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RESEARCH ARTICLE

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COMPARISON OF EXTRACTION TECHNIQUES FOR QUANTITATIVE DETERMINATION OF RUTIN FROM *Morus alba* LINN. BY REVERSE PHASE HIGH PERFORMANCE LIQUID CHROMATOGRAPHY.

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ABSTRACT

A simple, more efficient, less time consuming extraction technique has been developed for extraction of rutin, a flavonoid, from dry leaf powder of *Morus alba* Linn. Rutin was extracted from dried leaf powder of *Morus alba* Linn., using aqueous methanol as extracting solvent, using shaking extraction technique and ultrasonic extraction technique. The aqueous methanolic extracts obtained from these two techniques, were analysed by reverse phase HPLC method for quantitation of rutin. Chromatographic analysis was carried out on Cosmosil C_{18} column (150mm X 4.6mm, 5µm). The mobile phase used was a mixture of distilled water, methanol and triethylamine, in the volume ratio of 60:40:0.1, adjusted to pH 3.10, with orthophosphoric acid, at a flow rate of 1.0 mL/min. The UV detection was carried out at 256 nm.

KEYWORDS

Morus alba Linn.; Rutin; Shaking extraction technique; Ultrasonic extraction technique; Reverse phase high performance liquid chromatography; UV detection

1. INTRODUCTION

Morus alba Linn. (Family-Moraceae) White mulberry, is a moderate sized tree, extensively cultivated throughout the plains of India and hilly areas of Himalayas, upto an elevation of 3300m[1]. The *morus* plant is rich source of the isoprenoid substituted phenolic compounds, including flavonoids [2]. The leaves of *Morus alba* Linn. contain flavonoids like rutin [3], quercetin [3] and quercetin-3-triglucoside [3]. Rutin or Vitamin P (3,3/, 4/, 5,7-pentahydroxy flavone-3-rutinoside) [3], is the only flavone, which has a clinical use and its main biological property is to antagonize the increase of capillary fragility, associated with a hemorrhagic disease and is used to treat capillary bleeding [3]. The antioxidant property of rutin, its potential use as a food preservative, has been reported [4-5]. The hypoglycemic activity of rutin has also been reported [6] and is thus one of the active constituents in the leaves of *Morus alba* Linn, which also show the hypoglycemic effect [7]. Rutin is also used to provide protection against nuclear hazards [3].

The HPLC method has been reported in literature for the detection of rutin and quercetin from the leaves of *Morus alba* Linn.[8] and to study their antioxidant property, but the method is not used for quantitation of rutin from leaf powder of *Morus alba* Linn. The present HPLC method provides a comparative quantitation of rutin from aqueous methanolic extracts of dried leaf powder of *Morus alba* Linn, obtained by using shaking extraction technique and ultrasonic extraction technique and uses a simpler mobile phase than the reported method.

2. EXPERIMENTAL

2.1. MATERIALS

2.1.1. STANDARD AND REAGENTS

Rutin hydrate, was purchased from Sigma-Aldrich Chemie GmbH (Aldrich Division, Steinbeim, Germany).

Methanol and triethylamine used in the present work were of HPLC grade and were procured from Spectrochem Pvt. Ltd. (Mumbai, India). Water used in the present research work, was purified with a Milli-Q water purifying system (Millipore, USA). The solvents were filtered through 0.5µm (Millipore) membrane and degassed in an ultrasonic bath. Orthophosphoric acid (AR grade) was procured from Qualigens Fine Chemicals (Mumbai, India).

2.1.2.. PLANT MATERIAL

The leaves of *Morus alba* Linn, were collected from wild plants found in Keshav Srushti, Mumbai, India and were authenticated from Botanical Survey of India (BSI), Pune, India. The leaves of *Morus alba* Linn. were shade dried, powdered and then sieved through BSS mesh size 85 and stored at 25°C, in an airtight container.

2.1.2.1 PREPARATION OF STANDARD SOLUTIONS OF RUTIN

Stock solution of rutin (1000.0 µg/ mL), was prepared in 10 mL standard volumetric flask, by dissolving 10.88 mg of accurately weighed rutin hydrate, in about 5.0 mL of methanol, followed by sonication for 5 minutes and finally making up the volume of solution to 10.0 mL, with methanol. 1.0 mL of the above stock solution was diluted to 10.0 mL, with mobile phase comprising of mixture of distilled water, methanol and triethylamine, in the volume ratio

of 60:40:0.1, pH adjusted to 3.10, with orthophosphoric acid, to give standard solution of rutin, with concentration of 100.0µg/mL. The aliquots (0.05 mL to 2.50 mL) of 100.0 µg/mL solution of rutin, were transferred to 10.0 mL volumetric flasks and the volume of each flask was made upto 10.0 mL, with mobile phase, to obtain the working standard solutions of rutin, in the concentration range of 0.50 µg/mL to 25.00 µg/mL.

2.1.2.2. PREPARATION OF SAMPLE SOLUTIONS

Extraction of rutin from dried leaf powder of *Morus alba* Linn, was carried out using shaking extraction technique and ultrasonic extraction technique.

2.2. EXTRACTION TECHNIQUE

2.2.1. SHAKING EXTRACTION TECHNIQUE

About 0.10 g of leaf powder of *Morus alba* Linn, was accurately weighed in a stoppered tube. To the stoppered tube, 10.0 mL of aqueous methanol (40%) was added and the tube was shaken at 20 rpm on a rotary shaker (Best Engineering, Mumbai, India), for 60.0 min. The contents of the tube, were filtered through Whatman filter paper no. 41 (Merck, India). The filtrate was diluted to 10.0 mL with aqueous methanol (40%).

2.2.2. ULTRASONIC EXTRACTION TECHNIQUE

About 0.10 g of dry leaf powder of *Morus alba* Linn, was accurately weighed in a stoppered tube. To the stoppered tube, 10.0 mL of aqueous methanol (40%) was added and the tube was sonicated in an ultrasonic bath (Model: TRANS-O-SONIC, Frequency: 50 Hz, Pawan trading corporation, India), for 15.0 min. The contents of the tube, were filtered through Whatman filter paper no. 41(Merck, India). The filtrate was diluted to 10.0 mL with aqueous methanol (40%).

2.3. INSTRUMENTATION

HPLC analysis of both the extracts was performed with a Jasco HPLC system, consisting of PU-980 Isocratic Intelligent HPLC pump, AS 1555-10 autosampler, having 20 µl loop and UV-970 Intelligent detector. A pH meter (Labindia, Mumbai, India) was used for pH control.

2.3.1. CHROMATOGRAPHIC CONDITIONS

A Cosmosil C_{18} reversed phase column (150mm x 4.6mm, 5µm), was used for the chromatographic analysis of both the extracts. The mobile phase comprised of a mixture of distilled water, methanol and triethylamine, in the volume ratio of 60:40:0.1. The pH of the mobile phase was adjusted to 3.10 with orthophosphoric acid and flow rate of the mobile phase was maintained at 1.0 mL/min. The detection wavelength was 256 nm, in accordance to UV spectra of rutin [3]. The injection volume was 20 µL.

3. METHOD VALIDATION

3.1. LINEARITY

Linearity was evaluated by injecting six different concentrations in the range of 0.50µg/mL to 25.00µg/mL of working standard solutions of rutin. Each solution was injected five times and the values of peak areas of rutin and mean peak area of rutin for each concentration were recorded. The calibration curve was obtained by plotting a graph of mean peak areas vs. corresponding concentrations of rutin. The results indicated in Table 1.0, show that within the concentration range indicated, there was a good correlation between mean peak area and concentration of rutin.

3.2. LIMIT OF DETECTION (LOD) AND LIMIT OF QUANTITATION (LOQ)

The limit of detection was determined at a signal to noise ratio of 3:1. The limit of quantitation was determined at a signal to

noise ratio of 10:1. The LOD and LOQ values obtained are listed in Table 1.

3.3. PRECISION

The method was validated in terms of repeatability, intermediate precision (inter-day precision) and accuracy. The repeatability of the method was assessed by carrying out the Instrumental precision and Intra-assay precision (Intra-day precision).

The instrument precision was studied by injecting standard solution of rutin of concentration 15.0µg/mL, in ten replicates, in the chromatographic system under the specified conditions.

The Intra-day precision and the Inter-day precision of the method were evaluated, by injecting each concentration of 3.0 µg/mL, 15.0 µg/mL and 22.0 µg/mL of standard rutin solution thrice in the chromatographic system, on the same day and on three successive days.

The results expressed as % R.S.D. of peak area of rutin, are listed in Table 1. The results indicate that the method is precise and reproducible.

Table 1

Method validation parameters for the estimation of rutin by the proposed HPLC method

Parameter	Results
Linear range (n = 5) µg/mL	0.50 - 25.00
Correlation coefficient	0.9998
LOD µg/mL	0.025
LOQ µg/mL	0.50
Instrumental precision % R.S.D. (n=10)	0.58
Intra-day precision % R.S.D. (n=3)	0.07
Inter-day precision % R.S.D. (n=3)	0.09

3.4. SYSTEM SUITABILITY

System suitability was carried out to verify that the resolution and reproducibility of the system were acceptable for the analysis.

System suitability test was carried out by injecting 20 µL of rutin solution of concentration 10.0µg/mL in five replicates in the chromatographic system under specified conditions. The chromatograms were recorded. The values of percent relative standard deviation of peak area of rutin, retention time of rutin and peak asymmetry was taken as an indicator of system suitability and the results obtained are listed in Table 2.

Table 2
System suitability data for estimation of rutin, by the proposed HPLC method

Parameter	*Mean values	%RSD
Peak Area of rutin	46130	0.55
Retention time (Rt) of rutin (min)	8.27	0.14
Peak asymmetry of rutin	1.21	0.98

**Mean for n = 5 determinations*

All the values for standard solution of rutin, lie within the acceptable range, indicating suitability of the system.

3.5. DETERMINATION OF RUTIN FROM DRIED LEAF POWDER OF Morus alba Linn.

20 µL of each of the sample solutions, obtained by shaking extraction technique and ultrasonic extraction technique, from the dried leaf powder of *Morus alba* Linn., were injected in seven replicates, in HPLC system under the specified chromatographic conditions and peak areas of rutin were recorded for each sample. The amount of rutin was thus determined, for both the extraction techniques.

Table 3
Average contents of rutin in dried leaf powder Morus alba* Linn.*, with different extraction techniques

Sample	Weight of sample (g)	Extraction technique	Time for extraction	Average* contents of rutin found in sample (µg)	Average percent contents of rutin (%)	%RSD
Dried leaf powder of *Morus alba* Linn.	0.10	Shaking extraction technique	60.0 min	1.11	0.0111	1.02
		Ultrasonic extraction technique	15.0 min	1.52	0.0152	0.88

**Average value from n = 7 determinations.*

Figure 1

HPLC Chromatogram of standard rutin

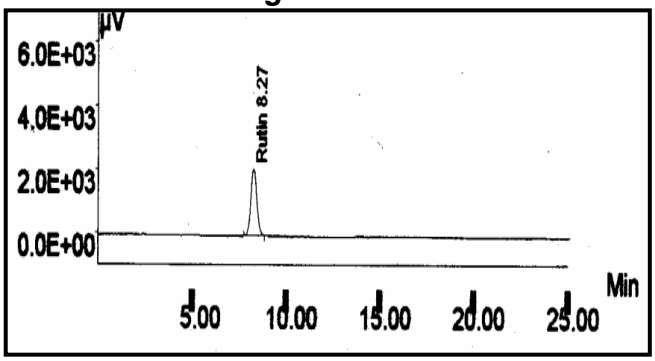
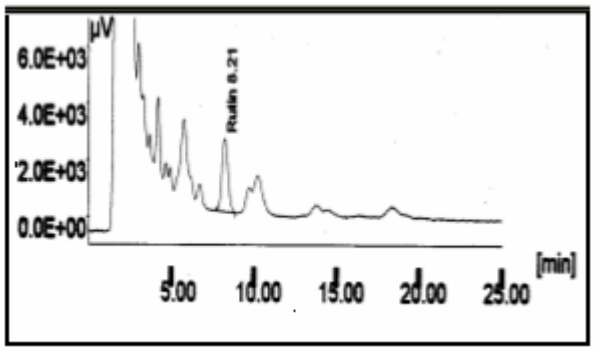


Figure 2

HPLC Chromatographic pattern of aqueous methanolic extract of dried leaf powder of Morus alba Linn., obtained by ultrasonic extraction technique.



3.6. ACCURACY

Accuracy of the method was tested by performing recovery studies at three different levels for rutin. To accurately weighed 0.10 g of leaf powder of *Morus alba*, known amounts of rutin (1.5 µg, 3.0µg and 4.5µg), were added, extracted using aqueous methanol (40%) by shaking extraction technique and ultrasonic extraction technique and the extracts were estimated by HPLC method, under the specified chromatographic conditions, as described above. The values of percent recovery and average percent recovery for rutin were calculated. The results are recorded in Table 4.

Table 4

Results for recovery experiment, using shaking extraction and ultrasonic extraction technique

Weight of leaf powder of *Morus alba Linn.* (g)	Amount of rutin found by shaking extraction (µg)	Amount of rutin found by ultrasonic extraction (µg)	Amount of rutin added (µg)	Amount of rutin recovered (µg)		Percent recovery (%)		Average percent recovery (%)	
				Shaking extraction	Ultrasonic extraction	Shaking extraction	Ultrasonic extraction	Shaking extraction	Ultrasonic extraction
0.1003	1.11	1.51	1.50	2.50	2.99	95.79	99.34		
0.1002	1.10	1.51	3.00	3.95	4.50	96.34	99.78	96.96	99.32
0.1004	1.13	1.52	4.50	5.56	5.95	98.76	98.84		

3.7. SOLUTION STABILITY

The stability of standard rutin solution was determined by comparing the peak areas of rutin solution, of concentration 10.0 µg/mL, at different time intervals, for a period of minimum 48 hrs, at room temperature. The results showed that the peak area of rutin almost remained unchanged (% R.S.D. was less than 2) over a period of 48 hrs, and no significant degradation was observed within the given period, indicating the stability of standard solution of rutin, for minimum 48 hrs.

4. RESULTS

The identity of peak of rutin in sample solutions, prepared by shaking extraction technique and ultrasonic extraction technique, was confirmed by comparing the retention times of rutin in these chromatograms, with that of retention time of standard rutin (8.27 min), (Figure 1). A good linear relationship was obtained for rutin, in the concentration range of 0.50 µg/mL to 25.00 µg/mL, with correlation coefficient of 0.9998. The average percent recoveries of rutin at three levels, for shaking extraction technique and ultrasonic extraction technique were 96.96 and 99.32 respectively (Table 4). The extraction by ultrasonic extraction technique required 15 min, while time required for shaking extraction technique was 60 min. Figure 2, represents a typical chromatogram of aqueous methanolic extract of dried leaf powder of *Morus alba* Linn. obtained by ultrasonic extraction technique. The assay results indicate that average percent contents of rutin estimated by shaking extraction techniques and ultrasonic extraction techniques, were 0.0111% and 0.0152% respectively, indicating greater extraction efficiency of rutin by ultrasonic extraction technique (Table 3).

5. DISCUSSION

HPLC method reported in literature uses a chromatographic system, coupled on-line with colorimetric detection and is used to study antioxidant properties of rutin and quercetin, from *Sophora japonica* and *Morus alba*. The method uses MeCN: MeOH: 25mM KH_2PO_4, in the volume ratio of 20: 15: 65, at a pH 3.0, as the mobile phase, with UV detection at 220 nm, using C_8 analytical column, combined with a C_{18} guard column 8. The quantitation of rutin is however not carried out in this method.

As extraction of phytochemicals from various plant parts, using different solvents, is a time consuming and laborious process, in the present research work, efforts are made to develop a simpler, more efficient and less time consuming extraction technique, for extraction of rutin from dried leaf powder of *Morus alba* Linn. The aqueous methanolic extracts of dried leaf powder of *Morus alba* Linn. were prepared by using shaking extraction technique and ultrasonic extraction technique and were analysed by HPLC, using a mixture of distilled water, methanol and triethylamine, as the mobile phase, in the volume ratio of 60: 40: 0.1, with pH adjusted to 3.10 with orthophosphoric acid. The column used was reversed phase, Cosmosil C_{18} (150mm X 4.6mm, 5µm), and detection was carried out at 256 nm. Methanol used in the present work is water miscible, has low viscosity, low surface tension and is readily obtained in pure form. The addition of triethylamine to mobile phase helped to improve the peak shape of rutin. The extraction times required for extraction of rutin from *Morus alba* Linn.leaves, using shaking extraction technique and ultrasonic extraction technique, were 60.0 min and 15.0 min respectively.

A comparison of average percent contents of rutin in extracts obtained by two techniques, indicated that ultrasonic extraction technique was a better extraction technique than shaking extraction technique and requires less time for extraction of rutin, with a better extraction efficiency.

6. CONCLUSION

Quantitative analysis of rutin by HPLC method, from aqueous methanolic extracts of dried leaf powder of *Morus alba* Linn., using shaking extraction technique and ultrasonic extraction technique, indicated that ultrasonic extraction technique has more extraction efficiency, in less time.

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