Electronic Supplementary Material (ESI) for RSC Advances.

Rutin phospholipid complexes confer neuro-protection in ischemic rats

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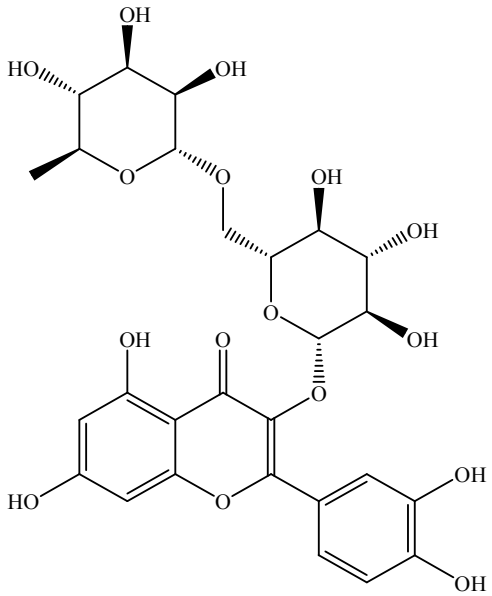
In the order in which the text appears in the manuscript

1. Introduction:



SF1: Chemical structure of rutin

2.3.2.7 Surface morphology studies:

Microscopic view of Ru-PLC's:

A small amount of Ru-PLC's was suspended in distilled water and a drop was placed on a slide and covered with a cover slip. Microscopic view of the complex was observed at a magnification of 10x, 20x and 40x and photographed with the help of Nikon Eclipse TS-100; Japan.

2.3.3 Oral pharmacokinetic assessment of Ru-PLC's:

Animals: Adult healthy male Sprague dawley (SD) rats (250±20 g) were used for this study. They were housed in national laboratory animal centre (NLAC) of CSIR-CDRI (Lucknow, India). The SD rats were kept in a controlled environment room temperature (22 ± 2°C), relative humidity 60 ± 5% and 12:12 hour light–dark cycle. Standard laboratory food and water were available at all times. The rats were fasted 12 hours but allowed free access to water prior to the experiment. Test and blank plasma were collected from (SD) rats.

LC-MS method for determining oral bioavailability of Ru-PLC's:

LC-ESI-MS/MS Condition: LC-MS/MS analysis was performed using a Shimadzu UFLC system (Kyoto, Japan) coupled to a mass spectrometer (MS) API 4000 (Q-trap) triple quadrupole instrument (AB-SCIEX, Toronto, Canada). The mass spectrometer was operated using an electro-spray ionization source in negative ion mode (ESI) with multiple reaction monitoring (MRM). The analytical column was a Agilent Zorbax SBC18, (50 x 2.1 mm, 3.5 µm) (Waters). The mobile phase consisted of methanol: 0.1% formic acid (90:10, v/v) in isocratic mode. The sample injection volume was 10µL and the total run time was 4.0 min using a flow rate of 1mL/min^{-1}. The column temperature was maintained at 40°C; both quadrupoles were maintained at unit resolution in order to assay all analytes. Zero air was used as the source gas while nitrogen was used as both the curtain and collision gas. The chromatograms were integrated using Analyst 1.4.6 software (Applied Biosystems, MDS Sciex, Toronto, Canada).

Preparation of calibration standards and quality control samples: Primary stock solutions of Rutin and Quercetin (I.S) were prepared by dissolving the standard compounds in methanol to achieve the concentration of 1.0 mg/mL. Appropriate dilutions of Rutin were prepared in methanol and spiked in compound free plasma to achieve the linear range of 1- 200.0 ng/mL. Quality control (QC) samples were low quality control (LQC) 4 ng/mL, medium quality control (MQC) 40.0 ng/mL and high quality control (HQC) 160.0 ng/mL. All the samples were stored at −20°C until the analysis.

Sample extraction procedure: Liquid-liquid extraction (LLE) technique was adopted to extract rutin from rat plasma by using various extraction solvents (chloroform, di-ethyl ether, ethyl acetate, tertiary butyl methyl ether (TBME) and their combination). Among these extraction solvents, ethyl acetate (EA) provides maximum recovery of rutin from rat plasma and a clean extract obtained from plasma sample with appropriate sensitivity at the LLOQ (1 ng/mL) level and the method was clinically applicable which was necessary for pharmacokinetic studies. Matrix effect was initially detected which was completely removed after adding 0.1% formic acid (FA) solution in the extraction method.

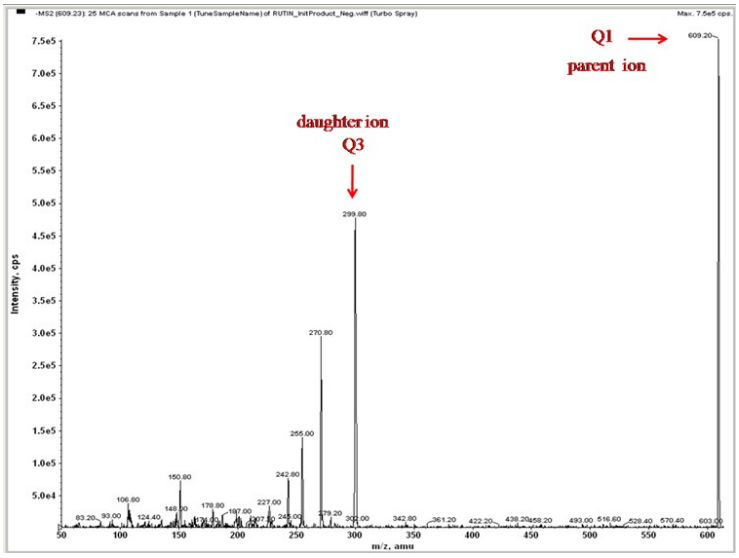
Rat plasma samples were retrieved from the sample storage area (-20ºC) and allowed to thaw at room temperature (25ºC). After vortexing, a 100 µL aliquot of plasma with 50 µL of internal standard solution (100 ng/mL) was transferred into polypropylene vial. In the mixed sample 150 µL of acetonitrile, 100µL of 0.1% FA solution was added and vortexed to mix well. In the each sample, 2.5 mL of EA (extraction solvent) was added and vortexed for 10 min at 2500 rpm on vibramax (Heidolph, Schwabach, Germany) followed by centrifugation (Eppendorf, Hamburg, Germany) at 5,000 rpm for 5 min. An aliquot of 2.0mL of supernatant organic layer was separated and evaporated on vacuum concentrator (Thermo scientific, Asheville, USA) to complete dryness. The residue was reconstituted with 100 µL of mobile phase and injected into the LC-MS/MS system for analysis.

Results:

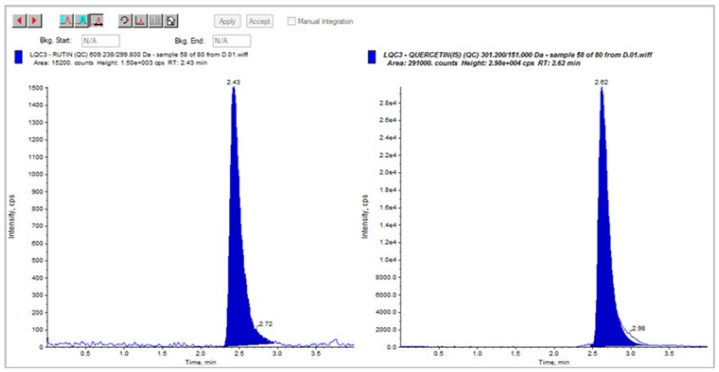
The transitions (precursor to daughter) monitored were m/z 609.2/299.8 for rutin and m/z 301.2/151.0 for Quercetin negative mode as shown in figure SF2. The source parameters, viz. curtain gas, CAD gas, Source temperature, Ion Spray Voltage, GS1 and GS2 as well as compound parameters viz. Declustering Potential (DP), Entrance Potential (EP), Collision Energy (CE) and Exit Potential (CXP) of the marketed compounds and internal standard were tabulated in the table ST1. Chromatograms of rutin with IS at low and high concentration spiked in plasma are depicted in figures SF3 (a) and (b). **Validation:** A calibration curve was constructed from a blank sample (a plasma sample processed without an I.S and rutin), a zero sample (plasma processed with I.S) and eight non-zero samples in the total range (1- 200.0 ng/mL). The linear equation obtained was, Y= 0.0123x + 0.00149 (r=0.99552).

ST1: Mass spectrometer parameters for LC-MS/MS analysis

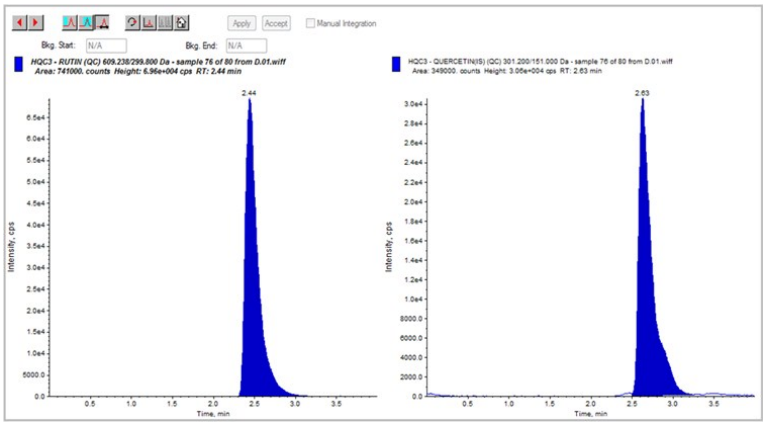
Parameter (MS/MS)	Compound	
	Rutin	**Quercetin (I.S)**
Parent Ion (Q1)	609.2	301.2
Daughter ion (Q3)	299.8	151.0
Dwell time (m sec)	150	150
Declustering potential (DP)	-140	-70
Collision energy (CE)	-54	-31
Collision exit potential (CXP)	-11	-10
Curtain gas (CUR)	30	
Ion source temperature (°C)	500	
Ion spray voltage (V)	-4500	
Ion source gas 1 (GS1)	50	
Ion source gas 2 (GS2)	50	
Collision (gas) CAD	High	



SF2: Mass spectrometer spectra of Rutin at negative mode



SF3(a): Chromatogram of Rutin and Quercetin (IS) (low quality control (LQC) concentration)

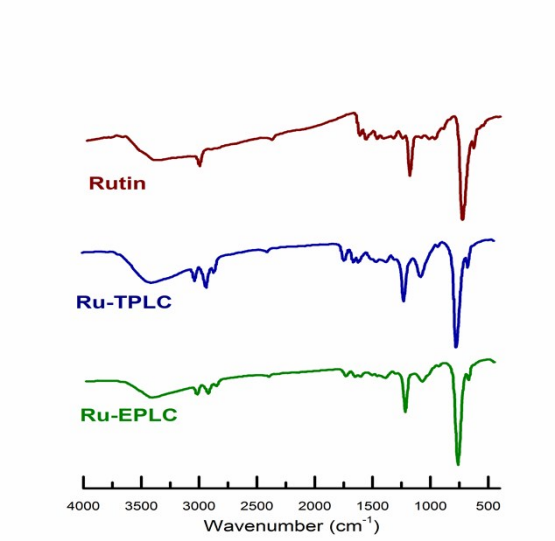


SF3(b): Chromatogram of Rutin and Quercetin (IS) (high quality control (HQC) concentration)

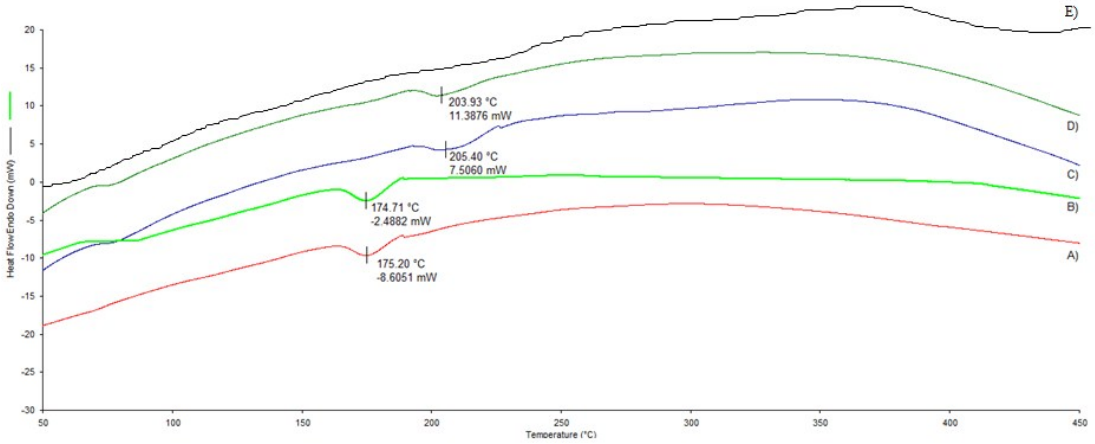
2.3.5.2 Surgical Procedure for induction of cerebral ischemia by MCAO:

Chloral hydrate (300mg/kg i.p.) was used to anaesthetize Sprague Dawley rats (260±20g) and then rats were placed in a supine position over a preheated operation table to maintain the body temperature at 37ºC±0.5ºC. Through a midline incision in the neck region the left common carotid artery (CCA) was exposed. The neck muscles were separated further to expose external carotid artery (ECA) and internal carotid artery (ICA). A silk suture knot was tied on the ICA loosely close to the bifurcation region. A (3-0) nylon monofilament suture (Ethicon, Johnsons & Johnsons Ltd, Mumbai) was introduced into the ECA lumen through a nick given in the external carotid artery. The intraluminal suture blocked the origin of the MCA, occluding all sources of the blood flow from the ICA, anterior cerebral artery (ACA) and posterior cerebral artery (PCA). The suture was pulled back after 2hrs of ischemia to re-establish the cerebral blood flow.

3.2.6 Compatibility studies:



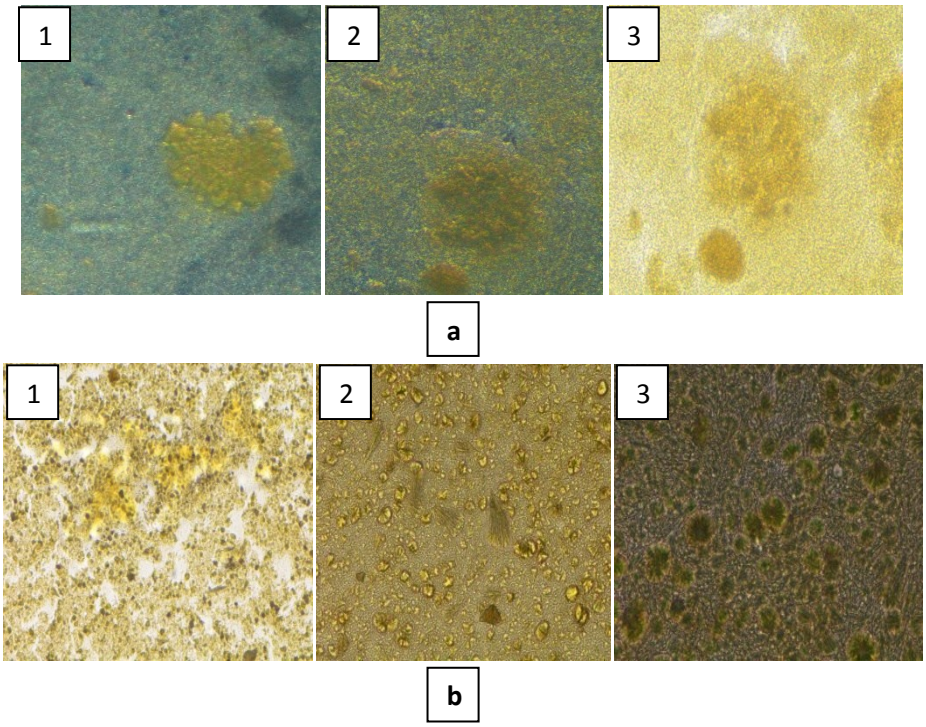
SF4a: FTIR spectra of rutin and Ru-PLC's



SF4b: DSC thermograms of **A)** Rutin, **B)** Rutin:EPC (1:1), **C)** Ru-EPLC, **D)** Ru-TPLC, **E)** EPC

3.2.7 Surface morphology studies:

The microscopic view as shown in SF5 indicated the presence of spherical structures of Ru-PLC's. Rutin was found to be intercalated in the lipid bilayer of EPC vesicles. The globular particles viewed in Ru-EPC's were more defined as compared to Ru-TPLC.



SF 5: Microscopic view of (a) Ru-TPLC (1) 10X (2) 20X (3) 40X
(b)Ru-EPLC (1) 10X (2) 20X (3) 40X

3.3Pharmacokinetic assessment of Ru-PLC's:

ST 2: Pharmacokinetic parameters for rutin and Ru-PLC's

Parameters	Units	Rutin	Ru-TPLC	Ru-EPLC
Kel	1/h	0.079±.004	0.06±.023	0.25±0.14
$t_{½}$	H	8.75±0.45	10.68±3.85	4.27±1.81
T_{max}	H	0.16	0.0833	0.0833
C_{max}	ng/mL	58.73±15.35	70.29±14.75	566.33±111.72
AUC 0-t	h*ng/mL	129.88±12.62	205.11±39.74	344.67±73.80
AUC 0- inf	h*ng/mL	142.57±12.0	252.84±33.27	362.95±88.72
CL	L/h/kg	704.88±62.37	400.40±56.0	285.9±64.10
MRT	H	8.6±1.9	14.04±2.35	6.16±3.2
Vss	L/kg	8872.28±346.37	7157.08±2457.97	1283.89±455.05

3.4 Brain distribution study:

ST3: Rutin concentration in brain after oral administration of pure rutin and Ru-PLCs at 100 mg/kg to SD rats.

Time (h)	Concentration (ng/g)		
	Pure Rutin	**RU-TPLC**	**RU-EPLC**
0.5	23.42±3.93	34.64±4.29	51.35±3.63
2	6.21±1.03	16.37±3.92	18.96±1.21
6	1.95±0.36	4.51±2.55	14.65±0.77