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The Bioavaliability of Hepatoprotective Flavoniods in Hypericum Japonicum Extract

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Abstract

Purpose: To study the absorption of main flavonoids in Hypericum japonicum extract (HJE) with liver protective property; Method: HPLC-ESI-MS was introduced to identify and evaluate the flavonoids in HJE; Caco-2 cell monolayer model was established and validated, and the compounds in HJE, including quercetin (Q), quercetin-3-O-rhamnoside (Q-3-R), quercetin-7-O-rhamnoside (Q-7-R) and quercetin-3-O-glucoside (Q-3-G) were administrated in individual, paired or mixed form of the compounds to the monolayer to evaluate their apparent permeability coefficients (P_{app} value). The transport of HJE was also investigated, mixture of pure components and HJE Inhibitor was added to investigate the transport mechanism of the compound mixture. The absorption of the four main ingredients in HJE was then investigated in vivo. Result: transportation of Q, Q-3-R Q-3-G but not Q-7-R trhough Caco-2 monolayer was observed when they were administrated individually. Increase of the transport of Q-3-G and Q-7-R and decrease in Q were observed when the four compounds were given in paired form; when the four flavonoids were given as a whole (either in mixture of pure compounds or in HJE), mass permeability of Q-3-R, Q-7-R and Q-3-G was found. In vivo study identified the in vitro investigation that the major active components of HJE could be absorbed after orally administrated to mice.

Conclusion: The increased transport of mixed active components in HJE gives rise to the enhanced hepatoprotetive effect of HJE, and therefore supports the use of botanical drugs.

Keywords: Flavonoids; Absorption; Interaction; Caco-2 cell Monolayer

Introduction

Hypericum japonicum (HJ) is a kind of herb which has been used for hepatitis therapy for more than hundreds years in southern China. Modern investigations reveal HJ's potent action of anti-microbial, liver protection and anti-hypertension (Ishiguro et al., 2002; Jiang et al., 1997; Liu et al., 2008). Previous study in our laboratory has established the chemical profile for the raw materials of *Hypericum japonicum* Thunb. (Yang et al., 2005). As well, our study showed quercetin and its aglycones, including quercetin-3-O-rhamnoside (Q-3-R), quercetin-3-O-glucoside (Q-3-G) and quercetin-7-O-rhamnoside (Q-7-R), are the main active components in *Hypericum japonicum* (Wang et al., 2008). Some preliminary data in our laboratory showed that the the total extract *Hypericum japonicum* (HJE) performs better hepatoprotective action than its component chemicals, quercetin and its analogs (Data not show). However, the underlying mechanism has not yet been documented. In this study, he intestinal absorption of components in HJE extract in different dose forms both *in vitro* and *in vivo* system were addressed. The mechanism of interaction on their transport was also investigated in this study.

Materials and Experiments

Chemicals and HJE preparation

Q, Q-3-R and Q-3-G were purchased from the Sigma. Q-7-R was extracted and purified from *Hypericum japonicum* in our laboratory. The purity of all chemicals above was above 95%. Verapamil, the P-glycoprotein inhibitor, was a kindly gift offered by Dr. Ma Yan in Guangzhou University of Chinese Medicine. HJE was prepared from *Hypericum japonicum* in our lab (Batch No. 20060524, 20060605, and 20060616). Briefly, *Hypericum japonicum* powder was extracted by 20-folder of boiled water (w/v) for 1 hour and then filtered. This step was repeated two times. The filtrate each time was then mixed and evaporated to dryness. Residue was triturated with distilled water and then extracted with 2-folder of ethyl acetate for two times. Ethyl acetate was then evaporated to dryness for preparing the HJE powder. Powders were diluted by proper solvent before use.

Cell line and cell culture

Caco-2 cells obtained from the Xiehe Medical School were cultured in Eagle's Minimum Essential Medium (Gbico) supplemented with 1% MEM nonessential amino acids (Mediatech), 10% fetal bovine serum (PAA), 100 units/mL of penicillin, and 0.1 mg/mL of streptomycin (Sigma) and were grown in a humidified atmosphere of 5% CO_2 at 37 degree centigrade.

For all transport studies, Caco-2 cells were seeded in 12 mm i.d. Transwell inserts (polycarbonate membrane, 0.4 mm pore size, Corning Costar Corp.) in 12-well plates at a density of 1.0×10^5 cells/cm^2. The basolateral side (serosal, BL side) and apical side (mucosal, AP side) compartments contained 1.5 and

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0.5 mL of culture medium, respectively. Culture medium was replaced per two days for the first ten days and daily thereafter until the 21st day.

Chemical identification and quantitative analysis on HJE by HPLC-ESI-MS

HPLC-ESI-MS (LCQ DECA XP, Thermo, USA) was introduced to identify and quantify ingredients in HJE. Chromatographic separation was achieved using a mobile phase consisting of methanol (A) and water (pH 2.5, B) at the gradient as follows: The ratio of A to B was constant at 36 to 64 during the first 65 min, and then changed linearly to be 65 to 35 at 100 min The flow rate was 1.0 mL/min. The column was kept at 25 degree centigrade. The mass spectra were recorded using ESI in both positive and negative mode with ion spray voltage at 3300 eV, source temperature at 350 degree centigrade, gas spray 1 at 60 psi, Gas spray 2 at 40 psi, current gas at 40 psi, desolvent voltage 1 at 40 eV, desolvent voltage 2 at 15 eV, focus voltage at 200 eV and scanning from 300 to 1000 amu. 0.05 g of HJE was dissolved by 50 mL methanol and then 10 μL of the solution was injected and analyzed. Q, Q-3-R, Q-3-G and Q-7-R were also analyzed as standards. Three batches of HJE were analyzed quantitatively as above. Then the content of Q, Q-3-R, Q-3-G and Q-7-R was calculated respectively through calibration curve method.

The establishment and evaluation of cell model

Caco-2 cells in Transwells were used for transport experiments after 21 days culturing. Transepithelial electrical resistance (TEER) values across the cell monolayer were measured using a Millicell-ERS voltohmmeter (Millipore Corp.). Only the inserts whose TEER values are more than 350 V/cm^2 in culture medium were used. Alkaline phosphatase (ALP) activity by commercial kit (Jiancheng biotechnical Co., Nanjing, P.R. China) on the 21st day was evaluated.

Propranolol was introduced as a marker to determine the transepithelial ability of the established model (Takahashi et al., 2002). Samples were collected from BL side of cell monolayer at 15, 30, 45, 60, 90 and 120 min respectively and analyzed for the propranolol content by high performance liquid chromatography (HPLC) under literature condition (Ma, 2005).

Transepithelial permeability evaluation of different dose-form agents

At the 21st day of the period, inserts were washed twice before 30 minutes incubation with 37 degree centigrade PBS buffer solution and then removed. Agents with different dose forms were added to apical (500 μL) or basolateral (1500 μL) side of the inserts, whereas the receiving chamber contained corresponding volume of transport medium. For evaluating of transepithelial

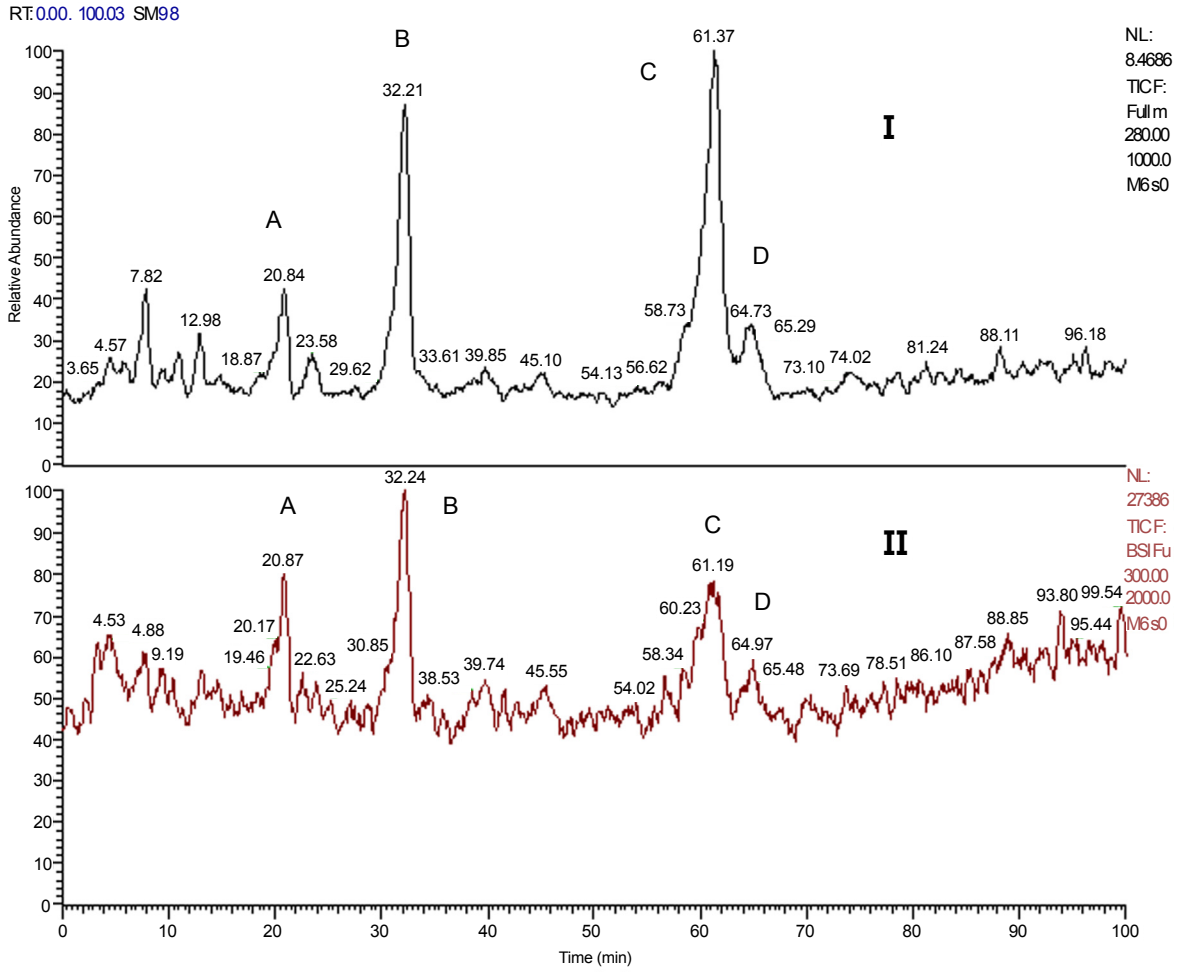
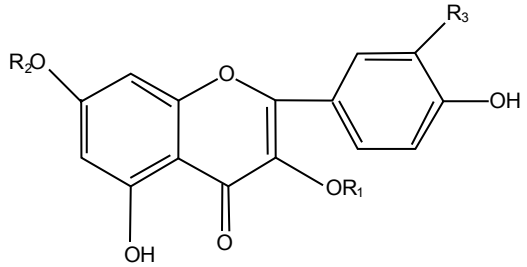


Figure 1: The total ion chromatogram (TICs) of HJE by HPLC-ESI-MS (A shows TIC btained in negative ionization mode; B shows TIC obtained in positive ionization mode).



	R1	R2	R3
Quercetin	H	H	H
Quercetin-3-O-rhamnoside	Rha	H	H
Quercetin-3-O-glucoside	Glu	H	H
Quercetin-7-O-rhamnoside	H	Rha	H

Figure 2: Chemical Structures of four flavonoids in HJE.

Batch No.	Q-3-G (%)	Q-3-R (%)	Q-7-R (%)	Q (%)
20060524	4.823	9.183	18.492	3.936
20060605	4.338	8.295	16.774	3.641
20060616	5.006	9.409	18.026	3.930
AVERAGE	4.722	8.962	17.764	3.836
RATIO	5	10	20	5

Table 1: Content Measurement of four flavonoid in HJE.

permeability of single pure compounds, chemicals (Q, Q-3-R, Q-3-G and Q-7-R) at dose of 25, 50 or 100 μM were added to the upper chambers. To determine the transport ability of co-occurring form of compounds, Q, Q-3-R, Q-3-G and Q-7-R were paired with one another with proper scales as in HJE, and added to upper chamber at the dose of 50 and 100 μg/mL. Mixture of Q, Q-3-R, Q-3-G and Q-7-R (with proper scale as in HJE) and HJE at the dose of 50 and 100 μg/mL were added to upper chamber to determine the permeability of components in mixed form. Samples were collected from receiving chambers of cell monolayer at 15, 30, 45, 60, 90 and 120 min respectively and analyzed by HPLC.

Influence of other flavonoids on efflux of Q-3-G and Q

Experiment was carried out as described in section 2.5. Transport mediums with chemicals (Q-3-G, Q, mixed flavonoids and mixed flavonoids with Verapamil) at the dose of 50 μg/mL were added to the basolateral (1500 μL) side of the inserts followed by the addition of 500 μL to the upper chambers.

In Vivo Observation on the Absorption of Main Ingredients in HJE

42 KM mice with either sex weighing 22~25 g were divided into seven groups. Animals were orally administrated with HJE at the dose of 486 mg/kg after 12-h fasting but free watering. Blood was collected at 0, 5, 15, 30, 60, 90 min after drug administration. Animals were then sacrificed by an overdose of pentobarbitone (Phenobarbital 200mg/kg, i.p). Plasma was then collected by centrifuging the blood at 6000 rpm at 25 degree centigrade for 10 min, and was extracted by 400 μL ethyl acetate for two times. The upper layers were collected and dried in room temperature. The residue was then dissolved by 50 μL methanol. Blank plasma was prepared as above from mice without HJE administration. Samples were then analyzed by HPLC. All animal experiments were conducted with the adherence to principles of laboratory animal care and proved by the Animal Ethics Committee of Sun Yat-sen University.

Statistical Analysis

Student T-test was introduced for statistical analysis. The result was expressed by means of mean ± SD.

Results

HPLC-ESI-MS was introduced to analyze the chemical composition of HJE. Our result shows that Q-3-G, Q-3-R. Q-7-R and Q compose as the majority of HJE (Figure 1), indicating that the hepatoprotective action of HJE is mainly contributed by the four compounds. Peak 1, 2, 3 and 4 were identified as Q-3-G, Q-3-R, Q-7-R and Q (Figure S1, Figure S2, chemical structure in Figure 2). The contents of Q, Q-3-R, Q-3-G and Q-7-R were measure as Table 1 shows. The contents of four compounds in HJE were mostly stable among different batches, and the ratio was approximately at 5:10:20:5.

To well establish the Caco-2 model in our study, TEER value between two sides of the inserts was measure and Figure 3 shows increasedTEER value of most inserts during incubation days, and the value reached more than 450 mΩ at the 21st day, which is suitable for transport study. Activity of ALP in AP side (1.063 ± 0.074 U/L) of Caco-2 monolayer is approximately 3.2 fold of that in BL side (0.333 ± 0.063 U/L), indicating the accumulation of ALP in AP side and the biochemical differentiation of the Caco-2 cell monolayer. Permeability of propranolol on monolayer was 26.88 ± 1.88×10^{-6} cm/s, similar to literature report (Ismael and Jibin, 1996).

Transepithelial Permeability, expressed as apparent permeability coefficients (P_{app}), was calculated by the following equation:

$$P_{app} = \frac{dQ}{dt \cdot A \cdot C_0}$$

where, dQ/dt is the change in drug concentration in the receiver solution (μM/s or μg/s), A denotes the membrane surface area (cm^2), and C_0 is the initial concentration in the donor compartment (μM or μg/mL). Results in Table 2 show potent differences of transepithelial permeability of quercetin and its analogs when they were transported through the Caco-2 monolayer in different dose forms, indicating a vast variety of absorption and bioavailability in different dose forms. When better addressing the inter-influence of quercetin analogs, Table 3 reveals that the efflux of Q-3-G is significantly inhibited when it was co-trans-

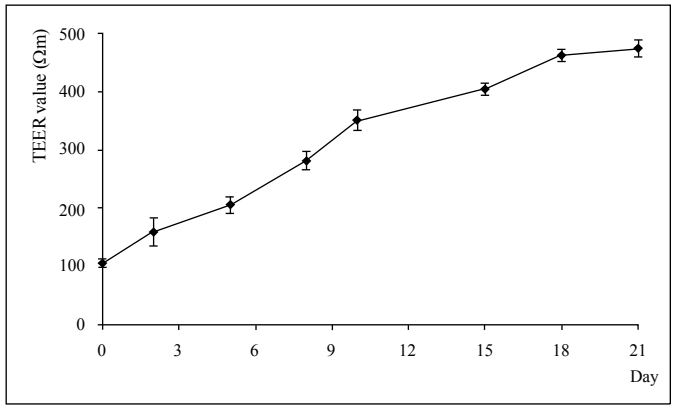


Figure 3: TEER value of Caco-2 cell monolayer in different days.

Compounds	Q-3-G	Q-3-R	Q-7-R	Q
Mixture Form of Flavonoids (50μg/mL)	3.235±0.201	2.109±0.192	0.432±0.069	0.000±0.000
Mixture Form of Flavonoids (100μg/mL)	2.273±0.174	2.180±0.169	0.445±0.106	0.000±0.000
HJE (50μg/mL)	3.432±0.336	2.470±0.273	0.224±0.087	0.000±0.000
HJE (100μg/mL)	2.339±0.287	1.923±0.184	0.486±0.044	0.000±0.000
Single Pure Form of Flavonoid (AP-BL) 25 μM	1.720±0.134	0.742 ±0.103	0.000±0.000	4.469±0.834
Single Pure Form of Flavonoid (AP-BL) 50 μM	1.781±0.191	0.795±0.094	0.000±0.000	3.528±0.792
Single Pure Form of Flavonoid (AP-BL) 100 μM	1.479±0.243	0.624±0.077	0.000±0.000	2.728±0.878
Single Pure Form of Flavonoid (BL-AP) 25 μM	4.135 ±0.588	1.378±0.089	0.000±0.000	11.508±1.885
Single Pure Form of Flavonoid (BL-AP) 50 μM	3.915 ±0.492	1.160 ±0.123	0.000±0.000	8.115±1.003
Single Pure Form of Flavonoid (BL-AP) 100 μM	3.476±0.570	1.019±0.177	0.000±0.000	5.994±0.730
Co-Occurring Form of Q-3-R and Q (50μg/mL)		1.579±0.064		1.189±0.238
Co-Occurring Form of Q-3-R and Q (100μg/mL)		1.449±0.098		1.074±0.203
Co-Occurring Form of Q-3-G and Q (50μg/mL)	2.131±0.283			1.104±0.175
Co-Occurring Form of Q-3-G and Q (100μg/mL)	1.762±0.254			0.943±0.086
Co-Occurring Form of Q-7-R and Q (50μg/mL)			0.796±0.137	1.817±0.208
Co-Occurring Form of Q-7-R and Q (100μg/mL)			0.688±0.193	1.462±0.153
Co-Occurring Form of Q-3-R and Q-3-G (50μg/mL)	3.090±0.455	2.016±0.203		
Co-Occurring Form of Q-3-R and Q-3-G (100μg/mL)	2.271±0.359	1.471±0.083		
Co-Occurring Form of Q-3-R and Q-7-R(50μg/mL)		2.623±0.444	0.784±0.126	
Co-Occurring Form of Q-3-R and Q-7-R(100μg/mL)		2.693±0.178	0.753±0.289	
Co-Occurring Form of Q-3-G and Q-7-R(50μg/mL)	2.852±0.366		1.092±0.192	
Co-Occurring Form of Q-3-G and Q-7-R(100μg/mL)	2.693±0.287		1.085±0.088	

Table 2: P_{app} values ($\times 10^{-6}$) of the four compounds in different dose forms (Mean±SD, n=3).

Papp/$\times 10^{-6}$cm·s-1	Single pure flavonoid	Mixure form of flavonoids	Mixure form of flavonoids with inhibitor
Q-3-G	2.126±0.492	0.303±0.107	
Q	13.592±1.175	23.019±0.753	17.937±1.084

Table 3: P_{app} values of flavonoids from BL side to AP side in different dose form (Mean±SD, n=3).

Time (min)	Q-3-G (μg/mL)	Q-3-R (μg/mL)	Q-7-R (μg/mL)	Q (μg/mL)
0	0.0000±0.0000	0.0000±0.0000	0.0000±0.0000	0.0000±0.0000
5	0.1769±0.2773	0.8288±0.6491	1.1872±0.9290	0.4643±0.3621
15	0.5565±0.2113	1.6182±0.2351	2.2186±0.2197	0.8046±0.3604
30	0.4976±0.2462	2.0432±0.4901	2.5770±0.4207	0.8817±0.1671
60	0.6518±0.4710	2.7631±0.6260	3.2999±0.6919	1.2265±0.1874
90	0.3856±0.3013	1.1675±0.9340	1.5003±1.1897	0.5747±0.4530

Table 4: Drug concentration in mice blood at different time after administrated HJE (Mean±SD, n=6).

port with its analogs, Q-7-G, Q-7-R or Q. The P_{app} value of Q-3-G fell to 0.303×10^{-6} cm/s from 2.126×10^{-6} cm/s when it was co-transported with any of its analogs. The results also show increased efflux of Q when it was co-transported, and this action could not be wholly eliminated by the addition of transporter inhibitor (10 μM Verapamil).

Chemical profile of plasma from mice treated with or without HJE reveals absorption of the four main flavonoids after oral administration to mice (Figure 4). Peak concentrations could be found 60 minutes after HJE treatment. Plasma drug concentrations at different time points are showed in Table 4.

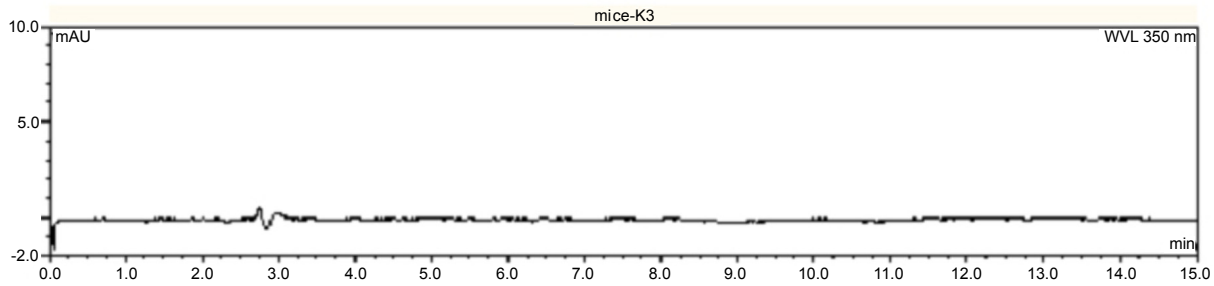
Discussion

The use of herbal medicine has a long tradition in China and other Asian countries. It is widely believed that herbal extract, the mixture form of particular pure compounds, exhibits better effect and less toxicity than each composing chemical therein. However, the proposed mechanism has never been documented. In our study, increased permeability of Q-3-G, Q-3-R and Q-7-R through Caco-2 cell monolayer in mixture form indicates the underlying inter-influence among different chemicals may significantly alter their absorption. Since the potent hepatoprotective action of Q-3-R, Q-3-G and Q-7-R have been reported and defined documented in mice (Li et al., 2007), the better hepatoprotective effect of HJE than quercetin and its analogs (Data not shown) may results from the increased absorption of Q-3-R, Q-3-G and Q-7-R in HJE.

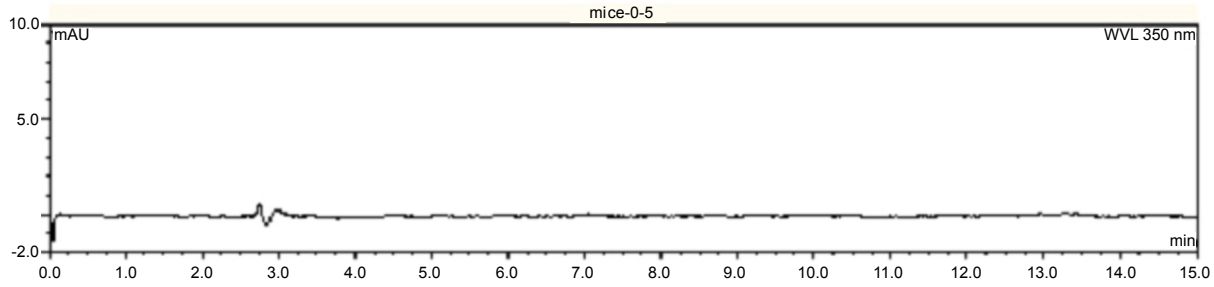
In order to further study the underlying inter-influence of Q-3-R, Q-3-G and Q-7-R in HJE, we investigated the transepithelial permeability of each compound when they were transported through Caco-2 monolayer in pair. Increased P_{app} value of Q-3-G and Q-7-R was observed, which gives evidence that co-transportation of quercetin analogs may increase their absorption. Our study also showed a collaborative inhibition of Q-3-R, Q-7-R and Q on the efflux of Q-3-G but enhanced output of Q by Q-3-R, Q-7-R and Q-3-G. Addition of P-gp inhibitor can not completely suppress the interinfluence among the four flavonoids, indicating that the interaction may not totally act via activating the transport related protein. Further investigation is necessary for elucidating the exact mechanism of the transport of HJE.

Conclusion

In conclusion, the intestinal absorption of four main compounds in HJE was investigated and the results indicate the absorption



(A)



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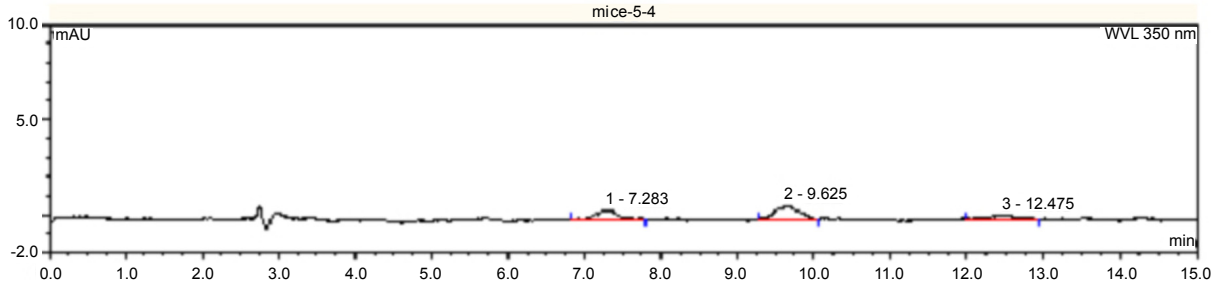

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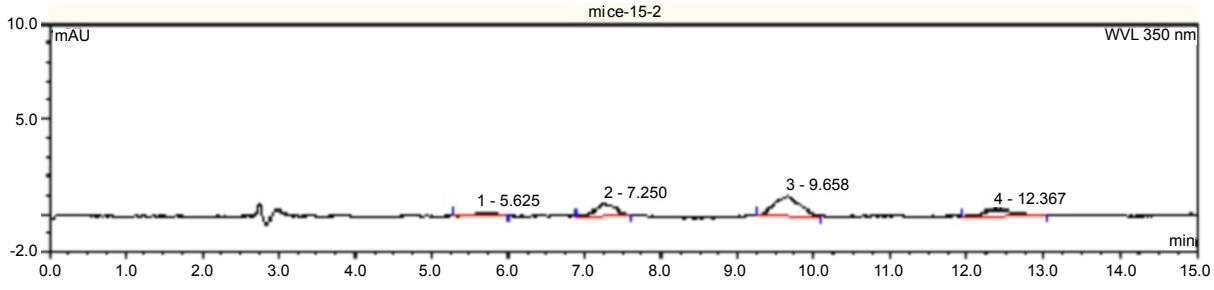

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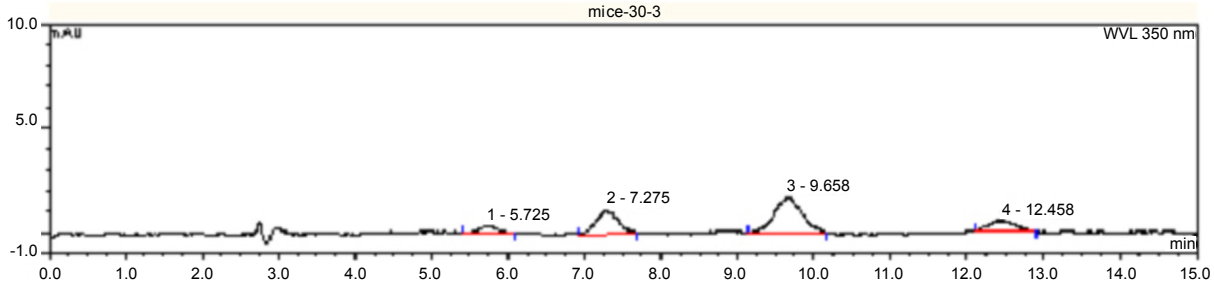

(E)

Figure 4: Chemical profile of mice plasma after administrated with or without HJE (A for blank plasma; B for plasma from mice treated with HJE at 0 min; C for plasma from mice treated with HJE at 5 min; D for plasma from mice treated with HJE at 15 min; E for plasma from mice treated with HJE at 30 min; F for plasma from mice treated with HJE at 60 min; G for plasma from mice treated with HJE at 90 min. In all fig.s, peak 1 was identified as Q-3-G, peak 2 as Q-3-R, peak 3 as Q-7-R, peak 4 as Q).

alters in different dose forms. The increased transport of Q-7-R and Q-3-G, which show potent liver protection when treated individually, as well as decreased transport of quercetin can be observed when the four compounds are co-transporting through the Caco-2 monolayer, revealing that integrated administration may improve the absorption of active components in HJE, which may be the possible reason why HJE show better liver protective action than its individual components. Utilization of herbal drugs and its extracts are often found with lack of scientific interpretation and experimental evidence. This present study combined with our previous investigation provides new perspective to validate the utilization of botanical drug. For further investigation on exact mechanism of botanical drug, more in vivo pharmacokinetic models and molecular approaches are expected in addition to the above mentioned study methods.

Acknowledgement

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