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HUMAN

PHYTOCHEMICAL SCREENING AND DEVELOPMENT AND VALIDATION OF HPLC METHOD FOR ISOLATED CONSTITUENT IN EXTRACT OF *Millingtonia hortensis linn*

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ABSTRACT

The present study aims, to develop analytical method of isolated constituent in methanolic extract of leaves of *Millingtonia hortensis linn*. Development of new methods for routine analysis of various chemical constituents present in plants is highly demanded by the industry. The extracts of *Millingtonia hortensis linn* were obtained by continuous heat extraction method by using Soxhlet extraction process containing methanol as solvents. Extract was further used for phytochemical study. Phytochemical screening showed presence of flavonoids and triterpenoid. TLC, IR, GCMS analysis of methanolic extracts shown new chemical constituents Rutin.

The developed HPLC method includes use of C18 Intersil, 4.6 (i.d.) x 250 mm column, mobile phase consist of mixture of methanol : Water (pH 5.5) in the ratio of 85: 15 % v/v at flow rate .5 ml/ min. Retention time was found to be 4.55, 4.52 for isolated Rutin and standard rutin respectively. Therotical plates was found to be 8045, 8234 for isolated and standard rutin . Method was validated with the help of parameter as linearity, range, accuracy, precision (intraday and interday's), LOD, LOQ. The developed method was found to be a relatively simple, precise and reproducible for the quantification of Rutin. The method does not employ any derivatization procedure and can be used as a quality control tool for the routine analysis of rutin from leaves extract.

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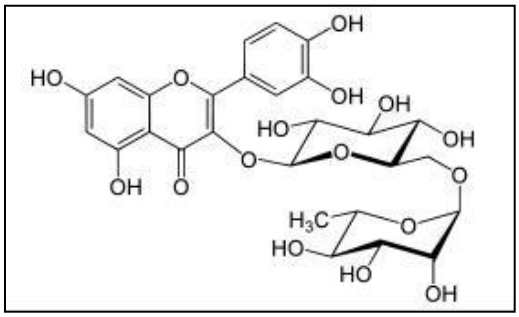
1. INTRODUCTION [1, 2]

In the last few decades there has been an exponential growth in the field of herbal medicine. It is getting popularized in developing and developed countries owing to its natural origin and lesser side effects. Cork tree is important medicinal plant in Southern Asia ranging from India, Burma, Thailand and South China. It is also called the Cork Tree, as an inferior cork is processed from its corky bark. The stem bark is used traditionally as mainly lung tonic, anti asthmatic and antimicrobial. The leaves of *Millingtonia hortensis* are used as antipyretic, sinusitis, cholagogue and tonic in folklore medicine.

Phytochemical evaluation is one of the tools for the quality assessment, which includes preliminary phytochemical screening, chemoprofiling and marker compound analysis using modern analytical techniques



Rutin

Rutin is 5, 7, 3, 4, tetrahydroxy flavonol -3-rhamanoglucoside and widely used in medicine for maintenance of capillary integrity. Both possess antioxidant activity and reduce low density lipoproteins [LDL] oxidation.

The literature revealed that no HPLC method is not yet reported for the estimation of rutin in methanolic extract of Millingtonia Hortensis leaves. Thus, this method is more accurate and cost effective. This paper describes simple, rapid, accurate, precise and economical method for determination of rutin in *Millingtonia Hortensis leaves extract.*

2. MATERIALS AND METHODS

2.1 Materials

Flavonoids standards (Rutin) purchased from SDFCL, Mumbai. All chemicals and reagents were purchased from Merck, Mumbai.

2.2 Plant material

The leaves of Millingtonia Hortensis were collected from Kodoli in Kolhapur district and authentified from Krishna College, Rethre (Karad).

2.3 Preparation of Extract [3]

The dried leaves were coarsely powdered and subjected to extraction by Soxlet. The extraction was done with Methanol.

2.4 Phytochemical screening [4, 5]

The phytochemical investigations revealed the presence of triterpenoids and Flavonoids in methanolic extract.

2.5 Isolation of Rutin by Thin layer chromatography

TLC was performed on TLC plates (20×10cm) The optimized mobile phase was Ethyl acetate: Toluene: Ethanol: Formic acid: Glacial acetic acid.

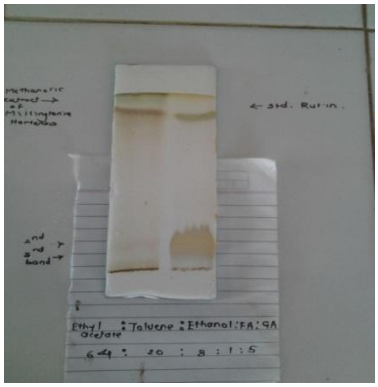


Fig 1: TLC for isolation of Rutin in Methanolic extract

2.6 Structural Elucidation of Isolated Constituent [6 -9]:

Structural elucidation of isolated constituent was done by FTIR Spectroscopy (Jasco 4100, FTIR) & GCMS analysis (Shimadzu, Japan, QP 2010).

2.7 HPLC METHOD DEVELOPMENT OF Rutin

Selection of Analytical wavelength:

Stock solution of both the marker and isolated constituent was prepared in methanol. Rutin showed maximum absorbance at 274 nm.

Instrumentation

The analysis was performed on JASCO Quaternary gradient pump system. It is equipped with four prominence LC Net II pump, and a UV – 2075 UV/Vis detector. Data acquisition was performed by using crome NAV software.

Chromatographic condition

Different mobile phases were tested in order of their polarity to find out the best conditions for separation of Rutin. The selected mobile phase containing Methanol: double distilled water (80:15) (pH-5.5) and gave acceptable retention time (RT). Detector was operated at 274 nm. The flow rate was maintained at 1.5 ml/min, with run time 10 min.

Method development

During the optimization cycle, several chromatographic conditions were attempted using Finepak sil C_{18} column (250 mm × 4.6 mm, 5 µm). Various mobile phase compositions containing different ratios of organic and aqueous phases were tried in an isocratic mode. Methanol was found optimum for the elution. Besides, double distilled water in different composition, at different pH values were attempted along with Methanol. Therefore, a mobile phase consisting of Methanol: double distilled water (80:15, *v/v*) (pH- 5.5) and pumped at a flow rate of 1.5 ml/ min, in an isocratic mode, gave good result.

2.9 VALIDATION OF THE DEVELOPED METHODS [10]:

a) Linearity

Appropriate dilutions from the above stock solution were taken in 6 different 10mL volumetric flask and the volume was made up to mark with Methanol to get a concentration ranging from 100 – 700 µg/mL. The absorbance of the resulting solutions was measured at 274 nm against reagent blank. A standard calibration curve was prepared by plotting absorbance Vs concentration and it was found to be linear over this concentration range to linear over this concentration range.

b) Recovery study

It was carried out by standard addition method at three different levels. Prepared the concentration of drug 50%, 100% and 150% and the concentration of sample solution i.e. methanolic extract 100%. Withdraw one ml from each concentration of drugs and mixed into two ml of sample solution and measured the absorbance on U.V. spectrophotometer and calculated recovery and % RSD.

c) Precision

From prepared stock solution the intraday precision were determined by estimating the corresponding response 3 times on same day , where as interday precision were determined by estimating the corresponding response on three different days over a period of one week. The results were reported in terms of Relative Standard Deviation (RSD).

d) Limit of Detection (LOD)

The detection limit of an individual analytical procedure is the lowest amount of analyte in a sample which can be detected but not necessarily quantitated as an exact value. The detection limit is usually expressed as the concentration of analyte (percentage parts per million) in the sample.

LOD = 3 × SD / slope of calibration curve, SD = Standard deviation of intercepts

e) Limit of Quantitation (LOQ)

The quantitation 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. Quantification limit is expressed as the concentration of analyte (e.g. - % ppm) in the sample**.**

LOD = 10 X SD / slope of calibration curve

Where, (SD = Standard deviation of intercept)

3) RESULT AND DISCUSSION

3.1 Qualitative chemical investigation

The Methanol extract of *Millingtonia Hortensis linn* were subjected to qualitative chemical investigation and it shown presence of Triterpenoid and Flavonoid.

3.2 Structural Elucidation of isolated compound by IR & GCMS

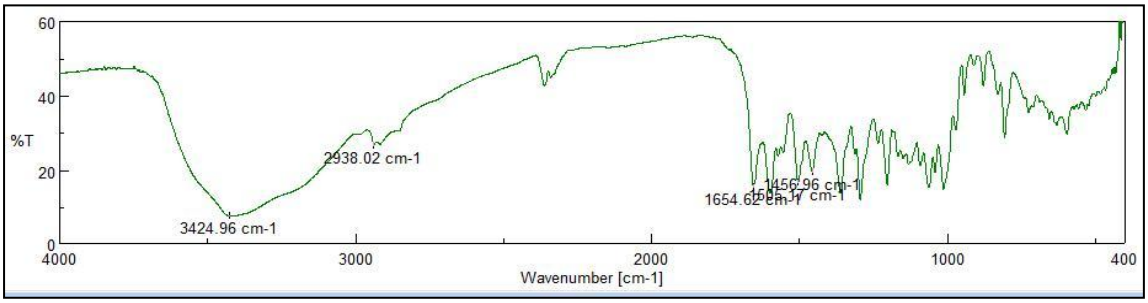


Fig 2: IR Spectra of isolated Rutin in methanolic extract

Table 1. Interpretation for IR spectra

Sr. no.	Values (cm^{-1})	Description
1	3424.96	(OH) Stretching
2	2938.02	(CH) Stretching
3	1505	(C=C)
4	1456.96	(CH) Bend
5	1654.62	(C=O)

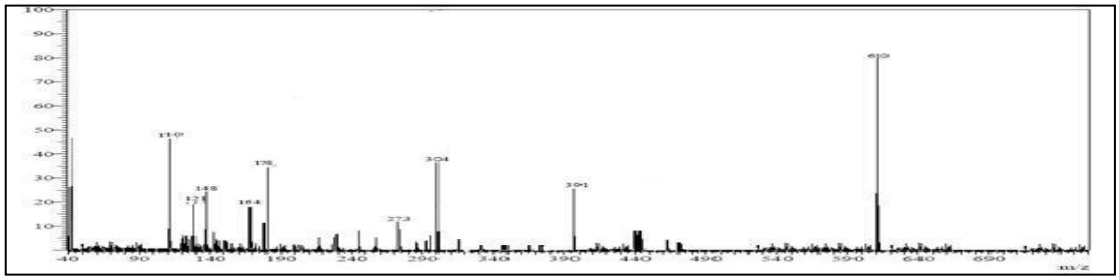


Fig. 3: MS of isolated rutin in Methanolic extract

On the results of TLC profile, FTIR studies and GC-MS studies, it confirms that isolated compound was “Rutin”.

3.3 HPLC METHOD DEVELOPMENT OF Rutin

Selection of Analytical wavelength:

Stock solution of both the marker and isolated constituent was prepared in methanol. Rutin showed maximum absorbance at 274 nm.

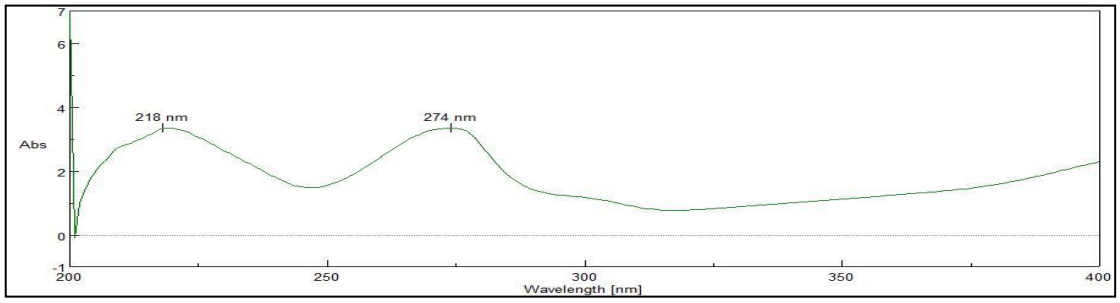


Fig 4: Spectra of Rutin

3.4 Chromatograms of isolated and standard Rutin:

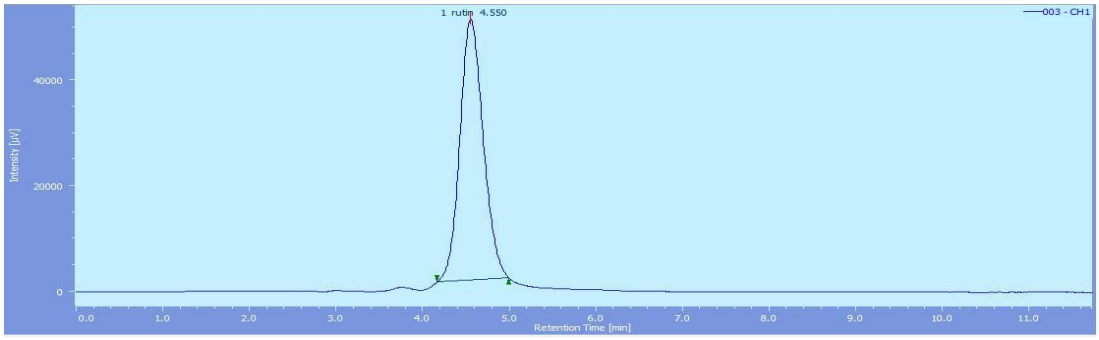


Fig 5: A Typical chromatogram of isolated Rutin

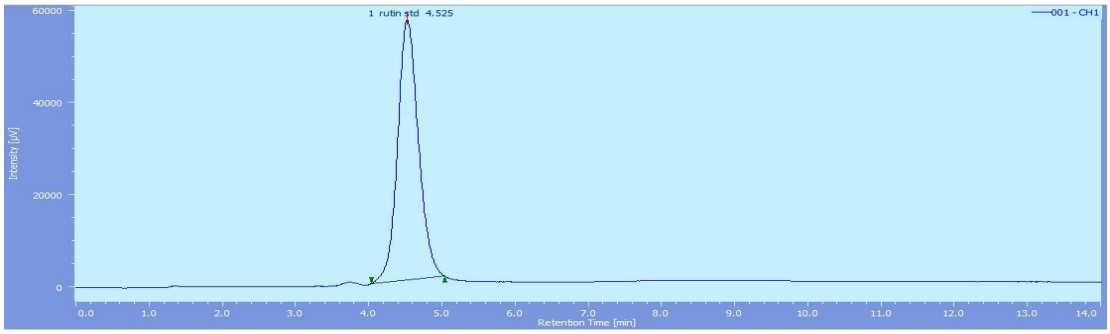


Fig 6: A Typical chromatogram of standard Rutin

3.5 Method Validation:

a) Linearity study:

Linearity was studied by preparing serial dilutions using standard stock solution as shown in dilution scheme. The linearity range for Rutin was found to be 100-700 µg/ml.

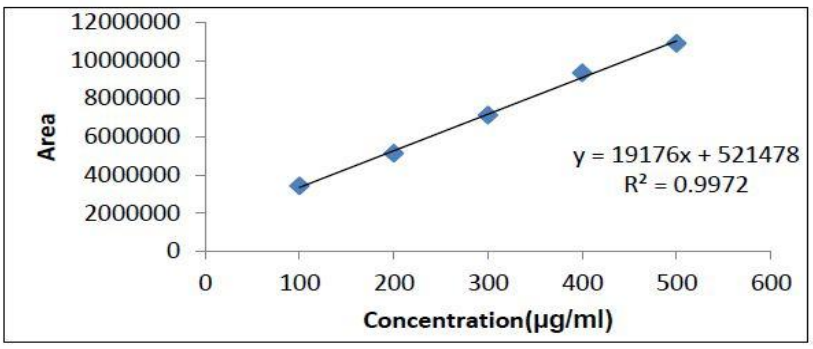


Fig 5: Plot of linearity curve for Rutin

Table 2. linear regression data for calibration curve of Rutin

Parameters	**Methods**
Drugs	Rutin
Wavelength range (nm)	274 nm
Slope (m)	19176
Intercept (b)	521478
Correlation coefficients(r2)	0.9972

b) LOD and LOQ

The LOD and LOQ were separately determined which is based on calibration curve.

Table 3. LOD and LOQ for Rutin

Parameters	Rutin
LOD	3.39 µg/ml
LOQ	10.29 µg/ml

b) Precision

Precision is determined by studying the interday and intraday precision.

Table 4. Interday and Intraday precision for Rutin

Interday Precision		Intraday precision	
%Amount found	**%RSD**	**% Amount found**	**% RSD**
82.03±0.4553	0.6497	82.17±0.5138	0.6434

c) Recovery

To check the accuracy of the proposed method, recovery studies were carried out 50, 100 and 150 % of the test concentration as per ICH guidelines. The recovery study was performed three times at each level.

Table 5. Recovery study of Rutin

Material used	**Standard Drug**	**Amount of Extract taken (%)**	**Amount of Standard drug Added (%)**	**% Mean Recovery ± S.D (n=3)**
Isolated Rutin in Methanolic extract	Rutin	100	50	85.01±0.191
		100	100	86.02±0.278
		100	150	86.23±0.504

4. CONCLUSION

In this study a simple and isocratic HPLC method was developed and validated for the quantification of rutin from the methanolic extract of leaves of *Millingtonia Hortensis linn*.

The method was validated in compliance with the International Conference on Harmonisation (ICH) guidelines (ICH-Q2) and is suitable for the determination of rutin from the extracts *Millingtonia Hortensis linn* with excellent precision, accuracy and linearity. The method is isocratic with an uncomplicated mobile phase, and the sample preparation and assay procedure are simple and rapid. Therefore, we suggest that this method can be suitable for the quantification of Rutin in quality control labs.

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