

Metabolic and Pharmacokinetic Investigation of Ilexsaponin A₁ in Normal and Antibiotic-treated Rats

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Abstract

Background: Ilexsaponin A₁ (IA₁) is a bioactive triterpene saponin derived from natural medicinal plants. IA₁ exhibits anti-inflammatory and proangiogenic activities and improves intestinal barrier function. It has been reported that IA₁ could be metabolized into a dominant metabolite, ilexgenin A (IA) by β -glucosidase enzymes in intestinal microflora.

Materials and Methods: Herein, an accurate, sensitive, and selective method based on ultra-performance liquid chromatography coupled with mass spectrometry was established to simultaneously profile the metabolism and pharmacokinetic behaviors of IA₁ in normal and antibiotic-treated rat plasma after intragastric administration of IA₁. The precursor-to-product ion pairs of IA and IA₁ were m/z 501.32→439.32 and m/z 663.38→501.32, respectively. For method validation, the specificity, matrix effect, accuracy, precision, and stability of the pharmacokinetic study were measured, and a calibration curve was created. The collaborative pharmacological target pathways of IA₁ and its metabolite IA were investigated using network pharmacology tools.

Results: The validated analytical method was successfully utilized to investigate the pharmacokinetics of IA₁ in normal and antibiotic-treated rats. The bioavailability of IA₁ and conversion from IA₁ to IA were significantly inhibited by antibiotic-treated rats after oral administration of IA₁. Fragment ions at m/z 483.3155, 455.3159, 439.3233, 421.3136, 395.3362, 152.9952, 113.0256, and 71.0531 were characteristic of the IA₁ moiety. IA₁ was metabolized in rat plasma by biotransformation routes involving deglycosylation, decarboxylation, isomerization, hydrogenation, dehydrogenation, and oxidation. Considering database analysis, IA and IA₁ play synergistic role in common pharmacological pathways, such as hypertrophic cardiomyopathy and dilated cardiomyopathy.

Conclusion: The experiments illustrated that β -glucosidase activity inhibited by antibiotics suppressed the hydrolysis reaction of IA₁ in the intestinal tract. IA₁ and IA play a synergistic role in exerting effects.

Keywords

Ilexsaponin A₁, ilexgenin A, UPLC–QTOF–MS, metabolic profiling, pharmacokinetic profiling

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Introduction

Triterpene saponins are widely distributed in natural medicinal plants and exhibit various bioactivities, such as anti-inflammatory, anti-obesity, anti-tumor, and anti-hypertensive activities (Biswas & Dwivedi, 2019). A significant characteristic of triterpene saponins is their restrictive bioavailability in the intestine, which results from their strong polarity. Ilexsaponin A₁ (IA₁) is an ursane-type and representative saponin in Traditional Chinese Medicine (TCM) with a long history, such as *Ilex pubescens* and *Ilex hainanensis* (Cao et al., 2018). It was reported that IA₁ exhibited potential for preventing cardiovascular or vascular insufficiency-related diseases, reducing inflammation, and improving intestinal barrier

function (Zhao et al., 2019). After oral administration, IA₁ inevitably contacts the gut microbiota and could be biotransformed in the gastrointestinal tract to increase its fat solubility before entering the bloodstream (Gong et al., 2020). The metabolization of IA₁ in intestinal microflora generates

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multiple metabolites with pertinent pharmacological effects. Studies have shown that IA_1 can be metabolized into the dominant metabolite, ilexgenin A (IA) (Zhao et al., 2011). Notably, IA and IA_1 are a pair of glycosides and aglycones generally found in natural medicinal plants (Zhao et al., 2018). The conversion process from IA_1 to IA was performed by the β -glucosidase enzyme in intestinal microflora (Tannock, 2001). The transformation of IA_1 to IA seems to be the pharmacodynamic basis for the effectiveness of IA_1 . To date, the conversion of IA_1 to IA after administration of IA_1 has never been calculated.

Antibiotics have been widely and globally applied to confront bacterial infections (Xu et al., 2023). Administration of antibiotics leads to a decrease in the abundance of beneficial symbiotic bacteria and an increase in the abundance of potentially harmful micro-organisms (Zimmermann & Curtis, 2019). Antibiotic administration triggers perturbations in the intestinal microbiota. The biotransformation of triterpene saponin processed by intestinal microflora could be strongly influenced by exposure to antibiotics, which are directly associated with their absorption and biological actions. The pharmacokinetic profile is the key means to monitor the dynamic processes of IA_1 and its metabolite IA *in vivo* after IA_1 administration. In early research, IA was quantitatively determined in rat plasma after oral administration or intravenous injection of IA (Li et al., 2012; Liu et al., 2010). However, few studies have demonstrated that abnormal intestinal microflora metabolism affects the pharmacokinetics of IA_1 and its metabolite, IA.

Literature reported that the maximum plasma concentration of triterpene saponin was confined to the nanogram level regardless of monomer ingredient or complex administration (Yang et al., 2018). A more sensible strategy was needed to measure the lower absorption of triterpenoid glycosides and aglycones. Multiple studies have confirmed the superiority of ultra-performance liquid chromatography coupled with quadrupole time-of-flight mass spectrometry (UPLC-QTOF-MS) techniques, which exhibit a higher mass resolution, sensitivity, and accuracy for drug metabolite determination (Hu et al., 2021; Xu et al., 2019). In our paper, the metabolic profile and pharmacokinetic behavior of IA_1 and its metabolite IA were comprehensively analyzed, as well as the striking speed, during continuous periods after intragastric administration of monomer IA_1 ; this analysis was based on obtaining accurate masses, elemental compositions, and product ions. The collaborative pharmacological target pathways of IA_1 and its metabolite, IA, were investigated using network pharmacology tools. Knowledge of the metabolism, transformation, and pharmacological action of IA_1 should provide a reference for *in vivo* pharmacodynamic mechanisms.

Materials and Methods

Chemicals

IA_1 and IA were obtained from *I. pubescens* with over 98% purity in our phytochemistry laboratory. Digoxin was purchased from Weikey Biotechnology Co., Ltd., (Sichuan, China). Acetonitrile and methanol (LC-MS grade) were manufactured by Merck (Darmstadt, Germany). LC-MS grade formic acid was obtained from Fluka (Sigma, USA). Deionized water was prepared by the Millipore system from Shanghai Hitech Instruments Co., Ltd. (Shanghai, China). The antibiotics, including neomycin sulfate and streptomycin, were both from Biotopped Science & Technology Co., Ltd. (Beijing, China). *p*-Nitrophenol and *p*-nitrophenyl β -D-glucopyranoside were purchased from Solarbio Corporation (Beijing, China).

Animal Experiments

Sprague Dawley rats (250–280 g) were purchased from the laboratory animal center at Guangzhou University of Chinese Medicine (Certificate No. SCXK 2013-0034). After the adaptation period, the animals were separately administered IA_1 at low (IA_1 -L group, 9.96 mg/kg) and high (IA_1 -H group, 99.6 mg/kg) doses in normal and antibiotic-treated rats. During modeling, the IA_1 -L and IA_1 -H groups were used as normal control groups (administered 0.5% CMC-Na orally per day), and antibiotic-treated groups (IA_1 -L-Anti group and IA_1 -H-Anti group) were administered neomycin sulfate (100 mg/kg) and streptomycin (100 mg/kg) orally per day for 7 days. Fecal samples were collected, and the body weights of the rats were recorded every day. Food was removed for 12 h before blood collection, but water remained freely available. All groups were intragastrically administered IA_1 on the seventh day. Blood samples (200 μ L) were collected into heparinized tubes at 0, 0.25, 0.5, 0.75, 1, 1.5, 2, 4, 6, 8, and 12 h from the orbital sinus using heparinized glass capillary tubes. The supernatants were obtained after centrifugation at 3,000 rpm for 15 min. All samples were stored at -80°C until analysis.

UPLC-QTOF-MS Conditions

The experiments were performed on a UPLC system (LC-30AD, Shimadzu, Japan) and an AB SCIEX Triple TOF 5600+ (Foster City, CA) mass spectrometer accompanied by a Duo Spray Ion Source. Common parameters of UPLC-QTOF-MS equipment were referred to previous research on triterpene saponin (Cao et al., 2018). The mobile phase was composed of 0.1% formic acid in water (A) and acetonitrile

(B). Metabolic and pharmacokinetic investigations were conducted using an Acquity BEH C₁₈ column (2.1 mm × 75 mm, 1.7 μm) and an Acquity BEH C₁₈ column (2.1 mm × 100 mm, 1.7 μm), respectively. The gradient elution program was set as 65%–50% A (0–4 min), 50–20% A (4–7 min), and 20%–10% A (7–10 min) for the metabolic profiling. A gradient elution program was set as 35%–20% A (0–4.2 min) for the pharmacokinetic study. The precursor-to-product ion pairs, collision energy, and declustering potential values of IA and IA₁ were m/z 501.32→439.32 (–64 eV, –90 eV) and m/z 663.38→501.32 (–37 eV, –100 eV), respectively. The target ion was monitored at m/z 779.43 for digoxin (internal standard, IS). The flow rate was 0.4 mL/min and the temperature was 40°C. The injection volume was 5 μL.

Determination of β-Glucosidase Activity

Fresh feces (0.2 g) collected from normal and antibiotic-treated rats were carefully mixed and suspended in 3.8 mL ice-cold phosphate buffer solution and then centrifuged at 200 rpm at 4°C for 5 min. The β-glucosidase activity of the supernatant was determined as previously described (Kang et al., 2016).

Sample Preparation and Data Processing

Stock solutions of two analytes (IA and IA₁) were prepared by dissolving two accurately weighed standards in methanol with final concentrations of 0.025 mg/mL for IA and IA₁. Calibration standards were prepared by spiking the standard working solutions into the blank plasma to obtain the final concentrations for IA and IA₁. Quality control samples were prepared at nominal concentrations of 1.14, 30.86, and 277.78 ng/mL. The detailed procedures for method validation are described in the supplementary material.

Frozen plasma samples were subjected to natural thawing at room temperature. The plasma samples from 0, 0.5, 1, 2, 4, 8, and 12 h were merged into equal 100 μL volumes, respectively. Plasma was precipitated with 300 μL methanol to determine the metabolites of IA₁. Then, 150 μL methanol containing IS of 0.1 μg/mL was added to 50 μL of plasma sample in a 1.5 mL tube. After vortexing for 5 min, the samples were centrifuged at 12,000 rpm for 15 min. The residue was reconstituted with 100 μL methanol containing 0.1% formic acid and injected into the UPLC-MS system for pharmacokinetic studies.

A pharmacokinetic study was performed using noncompartmental analysis on DAS 2.1.1 software supplied by the Mathematical Pharmacology Professional Committee of China (Shanghai, China). Experiment data were drawn and statistically analyzed by Prism 6.0. All the data are expressed as the arithmetic mean ± standard deviation (SD).

Network Pharmacology Study

IA₁- and IA-related targets were collected using databases including Swiss Target Prediction (<http://www.swisstargetprediction.ch/>) and TargetNet (<http://targetnet.scbdd.com/>). The SMILES representing IA and IA₁ were CC1CCC2(CCC3(C(=CCC4C3(CCC5C4(CCC(C5(C)C(=O)O)O)C)C)C2C1(C)O)C)C(=O)O and CC1CCC2(CCC3(C(=CCC4C3(CCC5C4(CCC(C5(C)C(=O)O)O)C)C)C2C1(C)O)C)C(=O)OC6C(C(C(C(O6)CO)O)O)O, respectively. Common intersecting genes were filtered by Venny 2.1.0 (<https://bioinfogp.cnb.csic.es/tools/venny/>). Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analysis was analyzed by the Database for Annotation, Visualization, and Integrated Discovery (DAVID) (<https://david.ncifcrf.gov/>). Based on the data accumulation, a compound-target (C-T) network and functional enrichment analysis were pictured by bioinformatics (<https://www.bioinformatics.com.cn/>) and Cytoscape software.

Results and Discussion

Common Pharmacological Targets of IA and IA₁

As shown in Figures 1A and B, 106 gene targets for IA were intersected with 49 gene targets for IA₁, revealing 17 common targets. The KEGG pathways were retrieved based on screening significant enrichment ($p < 0.05$) and contribution value, which were mostly focused on pathways in cancer and proteoglycans in cancer (Figure 1C). Proteoglycans are the major macromolecules within the extracellular matrix. Dysregulation of extracellular matrix remodeling can contribute to tumorigenesis development (Wei et al., 2020). IA₁ is among the most frequently studied proangiogenic phytochemicals, but increased rates of angiogenesis are tightly associated with cancer (Li et al., 2022). IA was reported to have anticarcinogenic activity and to obstruct the progression of cancer (Yun et al., 2021). The SwissADME online server (<http://www.swissadme.ch/>) was applied to evaluate the drug likeliness of IA₁ and IA. Unfortunately, IA₁ did not pass the drug likeliness criteria, including Lipinski, Ghose, Veber, Egan, Muegge, and Bioavailability Score. IA passed the criteria of Lipinski, Veber, and Egan.

Metabolite Profiling of IA₁ in Rat Plasma

Through MetabolitePilot™ and PeakView® 2.0 software, which are data processing and data mining techniques, a total of 17 metabolites of IA₁ were filtered in rat plasma. The metabolic pathways of IA₁ mainly refer to the phase I process. The corresponding MS² spectra and fragmentation pathways of IA₁ and IA are shown in Figures 2 and 3, respectively. Deglycosylation, isomerization, decarboxylation, hydrogenation, dehydrogenation, and oxidation occurred on the skeleton of IA₁ could be quickly recognized depending on the mass shift related to characteristic ions, including

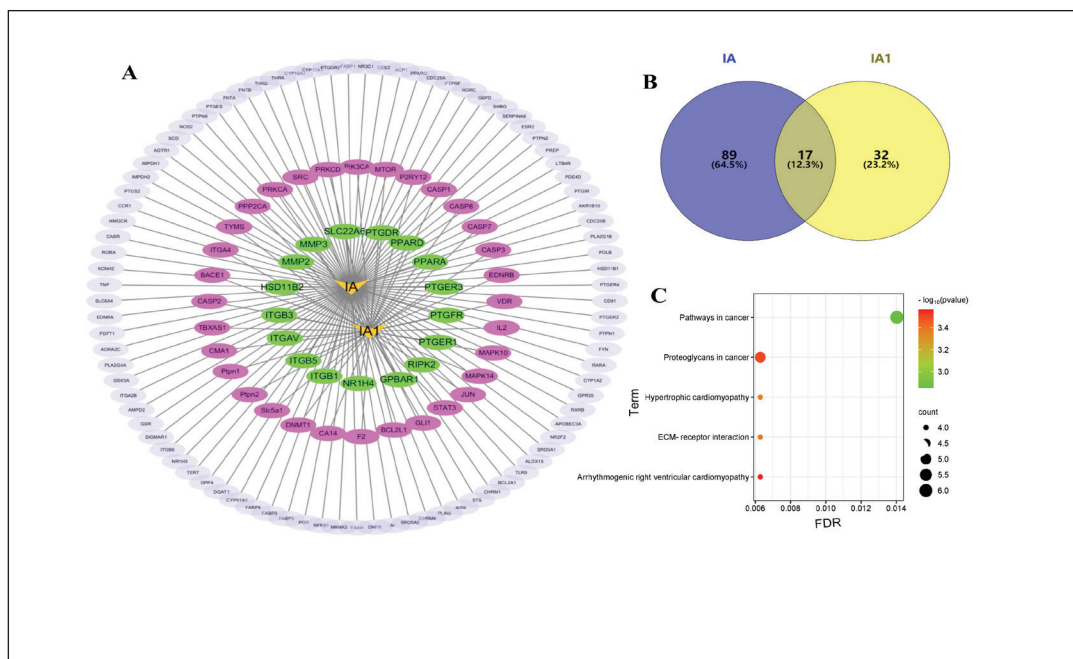


Figure 1. The Potential Targets of IA and IA₁ (A), Venn Diagram (B), and KEEG Pathway Analysis of DAVID Database (C).

Abbreviations: IA, ilexgenin A; IA₁, ilexgenin A₁; KEEG, Kyoto Encyclopedia of Genes and Genomes; DAVID, Database for Annotation, Visualization, and Integrated Discovery.

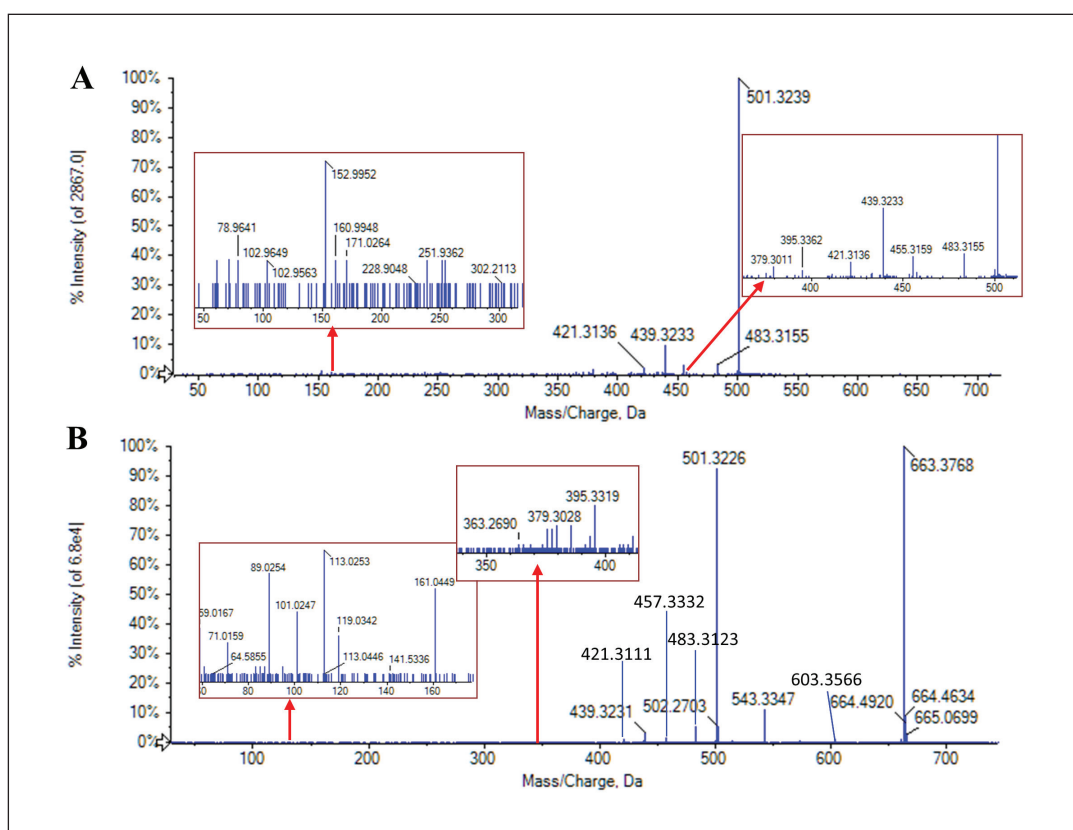


Figure 2. The MS/MS Spectra and Proposed Fragmentation of IA (A) and IA₁ (B).

Abbreviations: IA, ilexgenin A; IA₁, ilexgenin A₁; MS, mass spectrometry.

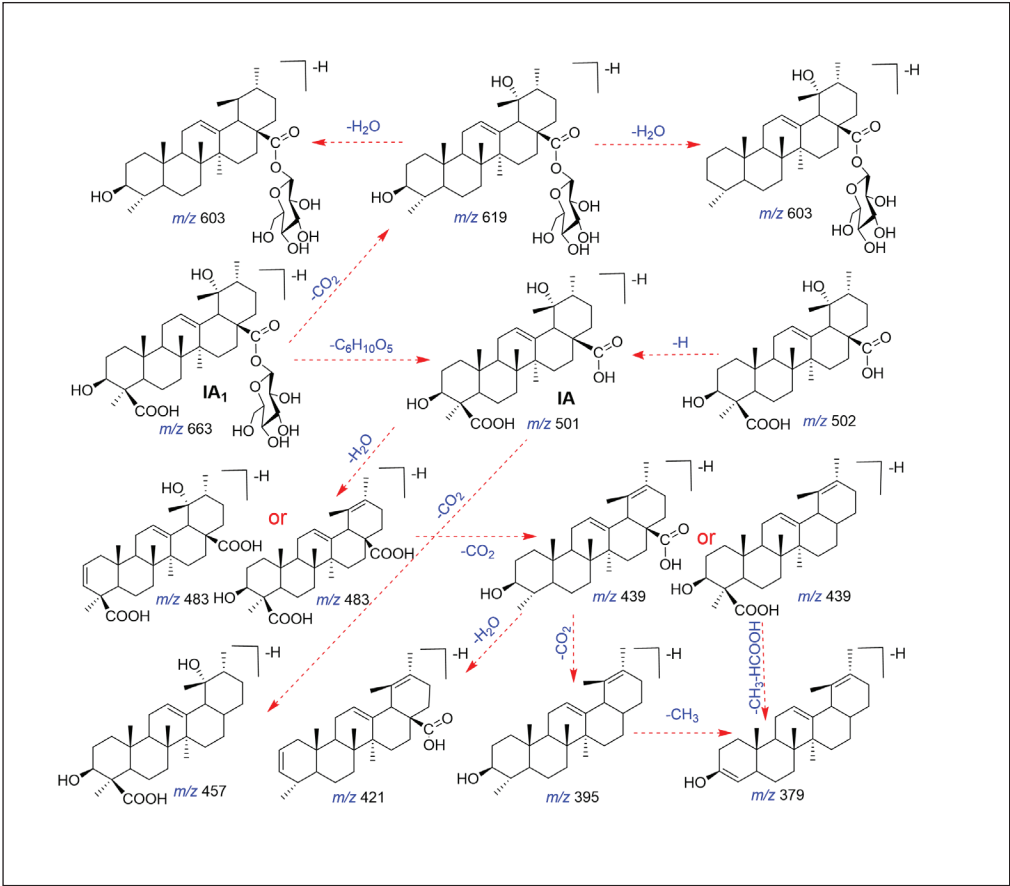


Figure 3. The Proposed Fragmentation of IA₁.

Abbreviations: IA, ilexgenin A; IA₁, ilexgenin A₁.

Table 1. UPLC-QTOF-MS Retention Times and Fragments Ions of IA₁ Metabolites in Rat Plasma.

Peak ID	t _R (min)	Predicted Formula	Adduct [M – H] [–]	Calculated (m/z)	Ppm	Description	MS/MS Fragments
IA ₁	3.61	C ₃₆ H ₅₆ O ₁₁	663.3782	663.3793	4.8	Parent	663.3768, 617.3665, 603.3566, 543.3347, 501.3226, 483.3123, 439.3231, 421.3111, 395.3319, 251.9362, 161.0449, 152.9952, 119.0342, 113.0446
M0 (IA)	6.49	C ₃₀ H ₄₆ O ₆	501.3228	501.3216	2.4	Deglycosylation	501.3239, 483.3155, 457.3500, 455.3159, 439.3235, 433.2953, 421.3136, 411.3276, 391.2887, 379.3011, 256.8691, 152.9952, 113.0253, 59.0196
M1	6.73	C ₂₉ H ₄₄ O ₄	455.3169	455.3167	0.5	Deglycosylation + Decarboxylation	455.3189, 411.3295, 409.3137, 395.2909, 353.2405, 204.9621, 152.9947, 160.9733

(Table 1 continued)

(Table 1 continued)

Peak ID	t_R (min)	Predicted Formula	Adduct $[M - H]^-$	Calculated (m/z)	Ppm	Description	MS/MS Fragments
M2	6.10	$C_{30}H_{44}O_6$	499.3074	499.3065	1.7	Deglycosylation + Dehydrogenation	499.3040, 453.3039, 431.3099, 409.3129, 393.2784, 391.3025, 353.2631
M3	6.50	$C_{30}H_{46}O_6$	501.3230	501.3222	1.7	Deglycosylation	501.3247, 483.3135, 455.3239, 439.3246, 421.3148, 379.3046, 304.2364
M4	6.73	$C_{30}H_{46}O_6$	501.3232	501.3222	2.1	Deglycosylation + Isomerization	501.1923, 455.3168, 411.3298, 385.2962, 391.2990, 307.1958, 297.1517, 171.0303, 152.9927, 74.0287
M5	7.23	$C_{30}H_{46}O_6$	501.3224	501.3222	0.4	Deglycosylation + Isomerization	501.2807, 455.3180, 411.3244, 433.3324, 391.3008, 395.3062, 304.2350, 152.9962, 102.9539, 78.9747
M6	8.73	$C_{30}H_{46}O_6$	501.3242	501.3222	4.0	Deglycosylation + Isomerization	455.3211, 453.8554, 433.2609, 411.3302, 395.3025, 393.3189, 391.3015, 160.9966, 152.9951, 103.0447, 78.9581
M7	4.45	$C_{30}H_{44}O_7$	515.3023	515.3014	1.8	Deglycosylation + Oxidation + Dehydrogenation	515.3041, 471.3027, 447.1369, 431.0970, 417.1199, 409.3039, 391.2956, 373.0124, 307.2392, 206.8937, 174.9552, 149.0001, 140.9988, 78.9591
M8	5.57	$C_{30}H_{44}O_7$	515.3020	515.3014	1.1	Deglycosylation + Oxidation + Dehydrogenation	515.3035, 469.2999, 409.3117, 397.7660, 332.8431, 174.9548, 130.9689
M9	5.35	$C_{30}H_{46}O_7$	517.3177	517.3171	1.3	Deglycosylation + Oxidation	517.2929, 471.3112, 427.3199, 312.8791, 213.1443, 186.9291
M10	3.90	$C_{30}H_{48}O_7$	519.3346	519.3327	3.5	Deglycosylation + Oxidation + Hydrogenation	519.3346, 473.3251
M11	5.39	$C_{30}H_{48}O_7$	519.3322	519.3327	-1.0	Deglycosylation + Oxidation + Hydrogenation	519.3322, 473.3262, 178.9014
M12	7.34	$C_{30}H_{48}O_7$	519.3345	519.3327	3.4	Deglycosylation + Oxidation + Hydrogenation	519.3345, 473.3276, 439.3386, 411.3292, 393.3170, 381.3138, 109.0699
M13	1.85	$C_{30}H_{44}O_8$	531.2963	531.2963	2.4	Deglycosylation + Dehydrogenation + Di-oxidation	531.2980, 503.1277, 485.2331, 469.4337, 452.8759, 441.1632, 421.7906, 405.2942, 363.2606, 96.9605, 71.0487, 43.0268

(Table 1 continued)

(Table 1 continued)

Peak ID	t_R (min)	Predicted Formula	Adduct $[M - H]^-$	Calculated (m/z)	Ppm	Description	MS/MS Fragments
M14	2.76	$C_{30}H_{44}O_8$	531.2963	531.2963	2.8	Deglycosylation + Dehydrogenation + Di-oxidation	531.3006, 485.2333, 387.2834, 174.9712, 79.9594
M15	2.80	$C_{30}H_{46}O_8$	533.3127	533.3120	1.4	Deglycosylation + Di-Oxidation	533.3160, 471.3154, 453.3034, 409.3131, 391.2996, 381.2447, 186.9272, 85.0353, 71.0523
M16	4.50	$C_{30}H_{46}O_8$	533.3109	533.3120	-2.0	Deglycosylation + Di-Oxidation	533.3279, 487.3090, 465.2429, 407.3004, 146.9245
M17	6.15	$C_{31}H_{48}O_8$	547.3281	547.3276	0.8	Deglycosylation + Isomerization	501.3224, 455.3136, 439.3305, 379.2978, 342.8903

Abbreviations: IA, ilexgenin A; IA₁, ilexgenin A₁; UPLC-QTOF-MS, ultra-performance liquid chromatography coupled with quadrupole time-of-flight mass spectrometry.

m/z 483.3155, 455.3159, 439.3233, 421.3136, 395.3362, 152.9952, 113.0256, and 71.0531. The formula, retention time (t_R), ppm, and MS/MS fragment data for IA₁ and M0–M17 are displayed in Table 1, and the extracted ion chromatograms of M0–M17 are illustrated in Figure S1. The proposed biotransformation pathways of IA₁ are shown in Figure S2. Fragmentation analysis for metabolite (M0–M17) identification is depicted in the supplementary material. Metabolic types, such as deglycosylation, decarboxylation, hydrogenation, dehydrogenation, isomerization, and oxidation, are typically identified for triterpenoid saponins (Lü et al., 2019; Yan et al., 2012). Additionally, exiguous significant differences in metabolic types were observed between normal and antibiotic-treated rats.

β -Glucosidase Activity and Rat Body Weight Trend

A comparison of β -glucosidase activity between the normal and antibiotic-treated groups was obtained by performing a *t*-test (Figure 4A). More days of modeling resulted in an increasing difference between the normal and antibiotic-treated groups. On the 7th day, the β -glucosidase activity of the normal group was approximately three times higher than that of the model group given antibiotics ($p < 0.01$). The β -glucosidase activity was regarded as an agreeable indicator of gut status (Lee et al., 2002). It was suggested that abnormal modifications of the intestinal flora were successfully triggered, which in turn led to a significant decrease in β -glucosidase activity. The type, quantity, proportion, location, and biological characteristics of the intestinal microbiota were altered once common intestinal flora disorders occurred (Patel et al., 2016). Additionally, the

growth rate of rat weight gain recorded before administration every day was calculated. The weights of the rats in the normal and antibiotic-treated groups increased steadily, which indicated no significant differences among them (Figure 4B). Therefore, antibiotic treatment did not induce organ toxicities or physiological changes.

Method Validation for Pharmacokinetic Studies

Under optimized conditions, the t_R of IA, IA₁, and IS were 3.71, 2.28, and 1.46 min, respectively. No interference at the t_R of IA, IA₁, or IS was discovered (Figure 5). The regression equations were $y = 4.87995e^{-5}x + 2.16161e^{-4}$ for IA and $y = 2.37018e^{-4}x + 0.00115$ for IA₁. The quantitative ranges of IA and IA₁ were 0.38–1040 ng/mL and 0.13–1060 ng/mL, respectively, with correlation coefficients of 0.9954 for IA and 0.9927 for IA₁. The detailed results of method validation are described in the supplementary material (Tables S1–S3).

Application in Pharmacokinetic Studies

The validated analytical method was successfully applied to investigate the pharmacokinetics of IA₁ and its metabolite IA after oral administration of IA₁. The mean plasma concentration–time profiles ($n = 6$) of IA₁ and its metabolite IA are shown in Figure 6, and the corresponding pharmacokinetic parameters of the tested compounds are listed in Tables 2 and 3. At the same dose, the pharmacokinetic parameters C_{max} , area under the plasma concentration versus time curve from 0 h to the last quantifiable concentration ($AUC_{(0-t)}$), and area under the plasma concentration versus time curve from 0 h to infinity

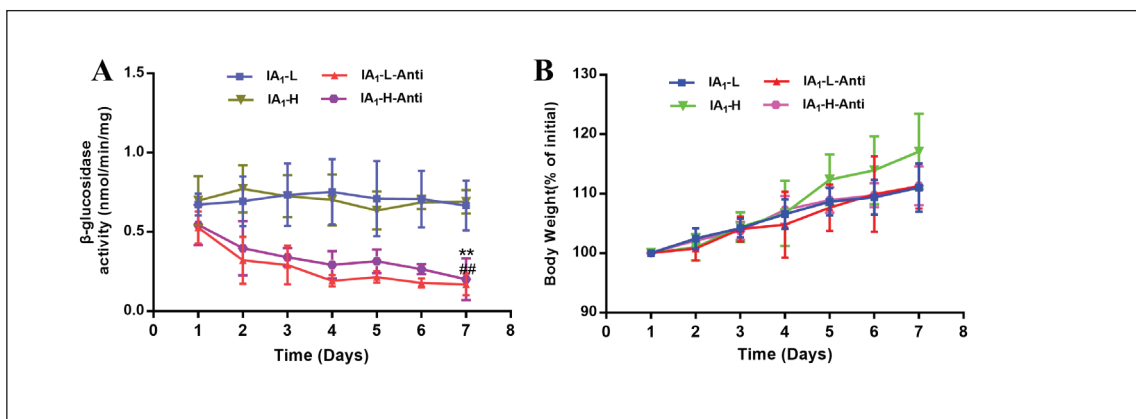


Figure 4. Fecal β -glucosidase Activity (A) and Body Weight Changes (B) of Rats in IA₁-L, IA₁-L-Anti, IA₁-H, and IA₁-H-Anti Groups.

Note: IA₁-L group: rats fed IA₁ (9.96 mg/kg) in normal rats; IA₁-L-anti group: rats fed IA₁ (9.96 mg/kg) in antibiotics-treated rats; IA₁-H group: rats fed IA₁ (99.6 mg/kg) in normal rats; IA₁-H-anti group: rats fed IA₁ (99.6 mg/kg) in antibiotics-treated rats. Data are expressed as mean $\pm$ SD ($n = 6$). $^{*}p < 0.01$ versus the IA₁-H group and $^{*}p < 0.01$ versus the IA₁-L group.

Abbreviations: IA, ilexgenin A; IA₁, ilexgenin A₁.

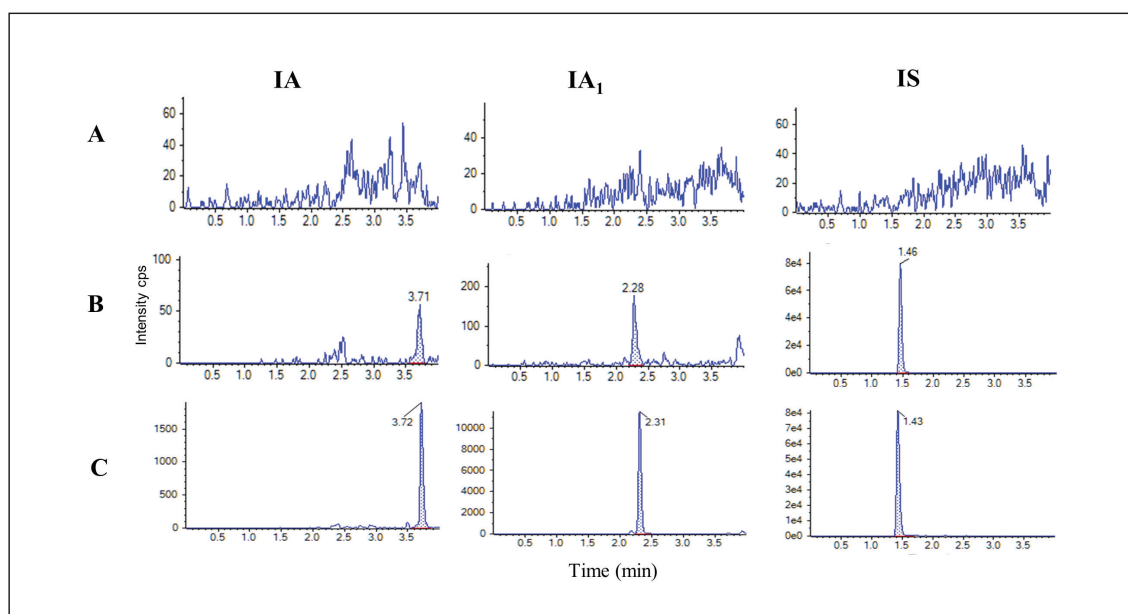


Figure 5. Representative Extracted Ion Chromatograms of IA, IA₁, and IS, Blank Plasma (A), Standard Sample (B), and Plasma Sample at 6 h After Administration (C).

Abbreviations: IA, ilexgenin A; IA₁, ilexgenin A₁; IS, internal standard.

(AUC_(0-∞)) of IA₁ in the IA₁-L dose group were considerably increased compared with those of the IA₁-L-anti group ($p < 0.01$). The differences in C_{max} , AUC_(0-t), and AUC_(0-∞) between the IA₁-H and IA₁-H-anti groups were dramatically significant ($p < 0.01$, $p < 0.05$) in Table 2. The amount of IA₁ taken up in the blood was relatively low, which is in accordance with earlier reports. The absorption of triterpenoid prototypes was severely limited by several properties, including low solubility, selectivity, and poor bioavailability (Zhou et al., 2017). The

plasma concentration of IA₁ at each time point in the intestinal flora disorder groups was lower than that in the normal group after intragastric administration, suggesting that the intestinal flora impeded the absorption of glycosides under the influence of antibiotics by monomer administration. However, herbal extracts can enhance the bioavailability of active compounds via ingredient–ingredient interactions, influence the activity of gut enzymes, and decrease antibiotic resistance, according to the overview (Su et al., 2020).

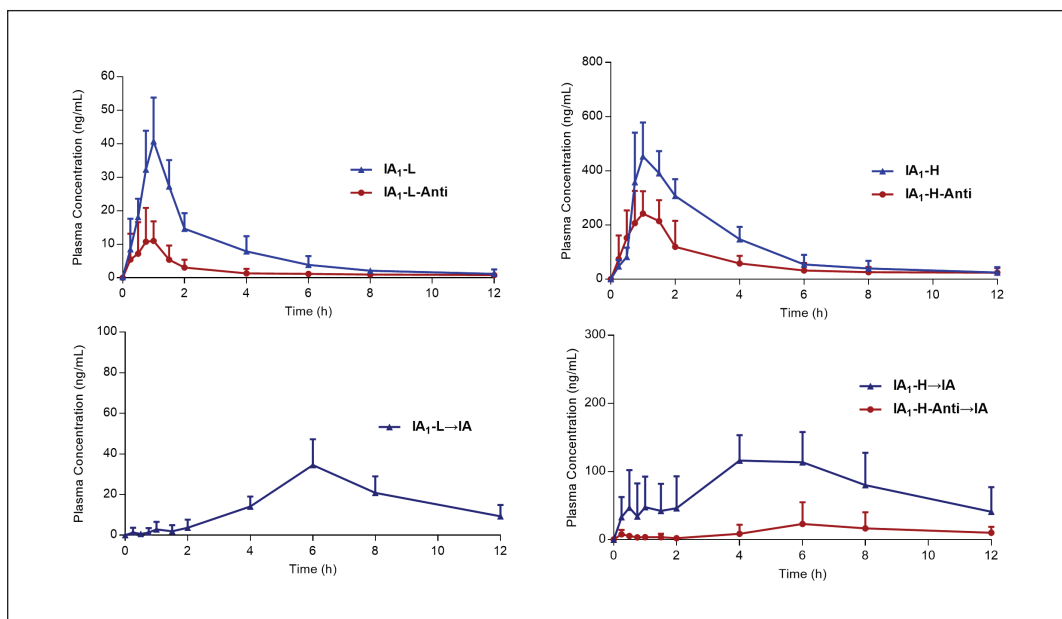


Figure 6. Plasma Concentration-time Curves of IA_1 and its Metabolite IA in Plasma from Normal and Model Rats After a Single Administration of IA_1 .

Note: IA_1 -L→IA group: IA converted from IA_1 (9.96 mg/kg) in normal rats; IA_1 -H→IA: IA converted from IA_1 (99.6 mg/kg) in normal rats; IA_1 -H-Anti→IA group: IA converted from IA_1 (99.6 mg/kg) in antibiotics-treated rats. Data are expressed as mean $\pm$ SD ($n = 6$).

Abbreviations: IA, ilexgenin A; IA_1 , ilexgenin A_1 .

Table 2. Main Pharmacokinetic Parameters of IA_1 in Rat Plasma Following Administration of IA_1 at 9.96 and 99.6 mg/kg (Mean $\pm$ SD, $n = 6$).

Parameters	IA_1 -L	IA_1 -L-Anti	IA_1 -H	IA_1 -H-Anti
$AUC_{(0-t)}$ (ng/L $\times$ h)	94.05 $\pm$ 26.65	30.60 $\pm$ 17.72**	1442.73 $\pm$ 313.32	759.24 $\pm$ 193.91###
$AUC_{(0-\infty)}$ (ng/L $\times$ h)	96.14 $\pm$ 26.07	38.00 $\pm$ 16.90**	1481.16 $\pm$ 305.07	839.90 $\pm$ 199.54###
$t_{1/2z}$ (h)	2.32 $\pm$ 0.94	4.69 $\pm$ 2.06*	2.02 $\pm$ 0.61	3.42 $\pm$ 1.93
T_{max} (h)	0.92 $\pm$ 0.12	0.83 $\pm$ 0.20	1.04 $\pm$ 0.25	1.17 $\pm$ 0.38
C_{max} (ng/L)	40.94 $\pm$ 12.97	15.32 $\pm$ 8.42**	483.51 $\pm$ 140.28	274.05 $\pm$ 81.81#

Notes: * $p < 0.05$ and ** $p < 0.01$ versus IA_1 -L group, # $p < 0.05$ and ### $p < 0.01$ versus IA_1 -H group.

Abbreviations: IA, ilexgenin A; IA_1 , ilexgenin A_1 ; $AUC_{0 \rightarrow t}$, area under the plasma concentration versus time curve from 0 h to the last quantifiable concentration; $AUC_{0 \rightarrow \infty}$, area under the plasma concentration versus time curve from 0 h to infinity; C_{max} , maximum plasma concentration; T_{max} , time to reach the maximum plasma concentration; $t_{1/2z}$, elimination half-life.

Table 3. Main Pharmacokinetic Parameters of IA Converted by IA_1 in Rat Plasma Following Administration of IA_1 at 9.96 and 99.6 mg/kg (Mean $\pm$ SD, $n = 6$).

Parameters	IA_1 -L→IA	IA_1 -H→IA	IA_1 -H-Anti→IA
$AUC_{(0-t)}$ (ng/L $\times$ h)	187.98 $\pm$ 61.05	913.34 $\pm$ 397.413	149.50 $\pm$ 175.11###
$AUC_{(0-\infty)}$ (ng/L $\times$ h)	238.25 $\pm$ 99.35	1658.53 $\pm$ 1132.17	231.39 $\pm$ 203.33#
$t_{1/2z}$ (h)	3.43 $\pm$ 1.14	7.71 $\pm$ 6.07	4.75 $\pm$ 2.61
T_{max} (h)	6.33 $\pm$ 0.82	4.67 $\pm$ 1.03	5.71 $\pm$ 4.21
C_{max} (ng/L)	35.27 $\pm$ 11.11	132.32 $\pm$ 41.30	26.58 $\pm$ 29.96###

Note: # $p < 0.05$ and ### $p < 0.01$ versus IA_1 -H group.

Abbreviations: IA, ilexgenin A; IA_1 , ilexgenin A_1 ; $AUC_{0 \rightarrow t}$, area under the plasma concentration versus time curve from 0 h to the last quantifiable concentration; $AUC_{0 \rightarrow \infty}$, area under the plasma concentration versus time curve from 0 h to infinity; C_{max} , maximum plasma concentration; T_{max} , time to reach the maximum plasma concentration; $t_{1/2z}$, elimination half-life.

The pharmacokinetic behavior of IA transformed from IA₁ was simultaneously investigated after oral administration of IA₁ (Figure 6). The value $T_{\max}$ of IA was observed at approximately 6.33 h in the IA₁-L group, while the amount of IA could not be measured because the content was less than the lower limit of the quantification standard in the IA₁-L-anti group in Table 3. The values of $C_{\max}$, $AUC_{(0-t)}$, and $AUC_{(0-\infty)}$ in antibiotic-treated rats were significantly lower than those in normal rats ($p < 0.01$, $p < 0.05$) at the same dose of 99.6 mg/kg. Biotransformation of triterpene saponins mediated by intestinal microflora occurred through deglycosylation, glycosylation, oxidation, and dehydrogenation reactions (Fu et al., 2019). It could be interpreted that β -glucosidase activity inhibited by antibiotics suppressed the hydrolysis reaction of IA₁ in the intestinal tract. Gut microbiota that expressed sundry enzymes could transform TCM compounds, which could generate active prodrugs with new bioactivity (Feng et al., 2019). Subsequently, the therapeutic effect could be altered as the enzymatic activities and diversity of the gut microbiota are affected by antibiotics (Li et al., 2019). The conversion product (IA) of IA₁ could be an important pharmacodynamic basis for exerting antithrombosis, reducing inflammation, and promoting angiogenesis (Yang et al., 2017). From another perspective, utilizing gut microbiota to enhance the efficacy of IA₁ should be a serious consideration.

Conclusion

The pharmacokinetics and metabolite profiles of IA₁ were investigated based on the UPLC–MS/MS technique. A total of 18 metabolites of IA₁ were identified in rat plasma by MetabolitePilot™ 1.5 and PeakView® 2.0 software. Isomerization, decarboxylation, hydrogenation, dehydrogenation, and oxidation occurred on the skeleton of IA, which was transformed from IA₁ through deglycosylation. The pharmacokinetic data demonstrated that the absorption of IA₁ and the conversion efficiency of IA₁ to IA were significantly impaired by disordered intestinal bacteria after monomer administration. Considering the database analysis, IA and IA₁ could play a synergistic role in common pharmacological pathway, such as hypertrophic cardiomyopathy and dilated cardiomyopathy. The converted product, IA, could be an important pharmacodynamic basis for IA₁ exerting an effect. However, the effects of antibiotics on the efficacy of IA₁ remain unclear. In addition, unsatisfactory drug likeness criteria of IA₁ and IA are adverse to clinical application. Therefore, these factors should be further considered in subsequent research.

Abbreviations

IA₁: Ilexsaponin A₁; IA: Ilexgenin A; TCM: Traditional Chinese Medicine; UPLC-QTOF-MS: Ultra-performance liquid chromatography coupled with quadrupole time-of-flight mass spectrometry; IS: Internal standard; PNPg: *p*-nitrophenol and *p*-nitrophenyl β -D-glucopyranoside; QC:

Quality control; C_{max}: Maximum plasma concentration; T_{max}: Time to reach the maximum plasma concentration; AUC_{0-t}: Area under the plasma concentration versus time curve from 0 h to the last quantifiable concentration; t_{1/2}: elimination half-life; AUC_{0-∞}: Area under the plasma concentration versus time curve from 0 h to infinity; SD: Standard deviation; KEGG: Kyoto Encyclopedia of Genes and Genomes; DAVID: Database for annotation, visualization, and integrated discovery; LLOQ: Lower limit of quantification.

Authors' Contributions

All authors made contributions to the completion of the article. Di Cao performed the material preparation, experiments, and writing original draft. Xiuting Shen, Zhengjiao Wang, and Xiaojun Song handled the data analysis and manuscript review. Zhongxiang Zhao executed the supervision and revision of manuscript.

Declaration of Conflicting Interests

The authors declared no potential conflicts of interest with respect to the research, authorship and/or publication of this article.

Statement of Informed Consent and Ethical Approval

The studies were approved by the ethics committee on the Care and Use of Laboratory Animals of Guangzhou University of Chinese Medicine.

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Summary

The amounts of IA₁ and its metabolite IA were simultaneously quantified in the plasma of normal and antibiotic-treated rats. β -Glucosidase activity inhibited by antibiotics suppressed the hydrolysis reaction of IA₁ in the intestinal tract. IA₁ was metabolized in rat plasma by biotransformation routes of deglycosylation, decarboxylation, isomerization, hydrogenation, dehydrogenation, and oxidation.

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