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ESTIMATION OF TWO BIOACTIVE COMPOUNDS FROM *AZADIRACTA INDICA* A.JUSS. LEAVES USING HPLC.

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ABSTRACT
Sensitive, simple, and accurate high-performance liquid chromatographic method has been established for determination of rutin and quercetin both simultaneously in *Azadiracta indica* A.Juss. leaf powder. The chromatographic separation was performed on Phenomenex C18 column (250 x 4.6, 5 µm) with a 60:40 *v/v* mixture of methanol and 0.1% O-phosphoric acid in water at the flow rate of 1.2 mL/min and detection at 258 nm, with a run time of 10.0 min. The developed method was then validated using statistical analysis. This will help in qualitative analysis of plant material using fingerprint pattern and quantitative analysis of rutin and quercetin.

KEY WORDS
HPLC, Rutin, Quercetin, *Azadiracta indica* A.Juss.

INTRODUCTION
Herbal medicine has been enjoying renaissance among the customers throughout the world. However, one of the implements in the acceptance of the Ayurvedic or Siddha formulations is the lack of standard quality control profile[1]. The quality of herbal medicine that is the profile of the constituents in the final product has implication in efficacy and safety. Due to the complex nature and inherent variability of the chemical constituents of the plant based drugs, it is difficult to establish quality control parameters and modern analytical techniques are accepted to help in circumventing this problem. Standardization of herbal formulations in terms of quality of raw materials, manufacturing practices and composition is important to ensure quality and optimum levels of active principles for their bio-potency. Recently, the concept of marker-based standardization of herbal drugs is gaining momentum. 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[2]. 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, etc [3].

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Azadirachta indica A. Juss (Meliaceae) popularly known as neem (Hindi), is a medicinal plant that grows freely all over Indian subcontinent. Neem has a role in the treatment of disorders like microbial infections, skin diseases, dental disorders, malaria, syphilis, leprosy and has antiseptic property[4–6]. Anti-inflammatory, immunostimulant and antiulcerogenic actions have also been reported in the extracts of *A. indica*[7–9]. The medicinal and industrial uses of various parts of Neem tree and the compounds isolated from it have been reviewed[10]. More than 135 compounds of diverse structure have been isolated from various parts of Neem[11,12] but few of them have been studied for their biological and pharmacological actions. Flavanoids like rutin and quercetin have been reported to possess antiulcer and anti-inflammatory activities[13].

MATERIALS AND METHODS

Plant material and Sample Preparation

Leaves of *Azadiracta indica* A.Juss. was collected from Matunga (Mumbai) region of India. It was authenticated from Blatter Herbarium, St. Xavier's College, Mumbai, India. After collection, The collected plant material was dried at room temperature in shade and then ground in a mixer to a fine powder, which was passed through an ASTM BSS 85 mesh size and stored in an airtight container, at room temperature. 100 mg of leaf powder of *Azadiracta indica* A.Juss. was extracted with 10 ml of methanol. The mixture was vortexed for 5 mins. and it was kept overnight for extraction. It was filtered through Whatman filter paper No. 1 and filtrate was diluted to ten fold by methanol and it was subjected to HPLC for simultaneous quantitation of rutin and quercetin

Chemicals and standard solutions Preparation

All the chemicals used in the experiments were of HPLC grade. Reference standard rutin and quercetin (purity 98%) were procured from Sigma Aldrich (Germany). The stock solutions of rutin and quercetin (10mg ml^{-1}) each were prepared separately in methanol. The stock solution were quantitatively transferred to give a solution of appropriate concentration range of rutin (10 µg mL^{-1} – 200 µg mL^{-1}) and quercetin (1 µg mL^{-1} – 20 µg mL^{-1}) respectively. Standard solutions were prepared by dilution of the stock solution.

Instrumentation and Chromatographic Conditions

Chromatographic separation was preformed with Merck Hitachi high performance liquid chromatograph equipped with L– 7100 pump fitted with L-7455 auto Sampler and HSM-LACHROM Multi HSM manager chromatographic software was used for data acquisition. The chromatographic separation was performed on Phenomenex C18 column (250 x 4.6, 5 µm) used for the analysis. The mobile phase comprising of 60:40 *v/v* mixture of methanol and 0.1% O-phosphoric acid in water was filtered through a 0.45 µm membrane filter (Millipore) and degassed by sonication. Throughout the run a flow rate of 1.2 mL min^{-1} was maintained. The column effluent was monitored at 258 nm with a L-2400 series multi-wavelength UV Detector. A typical HPLC chromatograms for simultaneous determination of rutin and quercetin form *Azadiracta indica* A.Juss. are shown in Figure 1 and Figure 2 respectively and the overlay is shown in Figure 3.

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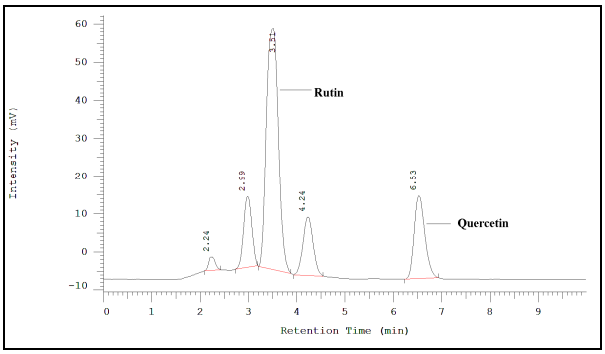

Fig 1. ***Chromatogram of leaves of Azadiracta indica A.Juss.***

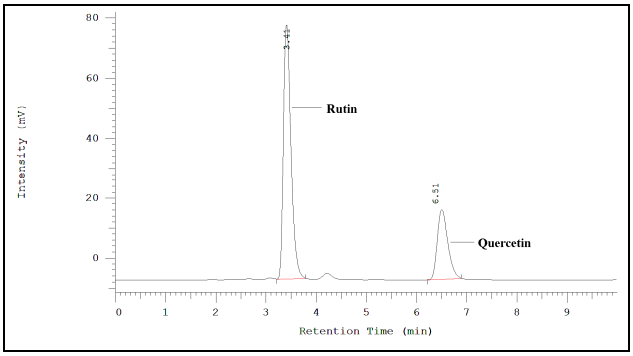

Fig 2. ***Chromatogram of Standards***

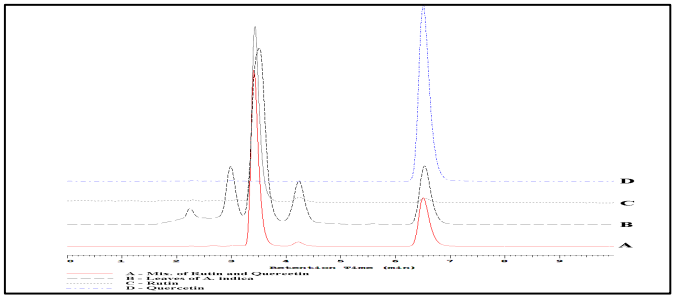

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Fig 3. ***Chromatographic overlay***

Method Validation

System Suitability

System suitability tests are used to ensure reproducibility of the equipment. The test was carried out by injecting 10 µL of mixture of standard solution of assay concentration of rutin and quercetin six times. The % RSD was found to be 0.12 for rutin and 0.36 for quercetin, which was acceptable as it is less than 2%.

Linearity

A good linearity was achieved in the concentration ranges of 63 µg mL^{-1} – 117 µg mL^{-1} for rutin and 7 µg mL^{-1} – 13µg mL^{-1} for quercetin. The regression equations and correlation coefficient for the reference were y = 10023x - 36045, R^2 = 0.9995 for rutin and y = 35260x - 11344, R^2 = 0.999 for quercetin respectively. The experiment was performed three times and the mean was used for the calculations. The data was analyzed by linear regression least squares fitting. The statistical data obtained is given in Table 1.

Table 1

Method validation summary

Parameters	Rutin	Quercetin
Linearity range [µg mL^{-1}]	63 µg mL^{-1} – 117 µg mL^{-1}	7 µg mL^{-1} – 13 µg mL^{-1}
Slope (*m*)[a)]	10023	35260
Intercept(*c*) [a)]	-36045	-11344
Correlation coefficient (R)	0.9995	0.999
LOD [µg mL^{-1}]	7	0.5
LOQ [µg mL^{-1}]	10	1.5
Intraday precision (n=3 COV)	0.12%	1.12%
Interday precision (n=3 COV)	0.14%	0.78%
System Suitability	0.11%	0.36%

[a)] *of the equation y = mx + c, where y is peak area, m is the slope, x is the concentration, and c is the intercept.*

Limit of Detection and Limits of Quantitation

The signal-to-noise ratio of 3:1 and 10:1 was used to establish LOD and LOQ, respectively. The LOD and LOQ of rutin was 7.0 µg mL^{-1} and 10.0 µg mL^{-1} and quercetin was 0.5 µg mL^{-1} and 1.5 µg mL^{-1} respectively.

Assay

The developed HPLC method was used for simultaneous determination of rutin and quercetin from leaf powder of *Azadiracta indica* A.Juss. The sample working solution (10 µL) was injected and the area of both rutin and quercetin peak was measured.

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From the calibration curve, the amount of rutin and quercetin in dry leaf powder of *Azadiracta indica* A.Juss. was calculated. The retention time of rutin and quercetin in sample solution was 3.51 and 6.53 and in the standard solution was found to be 3.48 and 6.51 respectively. The mean assay value of rutin was found to be 0.9079 mg per 100 mg of plant powder with % RSD as 0.29 and mean assay value of quercetin was found to be 0.1005 mg per 100 mg of plant powder with % RSD as 1.27.(Table 3)

Table 3
Assay Results

Sample Tested	Content of Marker compound* in mg	
	Rutin	**Quercetin**
Leaf powder of *Azadirachta indica* A. Juss with Rutin and Quercetin.	0.9079	0.1005

** Mean ± SD, n= 7*

Precision and Accuracy

The intra-day and inter-day precision was used to study the variability of the method. The % RSD for intra-day and inter-day precision for rutin were 0.12 and 0.14%, respectively and quercetin were 1.12 and 0.78 %, respectively. Accuracy of the method was studied using the method of standard addition. Standard rutin and quercetin solutions were added to the extract of the of *Azadiracta indica* A. Juss. leaves and the percent recovery was determined at two different levels 50% and 100%. Rutin and quercetin content was determined and the percent recovery was calculated. The results of recovery analysis are shown in Table 2 for both rutin and quercetin.

Table 2
Results of Recovery study

Standard	Level	Preanalysed sample in (µg mL^{-1})	Amount of std added to preanalysed sample in (µg mL^{-1})	Total amount of std found in (µg mL^{-1})	SD	RSD (%) (n = 7)	Recovery (%)
	0	90.8	0	90.72	0.01	0.148	99.95
	50%	90.8	45	135.69	0.006	0.058	100.06
Rutin	100%	90.8	90	180.94	0.029	0.207	99.08
						Mean	99.69
	0	10	0	10.015	0.04	1.02	99.7
Quercetin	50%	10	5	15.307	0.023	0.391	99.58
	100%	10	10	20.142	0.013	0.165	99.57
						Mean	99.61

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* **Mean ± SD, n= 7**

CONCLUSION

The application of a simple, rapid and accurate HPLC method for the simultaneous quantitation of rutin and quercetin in *Azadiracta indica* A. Juss leaf powder. The method was validated to track the active principles in the complex mixture of herbal ingredients. The method could be extended for the marker-based standardization of other herbal product containing rutin and quercetin. The method was found to be simple, precise, accurate, specific, sensitive and can be used for routine quality control of herbal raw materials also for the quantification of these compounds in plant materials.

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