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Cytochrome P450 Inhibition by Three Licorice Species and Fourteen Licorice Constituents

Guannan Li, **Charlotte Simmler**, **Luying Chen**, **Dejan Nikolic**, **Shao-Nong Chen**, **Guido F. Pauli**, and **Richard B. van Breemen***

UIC/NIH Center for Botanical Dietary Supplements Research, Department of Medicinal Chemistry and Pharmacognosy, University of Illinois College of Pharmacy, Chicago, IL 60612

Abstract

The potential of licorice dietary supplements to interact with drug metabolism was evaluated by testing extracts of three botanically identified licorice species (*Glycyrrhiza glabra* L., *Glycyrrhiza uralensis* Fish. ex DC. and *Glycyrrhiza inflata* Batalin) and 14 isolated licorice compounds for inhibition of 9 cytochrome P450 enzymes using a UHPLC-MS/MS cocktail assay. *G. glabra* showed moderate inhibitory effects against CYP2B6, CYP2C8, CYP2C9, and CYP2C19, and weak inhibition against CYP3A4 (testosterone). In contrast, *G. uralensis* strongly inhibited CYP2B6 and moderately inhibited CYP2C8, CYP2C9 and CYP2C19, and *G. inflata* strongly inhibited CYP2C enzymes and moderately inhibited CYP1A2, CYP2B6, CYP2D6, and CYP3A4 (midazolam). The licorice compounds isoliquiritigenin, licoricidin, licochalcone A, 18β-glycyrrhetinic acid, and glycycoumarin inhibited one or more members of the CYP2C family of enzymes. Glycycoumarin and licochalcone A inhibited CYP1A2, but only glycycoumarin inhibited CYP2B6. Isoliquiritigenin, glabridin and licoricidin competitively inhibited CYP3A4, while licochalcone A (specific to *G. inflata* roots) was a mechanism-based inhibitor. The three licorice species commonly used in botanical dietary supplements have varying potential for drug-botanical interactions as inhibitors of cytochrome P450 isoforms. Each species of licorice displays a unique profile of constituents with potential for drug interactions.

TOC Graphic

*Corresponding author. Richard B. van Breemen, Department of Medicinal Chemistry and Pharmacognosy, University of Illinois at Chicago, 833 S. Wood St., M/C 781, Chicago, IL 60612, Telephone: 312-996-9353, FAX: 312-996-7107, breemen@uic.edu.

1. Introduction

Many consumers of botanical dietary supplements also take prescription drugs and over the counter medications (Gardiner *et al.*, 2007a; Gardiner *et al.*, 2007b). Concomitant use of botanical dietary supplements and drugs might cause drug-botanical interactions, resulting in increased or decreased drug exposure and consequently leading to amplified adverse effects or drug inefficacy, respectively (de Lima Toccafondo Vieira and Huang, 2012; Gurley, 2012). Therefore, it is important to understand the potential of botanical dietary supplements to alter the metabolism and disposition of drugs. Major mechanisms of drug-botanical interactions include inhibition and induction of drug metabolizing enzymes or transporters (Liu *et al.*, 2011; Sprouse and van Breemen, 2016). Inhibition of metabolism can increase drug exposure and toxicity, whereas induction can lead to decreased drug exposure and loss of efficacy (Huang and Lesko, 2004; de Lima Toccafondo Vieira and Huang, 2012).

Hepatic metabolism is the primary clearance pathway for most prescription drugs. Over two-thirds of drug metabolism is mediated by cytochrome P450 (CYP) enzymes including CYP3A (46%), CYP2C9 (16%), CYP2C19 (12%), CYP2D6 (12%), CYP1A (9%), CYP2B6 (2%), and CYP2E1 (2%) (Pelkonen, *et al.,* 2008; Preissner, *et al.,* 2010; Rendic and Guengerich, 2015). Inhibition of CYP enzymes is the most common mechanism underlying drug-botanical interactions. Usually, CYP inhibition causes increased drug exposure and possible toxicity, except for drugs that require metabolic activation by CYP enzymes, when CYP inhibition may lead to loss of efficacy (Lin and Lu, 1998).

Licorice is the common name attributed to certain species of the genus *Glycyrrhiza* that are known to produce the saponin glycyrrhizin (Fig. 1), a natural sweetener responsible for the characteristic sweet taste of the roots. Traditionally, dried licorice roots have been used as a food flavoring agent and for various medicinal purposes since ancient times (Nassiri Asl and Hosseinzadeh, 2008; Hosseinzadeh and Nassiri-asl, 2015). As such, preparations of licorice roots have been reported to have significant anti-inflammatory, anti-viral, anti-carcinogenic, anti-allergic, hepatoprotective, and estrogenic effects (Fiore *et al.,* 2008; Hajirahimkhan *et al.*, 2013; Nassiri Asl and Hosseinzadeh, 2008). The genus *Glycyrrhiza* includes approximately 30 species, among which *Glycyrrhiza glabra* L., *G. uralensis* Fisch. ex DC. and *G. inflata* Batalin are the three most commonly used in various systems of traditional medicine. In the European and Chinese Pharmacopoeias, *G. glabra, G. uralensis* and *G. inflata* are used interchangeably (World Health Organization, 1999). Because of this botanical ambiguity, many licorice dietary supplements do not disclose which species are used in their formulation (Zhang and Ye, 2009; Simmler *et al.*, 2015a).

As in drug-drug interaction studies, in vitro models of drug-botanical interactions are usually evaluated to ascertain whether clinical investigations are indicated (van Breemen, 2015; Sprouse and van Breemen, 2016). Recombinant CYP enzymes, human hepatocytes or human liver microsomes are typically used with probe substrates to measure possible inhibition of enzyme activity caused by test compounds or botanical extracts (Li *et al.*, 2015; Walsky and Obach, 2004). The current FDA guidance for drug interaction studies recommends inhibition studies of CYP1A2, CYP2B6, CYP2C8, CYP2C9, CYP2C19, CYP2D6, and CYP3A (U.S. Food and Drug Administration, 2012). Although drug

interaction studies are not specified in the FDA guidance for CYP2A6 and CYP2E1, these enzymes may also cause drug-botanical interactions. CYP2A6 is involved in the metabolism of some tobacco compounds including nicotine, cotinine and *N*[1]-nitrosonornicotine (Pelkonen *et al.,* 2000), and CYP2E1 has been reported to metabolize ethanol, caffeine, acetaminophen, and chlorzoxazone (Hrycay and Bandiera, 2008; Dey 2013). In this study, we used a cocktail assay approach (Li *et al.*, 2015) with ultrahigh-pressure liquid chromatography-tandem mass spectrometry (UHPLC-MS/MS) to measure possible inhibition of all seven CYP isoforms recommended by the FDA as well as CYP2A6 and CYP2E1 by extracts of *G. glabra, G. uralensis* and *G. inflata* and 14 compounds (Fig. 1) isolated from these species.

2. Materials and Methods

2.1 Materials and Chemicals

Phenacetin was purchased from Tokyo Chemical Industry (Tokyo, Japan). Coumarin, bupropion hydrochloride, tolbutamide, chlorzoxazone, troleandomycin, glutathione, superoxide dismutase from bovine liver, catalase from bovine liver, β-nicotinamide adenine dinucleotide phosphate (NADPH), formic acid, potassium phosphate monobasic, and potassium phosphate dibasic were purchased from Sigma-Aldrich (St. Louis, MO). Dextromethorphan hydrobromide was obtained from MP Biomedicals (Santa Ana, CA). [d_6]-Hydroxybupropion was purchased from Santa Cruz Biotechnology (Dallas, TX), and midazolam and testosterone were obtained from Cerilliant Corporation (Round Rock, TX). [d_5]-7-Hydroxycoumarin, [d_7]-6β-hydroxytestosterone and [$^{13}C_3$]-1'-hydroxymidazolam were purchased from BD Gentest (Woburn, MA). Amodiaquine, [d_5]-*N*-desethylamodiaquine, (*S*)-mephenytoin, [d_4]-acetaminophen, [d_2]-6-hydroxychlorxazone, [d_9]-4-hydroxytolbutamide, [d_3]-(±)-4'-hydroxymephenytoin, and [d_3]-dextrorphan tartrate were purchased from Toronto Research Chemicals (Toronto, Canada). Glycycoumarin (92.3% w/w) was purchased from BioBioPha (Kunming Institute of Botany, China). The licorice compounds glycyrrhizin (95.0% w/w), 18β-glycyrrhetinic acid (97.0% w/w) glabridin (87.10% w/w), and licochalcone A (96.06% w/w) were purchased from Sigma-Aldrich (St. Louis, MO), whereas liquiritigenin (95.5% w/w), liquiritin (95.9% w/w), liquiritin apioside (88.0% w/w), isoliquiritigenin (95.5% w/w), isoliquiritin (89.7% w/w), isoliquiritin apioside/licuraside (74.8/23.8% w/w), and *p*-hydroxybenzylmalonic acid (90.0% w/w) were isolated as described previously (Simmler *et al.*, 2013, 2014, 2015b). Licoricidin (95.2% w/w) was isolated using another approach as described previously (Bodet *et al.*, 2008).

The purities of each purchased and isolated licorice compound were determined by 1D, ^{1}H quantitative NMR with the 100% method, as described previously (Pauli *et al.*, 2014).

Pooled mixed gender human liver microsomes from 200 donors were purchased from XenoTech (Lenexa, KS). Supersomes containing human recombinant CYP3A4, oxidoreductase and cytochrome b5 were obtained from Corning (Woburn, MA). LC-MS-grade acetonitrile and methanol were purchased from Thermo Fisher (Fair Lawn, NJ). Water was prepared using an Elga Purelab Ultra (Siemens Water Technologies, Woodridge, IL) water purification system.

2.2 Extraction and quantitative analysis of licorice compounds

Dried root materials of *Glycyrrhiza. uralensis* Fisch. ex. DC. and *Glycyrrhiza glabra* L. (Fabaceae) were purchased from a local supplier (Chicago, IL), and from Mountain Rose Herbs, respectively. *Glycyrrhiza inflata* Batalin was collected in Xinjiang province, China in 2012. Licorice powders were botanically identified through DNA-based identification, macroscopic and microscopic analyses, and comparison with voucher specimens at the Field Museum of Natural History (Chicago, IL) as well as DNA barcoding as described previously (Simmler *et al.*, 2015a). The root powders from each species were extracted by maceration at room temperature with a solvent mixture consisting of ethanol (200 USP proof), isopropanol and water (90:5:5, v/v/v) and a plant powder/volume of solvent ratio of 1:15 (Hajirahimkhan *et al.*, 2015). The quantitative analysis of 14 licorice constituents in the extracts was carried out using a UHPLC-MS/MS assay as described previously (Li, *et al.*, 2016).

2.3 IC_{50} determination using cocktail assay

Using a cocktail assay, IC_{50} values were determined according to the method of Li *et al.* (2015). Briefly, licorice extracts (*G. glabra, G. uralensis* and *G. inflata*) at 11 concentrations from 0.005–250 μg/mL or licochalcone A from 0.001–100 μM were incubated with 1.3 mM NADPH, 0.2 mg/mL human liver microsomes, and a cocktail of 10 probe substrates (100 μM phenacetin for CYP1A2, 1.5 μM coumarin for CYP2A6, 12 μM bupropion for CYP2B6, 1 μM amodiaquine for CYP2C8, 100 μM tolbutamide for CYP2C9, 50 μM (*S*)-mephenytoin for CYP2C19, 2.5 μM dextromethorphan for CYP2D6, 15 μM chlorzoxazone for CYP2E1, and 2.5 μM midazolam and 50 μM testosterone for CYP3A4) in potassium phosphate buffer (100 μL, 0.1 M, pH 7.4) at 37°C for 10 min. The incubation time of 10 min was selected so that the metabolic transformation of the probe substrates remained linear. The reactions were terminated by adding 20 μL of a stop solution consisting of water/acetonitrile/formic acid, (92:5:3; v/v/v) containing stable isotope-labeled surrogate standards of each metabolite. The samples were then centrifuged at 13000 × *g* at 4°C for 15 min prior to analysis using UHPLC-MS/MS as described previously (Li *et al.*, 2015). Probe substrates for CYP2B6 and CYP2E1 were used at initial concentrations of $0.1K_m$, while others were at K_m concentrations. As described previously (Li *et al.*, 2015), marker inhibitors of each of the cytochromes P450 were used as positive controls to confirm that the microsomes were susceptible to inhibition.

2.4 Single concentration inhibition potency screening assay

Fourteen licorice compounds (liquiritin, isoliquiritin, liquirtin apioside, liquirtigenin, isoliquiritigenin, glycyrrhizin, 18β-glycyrrhetinic acid, *p*-hydroxybenzylmalonic acid, licoricidin, glycycoumarin, licochalcone A, and glabridin and a mixture of isoliquiritin apioside/licuraside (74.76/23.75 % w/w)) (Fig. 1) at 10 μM each were incubated individually with the probe substrates cocktail as described above. Methanol (0.5 μL) was added as a solvent control, and percentages of control activity were calculated to determine inhibition potency.

2.5 Single point time-dependent inhibition screening assay

Licorice extracts and compounds showing inhibition of CYP3A4 were tested further to determine their potential to function as CYP3A4 time-dependent inhibitors. Human liver microsomes (0.5 mg/mL) were incubated with extracts (200 μg/mL or 400 μg/mL) or compounds (100 μM or 50 μM) when using midazolam or testosterone as the substrate, respectively, in potassium phosphate buffer (100 μL, 0.1 M, pH 7.4) at 37°C in the presence and absence of 1.3 mM NADPH. After 30 min, a 5 μL aliquot of the incubation mixture was added to 95 μL potassium phosphate buffer containing 1.3 mM NADPH and a probe substrate at $5K_m$ concentration. The reactions were terminated after incubating for 5 min and processed as described above. Addition of methanol solvent (0.5 μL) was used as a negative control and addition of 4 μM troleandomycin (a mechanism-based inhibitor of CYP3A4) (Polasek and Miners, 2006) was used as a positive control.

Two different calculations were used for evaluating the time-dependent inhibition data as indicated in equations (1) and (2). In equation (1), a ratio of the CYP3A4 activities in the experiment and negative control incubations was calculated (Atkinson *et al.*, 2005); and in equation (2), a difference was calculated between the CYP3A4 activities in the negative control and the experiment (Obach *et al.*, 2007). For both equations, the percentage of decrease in activity was determined with “A” defined as the activity of CYP3A4 (midazolam or testosterone).

$$\%\text{activity decrease}=100\times\{1-(\frac{A_{\text{compound(extract)+NADPH}}}{A_{\text{solvent control+NADPH}}})/(\frac{A_{\text{compound(extract)-NADPH}}}{A_{\text{solvent control-NADPH}}})\} \quad (1)$$

$$\%\text{activity decrease}=100\times\{(\frac{A_{\text{compound(extract)-NADPH}}}{A_{\text{solvent control-NADPH}}})-(\frac{A_{\text{compound(extract)+NADPH}}}{A_{\text{solvent control+NADPH}}})\} \quad (2)$$

2.6 K_I and k_{inact} determination

Incubations to determine K_i and k_{inact} values contained 0.5 mg/mL human liver microsomes or 20 pmol/mL cDNA expressed CYP3A4, 1.3 mM NADPH, and licochalcone A at various concentrations (0, 50, 100, 200, and 300 μM for human liver microsomes or 0, 2, 5, 10, 20, and 50 μM for recombinant CYP3A4) in potassium phosphate buffer (100 μL, 0.1 M, pH 7.4). After 0, 4, 8, 12, 20, or 30 min incubation at 37°C, 5 μL aliquots were transferred to 95 μL potassium phosphate buffer containing 1.3 mM NADPH and midazolam at saturating concentration. After 5 min, the reactions were terminated and processed as described above.

2.7 Effect of washing to restore CYP3A4 activity

Human liver microsomes (0.2 mg/mL) or recombinant CYP3A4 (1 pmol/mL) were incubated with 50 μM or 10 μM licochalcone A, respectively, for 30 min at 37°C in the presence and absence of 1.3 mM NADPH. Preincubated samples were centrifuged at 10,000×g at 4°C for 30 min, and the protein pellets were re-suspended in potassium phosphate buffer (100 μL, 0.1 M, pH 7.4). Washing was repeated 2 more times. After

washing, the remaining enzyme activity was measured using midazolam at the $5K_m$ concentration.

2.8 Effects of glutathione, superoxide dismutase and catalase on CYP3A4 inactivation

Licochalcone A (100 μM or 20 μM) was preincubated with 1.3 mM NADPH and 0.5 mg/mL human liver microsomes or 20 pmol/mL recombinant CYP3A4 for 30 min at 37°C in the presence and absence of 2 mM glutathione, 1000 U/mL superoxide dismutase or 1000 U/mL catalase. The remaining enzyme activity was determined using midazolam at the $5K_m$ concentration.

2.9 UHPLC-MS/MS of probe substrate metabolites

All metabolites and surrogate standards were analyzed by using UHPLC-MS/MS on a Shimadzu (Kyoto, Japan) Nexera UHPLC and LCMS-8050 triple quadrupole mass spectrometer as described previously (Li *et al.*, 2015). Briefly, separations were carried out using a Waters (Milford, MA) ACQUITY UPLC BEH C_{18} column (2.1 × 100 mm, 1.7 μm) at a flow rate of 0.5 mL/min with a column oven temperature of 40°C. The primary metabolites of the 10 probe substrates and their corresponding isotope-labeled internal standards were separated using a 3-min linear gradient from 20% to 75% acetonitrile in water containing 0.1% formic acid. Tandem mass spectrometry quantitative analysis of the probe substrate metabolites was carried out using electrospray with polarity switching, collision-induced dissociation, and selected reaction monitoring. For additional details regarding the UHPLC-MS/MS method, see Li *et al.* (2015).

2.10 Data analysis

Quantitative UHPLC-MS/MS data were analyzed using Shimadzu LabSolutions software. The IC_{50}, K_I and k_{inact} values were determined using the enzyme kinetics module of SigmaPlot (Systat Software, San Jose, CA). The percent of control activity, $k_{obs,app}$ and other calculations were carried out using Microsoft Excel (Seattle, WA).

3. Results

The inhibition curves (Fig. 2) and IC_{50} values (Table 1) for *G. glabra, G. uralensis* and *G. inflata* showed unique inhibitory effects indicating that not all species of licorice are equivalent with respect to drug interactions (pairwise comparison indicated that at least one pair of IC_{50} values was not the same with P-value<0.05). *G. glabra* extract displayed moderate inhibitory effects against CYP2B6, CYP2C8, CYP2C9, and CYP2C19, and weak inhibition against CYP3A4 (testosterone). In contrast, *G. uralensis* extract strongly inhibited CYP2B6 and moderately inhibited CYP2C8, CYP2C9 and CYP2C19, while *G. inflata* extract strongly inhibited enzymes in the CYP2C family and moderately inhibited CYP1A2, CYP2B6, CYP2D6, CYP3A4 (midazolam).

To help explain the differences in the inhibition profiles of the three studied licorice species, UHPLC-MS/MS was used to measure the levels of 14 characteristic marker compounds (Fig. 1 and Table 2). The profiles of these compounds were unique for each of the licorice species. For example, *G. glabra* contained relatively more flavonoid and chalcone apiosides

and was the only species to contain glabridin. Licoricidin and glycycoumarin were most abundant in *G. uralensis extract*, and only *G. inflata* contained licochalcone A.

All 14 licorice constituents were assayed for inhibition of 9 cytochrome P450 isoforms using the probe substrate cocktail. Six of these compounds, isoliquiritigenin, glabridin, 18β-glycyrrhetinic acid, licoricidin, glycycoumarin, and licochalcone A, showed more than 50% inhibition of one or more cytochrome P450 enzymes at a test concentration of 10 μM (Fig. 3). Occurring in *G. glabra, G. uralensis* and *G. inflata* extracts, isoliquiritigenin inhibited CYP2C8 and CYP2C9 and 18β-glycyrrhetinic acid was a weak inhibitor of CYP2C8 and CYP3A4 (testosterone). Found only in *G. glabra*, glabridin inhibited CYP3A4 (testosterone). Licoricidin, unique to the tested *G. uralensis* extract, inhibited CYP2B6, CYP2C8, CYP2C9, CYP2C19, and CYP3A4 (testosterone). Glycycoumarin (found in *G. uralensis* and *G. inflata* but not in *G. glabra*) inhibited CYP1A2, CYP2B6 and CYP2C. Licochalcone A, abundant only in the *G. inflata* extract (5.24% *w/w,* Table 2), strongly inhibited CYP2C8 and CYP2C9, was a moderate inhibitor of CYP2C19 and CYP3A4 (testosterone), and weakly inhibited CYP1A2. IC_{50} values were determined for licochalcone A (Table 1) and ranged from high nM levels for inhibition of CYP2C9 to less than 20 μM for CYP1A2.

Inhibitors of CYP3A4, *G. glabra* extract, *G. inflata* extract and licochalcone A were tested for possible time-dependent inhibition. The *G. inflata* extract showed time-dependent inhibition of CYP3A4 (midazolam) (Table 3), and licochalcone A, a major constituent of the *G. inflata* extract, also demonstrated time-dependent inhibition. However, neither lichochalcone A nor the *G. inflata* extract produced time-dependent inhibition of CYP3A4 metabolism of testosterone (Table 3).

The kinetic constants for the inactivation of CYP3A4 (midazolam) by licochalcone A were determined using human liver microsomes and recombinant CYP3A4. After estimating the initial rate constants ($k_{obs,app}$), K_I and k_{inact} were determined using nonlinear regression analysis (Fig. 4 and Fig. 5). The K_I and k_{inact} of licochalcone A for human liver microsomes were 116.13 μM and 0.38 min^{-1}, respectively, and using recombinant human CYP3A4, they were 2.22 μM and 0.047 min^{-1}. Efficiencies of inactivation were 0.0033 $\mu M^{-1}min^{-1}$ and 0.021 $\mu M^{-1}min^{-1}$ for human liver microsomes and recombinant CYP3A4, respectively. After washing out the inhibitor licochalcone A, the remaining enzyme activities were 57% for the human liver microsomes and 16.7% for recombinant CYP3A4, which indicated that washing did not restore CYP3A4 activity. Addition of glutathione, superoxide dismutase or catalase to the incubation did not prevent CYP3A4 inactivation by licochalcone A (Table 4). Therefore, licochalcone A was determined to be a mechanism based inhibitor of CYP3A4.

4. Discussion

Kent *et al.* (2002) found moderate inhibition of CYP3A4 by an acetone extract of *G. glabra*, and we found weak inhibition of this drug metabolizing enzyme as well as moderate inhibition of CYP2B6, CYP2C8, CYP2C9, and CYP2C19 by an ethanolic extract (Table 1). In previous studies of *G. uralensis*, Tsukamoto *et al.* (2005) reported moderate inhibition of CYP3A4 by a methanol extract, and Qiao *et al.,* (2014) reported strong inhibition of

CYP2C9 but no effects on CYP3A4 by an ethanol extract or an ethyl acetate extract. In our studies using an ethanolic extract of *G. uralensis*, the results of Qiao, *et al.* (2014) were confirmed with respect to inhibition of CYP2C9 and lack of inhibition of CYP3A4 (Table 1).

Variations between laboratories regarding the potential for inhibition of cytochrome P450 enzymes by botanical dietary supplements are probably due to several factors including differing plant materials, extraction procedures and assay procedures. These differences emphasize the need for chemically standardizing the licorice extracts prior to biological evaluation (van Breemen, 2015). Nevertheless, there are striking similarities between the results from various laboratories regarding the potential for the licorice species *G. glabra* and *G. uralensis* to cause drug-botanical interactions.

Not investigated previously by other laboratories, the *G. inflata* extract showed the most potent inhibition of cytochrome P450 enzymes (with IC_{50} values in the low μg/mL range) among the three licorice species commonly used in botanical dietary supplements. In addition, the *G. inflata* extract inhibited more cytochrome P450 enzymes including CYP1A2, CYP2B6, CYP2C8, CYP2C9, CYP2C19, CYP2D6, and CYP3A4 (Table 1). In assays of isolated licorice compounds, licochalcone A, which is a unique and abundant secondary metabolite of *G. inflata* (Table 2), was a major contributor to the inhibitory activity (Table 1).

In all, 14 licorice constituents were screened at 10 μM for possible inhibition of cytochrome P450 enzymes, and isoliquiritigenin, glabridin, 18*β*-glycyrrhetinic acid, licoricidin, glycycoumarin, and licochalcone A displayed the most significant inhibitory activities (Fig. 3). The mixture of isoliquiritin apioside/licuraside showed no inhibition, which is consistent with a previous report (Qiao *et al.*, 2014). In a study using recombinant enzymes instead of human liver microsomes, Kent *et al.,* (2002) found that glabridin irreversibly inhibited CYP3A4 and CYP2B6 and reversibly inhibited CYP2C9. Our data confirmed inhibition of CYP3A4 (testosterone) by glabridin but not inhibition of CYP2B6 or CYP2C9, which might be the result of differences between assays. In a human pharmacokinetics study, Aoki *et al.* (2007) reported that a single oral dose of a licorice dietary supplement containing 1% glabridin produced maximum plasma concentrations of up to 8 nM glabridin with a mean half-life of 10.7 h.

Our data indicate that isoliquiritigenin is a weak inhibitor of CYP3A4 (testosterone) and a moderate inhibitor of CYP2C9 (Table 1), and these results are consistent with those of Qiao *et al.* (2014). Like Qiao *et al.,* we also found that glycycoumarin inhibited CYP1A2, CYP2C9, CYP2C19, and that liquiritin, liquiritin apioside, isoliquiritin, and isoliquiritin apioside did not inhibit any cytochrome P450 enzymes. However, we did not observe significant inhibition of CYP2D6 by glycycoumarin or inhibition of CY1A2 by liquiritigenin. Note that Qiao *et al.* (2014) did not assay for inhibition of CYP2B6 or CYP2C8 and only tested compounds isolated from *G. uralensis*, which included 7 of the 14 licorice compounds reported here.

Abundant only in *G. inflata* (Table 2), licochalcone A contributed significantly to the inhibition activities of *G. inflata.* Licochalcone A strongly inhibited CYP2C and, like the *G. inflata* extract, was a mechanism-based inhibitor of CYP3A4, which is the most abundant isoform in the human liver and is responsible for the metabolism of many prescribed drugs. In vitro detection of time-dependent inhibition of CYP3A4 has been suggested to correlate well with clinically relevant drug-botanical interactions (Clarke and Jones, 2008; Grimm *et al.*, 2009).

Two equations were used to evaluate the experimental data for time-dependent inhibition of CYP3A4; note that equation 1 responds only to irreversible inhibition whereas equation 2 responds to both reversible and irreversible inhibition. For example, the positive control troleandomycin, known to only irreversibly inhibit CYP3A4, gave the same results using both equations (Table 4). However, time-dependent inhibition by *G. inflata* and licochalcone A showed different results using the two equations because they inhibited CYP3A4 (midazolam) both reversibly and time-dependently (Table 4). Further investigation, performed herein, confirmed that licochalcone A is a mechanism-based inhibitor of CYP3A4 (midazolam).

Licochalcone A, one of the major chalcones in *G. inflata* (5.24% *w/w*), contributed significantly to the cytochrome P450 enzyme inhibition observed for *G. inflata*. Previously, He *et al.* (2015) reported that licochalcone A strongly inhibited CYP1A2, CYP2C and CYP3A4 (testosterone). In our assay, licochalcone A strongly inhibited CYP2C and CYP3A4 (testosterone), and moderately inhibited CYP1A2, CYP2B6, CYP2D6 and CYP3A4 (midazolam). Importantly, we determined that licochalcone A inactivated CYP3A4 (midazolam) in a mechanism-based manner, although the underlying chemical reaction remains unclear. In a rat study, Choi *et al.* (2014) found that CYP3A4 metabolism of orally administered nifedipine was significantly inhibited by co-administration of licochalcone A at either 2.0 or 10 mg/kg, thereby increasing the serum half-life and the area under the plasma concentration-time curve for nifedipine.

Conclusions

Extracts from each of the three licorice species used most often in botanical dietary supplements, *G. glabra, G. uralensis* and *G. inflata*, were found to inhibit several drug-metabolizing cytochrome P450 enzymes. Several licorice characteristic compounds responsible for inhibiting these enzymes were identified, and among them were the species-specific compounds glabridin, glycycoumarin and more importantly licochalcone A. Because each of the licorice species showed a unique pattern of enzyme inhibition, different drug-botanical interactions may be expected for each. Therefore, it is important that the licorice raw materials used for the production of dietary supplements be botanically identified to the species level with a full disclosure of both the species identity and mode of preparation/extraction on the product labels.

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List of non-standard abbreviations

UHPLC-MS/MS	ultrahigh-pressure liquid chromatography/tandem mass spectrometry

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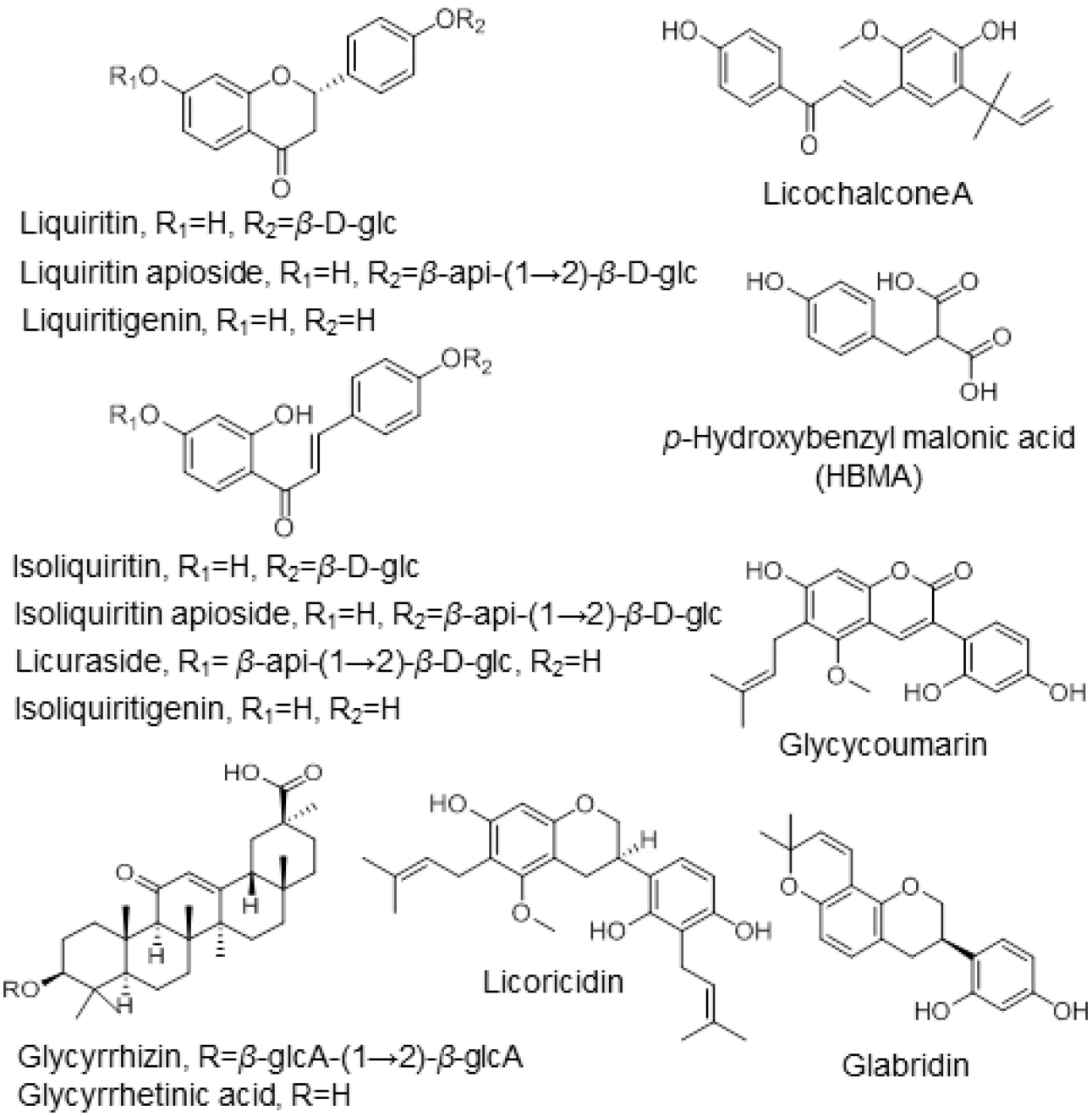

Fig. 1.

Chemical structures of the 14 licorice compounds tested individually for inhibition of cytochrome P450 enzymes.

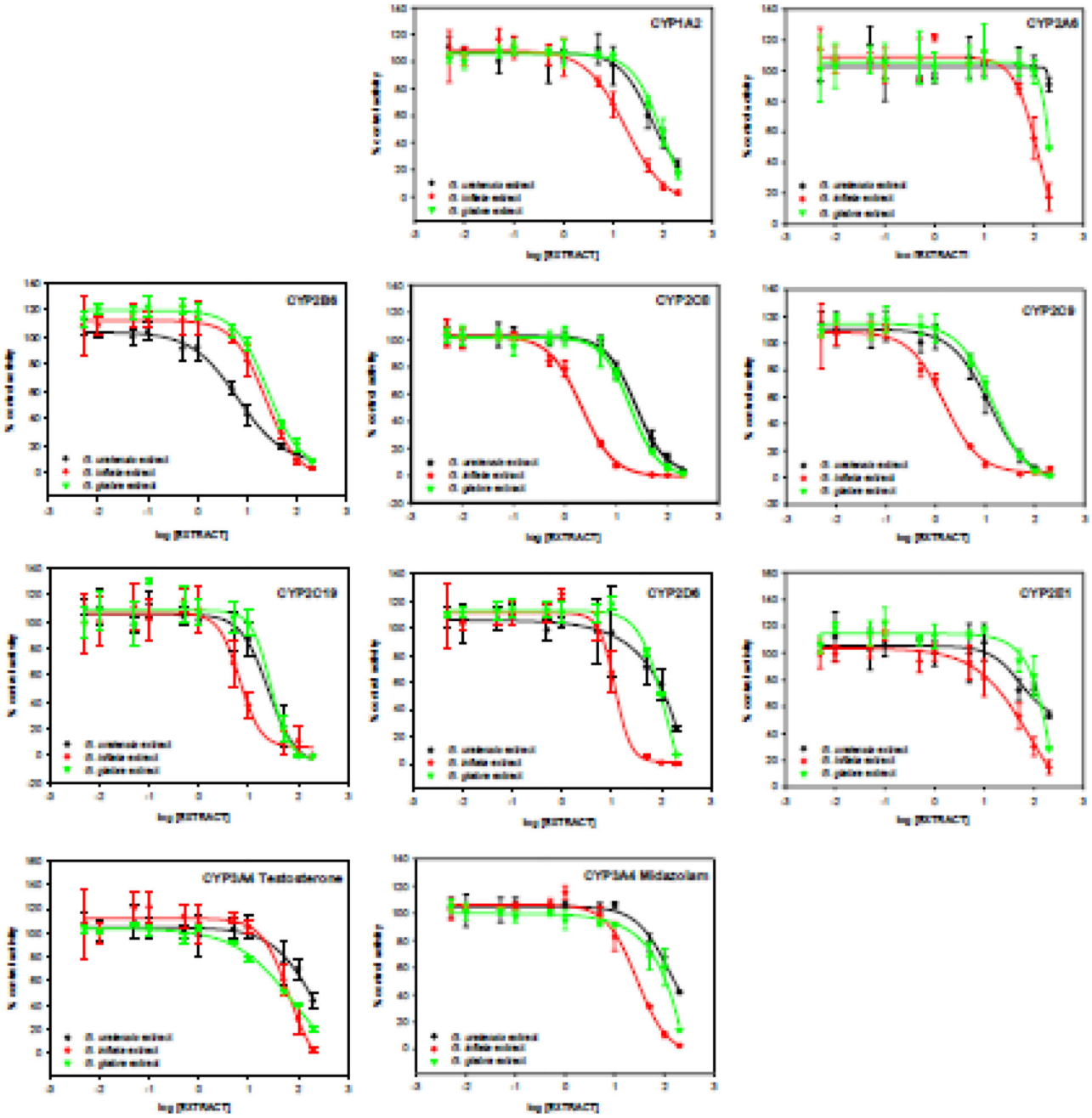

Fig. 2.

Inhibition curves for the inhibition of cytochrome P450 enzymes by extracts *G. uralensis, G. inflata* and *G. glabra* obtained using a cocktail assay. Activity is expressed as a percentage of remaining activity compared with the control containing no inhibitor. Experiments were carried out three times.

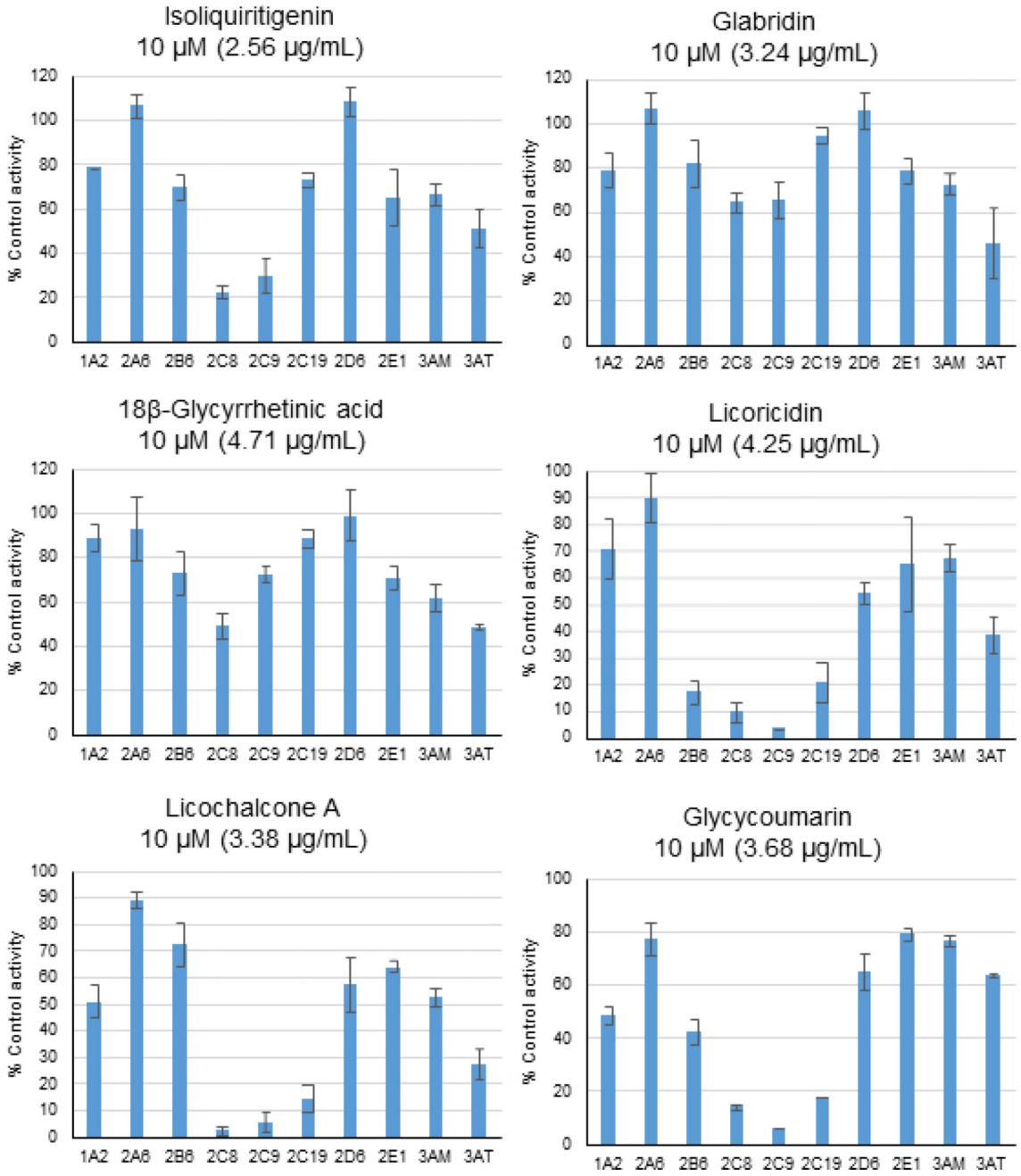

Fig. 3.

Inhibition of CYP enzymes by isoliquiritigenin, glabridin, glycyrrhetinic acid, licoricidin, glycycoumarin, and licochalcone A at 10 μM. Activity is expressed as a percentage of remaining activity compared with the control containing no inhibitor. Experiments were carried out in duplicate. 3AM: CYP3A4 (midazolam); 3AT: CYP3A4 (testosterone).

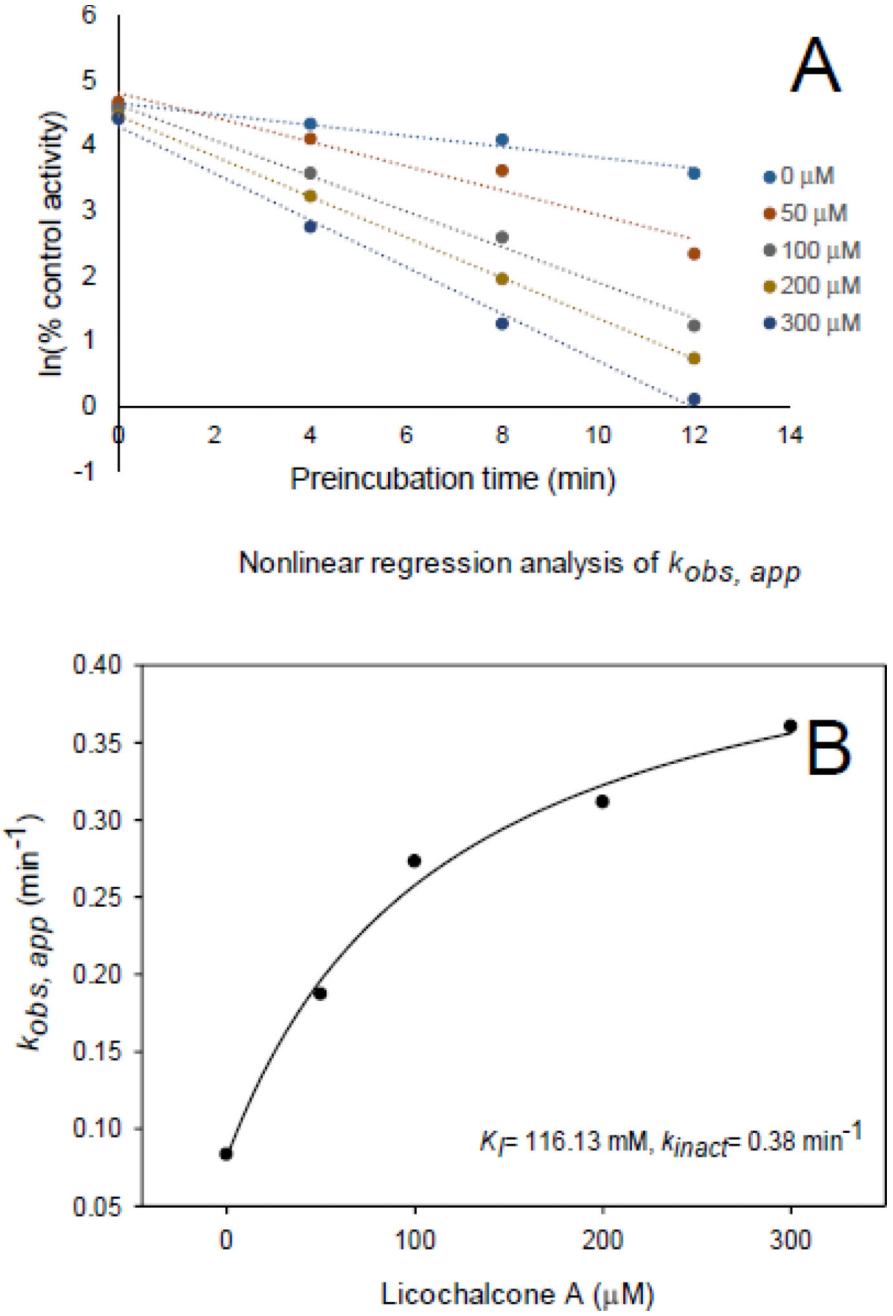

Fig. 4.

Determination of K_I and k_{inact} for inactivation of CYP3A4 (midazolam) by licochalcone A. (A) Time- and concentration-dependent inactivation of CYP3A4 (midazolam) by licochalcone A. (B) Non-linear regression analysis of $k_{obs,app}$. Activity is expressed as a percentage of remaining activity compared with the control containing no inhibitor. Experiments were carried out three times.

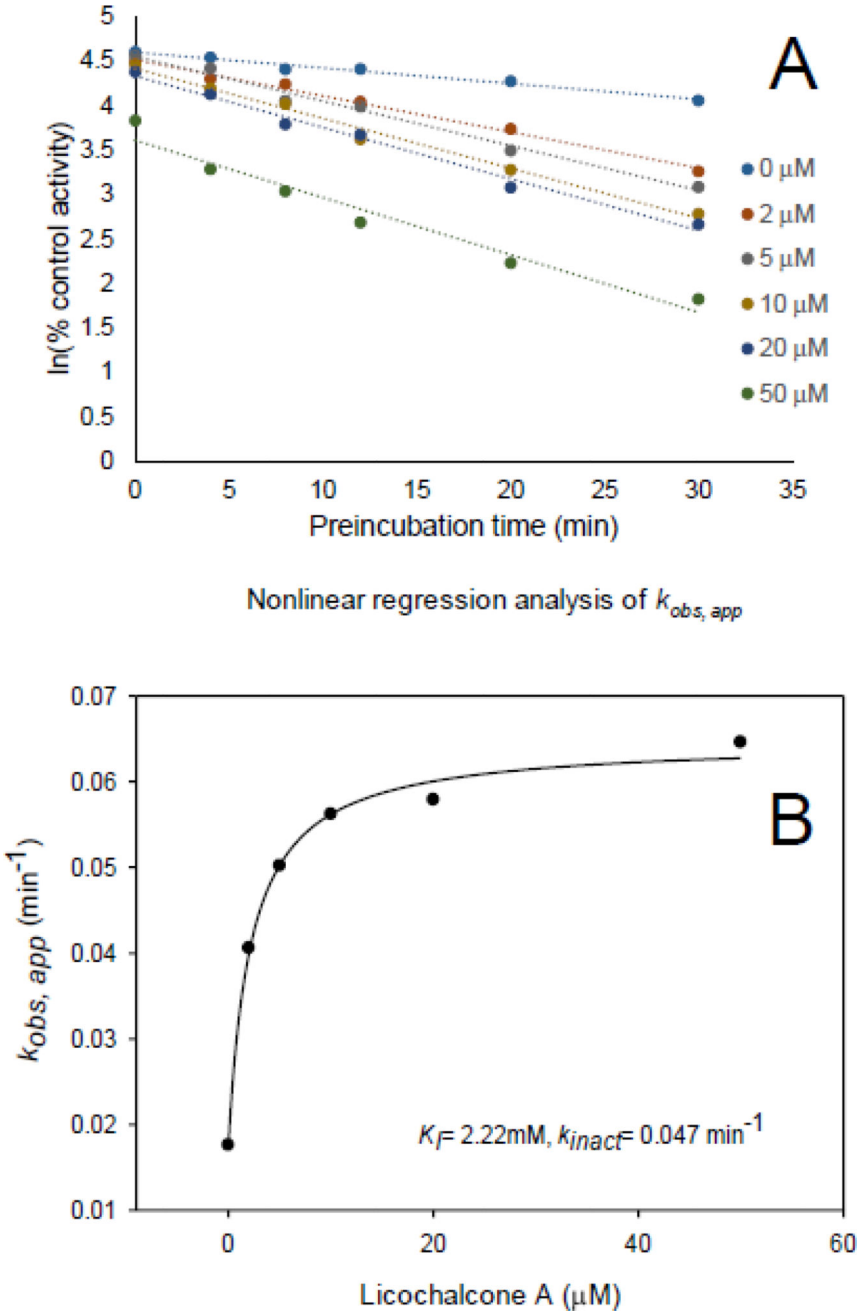

Fig. 5.

Determination of K_I and k_{inact} for inactivation of recombinant CYP3A4 (midazolam) by licochalcone A. (A) Time- and concentration-dependent inactivation of recombinant CYP3A4 (midazolam) by licochalcone A. (B) Non-linear regression analysis of $k_{obs,app}$. Activity is expressed as a percentage of remaining activity compared with the control containing no inhibitor. Experiments were carried out three times.

Table 1

IC_{50} values for inhibition of cytochrome P450 enzymes in human liver microsomes by licochalcone A and extracts of *G. uralensis, G. inflata* and *G. glabra* (mean ± standard deviation).

Enzyme	Substrate	*G. glabra* IC_{50} (μg/mL)	*G. uralensis* IC_{50} (μg/mL)	*G. inflata* IC_{50} (μg/mL)	Licochalcone A IC_{50} (μg/mL)
CYP1A2	Phenacetin	83.04 ± 16.96	68.25 ± 26.75	18.07 ± 4.14	12.40 ± 3.47
CYP2A6	Coumarin	>100	>100	>100	>25
CYP2B6	Bupropion	19.58 ± 1.80	6.51 ± 0.97	23.89 ± 4.55	17.96 ± 4.58
CYP2C8	Amodiaquine	17.06 ± 1.54	24.03 ± 3.00	2.08 ± 0.15	1.05 ± 0.08
CYP2C9	Tolbutamide	12.36 ± 1.14	12.65 ± 2.57	1.50 ± 0.19	0.78 ± 0.09
CYP2C19	(S)-Mephenytoin	19.68 ± 2.70	25.46 ± 5.12	6.75 ± 0.96	3.60 ± 0.79
CYP2D6	Dextromethorphan	>100	>100	11.73 ± 1.20	17.23 ± 1.68
CYP3A4	Midazolam	>100	>100	15.47 ± 2.19	16.05 ± 1.91
	Testosterone	55.64 ± 18.49	>100	70.22 ± 21.80	6.78 ± 1.27
CYP2E1	Chloroxazone	>100	>100	>100	>25

Table 2

Concentrations (% w/w) of the 14 licorice constituents in each tested licorice extract.

Licorice compound	*G. glabra*	*G. uralensis*	*G. inflata*
	% Weight compound / weight extract		
Liquiritin	0.31	3.83	1.33
Isoliquiritin	0.07	0.33	0.18
Liquiritin apioside	9.10	1.09	7.70
Isoliquiritin apioside	2.52	0.13	1.99
Licuraside	1.25	0.05	0.17
Liquiritigenin	0.07	0.23	0.26
Isoliquiritigenin	0.05	0.07	0.15
Glycyrrhizin	0.55	4.20	1.42
Glabridin	0.46	0	0
Glycyrrhetinic acid	0.03	0.01	0.20
Licoricidin	0	0.08	0
HBMA[a]	3.45	1.88	5.06
Glycycoumarin	0	0.13	0.005
Licochalcone A	0	0	5.24

[a]HBMA = *p*-hydroxy-benzylmalonic acid

Table 3

Percentage of CYP3A4 activity decrease after incubation with licorice extract or single compound using a single point time-dependent inhibition screening assay. Experiments were carried out in duplicate.

	Activity decrease %			
	3A4 (midazolam)		3A4 (testosterone)	
	Equation 1 (Obach *et al.* 2007)	Equation 2 (Atkinson *et al.* 2005)	Equation 1 (Obach *et al.* 2007)	Equation 2 (Atkinson *et al.* 2005)
G. glabra	Not determined	Not determined	−3.1	−6.6
G. inflata	10.8	34.7	−1.6	−2.9
Licochalcone A	23.2	58.0	0.9	1.4
Troleandomycin	75.6	72.8	26.8	28.3

Table 4

Effects of trapping agents on the inactivation of CYP3A4 by licochalcone A.

Assay components		% Control activity[a]
Human liver microsomes	Licochalcone A	100
	Licochalcone A, NADPH	49.5
	Licochalcone A, NADPH, glutathione	38.5
	Licochalcone A, NADPH, superoxide dismutase	46.9
	Licochalcone A, NADPH, catalase	42.4
Recombinant human CYP3A4	Licochalcone A	100
	Licochalcone A, NADPH	42.0
	Licochalcone A, NADPH, glutathione	32.4
	Licochalcone A, NADPH, superoxide dismutase	28.1
	Licochalcone A, NADPH, catalase	38.8

[a] mean of two experiments