After filtering through 0.45 µm nylon filter paper, the extract was diluted with mobile phase injected directly.

2.5 *Instrumentation and Chromatographic conditions*

The estimation of rutin was performed on a Shimadzu liquid chromatographic system equipped with LC-10AT *VP* solvent delivery system (pumps), SPD M-10A *VP* photodiode array detector and Rheodyne 7725i injector with 20 µl loop volume, LC Solution for data collection and processing (Shimadzu technologies, Japan). The mobile phase, MeOH: 0.05% Formic acid pH 3.2 (80:20 v/v) was pumped with a flow rate of 1 mL/min. The elution was monitored at 281 nm. Peak identity was confirmed by retention time and spectrum comparison. All the analysis was performed at ambient temperature.

3. RESULTS AND DISCUSSION

3.1 Method development and validation

Upon application of the developed method, single specific peak was obtained for rutin (Figure 2). Rutin was identified in *Prosopis cineraria* hydro-methanolic extract. The quantitative analysis revealed that (86.40 + 0.30 µg/mg) rutin in the hydro-methanolic (70% Methanol) extract. For validation of analytical methods, the guidelines of the International Conference on the Harmonization have recommended the accomplishment of linearity, accuracy tests, precision, detection and quantitation limit and robustness of the method.

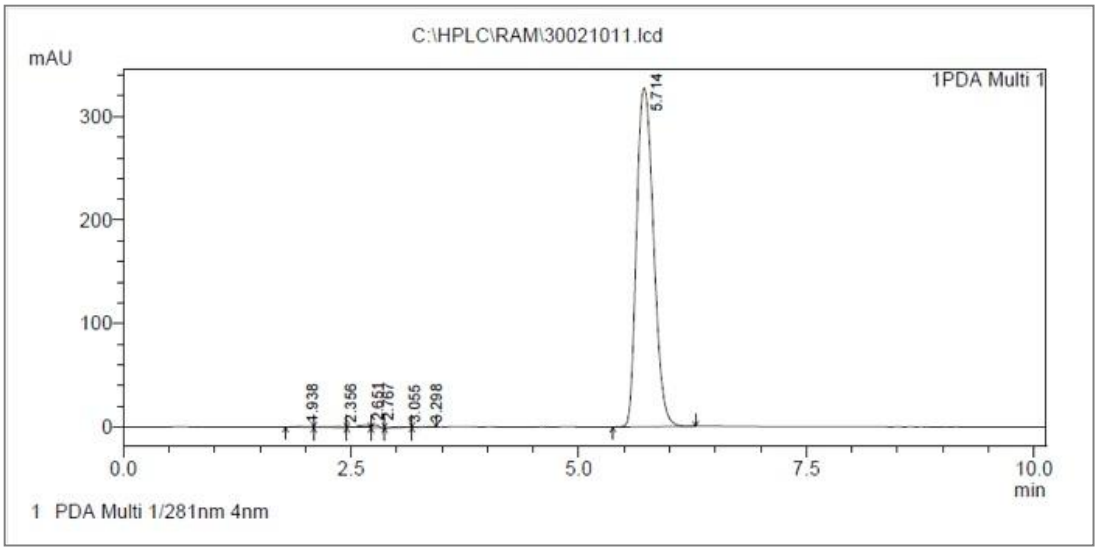

Figure 2: HPLC Chromatogram of standard Rutin

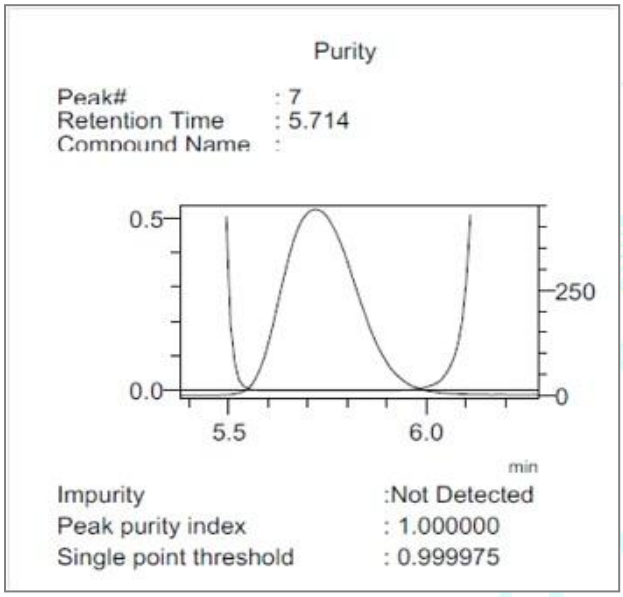

Figure 3: Peak purity of Rutin

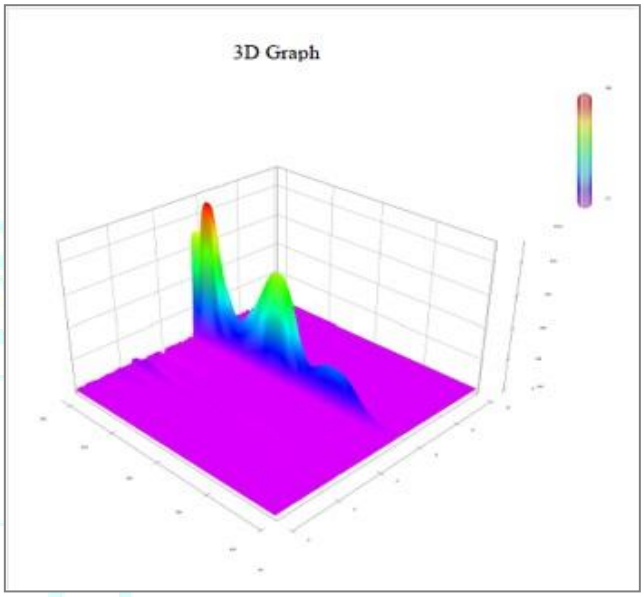

Figure 4: 3D-HPLC Chromatogram of standard Rutin

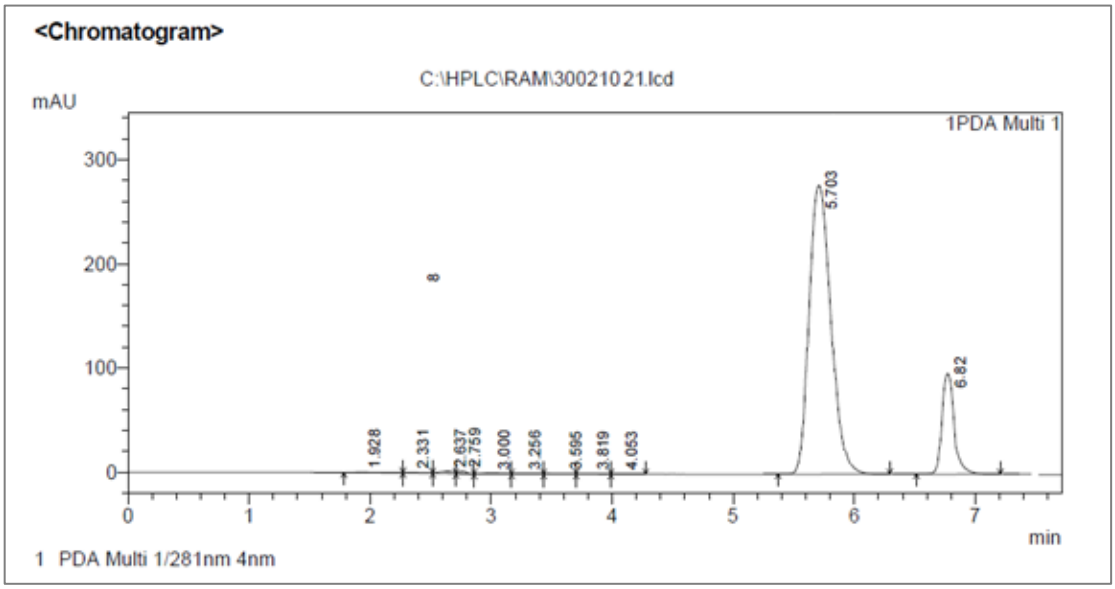

Figure 5: HPLC Chromatogram of crude hydro-methanolic extract of *Prosopis cineraria*

3.2 Linearity and range of the developed method

For linearity study, five solutions in the range of 2-10 µg/mL for rutin were analyzed. Each concentration was made and analyzed in triplicate. The peak areas obtained against each concentration of the analytes were used to build a linear regression equation and to determine value of correlation coefficient (Table 1). Good linearity was observed over the above-mentioned range with linear regression equation Y= 423462x for rutin (x is concentration of analytes in µg/mL and Y is peak area). The value of correlation coefficient was found to be 0.999 for rutin. The results indicate that the method is linear over the concentration range studied.

Table 1: Linearity data of Rutin

Conc. (µg/mL)	Area under curve (AUC) Replicates			Mean	S.D.	R.S.D (%)
	Rep-1	Rep-2	Rep-3			
2	849193	849893	849493	849526.33	351.1884	0.04133
4	1647586	1647086	1647044	1647238.67	301.5316	0.01830
6	2553479	2550690	2553268	2552479.00	1732.050	0.06785
8	3391572	3394572	3392572	3392905.33	1527.525	0.04502
10	4239465	4243588	4243140	4242064.33	2262.206	0.05332

3.3 Accuracy of the developed method

This study was performed by adding known amounts of rutin to the all dilution of linearity (2-10 µg/mL) in three replicates. The recovery range for rutin was found to be 99.67 to 99.81 %. The % relative standard deviation was found 0.0539 % for rutin (Table 2).

Table 2: Recovery and accuracy data

S. No.	Concentration (µg/mL)	Mean AUC	Response ratio
1	2	1695659	846755
2	4	2543560	846927
3	6	3391576	847034
4	8	4239365	847933
5	10	5086451	847086
Mean of RR			847147
S.D of RR			457.2608
% R.S.D of RR			0.053976

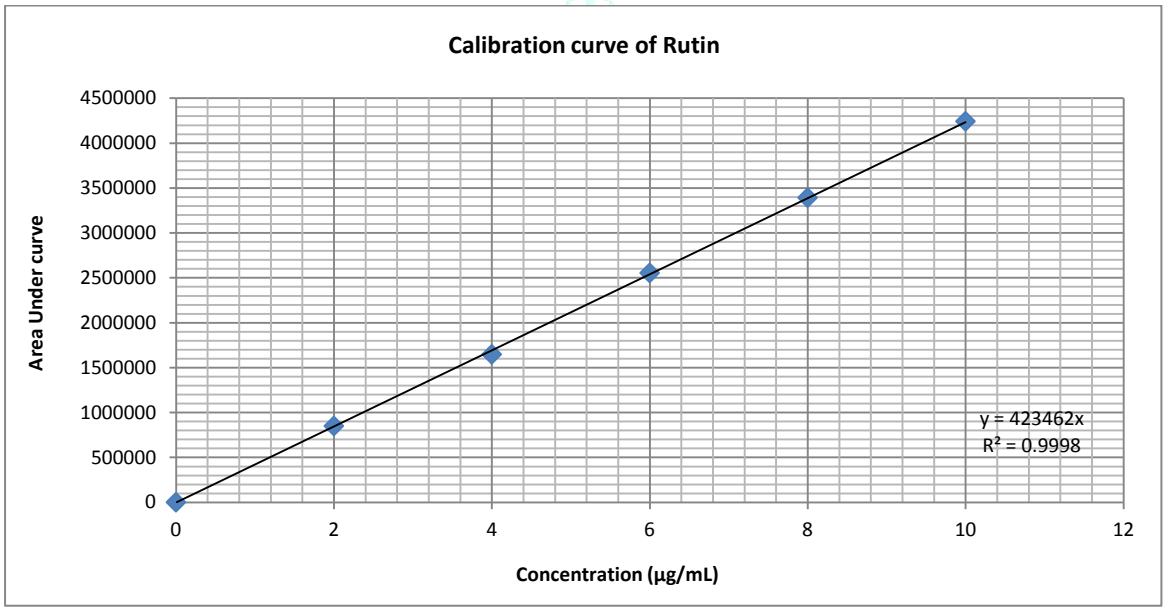

Figure 6: Calibration curve of Rutin

3.4 Precision of the developed method

Repeatability (Intra-day precision) was studied by calculating the relative standard deviation (RSD) for nine replicates of the concentration of 10 µg/mL, performed on the same day and under same experimental conditions. The results of rutin determinations in the working standard solution with the relative standard deviation were calculated (Table 3). Inter-day precision studies include the estimation of variations in analysis when a method is used within laboratories, on different day. The RSD values obtained for intraday & inter-day precision rutin were 0.03527 & 0.026154906 respectively.

Table 3 Precision data

S. No.	Concentration (µg/mL)	AUC (Same day)	AUC (Different day)
1	10	4243588	4240725
2	10	4239365	4240440
3	10	4243140	4241232
4	10	4241572	4241426
5	10	4242064	4241995
6	10	4240228	4242352
7	10	4241934	4243728
8	10	4242782	4242506
9	10	4239884	4243232
Mean of AUC		4241617.444	4241959.556
S.D. of AUC		1494.491143	1109.480521
% R.S.D of RR		0.035233992	0.026154906

3.5 Robustness of the developed method

The robustness of the proposed method was evaluated by deliberately changing the chromatographic conditions such as flow rate, solvent ratio and pH. The results showed that varying the chromatographic conditions had no appreciable effects on the chromatographic parameters (Table 4)

Table 4 Robustness study of the proposed HPLC method

Parameter	Conditions	Retention Time
Flow Rate (mL/min	0.9	6.1
	1	5.71
	1.1	5.25
Mobile Ratio (v/v)	75:25	6.15
	80:20	5.71
	85:15	5.32
pH	3.1	5.9
	3.2	5.71
	3.3	5.35

Table 5: System suitability studies for estimation of rutin by HPLC

S. No.	Parameters	Inference
1	Linearity range	2 µg/mL -10 µg/mL
2	Regression equation	y=42346x
3	Correlation coefficient	0.999
4	Asymmetric factor	1.948
5	Tailing factor	1.277
6	Theoretical plates	7134.155
7	Resolution	6.457
8	LOD (ng/mL)	100 ng/mL
9	LOQ (ng/mL)	ng/mL

3.6 Limit of detection and quantification

LOD & LOQ were calculated by using the following equations.

$$LOD = 3.3X\frac{\text{SD}}{\text{S}} \quad \text{And}$$

$$LOQ = 10X\frac{\text{SD}}{\text{S}}$$

Where SD = the standard deviation of the response,

S = Slope of the calibration curve.

The LOD value was found to be 100 ng/mL and the LOQ value was found to be 300 ng/mL for the rutin.

4. CONCLUSIONS

The proposed analytical method for estimation of rutin in the extracts of *Prosopis cineraria* is accurate, precise, linear, robust, reproducible and within the range. The results show that *Prosopis cineraria* contain considerable amounts of flavonoids which demonstrate that the plant could be considered as a potential source of natural health-promoting antioxidants for medicinal and food applications. This study established a quantitative method for the determination of rutin from *Prosopis cineraria*.

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