STUDY ON INTESTINAL TRANSPORT OF THE GLYCYRRHIZIC ACID IN THE GANCAOFUZI DECOCTION ACROSS THE CACO-2 CELL MONOLAYER MODEL BY HPLC-ESI-MS METHOD

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ABSTRACT

Objective: *To investigate the transport of Glycyrrhizic Acid in Gancaofuzi decoction ,which consists of R.glycyrrhizae, A. macrocephalae Rhizoma, Cinnamomum cassia Presl and Radix Aconiti Praeparata, and the interaction of the other compounds in the decoction.*

Methods: *In this study, HPLC-ESI-MS method was developed for the semiquantitative determination of Glycyrrhizic Acid on intestinal transport with Ginsenoside Re as internal standard. And this method was successfully applied to transport studies of Glycyrrhizic Acid and the interaction mechanism of the herbs in Gancaofuzi decoction.*

Results: *The results suggest that A. macrocephalae Rhizoma, Cinnamomum cassia Presl or Radix Aconiti Praeparata could increase the intestinal absorption of Glycyrrhizic Acid when mixed with R.glycyrrhizae decoction. And they could reduce the intestinal absorptions of Glycyrrhizic Acid when co-decocted with R.glycyrrhizae (no A. macrocephalae Rhizoma). It might be caused by the different chemical composition between the collaborative decoction and the mixed decoction. And the standard substance of flavonoids could reduce the intestinal absorption of Glycyrrhizic Acid while the standard substance of alkaloids could increase the intestinal absorption.*

Conclusion: *As we know, Chinese medicine is mainly taken orally and absorbed into the body through the small intestine to exert its efficacy. Caco-2 cell monolayer model was used to study the transport of Glycyrrhizic Acid by HPLC-ESI-MS method. And this study provided a new idea of Traditional Chinese Medicine.*

Keywords: *HPLC-ESI-MS, r. glycyrrhizae, saponins, transport, caco-2 cell monolayer model.*

DOI: 10.19193/0393-6384_2019_6_543

Received *November 30, 2018;* ***Accepted*** *February 20, 2019*

Introduction

Traditional Chinese Formula is an important traditional Chinese medicine's means which is used to treat the diseases in the clinical. The classic Chinese Formula Gancaofuzi decoction is well known for its treatment of rheumatic and rheumatoid arthritis. Gancaofuzi decoction is composed of R.glycyrrhizae, A.macrocephalae Rhizoma, Cinnamomum cassia Presl and Radix Aconiti Praeparata[(1)]. Recently, the study of Gancaofuzi decoction is mainly about its chemical composition and pharmacological effects. The compounds of Gancaofuzi decoction include flavonoids, saponins, alkaloids, phenolic acids and lactones. Its major active ingredients are flavonoids and saponins in R.glycyrrhizae, alkaloids in Radix Aconiti Praeparata. While Cinnamomum cassia Presl and macrocephalae Rhizoma mainly contain essential oil[(2-3)].

Gaos' study demonstrated that Gancaofuzi decoction contained 12 kinds of chemical constituents possessing bioactivities which came from Radix Aconiti Praeparata, A. macrocephalae Rhizoma, R.glycyrrhizae, Cinnamomum cassia Presl[(4)] Gancaofuzi decoction plays a role in the treatment of rheumatoid arthritis by restoring antioxidant activity, inhibiting the synthesis of inflammatory cytokines, regulating immune activity and reducing focal bone erosion and bone destruction[(5-7)]. Gancaofuzi decoction is mainly absorbed through the oral and then plays its pharmacological effects. Therefore, to study the absorptions of main chemical compositions of Gancaofuzi decoction by human intestine is very important. Currently, the Caco-2 cell monolayer model is an effective means to study drug absorption[(8-9)]. The Caco-2 cells are derived from human colon adenocarcinoma cells, and can be cultured in vitro to be epithelial differentiation and form tight junctions[(10)].

The cells can differentiate and cover the villus surface (apical side, AP, intestinal cavity side) and substrate surface (basolateral side, BL, intestinal wall side). After 21 days culture, the cells can fusion to form a continuous and complete single-cell layer. After morphological observation and transepithelial electrical resistance testing, the Caco-2 cell monolayer model was determined to be built successfully. At this time, their structure and biochemical properties are similar to the human intestinal epithelial cells with the same cell polarity and tight junctions. Also, the cells can express various transporters and metabolic enzymes as the intestinal epithelial cells[11-12]. As stated above, the Caco-2 cell monolayer model has been recognized as effective tools to be applied in the studies of drug uptake, metabolism, excretion and transport.

In recent years, the saponins being the main composition of Gancaofuzi decoction are concerned for their diversity and various pharmacological effects[13-16]. The main saponin of Gancaofuzi decoction is Glycyrrhizic Acid for its various farmercutical effects such as its in vivo anti-inflammatory activity[17].To investigate the transport of saponins, HPLC-ESI-MS method and internal standard method were established so that the content changes of several saponins through Caco-2 cell monolayer model could be detected. Such study could provide a theoretical basis for the further study of compatibility of traditional Chinese medicine from the perspective of intestinal absorption.

Materials and methods

Materials

Caco-2 human colon carcinoma cell line was obtained from Shanghai Cell Bank of Chinese Academy of Sciences; R.glycyrrhizae, A. macrocephalae Rhizoma and Cinnamomum cassia Presl were purchased from Tongrentang Pharmacy of Changchun; Radix Aconiti Praeparata was purchased from Jiangyou Pieces plant of Sichuan; Dulbecco's modified Eagle's medium (DMEM) was purchased from Corning Corporation(USA); Heat-inactivated fetal bovine serum(FBS) was purchased from Biological Industries Israel Beit Haemek ltd(USA); MTT, Trypsin and Penicillin were purchased from Dingguo Corporation (Beijing, China) DMSO was purchased from Xilong Chemical Corporation (Guangdong, China); streptomycin was purchased from Glenview Corporation (USA); Ginsenoside Re, Glycyrrhizic Acid, Liquiritin and Liquiritigenin were purchased from National Institutes for Food and Drug Control (Beijing, China); Hypaconitine(HA), Mesaconine (MA), Benzoylhypacoitine(BH), Benzoylmesaconine(BM), Benzoylaconitine(BA)were purchased from Biotechnology Co., Ltd. on Haiya Ji (Shanghai, China); methanol and acetonitrile were purchased from Fisher Scientic(America).Milli-Qwater (Millipore Inc, USA) was used in the study; all other chemicals were of analytical grade or better. MCO-175 CO2 incubator was purchased from SANYO (Japan); high speed refrigerated centrifuge was purchased from Eppendorf (Germany); enzyme mark instrument was purchased from TECAN (Australia); 96-well culture plate was purchased from Thermo (USA); transwell cell culture plate (1.12cm surface, 0.4μm pore size, 12mm diameter) was purchased from Corning Costar Corporation (USA).

Sample preparation

R. glycyrrhizae, compatibility with macrocephalae Rhizoma, Cinnamomum cassia Presl, Radix Aconiti Praeparata respectively and Gancaofuzi decoction were extracted twice with boiling water, 40 minutes for the first time and 30 minutes for the second time, and the solutions were filtered through centrifugation. Then, the filtrates were precipitated by 60 % alcohol to remove the polysaccharide. And the filtrates were concentrated to be freeze-dried. Then we got the mixed extracts of the decoction herbs.

Followed, these dried extracts were dissolved in DMEM to obtain the working standard solutions to be used in the study of MTT.

For the observation of transport, these dried extracts were dissolved in Hanks' balanced salt solutions (HBSS) to obtain the working solutions. To study the transport, we dissolved the standard substances with HBSS based on their content in Gancaofuzi decoction.

All samples of the transport study were freeze-dried for further application. Before detected by HPLC-ESI-MS, the samples were mixed with Ginsenoside Re (200 μg/L, dissolved in methanol) as internal standard to 100 μL (v/v = 50/50).

Cell culture

The Caco-2 cells were cultured in DMEM high-glucose medium containing 10 % fetal bovine serum, 100 U/mL penicillin and streptomycin. The cells were grown at 37 °C with 5 % CO^2.

Cytotoxicities of the single herb decoction, two herb collaborative decoction and Gancaofuzi decoction on Caco-2 cells

MTT colorimetric assay was widely used to determine cell growth and cell cytotoxicity[16]. The Caco-2 cells were seeded in 96-well culture plates at a density of 2×104 cells per well and were grown at 37 °C with 5 % CO^2 for 48 h. Then, the cells were treated with a series of working solutions for 2.5 h. The single herb decoction, two herb collaborative decoction or Gancaofuzi decoction were removed, and MTT were added, 100 μL per well (1mg/mL) for 4 h. Then, MTT was removed and DMSO was added, 100 μL per well. When all the blue crystallize generated through above procedure was dissolved by shaking for 10-15 minutes, followed, the absorbance of the plate was tested at 570 nm.

Transport

For transport studies, the Caco-2 cells were seeded in transwell polycarbonate insert filters at a density of 1×105 cells per well and were grown at 37°C with 5 % CO^2 for 21 days. In the first 7 days, the medium was replaced every 2 days, and then replaced every day. The transepithelial electrical resistance of the monolayer cells was measured using a DDS-12A Digital conductivity meter. If a cell could be used in the transport experiments, the transepithelial electrical resistance should exceed 500 $\Omega \cdot cm^{-2}$.

Before the experiments, the cell monolayers were rinsed twice using 37 °C HBSS and the cells were then incubated at 37 °C for 30min with HBSS. After that, the cell monolayers were treated with transport medium containing the single herb decoction, two herb collaborative decoctions, Gancaofuzi decoction or the mixed standard substances in either the AP side or the BL side.

When the transport from AP to BL was studied, 0.5 mL transport medium containing the single herb decoction, two herb collaborative decoction, Gancaofuzi decoction or the mixed standard substances was added to the AP side, and the BL side was treated with 1.5 mL HBSS, and vice versa. Then a 100 μL aliquot of the incubation solution was withdrawn at the times, 20, 40, 60, 90 and 120 mins, respectively, from the receiver chamber, and replaced with the same volume of 37 °C HBSS. At last, 100 μL transport medium was retrieved from the donation chamber and terminate the transportation with 4 °C HBSS. All the samples were freeze-dried for further application.

HPLC-ESI-MS method

HPLC-ESI-MS was used to analysis the samples in the transport study. The conditions of HPLC for analysis were as follows: system, Waters Acela U-HPLC with Acela temperature controlled auto sampler and column oven and Acela 1250 ultra-high pressure liquid pump; cchromatographic column, Kromasil C18(250×4.6 mm, 5 μm); mobile phase A, 0.1%aqueous acetic; mobile phase B, acetonitrile; gradient, 0 to 50 min, mobile phase A from 25 % to 50 %, mobile phase B from 75 % to 50 %; 0 to 5 min, mobile phase A 90 %, mobile phase B 10%; 5to 40 min, mobile phase A from 90 % to 75 %, mobile phase B from 10 % to 25 %; 40 to 70 min, mobile phase A from 75 % to 50 %, mobile phase B from 25 % to 50 %; 70 to 75 min, mobile phase A from 50 % to 30 %, mobile phase B from 50 % to 70 %; flow rate, 0.3 mL/min; injection volume, 10 μL. The conditions of MS for analysis were as follows: LTQ XL ion trap mass spectrometer with ESI electrospray ion source (Thermo, USA). Negative ion mode, mass scan ranged from m/z 150~1000, spray voltage was 4.5 kV, the metal capillary temperature was 250 °C, metal capillary voltage was 40 V, sheath gas flow rate of 30 L/h, sweep gas flow rate of 20 L/h, auxiliary gas flow rate of 5 L/h, lens voltage was 240 V.

Data analysis

The apparent permeabilitycoefficient (Papp) was calculated as follows:

$$P_{app} = (C_t \cdot V) / (C_0 \cdot A \cdot t)^{[18]}$$

where P_{app} is the apparent permeability coefficient in cm/s; C_t is the concentration of a compound got in the receiving chamber at indicated time points in mol/L; C_0 is the initial concentration of a compound in the transport samples on the donor chamber in moL/L; A is the surface area of the transwell in cm^2; V is the volume of receiving chamber in L; t is the time of each time point in min. In this study, C_t or C_0 is the concentration of the analytes at t time or the initial time. And the peak area was integrated by data processing software of LTQ.

Efflux ratio (P_{app} B-A/P_{app} A-B, Er) can intuitively reflect the actual absorption through the Caco-2 cells in the presence of the excretion. The efflux ratio (ER) was calculated as follows:

$$Er = P_{app}\ (BL\text{-}AP) / P_{app}\ (AP\text{-}BL)^{[19]}$$

Where P_{app} (BL-AP) is the BL-AP permeability; P_{app} (AP-BL) is the AP-BL permeability.

Results and discussion

HPLC-ESI-MS method established for the separation and identification of the main saponins

The transport samples were analyzed by HPLC-ESI-MS method and the total ion chromatogram (TIC) is presented in Fig. 1. The retention time and the [M-H] - value of the saponins are presented in Table.1.

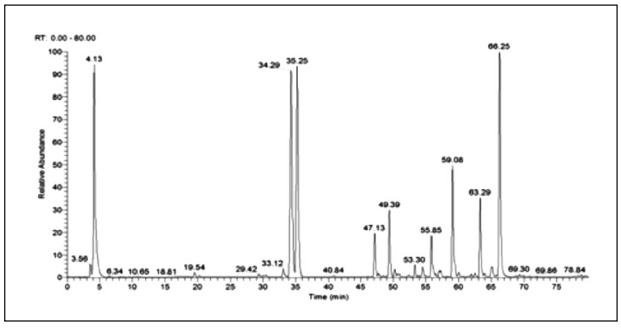

Figure 1: The total ion chromatogram of saponins.

No.	Rt (min)	$[M-H]^-$	Compound
1	63.29	837.37	Glycyrrhizin G2
2	64.08	819.33	Glycyrrhizin E2
3	66.25	821.85	Glycyrrhizic Acid
4	68.55	807.31	Glycyrrhizin B2

Table. 1: The retention times and identification of the saponins.

Cytotoxicities of the single herb decoction, two herbcollaborative decoction and Gancaofuzi decoction on Caco-2 cells

The concentration of each sample for the study of transport was determined according to MTT assay results, as shown in Table. 2. In this table, stands for the mixture of single herb decoctions, while & stands for two herbs collaborative decoction. And the standard substances were dissolved in HBSS based on their content in the compound of Gancaofuzi decoction for the transport study, as shown in Table. 3.

	Sample solution	The concentration of the crude drug (mg/mL)
Single herbs decoction	*R.glycyrrhizae* (A)	5
Mixed decoction	*R.glycyrrhizae* +*A. macrocephalae Rhizoma* (A+B)	5+5
	R.glycyrrhizae + *Cinnamomum cassia Presl* (A+C)	5+10
	R.glycyrrhizae + *Radix Aconiti Praeparata*(A+D)	5+10
	R.glycyrrhizae+ *A. macrocephalae Rhizoma* + *Cinnamomum cassia Presl*+ *Radix Aconiti Praeparata* (A+B+C+D)	5+5+10+10
Collaborative decoction	*R.glycyrrhizae* & *A. macrocephalae Rhizoma* (A &B)	5& 5
	R.glycyrrhizae & *Cinnamomum cassia Presl* (A &C)	5&10
	R. glycyrrhizae & *Radix Aconiti Praeparata* (A&D)	5&10
Gancaofuzi decoction	*R.glycyrrhizae* & *A. macrocephalae Rhizoma* *Cinnamomum cassia Presl* &*Radix Aconiti Praeparata*(A&B&C&D)	5&5&10&10

Table. 2: The concentrations of sample solution for transport study.

	Standard substances	Concentration in total decoction of *Gancaofuzi* decoction (mg/mL)	Concentration for transport study (mg/mL)
Saponins	Glycyrrhizic Acid	2.68	2.68
Flavonoids	Liquiritin	1.35	1.35
	Liquiritigenin	0.32	0.32
Alkaloids	HA	0.02	0.02
	MA	0.01	0.01
	BA	0.07	0.07
	BH	0.05	0.05
	BM	0.14	0.14

Table. 3: The concentrations for transport study of standard substances.

The transports of single R.glycyrrhizae decoction and mixed decoction with A. macrocephalae Rhizoma, Cinnamomum cassia Presl and Radix Aconiti Praeparata

The transports of single R.glycyrrhizae decoction and mixed decoction with A. macrocephalae Rhizoma, Cinnamomum cassia Presl and Radix Aconiti Praeparata were studied respectively. The values of P_{app} A-B, P_{app} B-A and Er (P_{app} B-A/P_{app} A-B) at 120 min are showed in Table. 4.

Compound	Name	P_{app}A-B ($\times 10^{-6}$cm/s)	P_{app}B-A ($\times 10^{-6}$cm/s)	Er
Glycyrrhizic Acid	*R.glycyrrhizae* (A)	5.10±0.24	5.00±0.31	0.98±0.09
	R.glycyrrhizae +*A. macrocephalae Rhizoma* (A+B)	3.00±0.15	2.42±0.24	0.81±0.11
	R.glycyrrhizae +*Cinnamomum cassia Presl* (A+C)	4.00±0.09	3.20±0.26	0.80±0.03
	R.glycyrrhizae +*Radix Aconiti Praeparata* (A+D)	3.85±0.12	3.39±0.17	0.88±0.06
	R.glycyrrhizae +*A. macrocephalae Rhizoma* +*Cinnamomum cassia Presl* +*Radix Aconiti Praeparata* (A+B+C+D)	3.83±0.27	3.45±0.19	0.90±0.04

Table. 4: The transports of Glycyrrhizin G2 and Glycyrrhizic Acid in single R.glycyrrhizae decoction and mixed decoction with A. macrocephalae Rhizoma, Cinnamomum cassia Presl and Radix Aconiti Praeparata (n=3).

Compared to the single R.glycyrrhizae decoction, after mixed with A. macrocephalae Rhizoma, Cinnamomum cassia Presl or Radix Aconiti Praeparata respectively, the values of P_{app} AP-BL, P_{app} BL-AP and Er were all reduced. Therefore, A. macrocephalae Rhizoma, Cinnamomum cassia Presl or Radix Aconiti Praeparata could inhibit the transport of Glycyrrhizic Acid both from AP to BL and from BL to AP. But they could increase the intestinal absorption of Glycyrrhizic Acid.

The transport of Glycyrrhizic Acid in collaborative decoction and Gancaofuzi decoction

To illustrate the importance of co-decocted herbs, we studied the effects of the collaborative decoction and the mixed decoction of single herbs decoction to the transport and absorption of Glycyrrhizic Acid. The transport of collaborative decoction containing R.glycyrrhizae and Gancaofuzi decoction were studied respectively. The values of Papp A-B, Papp B-A and Er (Papp B-A/Papp A-B) at 120 min are showed in Table. 5.

Compound	Name	P_{app}A-B ($\times10^{-6}$cm/s)	P_{app}B-A ($\times10^{-6}$cm/s)	Er
Glycyrrhizic Acid	*R.glycyrrhizae* (A)	5.17±0.13	5.09±0.12	0.98±0.05
	R.glycyrrhizae +A. macrocephalae Rhizoma (A+B)	6.20±0.09	4.42±0.16	0.71±0.06
	R.glycyrrhizae +Cinnamomum cassia Presl (A+C)	3.41±0.11	3.43±0.17	1.00±0.10
	R.glycyrrhizae +Radix Aconiti Praeparata (A+D)	3.52±0.27	11.83±0.31	3.37±0.11
	R.glycyrrhizae +A. macrocephalae Rhizoma +Cinnamomum cassia Presl +Radix Aconiti Praeparata (A+B+C+D)	4.73±0.16	5.91±0.19	1.26±0.16

Table. 5: The transports of collaborative decoction and Gancaofuzi decoction (n=3).

Compared to the single R.glycyrrhizae decoction, after co-decocted with A. macrocephalae Rhizoma, the Papp value from AP to BL of Glycyrrhizic Acid was increased, while the value of Papp from BL to AP and Er was reduced. Therefore, A. macrocephalae Rhizoma could promote the transport of Glycyrrhizic Acid from AP to BL and inhibit the transport of Glycyrrhizic Acid from BL to AP, so that A. macrocephalae Rhizoma increased the intestinal absorption of Glycyrrhizic Acid. And after co-decocted with Cinnamomum cassia Presl, the Papp values from AP to BL of and BL to AP were both reduced and the value of Er was reduced. Therefore, Cinnamomum cassia Presl could inhibit the transport of Glycyrrhizic Acid from AP to BL and BL to AP, and reduce the intestinal absorption of Glycyrrhizic Acid. While, after co-decocted with Radix Aconiti Praeparata, the Papp value from AP to BL of Glycyrrhizic Acid was increased, while the value of Papp from BL to AP and Er were reduced. Therefore, Radix Aconiti Praeparata could inhibit the transport of Glycyrrhizic Acid from AP to BL and promote the transport from BL to AP, and reduce the intestinal absorption of Glycyrrhizic Acid. Results showed that the process of co-decocted herbs changed the content of main components of extracts, which affected herbs' transport and absorption, thus demonstrated the importance of co-decocted herbs.

The results showed that the transport of Glycyrrhizic Acid was different from collaborative decoction and mixed decoction. This phenomenon may be related to the changes in the quality and quantity of the chemical compositions after co-boiling with each other. This also reflected the importance of traditional Chinese medicine compound compatibility application.

The transport of mixed standard substances in Gancaofuzi decoction

For further study of interaction mechanism, the main compounds in Gancaofuzi decoction were mixed based on their content in Gancaofuzi decoction and the transport of Glycyrrhizic Acid was studied. The results are showed in Table. 6.

Compound	Name	P_{app}A-B ($\times10^{-6}$cm/s)	P_{app}B-A ($\times10^{-6}$cm/s)	Er
Glycyrrhizic Acid	*Glycyrrhizic Acid*	27.67±0.11	12.25±0.07	0.44±0.03
	Glycyrrhizic Acid + Flavonoids	26.14±0.18	25.38±0.11	1.26±0.01
	Glycyrrhizic Acid + Alkaloids	20.76±0.12	2.67±0.15	0.13±0.06
	Glycyrrhizic Acid + Flavonoids + Alkaloids	9.01±0.14	15.97±0.21	1.77±0.04

Table. 6: The transport of the mixture of several standard substances in Gancaofuzi decoction (n=3).

When Glycyrrhizic Acid was mixed with the flavonoids (Liquiritin and Liquiritigenin), the values of P_{app} A-B, P_{app} B-A and Er were all increased. That is to say, the flavonoids could reduce the intestinal absorption of Glycyrrhizic Acid. On the other hand, When mixed with the alkaloids (HA, MA, BH, BM and BA), the values of P_{app}A-B, P_{app} B-A and Er were all reduced. That is to say, the alkaloids could increase the intestinal absorption of Glycyrrhizic Acid. And if the Glycyrrhizic Acid was mixed with the flavonoids and the alkaloids at the same time, its intestinal absorption was reduced. It seemed that the influence of the flavonoids to the absorption of Glycyrrhizic Acid is greater than the alkaloids. And these results were consistent with the results of collaborative decoction and Gancaofuzi decoction.

The transport results of the mixed standard substances in Gancaofuzi decoction indicated that the transport of Glycyrrhizic Acid in the decoction was influenced by both of flavonoids and alkaloids.

That is to say, the absorption and transport of the main components of Traditional Chinese medicine compound are mutual influence. This also reflected the importance of traditional Chinese medicine compound compatibility application. And A. macrocephalae Rhizoma, Cinnamomum cassia Presl and Radix Aconiti Praeparata worked together for the absorption of Glycyrrhizic Acid.

Conclusion

HPLC-ESI-MS method has been successfully applied to the analysis of trace amounts of biological sample, which had a good precision, repeatability and accuracy. In this study, a HPLC-ESI-MS method was developed for the separation and identification of the saponins in the Gancaofuzi decoction and detection of their transport through the Caco-2 cell model in vitro.

The results suggest that A. macrocephalae Rhizoma, Cinnamomum cassia Presl or Radix Aconiti Praeparata could increase the intestinal absorption of Glycyrrhizic Acid when they were mixed with the single R.glycyrrhizae decoction. And they could reduce the intestinal absorptions of Glycyrrhizic Acid when co-decocted with R.glycyrrhizae,but not A. macrocephalae Rhizoma. And the flavonoids could reduce the intestinal absorption of Glycyrrhizic Acid while the alkaloids could increase its intestinal absorption. This research provides a new method to study the saponins from biological samples which have importantly pharmacological effects on human body. By the transport study of saponins in Gancaofuzi decoction from single R.glycyrrhizae decoction, mixed decoction, collaborative decoction and mixed standard substances, we found that after co-boiling with each drug, the quality and quantity of the chemical compositions would changed, and this also influenced the transport and further pharmacological effects of chemical compositions.

And this study could also provide a theoretical basis for the further study of compatibility of traditional Chinese medicine from the perspective of intestinal absorption.

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Acknowledgement:
This study was supported by "Study on the Mechanism of "Rebellion" of Radix Paeoniae Rubra with Veratrum nigrum "Based on the Theory of" Eighteen Counteractions "of Traditional Chinese Medicine" in 2019 Jilin Provincial Department of Education(JJKH20191057KJ).

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