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ORIGINAL ARTICLE

Probe substrates assay estimates the effect of polyphyllin H on the activity of cytochrome P450 enzymes in human liver microsomes

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Abstract

Cytochrome P450 enzymes (CYPs) play a crucial role in phase I metabolic reactions. The activity of CYPs would affect therapeutic efficacy and may even induce toxicity. Given the complex components of traditional Chinese medicine, it is important to understand the effect of active ingredients on CYPs activity to guide their prescription. This study aimed to evaluate the effect of polyphyllin H on the activity of CYPs major isoforms providing a reference for the clinical prescription of polyphyllin H and its source herbs. The effects of polyphyllin H were evaluated in pooled human liver microsomes using probe substrates of CYP1A2, 2A6, 2C8, 2C9, 2C19, 2D6, 2E1, and 3A4 to determine their activities. The Lineweaver-Burk was used to model the inhibition, and a time-dependent inhibition experiment was performed to understand the characteristics of the inhibition. Polyphyllin H significantly suppressed the activity of CYP1A2, 2D6, and 3A4 with IC_{50} values of 6.44, 13.88, and 4.52 μM, respectively. The inhibition of CYP1A2 and 2D6 was best fitted with a competitive model, yielding the inhibition constant (K_i) values of 3.18 and 6.77 μM, respectively. The inhibition of CYP3A4 was fitted with the non-competitive model with the K_i value of 2.38 μM. Moreover, the inhibition of CYP3A4 was revealed to be time-dependent with the inhibition parameters inhibition constant (*KI*) and inactivation rate constant (K_{inact}) values of 2.26 μM^{-1} and 0.045 min^{-1}. Polyphyllin H acted as a competitive inhibitor of CYP1A2 and 2D6 and a non-competitive and time-dependent inhibitor of CYP3A4.

KEYWORDS
CYP450, drug–drug interaction, IC_{50}, *Rhizoma paridis* (Liliaceae), time-dependent inhibition

Abbreviations: CYPs, Cytochrome P450 enzymes; HLMs, human liver microsomes.

Erhao Wang and Mengxi Wang contributed equally to this work.

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1 | INTRODUCTION

Rhizoma paridis (Liliaceae) is common in the tropical and temperate regions of Eurasia, particularly in Southwest China. Known for its pharmacological activities of antitumor, antibacterial, hemostasis, and expelling insects, *R. paridis* has been medicated for a long history. Its pharmacological action is often attributed to active extractions like saponins, which are critical ingredients of *R. paridis*. Studies showed that *R. paridis* saponins can inhibit tumor metastasis, alleviate oxidative stress injury, and ameliorate hepatic fibrosis.[1–4] However, these saponins are also linked to the hepatic toxicity of *R. paridis*.[5] Polyphyllin H has been identified as a major steroidal saponin of *R. paridis* extracts.[6] In a former study focusing on polyphyllin H pharmacokinetics, it was reported that the maximum plasma concentration of polyphyllin H in beagle dogs was 20.72 ± 2.22 ng/mL after oral administration of 9.33 mg/kg *R. paridis* extracts, and polyphyllin H was identified as the most abundant component.[7] Polyphyllin H has been extensively researched for its pharmacokinetics, tissue distribution, and primary therapeutic effects.[8,9] However, its hepatic toxicity and potential risk when combined with other compounds are still unknown.

Cytochrome P450 (CYP), also known as mixed functional oxidase and monooxygenase, is a critical phase-I enzyme in biotransformation. It is responsible for metabolizing approximately 3/4 of phase I-dependent drugs.[10,11] CYPs are involved in the oxidation, demethylation, and other modifications of drug structures, enhancing their solubility and speeding up their excretion.[12–14] Liver CYPs contain major subfamilies, including CYP1A, CYP2A, CYP3A, CYP2C, CYP2D, and CYP2E.[15] These different CYP isoforms from various subfamilies show distinct characteristics and metabolize different xenobiotics. The inhibition or induction of CYP is a primary mechanism mediating drug–drug interaction.[16] Multiple drugs can be metabolized by the same CYP isoform, and a single drug can also influence the activity of multiple CYP isoforms.[17] Consequently, drug–drug interaction occurs when two or more drugs are co-administrated. Traditional Chinese medicine prescriptions usually contain more than two types of herbs, and the combination of traditional Chinese medicine and drugs is widely used in clinics. Previous studies have reported interactions between herbal active ingredients and co-administrated drugs due to the changed activity of CYPs. For example, the inhibitory effect of Shaoyao-Gancao-Fuzi decoction on CYP3A increased the systemic exposure of tofacitinib in rats with glycyrrhetinic acid, glycyrrhizic acid, and liquidity playing critical roles.[18] As the primary active ingredient of *R. paridis*, consideration should be given to the effect of polyphyllin H on the activity of CYPs for its safe clinical co-prescription and use as health products.

Liver microsomes are commonly used as experimental carriers in investigations of CYPs, due to their easy preparation, reproducibility, and short experimental period. Another significant advantage is their ability to assess the activity of multiple CYP isoforms simultaneously through a cocktail assay.[19] CYPs activities are evaluated using the probe reaction method, which measures the production of metabolites. This study explored the effect of polyphyllin H on the activity of major CYP isoforms in human liver microsomes and corresponding probe reactions (Table 1). The study also examined inhibition characteristics to reveal the potential mechanism underlying the effect of polyphyllin H.

2 | METHODS

2.1 | Study design

The probe reactions were performed in pooled gender-neutral human liver microsomes (HLMs, 22 donors, lot #4133007) obtained from BD Bioscience (USA). The concentrations of probe substrates and HLMs (added by protein concentration) were selected according to previous studies.[20,21] Three treatments were set: negative control (without inhibitors), positive control (with typical inhibitors of CYP isoforms, summarized in Table 1), and polyphyllin H (with 100 μM polyphyllin H). Key protein targets and ligands in this article are hyperlinked to corresponding entries in https://www.guidetopharmacology.org, the common portal for data from the IUPHAR/BPS Guide to PHARMACOLOGY,[22] and are permanently archived in the Concise Guide to PHARMACOLOGY 2019/20.[23]

2.2 | HLMs assay

The reaction systems were composed of 100 mM potassium phosphate buffer (pH = 7.4), NADPH generating system, HLMs, probe substrates, and polyphyllin H (100 μM) or typical inhibitors and the reaction volume was 200 μL. Probe substrates, inhibitors (except for dextromethorphan and quinidine were dissolved in water), and polyphyllin H were dissolved in methanol with a final concentration of 1%, and 1% neat methanol was added to the incubations without inhibitor (to exclude the effect of methanol on CYP450 activities). The relatively high concentration of polyphyllin H was primarily used to screen CYP450 isoforms responding to polyphyllin H according to previous studies.[20,24,25] The NADPH-generating system was prepared as follows: 5 mM $MgCl_2$ (Sigma, USA), 1 mM $NADP^+$ (Sigma, USA), 5 mM G-6-P (Sigma, USA), and 4 U/mL glucose-6-phosphate dehydrogenase (Sigma, USA). The reaction was initiated by the NADPH-generating system and conducted at 37 °C for 1 h after a 5-min preincubation. Ice-cold acetonitrile was added to terminate the reaction and the mixture was centrifugated at 13,000 rpm for 15 min after a 5-min ultrasonic mixing. Further incubation with 0, 5, 10, 15, 25, 50, and 100 μM polyphyllin H was performed with the substrates of the isoforms significantly inhibited by polyphyllin H with the same conditions as above to obtain the IC_{50} values assessing the inhibition strength. The IC50 values were calculated with the non-linear regression.

The time-dependent characteristics of CYP inhibition were evaluated with 20 μM polyphyllin H and 1 mg/mL HLMs (protein

TABLE 1 Reaction conditions of CYP isoforms tested, including maker reactions, incubation conditions, K_m, and inhibitors used in the Cocktail assay.

CYPs	Probe substrates	Substrate concentration (μM)	Marker reactions	Protein concentration (mg/mL)	Incubation time (min)	Estimated K_m (μM)	Positive control Inhibitors	Positive control Inhibitor concentration (μM)	References
1A2	Phenacetin	40	O-deethylation	0.2	30	48	Furafylline (Sigma, USA)	10	[12]
2A6	Coumarin (ICN Biomedicals, USA)	1.0	7-hydroxylation	0.1	10	1.5	Tranylcypromine (Sigma, USA)	10	[12]
2C8	Paclitaxel (Sigma, USA)	10	6α-hydroxylation	0.5	30	16	Montelukast (Beijing Aleznova Pharmaceutical, China)	5	[12]
2C9	Diclofenac (ICN Biomedicals, USA)	10	4′-hydroxylation	0.3	10	13	Sulphaphenazole (Sigma, USA)	10	[12]
2C19	S-mephenytoin (Sigma, USA)	100	4-hydroxylation	0.2	40	105	Tranylcypromine (Sigma, USA)	50	[12]
2D6	Dextromethorphan (ICN Biomedicals, USA)	25	O-demethylation	0.25	20	4.8	Quinidine (Sigma, USA)	10	[12]
2E1	Chlorzoxazone (Sigma, USA)	120	6-hydroxylation	0.4	30	126	Clomethiazole (Sigma, USA)	50	[12]
3A4	Testosterone (National Institute for the Control of Pharmaceutical and Biological Products, China)	50	6β-hydroxylation	0.5	10	53	Ketoconazole (ICN Biomedicals, USA)	1	[12]

CYP, cytochrome P450; K_m, the substrate concentration at half of the maximum velocity of the reaction (V_{max}).

concentration). The concentrations of probe substrates approximated to K_m are summarized in Table 1. The reaction system was prepared as described above and pre-incubated at 37°C for 30 min and then transferred to another tube for further incubation. The reaction was terminated after 0, 5, 10, 15, and 30 min by acetonitrile, and then centrifugated at 13,000 rpm for 15 min for HPLC analysis. Furthermore, the concentration of probe substrates was increased to approximately 4-fold K_m values and incubated with 0, 2, 5, 10, 20, and 50 μM polyphyllin H for 0, 5, 10, 15, and 30 min to obtain the time-dependent inhibition parameters inhibition constant (*KI*) and inactivation rate constant (K_{inact}) by linear fitting.

2.3 | Inhibition model fitting

The incubations with a series of concentrations of probe substrates were carried out to fit the inhibition model. The competitive inhibition was fitted with $v=(V_{max}\times S)/[K_m(1+I/K_i)+S]$, and the non-competitive inhibition model was fitted with $v=(V_{max}\times S)/[K_m+S(1+I/K_i)]$. Where V_{max} represents the maximum velocity of the reaction, K_m represents the substrate concentration at half of V_{max}, S represents the concentration of the substrate, I represents the concentration of polyphyllin H and K_i is the inhibition constant obtained from the nonlinear regression analysis. The values of V_{max} and K_m were calculated by the Lineweaver-Burk analysis.

2.4 | HPLC analysis

The HPLC analysis was conducted with the Agilent 1290 series. The supernatant of the reaction mixture was transferred to the C18 column with water: acetonitrile as the mobile phases. The temperature of the column was set as 30°C and the mobile rate was 0.2 mL/min. The gradient elution conditions were set as 10%–20% acetonitrile for 5 min, 20%–30% acetonitrile for 5 min, 30%–40% acetonitrile for 5 min, and 40% acetonitrile for 5 min. The total run time was 15 min per sample including a re-equilibration of 5 min. The quantitative detection method was constructed by our team based on previous studies.[26–28] The specific analysis conditions are summarized in Table 2. The inhibition ratio was calculated with the following equation:

$$I(\%) = \left[\left(C_{neg} - C_i\right) / \left(C_{neg}\right)\right] \times 100\%.$$

Where I represent the inhibition ratio, C_{neg} represents the production of metabolites in the negative control, C_i represents the production of metabolites in the positive control or polyphyllin H.

2.5 | Statistical analysis

Experimental data were analyzed by GraphPad Prism 9.0 software and expressed as mean ± SD ($n=3$). Difference comparison was performed with one-way ANOVA ($p<.05$).

TABLE 2 The detection conditions of HPLC for each CYP450 isoenzymes.

Isoenzymes	Internal reference	Mobile phase	Wavelength	References
CYP1A2	7-Hydroxycoumarin	Methanol: phosphate buffer (pH = 3.0, 50 mM) = 32:68	UV 245 nm	[21-23]
CYP2A6	-	Acetonitrile: acetic acid (0.1%, v/v) = 35:65	Fluo Ex/EM 340/456 nm	[21-23]
CYP3A4	Corticosterone	Methanol: water = 50:40, 0–15 min, 48% B-30% B; 15–22 min, 30% B-20% B	UV 254 nm	[21-23]
CYP2C8	-	Methanol: Water = 65:35	UV 230 nm	[21-23]
CYP2C9	Coumarin	Acetonitrile (A): phosphate buffer (pH = 7.4, 100 mM, B) = 32:68, 0–9 min, 68% B-32% B	UV 280 nm	[21-23]
CYP2C19	Tolbutamide	Methanol: potassium phosphate (pH 7.0, 10 mM) = 30:70	UV 204 nm	[21-23]
CYP2D6	-	Acetonitrile: phosphate buffer (pH = 3.0, 50 mM) = 25:75	Fluo Ex/EM 235/310 nm	[21-23]
CYP2E1	Phenacetin	Acetonitrile: acetic acid (0.5%, v/v) = 22:78, 1–10 min, 78% B-40% B	UV 287 nm	[21-23]

3 | RESULTS

3.1 | Effect of polyphyllin H on the activity of CYPs

The activity of all CYP isoforms was suppressed by corresponding inhibitors, with a decrease ranging from 94.35% to 83.63%. Polyphyllin H significantly suppressed CYP1A2, 2D6, and 3A4, reducing their activity to 24.49, 33.33, and 17.79% of the negative control, respectively (Figure 1A). The inhibitory effects of polyphyllin H were revealed to be concentration-dependent. As the concentration of polyphyllin H increased, the activities of CYP1A2 (Figure 1B), 2D6 (Figure 1C), and 3A4 (Figure 1D) decreased with IC_{50} values of 6.44, 13.88, and 4.52 μM, respectively.

3.2 | Inhibition models of CYP1A2, 2D6, and 3A4

With various concentrations of polyphyllin H and substrates of CYP1A2, 2D6, and 3A4, the inhibition of CYP1A2 (Figure 2A) and 2D6 (Figure 2B) was best fitted with the competitive inhibition model, as shown by the stable V_{max} in the double-reciprocal plots. In contrast, the inhibition of CYP3A4 was best fitted with the non-competitive inhibition model with a stable K_m (Figure 2C). The K_i values obtained for CYP1A2, 2D6, and 3A4 were 3.18, 6.77, and 2.38 μM, respectively.

3.3 | Time-dependent characteristics of CYP1A2, 2D6, and 3A4 inhibition

The activity of CYP3A4 decreased over the incubation period, while CYP1A2 and 2D6 remained relatively stable (Figure 3A). In the presence of 2, 5, 10, 20, and 50 μM polyphyllin H, a time-dependent manner was observed in the inhibition of CYP3A4 (Figure 3B). By fitting the slope of time-dependent inhibition, the related parameters KI and K_{inact} were obtained as 2.26 μM^{-1} and 0.045 min^{-1}, respectively (Figure 3C).

4 | DISCUSSION

Traditional Chinese medicine has the advantages of low toxicity and high efficiency.[29] However, its complex composition and lengthy treatment process would influence metabolic enzymes and induce drug–drug interactions. These interactions can alter the physicochemical properties, pharmacokinetics, and pharmacodynamics, and even lead to adverse reactions. The CYP1, CYP2, and CYP3 families account for 13%, 33%, and 30% of total CYPs and include several key isoforms.[30,31] Polyphyllin H was found to inhibit the activity of CYP1A2, 2D6, and 3A4 in the present study. The roles and divisions of these three isoforms vary slightly.

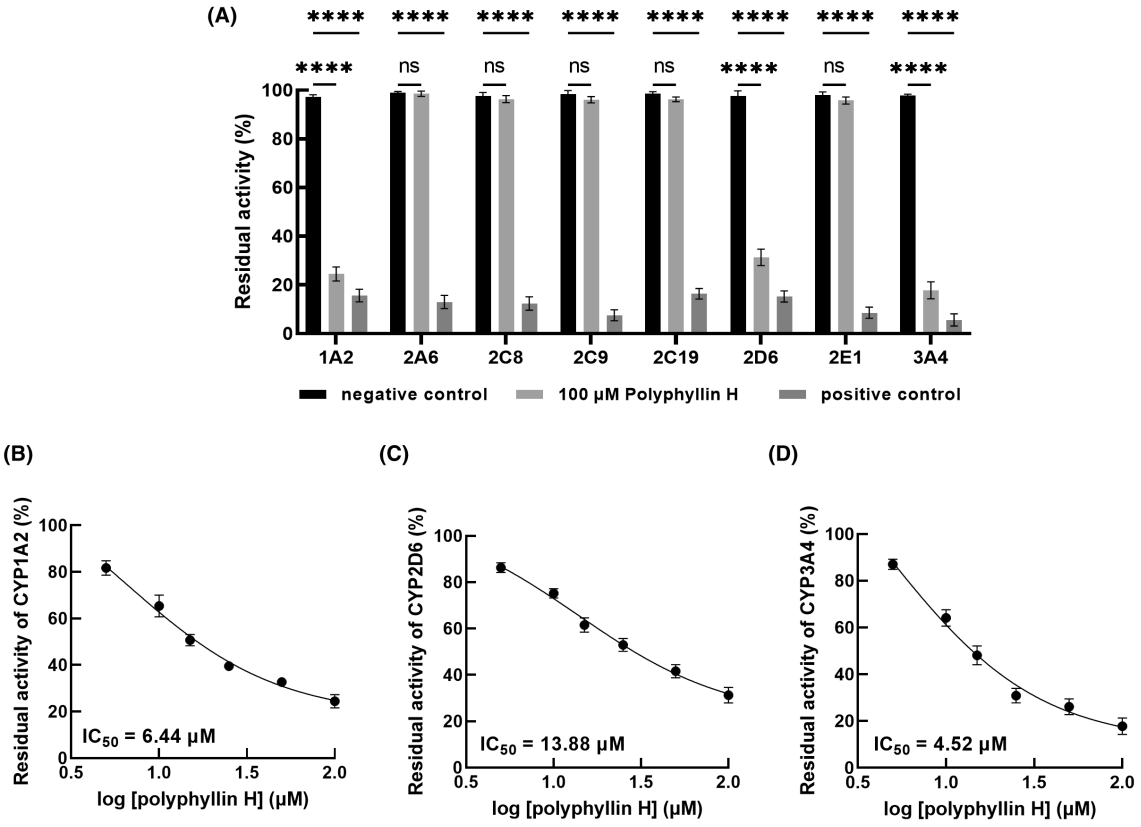

FIGURE 1 Polyphyllin H significantly suppressed the activity of CYP1A2, 2D6, and 3A4, but the inhibitory effects were weaker than typical inhibitors (A). The inhibition of CYP1A2 (B), 2D6 (C), and 3A4 (D) was enhanced with the increasing concentration of polyphyllin H. IC_{50}, Half maximal inhibitory concentration. $^{ns}p > .05$, $^{****}p < .0001$.

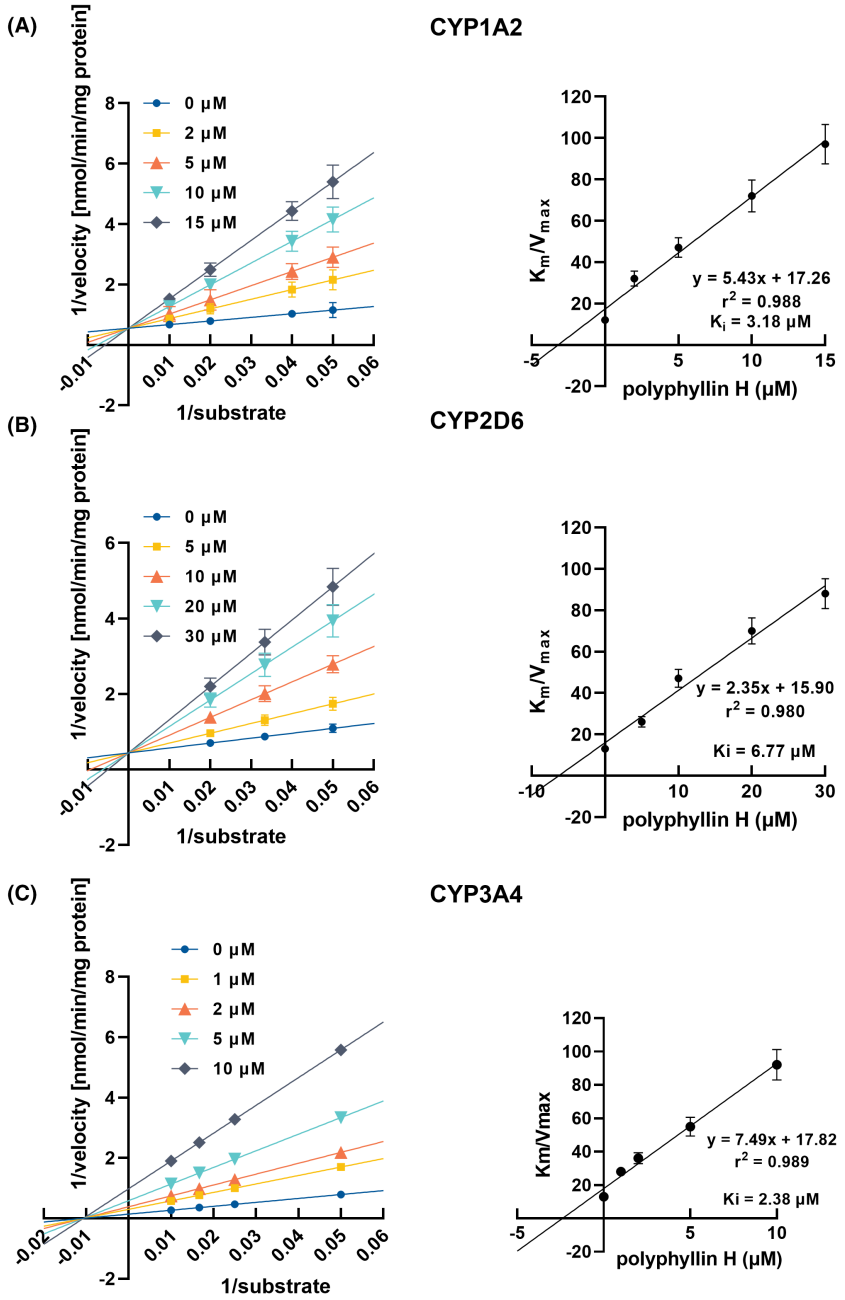

FIGURE 2 The inhibition of CYP1A2 (A) and 2D6 (B) was best fitted with a competitive model in the presence of 0–15 μM (for 1A2) and 0–30 μM (for 2D6) polyphyllin H concentrations. The inhibition of CYP3A4 was non-competitive in the presence of 0, 1, 2, 5, and 10 μM polyphyllin H (C). 1/velocity: The reciprocal of the reaction rate; 1/substrate: The reciprocal of substrate concentration; K_m: The substrate concentration at half of the maximum velocity of the reaction (V_{max}).

CYP1A2 is mainly responsible for the biotransformation of polycyclic aromatic hydrocarbons, aromatic amines, heterocyclic amines, and some halogenated hydrocarbons. CYP1A2 is also involved in the metabolism of xanthine drugs, such as phenacetin, propranolol, clozapine, and caffeine.[32,33] Although CYP2D6 only accounts for about 2% of CYPs, it participates in the metabolism of over a fifth of prescription drugs, involving antipsychotics, antidepressants, analgesics, and antiarrhythmic drugs.[34] The gene polymorphism of CYP2D6 affects drug metabolism and can induce adverse side effects. As such, the suppressed activity of CYP2D6 by polyphyllin H observed in this study would influence the metabolism of specific drugs and increase the risk of toxic reactions. CYP3A4 is widely expressed in the liver and gut and has extensive substrate specificity. According to statistical data, the substrates of CYP3A4 include 38 categories and over 150 drugs, which typically have a large molecular weight, aromatic rings, and less polarity.[35,36] Environmental factors can affect CYP3A4 activity and individual differences in CYP3A4 expression can cause unpredictable drug reactions and toxicity.[37] The altered activity of CYP1A2, 2D6, and 3A4 has been reported to mediate drug–drug interaction during drug co-administration. For instance, the inactivation of CYP1A2 by Xanthotoxin was suggested to induce the increasing systemic exposure of Tacrine.[38] A prospective observational study also demonstrated that the CYP2D6-mediated interactions could affect the efficiency of prescribed drugs in the emergency department.[39] Hence, the inhibition of these isoforms by polyphyllin H should draw attention to its co-administration with the substrates of these enzymes. Further clinical investigations are

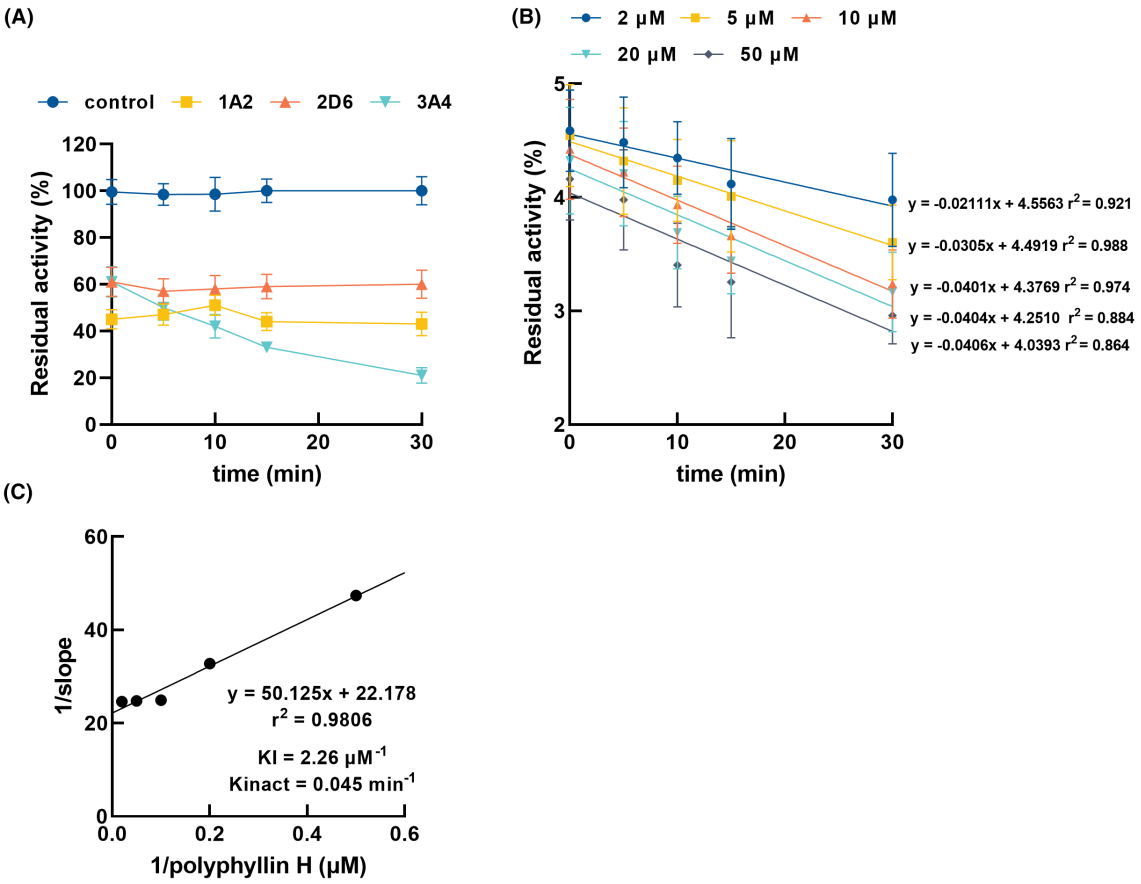

FIGURE 3 The inhibition of CYP3A4 was enhanced with incubation time (a), and the time-dependent inhibition parameters were obtained by fitting the inhibition under 2, 5, 10, 20, and 50 μM polyphyllin H (b and c).1/slope: The reciprocal of slopes under different concentrations of polyphyllin H in Figure 2B; 1/polyphyllin H: The reciprocal of polyphyllin H concentration in Figure 2B.

needed to specify the potential of polyphyllin H to induce interactions with co-administrated drugs. Additionally, the inhibitory effects of polyphyllin H appeared to be concentration-dependent. The IC_{50} values also provide a reference for its dosage in clinical prescriptions. However, there was a lack of in vivo validation for the metabolism and plasma concentration of polyphyllin H. Previously, pharmacokinetic studies reported the maximum plasma concentration of polyphyllin H was 11.75 ± 1.28 μg/L in rats and 20.72 ± 1.28 μg/L in beagle dog after oral administration of *R. paridis* extracts, which are both lower than the identified IC_{50} values.[7,40] Hence, further in vivo evaluation of the interaction of polyphyllin H with co-administrated drugs is necessary.

Inhibition characteristics are crucial in determining whether drug–drug interaction will occur. Both competitive and non-competitive inhibitions are reversible, mediated by weak forces such as hydrogen and hydrophobic bonds.[41,42] In competitive inhibition, inhibitors compete with substrates for binding sites, preventing substrate binding. In contrast, non-competitive inhibition allows enzymes to bind with substrates, but inhibitors bind to the enzyme substates complex, preventing the release of products. Herein, the inhibition of CYP1A2 and 2D6 was revealed to be competitive, while the inhibition of CYP3A4 was non-competitive. K_i, a critical parameter in reversible inhibition is linked with IC_{50} values according to previous studies. It was reported that IC_{50} is 2-fold of K_i value in competitive inhibition, which is consistent with our findings, and K_i is approximately equal to IC_{50} values in non-competitive inhibition.[43] However, recent CYP-related studies also observed that the K_i values were also half of IC_{50} values in non-competitive inhibition, which is similar to the present study.[44,45] Therefore, there is a controversy in the association between K_i value and IC_{50} values in non-competitive inhibition in experimental studies and modeling, which needs further investigation. The chemical structure of polyphyllin H includes several ring structures (Figure 4), which might be similar to the substrates of CYP1A2 and 2D6, and therefore induced competitive inhibition on these CYPs.

Time-dependent manner significantly influences the effect of polyphyllin H on CYPs. Time-dependent inhibition is subtle and presents a challenge in predicting drug–drug interaction. The parent drug must undergo various metabolic transformations before finally binding with CYPs.[46,47] In the inhibited isoforms, only the inhibition of CYP3A4 by polyphyllin H was found to increase with prolonged incubation time. This in vitro study primarily revealed the inhibitory effect of polyphyllin H on the activity of CYP1A2, 2D6, and 3A4 in human liver microsomes. It suggests that polyphyllin H could potentially affect the metabolism of substrate drugs of these CYP

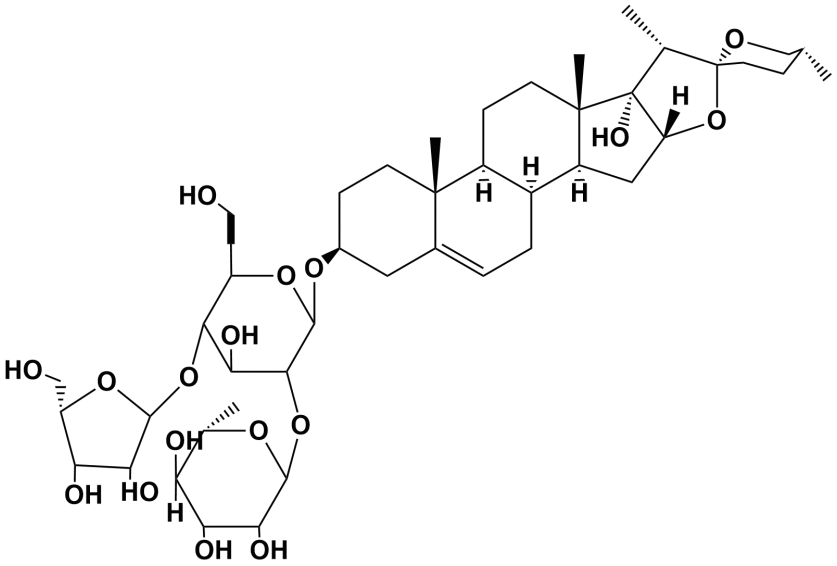

FIGURE 4 The chemical structure of polyphyllin H.

isoforms. Studies have developed prediction methods for the risk of drug–drug interaction.[48,49] However, due to the difference in bioavailability, there was a lack of in vivo validation close to the actual clinical situation. There are also several prediction models that could predict the risk of drug–drug interaction, such as dynamic model and static model. Therefore, future studies should pay more attention to evaluating the potential of polyphyllin H inducing drug–drug interaction with the help of prediction model and more in vivo models, and clinical validations. Additionally, with the development of mode fitting, in silico investigations could help to predict the potential binding sites between the compounds and CYP450s, providing more molecular evidence for revealing the affecting mechanisms of polyphyllin H on CYP1A2, 2D6, and 3A4, which should be taken into the study design in our future research.

5 | CONCLUSION

In conclusion, polyphyllin H has been found to inhibit the activity of CYP1A2, 2D6, and 3A4, in a manner that is directly correlated with its concentration. It causes competitive inhibition of CYP1A2 and 2D6 and non-competitive inhibition of CYP3A4. This inhibitory effect of polyphyllin H could potentially lead to drug–drug interactions. Therefore, this should be evaluated further in the context of clinical traits and considered in its prescription.

AUTHOR CONTRIBUTIONS

Erhao Wang: Conceptualization, Methodology, Formal analysis, Methodology, Writing - original draft. Mengxi Wang: Conceptualization, Methodology, Formal analysis, Methodology, Writing - original draft. Ming Gao: Conceptualization, Project administration, Supervision, Writing - review & editing.

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CONFLICT OF INTEREST STATEMENT

The authors report there are no competing interests to declare.

DATA AVAILABILITY STATEMENT

All data generated or analyzed during this study are included in this article. Further inquiries can be directed to the corresponding author.

ETHICS STATEMENT

Not applicable.

PATIENT CONSENT STATEMENT

Not applicable.

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