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A sensitive, rapid liquid chromatography tandem mass spectrometry (LC-MS-MS) method for simultaneous determination of rutin and quercetin in *Artocarpus lakoocha* Roxb. bark

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ABSTRACT

A rapid, sensitive and accurate liquid chromatographic tandem mass spectrometric method is described for simultaneous determination of rutin and quercetin in *A. lakoocha* Roxb. bark. Rutin and quercetin was extracted from the plant using methanol. The chromatographic separation was achieved using a Thermo Hypurity C18 (50×4.6 mm) 5.0 µ column interfaced with a triple quadrapole mass spectrometer. The mobile phase consisted of a mixture of Methanol: 10 mM ammonium acetate buffer whose pH was adjusted to 3.00 ± 0.05 with acetic acid (85:15, v/v) and was delivered at a flow rate of 05 mL min^{-1}. Electrospray ionization (ESI) source operated in the negative ion mode was used for the quantitation. Detection was performed using an Applied Biosystems Sciex API 5000 Mass spectrometer. The method was found to be simple, precise, accurate, fast, specific and sensitive and can be used for routine quality control analysis of Rutin and quercetin in *A. lakoocha* Roxb.

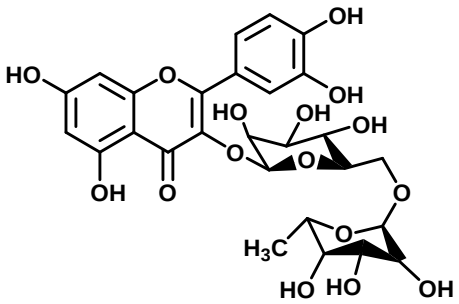
KEYWORDS

LC-MS-MS;
Rutin;
Quercetin;
Artocarpus lakoocha Roxb.

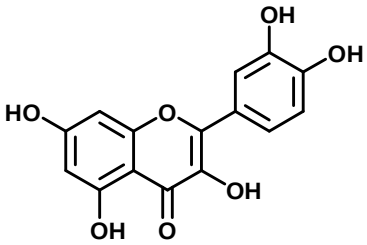
INTRODUCTION

Artocarpus lakoocha Roxb (Syn: *A. lacucha* Buch.-Ham.) is a member of the family Moraceae and is cultivated in Uttar Pradesh,Bengal, Khasi Hills and Western Ghats. It is called Monkey Jack in English and in Ayurveda it is called Lakuch, Kshudra Panas, Granthiphala and Pitanaasha. Bark when applied externally, draws out purulent matter; heals boils, cracked skin and pimples[1]. The brown powder called Puag-Haad in Thailand is a product of the aqueous extraction of *A. lakoocha* prepared by boiling the wood chips and then evaporating water away. This preparation has been used as a traditional anthelmintic drug for treatment of tapeworm infection in Thailand[2,3]. Phytochemical screening has revealed that it contains many phytochemical of which beta-sitosterol, cycloartenol, cycloartenone, a-amyrin acetate and lupeol acetate (bark); oxyresveratrol, lupeol, rutin and quercetin are few to name[4,5].

Rutin is a member of bioflavonoids, a large group of phenolic secondary metabolites of plants that include more than 2,000 different known chemicals. Bioflavonoids such as Quercetin, Rutin, and Hesperi-



(a)



(b)

Figure 1 : Structures, (a) Rutin, (b) Quercetin

din are important nutrients due to their ability to strengthen and modulate the permeability of the walls of the blood vessels including capillaries. Rutin may have antioxidant, anti-inflammatory, anticarcinogenic, antithrombotic, cytoprotective and vasoprotective activities. It is, however, much more soluble in water and methanol. Rutin's molecular formula is $C_{27}H_{30}O_{16}$, its molecular weight is 610.53 daltons, and its structural formula is shown in figure 1a[6]. Quercetin, a member of the flavonoids family, exerts many beneficial health effects, including improvement of cardiovascular health, reducing risk for cancer, protection against osteoporosis. This phytochemical has anti-inflammatory, anti-allergic and antitoxic effects. Most of these properties are linked to its strong antioxidant action of quercetin but quercetin also modulates the expression of specific enzymes. Quercetin induces apoptosis and influences protein and lipid kinase signaling pathways. Quercetin is a candidate for preventing obesity-related diseases. Its molecular formula and molecular weight is $C_{15}H_{10}O_7$ and 302.24 respectively. It is freely soluble in water and methanol its structural formula is shown in figure 1b[7].

The quality of herbal medicine that is the profile of the constituents in the final product has implication in efficacy and safety. Due to the complex nature and inherent variability of the chemical constituents of the plant

TABLE 1 : Results of recovery experiment

Standard	Level	Preanalysed Sample in (ng mL^{-1})	Amount of std added to preanalysed sample in (ng mL^{-1})	Total amount of std found in (ng mL^{-1})	SD	RSD (%) (n=7)	Recovery (%)
	0	38.044	0	38.001	0.753	1.982	99.89
Rutin	25 %	38.044	9.5	47.131	0.553	1.173	99.13
	50 %	38.044	19	56.496	0.086	0.151	99.04
						Mean	99.35
	0	30.588	0	30.564	0.541	1.769	99.92
Quercetin	25 %	30.588	7.5	37.443	0.357	0.954	98.31
	50 %	30.588	15	45.219	0.195	0.432	99.19
						Mean	99.14

based drugs, it is difficult to establish quality control parameters and modern analytical techniques are accepted to help in circumventing this problem[8]. Recently, the concept of marker-based standardization of herbal drugs is gaining momentum. Identification of major and unique compounds in herbs as markers and development of analytical methodologies for monitoring them are the key steps involved in marker-based standardization[9].

Quantitation of rutin and quercetin using different chromatographic techniques has been reported[10,11]. Literature survey, hence, revealed that there is no method available in the public domain for quantitation of rutin and quercetin from the bark of *A. lakoocha* Roxb. using an LC-MS-MS system. So, the aim of the present work was to develop a simple, fast, sensitive, precise, and accurate LC-MS-MS method for simultaneous determination of rutin and quercetin from the bark of *A. lakoocha* Roxb. The developed method was further validated as per ICH guidelines to indicate its suitability[12,13].

EXPERIMENTAL

(A) Chemicals and preparation of standard solutions

HPLC grade Methanol and acetonitrile were purchased from J.T.Baker, Mumbai, India. Extra pure Formic acid (99.9%) and ammonium formate was purchased from Fluka, Steinheim, Germany. High purity deionised water was prepared in-house using a Milli-Q water purification system obtained from Millipore,

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Bangalore, India. Standard rutin and quercetin (Purity 99 %) was procured from Sigma-Aldrich (Aldrich Division; Steinheim, Federal Republic of Germany).

Stock solutions of rutin and quercetin (1000 μg mL^{-1}) were prepared separately by accurately weighing 10 mg of both standards, transferring to two different 10 mL volumetric flask, dissolving in minimum quantity of methanol and diluting to volume with the same solvent. Further the solutions containing the mixture of rutin and quercetin were prepared using these stock solutions in methanol:water (80:20). The concentration ranges for both, rutin and quercetin in working standard solutions were 0.5 ng mL^{-1} to 100 ng mL^{-1}.

(B) Plant material and preparation of sample solution

The *A. lakoocha* Roxb. bark was collected from collected from Sadashivgad (Karwar) District of Karnataka, India during the flowering season. The plant was authenticated by the Botanical survey of India (Pune) Auth 08-137. The collected material was dried at room temperature, under shade and then ground in a mixer to a fine powder. This was then passed through an ASTM BSS mesh (size 85) and stored in an airtight container at room temperature. 5mg of the dried powder was accurately weighed, placed in a stoppered tube and 10 mL of Methanol was added. The sample was vortexed for 1-2 minutes and then left over night. The content was filtered through Whatmann No. 41 filter paper and the clear supernatant was collected in a dry tube. This solution was used for further experiments.

(C) Instrumentation and chromatographic conditions

A Hypurity C_{18}, (50×4.6mm), 5μ obtained from Thermo Electron, Mumbai, India was used for the compound retention. The mobile phase consisted of mixture of Methanol: 10mM ammonium acetate buffer pH adjusted to 3.00 ± 0.05 with acetic acid (85:15 *v/v*) and was delivered at a flow rate of 0.5 mL min^{-1} by employing a Shimadzu Prominence series (Kyoto, Japan) binary pump, at ambient temperature. Detection was achieved using an Applied Biosystems API 5000 MS-MS apparatus (Applied Biosystems, Ontario, and Canada) fitted with a Turbo Ion Spray source. The instrument was interfaced with a computer running Applied Biosystems Analyst version 1.4.2 software. Electrospray ionization (ESI) was performed in the negative ion mode. The spray voltage and source temperature were -4500 V and 550°C respectively. Nitrogen was used as the collision gas. The Declustering Potential (DP), Collision Energy (CE), Entrance potential (EP), Cell Exit Potential (CXP) were optimized during tuning as -255, -44, -10, -35 eV for rutin and -130, -30, -10, -19 for quercetin. The collision activated dissociation (CAD) gas was set at 5 psi, while the curtain gas was set at 15 psi. The Applied Biosystems API 5000 LC-MS-MS apparatus was operated at unit resolution in the multiple reaction monitoring (MRM) mode, monitoring the transition of the deprotonated molecular ion *m/z* 609.2 to the product ion *m/z* 301.0 for rutin and the transition of the deprotonated molecular ion *m/z* 301.0 to the product ion *m/z* 150.8 for quercetin. The instrument response was optimized for both rutin and quercetin by infusing a constant flow of a standard solution (100 ng mL^{-1}) via a T-piece into the stream of mobile phase eluting from the column. Figure 2 and 3 shows the product ion mass spectra obtained from collision-induced dissociation of the deprotonated molecular ions of rutin and quercetin respectively.

(D) Method validation

(a) System suitability

System suitability tests are used to ensure reproducibility of the equipment. The test was carried out by injecting 10 μL of mixture of standard solution of rutin and quercetin (25 ng mL^{-1}) six times. The % RSD was found to be 0.78 % and 1.60 % for rutin and quercetin respectively, which was acceptable as it is less than 2%.

(b) Linearity

In order to establish linearity, the mixture of standard solutions of Mimosine at eight different concentrations (0.5, 2.0, 5.0, 10.0, 25.0, 50.0, 80.0 and 100.0 ng mL^{-1}) were prepared in mobile phase. Each of these solutions (10 μL) was injected and the detector response for the different concentrations was measured. A graph was plotted of drug peak area against concentration. The plot was linear in the range 0.50 ng mL^{-1} to 100.0 ng mL^{-1} for both rutin and quercetin. The experiment was performed three times and the mean was used for the calculations. The equation of linear regression curve

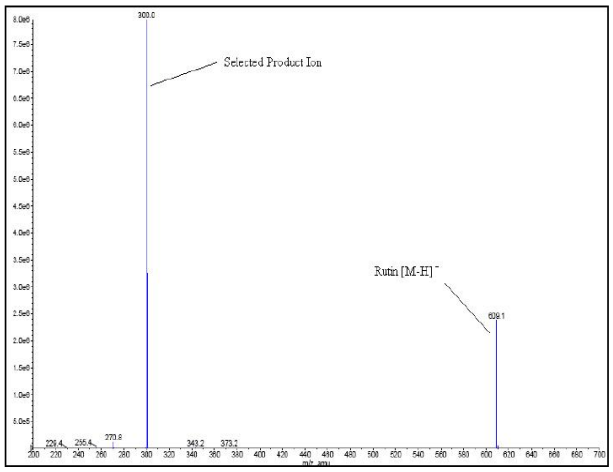

Figure 2 : Representative spectra of product ion of Rutin

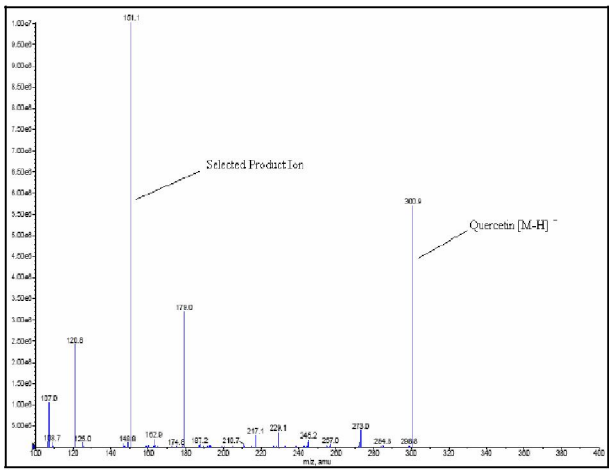

Figure 3 : Representative spectra of product ion of Quercetin

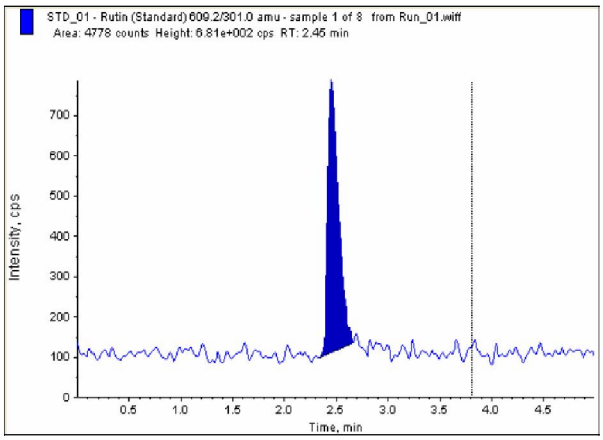

Figure 4 : Representative chromatogram of standard Rutin at LLOQ level (0.5 ng mL^{-1})

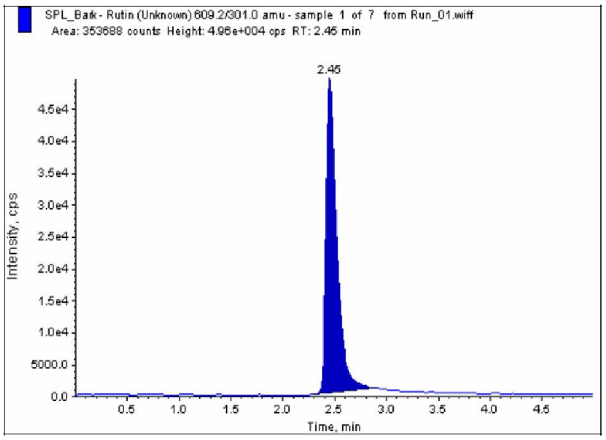

Figure 5 : Representative chromatogram of rutin in bark of *A. lakoocha* Roxb.

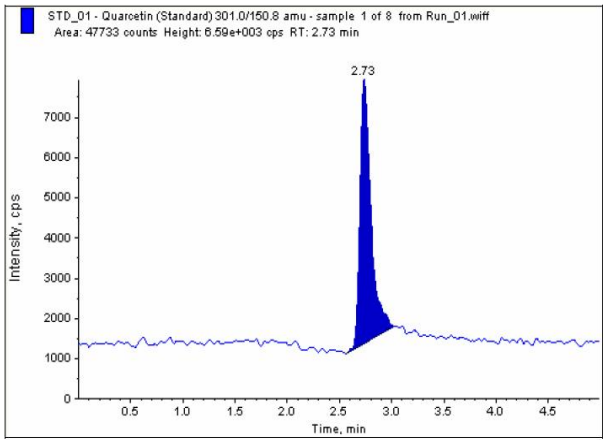

Figure 6 : Representative chromatogram of standard Quercetin at LLOQ level (0.5 ng mL^{-1})

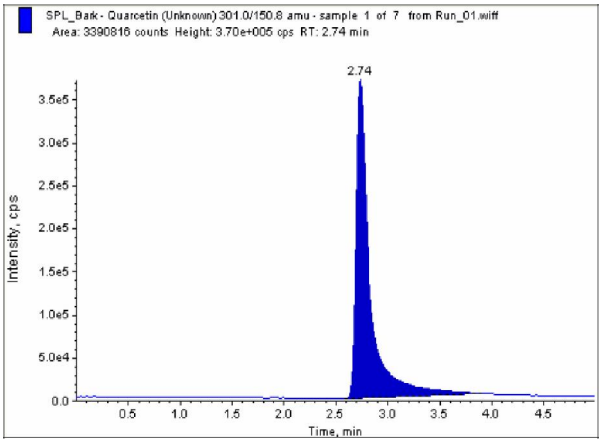

Figure 7 : Representative chromatogram of quercetin in bark of *A. lakoocha* Roxb.

obtained was y = 9725.9 x – 11804.5, where y = (peak area), x = (concentration of rutin in ng mL^{-1}) with a correlation coefficient 0.9965 for rutin and for quercetin the linear regression curve obtained was y = 115316.8 x – 147661.2 with a correlation coefficient 0.9984. Typical chromatograms of standards and plant

are shown in figure 4-7.

(c) Limit of detection and limits of quantization

The signal-to-noise ratio of 3:1 and 10:1 was used to establish LOD and LOQ, respectively. The LOD and LOQ of both rutin and quercetin were 0.1 ng mL^{-1} and 0.5 ng mL^{-1} respectively.

(d) Assay

The developed LC-MS-MS method was used for determination of with a correlation coefficient 0.9965 from the bark of *A. lakoocha* Roxb. . The sample working solution (10 µL) was injected and the area of rutin and quercetin peak was measured. From the calibration curve, the amount of rutin and quercetin in dry powder of *bark of A. lakoocha* Roxb. was calculated. The retention time of rutin in sample solution and in the standard solution was found to be 2.46 min. and that of quercetin was found to be 2.74 min. The mean assay value of rutin and quercetin was found to be 0.076 mg /g and 0.061 mg /g respectively with % RSD as 1.98 and 1.73% in bark of *A. lakoocha* Roxb.

(E) Precision and accuracy

The intra-day and inter-day precision was used to study the variability of the method. The % RSD for intra-day and inter-day precision for rutin were 0.91% and 1.31%, respectively and for quercetin were 1.19% and 1.06%. Accuracy of the method was studied using the method of standard addition. Standard solutions were added to the extract of the bark powder of *A. lakoocha* Roxb. and the percent recovery was determined at two different levels 25% and 50%. Rutin and quercetin content was determined and the percent recovery was calculated. The results of recovery analysis are shown in TABLE 1.

RESULTS AND DISCUSSION

The high selectivity of MS-MS detection allowed the development of a very specific and rapid method for simultaneous determination of rutin and quercetin in *A. lakoocha* Roxb. bark. During method development different options were evaluated to optimize, detection parameters and chromatography. Electrospray ionization (ESI) was evaluated to get better response of analytes as compared to atmospheric pressure chemical ionization (APCI) mode. It was found that the best signal was achieved with the ESI negative ion mode. A mobile phase containing formic acid solution and Methanol in varying combinations was tried during the initial development stages. But the best signal was achieved using a mobile phase containing 10mM ammonium acetate buffer pH adjusted to 3.00 $\pm$ 0.05 with acetic acid in combination with Methanol (15:85 *v/v*). Use of a short Hypurity C_{18}, (50mm × 4.6mm), 5μ column resulted in reduced run time of 5 min. Regression analysis of calibration data showed that the linearity of Mimosine was observer over a concentration range of 0.5ng mL^{-1} to 100 ng mL^{-1} with regression coefficient of 0.9965 and 0.9984 for rutin and quercetin respectively. The concentration of rutin and quercetin in 1.0 g of whole plant powder of *Mimosa pudica* L. was found to be 0.076 mg and 0.061 mg respectively.

When the method was validated in terms of instrumental precision, intra-assay precision and intermediate precision, the percent RSD values were found to be less then 2, indicating that the proposed method is precise and reproducible. The accuracy of the method was established by means of recovery experiments. The mean recovery was close to 100%, which indicates that method is accurate. The low values of %COV for replicate analyses are indicative of precision of the method. The method is specific because it resolved the standard rutin and quercetin well in presence of other phytochemicals of bark powder of *A. lakoocha* Roxb.

CONCLUSION

A new LC-MS-MS method has been developed for quantification of rutin and quercetin from the bark of *A. lakoocha* Roxb. The method developed with careful validation was found to be fast, simple, precise, sensitive and accurate. The linearity, precision, accuracy of the method proves that the method is easily reproducible in any quality control set-up provided all the parameters are followed accurately.

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