Guangzhou Institute of Traumatic Surgery[1]; Department of Orthopaedics[2], Guangzhou Red Cross Hospital, Jinan University; School of Pharmaceutical Sciences[3], Guangzhou Medical University, Guangzhou, China

Inhibition effects of eight anti-coronavirus drugs on glycosides metabolism and glycosidases in human gut microflora

Tianming Dai[1], Min Wang[2], Pengzhen Wang[1], Libing Dai[1], Renke Dai[3], Qingqi Meng[2,*]

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**Corresponding author: Qingqi Meng, Department of Orthopaedics, Guangzhou Red Cross Hospital, Jinan University, 396 Tongfu Zhong Road, Guangzhou 510220, China*
meng_qingqi@126.com

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The effects of eight oral anti-coronavirus drugs (lopinavir, ritonavir, chloroquine, darunavir, ribavirin, arbidol, favipiravir, oseltamivir) on the metabolism of four specific glycosides (polydatin, geniposide, quercitrin, glycyrrhizin) and on the activities of three major glycosidases (β-glucosidase, α-rhamnosidase, β-glucuronidase) from gut microflora were explored *in vitro* and determined by LC-MS/MS. The metabolism of polydatin, geniposide, quercitrin and glycyrrhizin was significantly inhibited by one or several anti-coronavirus drugs of 100 μM around 1 h and 4 h ($P<0.05$), among which darunavir could strongly reduce the production of genipin (70.6% reduction), quercitin (80.6% reduction) and glycyrrhetinic acid (37.9% reduction), which may cause a high risk of herb-drug interactions (HDI). Additionally, chloroquine reduced the production of genipin and quercitin by more than 75% ($P<0.05$), whereas arbidol had no significant influence on the metabolism of polydatin, quercitrin and glycyrrhizin ($P>0.05$) so that its risk may be lower. The inhibition of darunavir on β-glucosidase was relatively strong (IC_{50} = 193±23 μM), and the inhibition became weaker on β-glucuronidase and α-rhamnosidase (IC_{50}>500 μM). The consistency between gut microflora and glycosidase system indicated that the inhibition of darunavir on the activity of β-glucosidase and β-glucuronidase may be the main reason for affecting the metabolism of geniposide, glycyrrhizin and polydatin in gut microflora. However, for the inhibition of darunavir and chloroquine on the metabolism of quercetrin, there was no correlation between gut microflora and α-rhamnosidase system. Assessing the risk of HDI mediated by glycosidases in gut microflora may be conducive to the safety and efficacy of combining traditional herbal and Western medicine for the treatment of patients with Covid-19.

1. Introduction

Integrating the information in the treatment guidelines issued by the National Health Commission of P.R. China and the National Institutes of Health of the United States and the latest reports, oral anti-coronavirus drugs lopinavir, ritonavir, chloroquine, darunavir, ribavirin, arbidol, favipiravir and oseltamivir showed inhibition effects on the replication or invasion of SARS-CoV-2 *in vitro*. Most of these drugs are enzymes inhibitors (Cai et al. 2020; National Health Commission of China, 2020; National Institutes of Health of the United States, 2020; Vafaei et al. 2020). Clinically, the World Health Organization (WHO) and the U.S. Food and Drug Administration (FDA) state that, though there are several ongoing clinical trials of both western and traditional medicines, no medicines have been shown to prevent or cure COVID-19 (FDA, 2020; WHO, 2020).

In China, traditional medicines such as "AnGongNiuHuangWan", "LianHuaQingWenJiaoNang" and "HuoXiangZhengQiJiaoNang" are containing a large number of glycosides, including polydatin, geniposide, quercitrin and glycyrrhizin. These traditional medicines were recommended by the National Health Commission of China since they exhibited anti-coronavirus activity *in vitro* (Li et al. 2020), and immunomodulatory, antipyretic activities or relieved respiratory symptoms in patients with COVID-19 (National Health Commission of China, 2020; Teng et al. 2020). Combination of traditional Chinese and Western medicine in the treatment of COVID-19 has improved the cure rate and reduced the mortality rate in some hospitals, suggesting that the combination may be feasible for COVID-19 (Teng et al. 2020). But the combination may come with risks of herb-drug interaction (HDI). The report of treatment-related adverse effects of COVID-19 indicated that the adverse effects of the combination mainly occurred in the gastrointestinal tract leading to diarrhea, which did not appear or was milder when taking a single drug (Liu et al. 2020). So the efficacy and safety of the combination needed to be evaluated.

Research shows that HDI is related to gut microflora (Li et al. 2009). About 70% of the oral drugs are metabolized or activated by gut microflora after encountering commensal microorganisms in the small and large intestine (Zimmermann et al. 2019). Generally, the bioavailability of glycosides in traditional Chinese medicine is low and glycosides are stable in hepatic enzymes (Li et al. 2008). However, glycosides could be hydrolyzed by glycosidase from gut probiotic microflora to produce more pharmacologically active and absorbable aglycones (Li et al. 2009). When traditional Chinese and Western medicines are used in combination, some oral Western medicines may reach a high concentration (>1 mM) in the gastrointestinal tract, which could change the composition and number of gut microbiota (such as antibiotics), thereby affecting the metabolism and activation of traditional Chinese medicine, even leading to the accumulation of toxic products and induce adverse effects (Maier et al. 2018). Essentially, metabolism changes are due to enzyme changes in gut microflora. If Western medicines will directly change the activity of enzymes in gut microflora, this may lead to rapid alteration of metabolism and a high risk of HDI. However, the role of most Western medicines on the metabolism enzymes in gut microflora remains unknown.

Therefore, the effects of eight oral anti-coronavirus drugs (lopinavir, ritonavir, chloroquine, darunavir, ribavirin, arbidol, favipiravir, oseltamivir) on the metabolism of four glycosides (polydatin, geniposide, quercitrin, glycyrrhizin) and on the activities of three major glycosidases (β-glucosidase, α-rhamnosidase,

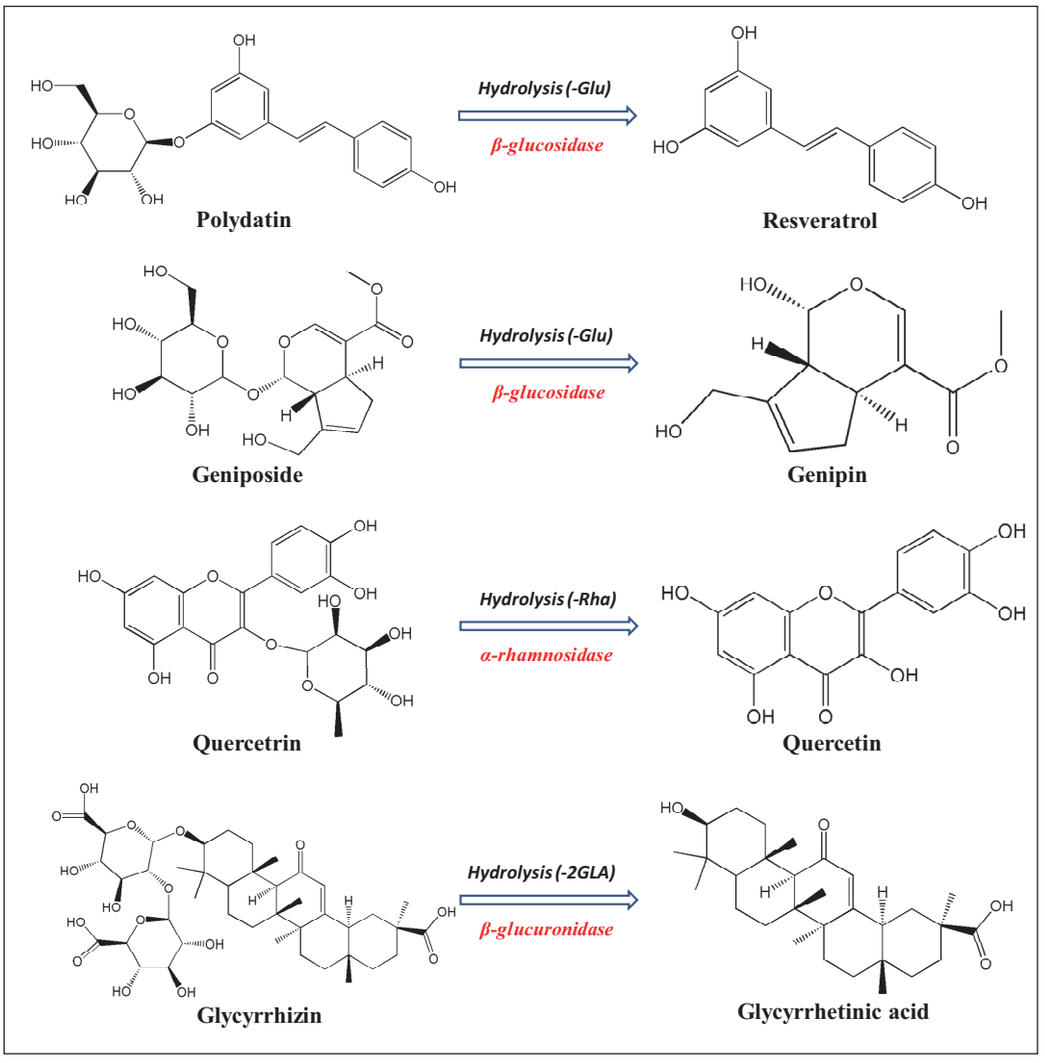

Fig. 1: Metabolic reactions of four specific substrates, their enzymes and corresponding metabolites. (Glu, glucose; Rha, rhamnose; GLA, glucuronic acid)

β-glucuronidase) from gut microflora will be explored in vitro metabolic system and determined by LC-MS/MS (Fig. 1). It is the first time to evaluate the risk of HDI mediated by glycosidases in gut microflora, which may be conducive to the safety and efficacy of integrating traditional Chinese and Western medicine.

2. Investigations and results

2.1. Establishment of analysis method

The developed method was successfully applied to simultaneously detect the four glycosides and four aglycones in human gut microflora or enzyme buffer. The standard curve and quality control (QC) in each analytical batch were within the acceptance criteria. Selectivity is shown in Fig. 2. No interference was found. The calibration curve of four glycosides and four aglycones was linear giving a correlation coefficient (r^2) >0.99.

2.2. In vitro inhibitory effects of anti-coronavirus drugs on glycosides metabolism in human gut microflora

The concentration of aglycones in the negative control group was taken as 100% to normalize the concentration of aglycones in the anti-coronavirus drugs groups. Since polydatin, geniposide and quercitrin were rapidly metabolized in human gut microflora, metabolism samples taken at 1 h and 4 h were used to evaluate the inhibitory effects on them. Glycyrrhizin was eliminated relatively slowly, thus metabolism samples at 4 h and 10 h were used.

The results in Fig. 3A show that resveratrol, the metabolite of polydatin, was significantly reduced at 1 h in the presence of ribavirin, lopinavir and castanospermine (P<0.05), whereas there was no decline with darunavir. Similarly, genipin was significantly decreased at 1 h under the inhibition of eight anti-coronavirus drugs (P<0.05), especially 100 μM chloroquine reduced the production of genipin by more than 80%. Significant reductions were also observed in quercetin at 1 h except for the arbidol and ribavirin group, while chloroquine and darunavir could reduce quercetin production by more than 70% (P<0.05). For glycyrrhetinic acid at 4 h, the metabolism was slightly activated by ribavirin, and it was significantly inhibited by darunavir (37.9% reduction) and the positive inhibitor amoxapine (P<0.05).

The results in Fig. 3B showed that a few incubation hours later, the inhibitory effects of anti-coronavirus drugs on the production of genipin, quercetin and glycyrrhetinic acid were still sustained. However, there was no significant difference between control and treatments for resveratrol at 4 h, which suggested that the inhibition was reversible and the metabolism could gradually return to normal.

Comparing different anti-coronavirus drugs, it was found that darunavir showed a relatively strong inhibition on the metabolism of the three substrates (geniposide, quercitrin and glycyrrhizin), which may have a higher risk of HDI, affecting the activation and

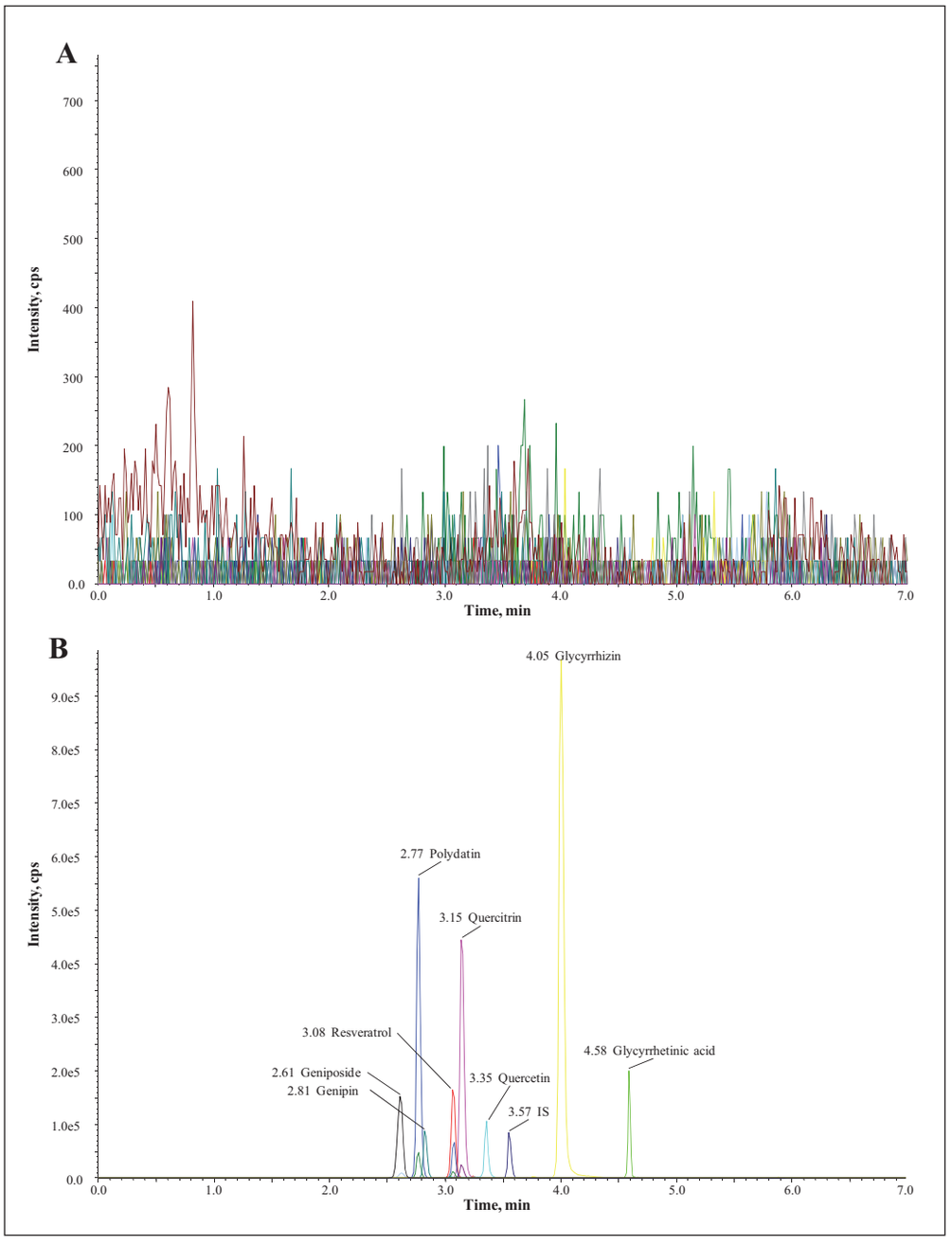

Fig. 2: The total ion chromatography (TIC) of four glycosides, four aglycones and IS apigenin in MRM mode for the blank human gut microflora solution (A) and the metabolism sample at 1 hour in gut microflora (B).

efficacy of glycosidic traditional Chinese medicines. Chloroquine also displayed strong inhibition on the metabolism of geniposide and quercitrin. Whereas arbidol had no significant influence on the metabolism of polydatin, quercitrin and glycyrrhizin (P>0.05) so its risk may be lower. Therefore, whether darunavir or chloroquine can inhibit the metabolism by glycosidase inhibition was explored in the next step.

2.3. In vitro enzyme activity inhibition of bacterial β-glucosidase, α-rhamnosidase and β-glucuronidase by darunavir or chloroquine

Under the same concentration of 100 μM substrates, similar concentration range of darunavir and similar metabolic rate as the gut microflora experiment, the enzyme activity inhibition assay was performed. The concentration of aglycones at 0 μM darunavir or chloroquine was taken as 100% to normalize the concentration of aglycones in the other groups. The relative concentration of aglycones represented the relative activity of glycosidase.

Fig. 4A shows that the inhibition was more significant in the genipin group (P<0.05) than in the resveratrol group for darunavir, though both resveratrol and genipin are markers for β-glucosidase activity. The production of quercitin indicated that darunavir had no inhibition effect on α-rhamnosidase. And β-glucuronidase was only weakly inhibited by darunavir. In Fig. 4B, chloroquine showed a weak inhibitory effect on β-glucosidase and no inhibition on α-rhamnosidase.

Since β-glucosidase was strongly inhibited by darunavir in geniposide metabolism, its IC_{50} value was determined to be 193±23 μM. The inhibition curve is presented in Fig. 5. The IC_{50} values of darunavir on α-rhamnosidase and β-glucuronidase were greater than 500 μM.

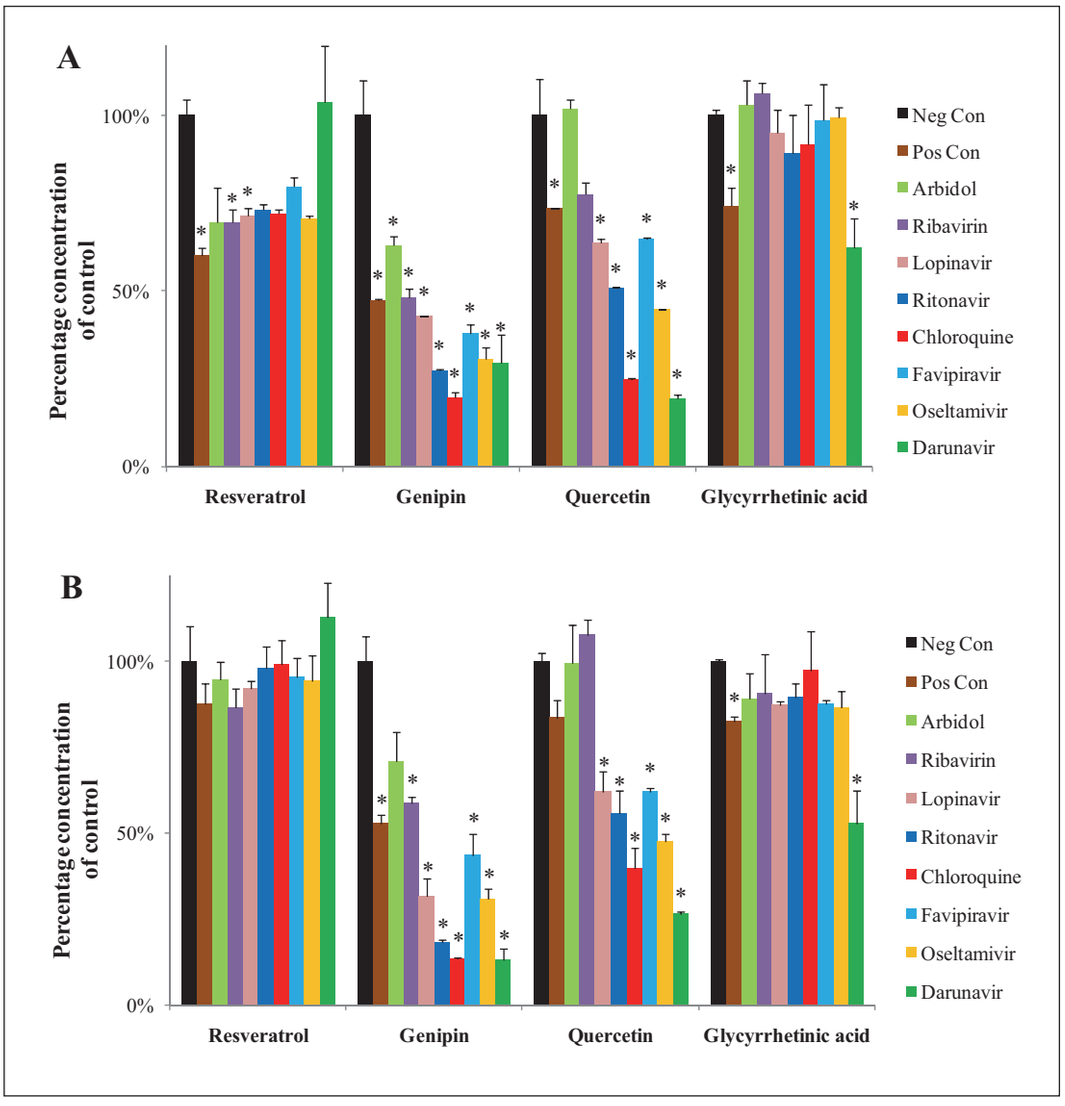

Fig. 3: Effects of eight anti-coronavirus drugs (100 μM) on the metabolism of polydatin, geniposide, quercitrin and glycyrrhizin in human gut microflora *in vitro*. (A) metabolites of polydatin, geniposide, quercitrin at 1 h and glycyrrhizin at 4 h. (B) metabolites of polydatin, geniposide, quercitrin at 4 h and glycyrrhizin at 10 h. (n=3) * $P<0.05$, compared to negative control.

3. Discussion

This method has successfully determined the eight compounds simultaneously and applied to the metabolism study of four glycosides in human gut microflora. The four "specific substrates" were selected as markers depending on their specific glycosyl groups. Both the gut microflora system and enzyme system were used to explore the inhibition of anti-coronavirus drugs on glycoside metabolism and glycosidases under similar conditions, such as the same substrate concentration of 100 μM, the same inhibitor concentration range from 50 μM to 500 μM, and similar metabolic rate (which means similar enzyme concentration) (Zimmermann et al. 2019).

Clinically, the oral dose of darunavir is 600 mg/tablet, and its estimated intestine concentration is about 330 μM (Arab-Alameddine et al. 2014; Maier et al. 2018). In human gut microflora, 100 μM darunavir could significantly inhibit the metabolism of geniposide, glycyrrhizin and polydatin which may induce inflammation or damage if the glycosides accumulate in the intestine, suggesting that oral high-dose darunavir may bring high risk of HDI (Xu and Li 2012; Wang et al. 2013). After further investigating the enzyme activity, it was observed that the inhibition effects of darunavir on the three glycosides were successively weakened (geniposide > glycyrrhizin > polydatin), which showed consistency between the gut microflora system and the enzyme system, indicating that the change in β-glucosidase and β-glucuronidase activity may be the main factor affecting the metabolism of the three glycosides. It should be noted that, although both geniposide and polydatin are substrates of β-glucosidase, they reflected different levels of inhibition on β-glucosidase by darunavir. Probably due to the different metabolic binding sites of geniposide and polydatin on β-glucosidase, darunavir mainly blocked the combination of geniposide and β-glucosidase resulting in stronger inhibition (Dopitova et al. 2008).

However, darunavir showed huge differences on the inhibition of quercitrin metabolism between the gut microflora system and the enzyme system. The inhibition was strong in the gut microflora system while almost no inhibition on α-rhamnosidase activity. It has been tested that darunavir has antibacterial and antifungal activities in the digestive tract, suggesting that darunavir may destroy certain microorganisms containing α-rhamnosidase and indirectly reduce the amount of α-rhamnosidase (Brilhante et al. 2020).

Interestingly, although it is reported that chloroquine did not show inhibitory effects on the growth of 40 major human gut bacterial strains at the estimated intestine concentration of 96 μM, it was observed that 100 μM chloroquine strongly inhibited the metabo-

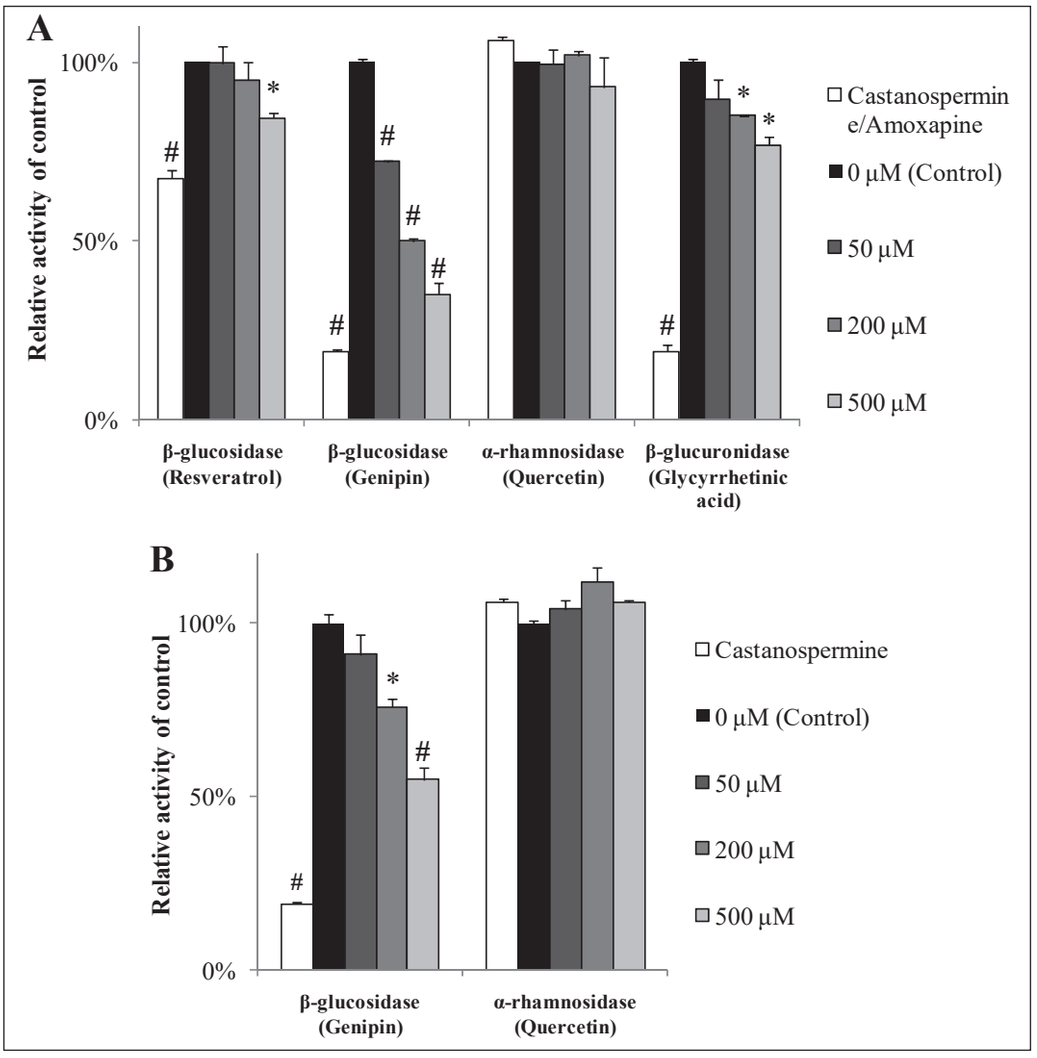

Fig. 4: Inhibition effects of different concentration of (A) darunavir on the activity of bacterial β-glucosidase, α-rhamnosidase and β-glucuronidase *in vitro*, and (B) chloroquine on the activity of bacterial β-glucosidase and α-rhamnosidase. (n=3) * P<0.05, # P<0.01, compared to 0 μM (Control).

lism of geniposide and quercetrin in human gut microflora, which may result in high risk of diarrhea or hepatotoxicity if geniposide or quercetrin accumulates in the intestine (Maier et al. 2018; Xu and Li 2012; Wang et al. 2013). After investigation on enzymes activity, chloroquine did not show significant inhibition on the activities of β-glucosidase or α-rhamnosidase (IC_{50}>500 μM).

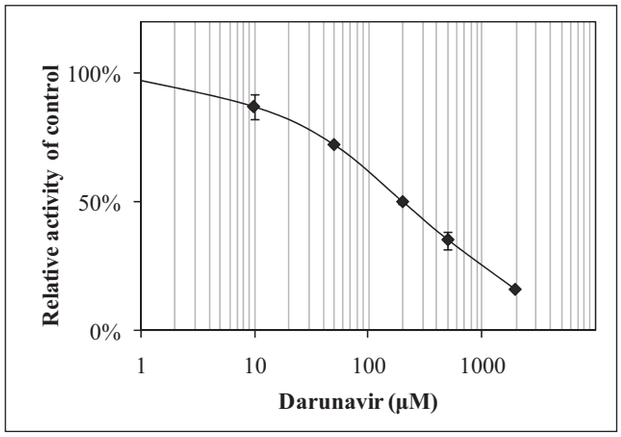

Fig. 5: Inhibition curve of darunavir on the activity of β-glucosidase in geniposide metabolism (n=3).

Since β-glucosidase is a secreted extracellular enzyme, α-rhamnosidase is a membrane-bound enzyme, and chloroquine could influence the membrane permeability in yeast cells, we hypothesized that chloroquine may obstruct the contact between substrate and enzyme by affecting the enzyme's extracellular secretion pathway or drug transporters (Kotal et al. 1988; Nunoura et al. 2014; Reisky et al. 2019). Besides, the impact of chloroquine on the expression of bacterial glycosidases will be studied in the further protein quantitative analysis in probiotic bacterial strains.

Among the eight drugs, arbidol displayed the weakest influence on the metabolism of glycosides, which was likely to associate with the lower incidence of adverse reaction for combined use of arbidol than chloroquine or lopinavir/ritonavir in clinical (Chen et al. 2020; Liu et al. 2020; Xue et al. 2020). Considering that the other seven anti-coronavirus drugs could significantly change the metabolism of glycosides in gut microflora, it may come with a high risk of HDI for the patients with COVID-19 who already has susceptible gut microbiota. Hence, arbidol may be more suitable for combining with traditional Chinese medicines in patients, which could reduce the impact on the efficacy and toxicity.

Moreover, it was found that the inhibition effect of anti-coronavirus drugs on the metabolism of traditional Chinese medicine was serious at 1 h and 4 h after incubation. It will be further explored whether proper extension of the interval time between the administration of Chinese medicine and Western medicine is beneficial to reduce the risk of HDI.

In conclusion, our research indicated that eight anti-coronavirus drugs of 100 μM had a rapid and varying influence on the metabolism of three different kinds of glycosides respectively, in particular, significantly inhibited the deglycosylation of compounds containing glucoside and rhamnoside *in vitro* human gut microflora. Glycosidase inhibition experiments revealed that the inhibition of darunavir on the activity of β-glucosidase and β-glucuronidase was the main reason for inhibiting the metabolism of geniposide, glycyrrhizin and polydatin in gut microflora.
Nowadays the Covid-19 epidemic is rebounding around the world. Regarding darunavir (with high HDI risk), when patients with Covid-19 use traditional herbal and anti-coronavirus medicines in combination, it is necessary to adjust the interval time to avoid alteration of efficacy and toxicity, so as to prevent/mitigate adverse effects. Another way may be choosing alternative drugs with the same indications and less impact on gut microflora, like arbidol.

4. Experimental

4.1. Chemicals and reagents

<exp>The standards (purity>99%) of glycosides (polydatin, geniposide, quercitrin, glycyrrhizin), aglycones (resveratrol, genipin, quercetin, glycyrrhetinic acid) and apigenin (internal standard, IS) were obtained from Chengdu Pufei De Biotech Co., Ltd (Chengdu, China). Lopinavir, ritonavir, chloroquine, oseltamivir, darunavir, ribavirin, favipiravir, arbidol, castanospermine (inhibitor of β-glucosidase) and amoxapine (inhibitor of β-glucuronidase) were purchased from Aladdin Biochemical Technology Co., Ltd (Shanghai, China) and their purity was more than 99% (Ahmad et al. 2012; Taylor et al. 1994). β-Glucosidase (EC No. 3.2.1.21, from *Agrobacterium* sp.) and α-rhamnosidase (EC No. 3.2.1.40, from prokaryote) were bought from Megazyme International Ireland Ltd (Ireland). β-Glucuronidase (EC No. 3.2.1.31, from *Escherichia coli*) was purchased from Sigma-Aldrich (St. Louis, MO, USA). Brain Heart Infusion (BHI) Broth was purchased from Qingdao Hope Bio-technology Co., Ltd. HPLC grade methanol and acetonitrile were obtained from Merck (Darmstadt, Germany). The other reagents were of analytical grade. The distilled water was purified by a Milli Q water purification system from Millipore Corporation (MA, USA).

4.2. Instrumentation and LC–MS/MS method

The LC–MS/MS analysis was performed using a Shiseido Nanospace 1312 HPLC system coupled with an AB Sciex 4000 Q Trap™. Data acquisition and quantification were conducted using Analyst 1.6 (AB Sciex, USA).
The four glycosides and four aglycones were simultaneously analyzed using a Welch XB-C_{18} column (2.1×50 mm, 3.0 μm, Lot number: 1101.23) at ambient temperature with a flow rate of 0.4 mL/min. The mobile phase A was composed of methanol and water at ratio of 5:95, v/v, and phase B, methanol and water (95:5, v/v). Both phases contained 0.1% formic acid. In the LC gradient profile, the mobile phase B was 10% (v/v) for 0.3 min and linearly increased to 100% from 0.3 to 3.0 min, it was held at 100% from 3.0 to 4.5 min, and it was then brought back to 10% at 4.6 min. The entire running time was 7.0 min. The retention times of polydatin, resveratrol, geniposide, genipin, quercitrin, quercetin, glycyrrhizin, glycyrrhetinic acid, and apigenin (IS) are shown in the Table.
MS/MS conditions were optimized as follows: source temperature, 550 °C; ion spray voltage, -4500 V; curtain gas, 30 psi; nebulizing gas, 55 psi; turbo ion spray gas, 50 psi; and collision gas, medium. The ion pairs, declustering potential (DP) and collision energy (CE) for the glycosides and aglycones in negative multiple reaction monitoring (MRM) are presented in the Table.

Table: Ion pairs, declustering potential (DP), collision energy (CE) and retention times for each analyte

Analyte	Ion pair (m/z)	Declustering potential (V)	Collision energy (eV)	Retention time (min)
Polydatin	389.0→227.0	-100	-20	2.77
Resveratrol	227.0→143.0	-110	-25	3.08
Geniposide	387.0→225.0	-60	-14	2.61
Genipin	225.0→123.0	-60	-20	2.81
Quercitrin	447.0→301.0	-100	-20	3.15
Quercetin	301.0→151.0	-100	-30	3.35
Glycyrrhizin	821.4→351.0	-110	-65	4.05
Glycyrrhetinic acid	469.4→355.0	-100	-50	4.58
Apigenin (IS)	269.0→117.0	-100	-25	3.57

4.3. Preparation of calibration samples

Stock solutions of the glycosides, aglycones, anti-coronavirus drugs and IS were prepared using dimethyl sulfoxide (DMSO). The working solutions containing eight compounds were serially diluted in methanol to obtain concentrations from 1 μM to 1,000 μM for glycosides and from 0.1 μM to 100 μM for aglycones. Standard curve and quality control (QC) samples in gut microflora solution or enzyme buffer were prepared with blank gut microflora solution or enzyme buffer, respectively. The final concentrations were 0.1, 0.2, 0.4, 2, 8, 40, 80 and 100 μM for glycosides and 0.01, 0.02, 0.04, 0.2, 0.8, 4, 8 and 10 μM for aglycones after mixing with the blank matrices. QC samples were at the concentrations of 0.2, 2 and 80 μM for glycosides and 0.02, 0.2 and 8 μM for aglycones. All working solutions, stock solutions, standard curve and QC samples were stored at 4 °C.

4.4. Sample preparation

An aliquot of gut microflora solution or enzyme buffer (50 μL), with 5 μL methanol or working solutions, were added to a 2 mL centrifuge tube and vortex-mixed. An amount of 450 μL ice-cool acetonitrile with IS (1 μg/mL) was immediately added to the mixture and vortex. The mixture was ultrasonicated for 1 min. After centrifugation at 14,000 g for 30 min at 4°C, 5 μL supernatant of each sample was injected into the LC-MS/MS system for analysis.

4.5. Preparation of human gut microflora solution

Fresh human fecal samples (2 g in total) were obtained from six healthy Chinese volunteers (29-37 years, three males and three females) from Guangzhou Red Cross Hospital (Guangzhou, China) according to a protocol approved by the Ethics Committee of Guangzhou Red Cross Hospital (approval number 2020-176-01). They were pooled and mixed with 20 mL sterile phosphate buffer (0.1 M, pH 7.4) in a sterile centrifuge tube. The mixture was centrifuged at 200×g and 4 °C for 10 min, and then the resulting supernatant was collected and centrifuged at 5000×g at 4 °C for 10 min. The obtained precipitate was resuspended in 40 mL of sterile Brain Heart Infusion (BHI) Broth (pH 7.4) to produce the human gut microflora solution (Lee et al. 2009).

4.6. In vitro inhibitory effects of anti-coronavirus drugs on glycosides metabolism in human gut microflora

To evaluate the inhibitory effects of anti-coronavirus drugs on glycosides metabolism, four glycosides with specific monosaccharide were used to indicate the influence on different enzymes (Fig. 1). The stock solutions of polydatin, geniposide, quercitrin and glycyrrhizin were mixed equally to produce the substrate solution (10 mM). A final concentration of 100 μM for the eight anti-coronavirus drugs or positive control (castanospermine or amoxapine) were mixed with the above human gut microflora solution respectively, and then pre-incubated at 37 °C for 5 min. DMSO was also mixed with the human gut microflora solution and used as negative control. The substrate solution was added into the pre-incubation solution to initiate the reaction (final concentration 100 μM for each glycoside). The reaction was anaerobically incubated at 37 °C in triplicate. At incubating time 0, 1, 4 and 10 h, 50 μL of the incubation solution were respectively transferred into 450 μL ice-cool acetonitrile (with IS) to stop the reaction. The resulting solutions were prepared according to the section '4.4 sample preparation' to quantified glycosides and their aglycones by LC-MS/MS. The effect of anti-coronavirus drugs on glycosides metabolism was assessed by comparing with the negative control using their aglycones as markers.

4.7. In vitro enzyme activity inhibition assay of bacterial β-glucosidase, α-rhamnosidase and β-glucuronidase by darunavir

Since darunavir showed significantly inhibition on geniposide, quercitrin and glycyrrhizin metabolism in human gut microflora, darunavir was selected for further enzyme activity inhibition assay. Polydatin and geniposide was used as probe substrates in β-glucosidase assay, while quercitrin was used in α-rhamnosidase assay, and glycyrrhizin was used in β-glucuronidase assay, respectively. Initial linear reaction time was investigated by metabolic stability experiments for the 4 probe substrates (Fig. 6). Each enzyme activity inhibition assay was conducted in the presence of 100 μM probe substrate, corresponding enzyme and positive inhibitor/darunavir in phosphate buffer (0.1 M, pH 7.4) anaerobically incubated at 37 °C in triplicate. The final concentrations of darunavir were 0 μM (control), 50 μM, 200 μM and 500 μM. The positive inhibitors were 100 μM castanospermine or 100 μM amoxapine. The enzyme concentrations were set to 12 μg/mL for β-glucosidase, α-rhamnosidase and β-glucuronidase, based

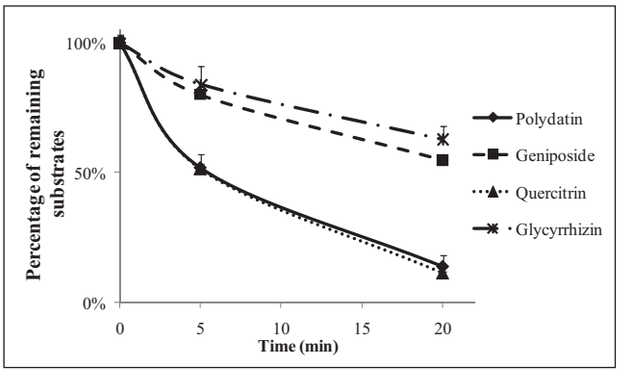

Fig. 6: Metabolic stability of 100 μM different glycosides in different glycosidase systems. (n=3)

on the depletion rate of substrates. In each assay, the probe substrate and inhibitor were mixed and pre-incubated for 5 min, and then the enzyme was added to start the reaction. After reaction for 20 min, 50 μL of the incubation solution were transferred into 450 μL ice-cool acetonitrile (with IS) to stop the reaction. The resulting solutions were prepared according to the section '4.4 sample preparation' to quantified glycosides and their aglycones by LC-MS/MS. Metabolites resveratrol and genipin were chosen as markers for β-glucosidase activity. Quercetin was chosen as markers for α-rhamnosidase activity. Glycyrrhetinic acid was chosen as markers for β-glucuronidase activity. The remaining activity after inhibition was assessed by comparing with the 0 μM (control) which served as 100%.

For the enzymes that strongly inhibited by darunavir, IC_{50} value (concentration of inhibitor to cause 50% inhibition of original enzymes activity) was determined. The serial concentrations of darunavir were 0 μM (control), 10 μM, 50 μM, 200 μM, 500 μM and 2000 μM in triplicate. Other reaction conditions were the same as mentioned above. The IC_{50} value was calculated by SPSS Statistics 22.0 (Chicago, USA) with Probit-Logit regression analysis.

4.8. Statistical analysis

Statistical analyses were performed with SPSS Statistics 22.0 (Chicago, USA). Differences of the remaining activity of β-glucosidase, α-rhamnosidase or β-glucuronidase between inhibition groups and negative control were compared using two-tailed t-test, respectively. *P* value of less than 0.05 was considered statistically significant.

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Conflicts of interest: none declared.

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