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DEVELOPMENT AND VALIDATION OF UFLC METHOD FOR QUANTIFICATION OF FLAVONOIDS IN *TECTONA GRANDIS* LEAVES

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ABSTRACT

A simple Ultra fast liquid chromatography (UFLC) method for the separation and quantitative determination of the flavonoids from Leaves of Tectona grandis, was developed. UFLC is a new technique in LC offering several potential advantages, especially the reduction of time. A special UFLC analysis performed on shimadzu LC- 30 AD liquid chromatograph equipped with column Aquity C18 (100 X 2.1mm) with a particle size of 1.7µm. The method was validated to demonstrate its specificity, Accuracy, Precision, LOD, LOQ, Linearity, Range, robustness. For rutin and quercetin the method was validated according to ICH guidelines. Method was highly precise and accurate because it shows low relative standard deviation as well as good % recovery values.

KEYWORDS: Tectona grandis, flavonoids, Rutin, Quercetin, UFLC, Validation.

1. INTRODUCTION

Tectona grandis linn .(Family - Verbenaceae) is one of the most famous timbers in the world and is renowned for its dimensional stability, extreme durability and hard which also resists decay even when unprotected by paints and preservatives. This plant is commonly called as teak and locally known as sagon, sagwan. It is one of the most important heart wood of the world over. Timber value of teak has been well known from decades.[1]

It is important to know that, which secondary plant metabolites are found in plant as it may provide a basis for its traditional uses. During more than 100 years of intensive research on

the chemistry of *Tectona grandis*, various compounds have been detected from different parts of the plant.[2]

Teak leaves contain haemostatic properties thus can be squeezed and applied on a cut skin to stop bleeding. Leaves are also useful in inflammations, leprosy, skin diseases, pruritus, stomatitis, ulcers. Leaves extract applied topically or given orally promoted the breaking strength, wound contraction and collegenation.[2] Several classes of phytochemicals like alkaloids, glycosides, saponins, steroids, flavonoids, proteins and carbohydrates have been reported in *Tectona grandis.*[3]

The major active nutraceutical ingredients in plants are flavonoids. As is typical for phenolic compounds, they can act as potent antioxidants and metal chelators. They also have long been recognized to possess anti-inflammatory, anti-allergic, hepatoprotective, antithrombotic, antiviral, and anticarcinogenic activities.[4]

The determination and quantification of flavonoid glycosides is a challenge. The Several methods have been described for the determination of flavonoids.[5]

The objective of this study was to development of Ultra fast liquid chromatography (UFLC) method for separation and quantitative determination of flavonoids. The method is used on reversed phase (RP) C18 columns. In comparison with the conventional HPLC method, UPLC offers many advantages including reduced run-time and less solvent consumption. In this paper, a rapid and reproducible UFLC method was developed for the simultaneous determination of significant flavonoids in *Tectona grandis* leaf.

2. EXPERIMENTAL

2.1 Plant Material

Tectona grandis leaf were used for this experiment. Leaves were collected from Dharmaj village of Gujarat. The leaves were air dried for 2 days and later crushed into smaller sizes and kept in tightly closed container in dark places until subjected to the extraction process.

2.2 Chemicals and reagent

Analytical grade methanol (Rankem) were used for HPLC analysis. Analytical grade formic acid (SDFCL) and De-ionised water was used. The reference standards Rutin (Loba chemie, India) and Quercetin (USP reference standard from Rockville, USA) was used for analysis. n-

hexane (merck), chloroform (Fisher Scientific), ethyl acetate (EMPLURA) were used for extraction. Aluminium chloride, Potassium acetate (Sigma aldrich).

2.3 Extraction Procedure

Dried leaves of *Tectona grandis* was powdered (100 gm) and extracted with ethanol (Fisher Scientific) for 3 hours at 50-60 ^{o}C. The extract was then filter and again extract with fresh solvent. Then extract was filtered using Whatmann filter paper and concentrated by using a rotary evaporator (superfit). The 10.60 gm of ethanolic extract was taken further for sequential liquid-liquid extraction. The ethanolic extract was dissolve in methanol:water (20:80) and mix it well. Then sequentially extracted with Hexane, chloroform and ethyl acetate in separating funnel. Collected the all fractions and concentrated on rotary evaporator. Ethyl acetate extract was concentrated (yield 1.31 gm) and used for further analysis.

2.4 Determination of Total Flavonoid Content

The total flavonoid content was determined using calibration curve of Quercetin. The solution of Quercetin 100 µg/ml concentrations were prepared in methanol and further dilutions of (6.25, 12.5, 25, 50, 80, 100 µg/ml) were prepared in methanol. The Stock solution of plant extracts was prepared by dissolving 10 mg of the extract transferred to 10 ml volumetric flask and made up the volume with methanol. The 10% aluminium chloride and 1M potassium acetate were prepared using distilled water. The assay was determined using 0.5ml of each extract stock solution and each dilution of standard quercetin taken separately in test tubes. To each test tube 1.5ml methanol, 0.1ml aluminium chloride solution, 0.1ml potassium acetate solution and 2.8ml distilled water were added and mixed well. The absorbance of this reaction mixture was recorded at 415 nm on UV spectrophotometer against the blank containing water instead of sample. The TFC was calculated using standard calibration curve of quercetin. The total flavonoid content was calculated as quercetin equivalents (mg/g) of dried extract.[6,7]

2.5 UFLC Analysis

analysis was performed on Shimadzu LC- 30 AD liquid chromatograph equipped with an SPD- 20A UV detector, SIL- 30AC auto sampler, DGU- 20A 5R degassing unit, CTO- 20AC column oven and separation was done by using column Acquity C18 (100mm X 2.1mm, 1.7µm). The mobile phase was a isocratic elution prepared from 0.1% formic acid in water: Methanol (50:50), with a flow rate of 0.20 mL/min. The injection volume was 1µL. UV

detection was performed at 360 and 254 nm. All the samples were filtered through Millipore filters 0.22 µm nylon filters from pall corporation.

The identification of each compound was carried out by comparing retention times and spiking standard with extract sample.

2.6 Validation parameters

Method validation was done according to ICH guideline on different parameters like specificity, Accuracy, Precision, LOD, LOQ, Linearity, Range, robustness.[8]

2.6.1 specificity

The specificity of the method was obtained by injecting the blank, standard and sample. For this chromatogram was taken by appropriate dilution and quantities of drug were determined and compared with blank solution. No interfering peaks for the determination of flavonoids were observed.

2.6.2 Accuracy

The accuracy of the method was determined by analysing the percentage of recovery of the main constituents in the extract. The samples were spiked with minimum of 3 different amounts of concentration level of standard compounds. The obtained average contents of the target compounds were used as the "real values" to calculate the spike recoveries.

2.6.3 Precision

Precision was studied by carrying out the analysis of six concentrations at 100% of the test concentration of rutin and quercetin were analysed. The intra-day experiment was obtained by six replicates for a day, and the inter-day was determined by six injections for 3 days for the retention time and the peak area. The precision was expressed as relative standard deviation (%RSD).

2.6.4 Linearity and range

The linearity is directly proportional to the concentration of the analyte in the sample. Stock solution of rutin and quercetin was suitably diluted with methanol to get concentrations in the linear range **74 to 226 µg/ml** for UFLC analysis. The Linearity graphs with correlation co-efficient for rutin and quercetin were plotted, using concentrations on X-axis and the respective peak areas on Y-axis.

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

The Detection limit of an individual analytical procedure is the lowest amount of analyte in a sample which can detected but not necessarily quantitated as an exact value. The quantification limit of an individual analytical procedure is the lowest amount of analyte in a sample which can be quantitatively determined with suitable precision and accuracy. Limits of detection and quantification were determined by calculation of the signal-to-noise ratio. Signal-to-noise ratios of approximately 3:1 and 10:1 were used for estimating the detection limit and quantification limit respectively of the method.

2.6.6 Robustness

The determination of the method's robustness a number of chromatographic parameters, such as flow rate, column oven temperature and detection wavelength, were varied to determine their influence in the quantitative analysis.

2.6.7 System suitability studies

System suitability parameters like number of theoretical plates (N), resolution (Rs) and tailing factor were studied.

3.0 RESULT AND DISCUSSIONS

3.1 Total flavonoid content determination

The total flavonoid content were determined from the **fig.1** shows calibration curves of quercetin (y=0.006x + 0.009, R^2= 0.990). The total flavonoid content of ethyl acetate extract was shown (4.61 ± 0.09) mg QE/g of dry material.

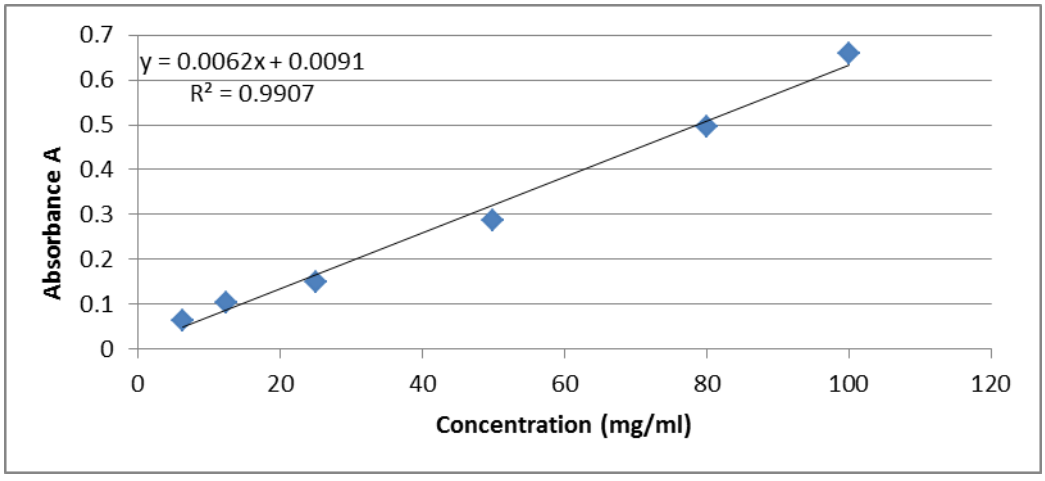

Fig. 1: Standard Curve of Quercetin (TFC).

3.2 Optimisation of UPLC conditions

Preliminary experiments were performed to obtain the best peak resolution and separation, for which both a mixture of standards and a real sample were used. The separation was carried out on C18 column and mobile phase selected was 0.1% formic acid in water and Methanol (50:50), The mobile phases were pumped into the column at a flow rate of 0.20ml/min. The detection wavelength used was 360 nm and the method was validated. Retention time for rutin and quercetin was found to be 2.93 and 7.24 min. respectively.

3.3 Method Validation

The UFLC method was validated by defining the specificity, Accuracy, Precision, LOD, LOQ, Linearity, Range, robustness.

Specificity

Specificity was evaluated by calculation of peak resolution. The selected mobile phase gave good peak shape and separation with no interference. The results indicate that the method is specific for each of the analyte.

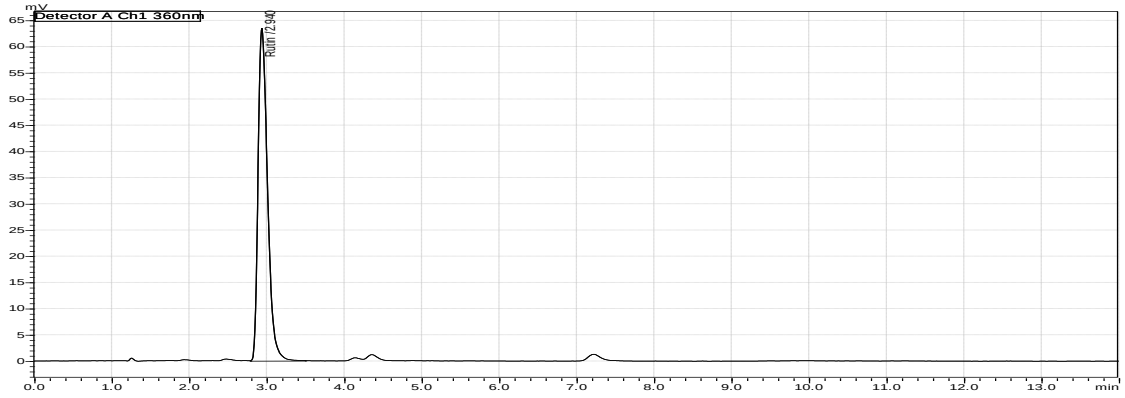

Fig. 2: UFLC chromatogram of standard rutin.

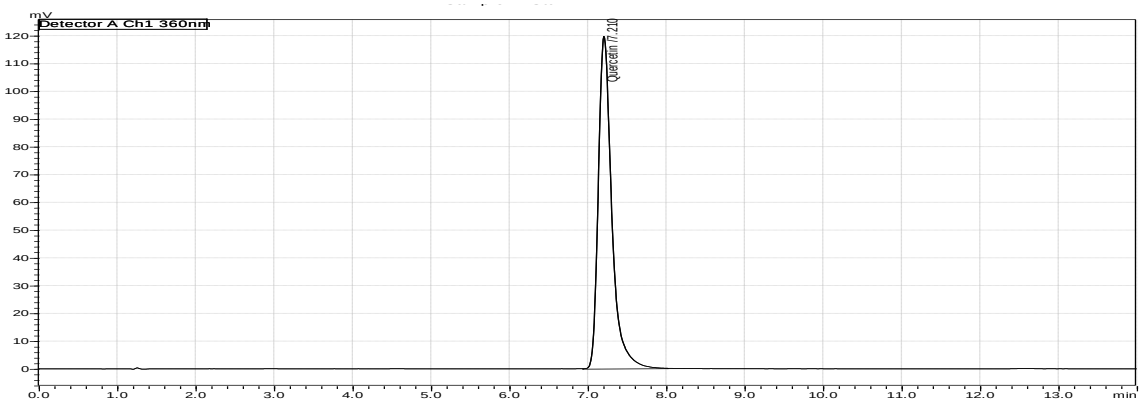

Fig. 3: UFLC Chromatogram of standard quercetin.

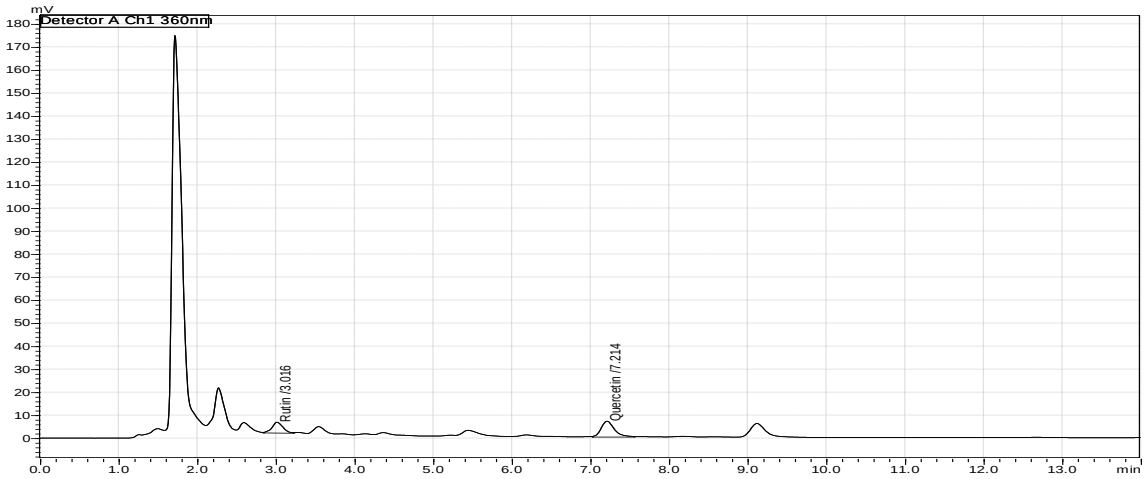

Fig.4: UFLC Chromatogram of Tectona grandis leaves ethyl acetate extract.

Precision and accuracy

The method precisions (repeatability) were determined by injecting six replicate samples preparation. The relative standard deviation (R.S.D) for sample preparation is 1.69 and 1.19 respectively of Rutin and Quercetin Intermediate precisions were calculated as % RSD was 1.24% for rutin and 1.48% for quercetin**.** (**Table 1**).

Accuracy was determined by spike recovery method. Spike recovery of Rutin was between 97%-104% and Quercetin was between 97%-103% which were within the acceptance criteria indicating method is accurate.

Table 1: Precision of method for determination of Rutin and Quercetin.

Sample name	Precision	
	Method Precision (% RSD)	Intermediate Precision (% RSD)
Rutin	1.69	1.24
Quercetin	1.19	1.48

Linearity

Calibration curves were obtained by plotting the peak areas of each analyte against the corresponding concentrations from the mixed stanadard solutions. Linear regression analysis of the calibration curves shows the assay was linear for all analytes over the concentration range 74-222µg/mL for rutin and 79-237 µg/mL for quercetin with correlation coefficients (r^2) for Rutin was found to be 0.994 and Quercetin was found to be 0.998.

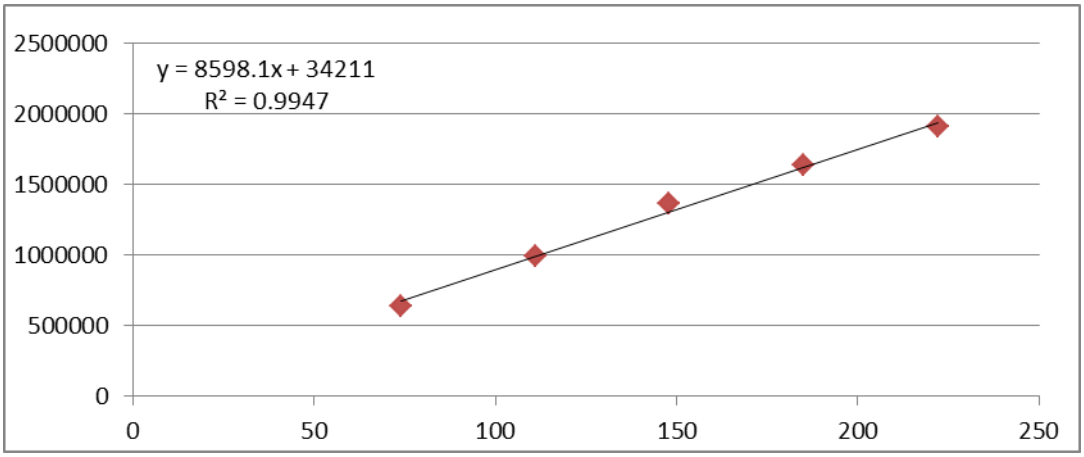

Fig. 5: Calibration curve of rutin.

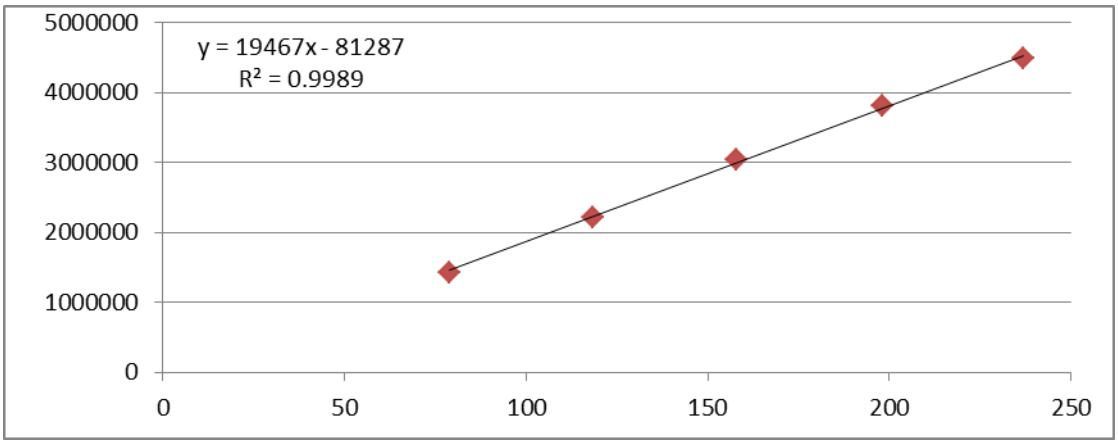

Fig. 6: Calibration curve of Quercetin.

Limit of detection and quantification

In present study the limit of detection was 0.0107 µg/ml for rutin and 0.125 µg/ml for quercetin. L1imit of Quantification for Rutin is 0.211 µg/ml and Quercetin is 0.108 µg/ml.

Robustness

Evaluation of robustness was done by deliberately change in individual factor such as change in flow rate, oven temperature and detection wavelength. In this study chromatographic parameters monitored were retention time, area, capacity factor (NLT 1.0), tailing factor (NMT 1.5) and theoretical plates (NLT 2000)

CONCLUSION

In this study a simple method was developed for estimation of rutin and quercetin in ethyl acetate fraction of ethanol extract of *Tectona grandis*. Validation was done as per ICH

guidelines. Result showed that the developed method is simple, fast, sensitive, suitable and specific.

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