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HIGH PERFORMANCE LIQUID CHROMATOGRAPHIC METHOD FOR SIMULTANEOUS ESTIMATION OF RUTIN AND MYRICETIN FROM DRIED LEAF POWDER OF *SYZYGIUM JAMBOS* (L.) ALSTON

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ABSTRACT

A simple RP-HPLC method has been developed, validated and used for simultaneous quantitative determination of two flavonoids viz. rutin and myricetin from the dried leaf powder of *Syzygium jambos* (L.)Alston. The chromatographic separation was performed on a Spinco C18 G column (250 mm x 4.6 mm, 5 µm) with mobile phase comprising of 0.3% trifluoroacetic acid in distilled water and methanol (55.0:45.0 v/v), delivered at the flow rate of 1.0 mL/min. The detection and quantitation of both the compounds was carried out using a PDA detector at λ= 254 nm. The method is precise as the value of percent RSD was found to be less than 2.The accuracy of the developed HPLC method was checked by carrying out the recovery experiment at three different levels, by using standard addition method. The percentage recovery was found to be 98.45 for rutin and 99.05 for myricetin indicating the accuracy of the method.

KEYWORDS: rutin, myricetin, *Syzygium jambos* (L.) Alston., HPLC.

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INTRODUCTION

Syzygium jambos (L.) Alston. is naturalized or cultivated in many parts of the world. It is a moderate sized evergreen tree from the family Myrtaceae, known for its edible, juicy fruits. Its leaves are glabrous, lanceolate, pointed, base cuneate with hardly any petiole, lively red when growing, but dark, glossy green on attaining full size.[1] All parts of the plant viz. stem bark, leaves, seeds are known to possess medicinal properties.[2] The leaves are given in decoction as a diuretic, as an expectorant, are used in the treatment of rheumatism and to treat sore eyes.[3, 4] The leaves of *Syzygium jambos* (L.) Alston. are known to possess antinociceptive,[5] anti-inflammatory[6] anti ulcer & anti oxidant[7] and hepatoprotective activity.[8] There are reports available confirming presence of flavonoids in various species of Myrtaceae family.[9] Rutin and myricetin belong to the group of flavonoids. Flavonoids are important for human beings due to their anti-oxidative and radical scavenging effects as well as their potential estrogenic and anticancer activities.[10] A large number of natural flavonoids possess anti-inflammatory activity.[11] Rutin is a natural flavonol, which possesses a wide range of pharmacological properties that have been exploited in human medicine and nutrition. Conventionally, it is used as an antimicrobial, antifungal, and anti-allergic agent.[12] It is reported to have antioxidant & anti-hyperglycemic[13] and anti-obesity activity.[14] The potential of rutin in mitigating radiation-induced mortality is reported, which may be attributed to the elevation in the antioxidant status.[15] Myricetin has antioxidant, antidepressant, anti-inflammatory and antidiabetic activities.[16,17] It is shown to have potent anticancer and chemopreventive effects.[18,19] Some HPLC methods were found to be reported for the simultaneous determination of rutin and myricetin from different plants. A reverse phase HPLC method with diode array detection is reported for evaluating the quality of *Ginkgo biloba* Linn. extract by establishing chromatographic fingerprint and simultaneously determining eight flavonoid compounds, including rutin and myricetin.[20] RP- HPLC analysis of flavonoids from curry leaf (*Murraya koenigii*. Linn.) by using diode array detector is reported for assessment of four major bioactive flavonoids including rutin and myricetin.[21] RP-HPLC method to quantify free phenolic acids and flavonoids from walnut leaves including rutin and myricetin has been reported.[22] Quantitative HPLC analysis of phenolic acids, ascorbic acid and flavonoids including rutin and myricetin is reported in four different solvent extracts of two wild edible leaves, *Sonchus arvensis* Linn. and *Oenanthe linearis* Wall. ex DC. from North-Eastern region in India.[23] However, no HPLC method has been reported in literature for simultaneous quantitation of rutin and myricetin from dried leaf powder of *Syzygium jambos* (L.) Alston. Hence, a simple, precise and accurate HPLC method has been developed and validated using International Conference on Harmonization (ICH) guidelines for simultaneous estimation of rutin and myricetin from dried leaf powder of *Syzygium jambos* (L.) Alston.

MATERIALS AND METHODS

Standards, Reagents and Chemicals

The reference standards rutin hydrate (purity ≥94.0% HPLC Grade) and myricetin (purity ≥96.0% HPLC Grade) were purchased from Sigma-Aldrich Chemie GmbH (Aldrich Division, Steinbeim, Germany).All the solvents used in the analysis were of HPLC grade. Methanol (purity- 99.7%), trifluoroacetic acid (purity- 99.8%) and distilled water used were procured from LiChrosolv Merck, India. HCl pure (35-38%) was procured from LOBA Chemie, Mumbai, India.

Plant material

Fully grown leaves of *Syzygium jambos* (L.) Alston. were collected from a domestic garden in Alibaug, District Raigad, Maharashtra, India, in the month of February. The leaves were washed with water to remove soil particles, dried in shade, and powdered. The powder was passed through BSS 85 mesh sieve and stored in an airtight container at room temperature (28 ± 2 °C).

Authentication

A herbarium of *Syzygium jambos* (L.) Alston. was prepared and authenticated from Botanical Survey of India, Pune, India. (Certificate No. BSI/WC/Tech/2012/70) Duplicate herbarium was prepared and preserved in Ramnarain Ruia College.

Preparation of solutions

Preparation of stock standard solution of rutin (1000.0 µg/mL)

About 51.50 mg of rutin hydrate equivalent to 50.0 mg of rutin was accurately weighed and transferred to 50.0 mL volumetric flask. 20.0 mL of methanol was added and the contents of the flask were sonicated in an ultrasonic bath (Model: TRANS-O-SONIC, Frequency: 50 Hz) for 10 minutes for complete dissolution of rutin. The contents were then diluted up to the mark with methanol to obtain stock solution of rutin with concentration of 1000.0 µg/mL.

Preparation of stock standard solution of myricetin (1000.0µg/mL)

About 50.0 mg of myricetin was accurately weighed and transferred to 50.0 mL volumetric flask. 20.0 mL of methanol was added and the contents of the flask were sonicated for 10 minutes for complete dissolution of myricetin. The contents were then diluted up to the mark with methanol to obtain stock solution of myricetin with concentration of 1000.0 µg/mL.

Preparation of sample solution

About 1.0 g of dried leaf powder of *Syzygium jambos* (L.) Alston. was accurately weighed and transferred to 50.0 mL stoppered conical flask. 9.9 mL of methanol and 0.1 mL of A.R. HCl were added to it. The flask was kept in a hot water bath (50 °C) for 10 minutes and then cooled to room temperature (28±2^{0}C). Further, sample solution was filtered through Whatman filter paper no. 41. The filtrate was then finally filtered using 0.45 µm nylon filters (Millipore) before the analysis.

Preparation of mobile phase

The mobile phase used in the present research work for simultaneous quantitation of rutin and myricetin from dried leaf powder of *Syzygium jambos* (L.) Alston was comprising of 0.3% trifluoroacetic acid in distilled water

and methanol (55.0:45.0 v/v). The mobile phase was degassed in an ultra-sonic bath ((Model: TRANS-O-SONIC, Frequency: 50 Hz) for 10 min.

HPLC conditions

Chromatographic separation was carried out with Shimadzu UFLC Prominence chromatograph, equipped with binary gradient pump (LC-20AD), fitted with auto sampler (SIL-20 AC HT) and oven (CTO-20 AC) having PDA detector (SPD-M20A). The chromatograms and data were recorded using LC solutions Software. Analysis was performed on a Spinco C18 G column (250 mm x 4.6 mm, 5 µm) at a temperature 40^0C and mobile phase comprising of 0.3% trifluoroacetic acid in distilled water and methanol (55.0:45.0 v/v), was used for the analysis. The system was run at a flow rate of 1.0 mL/min, 10.0 µL of sample was injected into the chromatographic system and the detection was done at 254 nm. The proposed HPLC method was validated and applied for the quantitative determination of rutin and myricetin from dried leaf powder of *Syzygium jambos* (L.) Alston.

METHOD VALIDATION

Linearity

Linearity of standard rutin was evaluated by injecting different concentrations in the range of 1.0 µg/mL to 500.0 µg/mL of rutin. Each solution was injected three times; the values of peak areas of rutin for each concentration were recorded and mean peak area was calculated. Similarly, linearity of standard myricetin was evaluated by injecting different concentrations in the range of10.0 µg/mL to 800.0 µg/mL of myricetin. Each solution was injected three times; the values of peak areas of myricetin for each concentration were recorded and mean peak area was calculated. The calibration curves of rutin and myricetin were obtained by plotting graphs of mean peak area against corresponding concentration of both the standards.

Limit of Detection and Limit of Quantitation

The limit of detection (LOD) and limit of quantitation (LOQ) were determined at signal to noise ratios of 3:1 and 10:1, respectively. Limit of Detection for rutin and myricetin were found to be 0.05µg/mL and 0.1µg/mL respectively and the Limit of Quantitation for rutin and myricetin were found to be 1.0 µg/mL and 10.0 µg/mL respectively**.**

System Suitability

The system suitability test was carried out to confirm that the chromatographic system used to carry out the analysis gives precise, accurate and reproducible results. System suitability was determined by injecting 10.0 µL of the 1:1 mixture of standard solutions of rutin and myricetin with the concentration 250.0 µg/mL each, six times into the chromatographic system, under the optimized chromatographic conditions. The chromatograms were recorded and the peak area values and the retention times of rutin and myricetin were noted for each injected concentration of both the standards. As the values of percent relative standard deviations for peak areas and retention times of both the standards were found to be less than 2, the system was found to be suitable. The system suitability parameters like peak tailing, resolution between two peaks and column efficiency were evaluated and found to be within acceptable limits for injected concentrations of both the standards rutin and myricetin.

Precision

The method was validated in terms of instrumental precision, repeatability, and intermediate precision. Instrumental precision was studied by separate, repetitive analysis (n=10) of the standard solutions of rutin and myricetin, using the proposed HPLC method. 10.0 µL of rutin solution with concentration 50.0µg/mL was injected ten times into the chromatographic system, under optimized chromatographic conditions and the values of peak areas of rutin for each replicate analysis were recorded. The method was repeated in the same way using myricetin standard solution of concentration 50.0 µg/mL. The values of mean peak area, standard deviation and percentage relative standard deviation were calculated for rutin and myricetin. The results are shown in Table 1.The repeatability was evaluated by analyzing six sample solutions of the dried leaf powder of *Syzygium jambos* (L.) Alston. A methanolic extract was prepared as described in the sample preparation. 10.0 µL of each of the sample solution was injected into the chromatographic system in triplicate on the same day, in the same laboratory, under the same specified chromatographic conditions. The peak areas of rutin and myricetin were recorded. The intermediate precision of the method was evaluated by analyzing six sample solutions in triplicate on three different days, in the same laboratory under the specified chromatographic conditions. The peak areas of rutin and myricetin were recorded. The values of mean peak area of rutin and myricetin, standard deviation (S.D.) and percent relative standard deviation (% R.S.D.) were calculated and are listed in Table 1. The precision results indicate that the proposed method is precise and reproducible.

Solution Stability

The stabilities of standard rutin and myricetin solution were determined by comparing the peak areas of rutin and myricetin solution, of concentration 10.0 µg/mL, at different time intervals, for a period of minimum 48 hrs. The results showed that the peak areas of rutin and myricetin almost remained unchanged (values of percent relative standard deviation were less than 2) over a period of 48 hrs, and no significant degradation was observed within the given period, indicating the stability of standard solutions of rutin and myricetin for minimum 48 hrs.

Table 1
Method validation data for simultaneous quantitation of rutin and myricetin

Parameters	Observations	
	rutin	myricetin
Linear Working Range	1.0 to 500.0 µg/mL	10.0 to 800.0 µg/mL
Correlation coefficient	0.999	0.999
Limit of Detection (LOD)	0.05µg/mL	0.1µg/mL
Limit of Quantitation (LOQ)	1.0 µg/mL	10.0 µg/mL
Stability of standard solution	Stable for minimum 48 hours	
Instrumental precision(n=10)	0.2384	0.2320
Repeatability % R.S.D. (n=6)	1.0795	1.0303
Intermediate precision % R.S.D. (n=6)	1.3565	1.2107

Specificity
Specificity is the ability of the method to measure the analyte response in the presence of other impurity. The specificity of the proposed HPLC method was ascertained by injecting 10.0 µL of blank into the chromatographic system under specified conditions to observe for interference, if any, with the peaks of interest in the chromatogram of the standard mixture. (Figure1). It was observed that there is no interference from the blank solution. Methanol was taken as blank solution since standard and sample solutions were prepared in methanol.

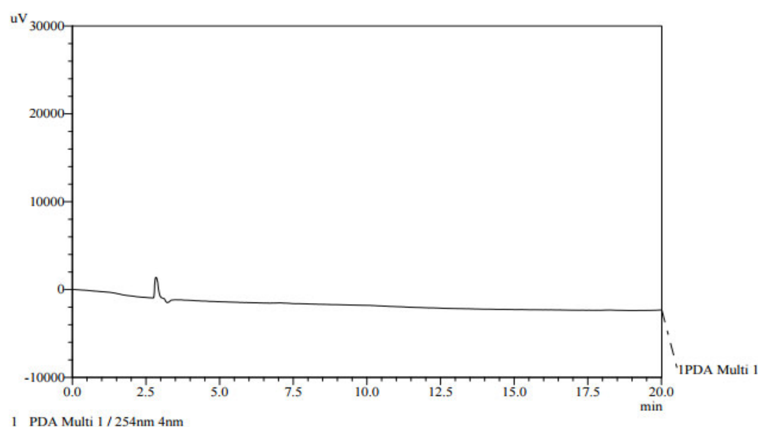

Figure 1
HPLC chromatogram obtained for blank (methanol)

Figure 2 represents HPLC chromatogram obtained for the mobile phase by injecting 10.0 µL into the chromatographic system under specified conditions.

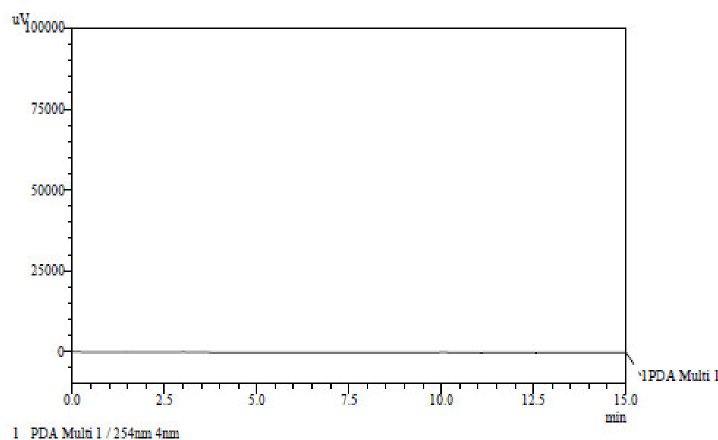

Figure 2
HPLC chromatogram obtained for mobile phase

Figure 3 represents HPLC chromatogram obtained for a mixture of standard rutin and myricetin by injecting 10.0 µL into the chromatographic system under the specified conditions.

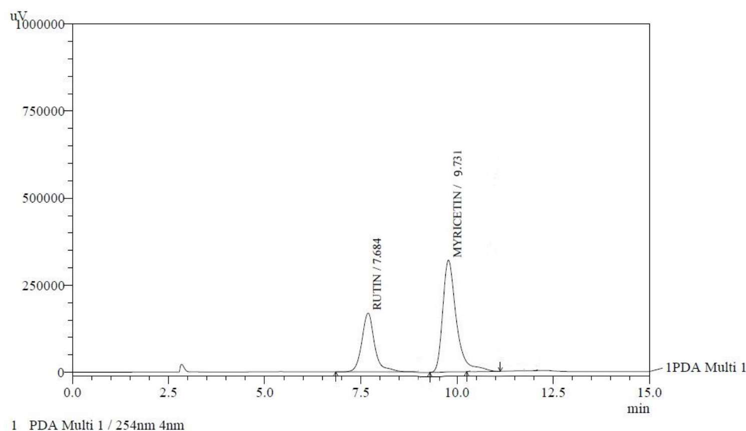

Figure 3
HPLC chromatogram obtained of standard rutin and myricetin

Robustness
Robustness means examining the effect of variations in the operational parameters on the analysis results. Robustness of the method was determined by making small deliberate changes in the chromatographic conditions utilized in present method. By introducing small changes in the mobile phase composition (± 2 mL of aqueous phase) and flow rate (± 0.1 mL/min.) the effects on the results were examined. The amounts of rutin and myricetin from dried leaf powder of *Syzygium jambos* (L.) Alston. obtained by altered method to that obtained by normal method were found to be similar. It was concluded that the method is robust as the above mentioned deliberate changes made in the method parameters did not affect the results significantly.

Assay procedure
The developed and validated HPLC method was used for quantitation of rutin and myricetin from the methanolic extract of dried leaf powder of *Syzygium jambos* (L.) Alston. 10.0 µL of methanolic extract of the dried leaf powder of *Syzygium jambos* (L.) Alston. (n=7) was injected separately into the chromatographic system under the specified conditions. Figure 4 represents HPLC chromatogram obtained for a sample solution of the dried leaf powder of *Syzygium jambos* (L.) Alston. by injecting 10.0 µL into the chromatographic system under specified conditions.

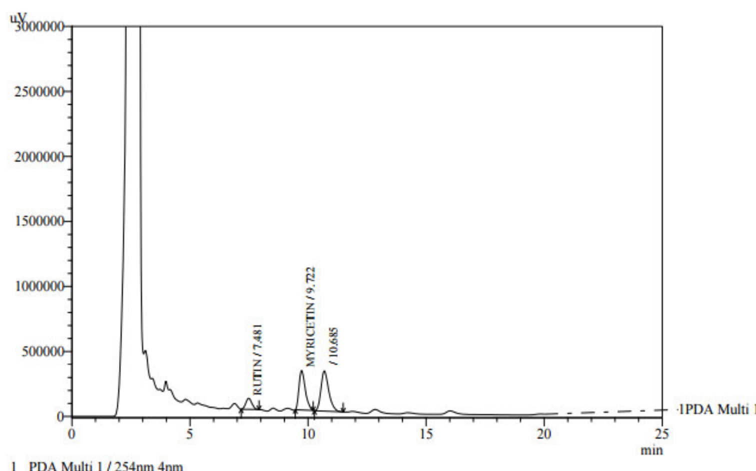

Figure 4
HPLC chromatogram of dried leaf powder of Syzygium jambos (L.) Alston.

Amounts of rutin and myricetin present in the sample solution were determined from the calibration curves, by using the peak area of rutin and myricetin in the sample solution. The results are presented in Table 2.

Accuracy
The accuracy of the method was established by performing recovery experiment by using standard addition method at three different levels. To accurately weighed about 1.0 g of dried leaf powder of *Syzygium jambos* (L.) Alston., known amounts of standard rutin (0.1 mg, 0.2 mg, 0.3 mg) and myricetin (0.1 mg, 0.2 mg, 0.3 mg), were added, and extracted using methanol as described earlier. Each of the three different levels containing sample solution and standards was injected in seven replicates. The samples were analyzed under the specified chromatographic conditions, as described above. The rutin and myricetin contents were quantified and the percentage recovery was calculated. The percent recovery values were then calculated. The results of accuracy are presented in Table 2.

Table 2
Results for Assay and Recovery

Parameters	Observations	
	rutin	myricetin
Assay (mg/g)	0.7648	2.2675
Percent Recovery	98.45	99.05

DISCUSSION

In literature, a reverse phase HPLC method with diode array detection was developed to evaluate the quality of *Ginkgo biloba* Linn. extract by simultaneous determination of eight flavonoid compounds, including rutin and myricetin. The chromatographic separation was performed on an Agilent SB-C18 column (250×4.6 mm, 5.0 µm) with a gradient elution program using a mixture of methanol and 0.1% formic acid (v/v) as mobile phase within 55 min at 360-nm wavelength.[19] In this method, a gradient elution program was used and the retention times of rutin and myricetin were about 18 min. and 24 minutes respectively. In the present research work, a simple isocratic elution is used and retention times of rutin and myricetin are about 7 min. and 9 min. respectively, so the developed method is simple and rapid.Reverse phase-high performance liquid chromatography-diode array detector (RP-HPLC-DAD) method was developed for assessment of four major bioactive flavonoids including rutin and myricetin, from curry leaf (*Murraya koenigii*. Linn.)[20] Separation was achieved on a reversed phase column (ZORBAX, Eclipse plus-C18,5µm, 4.6 × 150 mm, Agilent, USA) and methanol:acetonitrile:water:acetic acid (40:20:39:1, v/v/v/v) was employed as the eluent. In the present research work, the mobile phase is made up of only three components and more amount of water is used. Also, the amount of TFA needed to improve the peak shapes was only 0.3%.A RP- HPLC method to quantify free phenolic acids and flavonoids including rutin and myricetin from walnut leaves has been reported, using a Hypersil Gold C 18 column (5 µm particle size, 250 × 4.6 mm) with a gradient elution program using diode array detection. [21]A reversed-phase high-performance liquid chromatographic method using photodiode array detector with gradient elution has been developed and validated for the simultaneous estimation of ascorbic acid, free phenolic acids and flavonoids including rutin and myrecetin in four different solvent extracts of two wild edible leaves of *Sonchus arvensis* and *Oenanthe linearis*, collected from North-eastern region in India. The chromatographic separation was carried out on Acclaim C 18 column (5 µm particle size, 250 x 4.6 mm), Dionex Ultimate 3000 liquid chromatograph using a mobile phase of acetonitrile and 1% aqueous acetic acid solution with gradient elution.[22]In these methods reported above, gradient elution has been used, but in this work isocratic elution is used which is a simpler method. The mobile phase selected for the present research study is simple, containing only three components, 0.3% trifluoroacetic acid: methanol (55.0:45.0 v/v).Different mobile phases were tried for simultaneous HPLC separation of rutin and myricetin from other components of the dried leaf powder of *Syzygium jambos* (L.) Alston. and good separation was achieved by using mobile phase comprising of 0.3% trifluoroacetic acid in distilled water and methanol (55.0:45.0 v/v). Rutin and myricetin are strongly polar compounds hence mobile phase comprising of more amount of water which is more polar as compared to methanol was used. The addition of 0.3% trifluoroacetic acid to mobile phase helped to improve the peak shapes of rutin and myricetin. Detection was carried out at λ = 254 nm as both rutin and myricetin showed maximum response at this wavelength. The identity of the peaks of rutin and myricetin in the sample solutions was confirmed by comparing their retention times in sample with that of reference standards. The retention times for rutin and myricetin in the standard mixture were 7.684 minutes and 9.731 minutes respectively and in the sample solution of the dried leaf powder of *Syzygium jambos* (L.) Alston., 7.481minutes and 9.722 minutes respectively. The developed method provided a good separation of the phyto constituents with the resolution (Rs) of 4.556 whereas the tailing factors for rutin and myricetin are 1.124 and 1.408 respectively. The resolution and tailing factor values lie within the acceptable limits.A good linear relationship was observed for rutin and myricetin in the concentration range of 1µg/mL to 500µg/mL and 10 µg/mL to 800 µg/mL respectively with correlation coefficient 0.999 for both the components (Table 1). When the method was validated for instrumental precision, repeatability and intermediate precision, the values of percentage relative standard deviations were less than 2, indicating the proposed method is precise and repeatable (Table 1).The mean amounts of rutin and myricetin found in the dried leaf powder of *Syzygium jambos* (L.) Alston.were 0.7648 mg/g and 2.2675 mg/g respectively with percent recoveries 98.45 and 99.05 for rutin and myricetin. In the present work, the peaks of rutin and myricetin are well separated even when more quantity of aqueous phase is used and the retention times of rutin and myricetin are less compared to the reported methods for simultaneous quantitation of rutin and myricetin. The developed HPLC method is simple and fast as compared to the HPLC methods reported in the literature. The developed HPLC method was validated using ICH guidelines.

CONCLUSION

The developed HPLC method is precise and accurate and can be used for the routine quality control analysis and simultaneous estimation of rutin and myricetin from dried leaf powder of *Syzygium jambos* (L.) Alston.

CONFLICT OF INTEREST

Conflict of Interest declared none.

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