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Full Length Research Article

A Validated New RP-HPLC Method for Simultaneous Determination of Ferulic Acid, Quercetin, and Rutin in the Whole Plant Extract of *Dregea volubilis*

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Abstract

A new reverse-phase High-performance liquid chromatography (RP-HPLC) method was developed and validated for the simultaneous estimation of Ferulic Acid, Quercetin, and Rutin. In this research, an analytical C18 (4.6mm×25 cm), 5µm column was used for chromatographic separation with a mixture of methanol and water containing Potassium dihydrogen phosphate, and the mobile phase (pH 4.0 adjusted with ortho-phosphoric acid) at a flow rate of 1.5mL/min. The detection was carried out using a UV-Visible detector at 254 nm. The analytical method was validated prior to meeting the conditions specified by International Conference on Harmonization (ICH) and the parameters were specificity, linearity, the limit of detection (LOD), the limit of quantification (LOQ), accuracy, precision, robustness, and robustness. The calibration curve was found to be linear between the concentration ranges of 0.005-0.06 mg/mL, 0.006-0.072mg/mL and 0.009-0.108mg/mL for Ferulic Acid, Quercetin, and Rutin respectively. Furthermore, the LOD and LOQ of Ferulic Acid were 0.00456mg/mL and 0.013819mg/mL respectively. The LOD and LOQ of Quercetin were 0.001423mg/mL and 0.004311mg/mL respectively. Accordingly, LOD and LOQ of Rutin were 0.001343mg/mL and 0.004069mg/mL respectively. The accuracy of the optimized method was examined by recovery studies and the mean recovery was observed to be 100.24%, 99.52% & 99.44% for Ferulic Acid, Quercetin, and Rutin respectively, at 50% and 100% spiked levels. The repeatability testing for both standard and sample solutions revealed that the method is precise within the acceptable range and the % RSD of the precision was less than 2%. In addition, the readings of specificity, linearity, accuracy, precision, LOD, LOQ, and robustness of Ferulic Acid, Quercetin, and Rutin were within the criteria of acceptable limit as well.

***Key words*:** *Dregea volubilis*, Ferulic acid, Quercetin, Rutin, HPLC

Validation of analytical methods is mandatory in implementing a quality control system in any analytical laboratory. It provides an assurance of reliability during normal use and can be referred to as a process of providing documented evidence of quality for several herbal and traditional drugs. Separation techniques such as chromatography and electrophoresis have been extensively used for quality control of herbal medicine because of their high efficiency and speed [1]. *Dregea volubilis* is a large woody twining shrub of the family Apocynaceae found to be growing in high rainfall as well as in low rainfall regions [2]. This plant is known as Madhumalathi, Hema Jeevanti (Sanskrit), Nakchhikni (Hindi), Titakunga (Bengali), Dudhipaalateega (Telugu), in Indian traditional medicine. The preliminary phytochemical screening of the roots revealed the presence of alkaloids, glycosides, flavonoids, resins, saponins, tannins and carbohydrates [3-4]. The leaf paste is used to treat cough, fever, severe cold and rheumatic pain [5]. The preparations of leaf paste in combination with pepper and bark paste with hot milk is used to treat dyspepsia and primary urinary infections respectively [6]. According to published papers the plant exhibited bioactivities such as anti-inflammatory [7], Antibacterial [8-9], Antiradical [10], Antitumor [11], Antioxidant [12], and Larvicidal [13]. Chemical constituents present in the plant are Rutin, Quercetin, Ferulic acid, Lupeol, Ursolic acid and Oleanolic acid [14-15]. Sometimes excessive antibiotic intake may develop allergic reactions, hypersensitivity and immuno suppression in patients [16]. Furthermore, when antibiotics do not respond and control the illness, it may become more complicated which leads to use of stronger and more expensive

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drugs. Thus, the increase in emergence of antibiotic resistant bacteria increased the demand for alternative antibiotics from natural sources which are comparatively cheap with less adverse effects [17]. According to the references, no simultaneous determination of three phytocompounds from the plant has been published till date. In view of these wide therapeutic effects a need was felt for simultaneous quantitation of Ferulic acid, Quercetin and Rutin in the leaves of *Dregea volubilis*. In this research work, a simple, precise and accurate HPLC method has been established for simultaneous quantitation of Ferulic acid, Quercetin and Rutin in the dried leaf powder of *Dregea volubilis*. Further, the proposed method has been validated as per ICH guidelines. Such a study would not only facilitate standardization of the raw material and commercial products but also facilitate future pharmacological studies and quality control.

MATERIALS AND METHODS

Chemicals

HPLC grade Methanol was procured from Merck Life Science Private limited, Vikhroli, India. Analytical grade Potassium dihydrogen phosphate was procured from Merck Specialities Private Limited, Mumbai, India. Reference standard Rutin was purchased from the Tokyo Chemical Industry. Co. Ltd. The Quercetin reference standards were purchased from Yucca Enterprises, Mumbai, India. Ferulic acid was purchased from Sigma Aldrich.

Plant material

Whole plant of *Dregea volubilis* was collected from the Nalasopara region, Maharashtra, India. Herbarium samples were prepared in duplicate and authenticated by VIVA Herbarium, Virar, India. The leaves were washed with water to remove any dust particles, dried in a hot air oven at 37 °C, powdered, and then sieved through mesh size 0.22 micron and stored at 25 °C in an airtight container.

Preparation of stock solutions

Standard stock solutions of pure compounds were prepared separately by dissolving 10 mg of each compound in 10 mL of Methanol to get the concentration of 1000 µg/mL. For the calibration curve, solutions from 9-108 µg/mL, 6-72 µg/mL, and 5-60 µg/mL were prepared from the above stocks for Rutin, Quercetin, and Ferulic acid respectively.

Sample preparation

About 0.1 gm of dried whole plant powder of *Dregea volubilis* was weighed into a round bottom flask. 10 ml of methanol to the flask and the mixture was kept on the shaker overnight. The extract was then filtered through Whatman filter paper no. 41 (Merck, Mumbai, India). This solution was used for the assay.

Instrumentation

Jasco HPLC system was utilized for the development and validation of liquid chromatography, facilitated with a pump (model: PU 2080 plus), an autosampler (ALS) (model: AS2055 plus), and aC18 (250 cm×4.6 mm), 5µm column (Crest Pak), and the detector included UV/VIS (model: UV 2075 plus) operated at 254 nm. ChromNav Software (version) was used in order to process and evaluate the obtained results. Additionally, an analytical balance demonstrating four digits was used (Contech) for weighing purposes and a sonicator (PCI analytics, model: USB1.5L50H, India) was used prior to dissolving the reagents.

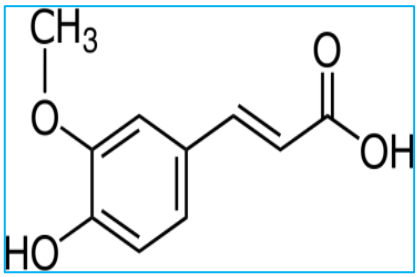

Fig 1 Ferulic acid

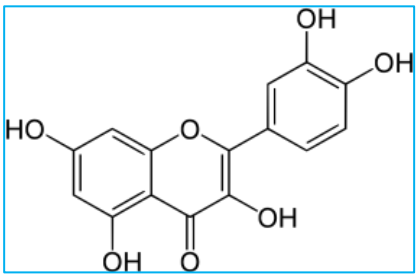

Fig 2 Quercetin

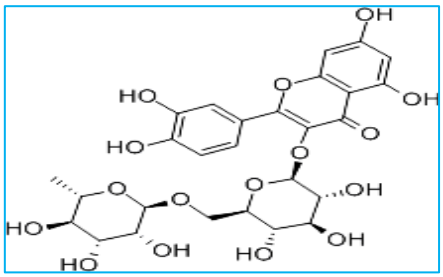

Fig 3 Rutin

Chromatographic conditions

The diluent was prepared by dissolving Potassium dihydrogen phosphate and the pH was adjusted to 4.0 with orthophosphoric acid and Methanol. The mobile phase was filtered through 0.45µm membrane filters and degassing was done by sonication for 20 min. analyses were carried out on analytical column C18, 5µm, 250×4.6 mm with a detection wavelength of 254 nm by a UV/VIS detector. The injection volume and flow rate were 10µL and 1.5 mL/min, respectively, in addition to the runtime of 15 minutes.

Validation of the method

The developed LC method was validated according to the ICH guidelines [18]. The validation parameters evaluated include specificity, linearity, precision, accuracy and robustness of the method.

Specificity: Specificity is considered the vital part of HPLC which deals with the potentiality of analytical techniques to differentiate between the analyte and other ingredients in the composite mixture [19]. The specificity of the proposed HPLC method was ascertained by analyzing standard compounds and samples. The peaks from sample solutions were confirmed by comparing the Rt of the peaks to those of the standards.

Linearity: Linearity is defined as the ability to find the test values which have direct relationship to the concentration of the analyte. Working standard solutions of Rutin 27-81µg/mL, Quercetin 24-60 µg/mL, and Ferulic acid 25-55 µg/mL were injected in triplicate. The peaks areas of Rutin, Quercetin, and Ferulic acid were plotted against concentrations. Subsequently, the linearity was examined with the help of a calibration curve to assess slope, correlation coefficient, and intercept on the Y axis.

Precision: Precision deals with the degree of closeness among individual tests, when repetitive technique was ap-plied in order to evaluate multiple replicates in three different occasions [20]. Variability of the method was studied by analyzing quality control samples of Rutin 27-81µg/mL,

Quercetin 24- 60 µg/mL, and Ferulic acid 25-55 µg/mL on the same day (intra- day precision) and on different days (interday precision) and the results were expressed as % RSD.

Limit of detection (LOD) and limit of quantification (LOQ)

The limit of detection (LOD) is defined as the lesser quantity of analyte in a sample which can be estimated but not inevitably assessed. Similarly, the limit of quantification (LOQ) deals with the minimum portion of analyte in a sample that can be quantifiably evaluated with appropriate precision. The calibration curve was repeatedly used for 7 times and SD of the intercepts was evaluated using the below-mentioned formula to calculate the values of LOD and LOQ.

$$LOD = (3.3 * SD) / Slope \quad \text{(1)}$$
$$LOQ = (10 * SD) / Slope \quad \text{(2)}$$

Where SD is the standard deviation of Y-intercept of 7 calibration curves and Slope is the average slope of the 7 calibration curves [21-22].

Accuracy / recovery:

Recovery tests were carried out to further investigate the accuracy of the method by adding two concentration levels (50% and 100%) of the mixed standard solutions to known amounts of *Dregea volubilis* samples. The resultant samples were then extracted and analyzed with the described method. The average percentage recoveries were evaluated by calculating the ratio of the detected amount versus added amount.

Robustness: According to the International Conference on Harmonization (ICH), the robustness of an analytical procedure is defined by its ability to remain unaffected by small and deliberate variations in method parameters [23]. This parameter was assessed by studying the impact of minor variations in the chromatographic conditions. The conditions evaluated were different flow rates and pH of the mobile phase. The retention time and peak response were observed.

RESULTS AND DISCUSSION

Method development and optimization

The various physicochemical characteristics of Ferulic acid, Quercetin and Rutin were acquired from the previously published literature. A suitable analytical method was developed prior to selecting preliminary reverse phase HPLC-UV chromatographic conditions such as stationary phase, mobile phase, determining wavelength and procedure of sample preparation. In order to achieve the goal, many mobile phase combinations were tried from which mixture of methanol and water containing Potassium dihydrogen phosphate (pH 4.0 adjusted with ortho-phosphoric acid). This mobile phase gave the resolution of Ferulic acid (3.70+0.02), Quercetin (14.05+0.02) and Rutin (5.02+0.02). These standards were also resolved from other components present in the sample extract enabling simultaneous quantification.

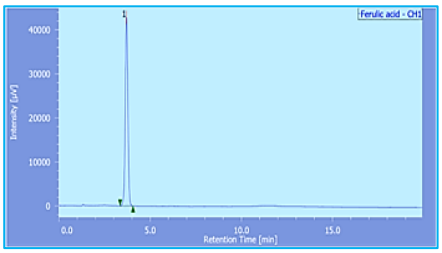

Fig 4 Chromatogram of ferulic acid

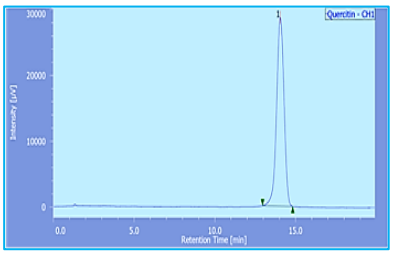

Fig 5 Chromatogram of quercetin

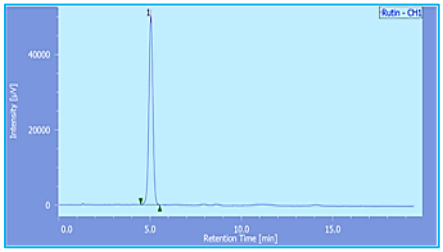

Fig 6 Chromatogram of rutin

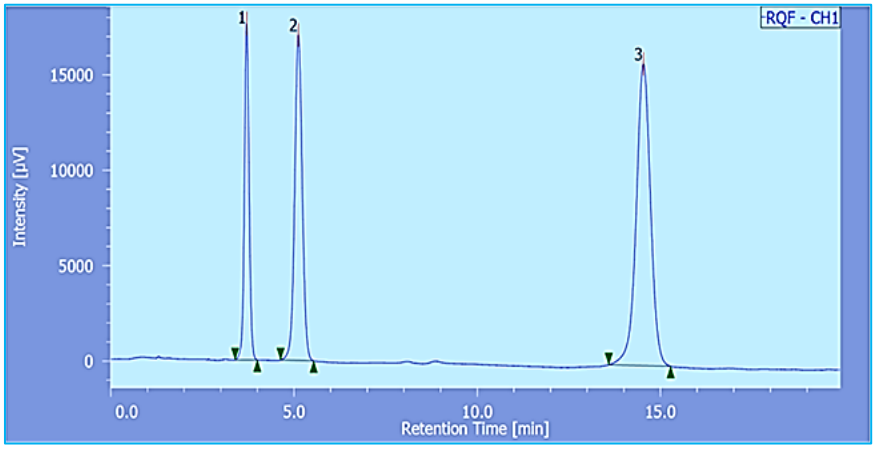

Fig 7 Chromatogram of mixture of ferulic acid, quercetin and rutin

Method validation

Specificity: The developed method was tested to determine specificity to make sure that no other interference from the solvent and matrix are present in the chromatograms of the three standards. The blank (mobile phase and extracting solvent), reference of the three standards and sample were injected and their chromatograms were compared and are shown in (Fig 1-5). No interference was observed due to the blank in the main peaks of the reference standard. Hence, it can be concluded that the developed method is specific for the simultaneous determination of Ferulic Acid, Quercetin, and Rutin.

Linearity: The linearity of the response (peak area) of the drugs was determined at six concentration levels ranging from 25% to 150% of the assay concentration for each of the three drugs. The assay concentration (100%) was 0.04,0.042 and 0.054 mg/ml for Ferulic Acid, Quercetin, and Rutin respectively. The seven concentration levels were in the range of 0.025 - 0.05 mg/ml for Ferulic Acid, 0.024 - 0.06 mg/ml for

Quercetin and 0.027 - 0.081 mg/ml for Rutin. The coefficients of determination (R2) values are greater than 0.999 for the three drugs. This indicates that the detector response was linear in the above specified concentration ranges.

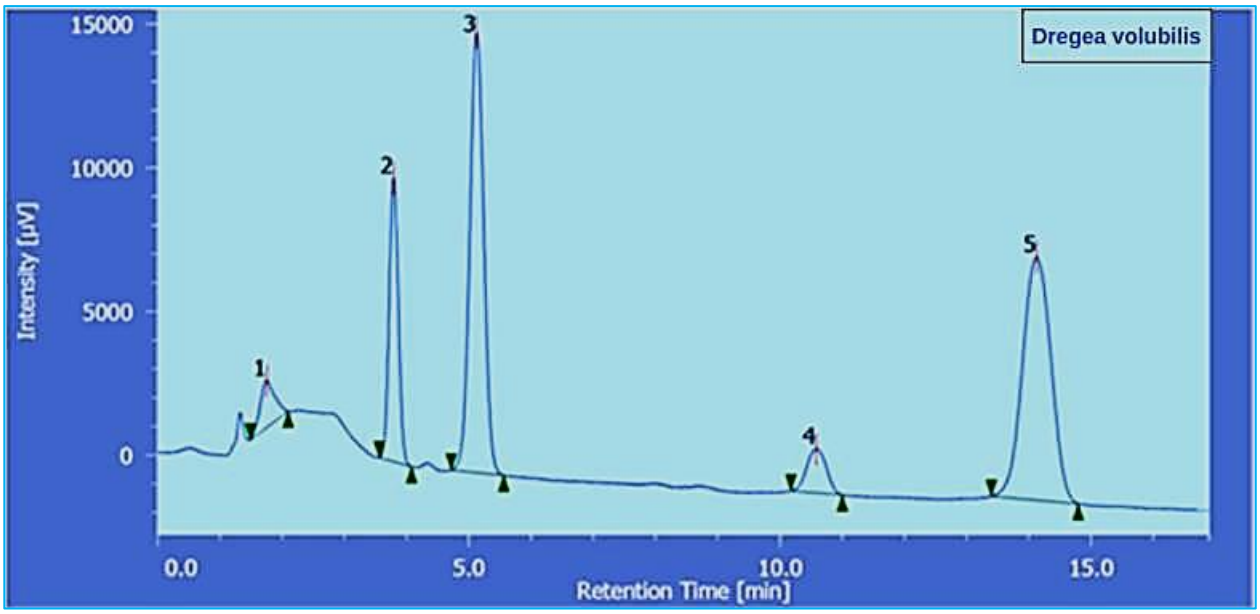

Fig 8 Chromatogram of *Dregea volubilis*

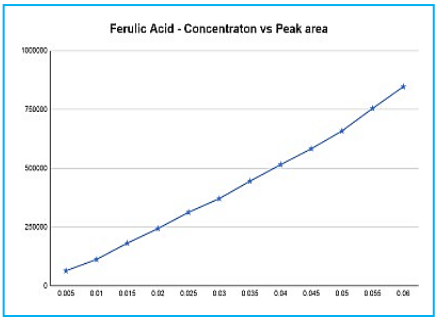

Fig 9 Calibration curve of ferulic acid

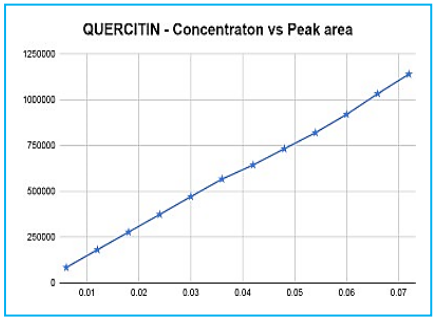

Fig 10 Calibration curve of quercetin

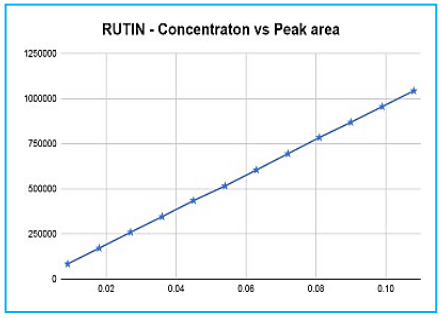

Fig 11 Calibration curve of rutin

Precision: Precision was determined as % RSDs of the peak areas of the drugs. The % RSD for the repeatability, intra- and inter- day precisions were less than 2% for the respective drugs at the assay concentration of 0.04,0.042 and 0.054 mg/ml for Ferulic Acid, Quercetin, and Rutin respectively. Results of the %RSD values of repeatability and intermediate precision studies showed that the method is precise for simultaneous determination of the three standards.

LOD and LOQ: These parameters were assessed by determining Ferulic Acid, Quercetin, and Rutin as per the formula illustrated in Section 2.6.5. In this study, the LOD and LOQ of Ferulic Acid were observed to be 0.00456 and 0.013819 mg/mL, respectively. LOD and LOQ of Quercetin were 0.001423 and 0.004311 mg/mL, respectively. Accordingly, LOD and LOQ of Rutin were 0.001343 and 0.004069 mg/mL, respectively.

Accuracy / recovery: Accuracy was evaluated as percent recovery of the added standards of the three drugs at the concentration levels of 50% and 100% of the assay concentration of each standard to the equivalent weights in the whole plant powder. The percentage recovery of Ferulic Acid, Quercetin, and Rutin were found to be 100.24, 99.52 and 99.44 respectively. The percentage recoveries of the three standards were in the range of 98–102% indicating the good accuracy of the optimized method for simultaneous determination of Ferulic Acid, Quercetin, and Rutin.

Robustness: This testing parameter was analyzed by assessing the impact of slight alteration in chromatographic conditions. The data of robustness evaluation demonstrated that a minor modification of method conditions including flow rate and pH of mobile phase was found to be robust within the desired range.

CONCLUSION

In this research, a new, novel, simple, accurate, precise, robust, and linear reverse phase HPLC method has been developed and validated for simultaneous assay evaluation of Ferulic acid, Quercetin and Rutin from *Dregea volubilis*. The newly developed method is validated as per the specification provided by ICH and proved to be appropriate for the intended application, and is able to give quantitative measurements accurately and precisely under slight variation of chromatographic conditions. The developed validated analytical method can support the pharmaceutical and ayurvedic industries and other researchers to analyze Ferulic acid, Quercetin and Rutin containing plants and dosage forms to evaluate quality in their products.

Acknowledgment

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