

RESEARCH ARTICLE



Investigation of the effective components of the flowers of *Trollius chinensis* from the perspectives of intestinal bacterial transformation and intestinal absorption

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ABSTRACT

Context: The flowers of *Trollius chinensis* Bunge (Ranunculaceae), used for respiratory tract infections, mainly contain flavonoids, phenolic acids, and alkaloids; however, the effective components are debatable because of their unclear *in vivo* activities.

Objective: This study investigates the effective components from the perspectives of biotransformation and absorption.

Materials and methods: Both single person derived- and multiple people-derived intestinal floras were used to investigate the biotransformation of aqueous extract of the flowers of *T. chinensis* (AEOF) at the concentrations of 15.0, 30.0, and 60.0 mg/mL, respectively, for 72 h. Both human colon adenocarcinoma cell line (Caco-2) monolayers and everted gut sacs were employed to evaluate the intestinal absorption of the intestinal bacterial transformed AEOF at the concentrations of 10, 20, and 30 mg/mL, respectively, for 180 min.

Results: 2''-O-β-L-Galactopyranosylorientin, orientin, vitexin, quercetin, veratric acid, proglobeflowery acid, and trolline in AEOF were not transformed by intestinal bacteria, while isoquercetin and trollioside were completely transformed. The P_{app} values of 2''-O-β-L-galactopyranosylorientin, orientin, and vitexin calculated based on the experimental data of intestinal absorption were at the levels of 10^{-5} , whereas those of veratric acid, proglobeflowery acid, and trolline were at 10^{-4} . The mass ratio of flavonoids to phenolic acids to alkaloids changed from 16:10:7 to 9:12:8 before and after absorption.

Discussion and conclusion: The dominant position of flavonoids was replaced by phenolic acids after absorption. In addition to flavonoids which are usually considered as the dominant effective ones, phenolic acids and alkaloids should be also very important for the efficacy of these flowers.

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Introduction

The flowers of *Trollius chinensis* Bunge (Ranunculaceae) are used as a drug in traditional Chinese medicine to treat infectious diseases of the upper respiratory tract, such as pharyngitis, tonsillitis, and bronchitis, due to their antibacterial, antiviral, and anti-inflammatory activities (Li et al. 2011; Wang et al. 2014; Qin et al. 2015). The constituents of these flowers have been extensively investigated during the past decades. It has been reported that these flowers mainly contain three groups of compounds, flavonoids, phenolic acids, and alkaloids (Wang et al. 2004a, 2004b; Qin et al. 2015). Among them, flavonoids constitute the dominant compounds in quantity which account for approximate 16% of the dried weight of the flowers (Wang & Chen 2008; Yan et al. 2008). The flavonoids in these flowers usually exist in the form of flavone C-glycosides, for example, vitexin, orientin, isoswertisin, isoswertiajaponin, 2''-O-β-L-galactopyranosylorientin, 2''-O-β-L-galactopyranosylvitexin, and 2''-O-(2'''-methylbutanoyl) isoswertisin (Li et al. 2007; An et al. 2015; Liu et al. 2015; Qin et al. 2015). These compounds are formed by the sugar moieties linking with the flavone aglycones at C-8 through C-C glycosidic bond (Cai et al. 2006; Liu et al. 2015; Qin et al. 2015). Phenolic acids are slightly less abundant than flavonoids, and most of

them are derivatives of *p*-hydroxybenzoic acid, such as veratric acid, proglobeflowery acid, globeflowery acid, and trollioside (Wang et al. 2004b; Liu et al. 2014; Wu et al. 2014; Yuan et al. 2016). As for alkaloids, only one compound named trolline in minimal content was isolated and structurally elucidated so far from the said flowers (Wang et al. 2004a; Liu et al. 2014).

It is well known that the therapeutic effect of Chinese medicine is ascribed to its special active compounds, which are usually called effective components. Although the chemical constituents of the flowers of *T. chinensis* have been well characterized, their main effective components are still disputable because all of the above three groups exhibit somewhat *in vitro* bioactivities related to the therapeutic effect of the flowers (Lin et al. 2004; Su et al. 2007; Wang et al. 2014). Most researchers considered the flavonoids as the main effective components because they are the most abundant ones in the flowers. However, it is inexplicable due to the following aspects. At first, the bioactivities of the extract of the title flowers do not positively correlate with its content of the total flavonoids, indicating that the flavonoids at least are not the only effective group of compounds in the flowers (Lin et al. 2001). Secondly, flavone C-glycosides are poorly absorbed by human body in the case of oral administration

(Lin et al. 2004; Yuan et al. 2016), which results in a poor bioavailability. Thirdly, the bioactivities of the major flavonoid compounds such as vitexin, orientin, and 2''-O- β -L-galactopyranosylorientin are less potent than the crude extract of the flowers, implying there may be other effective components besides flavonoids. Still others advocated that phenolic acids should be the main contributor to the efficacy of the flowers considering their good solubility and absorbability. In addition, the only alkaloid, trolline, isolated from the title flowers by our research group also displayed significant antiviral and antibacterial activities relevant to the therapeutic effect of the flowers (Wang et al. 2004a). Therefore, which one of the three groups contributes most to the efficacy of the flowers or the relative contribution degree of the three groups (if any) is still unknown. This problem has a significant influence on the pharmaceutical development of the title flowers because no one knows on which group of compounds high importance should be placed.

With the continuous in-depth investigation, it is recognized that the effective components of Chinese medicine are closely related to the *in vivo* processes such as absorption, distribution, metabolism, and elimination (ADME). Absorption is the most important aspect for the evaluation of effective components of the drugs used orally in that only the compounds absorbed through intestine and other organs or tissues into the circulation can be effective (Wang 2006; Yang et al. 2007). As a matter of fact, the compounds are usually structurally transformed by the intestinal bacteria before being absorbed into the blood. Based on this principle, in order to provide a basis for the assessment of the effective components of the flowers, we investigated the absorption including the intestinal bacterial transformation of the aqueous extract of the flowers of *T. chinensis* (AEOF) using the well-known *in vitro* models, i.e., the models of intestinal bacterial transformation, human colon adenocarcinoma cell line (Caco-2) monolayers, and everted gut sacs to ascertain the absorbed compounds.

Materials and methods

Intestinal bacterial transformation and intestinal absorption

Intestinal bacterial transformation

Each 3 g of fresh feces from 3 healthy men and 3 healthy women of 23–25 years old was suspended in 200 mL of general anaerobic medium (GAM, including L-cystine hydrochloride, soluble starch, pepsin, tryptone, peptone, soy peptone, serum powder, yeast extract, beef extract, and beef liver extract powder purchased from Beijing Aoboxing Biotechnology Co., Ltd, Beijing, China) broth prepared as per a literature (Yang & Xu 2011), respectively, and cultured in an anaerobic incubator (Shanghai Cimomedical Appliance Co., Ltd., Shanghai, China) at 37 °C for 24 h (Tsuchihashi et al. 2009). The supernatant (0.5 mL) was taken out, diluted 20-fold with GAM, and incubated at 37 °C for 24 h in the anaerobic incubator again to obtain the activated intestinal bacteria. Each 100 μ L of the activated intestinal bacteria from the 6 volunteers were added into 11.4 mL of GAM, mixed adequately, and incubated in the same conditions as previously to obtain the mixture of activated intestinal bacteria. About 0.4513, 0.9026, and 1.8052 g of AEOF were dissolved in 30 mL of GAM on a super clean bench (Beijing Great Wall Air Purification Co. Ltd., Beijing, China), respectively, to generate the experimental groups. Then, the triplicate GAM containing AEOF was divided into 24 screw-cap test tubes, and each screw-cap test tube had 3 mL of the GAM and 100 μ L of the activated intestinal bacteria and the mixture of activated intestinal bacteria, respectively.

They were incubated under the same condition, and the test tubes of each concentration in triplicate were removed from the anaerobic incubator at 0, 2, 4, 6, 12, 24, 48, and 72 h. About 100 μ L of the bacterial suspension was replaced with GAM to obtain the control group. The GAM absent of the AEOF was used as the blank group. The samples were centrifuged at 16162 g for 15 min at 4 °C to remove the intestinal bacteria; then, 3 volumes of methanol was added into 200 μ L of the supernatant, mixed adequately, and centrifuged at 16162 g for 15 min at 4 °C to remove protein. About 300 μ L of the supernatant was collected, filtered through a 0.45 μ m membrane filter, and stored at –20 °C before HPLC analysis.

HPLC conditions

The HPLC analysis was performed using a Waters 1500 series (Waters Business Operations, Milford, MA) equipped with a 1525 binary HPLC pump, an on-line degasser, a manual injector and a 2489 UV/Visible detector. A Phenomenex C₁₈ column (4 μ m, 250 mm $\times$ 4.6 mm) was used to analyze the resultant solutions. The temperature was set at 35 °C and the UV detecting wavelengths were 254 and 365 nm. The mobile phase consisted of acetonitrile (HPLC grade, Fisher Co., Pittsburgh, PA) (A) and 1.0% acetic acid (Beijing Chemical Works, Beijing, China) in purified water (v/v) (B), and the gradient elution program was as follows: 5–10% A (0–5 min), 10–20% A (5–10 min), 20–25% A (10–22 min), 25–40% A (22–35 min), 40–50% A (35–40 min), and 50–5% A (40–42 min) at a flow rate of 1.0 mL/min. The volume injected was 5 μ L.

Absorption experiment with Caco-2 model

Cell culture. The Caco-2 cells (No. 3111C0001CCC000100, passage 34) were provided by the Cell Resource Center, Peking Union Medical College (the headquarters of National Infrastructure of Cell Line Resource, NSTI). The cell line's species origin identified by PCR was verified by STR profiling (FBI, CODIS), and it was free of mycoplasma contamination by PCR and culture. The Caco-2 cells were cultured in the culture medium consisting of Dulbecco's Modified Eagle Medium (DMEM, Gibco Inc., Grand Island, CA)-fetal bovine serum (FBS, Gibco Inc., Grand Island, CA)-nonessential amino acids (NEAA, Corning Costar, Cambridge, MA)-'penicillin-streptomycin' (Corning Costar, Cambridge, MA) (100:10:1:1, v/v/v/v) in 25 cm² flasks, which were incubated in 5% CO₂ and constant temperature (37 °C) and humidity in the incubator. The culture medium was replaced every 2 or 3 days, and the cells were split 1:2 or 1:3 to conduct passage when they reached 80% confluence.

MTT assay. The Caco-2 cells were seeded into 96-well plates as they were in logarithmic growth phase. Each well was added with 100 μ L of the cell suspension (8.0×10^3 cells/mL) and incubated in 5% CO₂ and constant temperature (37 °C) and humidity in the incubator. After the cells were confluent, the culture medium was replaced with 100 μ L of Hanks balanced salt solution (HBSS, including sodium chloride, potassium chloride, sodium bicarbonate, dipotassium hydrogen phosphate, disodium hydrogen phosphate dodecahydrate, and D-glucose manufactured by Beijing Chemical Works, Beijing, China) (control group), HBSS containing the intestinal bacterial transformed AEOF at the concentrations of 10, 20, and 30 mg/mL (experiment groups), and paclitaxel (Beijing SL Pharmaceutical Co., Ltd., Beijing, China) (positive control) at the concentrations of 0.02, 0.04, and

0.08 mg/mL in HBSS in quadruplicate, respectively. The cells were incubated under the same conditions for 4 h; then, 100 μ L of 3-(4,5-dimethylthiazolyl)-2,5-diphenyl tetrazolium bromide (MTT, Amresco, Radnor, PA) solution at the concentration of 0.5 mg/mL took the place of HBSS. After the cells were incubated under the same conditions for 4 h, the supernatant was replaced by 150 μ L of dimethyl sulphoxide (DMSO, Sigma Chemical Co., Deisenhofen, Germany); then, the cells were shaken for 10 min. The absorption of the samples was measured on a microplate reader (BioTek Instruments, Inc., Winooski, VT) at the wavelength of 570 nm.

Cell differentiation. About 1.5 mL of the culture medium was added into the basal (BL) side of each well of the 12-well Transwell plates (insert diameter 12 mm, pore size 3.0 μ m, membrane growth area 1.12 cm², Corning Costar, Cambridge, MA), and 0.5 mL of the Caco-2 cell suspension at the density of 2.0×10^5 cells/mL was seeded into the apical (AP) side of each insert. The cells were incubated in 5% CO₂ and constant temperature (37 °C) and humidity in the incubator. The culture medium in both AP and BL sides was replaced every other day in the first week, then that in AP side was replaced every day and that in BL side was replaced every other day. The cells would differentiate and form a confluent monolayer. The integrity and viability of the cell monolayers were evaluated by measuring transepithelial electrical resistance (TEER) values in the culture medium at 37 °C using a Millicell[®]-ERS system (Millipore Corp., Bedford, MA), and only the inserts with the TEER above 600 Ω /cm² on day 21 were selected to perform the transport experiment.

Transport experiment

Culture medium in both AP and BL sides was removed on day 21, and the monolayers were rinsed twice with HBSS at 37 °C. Then, 0.5 and 1.5 mL of HBSS were added into the AP and BL sides, respectively, and the cells were cultured in a thermostatic shaker (Beijing Jiayuan Xingye Technology Ltd., Beijing, China) at 50 rpm and 37 °C for 30 min. The intestinal bacterial transformed AEOF at the concentrations of 10, 20, and 30 mg/mL in HBSS were added into AP (0.5 mL) or BL (1.5 mL) side in triplicate, respectively, and the receiving chambers were added with the corresponding volume of HBSS. Propranolol and atenolol (Sigma Chemical Co., Deisenhofen, Germany), which are the markers for good and poor permeability, respectively, were employed as the compounds of reference group. About 200 μ L of the solution in the receiving chambers was collected at 30, 60, 90, 120, 150, and 180 min, respectively, and then the same volume of HBSS was added. All samples were stored at –20 °C and filtered through a 0.45 μ m membrane filter for HPLC analysis.

HPLC conditions

The HPLC conditions used for the analysis of the intestinal bacterial transformed AEOF before and after transport experiment was the same as above. An Agilent TC-C₁₈ column (5 μ m, 250 mm $\times$ 4.6 mm) was used to analyze the resultant solutions of propranolol and atenolol at 30 °C, and the isocratic elution was carried out using methanol (HPLC grade, Fisher Co., Pittsburgh, PA)-monopotassium phosphate (Beijing Chemical Works, Beijing, China) (0.02 mol/L) (1:1, v/v) at the flow rate of 0.9 mL/min and acetonitrile-monopotassium phosphate (0.05 mol/L) (1:9, v/v) at 0.8 mL/min for the resultant solutions of propranolol and atenolol, respectively. The UV detecting

wavelengths were 290 and 277 nm for propranolol and atenolol, respectively, and the injection volumes were all 10 μ L.

Absorption experiment with everted gut sacs

Processing of intestinal bacterial transformed AEOF

About 150 μ L of the intestinal bacterial suspension was added into 5.0 mL of GAM containing 400 mg of AEOF in sextuplicate and incubated at 37 °C for 24 h in the anaerobic incubator. Then, the resulting solutions were centrifuged at 16262 g for 15 min at 4 °C to get rid of the intestinal bacteria. The supernatant was collected, added with 2 volumes of methanol, and centrifuged at 16262 g for 15 min at 4 °C to remove proteins. The supernatant was concentrated at 55 °C and lyophilized in a lyophilizer (Beijing Boyikang Experimental Instrument Co., Ltd., Beijing, China). The resultant powder was stored at –20 °C until the next test.

Preparation of everted gut sacs

Sprague-Dawley (SD) rats (200 $\pm$ 20 g) provided by Beijing Vital River Laboratory Animal Technology Co., Ltd. (Beijing, China) were fed with standard rodent diet in plastic cages at 25 °C in the light-controlled environment. The experiment was conducted after the animals were fasted for 24 h while they were free to drink water. All animal experiments were carried out according to the protocol approved by the Animal Ethics Committee of Beijing University of Chinese Medicine. The SD rats were divided into 3 groups randomly in triplicate and sacrificed by cervical dislocation. A fragment of 10 cm jejunum was taken out from each sacrificed rat, and the mesentery was removed carefully. The fragments of jejunum were washed in Tyrode solution (sodium chloride, potassium chloride, calcium chloride, sodium dihydrogen phosphate, sodium bicarbonate, magnesium chloride, and D-glucose bought from Beijing Chemical Works, Beijing, China) at 0 °C as per a literature (Surya et al. 2014) until insoluble substances were removed completely; then, they were everted with a latex tube. One end of the everted fragments was tied tightly, and the other one was fixed on the sampler to form everted intestinal sack. In the blank control group, each sack was added with 2.0 mL of 37 °C Tyrode solution and put into a container with 30 mL of Tyrode solution bubbled with CO₂–O₂ (5:95, v/v) at 37 °C for 5 min to obtain the blank intestinal Tyrode solution (Liu et al. 2014; Surya et al. 2014). In the experiment groups, the Tyrode solution in the sack was replaced by the same volume of Tyrode solution containing intestinal bacterial transformed AEOF at the concentrations of 10, 20, and 30 mg/mL, respectively, and incubated under the same conditions as the blank control group. Each 200 μ L of the samples was collected at 30, 60, 90, 120, 150, and 180 min, and replaced by 200 μ L of Tyrode solution. All samples and the blank intestinal Tyrode solution were stored at –20 °C and filtered through 0.45 μ m membrane filter previous to HPLC assay.

Methodological test

Preparation of samples for standard curve and quality control

The reference compounds, 2''-O- β -L-galactopyranosylorientin, orientin, vitexin, quercetin, isoquercetin, trollioside, veratric acid, proglobeflowery acid, and trolline (The compounds were isolated from the flowers of *T. chinensis* by our group, and their purities were above 98% determined by HPLC assay) were prepared into the stock solutions of 512.50, 72.20, 287.50, 250.00, 26.25, 262.50,

362.50, 33.89, and 287.50 µg/mL, respectively, and then they were diluted with methanol to obtain the working solutions. The quality control (QC) samples of above references were prepared with GAM, HBSS, and Tyrode solutions at three different concentrations. The concentration-time relationship of the compounds was analyzed by linear regression, and the linear regression equations and the linearity ranges of all analytes are shown in Table 1. In the chromatogram obtained with the reference solutions, no significant interfering peak adjacent to the peaks of 2''-O-β-L-galactopyranosylorientin, orientin, vitexin, quercetin, isoquercetin, trollioside, veratric acid, proglobeflowery acid, and trolline was detected. The retention times, tailing factors, limits of detection (LOD) and quantification (LOQ) of above compounds are shown in Table 1. These data showed that the HPLC conditions were suitable for the analysis of all analytes.

Repeatability and precision

Six replicate QC samples of three concentrations were used to estimate the repeatability of all analytes in GAM, HBSS, and Tyrode solutions, respectively. The inter- and intra-day precisions were evaluated by QC samples of three concentrations of all analytes in GAM, HBSS, and Tyrode solutions with 6 replicates on the same day and 3 continuous days, respectively. Both repeatability and precision were expressed as relative standard deviation (RSD). The results of repeatability and inter- and intra-day precisions provided in Tables 2–4 were in the acceptable ranges, demonstrating that the method used was repeatable and reliable.

Stability and accuracy

The stability was assessed with triplicate QC samples of 3 concentrations of all analytes in GAM, HBSS, and Tyrode solutions under the following conditions: (1) the QC samples were stored

at ambient temperature for 24 h; (2) the QC samples were treated with 3 freeze-thaw cycles; (3) the QC samples were stored at −20 °C for 15 days. The accuracy was estimated by six replicate QC samples of 3 concentrations on the same day. The stability and accuracy were expressed as RSD and relative error (RE), respectively. The results of stability and accuracy shown in Tables 2–4 indicated that the samples under the analytical conditions were stable and the method was accurate.

Calculation

The P_{app} was calculated using the formula $P_{app} = (dQ/dt) \times (1/A) \times (1/C_0)$, wherein, dQ (µg) represented the accumulation in the receiver chamber; dt (s) represented the time of transport; A (cm²) represented the surface area of the Caco-2 monolayers or the effective intestines (Liu et al. 2014; Ding et al. 2015); C_0 (µg/mL) represented the initial concentration of the corresponding compound in the donor chamber. The transport percentage (%) was calculated using the formula transport percentage (%) = $dQ/(C_0 \times V) \times 100$, wherein, V (mL) represented the volume of the donor compartment.

The following formula was used to calculate the cell survival rate = $\bar{A}/\bar{A}_0 \times 100\%$, wherein, $\bar{A}$ represented the average absorbance value of the experiment groups, and $\bar{A}_0$ represented the average absorbance value of the control group.

Results

Intestinal bacterial transformation

The intestinal bacterial transformation model was employed to investigate the transformation of the constituents of AEOF. As shown in Figures 1(A–D), 2 and 3, and Table 5, the major compounds, e.g., orientin, vitexin, 2''-O-β-L-galactopyranosylorientin,

Table 1. The results of methodological evaluation (GAM).

Compounds	Concentration (µg/mL)	Precision (%RSD, $n = 6$)		Accuracy (%RE, $n = 6$)	Repeatability (%RSD, $n = 6$)	Stability (%RSD, $n = 3$)		
		Intra-day	Inter-day			Freeze-thaw	Short-term	Long-term
Orientin	0.06	2.76	4.42	6.03	1.16	7.50	3.70	6.75
	9.27	8.01	3.06	7.87	2.81	9.88	3.22	3.36
	37.06	2.55	1.26	1.87	1.57	8.49	5.42	1.83
Vitexin	0.22	9.64	5.13	7.99	8.02	5.10	4.45	2.77
	4.19	6.02	6.17	3.49	4.59	7.17	5.84	4.19
	16.74	3.02	2.95	−2.43	4.40	3.08	7.62	3.13
2''-O-β-L-Galactopyra-nosylorientin	0.05	8.33	7.91	−9.74	7.49	3.16	8.41	8.94
	35.58	1.81	1.11	2.28	1.70	1.46	5.52	1.35
	142.30	5.95	7.93	2.83	3.25	4.41	9.11	2.19
Quercetin	0.22	2.33	2.29	2.95	3.29	1.74	2.88	4.36
	3.57	1.62	1.28	−5.09	1.34	1.46	1.76	9.57
	14.28	2.27	1.97	1.78	1.89	2.57	1.78	1.76
Isoquercetin	0.11	3.70	3.45	5.51	5.09	2.57	3.06	5.00
	0.93	1.38	1.43	6.06	2.12	5.87	4.99	2.79
	3.72	3.74	4.52	3.73	4.85	5.19	3.72	6.94
Veratric acid	0.12	7.78	4.7	8.75	2.51	7.17	1.37	8.54
	10.55	3.09	1.94	−8.49	2.37	3.09	4.24	8.30
	42.20	2.38	2.93	−6.03	2.87	2.58	3.52	2.80
Proglobeflowery acid	0.88	5.27	2.57	8.05	1.86	7.35	3.06	4.57
	28.27	9.26	5.22	5.61	8.14	5.37	3.43	7.97
	113.08	5.42	5.50	3.31	4.21	5.85	5.56	3.95
Trollioside	0.37	5.73	6.31	5.56	8.92	2.26	6.26	9.42
	7.81	6.66	5.07	−7.37	6.08	8.90	7.05	4.64
	31.24	5.77	3.97	1.76	5.90	0.96	5.60	5.27
Trolline	2.27	5.73	6.32	5.56	5.11	2.26	6.26	9.42
	61.25	9.42	5.28	6.40	8.22	4.63	1.15	5.68
	245.00	2.25	3.07	−2.99	8.89	1.55	2.04	1.39

Table 2. The results of methodological evaluation (HBSS solution).

Compounds	Concentration ($\mu\text{g/mL}$)	Precision (%RSD, $n = 6$)		Accuracy (%RE, $n = 6$)	Repeatability (%RSD, $n = 6$)	Stability (%RSD, $n = 3$)		
		Intra-day	Inter-day			Freeze-thaw	Short-term	Long-term
Orientin	0.06	6.56	9.59	-2.51	7.19	7.88	6.68	9.55
	9.27	2.71	3.06	4.48	3.45	3.57	2.07	2.07
	37.06	1.50	2.54	1.61	1.92	1.11	4.49	3.95
Vitexin	0.22	8.29	9.96	-7.07	8.06	8.36	7.07	3.53
	4.19	3.01	6.17	3.49	4.11	1.98	4.85	4.77
	16.74	3.81	4.07	2.06	6.10	1.12	5.49	4.07
2 ² -O- β -L-Galactopyranosylorientin	0.05	8.45	6.38	2.44	9.07	9.89	5.79	8.36
	35.58	3.78	1.11	-7.45	3.76	1.86	1.45	4.76
	142.30	8.66	4.96	-1.52	4.74	8.58	3.99	3.87
Veratric acid	0.12	8.14	8.51	8.91	4.98	9.64	8.06	9.01
	10.55	1.10	1.94	-7.45	1.98	1.03	2.56	2.07
	42.20	1.73	1.88	5.47	2.23	2.67	2.36	1.02
Proglobeflowery acid	0.88	4.44	2.97	7.38	5.83	5.29	1.01	8.83
	28.27	6.49	5.22	4.17	5.82	1.69	6.56	8.04
	113.08	3.64	3.45	1.09	7.45	4.31	7.30	1.52
Trolline	2.27	5.19	5.25	9.58	5.94	2.12	3.94	8.61
	61.25	5.91	5.28	3.21	6.78	7.26	1.52	7.83
	245.00	3.57	2.56	0.93	4.33	4.51	2.27	4.93

Table 3. The results of methodological evaluation (Tyrode solution).

Compounds	Concentration ($\mu\text{g/mL}$)	Precision (%RSD, $n = 6$)		Accuracy (%RE, $n = 6$)	Repeatability (%RSD, $n = 6$)	Stability (%RSD, $n = 3$)		
		Intra-day	Inter-day			Freeze-thaw	Short-term	Long-term
Orientin	0.06	9.73	7.12	4.16	6.69	9.76	6.25	4.50
	9.27	2.45	2.45	-1.12	4.45	1.24	3.24	5.01
	37.06	1.69	1.36	1.02	1.49	2.40	2.82	6.29
Vitexin	0.22	9.04	9.61	-3.78	5.76	5.11	9.34	0.87
	4.19	2.74	1.25	2.68	2.87	0.33	1.68	3.58
	16.74	5.89	5.42	-2.32	7.16	3.46	6.63	8.69
2 ² -O- β -L-Galactopyranosylorientin	0.05	8.45	2.84	5.72	9.83	8.31	4.23	1.02
	35.58	3.78	1.25	0.91	-3.03	0.87	1.15	1.46
	142.30	8.66	0.30	0.96	-0.64	0.20	4.18	4.71
Veratric acid	0.12	7.82	9.26	4.89	13.30	8.66	6.02	6.32
	10.55	2.74	3.46	-4.48	2.07	4.28	3.01	0.20
	42.20	2.66	2.84	-6.50	2.81	3.11	1.80	2.99
Proglobeflowery acid	0.88	5.54	8.05	8.82	7.18	7.60	5.54	5.17
	28.27	5.07	4.72	-3.47	4.69	4.15	5.07	6.65
	113.08	1.87	1.49	2.86	3.64	7.46	2.27	4.34
Trolline	2.27	5.58	5.95	8.03	6.28	3.51	3.70	7.36
	61.25	7.55	1.25	7.47	3.19	0.79	4.09	9.77
	245.00	3.04	4.16	1.35	3.60	2.72	5.95	4.52

Table 4. The results of system suitability test.

Compounds	Retention time (min)	Tailing factor	LOD (ng)	LOQ (ng)	Calibration curve
Orientin	15.876	1.01	0.08	0.28	$y = 21994x + 1469.4$ ($r = 0.9996$, 0.06–37.06 $\mu\text{g/mL}$)
Vitexin	17.673	1.14	0.07	0.24	$y = 14992x + 2103.4$ ($r = 0.9998$, 0.22–16.74 $\mu\text{g/mL}$)
2 ² -O- β -L-Galactopyranosyl-orientin	14.054	0.98	0.08	0.26	$y = 15176x - 1132.4$ ($r = 0.9998$, 0.05–142.30 $\mu\text{g/mL}$)
Quercetin	34.402	1.05	0.18	0.62	$y = 16809x - 1867.9$ ($r = 0.9987$, 0.22–14.28 $\mu\text{g/mL}$)
Isoquercetin	18.667	1.17	0.16	0.54	$y = 47596x - 2454.9$ ($r = 0.9994$, 0.11–3.72 $\mu\text{g/mL}$)
Trollioside	21.102	0.97	0.76	2.53	$y = 7321.7x - 2599.8$ (0.9995, 0.37–31.24 $\mu\text{g/mL}$)
Veratric acid	20.490	1.03	0.10	0.34	$y = 31588x + 60.7$ ($r = 0.9995$, 0.12–42.20 $\mu\text{g/mL}$)
Proglobeflowery acid	39.799	0.92	2.33	7.76	$y = 1204.1x - 1052.6$ ($r = 0.9998$, 0.88–113.08 $\mu\text{g/mL}$)
Trolline	14.619	1.09	5.90	19.67	$y = 638.6x - 1964.2$ ($r = 0.9998$, 2.27–245.00 $\mu\text{g/mL}$)

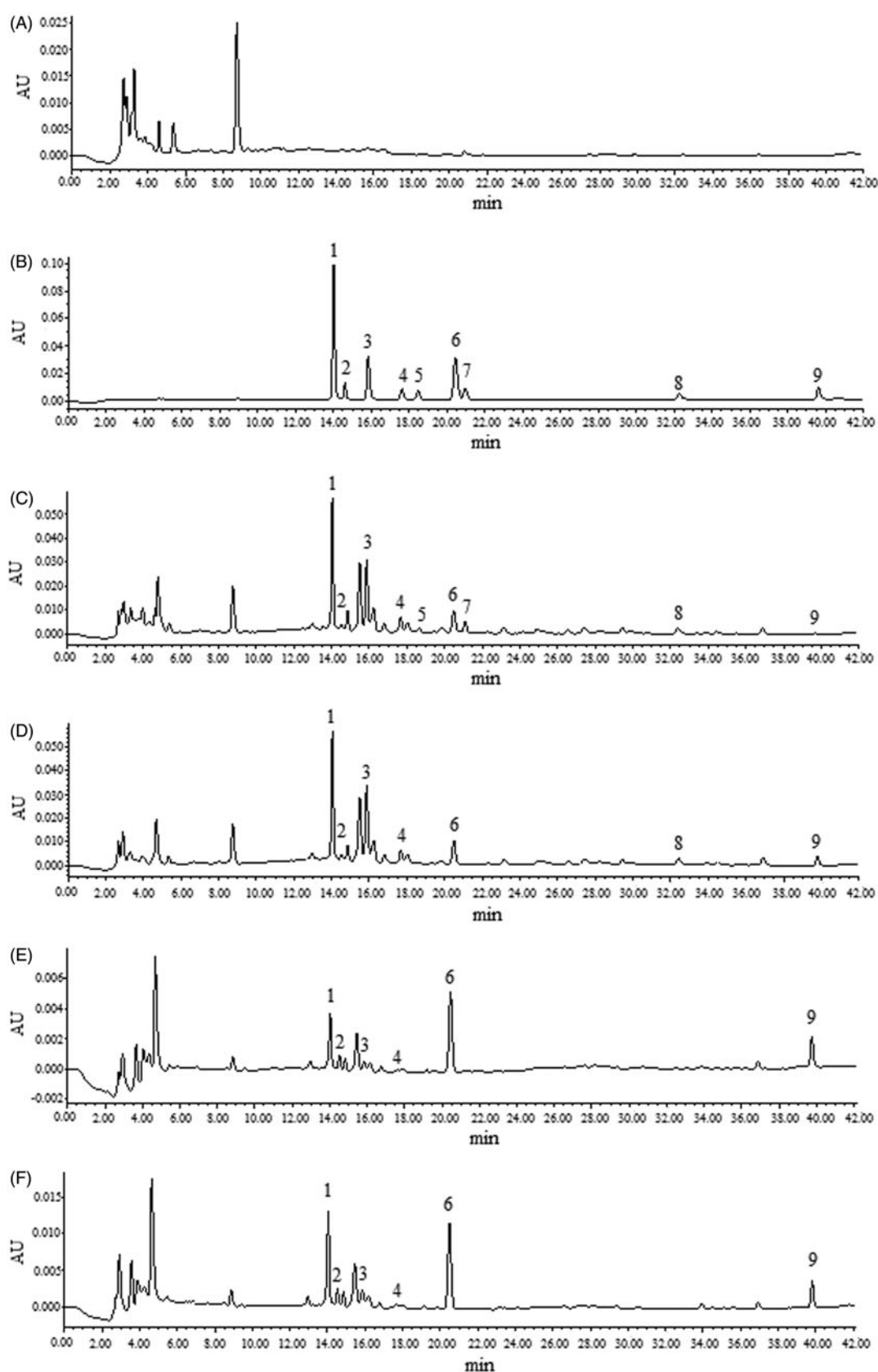


Figure 1. The HPLC fingerprints of various samples. A: GAM; B: references in methanol; C: AEOF in GAM; D: intestinal bacterial transformed AEOF in GAM; E: intestinal bacterial transformed AEOF transported by Caco-2 cell monolayers; F: intestinal bacterial transformed AEOF transported by everted gut sacs. 1: 2"-O- β -L-galactopyranosylorientin; 2: trolline; 3: orientin; 4: vitexin; 5: isoquercetin; 6: veratric acid; 7: trollioside; 8: quercetin; 9: proglobefloweric acid.

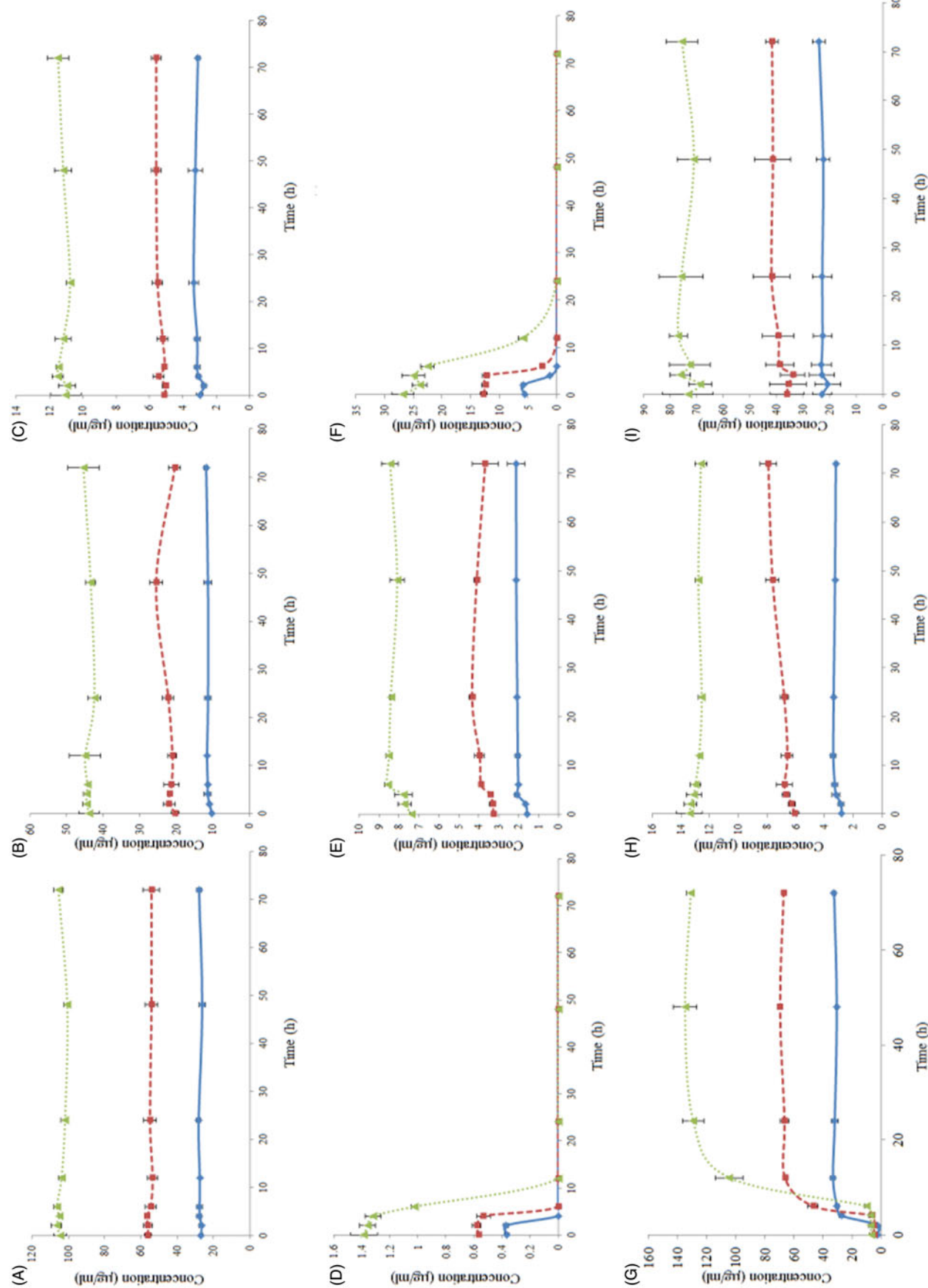


Figure 2. The concentrations of the compounds in AEOF transformed by intestinal bacteria from a single volunteer as a function of time. Solid line: the concentration of the extract was 15 mg/mL; dashed line: the concentration of extract was 30 mg/mL; dotted line: the concentration of the extract was 60 mg/mL. A: 2"-O-β-L-galactopyranosylorientin; B: orientin; C: quercetin; D: isoquercetin; E: vitexin; F: trolox; G: trolox; H: veratric acid; I: trolline.

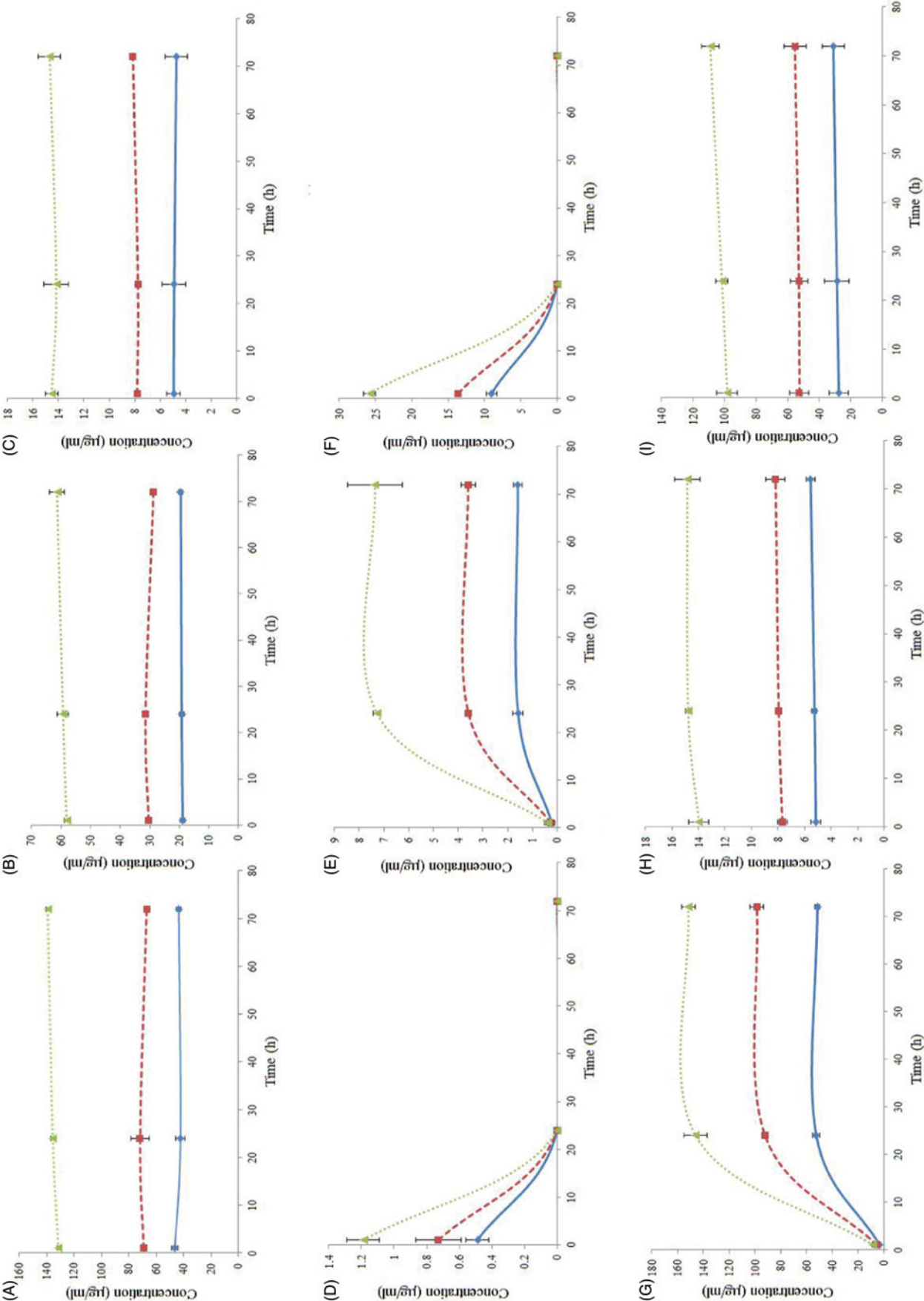
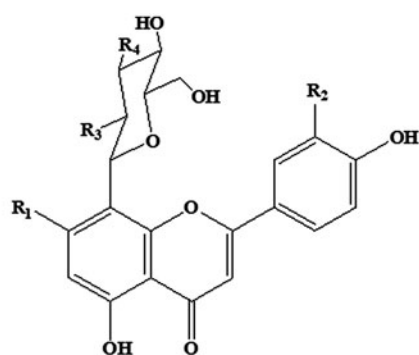


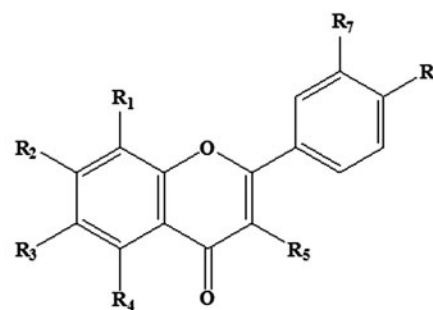
Figure 3. The concentrations of the compounds in AEOF transformed by the mixture of intestinal bacteria from 6 volunteers as a function of time. Solid line: the concentration of the extract was 15 mg/mL; dashed line: the concentration of extract was 30 mg/mL; dotted line: the concentration of the extract was 60 mg/mL. A: 2"-O- β -l-galactopyranosylorientin; B: orientin; C: vitexin; D: isoquercetin; E: quercetin; F: trollose; G: progibeflowery acid; H: veratric acid; I: trollose.

Table 5. The contents of the six compounds in the extract in various stages.

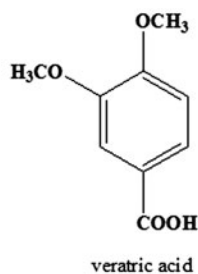
Compounds	Before transformed by intestinal bacteria (μg/mL)	After transformed by intestinal bacteria (μg/mL)	After transported with Caco-2 cell monolayers (μg/mL)	After transported with everted gut sacs (μg/mL)
Orientin	10.27	11.83	0.33	0.32
	20.33	20.38	0.35	0.49
	43.87	43.42	0.82	1.04
Vitexin	2.98	3.10	0.19	0.11
	5.11	5.70	0.23	0.14
	11.04	11.19	0.39	0.50
2 ² -O-β-L-Galactopy-ranosylorientin	26.86	28.06	0.41	1.57
	56.18	56.34	1.44	1.69
	104.39	100.57	4.78	5.38
Quercetin	1.56	2.12	0.00	0.00
	3.25	4.02	0.00	0.00
	7.38	8.45	0.00	0.00
Isoquercetin	0.37	0.00	0.00	0.00
	0.56	0.00	0.00	0.00
	1.38	0.00	0.00	0.00
Veratric acid	2.81	3.22	0.92	0.59
	6.10	7.44	1.62	1.64
	13.39	12.78	2.79	2.02
Trollioside	5.62	0.00	0.00	0.00
	12.76	0.00	0.00	0.00
	26.84	0.00	0.00	0.00
Progloboflowery acid	2.55	32.40	11.11	1.95
	4.78	67.09	17.18	4.95
	6.67	134.85	23.46	7.74
Trolline	22.80	22.37	8.10	4.33
	35.96	36.28	13.59	6.97
	73.23	71.00	19.15	14.76



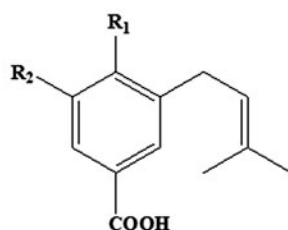
orientin: R₁=OH; R₂=OH; R₃=OH; R₄=OH;
 vitexin: R₁=OH; R₂=H; R₃=OH; R₄=OH;
 2²-O-β-L-galactopyranosylorientin: R₁=OH; R₂=OH; R₃=O-β-L-galactopyranosyl; R₄=OH;



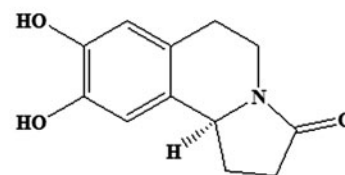
quercetin: R₁=H; R₂=OH; R₃=H; R₄=OH; R₅=OH; R₆=OH; R₇=OH;
 isoquercetin: R₁=H; R₂=OH; R₃=H; R₄=OH;
 R₅=O-β-D-glucopyranosyl; R₆=OH; R₇=OH;



veratric acid



trollioside: R₁=O-β-D-glucopyranosyl; R₂=OCH₃;
 progloboflowery acid: R₁=OH; R₂=OCH₃;



trolline

Figure 4. The structures of the nine compounds.

quercetin, veratric acid, progloboflowery acid, and trolline were not changed until 72 h during the intestinal bacterial transformation using either the single person derived- or the multiple people-derived intestinal bacteria, while isoquercetin and trollioside were completely transformed to quercetin and progloboflowery acid in 24 h, respectively. The structures of above nine compounds are shown in Figure 4.

Transport of intestinal bacterial transformed AEOF through Caco-2 cell monolayers and everted gut sacs

The intestinal absorption of the intestinal bacterial transformed AEOF was studied using the famous transport models of Caco-2 cell monolayers and everted gut sacs. The results are shown in Figure 5.

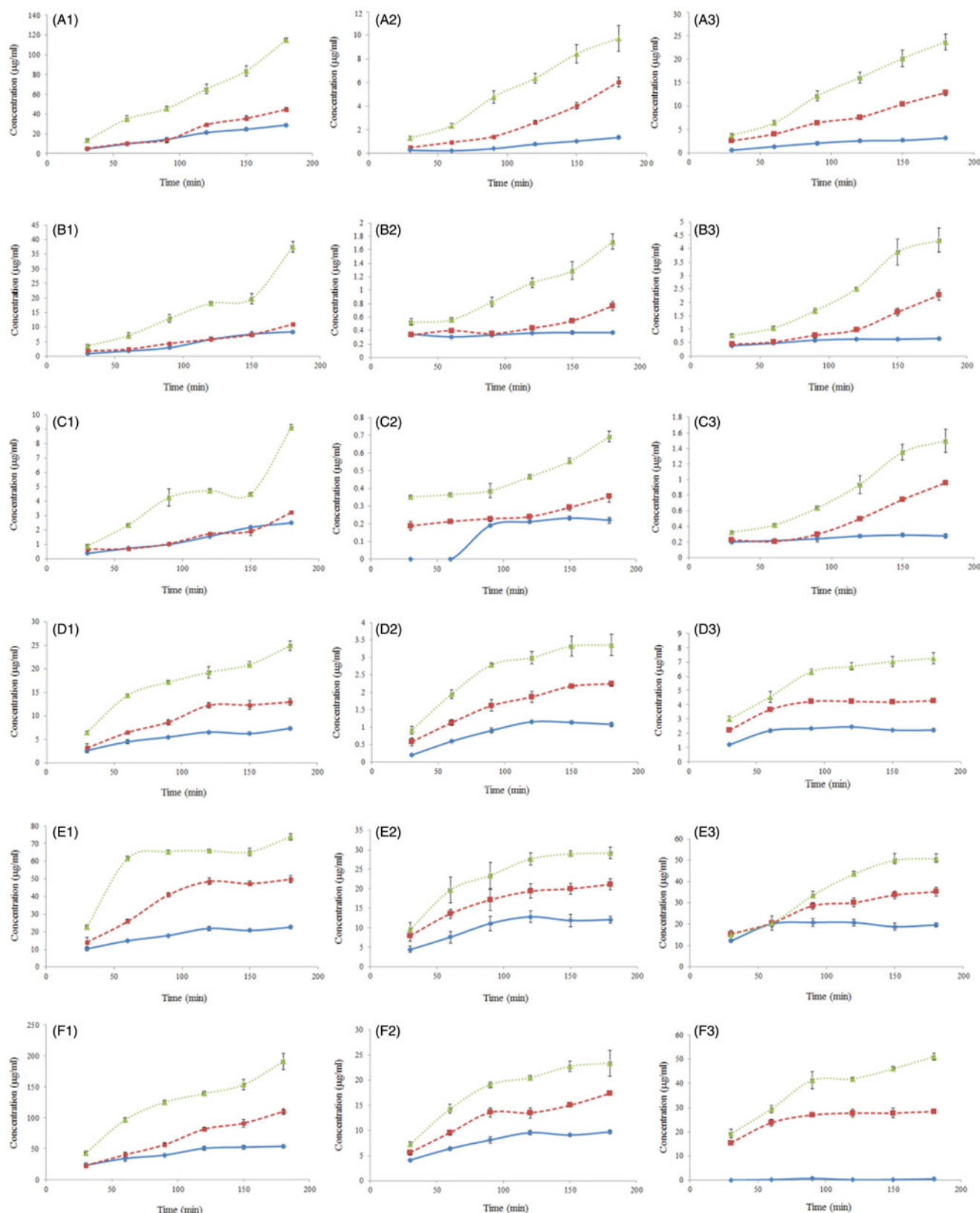


Figure 5. The concentrations of the 6 compounds in AEOF transformed by the intestinal bacteria from a single volunteer tested with Caco-2 cell monolayers and everted gut sacs as a function of time. Solid line: the concentration of extract was 10 mg/mL; dashed line: the concentration of extract was 20 mg/mL; dotted line: the concentration of extract was 30 mg/mL. A, B, C, D, E, and F represented the results of compounds 2''-O-β-L-galactopyranosylorientin, orientin, vitexin, veratric acid, pro-globeflowery acid, and trolline, respectively. 1, 2, and 3 represented the results obtained with everted gut sacs, Caco-2 cell monolayers AP→BL, and BL→AP, respectively.

Validation of Caco-2 cell monolayer model

The TEER values of the Caco-2 monolayers were increased steadily over time, and above 600 Ω/cm^2 on day 21 after seeding. The apparent permeability coefficient (P_{app}) values of the reference compounds, propranolol and atenolol, detected with the Caco-2 monolayers were $(4.12 \pm 0.17) \times 10^{-5}$ and $(2.38 \pm 0.24) \times 10^{-7}$ cm/s, respectively, which were basically consistent with the acceptable values reported in the literatures (Yee 1997; Liu et al. 2015). Therefore, this model was validated for the evaluation of the intestinal absorption potential of the compounds of interest.

Non-toxic dose range of test compounds

The survival rates of the Caco-2 cells treated with the intestinal bacterial transformed AEOF at the concentrations of 10, 20, and 30 mg/mL for 4 h were 95.2, 90.4, and 88.8%, respectively, whereas those of the Caco-2 cells treated with paclitaxel, which was used as a positive control for cytotoxicity, at the concentrations of 0.02, 0.04, and 0.08 mg/L for 4 h were 67.3, 45.5, and 37.4%, respectively. These results demonstrated that the extract at the concentrations of 0–30 mg/mL had no toxic effect on the Caco-2 cells; thus, 10, 20, and 30 mg/mL were selected as the test concentrations in the experiment.

Results of transport

As displayed in the HPLC chromatographic fingerprints of the intestinal bacterial transformed AEOF (Figure 1(D–F)), the contents of 2''-O- β -L-galactopyranosylorientin, orientin, and vitexin decreased significantly, while the contents of veratric acid, proglobeflowery acid, and trolline increased remarkably after transported through the Caco-2 cell monolayers and everted gut sacs (Table 5). The P_{app} values and the transport percentages of the main compounds (Tables 6 and 7) were also employed to assess the intestinal absorption of the compounds. The results showed that the P_{app} values of 2''-O- β -L-galactopyranosylorientin, orientin, and vitexin were at the levels of 10^{-5} , whereas those of

veratric acid, proglobeflowery acid, and trolline were at the levels of 10^{-4} . This suggests that 2''-O- β -L-galactopyranosylorientin, orientin, and vitexin are relatively more difficult to be absorbed than veratric acid, proglobeflowery acid, and trolline.

Discussion

Intestinal bacteria constitute a special microbial population in the human intestine, which can transform the compounds of the Chinese medicine using their specific enzymes (Yang & Xu 2011). One of the most common reactions catalyzed by the enzymes generated in the intestinal bacterial flora is the deglycosylation (Wang et al. 2013). Through this reaction, the intestinal bacteria obtain sugars as their nutrition; thus, the glycosides are usually hydrolyzed by these microorganisms to form their respective aglycones. In this regard, most of the compounds in the title flowers should be hydrolyzed. However, only the compounds with O-glycosidic bonds including isoquercetin and trollinose were transformed into their aglycones, i.e., quercetin and proglobeflowery acid in this experiment. With respect to 2''-O- β -L-galactopyranosylorientin, orientin, and vitexin, they were hardly transformed by the intestinal bacteria due to their solid C-glycosidic bond in this experiment.

Both Caco-2 cell monolayers and everted gut sacs are well-known models used for the evaluation of the absorbability of the orally administrated drugs. The results obtained with both models consistently demonstrated that the phenolic acids and alkaloids in the intestinal bacterial transformed AEOF, such as veratric acid, proglobeflowery acid, and trolline were more easily absorbed than the flavonoids such as orientin, vitexin, and 2''-O- β -L-galactopyranosylorientin because the P_{app} values of the phenolic acids and alkaloid were in the magnitude of 10^{-4} which represents the relatively easy absorbable compounds while flavonoids were in the magnitude of 10^{-5} which represents the relatively difficult absorbable compounds (Table 6).

The absorbability of the compounds closely correlates with their absorptive mechanism and structure. Our previous study has evidenced that the absorption of the flavonoids with 7-OH

Table 6. The P_{app} values of the six compounds.

	Concentration (mg/mL)	2''-O- β -L-Galactopyranosylorientin ($\times 10^{-5}$ cm/s)	Orientin ($\times 10^{-5}$ cm/s)	Vitexin ($\times 10^{-5}$ cm/s)	Veratric acid ($\times 10^{-4}$ cm/s)	Proglobeflowery acid ($\times 10^{-4}$ cm/s)	Trolline ($\times 10^{-4}$ cm/s)
Caco-2 cell monolayers	10 _(AP→BL)	1.11 $\pm$ 0.09*	2.53 $\pm$ 0.03*	3.96 $\pm$ 0.05	1.04 $\pm$ 0.05*	1.05 $\pm$ 0.04*	1.12 $\pm$ 0.07*
	20 _(AP→BL)	1.90 $\pm$ 0.09*	1.35 $\pm$ 0.02*	1.36 $\pm$ 0.15*	1.05 $\pm$ 0.15*	1.23 $\pm$ 0.26*	1.27 $\pm$ 0.12*
	30 _(AP→BL)	4.07 $\pm$ 0.40*	1.95 $\pm$ 0.14*	3.38 $\pm$ 0.28*	1.09 $\pm$ 0.04*	1.81 $\pm$ 0.22	1.07 $\pm$ 0.06*
	10 _(BL→AP)	2.16 $\pm$ 0.24	1.92 $\pm$ 0.14	2.83 $\pm$ 0.27	2.36 $\pm$ 0.05	2.23 $\pm$ 0.16	2.31 $\pm$ 0.07
	20 _(BL→AP)	3.49 $\pm$ 0.09	1.18 $\pm$ 0.02	1.64 $\pm$ 0.05	2.10 $\pm$ 0.04	1.42 $\pm$ 0.05	1.94 $\pm$ 0.02
	30 _(BL→AP)	4.17 $\pm$ 0.28	1.63 $\pm$ 0.06	2.16 $\pm$ 0.05	1.99 $\pm$ 0.04	1.05 $\pm$ 0.04	1.84 $\pm$ 0.11
Everted gut sacs	10 _(AP→BL)	4.11 $\pm$ 0.25	2.19 $\pm$ 0.24	2.60 $\pm$ 0.28	1.27 $\pm$ 0.04	4.50 $\pm$ 0.11	1.38 $\pm$ 0.01
	20 _(AP→BL)	2.23 $\pm$ 0.41	1.85 $\pm$ 0.01	1.55 $\pm$ 0.11	1.11 $\pm$ 0.07	5.34 $\pm$ 0.14	1.07 $\pm$ 0.05
	30 _(AP→BL)	4.67 $\pm$ 0.25	3.40 $\pm$ 0.34	3.81 $\pm$ 0.50	1.42 $\pm$ 0.03	5.86 $\pm$ 0.08	1.51 $\pm$ 0.04

* $p < 0.05$, P_{app} (AP→BL) versus P_{app} (BL→AP).

Table 7. The transport percentage of the six compounds.

	Concentration (mg/mL)	2''-O- β -L-Galactopyranosylorientin (%)	Orientin (%)	Vitexin (%)	Veratric acid (%)	Proglobeflowery acid (%)	Trolline (%)
Caco-2 cell monolayers	10 _(AP→BL)	13.41	30.53	47.97	109.67	127.39	135.43
	20 _(AP→BL)	22.93	16.39	16.41	127.58	148.57	153.03
	30 _(AP→BL)	49.29	23.54	40.90	131.58	219.09	130.00
	10 _(BL→AP)	8.72	7.73	11.43	95.04	89.93	93.13
	20 _(BL→AP)	14.08	4.76	6.61	84.75	57.32	78.21
	30 _(BL→AP)	16.83	6.56	8.74	80.33	42.47	74.30
Everted gut sacs	10 _(AP→BL)	8.76	4.67	5.55	27.04	9.60	29.42
	20 _(AP→BL)	4.23	3.50	2.94	21.02	10.58	20.32
	30 _(AP→BL)	9.25	6.75	7.55	28.12	11.61	30.00

including 2''-O- β -L-galactopyranosylorientin, orientin, and vitexin involved transporter mediated efflux in addition to passive diffusion (Liu et al. 2015), the phenolic acids such as veratric acid and proglobeflowery acid were absorbed mainly through passive diffusion, and the alkaloid, trolline, was absorbed through an associative mechanism including both active and passive transports (Liu et al. 2014; Wu et al. 2014). Hence, the involvement of efflux transporters, e.g., P-glycoprotein (P-gp) and multi-drug resistance related proteins (MRPs) may slow the transport of flavonoids so as to lower their absorption and bioavailability accordingly. Two other key factors influencing the absorption of the compounds are molecular weight and polarity. Both phenolic acids and alkaloids of interest are smaller molecules compared with flavonoids, resulting in a faster passive transport.

The absorption of the compounds has an important impact on the assessment of effective components of the flowers of *T. chinensis* because the mass ratio of flavonoids to phenolic acids to alkaloids in the intestinal bacterial transformed AEOF changed significantly from 16:10:7 to 9:12:8 before and after absorption (Table 5). That is to say, the flavonoids in the intestinal bacterial transformed AEOF changed from the major components to the minor ones after absorption; in contrast, the phenolic acids changed from the minor ones to the major ones as far as the quantity was concerned.

The identification of the effective components of Chinese medicine is a crucial issue for its research and development. The present investigation suggests that the assessment of the effective components should not only consider the quantity of the compounds in the crude drugs, but also take into account the *in vivo* process especially the absorbability of the compounds.

Conclusions

The results of this study improve our understanding of the effective components of the flowers of *T. chinensis*. In addition to flavonoids which used to be considered as the dominant effective ones, phenolic acids and alkaloids, are also very important for the efficacy of these flowers.

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Disclosure statement

No potential conflict of interest was reported by the authors.

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