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Effects of Gut Microbiota and Ingredient-Ingredient Interaction on the Pharmacokinetic Properties of Rotundic Acid and Pedunculoside

Authors
Bao Yang[1], Hui Li[1], Qingfeng Ruan[1], Shenxin Xuan[1], Xiaojing Chen[1], Hui Cui[1], Zhongqiu Liu[1], Jing Jin[2], Zhongxiang Zhao[1]

Affiliations
1 School of Pharmaceutical Sciences, Guangzhou University of Chinese Medicine, Guangzhou, China
2 School of Pharmaceutical Sciences, Sun Yat-Sen University, Guangzhou, China

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Correspondence
Prof. Dr. Zhongxiang Zhao
School of Pharmaceutical Sciences, Guangzhou University of Chinese Medicine, Guangzhou Higher Education Mega Center
232 Waihuan East Road, Guangzhou 510006, China
Phone: + 86 20 39 35 83 25, Fax: + 86 20 39 35 82 53
zzx37@163.com

Correspondence
Associate Professor Dr. Jing Jin
School of Pharmaceutical Sciences, Sun Yat-Sen University, Guangzhou Higher Education Mega Center
132 Waihuan East Road, Guangzhou 510006, China
Phone: + 86 20 39 94 30 34, Fax: + 86 20 39 94 30 00
jinjing@mail.sysu.edu.cn

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ABSTRACT

Rotundic acid and pedunculoside are the most abundant constituents in Ilicis Rotundae Cortex, and possess lipid-lowering activity. In this study, we evaluated the pharmacokinetic interactions of rotundic acid with pedunculoside and other ingredients from Ilicis Rotundae Cortex with rotundic acid and pedunculoside, and preliminarily investigated the effects of gut microbiota on their pharmacokinetics using a pseudo-germ-free rat model. After a single oral administration of each monomer, a monomer mixture, and Ilicis Rotundae Cortex extract to the conventional and pseudo-germ-free rats, rotundic acid and pedunculoside were quantified in plasma by an UPLC/Q-TOF-MS/MS method. The systemic exposure (maximum plasma concentration and area under concentration-time curve) of two analytes in conventional rats were increased in an approximately dose-dependent manner. Oral administration of rotundic acid and pedunculoside in the forms of a monomer mixture and Ilicis Rotundae Cortex extract to the conventional rats significantly decreased the systemic exposure compared with the monomer groups, which demonstrated the existence of significant pharmacokinetic interactions. The pseudo-germ-free rats were prepared by nonabsorbable antibiotic treatment, and the systemic exposure of two analytes were significantly decreased and most of the "time to reach the maximum" values were delayed in comparison to conventional rats, therefore gut microbiota might serve as an efficient absorption promoter. These results provide a scientific basis for the clinical application of the two bioactive constituents and Ilicis Rotundae Cortex.

Introduction

Ilex rotunda Thunb., a medically woody plant in the Aquifoliaceae family, is widely distributed throughout southern China [1, 2]. In the Chinese Pharmacopoeia (2015 edition) [3], its bark is officially recorded as IRC and is used for the treatment of gastrointestinal and cardiovascular diseases. IRC is a rich source of triterpenoids, which exhibit significant cardiovascular protection activities [1, 4–7]. Both PDC and its aglycone RA are by far the most abundant (approximately 23.5% of the IRC extract) and are representative medicinal triterpenoids in IRC that have been selected as the quality control markers [8, 9]. Substantial lipid-lowering effects for PDC and RA have also been reported [7, 10]. In this context, it is of great importance to investigate the pharmacokinetic properties of RA and PDC for a deep understanding of the pharmacological effects of the pure compounds as well as IRC.

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ABBREVIATIONS

AEH	pseudo-germ-free rats administered with Ilicis Rotundae Cortex extract at 2000 mg/kg
AEL	pseudo-germ-free rats administered with Ilicis Rotundae Cortex extract at 200 mg/kg
AMH	pseudo-germ-free rats administered with monomer mixture at 470 mg/kg
AML	pseudo-germ-free rats administered with monomer mixture at 47 mg/kg
APH	pseudo-germ-free rats administered with pedunculoside monomer at 301 mg/kg
APL	pseudo-germ-free rats administered with pedunculoside monomer at 30 mg/kg
ARH	pseudo-germ-free rats administered with rotundic acid monomer at 226 mg/kg
ARL	pseudo-germ-free rats administered with rotundic acid monomer at 23 mg/kg
ATM	antimicrobial
AUC	area under concentration-time curve
C_{max}	maximum plasma concentration
IRC	Ilicis Rotundae Cortex
IS	internal standard
LLOQ	lower limit of quantification
MRT	mean residence time
NEH	conventional rats administered with Ilicis Rotundae Cortex extract at 2000 mg/kg
NEL	conventional rats administered with Ilicis Rotundae Cortex extract at 200 mg/kg
NMH	conventional rats administered with monomer mixture at 470 mg/kg
NML	conventional rats administered with monomer mixture at 47 mg/kg
NPH	conventional rats administered with pedunculoside monomer at 301 mg/kg
NPL	conventional rats administered with pedunculoside monomer at 30 mg/kg
NRH	conventional rats administered with rotundic acid monomer at 226 mg/kg
NRL	conventional rats administered with rotundic acid monomer at 23 mg/kg
PDC	pedunculoside
QC	quality control
RA	rotundic acid
SRM	selected reaction monitoring
TCM	traditional Chinese medicine
T_{max}	time to reach the maximum
$t_{1/2}$	half-life

Due to the complexity of chemical constituents in TCMs, to some extent, herbal ingredient-ingredient and substance-ingredient pharmacokinetic interactions cannot be neglected, and that may significantly affect oral absorption and biological activity [11–13]. Therefore, it is important to illustrate those possible pharmacokinetic interactions after the administration of herbal ingredients or raw herbs. However, until now, the possible pharmacokinetic influences of other ingredients in IRC on RA and PDC, as well as the potential pharmacokinetic interaction between RA and PD, are seldom reported.

IRC and its ingredients are often given via the oral administration route, and are largely absorbed though the intestinal tract, so they will inevitably interact with the gut microbiota. A wealth of information shows that symbiotic gut microbiota represents an important bridge between host metabolism and environmental substances [14]. It is one of the most important places for the metabolism of TCMs before absorption into the blood due to the presence of numerous enzymes. Among the various reported enzymes, β-glucosidase is the most studied and highly produced intestinal bacterial enzyme [14–16]. Thus, compounds with a β-glucoside bond in IRC may be strongly affected after oral administration. Moreover, it is emphasized that gut microbiota can also indirectly influence the *in vivo* process of TCMs by affecting the intestinal absorption and the expression profiles of liver metabolic enzymes, which may influence their pharmacokinetics and pharmacodynamics [14–17].

Therefore, in this study, we established a pseudo-germ-free rat model to systemically investigate the potential effects of gut microbiota on the pharmacokinetic behaviors of RA and PDC. Moreover, after oral administration of each monomer, monomer mixture, and IRC extract to the conventional rats, we also compared the pharmacokinetic parameters of RA and PDC to evaluate the presence of possible pharmacokinetic interactions. It was expected that this experiment would contribute to improving clinical therapeutic effects and further pharmacological studies of RA, PDC, and IRC.

Results and Discussion

In our previous work [18], a UPLC/Q-TOF-MS/MS method was established for quantification analysis of six triterpenoids in rat plasma after oral administration of IRC extract. Here, using the developed LC-MS method as the starting point, the chromatographic conditions were further modified to shorten the run time. The mass spectrometer was performed in the SRM model, and the ion transition and collision energy values for RA, PDC, and IS were m/z 487.34 → 469.33 and −47 eV, m/z 695.40 → 487.34 and −38 eV, and m/z 825.40 → 649.36 and −55 eV, respectively. The representative MS/MS spectra of two analytes under the optimized conditions are shown in ▶ **Fig. 1B, C**.

As shown in ▶ **Fig. 2**, the retention times of RA, PDC, and IS were about 2.83, 1.68, and 1.44 min, respectively, and no interfering peaks were observed. The calibration curves were $y = 0.00731x + 0.01632$ for RA and $y = 0.01185x + 0.05717$ for PDC. Linearity was confirmed over the whole calibration range (2.88–2400 ng/mL for RA and 1.52–800 ng/mL for PDC) with correlation coefficient values of 0.9920 for RA and 0.9978 for PDC. The LLOQs for RA and PDC were 2.88 and 1.52 ng/mL, respectively. The precisions of two analytes (**Table 1S**, Supporting Information) were less than 12.2 and 13.3%, whilst the accuracy ranged from −12.6 to 12.3%. The extraction recovery of two analytes varied from 86.3 to 110.3%, while the matrix effect ranged from 86.6 to 109.7% (**Table 2S**, Supporting Information). The results of the stability tests are summarized in **Table 3S**, Supporting Information, within the ac-

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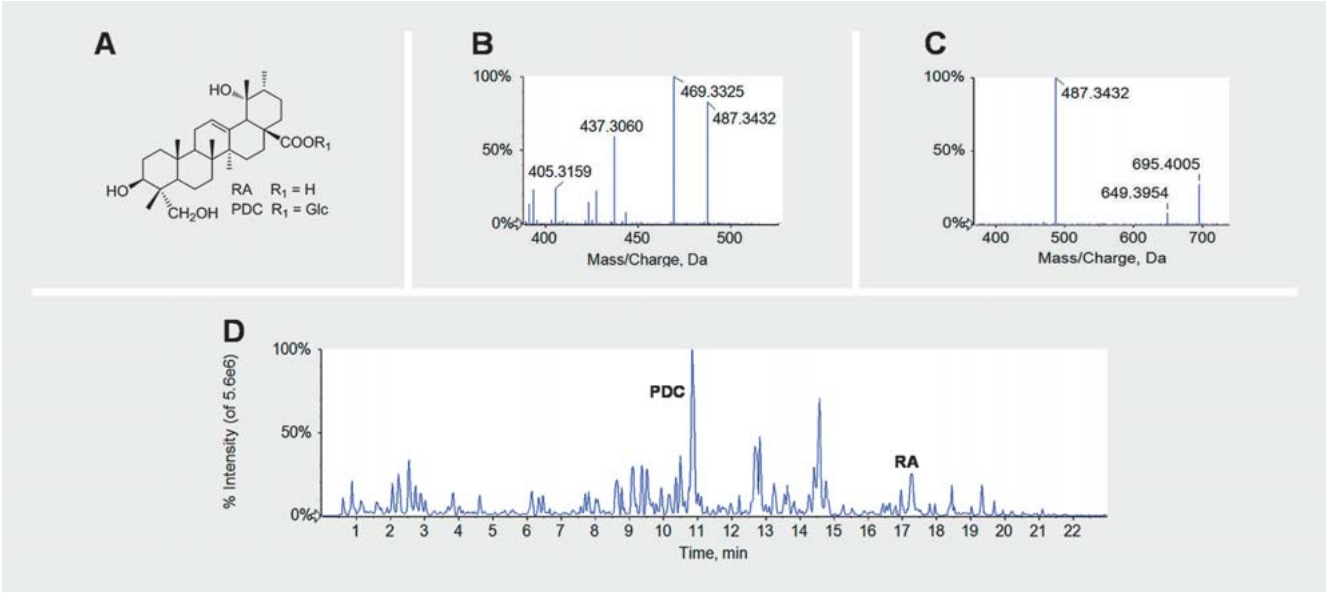
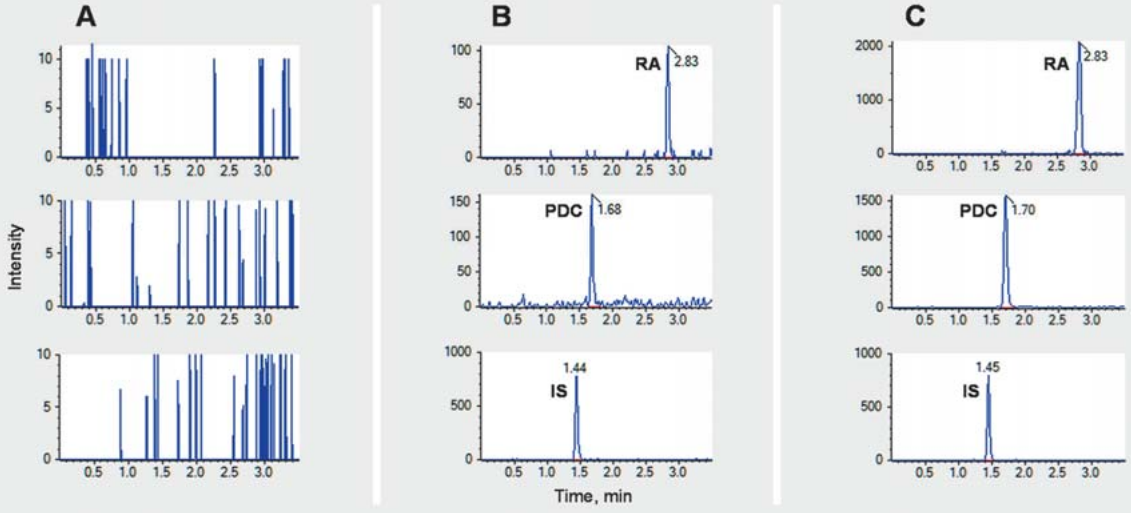


▶ **Fig. 1** **A** Chemical structures of RA and PDC; representative MS/MS spectra of RA (**B**) and PDC (**C**). **D** Representative total ion chromatogram of IRC extract in the negative ESI mode. IRC, Ilicis Rotundae Cortex; PDC, pedunculoside; RA, rotundic acid.



▶ **Fig. 2** Representative extract ion chromatograms of RA, PDC, and IS in rat plasma. **A** Blank plasma; **B** blank plasma spiked with two analytes in LLOQ and IS; **C** plasma sample from a rat after a single oral administration of IRC extract. IRC, Ilicis Rotundae Cortex; IS, internal standard; LLOQ, lower limit of quantification; PDC, pedunculoside; RA, rotundic acid.

ceptable criteria of ± 15 %. All results demonstrated that the optimized analytical method was sensitive and reproducible.

Compared with the conventional rats, most of the ATM-treated rats developed diarrhea after oral administration with the antibiotic mixture for 7 consecutive days. The metabolic activity of gut microbiota was directly evaluated by analysis of the β-glucosidase activity in feces samples. Experimental data showed that the β-glucosidase activity in the conventional group (0.93 ± 0.21 nmol/min/mg) was significantly higher ($p < 0.01$) than in the ATM-treated group (0.27 ± 0.06 nmol/min/mg), which confirmed the validity of the pseudo-germ-free rat model.

This developed UPLC/Q-TOF-MS/MS method was successfully employed to quantify the plasma concentrations of RA and PDC. The mean plasma concentration-time profiles of RA and PDC are presented in ▶ **Figs. 3** and **4**, and part of them showed two or three peaks. The most reasonable explanation might be the enteric circulation, inhomogeneity of intestinal absorption, gastric motility and so on, and this phenomenon was common in the pharmacokinetic studies of TCMs [11, 19]. The major pharmacokinetic parameters of C_{max}, T_{max}, $t_{1/2}$, AUC, and MRT are summarized in ▶ **Tables 1** and **2**.

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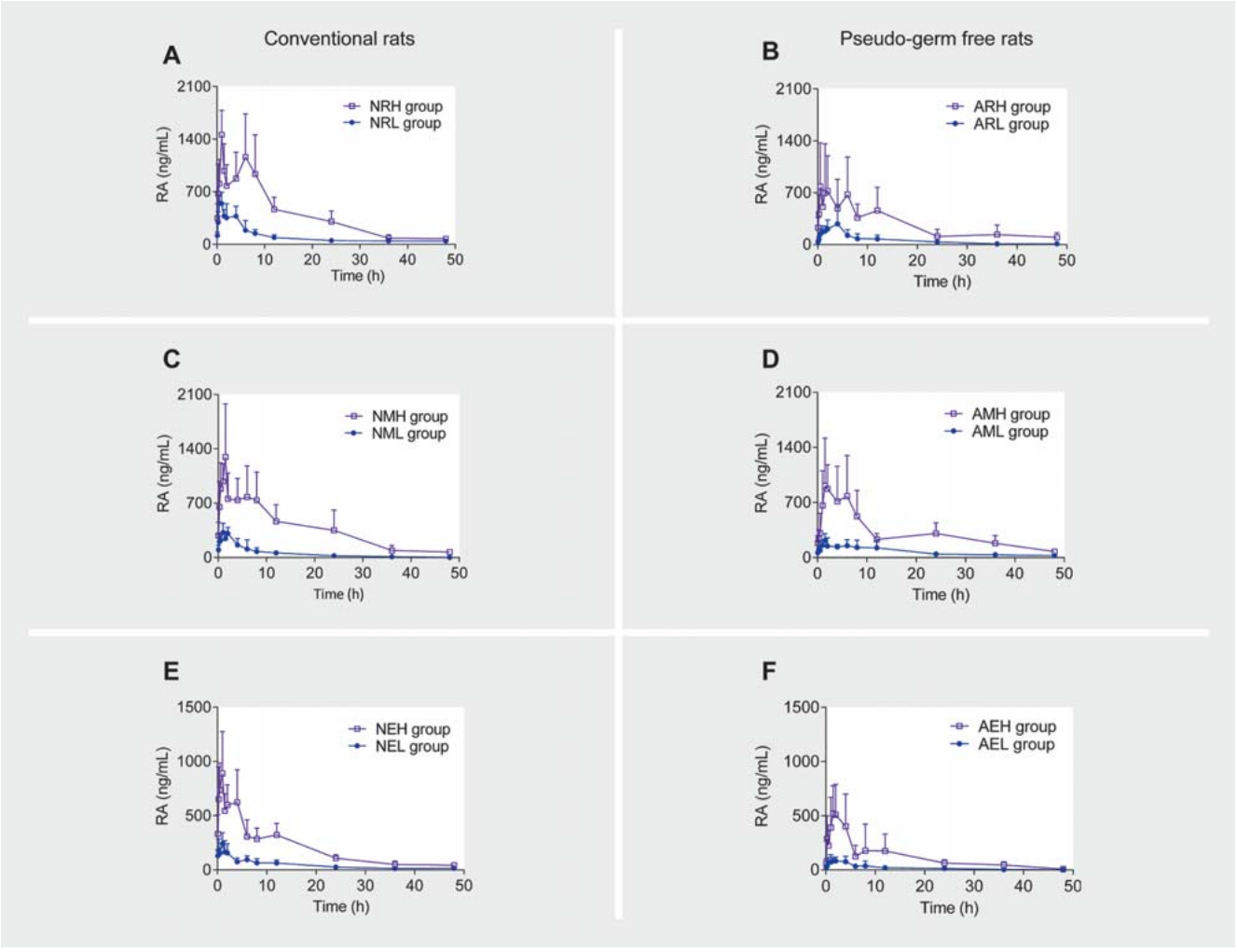

▶ **Fig. 3** Mean plasma concentration-time curves of RA in conventional and pseudo-germ-free rat plasma after a single oral administration of RA (**A** and **B**), the monomer mixture (**C** and **D**), and IRC extract (**E** and **F**) at two dose levels. IRC, Ilicis Rotundae Cortex; RA, rotundic acid.

We first investigated the pharmacokinetic behaviors of RA and PDC in conventional rats after oral administration of each monomer, monomer mixture, and IRC extract at two dose levels. Overall, the systemic exposure (C_{max} and AUC) of RA and PDC in conventional rats seemed to increase in a dose-dependent manner over the two dose levels, although not necessarily dose proportional, while no clear trend was observed for T_{max}, $t_{1/2\,z}$, and $MRT_{(0\text{-}t)}$. After oral administration of the RA monomer to the conventional rats, the mean C_{max} values were reached within 1.3 h and amounted to 1689.5 ± 402.6 and 627.2 ± 110.4 ng/mL at the doses of 462 µmol/kg and 46 µmol/kg, respectively, and the corresponding mean AUC_{0-t} values were 21 024.2 ± 5661.6 and 4908.4 ± 1150.3 ng h/mL, respectively. When the PDC monomer was orally administered to the conventional rats at doses equivalent to the RA monomer, the mean C_{max} values were reached within 1.8 h and amounted to 513.5 ± 100.6 and 115.8 ± 34.6 ng/mL, respectively, and the corresponding mean AUC_{0-t} values were 4811.9 ± 785.9 and 673.6 ± 319.4 ng h/mL, respectively. A longer mean T_{max} and lower systemic exposure for PDC were observed compared with RA. This phenomenon may be explained by their different chemical structures, which the attachment of one sugar moiety remarkably reduced the rate of absorption and decreased the systemic exposure, and the molecular mass of PDC was about 650 Da, which was slightly greater than the favorable value [20, 21].

In the comparative pharmacokinetics studies, the systemic exposure of RA and PDC in conventional rats was remarkably decreased when orally administrated the monomer mixture or IRC extract (▶ **Tables 1** and **2**). Specifically, in comparison with the relevant monomer groups, when administered with monomer mixture at 470 and 47 mg/kg, the mean C_{max} values of RA were decreased by 14 and 41%, respectively, and the corresponding mean AUC_{0-t} values were decreased by 18 and 49%, respectively. The dose-normalized mean C_{max} values of PDC were decreased by 45 and 19%, respectively, and the corresponding dose-normalized mean AUC_{0-t} values were decreased by 46 and 14%, respectively. As compared with the relevant monomer groups, when administered with the IRC extract at 2000 and 200 mg/kg, the mean C_{max} values of RA were decreased by 38 and 54%, respectively, and the corresponding AUC_{0-t} values were decreased by 55 and 58%, re-

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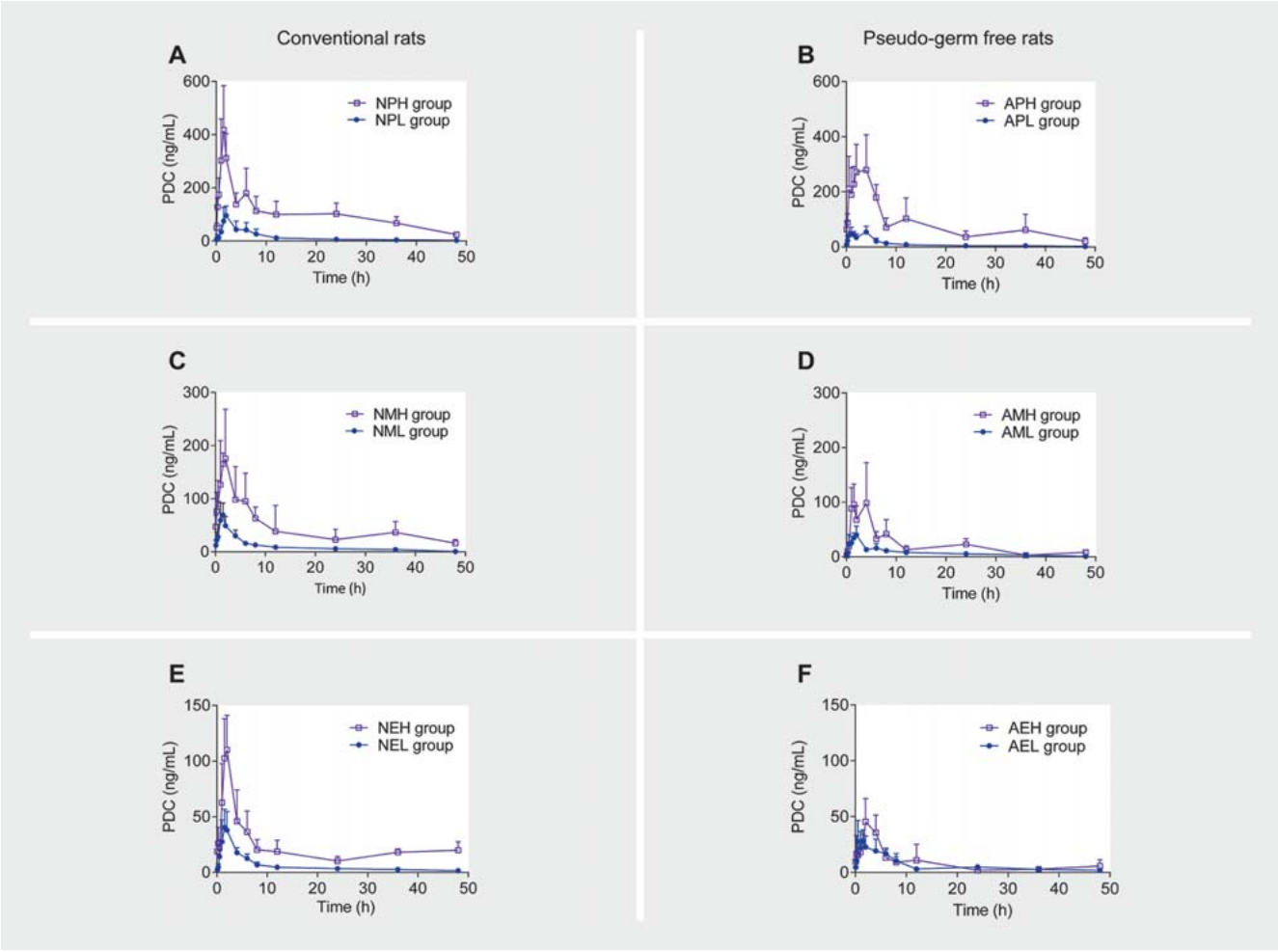

▶ **Fig. 4** Mean plasma concentration-time curves of PDC in conventional and pseudo-germ-free rat plasma after a single oral administration the PDC monomer (**A** and **B**), monomer mixture (**C** and **D**), and IRC extract (**E** and **F**) at two dose levels. IRC, Ilicis Rotundae Cortex; PDC, pedunculoside.

spectively. The dose-normalized mean C_{max} values of PDC were decreased by 69 and 47%, respectively, and the corresponding dose-normalized mean AUC_{0-t} values were decreased by about 73 and 48%, respectively. Consequently, the above results not only indicated a remarkable pharmacokinetic interaction between the RA and PDC monomers, but also provided evidence suggesting that some other ingredients from the IRC might have a negative impact on the systemic exposure of RA and PDC, and might subsequently degrade their bioavailability. The reason might be that RA, PDC, and other ingredients in the IRC had very similar chemical structures, resulting in the competitive absorption that appeared in the small intestine.

An increasing number of studies suggest that gut microbiota plays an important role in the absorption and metabolism of TCMs after oral administration [14–16]. Thus, we established a pseudo-germ-free rat model to evaluate whether the pharmacokinetics of RA and PDC were affected by the gut microbiota. As shown in ▶ **Tables 1** and **2**, it was obvious that the mean C_{max} and AUC values of RA and PDC in pseudo-germ-free rats were decreased and most of the mean T_{max} values were delayed (about 0.5 h, except for PDC in the APL and AMH groups) by comparison with the conventional rats, while the other pharmacokinetic parameters did not show significant differences. The mean C_{max} values of RA in the ARH, ARL, AMH, AML, AEH, and AEL groups were decreased by 26, 38, 12, 35, 21, and 56% compared with the conventional groups, respectively, and the corresponding mean AUC_{0-t} values were decreased by 40, 44, 15, 8, 41, and 57%, respectively. Similarly, in comparison with the conventional groups, the mean C_{max} values of PDC in the APH, APL, AMH, AML, AEH, and AEL groups were decreased by 32, 48, 41, 35, 64, and 18%, respectively, and the corresponding mean AUC_{0-t} values were decreased by 19, 31, 51, 25, 63, and 19%, respectively. Therefore, with regard to the potential ingredient-ingredient pharmacokinetic interactions, it was confirmed that the gut microbiota could significantly affect the pharmacokinetic behaviors of RA and PDC, which might be directly correlated with marked changes in biological activity. The exact mechanisms were unclear, but it was speculated that the gut microbiota might serve as an efficient absorption promoter contributing to the high systemic exposure of RA and PDC. In addition, considering that the gut microbiota was susceptible to the antibiotics, if not absolutely necessary, the combined application of antibiotics with IRC, RA, and PDC should be avoided.

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▶ **Table 1** Pharmacokinetic parameters of RA after a single oral administration of the RA monomer, monomer mixture, and IRC extract to conventional and pseudo-germ-free rats at two dose levels (mean ± SD, n = 6).

Parameters	Unit	RA monomer		Monomer mixture of RA and PDC[a]		IRC extract[a]	
		226 mg/kg (462 µmol/kg)	23 mg/kg (46 µmol/kg)	470 mg/kg	47 mg/kg	2000 mg/kg	200 mg/kg
Conventional rats		**NRH group**	**NRL group**	**NMH group**	**NML group**	**NEH group**	**NEL group**
C_{max}	ng/mL	1689.5 ± 402.6	627.2 ± 110.4	1458.8 ± 538.6	368.6 ± 75.6$^{\#\#}$	1041.5 ± 293.6$^{\#}$	289.1 ± 77.4$^{\#\#\triangle}$
T_{max}	h	1.3 ± 0.4	1.1 ± 0.3	1.4 ± 0.4	1.2 ± 0.3	1.0 ± 0.6	1.1 ± 0.4
$t_{1/2z}$	h	12.3 ± 2.1	10.6 ± 2.2	9.0 ± 3.3	8.5 ± 2.0	10.4 ± 4.6	16.3 ± 2.3
$AUC_{(0-t)}$	ng h/mL	21 024.2 ± 5661.6	4908.4 ± 1150.3	17 280.1 ± 6063.4	2492.5 ± 606.9$^{\#\#}$	9360.9 ± 1485.3$^{\#\#\triangle}$	2083.7 ± 587.6$^{\#\#}$
$AUC_{(0-\infty)}$	ng h/mL	23 274.5 ± 5363.3	6181.5 ± 1323.2	17 931.7 ± 6012.4	2530.2 ± 594.5$^{\#\#}$	9852.0 ± 1299.1$^{\#\#\triangle}$	2383.9 ± 579.7$^{\#\#}$
$MRT_{(0-t)}$	h	12.9 ± 1.4	13.6 ± 1.9	13.8 ± 1.9	9.5 ± 2.3	12.2 ± 2.3	12.5 ± 2.7
Pseudo-germ-free rats		**ARH group**	**ARL group**	**AMH group**	**AML group**	**AEH group**	**AEL group**
C_{max}	ng/mL	1242.4 ± 528.9	389.4 ± 178.4*	1287.9 ± 343.4	238.6 ± 72.3*	823.2 ± 106.7$^{\#\#\triangle}$	128.1 ± 29.5$^{**\#\#\triangle}$
T_{max}	h	2.1 ± 1.5	2.5 ± 1.4	2.0 ± 1.2	2.1 ± 1.0	2.1 ± 1.1	1.9 ± 1.2
$t_{1/2z}$	h	15.2 ± 8.2	12.8 ± 7.7	15.2 ± 5.2	16.1 ± 6.5	7.3 ± 2.0	16.8 ± 7.3
$AUC_{(0-t)}$	ng h/mL	12 531.9 ± 5627.1*	2757.9 ± 1485.3*	14 747.9 ± 4064.7	2283.6 ± 856.7	5560.9 ± 1752.1$^{**\#\triangle\triangle}$	904.3 ± 528.8$^{**\#\triangle\triangle}$
$AUC_{(0-\infty)}$	ng h/mL	14 829.5 ± 8035.6*	2931.6 ± 1477.5*	16 424.1 ± 3094.4	2198.6 ± 782.1	5595.9 ± 1350.4$^{**\#\triangle\triangle}$	980.7 ± 478.8$^{**\#\triangle\triangle}$
$MRT_{(0-t)}$	h	14.86 ± 2.6	11.3 ± 1.5	16.5 ± 2.2	15.4 ± 3.7	12.2 ± 2.2	9.9 ± 1.8

[a] Oral administration of the monomer mixture at 470 and 47 mg/kg, and IRC extract at 2000 and 200 mg/kg were equivalent to that of the oral administration of the RA monomer at 226 and 23 mg/kg, respectively. $^{*}P < 0.05$ and $^{**}p < 0.01$ when compared with the level of the corresponding group of conventional rats; $^{\#}p < 0.05$ and $^{\#\#}p < 0.01$ when compared with the corresponding group that was orally administrated the RA monomer; $^{\triangle}p < 0.05$ and $^{\triangle\triangle}p < 0.01$ when compared with the corresponding group that was orally administrated the monomer mixture

▶ **Table 2** Pharmacokinetic parameters of PDC after a single oral administration of the PDC monomer, monomer mixture, and IRC extract to conventional and pseudo-germ-free rats at two dose levels (mean ± SD, n = 6).

Parameters	Unit	PDC monomer		Monomer mixture of RA and PDC[a]		IRC extract[a]	
		301 mg/kg (462 µmol/kg)	30 mg/kg (46 µmol/kg)	470 mg/kg	47 mg/kg	2000 mg/kg	200 mg/kg
Conventional rats		**NPH group**	**NPL group**	**NMH group**	**NML group**	**NEH group**	**NEL group**
C_{max}	ng/mL	513.5 ± 100.6	115.8 ± 34.6	228.3 ± 58.8$^{\#\#}$	75.7 ± 22.7	128.7 ± 28.8$^{\#\#\triangle\triangle}$	50.2 ± 12.8$^{\#\#}$
T_{max}	h	1.5 ± 0.4	1.8 ± 0.3	1.6 ± 0.4	1.5 ± 0.6	1.6 ± 0.4	1.6 ± 0.4
$t_{1/2z}$	h	14.2 ± 1.8	15.3 ± 7.0	15.8 ± 6.2	10.7 ± 4.0	21.4 ± 4.5	20.2 ± 4.1
$AUC_{(0-t)}$	ng h/mL	4811.9 ± 785.9	673.6 ± 319.4	2121.5 ± 608.7$^{\#\#}$	468.7 ± 119.6	1071.4 ± 142.7$^{\#\#\triangle\triangle}$	283.9 ± 66.9$^{\#\triangle}$
$AUC_{(0-\infty)}$	ng h/mL	6228.7 ± 1293.6	753.6 ± 287.1	2980.9 ± 458.5$^{\#\#}$	483.2 ± 113.9$^{\#}$	2327.5 ± 879.3$^{\#\#}$	340.6 ± 64.1
$MRT_{(0-t)}$	h	17.1 ± 1.7	11.2 ± 2.2	16.3 ± 3.5	11.5 ± 2.2	18.8 ± 2.1	11.8 ± 1.6
Pseudo-germ-free rats		**APH group**	**APL group**	**AMH group**	**AML group**	**AEH group**	**AEL group**
C_{max}	ng/mL	349.9 ± 75.7*	60.1 ± 19.2*	133.9 ± 62.9$^{*\#\#}$	48.9 ± 8.1*	45.5 ± 20.8$^{**\#\#\triangle\triangle}$	40.9 ± 7.7$^{\#}$
T_{max}	h	2.1 ± 1.2	1.7 ± 1.4	1.8 ± 1.1	2.3 ± 0.7	2.3 ± 0.6	2.2 ± 0.8
$t_{1/2z}$	h	18.6 ± 6.8	12.4 ± 6.3	11.6 ± 3.9	14.4 ± 7.5	15.6 ± 4.4	23.1 ± 7.0
$AUC_{(0-t)}$	ng h/mL	3904.2 ± 1627.1	463.3 ± 141.7	1035.5 ± 334.1$^{**\#\#}$	352.2 ± 69.3	396.1 ± 207.3$^{**\#\#\triangle\triangle}$	228.9 ± 39.3$^{\#}$
$AUC_{(0-\infty)}$	ng h/mL	4963.6 ± 2064.9	529.7 ± 119.7	1100.4 ± 393.2$^{**\#\#}$	372.4 ± 62.5	498.7 ± 254.3$^{**\#\#\triangle}$	353.4 ± 219.5
$MRT_{(0-t)}$	h	14.4 ± 4.4	11.1 ± 3.4	13.5 ± 2.6	12.8 ± 3.3	13.5 ± 2.0	12.7 ± 2.2

[a] Oral administration of the monomer mixture at 470 and 47 mg/kg, and IRC extract at 2000 and 200 mg/kg were equivalent to that of the oral administration of the PDC monomer at 244 and 24 mg/kg, respectively. $^{*}P < 0.05$ and $^{**}p < 0.01$ when compared with the level of the corresponding group of conventional rats; $^{\#}p < 0.05$ and $^{\#\#}p < 0.01$ when compared with the corresponding group that was orally administrated the PDC monomer, and the AUC and C_{max} values were normalized to the dose when statistics were performed. $^{\triangle}P < 0.05$ and $^{\triangle\triangle}p < 0.01$ when compared with the corresponding group that was orally administrated the monomer mixture

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Moreover, in view that the PDC could be partially converted to RA by the β-glucosidase of gut microbiota [22], the plasma concentrations of RA in the NPH, NPL, APH, and APL groups were quantified. As shown in ▶ **Table 3** and **Fig. 5**, the rate of conversion by gut microbiota seemed relatively slow, which exhibited significantly later mean T_{max} values of 6.1 ± 3.5 and 8.4 ± 2.2 h in the NPH and NPL groups, respectively. The corresponding mean C_{max} values were 303.1 ± 111.2 and 133.5 ± 33.1 ng/mL, respectively, and the mean AUC_{0-t} values were 3716.7 ± 1113.3 and 1926.4 ± 454.9 ng h/mL, respectively. The β-glucosidase activity of gut microbiota was markedly reduced after the antibiotic treatment, resulting in the systemic exposure of RA in the APH and APL groups being significantly decreased by more than 58% compared with the NPH and NPL groups, and the mean T_{max}, $t_{1/2z}$, and $MRT_{(0-t)}$ values were all delayed.

However, there was no evidence to rule out the potential effects of these two selected antibiotics on the drug transporters in this study as well as in other reports, but its significant impact on the function of gut microbiota has been proven. Therefore, the potential effects of streptomycin and neomycin on the drug transporters should be illustrated in further study, and a germ-free rat model should be adopted to acquire more accurate results. In summary, this is the first report on the pharmacokinetic comparisons of RA and PDC in conventional and pseudo-germ-free rats after a single oral administration of each monomer, monomer mixture, and IRC extract. These results might provide significant contributions for the clinical use of RA, PDC, and IRC.

▶ **Table 3** Pharmacokinetic parameters of RA after a single oral administration of the PDC monomer to conventional and pseudo-germ-free rats at two dose levels (mean ± SD, n = 6).

Parameters	Unit	PDC monomer	
		301 mg/kg	30 mg/kg
Conventional rats		**NPH group**	**NPL group**
C_{max}	ng/mL	303.1 ± 111.2	133.5 ± 33.1
T_{max}	h	6.1 ± 3.5	8.4 ± 2.2
$t_{1/2z}$	h	8.6 ± 2.5	13.0 ± 3.7
$AUC_{(0-t)}$	ng h/mL	3716.7 ± 1113.3	1926.4 ± 454.9
$AUC_{(0-\infty)}$	ng h/mL	3828.8 ± 1063.6	2053.7 ± 446.8
$MRT_{(0-t)}$	h	14.5 ± 2.7	15.2 ± 1.9
Pseudo-germ-free rats		**APH group**	**APL group**
C_{max}	ng/mL	35.2 ± 9.5**	30.0 ± 5.3**
T_{max}	h	19.9 ± 5.8	10.4 ± 8.4
$t_{1/2z}$	h	24.6 ± 4.2	20.0 ± 7.8
$AUC_{(0-t)}$	ng h/mL	793.4 ± 282.8**	633.3 ± 194.0**
$AUC_{(0-\infty)}$	ng h/mL	1132.2 ± 296.9**	855.0 ± 344.8**
$MRT_{(0-t)}$	h	22.7 ± 5.3	21.5 ± 1.6

*P < 0.05 and **p < 0.01 when compared with the level of the corresponding group of conventional rats

Materials and Methods

Chemicals and reagents

RA (purity > 99.0%) and PDC (purity > 99.0%) were isolated in our laboratory from IRC, and their chemical structures (▶ **Fig. 1A**) were confirmed with HR-MS and NMR analysis [5,6]. Digoxin (IS, purity > 99.0%) was obtained from Weikeqi Biological Technology Co. Ltd. Neomycin sulfate (USP grade) and streptomycin sulfate (USP grade) were purchased from Beijing Biotopped Science and Technology Co., Ltd. p-Nitrophenyl-β-D-glucopyranoside (purity > 98.0%) and p-nitrophenol (purity > 98.0%) were purchased from Dalian Meilun Biology Technology. Sodium hydroxide (NaOH, purity > 99.0%) was bought from Sigma-Aldrich. MS grade methanol and acetonitrile were acquired from Merck. Ultrapure water used throughout all experiments was purified with a Milli-Q system. The 70% ethanol extract of IRC was prepared using a previously published method, and the contents of RA (113.2 mg/g) and PDC (121.7 mg/g) were determined by a UPLC-MS method [8]. The representative total ion chromatogram of the IRC extract is shown in ▶ **Fig. 1D**. The RA and PDC monomer mixtures were prepared by mixing 1130 mg RA monomer with 1220 mg PDC monomer.

Apparatus and operation conditions

The multistage MS experiments were conducted on an AB Sciex Triple-TOF 5600$^+$ mass spectrometer coupled to a Shimadzu LC-30 AD chromatography system. Separations were accomplished on a Waters Acquity UPLC BEH C_{18} column (100 × 2.1 mm, 1.7 µm) at 50 °C with a mobile phase composed of 0.1% formic acid-acetonitrile (v/v, solvent A) and 0.1% formic acid-water (v/v, solvent B). The gradient elution program was as follows: 0–2.5 min, 35–65% A; 2.5–3.0 min, 65–70% A; 3.0–3.5 min, 70% A; 3.5–4.0 min, 70–100% A; 4.0–4.5 min, 100–35% A; 4.5–7.5 min, 35% A. The flow rate was 0.4 mL/min, and the injection volume was 5 µL. The mass spectrometer was operated in the negative ESI mode. The optimized instrument settings were as follows: ion spray voltage, −4500 V; source temperature, 550 °C; nebulizer gas pressure and heater gas pressure, 55 psi; curtain gas pressure, 30 psi; declustering potential, 100 eV.

Sample preparation

Preparation of calibration and quality control samples

A mixed stock solution containing 1.90 mg/mL of RA and 0.26 mg/mL of PDC was prepared in methanol. Then a series of working standard solutions were prepared by serially diluting the mixed stock solution with methanol. The calibration standard samples were freshly prepared by spiking the relevant working standard solutions with blank rat plasma to obtain final concentrations of 2400, 1200, 600, 150, 75, 37.5, 18.75, 9.38, 4.69, and 2.88 ng/mL for RA, and 800, 400, 200, 100, 50, 25, 12.5, 6.25, 3.13, and 1.52 ng/mL for PDC. QC samples were at 2400, 150, and 4.69 ng/mL for RA, and 800, 50, and 3.13 ng/mL for PDC. The IS working solution (1000 ng/mL) was prepared in methanol.

Preparation of plasma samples

For preparing the plasma samples, 100 µL of thawed plasma samples were vortex-mixed with 10 µL of IS working solution and 400 µL of methanol for 3 min, and then centrifuged at 10 008 × *g* for 15 min. The supernatant was transferred into another Eppen-

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▶ **Fig. 5** Mean plasma concentration-time curves of RA in conventional (**A**) and pseudo-germ free (**B**) rat plasma after a single oral administration of the PDC monomer at two dose levels. PDC, pedunculoside; RA, rotundic acid.

dorf tube and evaporated to dryness under vacuum at 30 °C. Each dried residue sample was reconstituted in 100 µL of methanol (containing 0.1% formic acid), followed by centrifugation at 10 008 × *g* for 15 min. Finally, 5 µL of the supernatant was used for analysis.

Qualitative method validation

Selectivity was ascertained by comparing the chromatograms of six blank rat plasma samples, blank plasma spiked with the analytes and IS, and plasma samples from rats after oral administration of the IRC extract. The calibration curves were determined by plotting the peak area ratio (*y*) of the analytes to the IS versus the nominal concentration (*x*) of analytes with weighted ($1/x^2$) least square linear regression. The LLOQ was determined as the concentrations with a signal-to-noise ratio of 10. For precision and accuracy studies, QC samples were prepared in six replicates and tested on the same day and three consecutive days, respectively. The extraction recovery was calculated by comparing the peak area ratios of the analytes in regularly pretreated QC samples with those in post-extracted plasma samples. The matrix effect was assessed via comparison of the peak area ratios in the post-extracted samples to those in neat solution at the same concentration. The stability of the analytes at long-term storage (30 days at − 80 °C), short-term storage (at room temperature for 24 h), and three freeze-thaw cycles (− 20 °C to room temperature as a cycle) was investigated by analyzing six replicates of the QC samples.

Animal model

A total of 96 specific pathogen-free Sprague-Dawley rats, male, and weighing 220–240 g were purchased from the Animal Laboratory Center, Guangzhou University of Chinese Medicine. All rats were housed under standard conditions of temperature, humidity, and light, and were provided with a standard diet and water. The experiments were approved by the Animal Ethics Committee of Guangzhou University of Chinese Medicine (May 16th, 2017, No. S20170516002), and were conducted in compliance with the Guidelines for Animal Experimentation of the university. All rats were acclimatized for 1 week before the experiment and then randomly divided into two groups (48 rats per group): ATM-treated and conventional groups. The ATM-treated group rats were orally administered with a nonabsorbable antibacterial mixture consisting of streptomycin (100 mg/kg/day) and neomycin (100 mg/kg/day) for 7 consecutive days, while the conventional group rats received the same volume of 0.9% normal saline [23, 24]. The feces samples were collected separately on the 7th day and stored at − 80 °C.

Assay of β-glucosidase activity

The assay was performed according to a previously reported method [25]. Briefly, approximately 0.2 g of feces were suspended in 3.8 mL of precooled sterile phosphate-buffered saline (4 °C). After centrifugation at 2 × *g* for 5 min (at 4 °C), 0.2 mL of the supernatant was transferred to another Eppendorf tube, and then 0.4 mL of 2 mM p-nitrophenyl-β-D-glucopyranoside and 0.4 mL of 0.1 M phosphate-butter were added. The reaction mixture was incubated for 20 min at 37 °C, followed by the addition of 200 µL of 0.5 N NaOH to stop the reaction. Subsequently, the mixture was centrifuged at 6950 × *g* for 5 min, and the supernatant was applied to measure the enzyme activity using an enzyme immunoassay analyzer at 405 nm.

Pharmacokinetic studies

Once the pseudo-germ-free rat model was successfully established, the ATM-treated rats were randomly assigned into eight subgroups (6 rats per group): ARH, ARL, APH, APL, AMH, AML, AEH, and AEL groups. The conventional rats were randomly divided into eight subgroups as well (6 rats for per group): NRH, NRL, NPH, NPL, NMH, NML, NEH, and NEL groups. The daily adult dose of IRC is 9.0–30.0 g in the Chinese pharmacopoeia, which means the daily intake of IRC extract for a rat is 0.3–1.0 g accordingly. Therefore, the two dosage levels of IRC extract in this study were determined as 2000 and 200 mg/kg, which were equivalent to 226 and 23 mg/kg of RA, and 244 and 24 mg/kg of PDC, respectively. Moreover, to compare the pharmacokinetics of RA and PDC, the dosages of PDC monomer were adjusted to 301 and 30 mg/kg, with an equivalent molar concentration to RA. The dosages of the different groups were as follows: AEH and NEH groups: administered IRC extract at 2000 mg/kg; AEL and NEL groups: administered IRC extract at 200 mg/kg; ARH and NRH groups: administered the RA monomer at 226 mg/kg (462 µmol/kg); ARL and NRL groups: administered the RA monomer at 23 mg/kg (46 µmol/kg); APH and NPH groups: administered the PDC monomer at 301 mg/kg (462 µmol/kg); APL and NPL groups: administered the PDC monomer at 30 mg/kg (46 µmol/kg); AMH and

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NMH groups: administered the monomer mixture at 470 mg/kg; AML and NML groups: administered the monomer mixture at 47 mg/kg.

The pharmacokinetic studies were conducted on the 8th day, and food was prohibited for 12 h before the experiment, but water was freely available. Heparinized blood samples (0.5 mL) were obtained from the suborbital venous plexus at 0, 0.083, 0.25, 0.5, 0.75, 1, 1.5, 2, 3, 4, 6, 8, 12, 24, 36, and 48 h, and immediately centrifuged at 1114 × *g* for 10 min to obtain the plasma. The supernatants were collected and frozen at − 80 °C until analysis. The major pharmacokinetic parameters were calculated using non-compartmental analysis with DAS 2.0 software and are presented as means ± standard deviation. Statistical comparisons for the pharmacokinetic parameters were determined by unpaired two-tailed Student's t-test using GraphPad Prism 5 software, with $p < 0.05$ considered significant.

Supporting information

Intraday and inter-day precisions, accuracy, extraction recovery, matrix effect, and stability of PDC and RA in rat plasma are available as Supporting Information.

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Conflict of Interest

The authors have declared no conflict of interest.

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