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Predicting the potential toxicity of 26 components in *Cassiae* semen using *in silico* and *in vitro* approaches

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ABSTRACT

Cassiae semen are dried and ripe seeds of *Cassia obtusifolia* L. or *Cassia tora* L. (Fabaceae) and have been made into roasted tea or used as a traditional medicine in Asian countries. However, it was reported to result in liver and renal toxicity. The components of *Cassiae* semen that induce hepatotoxicity or nephrotoxicity remain unknown. In the present study, we evaluate the potential toxicity of 26 newly isolated compounds from *Cassiae* semen using quantitative structure–activity relationship (QSAR) methods and co-culture of hepatic and renal cell approaches, and we aim to illustrate the relationship between the structural characteristics and cytotoxicity by general linear models (GLMs). Both the QSAR models and co-culture of hepatic and renal cell systems predicted that 6 compounds were potentially hepatotoxic, 10 compounds were potentially nephrotoxic, and specific anthraquinones and anthraquinone-glucosides were potential toxicants in *Cassiae* semen. Specific groups such as –OH and $–OCH_3$ at the R1, R2, R3, and R7 positions influenced the cytotoxicity.

1. Introduction

Cassiae semen are dried and ripe seeds of *Cassia obtusifolia* L. or *Cassia tora* L. (Fabaceae). It has been made into roasted tea or used as a traditional medicine in Asian countries due to its various pharmacological effects, including neuroprotection (Yi et al., 2016), antidiabetic activities (Kim et al., 2014), antibacterial activities (Sung et al., 2004), antioxidant activities (Liu et al., 2015) and blood lipid regulation (Chen et al., 2011). In China, *C.* semen was primarily used to treat hyperlipidemia and to protect against hepatoxicity (Niu et al., 2010). However, a case study reported that extracts of *C.* semen could cause hepatocellular damage and cholestasis in humans (Peng et al., 2016). Irreversible damage also occurred in the kidneys of rats after administration of 10.0 $g{\cdot}kg^{-1}$ freeze-dried powdered *C.* semen for 26 weeks (Pei et al., 2017). Nevertheless, the components of *C.* semen that induce hepatotoxicity or nephrotoxicity remain unknown.

The major active components in *C.* semen have been determined to be anthraquinones (AQs) and naphthopyrones (Dong et al., 2017). AQs are aromatic compounds characterized by a cyclic diketone structure (Zhao et al., 2016). AQs in *C.* semen were divided into anthraquinone type Ⅰ (where R1, R2, and R3 were $–OCH_3$, –OH, and $–CH_3$), while others were anthraquinone type Ⅱ. AQs, especially rhein, chrysophanol, emodin, physcion, and aloe-emodin (and their glycosides) are the active components of several traditional medical plants, such as *Polygonum multiflorum* Thunb, *Rheum officinale* Baill, *Rheum palmatum* L., and *Rheum tanguticum* Maxim (Cai et al., 2004). Previous studies focusing on the toxicity of AQs isolated from different plant sources indicated that some AQs could exert toxicity by forming epoxidized metabolites via metabolic activation (Jiang et al., 2017). A few AQs, such as rhein and emodin, have been studied (Malik and Muller, 2016; Mao et al., 2017; Panigrahi et al., 2015), while other AQs, such as obtusifolin, aurantio-obtusin, and

Abbreviations: C. semen, *Cassiae* semen; TCM, Traditional Chinese medicine; AQ, Anthraquinone; QSAR, Quantitative structure-activity relationship; IdMOC, Integrated discrete multiple organ co-culture; GLM, General linear models; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; CYP, Cytochrome P450.

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obtusin, present at higher contents in *C.* semen, remain uninvestigated.

There have been over 70 components isolated and identified from *C.* semen thus far (Dong et al., 2017), and 26 of them have been identified and a small number of pure compounds obtained. Examining the conventional toxicity of a variety of phytochemicals with multiple complex tests is time-consuming and labor-intensive. A simple, high-throughput approach for the screening of potential nephrotoxic and hepatotoxic compounds is needed.

As an *in silico* method, quantitative structure–activity relationship (QSAR) models focus on correlating chemical structure features to pharmacological activity or properties in a quantitative manner for a component (Roy et al., 2015). Due to the advantages of low cost and high throughput, the QSAR model has been used as an alternative and complementary method for predicting drug toxicity (Benfenati, 2016).

The integrated discrete multiple organ co-culture (IdMOC) system is another tool for evaluating the cytotoxicity of compounds towards multiple cell types quantitatively at the same time. It is a co-culture model that employs the wells-in-a-well concept (Li et al., 2004). Metabolic interactions could also be explored when cells within the metabolic capacity are present in the co-culture system (Li, 2008). Therefore, establishing a liver and kidney cell co-culture system by using IdMOC plates might be an efficient way to predict the potential hepatic and renal toxicity of the compounds *in vitro*.

In the present study, we evaluated the potential toxicity of the 26 compounds in *Cassiae* semen using *in silico* and *in vitro* approaches. First, *in silico* QSAR models were used to predict the nephrotoxicity and hepatotoxicity of the 26 phytochemicals isolated from *C.* semen, including 20 AQs, 4 naphthopyrones, and 2 phenols. A co-culture of the hepatic and renal cell system using IdMOC plates was then established, validated, and applied to assess the potential hepatotoxicity and nephrotoxicity of the 26 compounds. The general linear model was applied to explore the relationship between structural characteristics and cytotoxicity.

2. Materials and methods

2.1. Materials

The 26 compounds isolated from *C.* semen with a chemical purity of over 95.0% (Pang et al., 2019; Pang et al., 2018; Wang et al., 2019) were provided by Tianjin University of Traditional Chinese Medicine. The CAS numbers and classifications based on the chemical structure of the 26 compounds are shown in Table 1.

HK-2 cells and HepG2 cells were purchased from Kaiji Biological Technology Co., Ltd. (Nanjing, China). HepaRG cells, Hoechst 33,342 (#H1399), MitoTracker deep red (#M22426), and BOBO-3 (#B3586), were obtained from Invitrogen (New York, USA). Fetal bovine serum (FBS), penicillin, and streptomycin were all purchased from Gibco BRL (Grand Island, NY). IdMOC co-culture plates, formic acid, methanol, and acetonitrile (chromatographical grade) were obtained from Sigma-Aldrich (Saint Louis, USA).

Glutamine, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), and trypsin were purchased from Invitrogen (Carlsbad, CA, USA). CYP isozyme probe substrates and their metabolites (phenacetin, tolbutamide, mephenytoin, dextromethorphan, and midazolam) were purchased from the China Institute of Drug and Biological Products (Beijing). Paracetamol, 4-hydroxytolbutamide, 4-hydroxymephenytoin, dextrorphan, and 1′-midazolam were purchased from BD Gentest (Woburn, MA).

2.2. QSAR models for nephrotoxicity and hepatotoxicity prediction

Hepatotoxicity prediction: The ADMET Predictor™ (version 10.0.0.11, Simulations Plus, Lancaster, CA, USA) is software that can predict chemical indicators and toxicity using its prediction module or self-built QSAR models. Its prediction module was used for predicting the hepatotoxicity of the 26 phytochemicals isolated from *C.* semen. Five liver injury indicators (AlkPhos, GGT, LDH, SGOT, and SGPT) were used as indicators to determine hepatotoxicity. The compound was thought to produce adverse effects on the liver when three hepatotoxic indicators were positive.

Nephrotoxicity prediction: A self-built QSAR model based on the ADMET Modeler™ module, the model-building tool in the ADMET Predictor™ software, was applied to predict the potential nephrotoxicity of 26 compounds. The accuracy (Acc) of this model was 76.7%. The prediction outcome was directly defined as nephrotoxicity “positive” or “negative” (Sun et al., 2019).

2.3. Cell culture

HK-2 cells, HepaRG cells, and HepG2 cells were incubated in the cultures shown in Table 2. The generation of all types of cells ranged between 10 and 15.

2.4. Establishment and validation of co-culture of the hepatic and renal cell system

2.4.1. Detection of cytochrome P450 metabolic capacity of HepaRG/HepG2 cells

To investigate the cytochrome P450 metabolic capacity of HepaRG cells, HepaRG and HepG2 cells (used as a control) were plated at a density of 2×10^4 cells/well in 96-well plates. After reaching 80–90% confluency, the medium was replaced with new medium without FBS, penicillin, streptomycin, and phenol but supplemented with different probe substrates for CYP isoforms, including phenacetin (CYP1A2, 25 μmol/L), tolbutamide (CYP2C9, 25 μmol/L), dextromethorphan (CYP2D6, 10 μmol/L), midazolam (CYP3A4, 10 μmol/L) and mephenytoin (CYP2C19, 100 μmol/L). After incubation for different periods (1, 2, 4, 8, 12, and 24 h), the medium was centrifuged for 5 min at 10,000 × *g*, and the supernatant was collected for further analysis. Paracetamol, dextrorphan, 4-hydroxytolbutamide, 4-hydroxymephenytoin, and 1′-hydroxymidazdam are metabolites of specific probe substrates of phenacetin, dextromethorphan, tobutamide, mephenytoin, and midazolam, respectively. Their contents were detected by a UHPLC-MS/MS system. Conditions of the UHPLC system and mass spectrometry were as previously described (Shen et al., 2013).

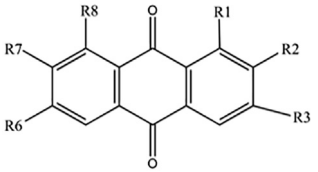
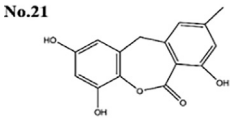
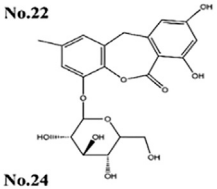
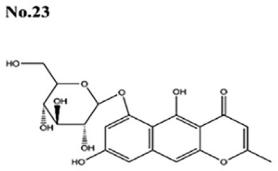
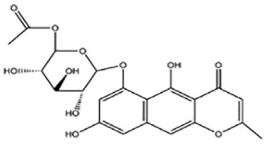
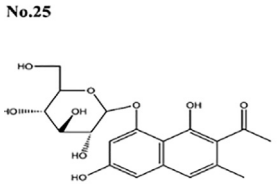
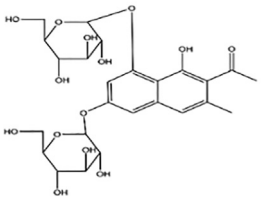
2.4.2. Comparison of the 24 h toxicity difference between single cell culture and co-culture by using the MTT assay

Eight components in *Cassiae* semen, including the 5 AQs (Nos. **8, 9, 10, 11 & 12**) in the process of metabolic transformation (Fig. 1) (Bachmann and Schlatter, 1981; Qin et al., 2018; Song et al., 2009; Sun and Chen, 1986; Sun et al., 2018; Tian et al., 2012; Yu et al., 2016), 2 glycosides (Nos. **14 & 15**) and 1 AQ that only exists in *C.* semen (No. **5**) (Wang et al., 2012; Yuan et al., 2012) were tested in single culture (HK-2 cells alone) and co-culture of renal and hepatic cells (HK-2 and HepaRG cells) in IdMOC plates to validate the co-culture system. HK-2 cell viabilities were compared between single cultures and co-cultures to illustrate the metabolic capacity of the co-culture system, which might help clarify the contribution of hepatic metabolism to renal cytotoxicity.

Single-culture HK-2 cells were exposed to the components at various concentrations for 24 h. Components were dissolved in DMSO at a final concentration of no > 0.5%. Cells in the vehicle control group were treated with 0.5% DMSO alone.

Co-culture of renal and hepatic cells using IdMOC culture plates was conducted essentially as described (Li et al., 2012). Briefly, 40 μL of cell suspension, 5.0×10^5 HepaRG cells/mL, and 4.0×10^5 HK-2 cells/mL were added to 3 inner wells of a chamber. The plates were

Table 1
The CAS number and structure of 26 compounds isolated from *Cassiae* Semen.

No.	Name	CAS	Structure	R1	R2	R3	R6	R7	R8
Anthraquinone-Type I									
1	Chryso-obtusin	70588-06-6		OCH_3	OH	CH_3	OCH_3	OCH_3	OCH_3
2	Obtusin	70588-05-5		OCH_3	OH	CH_3	OCH_3	OCH_3	OH
3	Obtusifoline	477-85-0		OCH_3	OH	CH_3	H	H	OH
4	1,7,8-methoxyl-2-hydroxyl-3-methyl-anthraquinone	2195434-05-8		OCH_3	OH	CH_3	H	OCH_3	OCH_3
5	Aurantio-obtusin	67979-25-3		OCH_3	OH	CH_3	OH	OCH_3	OH
6	1,2,3,7-tetrahydroxy-8-methoxy-6-methyl-anthraquinone	130018-57-4		OCH_3	OH	CH_3	OH	OH	OH
7	1,2,7-trihydroxy-8-methoxy-6-methyl-9,10-anthraquinone	38393-73-6		OCH_3	OH	CH_3	H	OH	OH
Anthraquinone-Type II									
8	Emodin	518-82-1		OH	H	CH_3	OH	H	OH
9	Chrysophanol	481-74-3		OH	H	CH_3	H	H	OH
10	Aloe-emodin	481-72-1		OH	H	CH_2OH	H	H	OH
11	Rhein	478-43-3		OH	H	COOH	H	H	OH
12	Physcion	521-61-9		OH	H	CH_3	OCH_3	H	OH
13	Alaternin (quinone)	641-90-7		OH	OH	CH_3	OH	H	OH
Anthraquinone-Glucoside									
14	Physcion-8-*O*-*β*-D-glucoside	26296-54-8		OH	H	OCH_3	CH_3	H	O-glu
15	Rhein-8-*O*-*β*-D-glucoside	113443-70-2		OH	H	COOH	H	H	O-glu
16	Obtusifoline-2-*O*-*β*-D-glucoside	120163-18-0		OCH_3	H	O-glu	CH_3	H	OH
17	9,10-Anthracenedione	1193512-20-7		OCH_3	O-glu-$COCH_3$	CH_3	H	H	OH
18	Gluco-obtusin	960135-79-9		OCH_3	O-glu	CH_3	OCH_3	OCH_3	OH
19	Chryso-obtusin-2-*β*-D-glucoside	96820-54-1		OCH_3	O-glu	CH_3	OCH_3	OCH_3	OCH_3
20	Glucoaurantio-obtusin	129025-96-3		OCH_3	OH	CH_3	O-glu	OCH_3	OH
Phenol									
21	9-Dehydroxyeurotinone (EDG)	1360606-85-4							
22	2-*O*-*β*-D-Glucoside-9-dehydroxyeurotinone	2236114-61-5							
Naphthopyrone									
23	Cassiaside	13709-03-0							
24	Nor-rubrofusarin-6-*O*-*β*-D-(6'-*O*-acetyl) glucopyranoside	1253053-49-4							
25	6-Hydroxymusizin-8-*O*-*β*-D-glucopyranoside	23566-96-3							
26	Cassia-glycoside Π	2241081-56-9							

cultured at 37 °C with 5% CO_2 for 4 h. Then, 1.2 mL of culture medium containing a different concentration of phytochemical was added to each chamber to cover the 6 inner wells to realize 24 h exposure at 37 °C with 5% CO_2.

2.5. MTT assay to screen renal and hepatic cytotoxicity of 26 phytochemicals in a co-culture system

The co-culture of HK-2 and HepaRG cells was exposed to the 26 compounds at various concentrations for 24 h and 48 h, respectively. The compounds were dissolved in DMSO at a final concentration of no > 0.5%. Triptolide (10 μmol/L and 25 μmol/L) were used as positive controls for renal and hepatic cytotoxicity, respectively.

Table 2
Conditions of Cell culture.

Cells	Medium	Supplement	Incubation
HK-2	RPMI-1640 medium	10% (v/v) FBS; 100 U/mL penicillin; 100 mg/mL streptomycin	5% CO2; 37 ℃.
HepaRG	William's E medium	10% (v/v) FBS; 100 U/mL penicillin; 100 mg/mL streptomycin; Tox medium supplement (Invitrogen)	
HepG2	DMEM medium	10% (v/v) FBS; 100 U/mL penicillin; 100 mg/mL streptomycin	

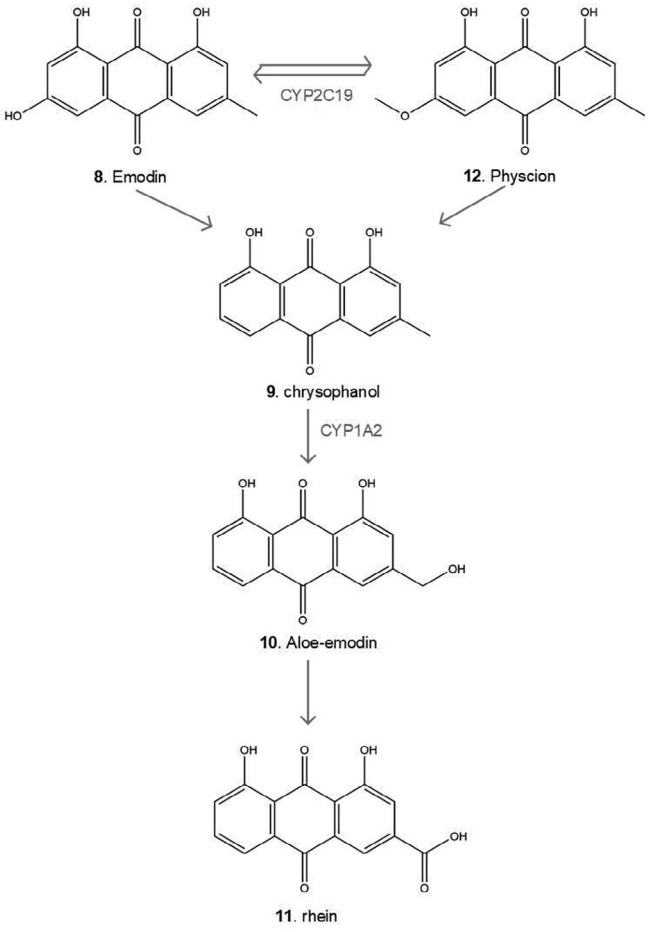


Fig. 1. The proposed transformation of emodin to rhein in *in vivo* metabolism (Bachmann and Schlatter, 1981; Qin et al., 2018; Song et al., 2009; Sun and Chen, 1986; Sun et al., 2018; Tian et al., 2012; Yu et al., 2016).

2.6. High-content analysis of potential hepatotoxic and nephrotoxic phytochemicals

After the co-culture of HK-2 and HepaRG cells was exposed to the 26 compounds for 24 h, 1.2 mL of RPMI 1640 media containing Hoechst 33,342 (nuclear fluorescent dye, final concentration 1 μM), MitoTracker® deep red (mitochondrial fluorescent dye, final concentration 0.3 μM), and BOBO-3 (nucleic acid fluorescent dye, final concentration 0.75 μM) (Table S7) was added. Plates were placed in an incubator for 30 min. After removing the media and washing the chambers with PBS twice, image analysis was conducted using the Operetta and Columbus systems. Images were captured in a 20x objective lens, with 8 fields per well. Cells were identified using nuclear Hoechst 33,342 staining.

2.7. The relationship between the functional groups in the chemical structures and cytotoxicity

A general linear model of categorical variables was applied to the 50% inhibition concentration (IC_{50}) and functional groups of anthraquinone derivatives using Rx64 4.0.1. The IC_{50} values shown as "> 500" were valued as 501. Stepwise regression was employed as a variable selection method.

2.8. Statistical analysis

SPSS 22.0 (IBM, New York, USA) was used for data analyses. All data are presented as the mean ± standard deviation (SD), and statistical significance was assessed by one-way analysis of variance (ANOVA) followed by the least-significant difference test (LSD) for multiple comparisons. $P < 0.05$ was considered statistically significant. The IC_{50} values were calculated following nonlinear regression analysis by Prism 6 software.

3. Results

3.1. QSAR prediction for potential hepatotoxicity and nephrotoxicity

A total of 26 compounds were analyzed using QSAR models for potential hepatotoxicity and nephrotoxicity (Table 3). Three compounds (2 anthraquinone-type Is and 1 anthraquinone-glucoside) were predicted to be hepatotoxic. Eight compounds (1 anthraquinone-type I, 4 anthraquinone-type IIs, and 3 anthraquinone-glucosides) were predicted to be nephrotoxic.

3.2. Establishment and validation of the co-culture system

3.2.1. Detection of cytochrome P450 metabolic capacities of HepaRG/HepG2 cells

As shown in Fig. 2, the metabolic capacities of CYP2C9, CYP2C19, CYP3A4, and CYP1A2 in HepaRG cells were higher than those in HepG2 cells. Therefore, HepaRG cells were incorporated into the co-culture system.

3.2.2. Comparison of the 24 h cytotoxicity difference between single-cell culture and co-culture of 8 major components in Cassiae semen

The cytotoxicities of 8 major components (Nos. **5, 8, 9, 10, 11, 12, 14 & 15**) were compared between single-cell culture and co-culture (Table S1). In particular, emodin (No. **8**) was cytotoxic to HK-2 cells in the single-culture and co-culture systems (Fig. 3). The IC_{50} value of emodin decreased from 139.90 μM (single culture) to 88.97 μM (co-culture), which is consistent with an *in vivo* study (Jiang et al., 2018). This suggests that the co-culture system in the present study allowed the evaluation of renal toxicity in the presence of hepatic metabolism.

Table 3
The potential hepatotoxicity and nephrotoxicity of the 26 compounds isolated from *Cassiae* Semen.

No.	Hepatotoxicity			Nephrotoxicity		
	*In vivo**	QSAR model	Cytotoxicity	*In vivo**	QSAR model	Cytotoxicity
Anthraquinone-Type I						
1	–	P	N	–	N	P
2	P	N	P	–	N	P
3	P	N	P	–	N	N
4	–	P	N	–	P	P
5	P	N	P	–	N	N
6	–	N	N	–	N	N
7	–	N	P	–	N	N
Anthraquinone-Type II						
8	P	N	P	–	P	N
9	–	N	N	–	P	P
10	P	N	P	P	P	N
11	P	N	P	–	P	P
12	–	N	P	–	N	N
13	–	N	N	–	N	N
Anthraquinone-Glucoside						
14	–	N	P	–	N	P
15	–	N	N	–	P	N
16	–	N	P	–	P	N
17	–	N	P	–	P	N
18	–	N	N	–	N	N
19	–	P	N	–	N	N
20	–	N	N	–	N	N
Phenol						
21	–	N	N	–	N	N
22	–	N	N	–	N	N
Naphthopyrone						
23	–	N	P	–	N	N
24	–	N	N	–	N	N
25	–	N	N	–	N	N
26	–	N	N	–	N	N

"N" = negative, "P" = positive, "-" = not found.
**in vivo* results cited from the literature (Program, 2001; Wang et al., 2009; Xu et al., 2019; Yang et al., 2019b).

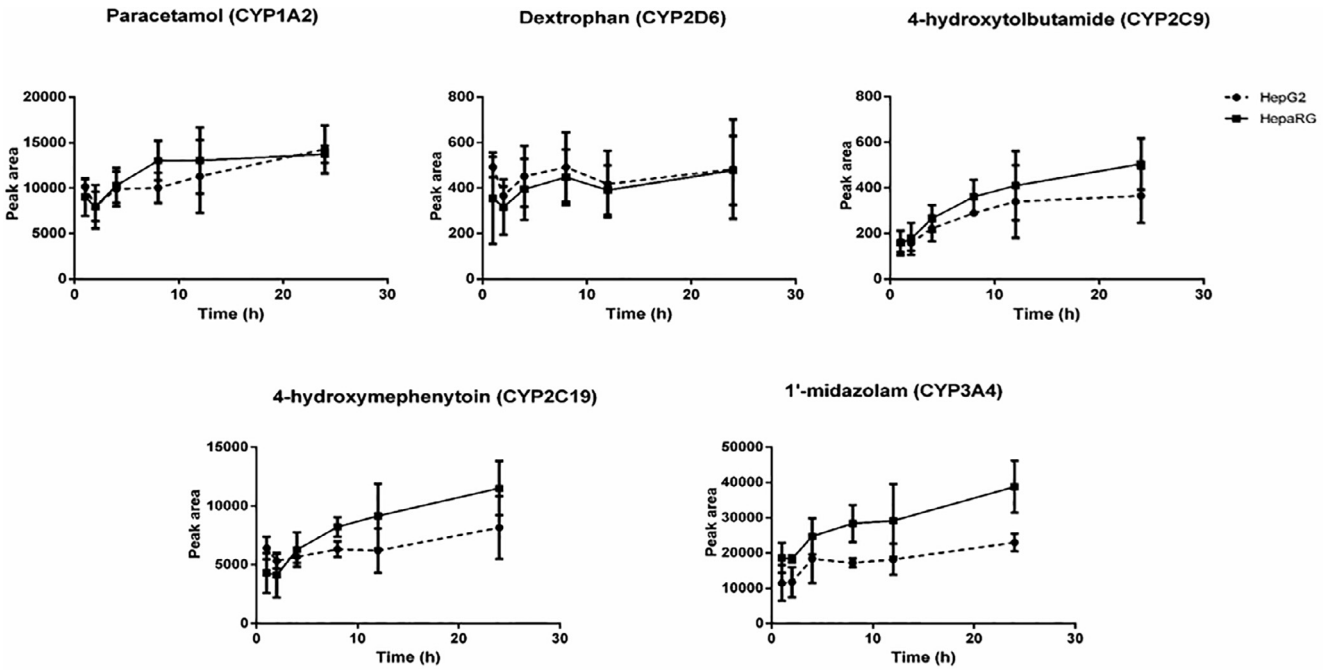


Fig. 2. The average peak area of the metabolites of CYP isozyme probe substrate after metabolization by HepaRG or HepG2 cells. Paracetamol, dextrorphan, 4-hydroxytolbutamide, 4-hydroxymephenytoin, and 1′-hydroxymidazdam were the metabolites of specific probe substrates of phenacetin (CYP1A2), dextromethorphan (CYP2D6), tobutamide (CYP2C9), mephenytoin (CYP2C19) and midazolam (CYP3A4), respectively.

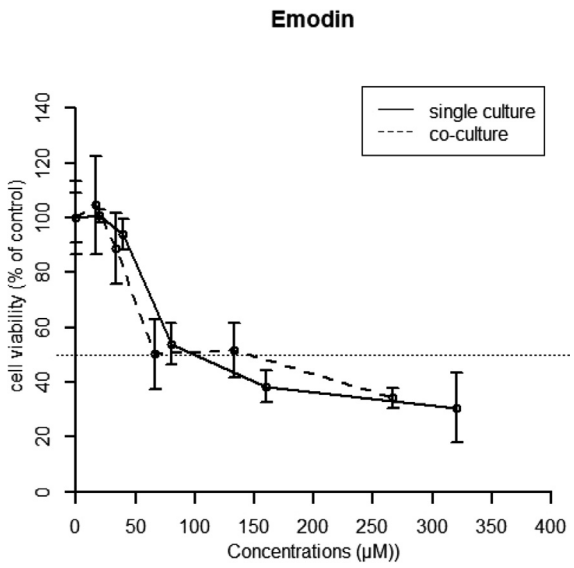


Fig. 3. Effects of emodin cytotoxicity assessed by the MTT assay in single HK-2 cell culture and HepaRG & HK-2 cell co-culture for 24 h. For the single culture and co-culture, the concentrations of emodin were 20, 40, 80, 160, and 320 μM and 16.67, 33.33, 66.67, 133.33, and 266.67 μM, respectively.

3.3. Hepatic and renal cytotoxicity of 26 phytochemicals by using a co-culture system

The cytotoxicities of 26 compounds on HK-2 cells and HepaRG cells are shown in Tables S2, S3, S4, and S5. As expected, the viabilities of HK-2 and HepaRG cells in the positive group were 43.65 ± 2.26% and 50.55 ± 5.68%, respectively.

Based on the renal and hepatic cytotoxicity for 48 h, the compounds were determined to be potentially hepatotoxic or nephrotoxic when the IC_{50} values were under 100 μM (Persson et al., 2013). The results of *in vitro* cytotoxicity and their IC_{50} values are presented in Table 3 and Table 4, respectively. After treatment with these compounds for 24 h or 48 h, the viability of HK-2 and HepaRG cells decreased in a dose-dependent manner. Moreover, the cytotoxicity of anthraquinone-glucoside was lower than that of other AQs.

Four anthraquinone-type I compounds (Nos. **2, 3, 5 & 7**), four anthraquinone-type II compounds (Nos. **8, 10, 11 & 12**), three anthraquinone-glucosides (**Nos. 14, 16 & 17**), and one naphthopyrone (No. **23**) decreased the viability of HepaRG cells (Table 3).

Three anthraquinone-type Is (Nos. **1, 2 & 4**), two anthraquinone-type IIs (Nos. **9 & 11**), and one anthraquinone-glucoside (No. **14**) reduced the viability of HK-2 cells (Table 3).

3.4. Potential hepatotoxic and nephrotoxic compounds in Cassiae semen

As shown in Table 3, Nos. **2**, **3**, **5**, **8**, **10,** and **11** were reported to be hepatotoxic *in vivo* (Program, 2001; Wang et al., 2009; Xu et al., 2019; Yang et al., 2019b), while only No. **10** was known to be nephrotoxic (Wang et al., 2009). In the present study, all the liver cytotoxicities of these compounds were consistent with the *in vivo* results (6/6, 100%), while few of the results predicted by QSAR models were consistent with them, demonstrating that the co-culture cell system was more sensitive in predicting the hepatotoxicity of those compounds. In addition, the QSAR model correctly predicted the nephrotoxicity of No. **10**. However, such results cannot conclude that the QSAR model was more sensitive than the co-culture system in nephrotoxicity.

The co-culture cell system produced more credible results for hepatotoxicity. Hence, the potential hepatotoxic compounds were predicted by the cytotoxic results. Apart from the 6 compounds known to cause hepatotoxicity (Nos. **2**, **3**, **5**, **8**, **10,** and **11**), the other 6 components (Nos. **7**, **12**, **14**, **16**, **17** and **23**) were potentially hepatotoxic.

Table 4
Hepatic and renal cytotoxicity (IC_{50}, μM) of 26 compounds isolated from *Cassiae* Semen.

No.	HepaRG		HK-2	
	24 h	48 h	24 h	48 h
Anthraquinone-Type I				
1	71.85	387.90	52.99	33.61
2	298.25	81.23	50.54	57.76
3	117.40	34.95	310.11	389.00
4	420.20	191.70	332.50	37.51
5	332.90	75.49	>500	117.05
6	>500	209.10	>500	282.80
7	>500	44.24	>500	>500
Anthraquinone-Type II				
8	125.30	31.95	88.97	122.00
9	>500	170.50	>500	93.90
10	>500	96.17	>500	133.80
11	306.60	36.09	229.40	44.08
12	>500	73.61	>500	196.30
13	>500	>500	>500	>500
Anthraquinone-Glucoside				
14	>500	85.34	>500	93.55
15	>500	208.4	>500	108.00
16	108.40	98.47	270.16	251.10
17	93.37	84.38	>500	137.90
18	178.00	125.40	254.68	>500
19	260.90	NA	>500	NA
20	154.30	NA	>500	NA
Phenol				
21	448.30	331.00	379.93	111.30
22	>500	>500	>500	>500
Naphthopyrone				
23	>500	90.44	>500	390.80
24	>500	225.70	>500	>500
25	>500	316.60	>500	>500
26	>500	>500	>500	>500

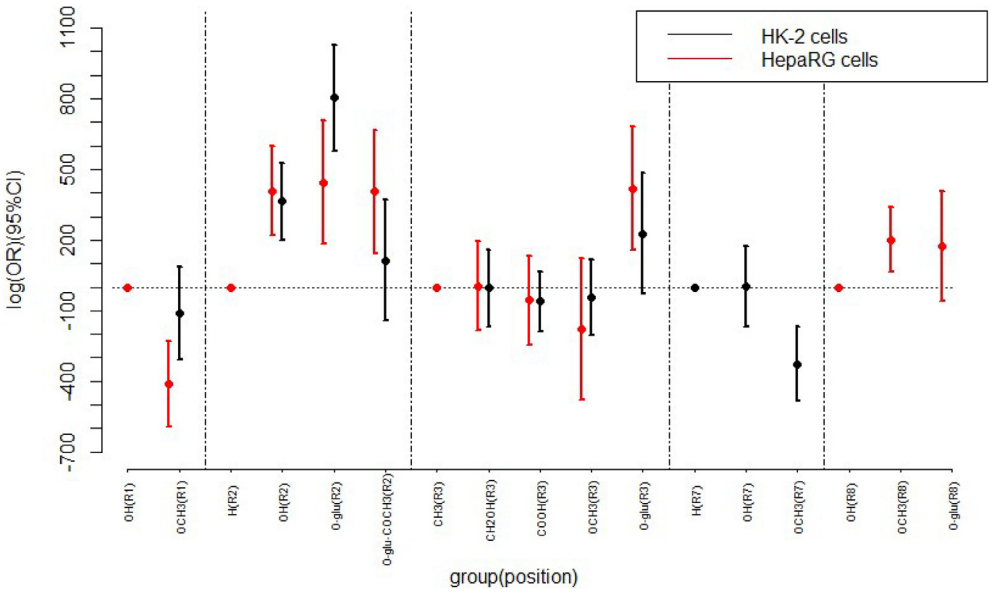


Fig. 4. Functional group (position) and odds ratio (OR) (95% CI) based on the IC_{50} in HK-2 and HepaRG cells exposed for 48 h. Only meaningful groups are presented.

The potential nephrotoxic compounds were predicted by the positive results of either the QSAR model or co-culture system. Nos. **1**, **2**, **4**, **8**, **9**, **11**, **14**, **15**, **16** and **17** were potentially nephrotoxic. Our results also suggest that Nos. **6**, **13**, **18**, **20**, **21**, **22**, **24**, **25** and **26** can be regarded as nontoxic to the liver or kidney.

3.5. High-content analysis of potential hepatotoxic and nephrotoxic phytochemicals

Six hepatotoxic and ten nephrotoxic components were further tested by high-content analysis (Figs S1-S5). The results showed that most of them could cause a decrease in the number of cells and increases in the permeability of the plasma membrane and mitochondria. The nuclei of cells also shrank. Nos. **2**, **8**, **11** can cause both hepatotoxicity and nephrotoxicity. Although the viability of HepaRG cells did not significantly decrease after exposure for 24 h, high-content analysis indicated that Nos. **2**, **8**, and **11** at low concentrations (16.67 or 33.33 μM) could cause an increase in mitochondria.

3.6. The relationship between the functional groups in chemical structures and cytotoxicity

The relationships between functional group substitutions in the cyclic diketone structure and their cytotoxicity are shown in Fig. 4 and Table S6. The adjusted R-squared values of the models are 0.8137, 0.5076, and 0.5589. Specific –OH and -O-glu groups at the R1, R2, R3, and R8 positions influence liver toxicity, and the –OH and -O-glu groups at the R2 and R7 positions influence kidney toxicity.

4. Discussion

Each medicinal herb contains multiple phytochemicals, and pure compounds in medicinal herbs are difficult to obtain in sufficient amounts, posing a great challenge to the screening of potential toxic compounds. We integrated *in vitro* and *in silico* methods to predict the toxicity of compounds in *C.* semen. *In silico* methods are tools for the screening, prioritization, and decision-making in chemical hazard assessment (Hung et al., 2020). *In vitro* methods could provide experimental evidence as an alternative method. A combination of *in silico* and *in vitro* methods can provide more reliable prediction results.

QSAR modeling is used to predict the potential adverse effects of a chemical based on its structure. Therefore, an adequately sized dataset, appropriate descriptors, appropriate endpoints, and their value were vital to a successful QSAR model. We predicted the potential toxicity of 26 compounds in *C.* semen using QSAR modeling and a co-culture cell system. Our results indicated that anthraquinones and their glucosides were major potential toxic components, and the co-culture system was more credible than that of QSAR, as verified by *in vivo* results. More differences between *in silico* and *in vitro* results were found for hepatotoxicity. The reason might be partly explained by the dataset of the hepatotoxicity model in the ADMET predictor. The training dataset comes from the US Food and Drug Administration's Center (Matthews et al., 2004), and most of them are chemical drugs. The hepatotoxicity model, which was based on these chemicals, might not be fit for predicting natural products that have different structures and chemical spaces (Wetzel et al., 2007). The false-negative hepatotoxicity prediction of phytochemicals in *C.* semen might be due to insufficient data on natural products. Moreover, the selection of hepatotoxic endpoints influences the prediction. The hepatotoxicity model used 5 liver injury indicators (AlkPhos, GGT, LDH, SGOT, and SGPT), which were symbols for hepatitis and hepatocyte necrosis. However, the bile acid change, which was the main reason for hepatotoxicity caused by *C.* semen and AQs (Kang et al., 2017), is not indicated in this model. These factors contributed to the inaccurate prediction of components in *C.* semen using the hepatotoxicity model.

The AQs for the compounds isolated from *C.* semen, some AQs and their glycosides showed a great possibility to be potential toxicants. After oral administration of *C.* semen to rats, most AQs were mainly absorbed in the ileum (Kong et al., 2011), metabolized by phase I and phase II reactions in the liver, and then eliminated from the kidney (Panigrahi et al., 2015; Song et al., 2010). Chrysophanol exists with the highest content in plasma among these AQs, followed by obtusifolin, aurantio-obtusin, obtusin, chrysoobtusin, emodin, and rhein (Yang et al., 2019a). Moreover, obtusifolin, aurantio-obtusin, and obtusin showed potential accumulation and might have potentially toxic effects (Yang et al., 2019b). *In vivo* studies also indicated that emodin and rhein had potential hepatotoxicity (Program, 2001; Yang et al., 2019b). In the present study, these high-content AQs in plasma were all predicted to have hepatotoxicity or nephrotoxicity.

Prioritization to test their hepatotoxicity or nephrotoxicity is warranted.

GLM models in the present study suggest that specific group substitutions would influence the toxicity of AQ derivatives. A few studies have focused on the relationship between the toxicity of AQs and their structure. Zhou et al. found that anthraquinones would have similar cytotoxicity when the R1 position was replaced by –OH or $–OCH_3$, comparing two AQs whose groups in R1 sites differ from each other (Zhou et al., 2008). In contrast, we found that there was an obvious difference in hepatotoxicity between –OH and $–OCH_3$ at R1 sites. Feng et al. found that hydroxylation at the R1 position could significantly enhance the cytotoxicity of AQs (Feng, Hao, Chen, & Samuel, 2011). Our GLM models demonstrated that $–OCH_3$ at the R1 position could be more hepatotoxic than –OH at R1. Previous studies are based on simple comparisons and qualitative analyses. GLM models can provide quantitative analysis. However, *in vivo* experimental verifications are needed, and the mechanism requires further exploration.

5. Conclusion

The present study attempts to evaluate the potential toxicity of 26 compounds in *Cassiae* semen using *in silico* and *in vitro* approaches. In addition to predicting the compounds known to cause toxicity successfully, we found an additional 6 hepatotoxic and 10 nephrotoxic candidates for compound prioritization. Ten compounds were predicted to be nontoxic to the liver and kidney. Specific group substitutions, such as –OH and -O-glu, in the AQ structure influence hepatic or renal cytotoxicity.

CRediT authorship contribution statement

Jinlan Yang: Methodology, Investigation, Writing - original draft. **Shuo Wang:** Writing - original draft, Visualization. **Tao Zhang:** Formal analysis. **Yuqing Sun:** Formal analysis, Validation. **Lifeng Han:** Resources, Writing - review & editing. **Prince Osei Banahene:** Investigation. **Qi Wang:** Conceptualization, Supervision, Resources, Writing - review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.crtox.2021.06.001.

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