Journal of Ethnopharmacology 179 (2016) 356–366

Contents lists available at ScienceDirect

Journal of Ethnopharmacology

journal homepage: www.elsevier.com/locate/jep

Stereoselective metabolism of amygdalin-based study of detoxification of Semen Armeniacae Amarum in the Herba Ephedrae–Semen Armeniacae Amarum herb pair

Shuai Song [a,b,1], Qinhai Ma [a,b,1], Qingfa Tang [a,b], Feilong Chen [a,b], Xuefeng Xing [a,b], Yang Guo [c], Shenshen Guo [a,b], Xiaomei Tan [a,b,**], Jiabo Luo [a,b,*]

[a] School of Traditional Chinese Medical Science, Southern Medical University, Guangzhou 510515, PR China
[b] Guangdong Provincial Key Laboratory of Chinese Medicine Pharmaceutics, Southern Medical University, Guangzhou 510515, PR China
[c] Department of Pharmacy, The Renhe Affiliated Hospital to China Three Gorges University, Yichang 443002, PR China

ARTICLE INFO

Article history:
Received 16 May 2015
Received in revised form
8 September 2015
Accepted 14 December 2015
Available online 21 December 2015

Keywords:
Detoxification
Herb pair
Mahuang
Xingren
Pharmacokinetics

Chemical compounds studied in this article:
d-amygdalin (PubChem CID: 656495)
l-amygdalin (PubChem CID: 441462)
d,l-amygdalin (PubChem CID: 39999)
Prunasin (PubChem CID: 120639)
Ephedrine (PubChem CID: 9294)
Pseudoephedrine (PubChem CID: 7028)
l-prunasin (PubChem CID: 91434)
d-prunasin (PubChem CID: 119033)

ABSTRACT

Ethnopharmacological relevance: The *Mahuang–Xingren* (*MX*) herb pair, the combination of Herba Ephedrae (*Mahuang* in Chinese) and Semen Armeniacae Amarum (*Xingren* in Chinese), is a core component of traditional Chinese medicine formulations used to treat asthma and bronchitis. Although *Xingren* is considered to be toxic, *MX* is widely used in the clinic and has few adverse effects. The mechanism underlying detoxification of *Xingren* by *Mahuang* in *MX* remains unknown and merits investigation.
Aim of the study: To determine the mechanism underlying detoxification of *Xingren* by *Mahuang* in *MX*.
Materials and methods: Acute toxic effects were evaluated in mice after oral administration of *Mahuang*, *Xingren*, and *MX* aqueous extracts. Synergism, additivity, and antagonism were quantified by determining the CI (combination index) and DRI (dose-reduction index), which were calculated by the median effect method. High performance liquid chromatography analysis of bioactive compounds (ephedrine, pseudoephedrine and amygdalin) in aqueous extracts and data from previous pharmacokinetic studies in rats were combined to explore the potential mechanism of toxicity antagonism by the components of *MX*. Moreover, the cytotoxic effects of amygdalin and amygdalin activated by β-glucosidase (including different proportions of L-amygdalin and D-amygdalin) were also investigated.
Results: *Mahuang* prevented and antagonized the acute toxicity of *Xingren* and allowed escalation of the *Xingren* dose. Pearson correlation analysis indicated that the proportion of D-amygdalin was closely correlated with the antagonism of *Xingren* toxicity. The antagonism of its acute toxicity was primarily attributed to stereoselective metabolism of amygdalin. Interestingly, the process was facilitated by *Mahuang*, which led to reduced levels of the D-prunasin *in vivo* and thus reduced toxicity. Furthermore, the mechanism was also evaluated by testing the cytotoxicity of amygdalin. Metabolism of D-amygdalin was a major cause of cytotoxicity and no stereoselective metabolism occurred in culture medium.
Conclusions: A comprehensive study of *Xingren* detoxification in the context of the *MX* combination suggested that stereoselective metabolism of amygdalin facilitated by *Mahuang* may be the crucial mechanism underlying detoxification of *Xingren* in the *MX* combination. Therefore, *Mahuang* acts to enhance and control the effects of *Xingren* in the *MX* combination. These results illustrate the rationale behind the combination of *Mahuang* and *Xingren*.

1. Introduction

Herb pairs, consisting of 2 herbs prescribed together to decrease toxicity and increase therapeutic efficacy, are the most fundamental and simplest form of multi-herb therapy. Herb pairs are based on established principles known as the "seven compatibilities": single action, mutual reinforcement, mutual assistance, mutual restraint, mutual detoxification, mutual inhibition and mutual antagonism (Wang et al., 2012). Although composed of only 2 herbs, herb pairs are the basis of more complex multi-herb formulations and possess most of the basic therapeutic features of such derived formulations (Liu et al., 2012). In traditional Chinese medicine (TCM), Herba Ephedrae (*Mahuang* in Chinese, the dried herbaceous stems of *Ephedra sinica Stapf.*) and Semen Armeniacae

* Correspondence at: School of Traditional Chinese Medical Science, Southern Medical University, 1838 North Guangzhou Avenue, Guangzhou 510515, PR China. Tel./fax: +86 20 61648266.
** Co-corresponding author.
E-mail address: ljb@smu.edu.cn (J. Luo).
[1] These authors contributed equally to this work.

http://dx.doi.org/10.1016/j.jep.2015.12.019
0378-8741/

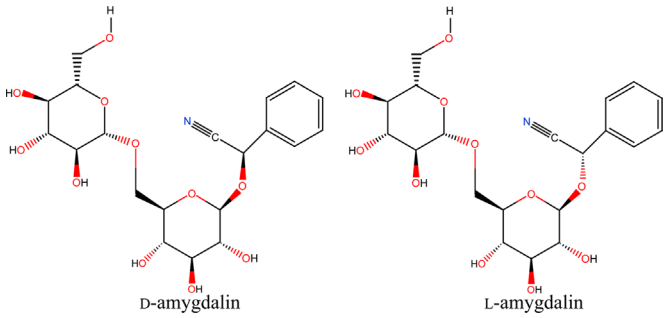


Fig. 1. Chemical structure of amygdalin.

Amarum (*Xingren* in Chinese, the dried, ripe seeds of *Prunus armeniaca* L. var. *ansu* Maxim.) constitute an herb pair known as *Mahuang-Xingren* according to the principles of mutual reinforcement and mutual assistance, which is used to treat asthma and bronchitis (Li et al., 2010).

Ephedrine (E) and pseudoephedrine (PE) are the main bioactive compounds of *Mahuang*. In contrast to the ephedra-containing dietary supplements used in the United States, *Mahuang* has been used widely without significant adverse effects under proper TCM medical supervision (Mehendale et al., 2004). However, *Xingren* is designated as a toxic herb by *Shennong's Classic of Materia Medica*. In 2014, a case of fatal cyanide poisoning secondary to *Xingren* use as an anticancer supplement was reported in Changshu City, Jiangsu Province, China. (China Jiangsu, 2014). Amygdalin (Fig. 1), a major effective component of *Xingren* and a source of its toxicity, can be metabolized into cyanide, leading to fatal cyanide poisoning, especially after oral administration (Park et al., 2013). Although the beneficial effects of *Xingren* (or amygdalin) as a treatment for cancer are controversial (Milazzo et al., 2011; Moertel et al., 1982), it is the most popular single herb used to treat asthma in clinics in China (Huang et al., 2013). As a classical TCM combination, *MX* is a core component of many herbal prescriptions and Chinese patent drugs for asthma and cough, due to the synergistic efficacy of its components and its few associated side effects (Wang et al., 2013; Zeng and Jiang, 2010). The bioactive constituents (Ma et al., 2014; Shu et al., 2012), pharmacodynamics (Zhang et al., 2009) and mechanism of action (Li et al., 2010) of the *MX* have been investigated, but a comprehensive study of *Xingren* detoxification in the context of the *MX* combination has not been performed. Providing a scientific explanation for *Xingren* detoxification would promote understanding of its safe application and provide a rational basis for herb-pair theory in TCM. Therefore, we hypothesized that *Xingren* detoxification is achieved by the principle of "mutual restraint" (or "mutual detoxification") underlying its synergistic anti-asthma effects.

We conducted several experiments using *MX* to test our hypothesis: (a) synergism and antagonism of the acute toxic effects of orally administered *Mahuang*, *Xingren*, and *MX* extracts were evaluated by the median effect method; (b) high performance liquid chromatography (HPLC) analysis of the bioactive compounds (E, PE, and amygdalin) in *Mahuang*, *Xingren* and *MX* aqueous extracts was performed to explain the *Xingren* detoxification mechanism *in vitro*; (c) a pharmacokinetics study in rat was performed in rats to explore the potential mechanism of antagonism of *Xingren* toxicity *in vivo*; (d) finally, the cytotoxic effects of amygdalin and amygdalin activated by β-glucosidase (including different ratios of D-amygdalin) were also investigated. In previous studies, we developed and validated a UPLC-MS/MS method and applied it to study the comparative pharmacokinetics of *MX*. Stereoselective metabolism of amygdalin was found in SD rats after a single oral administration of the *MX* extract (Song et al., 2015). This phenomenon led to our interest in performing a comprehensive study to determine whether stereoselective metabolism *in vivo* is associated with *Xingren* detoxification.

In the present study, a comprehensive detoxification study of *Xingren* in *MX* herb pair was performed for the first time. Assays of potential herb–herb interactions that may alter acute toxicity, quantitative analysis of the bioactive compounds in the extracts, evaluation of *in vivo* pharmacokinetics and cytotoxicity tests were combined to investigate the possible mechanisms of *Xingren* detoxification. Moreover, preliminary evidence indicated that stereoselective metabolism of amygdalin may play a key role in *Xingren* detoxification upon oral administration of *MX* extract.

2. Materials and methods

2.1. Chemicals and reagents

Standards for E hydrochloride, PE hydrochloride, D-amygdalin and benzaldehyde (purity, ≥99%) were provided by the National Institutes for Food and Drug Control (Beijing, China). D-Prunasin (purity, ≥96%) was purchased from BioBioPha Co., Ltd. (Kunming, China). HPLC-grade acetonitrile, methanol (Merck, KGaA, Darmstadt, Germany) and phosphoric acid (Kermel Chemical Reagent Co., Ltd.) were used for HPLC analysis. Distilled deionized water was freshly generated using a MilliQ Ultra-Pure Water System (Millipore, Billerica, MA, USA).

2.2. Plant materials and extracts preparation

Semen Armeniacae Amarum (*Xingren* in Chinese, the dried, ripe seeds of *Prunus armeniaca* L. var. *ansu* Maxim.; including endocarp; Gansu Province; batch number: 20130901) and Herba Ephedrae (*Mahuang* in Chinese, the dried herbaceous stems of *Ephedra sinica* Stapf.; Inner Mongolia Autonomous Region; batch number: 20110901) were purchased from Zisun Medicinal Material Co., Ltd. (Guangzhou, China). The plant materials were authenticated by Professor Ji Ma (Department of Chinese Medicine Authentication, School of Traditional Chinese Medical Science, Southern Medical University, Guangzhou, China). The plant names were checked with www.theplantlist.org on April 14th, 2015.

Because the proportions of herbs used in *MX* prescriptions in TCM clinics vary, the assays were performed using different ratios of the *MX* constituents. According to an extraction method described in the *Formula of Traditional Chinese Medicine*, *Mahuang* (90 g) and *Xingren* (90 g) were immersed in 1440 mL water (1:8, weight/volume) for 30 min and decocted twice for 60 min. The solution was filtered and concentrated to obtain the *MX* (1:1) decoction. The obtained solution was concentrated to 100 mL using a rotary evaporator and stored at −20 °C overnight. The frozen decoction was freeze-dried using a lyophilizer (Labconco, Kansas City, MO, USA) to obtain the lyophilized powder form of the extract. The lyophilized *MX* (1:1) extract powder was stored at −20 °C. The same procedure was followed for the preparation of the single herb, *MX* (4:1), *MX* (2:1), *MX* (1:2), and *MX* (1:4) extracts.

2.3. Acute toxicity study

2.3.1. Animals

Adult male and female Kunming mice, weighing 18–22 g, were obtained from the Lab Animal Center of Southern Medical University (certificate: SCXK (Guangdong Province) 2011-0015, No. 44002100001782). Animal subjects were housed at the SPF Animal Center of The First Affiliated Hospital to Southern Medical University (Guangzhou, China) according to the regulations of the animal care committee. The animals were housed in stainless steel

cages with a 12-h light cycle (7:00 AM–7:00 PM). The temperature was maintained at 21.5–25.5 °C with a relative humidity of 47.4–70.3% and 6–20 air changes/h.

2.3.2. Experimental design

The experimental protocol was approved by the Animal Ethics Committee of Southern Medical University. The animal studies were designed according to *Guidance for Acute Toxicity of TCM and Natural Medicine* (China Food and Drug Administration, 2005).

Mice were acclimatized to the facilities and environment for 5 days before the experiments. Tap water and food were readily accessible to the mice throughout the study. In this method, the dose levels of the extracts were detected using acute oral toxicity pretests to obtain the minimal effective dose and maximal effective dose. Mice (N=360) were randomly assigned to 8 groups, including *Mahuang*, *Xingren*, *MX* (4:1), *MX* (2:1), *MX* (1:1), *MX* (1:2), *MX* (1:4) and control groups (5 dose levels in each group; 5 males and 5 females per dose level). Lyophilized powder samples were reconstituted with distilled deionized water and 5 dose levels were obtained by geometric dilution (data for each group shown in Supplementary table 1). After being fasted for 12 h, animals were administered a single dose of one of the extracts at 0.35 mL/10 g via oral gavage. Animals found in a moribund condition and animals showing severe pain or enduring signs of severe distress were humanely killed.

2.3.3. Data analysis

General behaviors were continuously monitored for 5 h after dosing, and each mouse was submitted to necroscopic examination immediately after death. Body weights were recorded before, during, and after the test. Mortalities in 24 h were used for herb combination analysis by the median-effect method. Combination index (CI) and dose-reduction index (DRI) were plotted as a function of the affected fraction (Fa), which represented the mortality (Fig. 2A). This plot allows quantification of synergism (CI < 1), antagonism (CI > 1) and additive effects (CI=1), as well as how much the dose of each drug in a combination may be reduced at a given effect at various dose levels. D_m, CI and DRI were calculated using CalculSyn 2.0 (Biosoft, Cambridge, UK) based on the median-effect method (Chou, 2011). The linear correlation coefficient (r) of the median-effect plot is considered to be a measure of the data conformity according to the mass-action law principle when an experimental measurement is assumed to be accurate. An r-value of 1 indicates flawless conformity, while a low value may be the result of biological variability or experimental deviation.

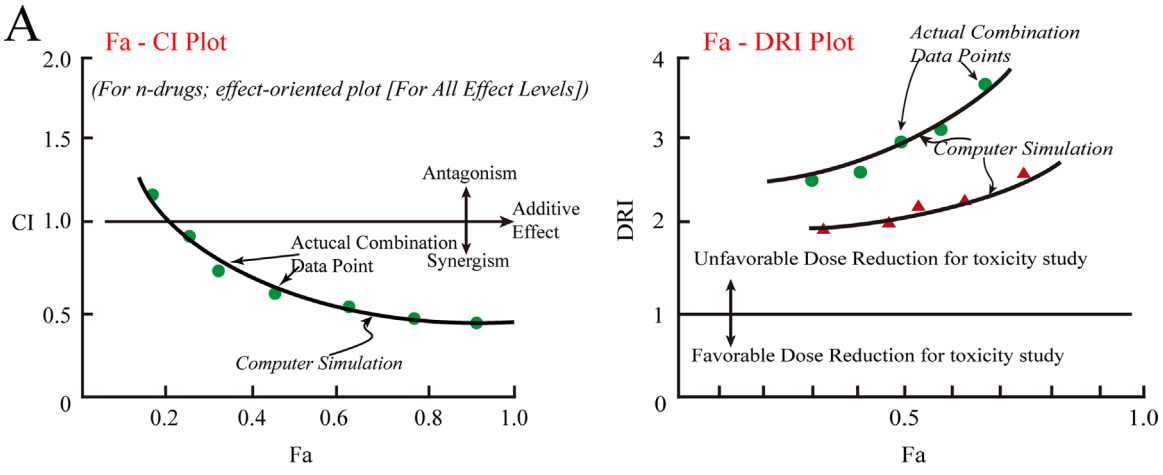
2.4. HPLC Analysis of the extracts

2.4.1. Sample preparation for HPLC analysis

The lyophilized powders of *Mahuang*, *Xingren* and *MX* (equivalent to 3.00 g raw material) were dissolved separately in 50 mL deionized water and solubilized by ultrasonic treatment for 5 min. Next, 10 mL of each suspension was diluted to a final volume of 25 mL with methanol. The solutions were shaken by hand to mix them well and left to stand overnight. The supernatants were transferred to 1.5 mL Eppendorf tubes and centrifuged at 17,760*g* for 10 min. The final supernatant was filtered through a 0.22 μm syringe filter before use. The resulting solutions (2 μL each) were injected into the LC system for quantitative analysis.

2.4.2. Instruments and conditions

Extracts and quality control samples were analyzed using an Agilent 1200 Series HPLC System (Agilent Technologies, Cary, NC, USA) equipped with a Proshell 120 EC-C18 column (2.1 mm × 150 mm, 2.7 μm; Agilent Technologies) and a photodiode array detector. Acetonitrile and 0.15% phosphoric acid aqueous solution were used as mobile phases A and B, respectively, with a flow rate of 0.5 mL/min at 25 °C. The solvent gradient was: 4% A at 0–1.0 min; 4% A→6% A at 1.0–6.0 min; 6% A→8% A at 6.0–



B

$$CI = \frac{D_1}{(Dx)_1} + \frac{D_2}{(Dx)_2} = \frac{1}{(DRI)_1} + \frac{1}{(DRI)_2}$$

$(Dx)_i$: doses of each drug "alone" that gives x% effect;

Di : doses of each drug in combination that also gives x% effect.

Fig. 2. Diagnostic plots and algorithms for drug combination studies based on median effect method. (A) The typical quantitative diagnostic graphics generated by the computer simulation: the classic Fa (fraction affected)-CI (combination index) plot and the Fa-DRI (dose-reduction index) plot for the constant ratio combination design. (B) CI equation and its rearrangement.

8.0 min; 8% A at 8.0–13.0 min; 8% A→10% A at 13.0–15.0 min; 10% A→12% A at 15.0–19.0 min; 12% A at 19.0–22.0 min; 12% A→15% A at 22.0–25.0 min; 15% A→4% A at 25.0–35.0 min. Analytes were monitored at 215 nm. The data were analyzed using Agilent Chemstation software for LC-3D system (Rev. B.04.03-SP1).

2.4.3. Analytical method validation

The method described herein was validated for selectivity, linearity, precision, accuracy, recovery and stability according to the Guideline for the Analytical Method Validation in Pharmaceutical Analysis (Chinese Pharmacopoeia Commission, 2010). Intra-day (within a single day) and inter-day (over 3days) precisions were determined and expressed as relative standard deviation (RSD). A method is considered precise, if the RSD is within ±3%. Instrumental repeatability was assessed by analyzing the same sample (196.2 μg/mL E, 62.7 μg/mL PE and 243.4 μg/mL amygdalin) 7 times consecutively. The accuracy was evaluated by the recovery test. By adding known quantities of the mix standard solutions to the sample solution at three different concentration levels, recovery of each compound was calculated by comparing the amount subtracted before spiking from that after spiking with the actual amount added. The method is considered accurate if the RE is within ±3%. Stability was evaluated by comparing *MX* samples stored for 0, 3, 6, 12 and 24 h with fresh samples and expressed as RSD.

2.4.4. Determination of bioactive compounds

Three portions of working extract were prepared for each type of extract. Two samples were obtained from each type of working extract according to procedures described in "*Sample preparation for HPLC analysis*". The external standard method was used for the quantitative determination of E, PE and amygdalin (including L-amygdalin and D-amygdalin). The content of each compound was expressed as extraction efficiency (ratio of compound transferred from the herb into the extract).

2.4.5. Correlation analysis of potential bioactive compounds and acute toxicity

Due to the complexity of the compounds in TCM formulations, it is difficult to elucidate the specific mechanism underlying their acute toxic effects. Pearson correlation analysis was used to assess the relationships between differences in bioactive compounds and CI.

2.5. Pharmacokinetics analysis

As described in detail in our previous work, a comparative pharmacokinetics studies in rats were performed after oral administration of *Mahuang*, *Xingren*, and *MX* extracts (Song et al., 2015). Specific pathogen-free male Sprague–Dawley rats (200 ± 20 g) were supplied by the Lab Animal Center of Southern Medical University (certificate No. 44002100002402). The experimental protocol was approved by the Animal Ethics Committee of Southern Medical University, and animal studies were performed according to the Guide for the Care and Use of Laboratory Animals. In brief, SD rats were randomly divided into 3 groups (6 rats per group): a *Mahuang* group, a *Xingren* group, and a *MX* (1:1) group. Rodents in the *Mahuang* group were administered an oral dose of 3 g/kg (equal to the herbal preparation extracted from 3 g herb materials) *Mahuang*, *Xingren*, and *MX* extracts. Blood samples were collected from the retro-orbital plexus at designated time points after dosing. The plasma samples, pretreated by a one-step direct protein precipitation with acetonitrile, were analyzed by our validated UPLC-MS/MS method (Song et al., 2015). A non-compartmental analysis was adopted in this study, and DAS software version 3.2.2 (Mathematical Pharmacology Professional Committee of China, Shanghai, China) was used to calculate the pharmacokinetic parameters. The previous pharmacokinetics studies demonstrated that stereoselective metabolism of amygdalin occurred *in vivo*. The present study together with our previous work further highlights the correlation between stereoselective metabolism of amygdalin and *Xingren* detoxification. Plasma concentrations of prunasin (the primary metabolite of amygdalin) were reanalyzed in terms of the ratio of L-prunasin to D-prunasin to better understand the decrease in *Xingren* toxicity.

2.6. Cytotoxicity test

2.6.1. Cell culture and materials

β-Glucosidase (lyophilized powder, ≥6 U/mg) was obtained from Sigma-Aldrich Chemical (St. Louis, MO, USA). MDCK (Madin-Darby canine kidney cell line) cells and PC12 (rat pheochromocytoma cell line) cells were obtained from Type Culture Collection of the Chinese Academy of Sciences (Shanghai, China). The cells were cultured in Dulbecco's modified Eagle's medium-Ham's F-12 nutrient mixture (1:1) containing 10% fetal bovine serum at 37 °C in a humidified atmosphere of 5% CO_2–95% air.

2.6.2. Experimental design

The D-amygdalin content of herbal extracts varies greatly in response to boiling water and combination with *Mahuang* (Hwang et al., 2002; Isoza et al., 2001; Xie et al., 2012). In the intestine and liver, enzyme complexes containing β-glucosidase can degrade amygdalin into many different metabolites (Berrin et al., 2002). Therefore, amygdalin cytotoxicity was evaluated in terms of different chemical configurations. Similar to the decocting of herbs, epimerization of amygdalin was carried out as follows: D-amygdalin stock solutions (150 mM) were placed in tubes in a boiling water bath for 0 min, 4 min, 8 min, or 25 min to obtain a. 100%, b. 81.9%, c. 68.8% and d. 49.9% D-amygdalin respectively (ratios calculated by D-amygdalin/L-amygdalin + D-amygdalin). Working concentrations were obtained by gradual dilution with culture medium. Growth inhibition assays were carried out with MDCK and PC12 cells in the presence of different proportions of D-amygdalin to determine whether changing the proportion of D-amygdalin affected the cytotoxic activity. Moreover, in order to stimulate its degradation after oral administration, growth inhibition by amygdalin activated by β-glucosidase was also investigated. Therefore, the objective of the current study was to examine the effects of amygdalin and amygdalin activated by β-glucosidase at different configuration ratios on proliferation of PC12 and MDCK cells. The experimental protocol is shown in Fig. 3A.

2.6.3. Cell proliferation assay

Cell proliferation was measured using the Cell Counting Kit-8 (CCK-8, Dojindo Laboratories, Japan). For the assays, the cells (1×10^4 cells/well in 100 μL of culture medium) were seeded into 96-well plates and left to adhere overnight, after which medium were removed and replaced by 90 μL of different concentrations of the samples and 10 μL β-glucosidase solution (1 mg/ mL) (or blank culture medium). After 48 and 72 h of incubation, the media were replaced with 90 μL of serum-free culture medium. Subsequently, 10-μL CCK-8 was added prior to incubation in the dark at 37 °C for 1 h. The absorbance was determined using Infinite M200 plate reader (Tecan Research Triangle Park, NC, USA). The concentration of compound required to inhibit proliferation by 50% (IC_{50}) was determined by plotting the percentage of cell growth inhibition against the compound concentration. All experiments were repeated 3 times, and the results are expressed mean ± SEM (standard error of mean).

2.6.4. Characterization of enzymatic hydrolysis time profile

Qualitative analysis of enzymatic hydrolysis time profiles was performed by HPLC. PC12 cells at 80% confluence were exposed to 5.0 mM amygdalin and amygdalin activated by β-glucosidase (at

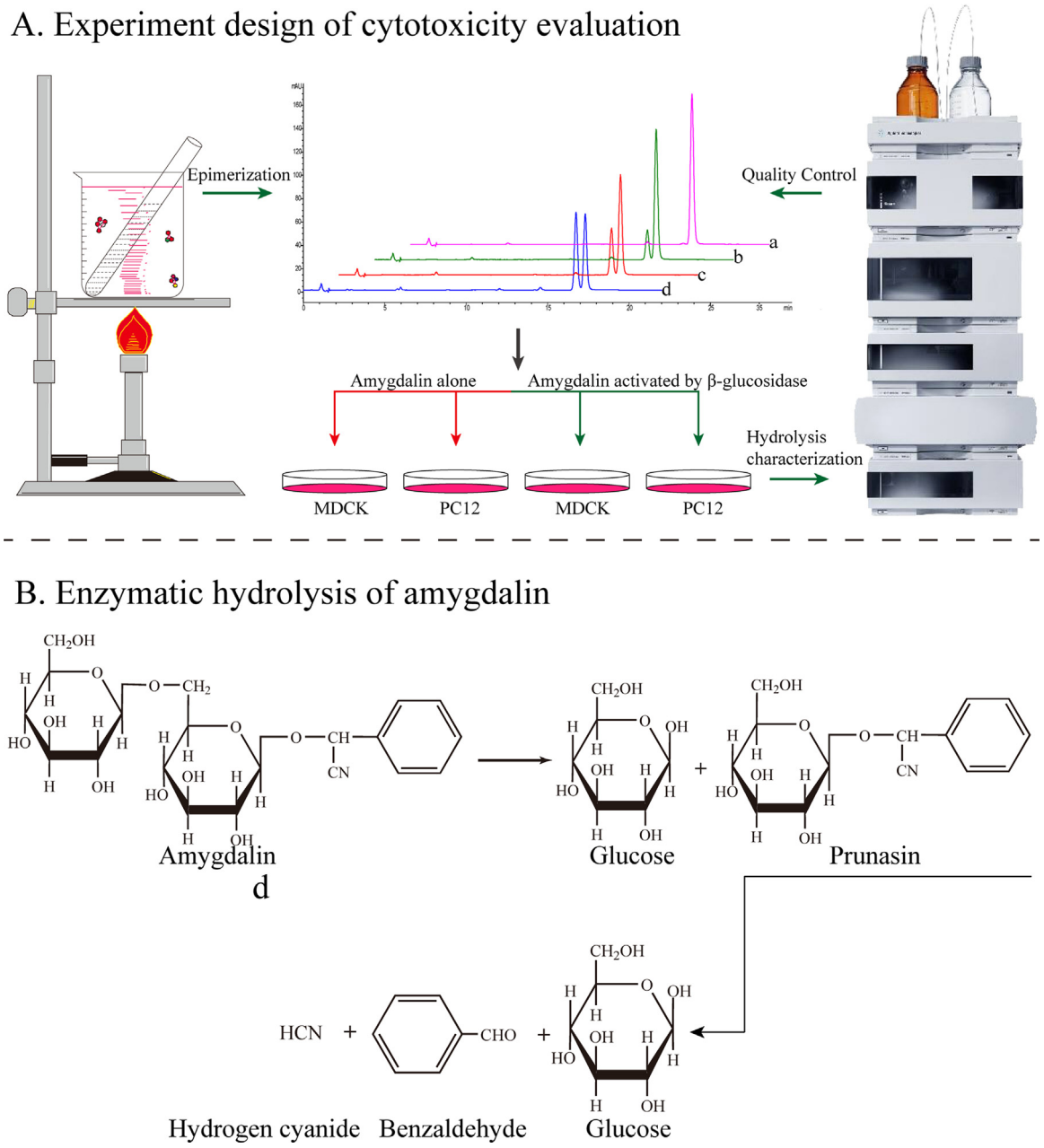


Fig. 3. Graphical protocol of cytotoxicity evaluation (A) and hydrolysis process of amygdalin (B).

different configuration ratios) for 0, 60, 120 and 240 min. Culture medium samples (500 μL) were collected at the indicated times and filtered through a 0.2-μm Acrodisc 25 mm syringe filter. The enzyme reaction was terminated by the addition of 1500 μL of ice-cold acetonitrile. Each sample was vortexed for 5 min and centrifuged at 17,760*g* for 15 min. The supernatant was filtered through a 0.22 μm syringe filter and dried under a gentle nitrogen stream. The residue was reconstituted in 5000 μL of 50% methanol and centrifuged at 20,385*g* for 20 min at 4 °C. The supernatant was obtained and analyzed by HPLC based on the method described in "*2.4.2. Instruments and conditions*".

2.7. Statistical analysis

The results (except cytotoxicity tests) are presented as mean ± SD. The data were analyzed by one-way analysis of variance (ANOVA) followed by least-significant difference post-hoc (LSD) tests. Pearson's test was applied to assess the correlations between the constituents of amygdalin and combination analysis of acute toxicity. Statistical analysis was performed using SPSS software (version 16.0, IBM Corp., Armonk, NY, USA). A probability value of $P < 0.05$ was considered to be statistically significant.

3. Results and discussion

3.1. Acute toxicity study

3.1.1. Obvious antagonistic effect of MX

In the *Mahuang*, *MX* (4:1) and *MX* (2:1) groups, death occurred 10–40 min after administration. Toxicity was manifested predominantly as profuse sweating, Straub tail reaction, trembling, convulsions, and spasms. No obvious toxic signs were observed in the *MX* (1:1), *MX* (1:2), *MX* (1:4) and *Xingren* groups within 50 min

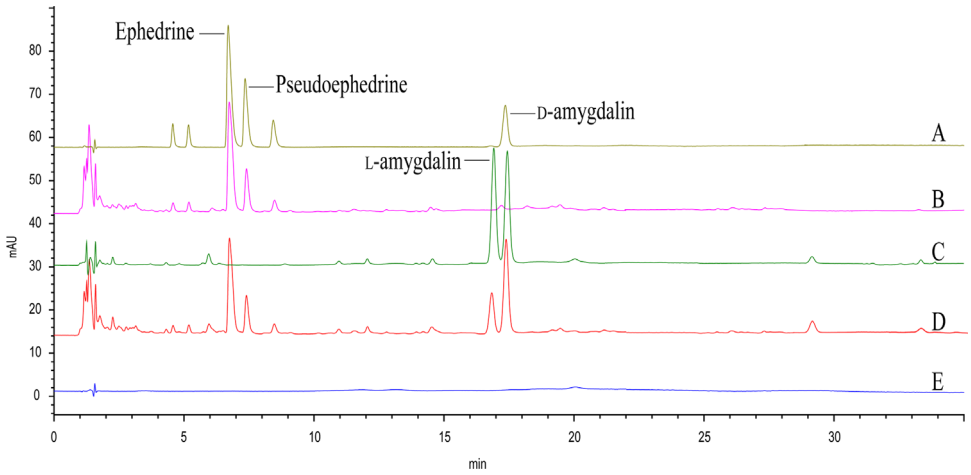


Fig. 4. Representative HPLC-DAD chromatograms of mixed standards (A), *Mahuang* (B) *Xingren* (C), *Mahuang-Xingren* herb pair (1:1) (D) and blank (E). Ephedrine, pseudoephedrine and amygdalin were used to quantify the aqueous extracts.

of administration of the extracts; however, dramatic changes occurred within the period from 60 to 100 min after administration of the extracts, including lying prostrate, catalepsy, shortness of breath, respiratory distress, opisthotonos and death. The major organs showed no obvious lesions at necropsy under observation by the naked eye (data not shown) and there were no significant changes in body weight during the experiment in the surviving mice (Supplementary table 2). The median lethal dose (LD_{50}) and 95% confidence intervals of the extracts in mice after oral administration are summarized in Table 1. The results indicated that *Xingren* was more toxic than *Mahuang* and demonstrated that the safety profile of the *MX* combination had been improved significantly in comparison with that of *Xingren* alone (the *MX* treatments had LD_{50} concentrations greater than that of *Xingren*). Using median effect analysis, we explored *MX* interactions by testing the combination with different ratios of components. In all groups, *r*-values were between 0.962 and 0.998, indicating that the data conformity were acceptable. Obvious antagonistic effects with high CI_{50} (CI value at EC_{50}) values were observed when the acute toxicity of the *MX* combinations was tested (Table 1 and Supplementary Fig 1). Moreover, the results of these experiments were consistent with the herb combination ratios: higher proportions of *Mahuang* were associated with stronger antagonistic effects.

3.1.2. Antagonistic action mainly led to the favorable DRI of Xingren

Rearrangement of the equation for CI also provides algorithms for solving the DRI for each drug in a particular combination (Fig. 2B). A DRI greater than 1 indicates favorable dose reduction that can reduce toxicity in therapeutic applications (Chou, 2011). Conversely, in toxicity study, a DRI less than 1 indicates a higher tolerance that increases the therapeutic index. After combined treatment in mice, the DRI_{50} (DRI at LD_{50}) of *Xingren* was decreased by 7.94- to 1.36-fold (except *MX* 1:4) and the DRI_{50} of *Mahuang* was increased by 1.57–1.06-fold when compared with the two herbs taken individually (Table 1). Generally speaking, the results of the combination analysis suggested that improvement of the *MX* safety profile was mainly due to *Xingren* detoxification. *Mahuang* could antagonize the acute toxicity of *Xingren* and significantly decrease the DRI_{50} of *Xingen*.

3.2. HPLC analysis of the extracts

3.2.1. Validation of the HPLC method

A reliable HPLC method was developed and validated to simultaneously determine E, PE, and amygdalin. The calibration curves for all analytes exhibited good linearity ($r^2 > 0.999$, Supplementary table 3). The LOQs of E, PE, and amygdalin were 10.1 μg/mL, 5.05 μg/mL, and 11.1 μg/mL, respectively. Representative chromatograms of the blank sample, standards, *Mahuang*, *Xingren* and *MX* (1:1) solutions are shown in Fig. 4 (chromatograms of *MX* of different combination ratios shown in Supplementary Fig. 2). No peaks that could interfere with the measurements of the studied compounds were observed. L-Amygdalin and D-amygdalin were characterized by high-resolution electron

Table 1
Mahuang –*Xingren* (*MX*) combination induced an antagonistic effect on acute toxicity compared to treatment with herbs oral administered individually as demonstrated by median drug effect analysis calculating the combination index (CI) and the dose-reduction index (DRI).

	D_m value (g/kg)		Combination index		DRI_{50}		*r*
Group	LD_{50}	95% confidence intervals	CI_{50}	95% confidence intervals	*Mahuang*	*Xingren*	
Mahuang	93.2	85.2–102.0	–	–	–	–	0.970
Xingren	29.9	25.3–35.3	–	–	–	–	0.962
MX (4:1)	87.9	81.7–94.5	8.57	6.90–10.3	1.57	0.13	0.980
MX (2:1)	81.6	77.9–85.3	5.01	4.28–5.74	1.44	0.23	0.996
MX (1:1)	81.4	75.5–87.9	3.60	3.07–4.13	1.14	0.38	0.991
MX (1:2)	64.6	62.9–66.3	2.24	1.98–2.50	1.14	0.73	0.998
MX (1:4)	59.3	52.6–66.9	1.68	1.48–1.87	1.06	1.36	0.978

D_m: Median-effect dose or concentration.
LD_{50}: Median lethal dose.
CI_{50}: CI at LD_{50}.
DRI_{50}: DRI at LD_{50}.
r: The linear correlation coefficient of the median-effect plot.

Table 2
Precision, accuracy and stability of HPLC method for determination of analytes.

Analytes	Spiked conc. (μg mL^{-1})	Observed conc. (μg mL^{-1})	Precision (RSD%)		Accuracy (RE%)		Instrument	Stability
			Intraday	Interday	Intraday	Interday	Repeatability RSD (%)	RSD (%)
Ephedrine	40.4	236.8 ± 1.1	2.7	2.6	0.12	0.75		
	101	296.6 ± 2.6	2.4	2.8	−0.56	−0.59	0.99	2.22
	253	449.6 ± 2.7	0.9	1.2	0.14	0.19		
Pseudoephedrine	20.2	82.9 ± 0.5	2.7	2.7	−0.75	0.02		
	50.5	113.4 ± 1.1	2.4	2.2	0.53	0.17	0.87	1.92
	126.5	189.1 ± 2.0	1.3	1.8	−0.24	0.51		
Amygdalin	22.2	265.6 ± 0.6	2.6	2.7	−0.18	0.06		
	88.8	333.7 ± 2.2	2.4	2.5	1.53	2.20	0.50	2.06
	555	801.3 ± 6.0	1.1	1.1	0.52	0.64		

RSD: relative standard deviation RE: relative error.

Table 3
Contents of the three bioactive compounds in *Mahuang*, *Xingren*, and *Mahuang-Xingren* (*MX*).

Group	Compound[a] (mean ± SD)				
	Ephedrine	Pseudoephedrine	L-amygdalin	D-amygdalin	Amygdalin[b]
Mahuang	6.69 ± 1.16	2.02 ± 0.42	–	–	–
Xingren	–	–	5.62 ± 0.36	6.15 ± 0.45**	11.77 ± 0.72
MX (4:1)	7.50 ± 0.53	2.38 ± 0.18	2.75 ± 0.20**	10.08 ± 0.50**	12.83 ± 0.70
MX (2:1)	8.00 ± 0.54	2.43 ± 0.22	2.97 ± 0.34**	9.87 ± 1.23**	12.84 ± 1.52
MX (1:1)	8.23 ± 0.30*	2.56 ± 0.13*	3.69 ± 0.10**	10.47 ± 0.89**	14.16 ± 0.98**
MX (1:2)	7.26 ± 1.05	2.10 ± 0.31	3.47 ± 0.48**	7.91 ± 0.74**	11.38 ± 1.20
MX (1:4)	5.57 ± 0.64	1.66 ± 0.27	3.48 ± 0.44**	6.72 ± 0.52	10.19 ± 0.94

[a] The content unit was mg/g that expressed as extraction efficiency by decocting.
[b] L-amygdalin and D-amygdalin.
* $P < 0.05$.
** $P < 0.01$, vs. single herbs (*Mahuang* or *Xingren*).

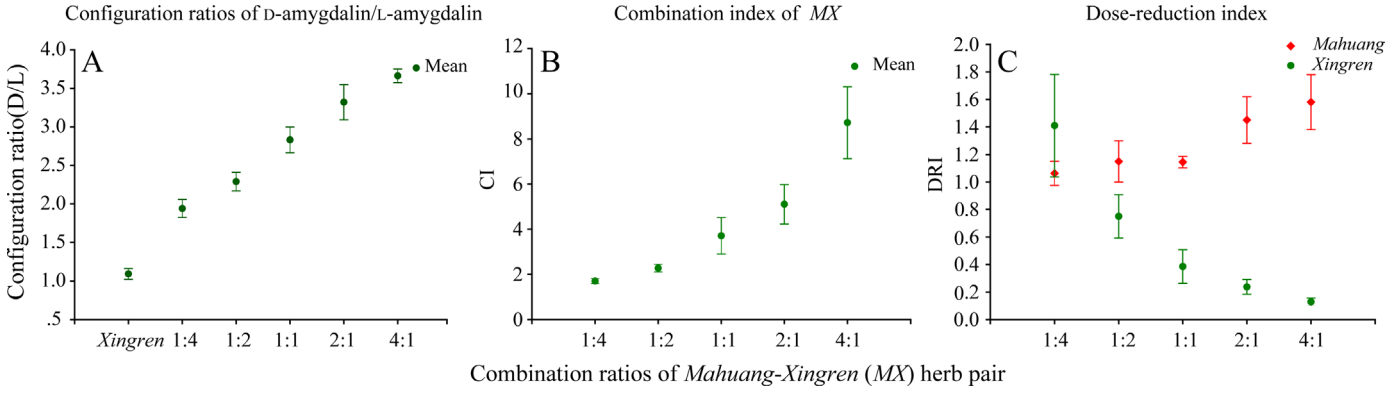


Fig. 5. The change in constituents of amygdalin of different combination ratios (A) and its association with combination index (B) and dose-reduction index (C).

spray ionization massspectrometry (Supplementary Fig. 3). Two compounds with identical protonation and product ion mass spectra were observed, indicating likely epimer formation. The precision of the method, as evaluated by performing 6 replicate assessments, was acceptable, with both intra-day and inter-day RSD of less than 3% (Table 2). The repeatability of the assay was good, because the peak area RSD of each compound in the *MX* (1:1) extract was less than 1%. Accuracy is calculated as the percentage of recovery. Table 2 illustrates the RE, which ranged between −0.75 and 2.20% and was thus within the pre-established range of ±3%. Satisfactory stability of the compounds in the *MX* extracts was also observed for at least 24 h at room temperature, because each peak area RSD was less than 3% (Table 2).

3.2.2. Antagonism of acute toxicity was closely correlated with D-amygdalin

The validated HPLC method was successfully applied to determine the compounds in the *Mahuang*, *Xingren*, and *MX* extracts. As shown in Table 3, the extraction efficiencies (mg/g) of E, PE, and amygdalin (including L-amygdalin and D-amygdalin) in combination (except *MX* 1:1) were not significantly different ($P > 0.05$) from those of the single herbs. However, the extraction efficiency of D-amygdalin in *MX* was increased significantly ($P < 0.05$) in comparison with that of *Xingren* alone. The epimeric forms of a drug may display different pharmacological or toxic effects (Food and Drug Administration, 1992). Combined with results obtained from acute toxicity studies, these findings suggest that changes in the amygdalin composition could be associated with the favorable

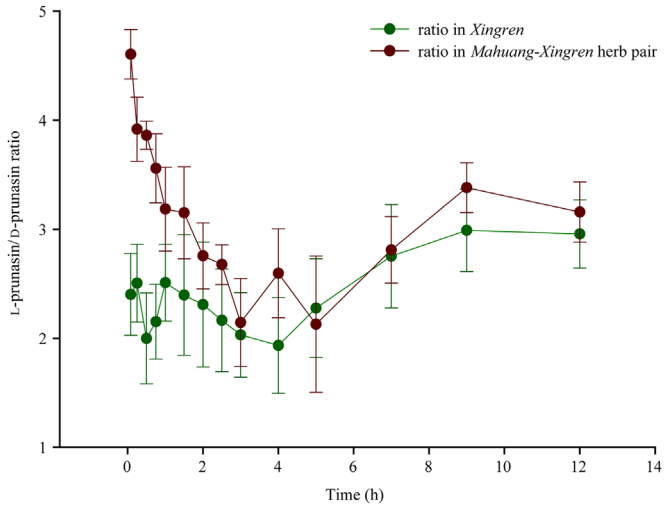


Fig. 6. Mean concentration ratio-time profiles of L-prunasin/D-prunasin in rat plasma after oral administration of *Xingren* and *Mahuang-Xingren* extracts. Each point represents the mean ± SD ($n=6$).

DRI of *Xingren* (Fig. 5A and C). Therefore, Pearson correlation analysis was used to identify relationships between the CIs and the ratios of D-amygdalin/L-amygdalin. The Pearson correlation coefficient ($r=0.901$, $P<0.01$) indicated that the ratio of D-amygdalin/L-amygdalin and CI were very strongly related (Fig. 5A and B). In other words, the proportion of D-amygdalin was closely correlated with the level of antagonism. However, this result seemed to be contradictory to the favorable DRI of *Xingren*. D-Amygdalin has been reported as the cytotoxic component of *Xingren*, which exhibits a selective killing effect on cancer cells (Chang et al., 2006). This contradiction may be explained by the *in vivo* nature of drug disposition, in contrast with the *in vitro* nature of the studies of amygdalin content variation. Therefore, antagonism of D-amygdalin metabolism may have contributed to the antagonism of acute *Xingren* toxicity observed in this study.

3.3. Interpretation of pharmacokinetics data

In comparison with previous UPLC-MS/MS determination of bioactive compounds in rat plasma and comparative pharmacokinetics analysis after oral administration of *Mahuang, Xingren, and MX* extracts, more attention was paid to the metabolic characteristics of amygdalin. D-Amygdalin absorbed in the blood would be metabolized to D-prunasin with few parent compounds after oral administration of pure amygdalin (Fang et al., 2010). The ratio of L-prunasin to D-prunasin in plasma was not consistent with that of the parent drug in aqueous extracts, indicating stereoselective amygdalin metabolism *in vivo*. As illustrated in Fig. 6 and Supplementary table 4 (published data in *Journal of Pharmaceutical and Biomedical Analysis*), D-prunasin was less abundant than L-prunasin in rat plasma, although there was more D-amygdalin than L-amygdalin in the extract (Fig. 4C and D). Interestingly, the process was facilitated by *Mahuang*, which led to virtual dose reduction of the D-prunasin dose *in vivo* and thus reduced toxicity. To clarify the relationship between the *MX* combination and the reduction in toxicity, a graphical description of our conjecture based on the chemical equilibrium of amygdalin *in vitro* and prunasin *in vivo* is illustrated in Fig. 7. In brief, the aqueous extract of *Xingren* is a racemic mixture that consists of equivalent amounts of each of a pair of epimers (50% of D-amygdalin and 50% of L-amygdalin). The chemical equilibrium *in vitro* (L-amygdalin⇌D-amygdalin) was disrupted by *Mahuang*, increasing the efficacy of D-amygdalin. In response, the metabolite equilibrium (L-prunasin⇌D-prunasin) should shift towards L-prunasin to restore the chemical balance *in vivo*, especially upon administration of the *MX* extract.

A previous study indicated that the lower metabolic rate (lower CLz/F and longer MRT and $t_{1/2}$) and limited distribution (smaller V_z/F values) of amygdalin and prunasin upon administration of the *MX* extract also contributed to reducing the toxicity reduction of *Xingren* in comparison with that *Xingren* alone (Song et al., 2015). Considering stereoselective metabolism-related characteristics, reducing the dose of D-prunasin, limiting its biodistribution and lowering its metabolic rate are also recognized as the most likely mechanism underlying the detoxifying effect of *Mahuang* on

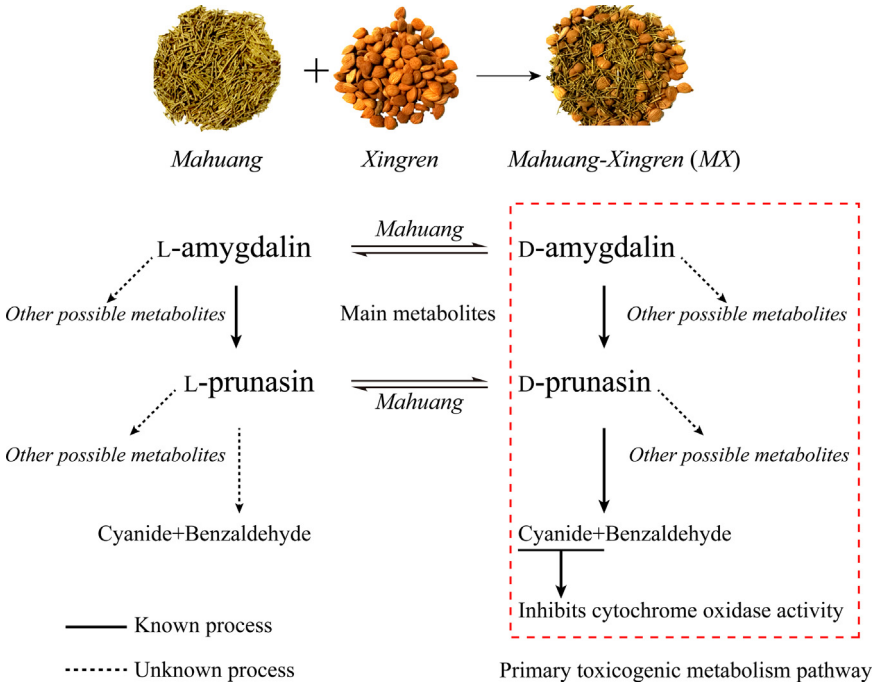


Fig. 7. Conjecture on the explanation for stereoselective metabolism of amygdalin based on chemical equilibrium.

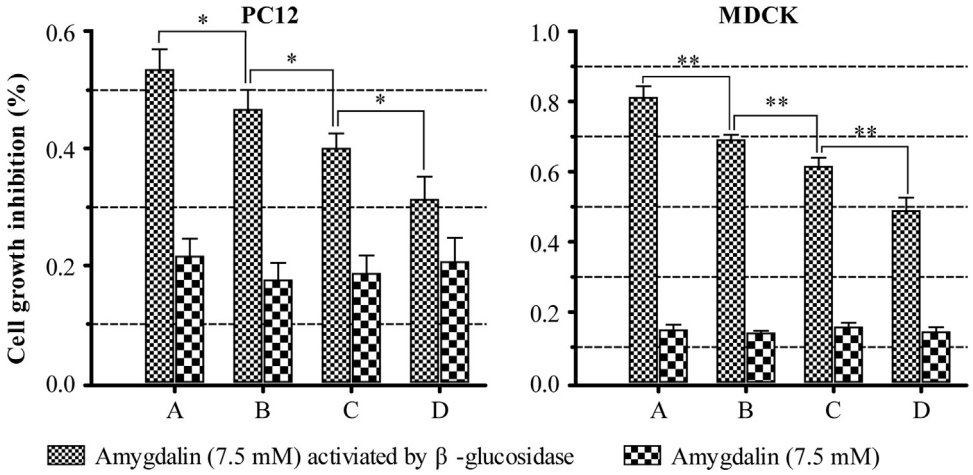


Fig. 8. Effect of amygdalin (including 100% (A), 81.9% (B), 68.8% (C), and 49.9% (D) D-amygdalin) with or without activation by β-glucosidase on inhibition of PC12 and MDCK cell proliferation after 48 h incubation (LSD test, $^{*}P<0.05$, $^{**}P<0.01$).

Table 4
The half maximal inhibitory concentrations, IC_{50} (μM),of A (100% D-amygdalin), B (81.9% D-amygdalin), C (68.8% D-amygdalin), and D (49.9% D-amygdalin) estimated by the CCK-8 assays on rat pheochromocytoma (PC12) and Madin-Darby canine kidney cell line (MDCK), means ± SEM ($N=3$).

Cell line	IC_{50} (μM, Mean ± SEM)			
	A	B	C	D
PC12	38.83 ± 5.96^{a}	34.99 ± 3.67^{a}	40.16 ± 2.69^{a}	39.57 ± 2.10^{a}
PC12*	5.97 ± 0.45^{d}	7.06 ± 0.42^{c}	8.14 ± 0.48^{b}	9.63 ± 0.82^{a}
MDCK	63.97 ± 7.36^{a}	62.39 ± 6.50^{a}	64.95 ± 5.21^{a}	64.10 ± 6.04^{a}
MDCK*	3.93 ± 0.47^{d}	5.26 ± 0.42^{c}	6.82 ± 0.2^{b}	8.42 ± 0.94^{a}

The different superscripts (a–d) within a line indicate significant differences between the drugs ($P<0.05$).

* Treatment with amygdalin activated by β-glucosidase.

Xingren in the *MX* combination. The molecular mechanisms underlying the detoxifying effect of *Mahuang* may involve enzyme-mediated reactions and merit further study.

3.4. Cytotoxicity assay

Stereoselective metabolism, a key mechanism of toxicity reduction, was also demonstrated in the cytotoxicity test. The inhibitory effects of treatment with 7.5 mM amygdalin (including 49.9%, 68.8%, 81.9% or 100% D-amygdalin) (with or without activation by β-glucosidase) for 48 h on the proliferation of PC12 and MDCK cells is shown in Fig. 8. The median effective dose (IC_{50}), the drug concentration causing 50% inhibition of the desired activity, was calculated graphically for each cell proliferation curve using GraphPad Prism Version 5.01 for Windows (GraphPad Software

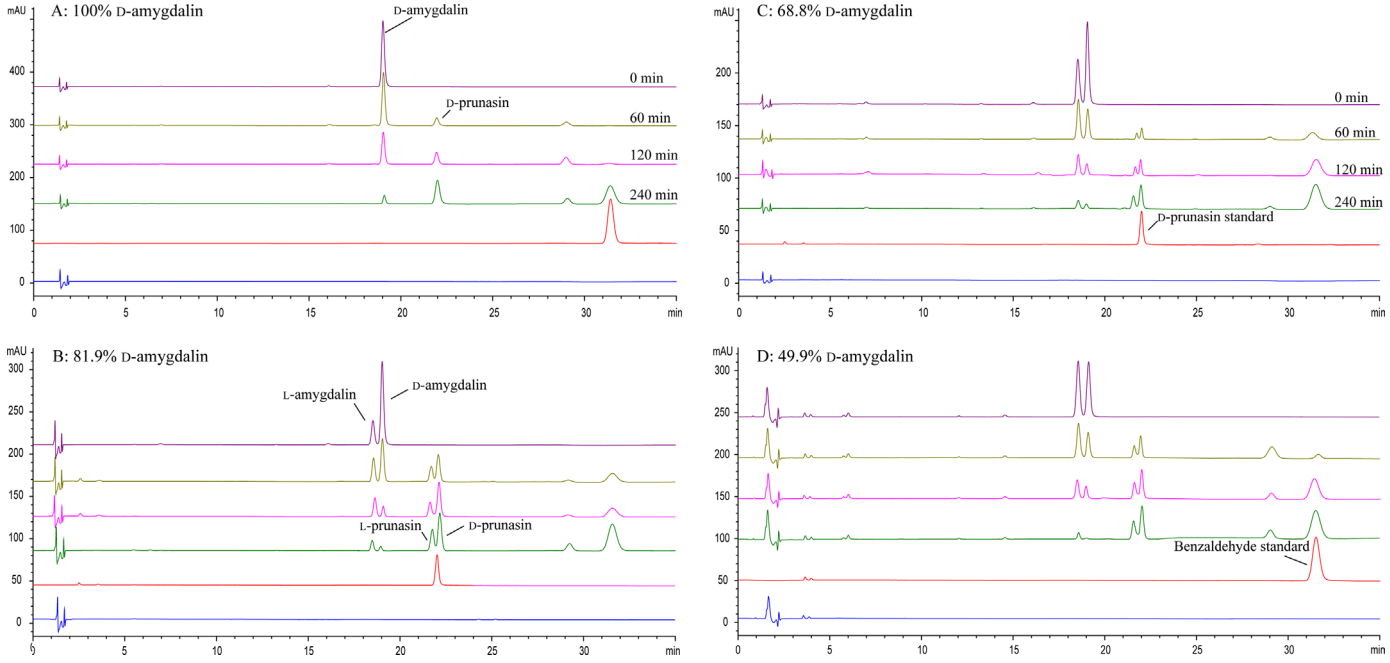


Fig. 9. Representative HPLC-DAD chromatograms ($\lambda=215$ nm) of amygdalin (including 100% D-amygdalin: A, 81.9% D-amygdalin: B, 68.9% D-amygdalin: C, and D-amygdalin: D) and its metabolites hydrolyzed by β-glucosidase in PC12 cell culture medium at indicated times.

Inc., San Diego, CA). The IC_{50} values of amygdalin and amygdalin activated by β-glucosidase on the proliferation of PC12 and MDCK cells for 48 h are summarized in Table 4. Similar to other recent studies (Nie et al., 2013; Zhou et al., 2012), β-glucosidase-mediated hydrolysis of amygdalin significantly enhanced its inhibitory effects on growth. The D-amygdalin ratio did not alter cytotoxicity in the absence of β-glucosidase. However, a higher proportion of D-amygdalin could enhance cytotoxicity in the presence of β-glucosidase. These results suggested that benzaldehyde and hydrogen cyanide released during D-amygdalin hydrolysis contribute to amygdalin cytotoxicity.

3.4.1. Metabolism of D-amygdalin had a major contribution to cytotoxicity

Amygdalin consists of a gentiobiose, a disaccharide composed of 2 units of D-glucose, and mandelonitrile. β-Glucosidase catalyzes hydrolysis of β-glycosidic bonds, resulting in dissociation of mandelonitrile and formation of benzaldehyde and hydrogen cyanide (Fig. 3B), which are toxic to living cells. As shown in Fig. 9, amygdalin, pruasin and benzaldehyde were detected in the culture medium. Contrary to the results of the *in vivo* metabolism study, the ratio of L-prunasin/D-prunasin was approximately consistent with that of the parent drug, indicating that no stereoselective metabolism occurred. Moreover, in contrast to the situation *in vivo*, hydrolysis of D-amygdalin *in vitro* was a simple process that was not influenced by biodistribution and clearance, enhancing D-amygdalin toxicity. These results suggest that D-amygdalin metabolism was the major source of hydrogen cyanide in this experiment (Fig. 7). Stereoselective metabolism, in which the metabolic pathway is manipulated to some extent, may be critical for detoxification of *Xingren* in the *MX* combination.

3.4.2. Cell selection in the cytotoxicity study

Cell culture can be used in preliminary toxicology evaluations as a means of saving time and animals (Vanparys, 2002). Cytotoxicity tests using specialized cells are useful when the *in vivo* toxicity of a chemical is already well established. Chronic oral administration of the cassava plant (which contains amygdalin) causes neurological damage in humans similar to that seen in persons suffering from tropical ataxic neuropathy, a disorder attributed to chronic cyanide exposure (Kalyanaraman et al., 1983; Newton et al., 1981). In the present study, we used PC12 cells, a standard *in vitro* model for evaluation of the developmental neurotoxicity of a diverse range of compounds. Although beneficial effects of amygdalin for cancer patients are not currently supported by sound clinical data (Milazzo et al., 2011), there are a few reports of pharmacological concentrations of amygdalin selectively killing malignant cells, but not normal cells, suggesting that it could be useful as a treatment for cancer (Chen et al., 2013). MDCK cells, a normal canine kidney cell line, are used to assess cytotoxicity in research on antineoplastic agents (Dharmatti; et al., 2014; Petinari et al., 2004). To better understand the cytotoxicity of amygdalin, its effect on normal MDCK cells were also evaluated.

4. Conclusion

The method used to combine herb pairs is the summation of millennia of clinical experience. The *MX* herb pair is widely used in the clinic due to its efficacy and few adverse effects. According to TCM theory, *Mahuang* is combined with *Xingren* to maintain the balance of the nature of the herbs and recover the harmony of lung *Qi*. In this paper, a comprehensive detoxification study of *Xingren* in the context of the *MX* herb pair was undertaken. In comparison with *Xingren* alone, the *MX* combination had a favorable therapeutic index due to the clear antagonistic effect of *Mahuang* on *Xingren* toxicity. Moreover, analysis of bioactive compounds, pharmacokinetics experiments in rats, and cytotoxic assessment provided further evidence that stereoselective metabolism of amygdalin may be a crucial mechanism underlying detoxification upon oral administration of *MX* extracts. This study provides a preliminary explanation for the hypothesis regarding the "mutual restraint" relationship of *Mahuang* and *Xingren* in *MX* proposed above, and offers a new mode of thinking regarding research into *MX* combination theory and the mechanisms underlying detoxification effects in TCM herb pairs.

Acknowledgments

S. Song wishes to acknowledge Q.-H. Ma for his technical assistance in the cytotoxicity evaluation. The first author also sincerely acknowledges the other authors of the study for their support. This work was supported by a grant from the National Natural Science Foundation of China (No. 81030066) and Natural Science Foundation of Guangdong Province (No. 2015A030313282).

Appendix A. Supplementary material

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.jep.2015.12.019.

References

Berrin, J.G., et al., 2002. Functional expression of human liver cytosolic beta-glucosidase in *Pichia pastoris*. Insights into its role in the metabolism of dietary glucosides. Eur. J. Biochem. 269, 249–258.

China Food and Drug Administration, 2005. Guidance for Acute Toxicity of TCM and Natural Medicine ⟨http://www.gdda.gov.cn/publicfiles/business/cmsresources/gddec/cmsrsdocument/doc7567.pdf⟩ (accessed 20.12.13).

Chang, H.K., et al., 2006. Amygdalin induces apoptosis through regulation of Bax and Bcl-2 expressions in human DU145 and LNCaP prostate cancer cells. Biol. Pharm. Bull. 29, 1597–1602.

Chen, Y., et al., 2013. Amygdalin induces apoptosis in human cervical cancer cell line HeLa cells. Immunopharmacol. Immunotoxicol. 35, 43–51.

Chou, T.C., 2011. The mass-action law based algorithms for quantitative econo-green bio-research. Integr. Biol. 3, 548–559.

Chinese Pharmacopoeia Commission, 2010. The Pharmacopoeia of People's Republic of China. Vol.1. China Medical Science and Technology Press, Beijing, China, pp. 187–188.

Dharmatti, R., et al., 2014. Surface orchestration of gold nanoparticles using cysteamine as linker and folate as navigating molecule for synaphic delivery of doxorubicin. J. Nanomed. Res. 1, 1–7.

Fang, M., et al., 2010. Effect of processing on metabolism of amygdalin from bitter almond in rat. China J. Chin. Mater. Med. 35, 2684–2688.

Food and Drug Administration, 1992. Development of New Stereoisomeric Drugs. ⟨http://www.fda.gov/Drugs/GuidanceComplianceRegulatoryInformation/Guidances/ucm122883.htm⟩ (accessed 07.12.14).

Huang, T.P., et al., 2013. Characteristics of traditional Chinese medicine use in children with asthma: a nationwide population-based study. Allergy 68, 1610–1613.

Hwang, E.Y., et al., 2002. Reverse-phase HPLC separation of D-amygdalin and neoamygdalin and optimum conditions for inhibition of racemization of amygdalin. Chem. Pharm. Bull. 50, 1373–1375.

Isoza, T., et al., 2001. Quantitative determination of amygdalin epimers by cyclodextrin-modified micellar electrokinetic chromatography. J. Chromatogr. A 923, 249–254.

Kalyanaraman, U.P., et al., 1983. Neuromyopathy of cyanide intoxication due to "laetrile" (amygdalin). A clinicopathologic study. Cancer 51, 2126–2133.

Li, Y., et al., 2010. Effect of San-ao Decoction, a traditional Chinese prescription, on IL-4 treated normal human bronchial epithelium. J. Ethnopharmacol. 131, 104–109.

Liu, L., et al., 2012. Taoren-Honghua herb pair and its main components promoting blood circulation through influencing on hemorheology, plasma coagulation and platelet aggregation. J. Ethnopharmacol. 139, 381–387.

Ma, C., et al., 2014. Using UPLC–QTOF–MS to analyze the chemical changes between traditional and dispensing granule decoctions of San-Ao-Tang. J. Chromatogr. Sci. 52, 277–292.

Mehendale, S.R., et al., 2004. Ephedra-containing dietary supplements in the US versus ephedra as a Chinese medicine. Am. J. Chin. Med. 32, 1–10.

Milazzo, S., et al., 2011. Laetrile Treatment for Cancer, Cochrane Database of Systematic Reviews. John Wiley & Sons, Ltd..
Moertel, C.G., et al., 1982. A clinical trial of amygdalin (Laetrile) in the treatment of human cancer. N Engl. J. Med. 306, 201–206.
Newton, G.W., et al., 1981. Amygdalin toxicity studies in rats predict chronic cyanide poisoning in humans. West J. Med. 134, 97–103.
Nie, Z., et al., 2013. Bladder cancer cells apoptosis induced by amygdalin fonowing specific activation by β-glucosidase. J. Mod. Urol. 18, 113–116.
Park, J.H., et al., 2013. Single oral dose toxicity study of prebrewed armeniacae semen in rats. Toxicol. Res. 29, 91–98.
Petinari, L., et al., 2004. Cytotoxicity of tamoxifen in normal and tumoral cell lines and its ability to induce cellular transformation in vitro. Cell Biol. Int. 28, 531–539.
Shu, X., et al., 2012. Comparative analysis of the main bioactive components of Sanao decoction and its series of formulations. Molecules 17, 12925–12937.
Song, S., et al., 2015. Concurrent quantification and comparative pharmacokinetic analysis of bioactive compounds in the Herba Ephedrae–Semen Armeniacae Amarum herb pair. J. Pharm .Biomed. Anal. 109, 67–73.
Vanparys, P., 2002. ECVAM and pharmaceuticals. Altern. Lab. Anim. 30 Suppl 2, 221–223.
Wang, S., et al., 2012. Compatibility art of traditional Chinese medicine: from the perspective of herb pairs. J. Ethnopharmacol. 143, 412–423.
Wang, Z., et al., 2013. Study on prescription characteristics of Chinese patent medicines for antitussive effect. Modermiz Tradit. Chin. Med. Mater. Med. 15, 1759–1765.
China Jiangsu Website, 2014. Changshu Woman Poisoned by Bitter Almond. ⟨http://english.jschina.com.cn/TodayJiangsu/201412/t1929232.shtml⟩ (accessed 24.12.14).
Xie, Y., et al., 2012. Content of bioactive compounds in aqueous extracts of Ephedra Herba with Semen Armeniacae Amarum by compatibility of different ratios. Lishizhen Med. Mater. Med. Res. 23, 2686–2688.
Zeng, Z.P., Jiang, J.G., 2010. Analysis of the adverse reactions induced by natural product-derived drugs. Br. J. Pharmacol. 159, 1374–1391.
Zhang, Y., et al., 2009. Effects of San'ao decoction and its analogous prescriptions on airway inflammation in mice with respiratory syncytial virus- and ovalbumin-induced asthma. J. Intergr. Med. 7, 354–359.
Zhou, C., et al., 2012. Enhancement of amygdalin activated with beta-D-glucosidase on HepG2 cells proliferation and apoptosis. Carbohydr. Polym. 90, 516–523.

Glossary

Mahuang-Xingren herb pair: A classical combination in traditional Chinese medicine consisting of Herba Ephedrae (*Mahuang* in Chinese) and Semen Armeniacae Amarum (*Xingren* in Chinese) that is used to treat asthma, and bronchitis;

D_m value: The median-effect dose or concentration. It is usually represented by ED50 or IC50. Depending on the endpoint of the measurement, it can also be represented by LD50 or TD50 if lethality or toxicity is measured;

Combination index (CI): A quantitative measure of the degree of drug interaction in terms of additive effect ($CI=1$), synergism ($CI<1$), or antagonism ($CI>1$) for a given endpoint of the effect measurement;

Dose-reduction index (DRI): A measure of how much the dose of each drug in a synergistic combination may be reduced at a given effect level compared with the doses of each drug alone.