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RESEARCH ARTICLE

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LIQUID CHROMATOGRAPHY- TANDEM MASS SPECTROMETRIC METHOD FOR SIMULTANEOUS DETERMINATION OF RUTIN AND QUERCETIN FROM LEAVES OF *ARTOCARPUS LAKOOCHA* ROXB.

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ABSTRACT

Artocarpus lakoocha Roxb. is a valuable tropical tree species native to India and used for fruit, furniture, timber, and feed. *Artocarpus lakoocha* Roxb. contains a crude protein, crude fiber and mineral contents. A rapid, reproducible and efficient liquid chromatography-tandem mass spectrometry (LC-MS/MS) method has been developed to quantify Rutin and Quercetin simultaneously from *Artocarpus lakoocha* Roxb. leaves. The analytes were chromatographed on a Hypurity C18 (50mm x 4.6mm i.d., 5 µ particle size) column using 10µL injection volume with a run time of 5 min. The precursor and product ion of analytes were monitored on a triple quadrapole mass spectrometer, operating in the multiple reaction monitoring mode (MRM) with negative polarity. The proposed method was validated over the range of 0.5 ng/ml to 100.0 ng/ml. The proposed LC-MS/MS method was validated for linearity, accuracy, precision and limit of quantitation.

KEYWORDS

Artocarpus lakoocha Roxb., Rutin, Quercetin, LC-MS/MS

INTRODUCTION

Artocarpus lakoocha Roxb. [1] BADAHAR trees of the Moraceae family, originating in south east Asia[2], have a relatively short period of deciduousness beginning earlier at lower altitudes. Farmers plant this species on their own land as a source of green fodder for their cattle and buffaloes. The green leaves are available during the critical period of fodder shortage. Leaves of *Artocarpus lakoocha* Roxb. proved to have relatively constant nutrient values throughout the lopping season[3]. The edible fruit pulp is believed to act as a tonic for the liver. Raw fruits and male flower spike (acidic and astringent) are utilized in pickles and chutney (sauce).

The brown powder called Puag-Haad in Thailand is a product of the aqueous extraction of *A. lakoocha* Roxb. 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 [4,5]. 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[6,7].

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 Hesperidin 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 Fig. 1 [8].

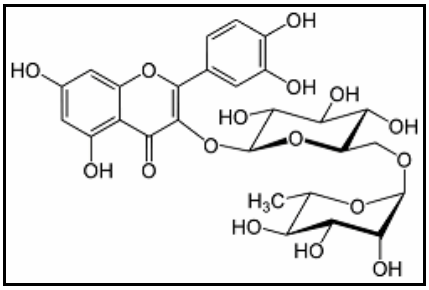

Figure 1
Structure of Rutin

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 it 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 Fig. 2. [9].

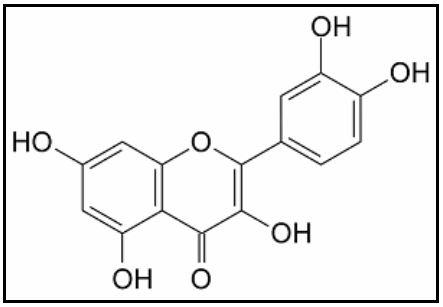

Figure 2
Structure of Quercetin

Literature survey revealed that there is no method available for simultaneous quantitation of Rutin and Quercetin from the leaves of *A. lakoocha* Roxb. using an LC-MS-MS system. The aim of this study was to develop a selective, rapid, precise, and accurate method for simultaneous determination of Rutin and Quercetin in *Artocarpus lakoocha* Roxb.leaves.

Experimental

Standard Rutin and Quercetin were procured from Sigma-Aldrich Chemie (Steinheim, Germany). AR grade Ammonium formate was procured from Quligens Ltd. (Mumbai, India). HPLC grade Acetonitrile and Methanol were purchased from J.T. Baker Inc. (Phillipsburg, NJ, USA). Purified water was obtained from Milli Q A10 gradient water purification system (Millipore, Bangalore, India).

Leaves of *Artocarpus lakoocha* Roxb. were collected from Madgoan (Goa) region of India. It was authenticated from Botanical survey of India (Pune).

Standard and sample preparation

The stock solutions of Rutin and Quercetin (1000 µg mL^{-1}) were separately prepared in Methanol. Working solutions in the required concentration range were prepared by appropriate dilution of the stock solutions in Methanol: Water (50:50 v/v). All the solutions were stored at 2-8°C and brought to room temperature before use. The concentration ranges for both, Rutin and Quercetin in working standard solutions were 0.5 ng mL^{-1} to 100.0 ng mL^{-1}.

Leaves of *Artocarpus lakoocha* Roxb. were collected, washed, dried in the shade, and powdered. The powder was passed through an 80-mesh sieve and stored in an airtight container at room temperature. 5 mg of the accurately weighed, dried powder was transferred to a 10 mL standard volumetric flask and made upto the mark with the Methanol. The solution was vortexed for five minutes and then left to stand overnight at room temperature. The solution was filtered through Whatman filter paper no 41 (E. Merck, Mumbai, India). The filtrate was collected in a dry stoppered test tube. This sample solution was used for the assay.

Liquid chromatography and mass spectrometric conditions

An HPLC system (Shimadzu, Kyoto, Japan) consisting of a binary LC-10AD prominence pump, autosampler (SIL-HTc) and solvent degasser (DGU-14) were used for the analysis. For separation, the samples were analysed without any guard column on Hypersil Hypurity C18 (50mm x 4.6mm i.d., 5 µ particle size) analytical column of Thermo (I) Pvt. Ltd. (India). The flow rate of the mobile phase under isocratic condition was kept at 0.5 mL/min. The autosampler temperature was set at 4°C and the injection volume was 10µL. Column oven temperature was set at 40°C. The mobile phase consisted of 10mMol/L ammonium acetate (pH 3.0±0.5) and Methanol (20:80 v/v). The total run time was 5.0 min. Detection of analyte and IS was performed on a triple Quadrapole mass spectrometer, API-5000, (MDS SCIEX, Toronto, Canada) equipped with Turbo ion spray ionization source and operating in negative ion mode. Analyst software version 1.4.2 was used to control all parameters of

LC and MS. Quantitation was performed using multiple reaction monitoring (MRM) mode to study parent → product ion (m/z) transitions for Rutin (609.2/301.0) and Quercetin (301.0 /150.8).

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. Quadrapole 1 and Quadropole 3 both were maintained at unit resolution and dwell time was set at 400msec for both.

Analytical data processing

Chromatographic data was collected and integrated using Analyst software version 1.4.21. Peak area was utilized for the construction of calibration curve. Weighing of 1/x (linear regression analysis where x is the analyte concentration) was used for curve fitting. Concentration of the analytes in plant sample was calculated from the equation (y=mx+c), where y is the peak area ratio.

Method validation

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 5%.

The linearity of the method was determined by analysis of standard plots associated with a 8-point standard calibration curve. Three linearity curves containing eight non-zero concentration (0.5, 2.0, 5.0, 10.0, 25.0, 50.0, 80.0 and 100.0 ng mL^{-1}) were analyzed. Calibration curves of peak area ratio versus concentration were drawn. In each case, 10 µL of the solutions were injected. A linear relationship between the peak area and the concentration was observed for Rutin and Quercetin in the range of 0.5 ng mL^{-1} to 100.0 ng mL^{-1}. The experiment was performed thrice and the mean of the peak area was used for the calculation. Typical Chromatograms of Rutin and Quercetin are shown in Figure 3A and 3 B respectively.

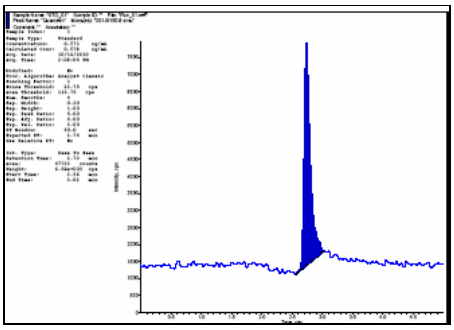

Figure 3A

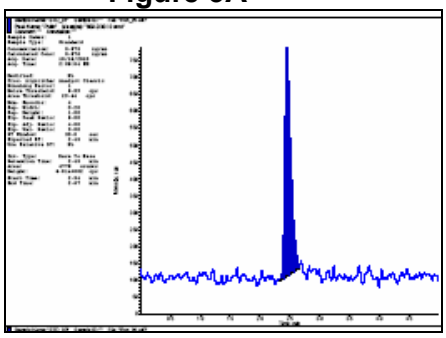

Figure 3B

The signal-to-noise ratio of 3:1 and 5:1 was used to establish LOD and LOQ respectively. The LOD and LOQ of Rutin and Quercetin were 0.3 ng mL^{-1} and 0.5 ng mL^{-1} respectively. The linearity data is given in **Table 1**.

Table 1
Linearity Data for Rutin and Quercetin

Data	Rutin	Quercetin
Linearity range ng mL^{-1}	0.5 to 100.0	0.5 to 100.0
Slope (m)*	9725	115316
Intercept (c)*	-11804	-147661
Correlation coefficient (R)	0.9965	0.9984
LOD ng mL^{-1}	0.3	0.3
LOQ ng mL^{-1}	0.5	0.5
Intraday Precision (RSD[%], n = 3)	0.9	1.2
Interday Precision (RSD[%], n =3)	1.3	1.1

**- Of the equation y = mx + c, where y is peak area, m is the slope, x is the concentration and c is the intercept.*

10 µL of plant extract was injected and peak area of Rutin and Quercetin were measured using the linearity equation. The assay procedure described was repeated seven times starting from weighing of the plant powder. The retention time of Rutin and Quercetin were 2.45 min and 2.73min respectively. The mean assay values of Rutin and Quercetin in plant were found to be 0.009 and 0.010%.

RESULTS AND DISCUSSION

Chromatographic analysis of Rutin and Quercetin were initiated under isocratic condition with an aim to develop a simple separation process with short run time. Separation was tried using various combinations of Methanol and buffer with varying contents of each component on variety of columns like C8 and Betasil C18 to identify the optimal mobile phase that produce the best sensitivity, efficiency and peak shape. Use of buffer of pH-3.0 helped in achieving good response for MS detection operating in positive mode. Thus the mobile phase consisting of 10mM Ammonium acetate with pH adjusted to 3.0 with glacial acetic acid: Methanol 20:80 *v/v* was found suitable for analysis. High content of Methanol (85%) in Mobile phase helped in eluting the analytes within 5 minutes at flow rate of 0.5 ml/min. Hypersil Hypurity C18 (50mm x 4.6mm i.d., 5 µ particle size) column gave good peak shape and response even at LOQ level. For both the analytes low injection volume of 10µl reduced overloading of column with analyte there by ensuring more number of analyses on the same column.

The response to Rutin and Quercetin was found to be linearly dependent on concentration in the range 0.5 ng ml^{-1} to 1000 ng ml^{-1}, with correlation coefficient of 0.9965 and 0.9984 respectively. The variability of the method was studied by analyzing aliquots of the different concentrations of puerarin solutions on the same day (intra-day precision) and on different days (inter-day precision). The results were expressed as % RSD. The % RSD values were found to be less than 5%, indicating that the selected method is precise and reproducible.

The robustness of the method was studied, during method development, by determining the effects of small variation, of mobile phase composition (±2%). No significant change in Retention time or in response of both the analytes was observed, indicating the robustness of the method.

CONCLUSION

The developed LC-MS/MS method is selective, rugged, rapid, precise, and accurate which can be used for simultaneous quantitative determination of Rutin and Quercetin from *Artocarpus lakoocha* Roxb. leaf powder.

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