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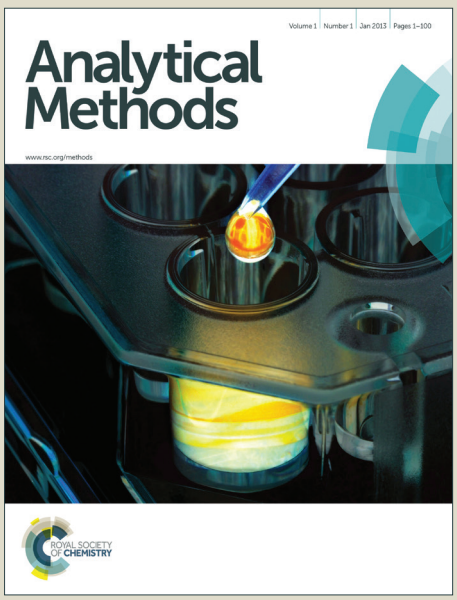

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Simultaneous determination of eight active components in chloroform extracts from raw and vinegar-processed *Genkwa Flos* using HPLC-MS and identification of the hepatotoxic ingredients with HL-7702 cell

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Abstract

Genkwa Flos, a traditional Chinese medicine ~~(TCM)~~, displays severe hepatotoxicity when excessively or chronically used in raw form. It was proved that the chloroform extracts were the major hepatotoxic parts. The vinegar process procedure may weaken the toxicity and enhance the therapeutic effects. This study was conducted to investigate a quality control method of the chloroform extracts of *Genkwa Flos* and identify the potential hepatotoxic ingredients with HL-7702 cells. An LC-MS method was developed and fully validated to simultaneously determine three flavonoids (apigenin, genkwanin and hydroxygenkwanin), three lignans (syringaresinol, medioresinol and matairesinol) and two diterpene esters (yuanhuacine and genkwadaphnin) in the chloroform extracts. With satisfactory linearity, precision, repeatability, stability and recovery, the method was applied to compare the content changes of the eight compounds in raw and processed herbs. After processing, the content of flavonoids increased, the lignans did not obviously change, while the diterpene esters decreased. Compared with the blank control group, the morphology change, viability decrease and the hepatic marker enzymes ~~(AST and ALT)~~ increase were found in cell culture supernatant of HL-7702 cells after given the two diterpene esters. The results provided a comprehensive quantitative method of the chloroform extracts from *Genkwa Flos* and indicated that yuanhuacine and genkwadaphnin could be two of the potential hepatotoxic substances of the herb.

Keywords: *Genkwa Flos*, hepatotoxicity, simultaneous determination, HL-7702 cells

Introduction

Genkwa Flos (GF), the flower bud of *Daphne genkwa* Sieb. et Zucc. (Thymelaeaceae), is listed in the Chinese Pharmacopoeia [1] as a purgation herb medicine. Early chemical researches indicated that flavonoids, lignans and diterpene esters are the main constituents of GF and also the bioactive components for diuretic, antitussive, expectorant, analgesic, abortifacient, sedative and anticonvulsant, antileukemia, antioxidant and antitumor [2-9].

GF has a long history in clinical practice. However, the hepatotoxicity induced by GF has been reported [10-13] in recent years and the previous studies indicated that raw GF should not be used directly unless the toxicity has been decreased or removed. Traditionally, vinegar processing, documented in Chinese Pharmacopoeia [1], is the best choice to weaken the toxicity and relief the symptom of vomiting and abdominalgia.

In our previous study [12, 13], Sprague-Dawley (SD) rats were orally administrated with GF extracts processed by different solutions. Based on the results of histopathology and classical liver biochemical indicators (aspartate aminotransferase, AST and alanine transarninase, ALT), it was found that the hepatotoxic ingredients of GF were major in chloroform extracts and the vinegar-processing can indeed reduce hepatotoxicity.

Although the hepatotoxicity of GF was recognized for years, there was few study on the quality control of the hepatotoxic parts. The theoretical foundation of vinegar processing are still unclear and the potential hepatotoxic ingredients are uncertain. Up to now, only several reports described quantitative methods (TIC, LC) [14-17] to determine one or two kinds of the main components of GF. There is a lack of effective method to simultaneously determine the three types (flavonoids, lignans and diterpene esters) of constituents in GF, and it is dangerous for patients to take GF without knowing the exact contents. Therefore, it is important and necessary to develop a method for the simultaneous assay of the major constituents in the hepatotoxic parts of GF.

In this study, a total of eight representative active substances in chloroform

extracts from GF were simultaneously assayed by a sensitive and robust LC-MS method, including three flavonoids (apigenin, genkwanin and hydroxygenkwanin), three lignans (syringaresinol, medioresinol and matairesinol) and two diterpene esters (yuanhuacine and genkwadaphnin). It is the first time to build a quantitative method for the comprehensive analysis of the three main kinds of bioactive components from the hepatotoxic parts of GF. Since the underlying mechanisms of herb processing were found mainly related to the changes in the composition and/or activity of the components in the herbs [18], the content changes between raw and vinegar-processed GF were observed, and the experiments on HL-7702 cell, including cell morphology, cell viability, the hepatic marker enzymes (ALT and AST) in cell culture supernatant, were carried out on the content-decreased components to assure the hepatotoxic substances. The results may improve the quality control of the hepatotoxic parts of GF, confirm the hepatotoxic substances and contribute to the safety application in clinical.

Experimental

Chemicals and materials

A total of fourteen batches of GF were purchased from drug stores in different provinces of China and authenticated by Professor Ying Jia. (School of Traditional Chinese Materia Medica, Shenyang Pharmaceutical University). The collection locations are listed in Table 1. The reference standards of syringaresinol (1), medioresinol (2), matairesinol (3), apigenin (4), genkwanin (5), hydroxygenkwanin (6), genkwadaphnin (7) and yuanhuacine (8) were isolated and identified previously from the raw GF in our lab. Their structures were elucidated by IR, MS, NMR analyses, and the purities were determined to be more than 98% by HPLC-DAD (Shimadzu, Tokyo, Japan). Mangiferin (internal standard, IS) and diosbulbin-B (positive control for cell) were supplied by the National Institutes for Food and Drug Control (Beijing, China). Structures of the eight compounds and IS are shown in Figure 1 Methanol (HPLC grade) was obtained from Fisher Scientific (Fair Lawn, NJ, USA). Distilled water was purchased from Wahaha Co., Ltd (Hangzhou, China) and used throughout the study. Rice vinegar was purchased from Jiangsu Hengshun

Vinegar Co., Ltd (Zhenjiang, China). Dimethyl sulfoxide (DMSO) was purchased from Yuwang Industrial Co. Ltd. (Shandong, China). The commercial kits used in biochemical assays of AST and ALT were purchased from Nanjing Jiancheng Bioengineering Institute (Nanjing, China).

Preparation of Stock Solutions

The reference standards of compounds 1-8 were accurately weighed and dissolved in methanol at the concentrations of 0.265, 0.0696, 0.289, 0.565, 0.496, 0.515, 0.0556 and 0.117 mg/mL, respectively. A proper amount of the eight standard solutions above were pipetted accurately into one volumetric flask with methanol making total capacity to 10 mL for the mixed standard solution.

The stock solution of IS (1.01 mg/mL) was diluted to concentration 0.0404 mg/mL with methanol as working solution. All the solutions were stored at 4 °C.

Preparation of Genkwa Flos

The dried and powdered GF were accurately weighed (approximately 0.6 g) and ultrasonic-extracted with 20 mL of methanol for 30 min. The solutions obtained were removed under reduced pressure; the methanol extracts were redissolved in water and extracted by chloroform of the same volume twice. Then the extract solutions were collected and removed under reduced pressure and the chloroform extracts were redissolved in 5 mL methanol and filtered through a 0.22 μm filter. An aliquot of 100 μL filtrate was mixed with 100 μL of IS, 20 μL of which was used for LC-MS analysis.

The processed drugs were prepared according to Chinese Pharmacopoeia [1]. The dried GF (100 g) were soaked in a mixture of rice vinegar (30 mL) and water (60 mL) for 12 h. When the solvent was almost absorbed, the mixture was stir-heated to nearly dry with gentle heat, and finally air dried and powdered. The preparations of the processed samples were the same as the crude samples.

Instruments and LC-MS conditions

A Shimadzu LC-MS 2010 (Japan) equipped with an ESI interface was applied to this assay. Liquid chromatographic separation was performed on a Kromasil C_{18} column (200 mm × 4.6 mm, 5 μm) at 30 °C. The flow rate was 0.8 mL/min, and 25% of the eluent was split into the inlet of the mass spectrometer. A gradient elution was applied with the initial mobile phase of 55% A (methanol) - 45% B (water). The gradient elution was as follows: 0-8 min, 55% A→65% A; 8-13 min, 65% A→95% A; 13-15 min, 95% A→100%A; 15-20 min, 100% A→100% A. The injection volume was 20 μL.

All the analytes and IS were ionized by ESI source in positive mode under the following conditions: nebulizing gas, 1.5 L/min; curved desolvation line (CDL) temperature, 250 °C; heat block temperature, 200 °C; detector voltage, 1.75 kV; the other parameters were modified as the tuning file. Analysis was conducted in SIM with $[M+Na]^+$ at *m*/*z* 441.05 for syringaresinol, *m*/*z* 411.05 for medioresinol, *m*/*z* 381.05 for matairesinol, m/z 293.00 for apigenin, *m*/*z* 307.00 for genkwanin, *m*/*z* 625.00 for genkwadaphnin, m/z 301.00 for hydroxygenkwanin, *m*/*z* 671.20 for yuanhuacine and the *m*/*z* 445.00 for IS, respectively. The data acquisition was performed by LC-MS Solution Version 3.0.

Method validation

According to the guideline of International Conference on Harmonization [19], the established method was validated for linearity, limit of detection (LOD) and limit of quantification (LOQ), precision (inter- and intra-day precision), repeatability, accuracy and stability. The herbs from Shanxi province were chosen for the method validation.

Calibration, LOD and LOQ

Mixed stock solution was diluted with methanol to appropriate concentrations for establishing calibration curves (dilution factor = 1, 2, 5, 10, 15, 20) and each testing solution was analyzed in triplicates. The calibration ranges for compound 1-8 were 0.2120 - 4.240, 5.568×10^{-2} - 1.114, 0.4624 - 9.25, 2.260 - 45.20, 3.174 - 63.49, 3.296

- 65.92, 8.90×10^{-2} - 1.779 and 0.1872 - 3.744 μg/mL. The calibration curves were constructed by plotting the ratio of peak areas between analytes and IS (*Y*) against the the concentration of each component (*X*, μg/mL) using the linear regression analysis. The LOD and LOQ were measured as the signal-to-noise ratios (S/N) of 3 and 10, respectively.

Precision

Measurement of intra-day and inter-day variability with mixed standard solutions (low, medium, and high concentration) was utilized to assess the precision of the instrument. For intra-day variability, the standard solutions were examined for six injections within one day and for inter-day variability, the standard solutions were analyzed in three consecutive days. Relative standard deviation (RSD) were calculated.

Repeatability

The method's precision was evaluated by the analysis of sample solutions at three concentrations (low, medium and high). Among those, a aliquot of 0.3 g GF powder was prepared for the low concentration, 0.6 g for medium concentration and 0.9 g for high concentration. Each concentration was tested by three samples and RSD value was calculated among the 9 samples.

Accuracy

The accuracy was determined by recovery test performed by spiking three concentration levels (50%, 100%, 150%) of mixed standards into GF powder sample (0.3 g) and then extracted using the method mentioned above. The recovery for each analyte was calculated as follows: recovery (%) =100 × (amount found – original amount)/ amount spiked.

Stability

The stability was tested with sample solutions at 30 ℃ and analyzed at 0, 2, 4, 6, 8, 12 h, respectively.

Sample determination

The validated method was applied to simultaneously determine compounds 1-8 in chloroform extracts from GF covering 14 batches. The contents of the eight compounds in the samples were quantified with the mean values of three replicate injections. The content changes between raw and vinegar processed herbs were observed and the content-decreased compounds, yuanhuacine and genkwadaphnin, were chose to conduct cell experiments.

Cell culture

HL-7702, a kind of normal human liver cell, was purchased from the Institute of Biochemistry and Cell Biology Sciences, Chinese Academy of Sciences (Shanghai, China). The cells were cultured in RPMI 1640 medium (Hyclone, USA) supplemented with 20% fetal bovine serum (FBS), and incubated at 37 ℃ in a humidified incubator with an atmosphere of 5% CO_2. Cells were subcultured every third day (1:3) using trypsinization (0.25%, *w/v*, trypsin in D-Hanks sodium with 0.2% EDTA–2Na) and used within 20 passages of the initial stock culture.

The stock solutions of genkwadaphnin, yuanhuacine and diosbulbin-B were prepared in DMSO fresh, and then were diluted to the desired concentration with culture medium. The final DMSO concentration in the medium was less than 0.1%.

Cell treatment and morphology observation

The HL-7702 cells were seeded in 96-well plates (10^4 cells/well, 100 μL/well) and cultured at 37 ℃ for 12 h. The supernatant was removed and the cells were washed with PBS twice. Medium containing different concentrations of genkwadaphnin (5, 10, 20, 50, 100 μg/mL), yuanhuacine (5, 10, 20, 50, 100 μg/mL) and diosbulbin-B (5, 10, 20, 50, 100 μg/mL) as a positive control were added, respectively. The cells were incubated at 37 ℃ for 24 h followed by an observation

under an inverted microscope.

Cell viability

The HL-7702 cells were seeded in 96-well plates (10^4 cells/well, 100 μL/well) and cultured at 37 °C for 12 h. Then cells were prepared with different concentrations of yuanhuacine, genkwadaphnin and diosbulbin as a positive control. In addition, one blank and one control containing DMSO were included in each experiment. Each treatment was repeated five times. After incubation for 24, 48 and 72 h, the cytotoxicity of samples on the HL-7702 cells was measured by 3-(4,5-dimethylthiazol-z-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay [20]. The supernatant was discarded and the cells were washed with PBS twice. 100 μL of culture medium containing 0.1% MTT (0.5 mg/mL) was added to each well and then the cells were incubated for 4 h. At the end of incubation period, the medium was removed and 150 μL of dimethylsulfoxide (DMSO)/well was added to solubilize formazan crystals. The absorbance of each well at 570 nm was detected with an ELISA plate reader.

ALT and AST measurement

The activities of The hepatic marker enzymes (ALT and AST) in cell culture supernatant after incubation for 72 h were determined using commercial kits according to the manufacturer's protocols.

Statistical analysis

The data were expressed as mean ± standard deviation (mean ± SD). Statistical analysis was performed using SPSS 16.0. Paired-sample *t* test was adopted in content determination and unpaired *t* test was adopted in cell experiments. The significant difference was judged as $p<0.05$.

Results and Discussion

Optimization of LC-MS conditions

Since it is quite difficult to determine lignans and diterpene esters with HPLC for their low inherent UV absorbance and low contents in GF, an LC-MS method was established for its excellent sensitivity to comprehensively determine the three main kinds of components in the chloroform extracts of GF. Both positive and negative ion modes were tested, and the three flavonoids ingredients (apigenin, genkwanin and hydroxygenkwanin) can be detected in two modes, while the lignans and diterpene esters showed better response in positive mode, especially the $[M+Na]^+$. What's more, when in positive mode, the response of $[M+Na]^+$ is much better than that of $[M+H]^+$ for the flavonoids. Finally, the positive mode and SIM of $[M+Na]^+$ of eight analytes were selected, and the ions showed good stability and reproducibility during the method development and validation. Methanol provided lower background than acetonitrile. While gradient elution was adopted for the less time-consuming and better peak shape than isocratic elution. Representative chromatograms are shown in Figure 2.

Method validation

The calibration curves, LOD and LOQ are listed in Table 2. All the analytes showed good linearity ($r > 0.9992$) in the test concentration range. The precision and accuracy obtained for the calibration points used for the calibration curve were calculated. All of the RSD (%) for precision were less than 1.5%, and RE (%) for accuracy were no more than 3.2% with RSD (%) less than 2.0%, which indicate the linearities of the method were reliable. The established method showed a good reproducibility with intra- and inter-day variabilities less than 3.4% and 4.2%, respectively. The repeatabilities of the method were not more than 4.6%, and the recoveries were in the range of 96.6% - 98.8% with RSD < 3.3% (Table 3). All the analytes in the sample were stable within 12 h with RSD < 4.1%.

Sample determination

All the eight compounds were detected in 14 batches of samples. The flavonoids showed the highest amount, the lignans the second and the diterpene esters the last.

The contents of the analytes were quantified and the results are summarized in Table 1.

It was found that there is a variability in the contents of raw GF among the 14 batches of samples. The content of lignans showed a maximum difference about 5 times; flavonoids about 3 times and diterpene esters about 2 times. Several reasons may contribute to the differences, such as plant origin, picking time, drying process and storage conditions. Those all suggested that each procedure dealing with the herbs should be standardized in the future to control the quality of GF.

After the vinegar-processing procedure, the eight represented compounds showed different changes: the flavonoids (apigenin, genkwanin and hydroxygenkwanin) increased and the lignans (syringaresinol, medioresinol and matairesinol) showed no obviously change, while the diterpene esters (genkwadaphnin and yuanhuacine) decreased.

There are several kinds of flavonoid glycosides in GF, such as genkwanin-5-*O*-*β*-D-glucoside, genkwanin-5-*O*-*β*-D-primeveroside, hydroxygenkwanin-3'-*O*-*β*-D-glucoside, apigenin-7-*O*-*β*-D-glucopyranoside and apigenin-7-*O*-*β*-D-glucuronide [2]. Glycosides are easy to hydrolyze under acidic conditions or heating, and therefore the flavonoid glycosides in GF might convert to corresponding aglycones during the vinegar processing procedure. Besides, it's reported that the inherent subacid flavonoids are easily extracted at the condition of acid [21, 22]. That might be the responsible reason for the increase of genkwanin, hydroxygenkwanin and apigenin. As for genkwadaphnin and yuanhuacine, their ortho-ester structure may open loop with acid, which may lead to the decrease.

The vinegar processing procedure consists of two main steps: moistening and stir-frying. During the moistening, the herb was soaked in vinegar and water for 12 h, while the flavonoid glycosides, hydrophilic and irritative to gastrointestinal tract, may dissolved in the solution. When stir-frying, the flavonoid glycosides were transformed into flavonoid aglycones, which are reported as bioactive ingredients for antibacterial, autoxidation and antiinflammatory of GF. Hence, it explained that vinegar processing can enhance the therapeutic effect of GF and the amount and ratio of vinegar and

water may play a major impact on the effect of the processing.

The underlying mechanisms of herb processing were found mainly related to the changes in the composition and/or activity of the components in the herbs [18]. As vinegar processing procedure was confirmed to reduce the hepatotoxicity of GF, and the content-increased ingredients, the three flavonoids, were reported to be bioactive. We inferred that the content-decreased ingredients, genkwadaphnin and yuanhuacine, may be related to the hepatotoxic substances. Thus, the experiments on HL-7702 cells, norman human liver cells, were conducted to prove whether yuanhuacine and genkwadaphnin are hepatotoxic.

Cell morphology observation and viability assay

Compared with normal cells, the cells treated with genkwadaphnin and yuanhuacine became smaller, round, and some cells burst and float (Figure 3). The higher the concentration of treated compounds, the more the number of cells showing abnormal morphology.

Cell viability showed both dose- and time-dependent relationship in the tested range for both compounds. As shown in Figure 4, at the same concentration, the positive control compound, diosbulbin-B, showed the highest inhibition rate, yuanhuacine the second and genkwadaphnin the last. Genkwadaphnin with high dose(50 and 100 μg/mL) and yuanhuacine with middle dose (20, 50 and 100 μg/mL) can obviously reduce the cell viability (inhibition rate > 50% in 72 h). The maximum inhibition rates and IC_{50} value after incubated for 24, 48 and 72 h were listed in Table 4.

Effect on AST and ALT

Due to the change of cell membrane permeability induced by the potential hepatotoxic substances, AST and ALT, which are normally in the cytoplasm, were leaked out from hepatocytes to culture medium. The leakage of AST and ALT were measured to evaluate the degree of cellular injury. As shown in Figure 5, both

genkwadaphnin and yuanhuacine could caused the increase of AST and ALT with dose dependent manner. Compared with normal cells, samples incubated with genkwadaphnin and yuanhuacine showed significant differences in the concentration ranges of 50-100 μg/mL and 20-100 μg/mL, respectively.

According to the results of cell experiments, it was summarized that genkwadaphnin with high dose and yuanhuacine with middle dose can change the cell morphology of HL-7702, reduce the cell viability and increase the hepatic marker enzymes level, indicating that genkwadaphnin and yuanhuacine may be toxic to liver cells.

Conclusion

An LC-MS quality control method were developed to simultaneously determine three flavonoids (apigenin, genkwanin and hydroxygenkwanin), three lignans (syringaresinol, medioresinol and matairesinol) and two diterpene esters (yuanhuacine and genkwadaphnin) in the hepatotoxic parts of Genkwa Flos. The content variation between raw and vinegar-processed herbs was observed and the content-decreased ingredients, yuanhuacine and genkwadaphnin, were identified as two of the hepatotoxic substances of Genkwa Flos combining experiments on HL-7702 cells.

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Table 1. Determination of sample content in raw and processed Genkwa Flos ($n = 3$, μg/g)

Origins	1	1*	2	2*	3	3*	4	4*	5	5*	6	6*	7	7*	8	8*
Shanxi	191.0	170.0	19.60	19.20	186.0	145.0	1.090E+03	1.240E+03	2.540E+03	3.910E+03	2.770E+03	3.030E+03	54.10	49.70	154.0	115.0
Zhejiang	184.0	162.0	22.40	24.90	58.20	42.30	1.240E+03	1.420E+03	3.580E+03	4.740E+03	1.610E+03	2.210E+03	75.40	72.80	73.40	57.60
Anhuiwuhu	233.0	243.0	24.10	31.40	68.80	49.50	1.500E+03	1.710E+03	4.300E+03	5.080E+03	2.030E+03	2.640E+03	74.20	69.10	76.20	57.30
Henan	277.0	259.0	18.50	12.20	474.0	456.0	1.630E+03	1.770E+03	4.970E+03	5.230E+03	4.940E+03	5.410E+03	93.0	84.0	136.0	109.0
Liaoningshenyang	196.0	217.0	32.90	41.00	93.0	82.0	8.90E+02	1.140E+03	3.020E+03	4.310E+03	2.020E+03	2.930E+03	74.30	67.40	60.20	47.10
Hubeiwuhan	193.0	185.0	21.70	17.30	434.0	447.0	1.420E+03	1.720E+03	4.900E+03	5.170E+03	4.710E+03	5.650E+03	63.50	54.80	123.0	105.0
Jiangsunanjing	294.0	271.0	18.80	12.50	279.0	269.0	1.110E+03	1.320E+03	4.290E+03	5.150E+03	4.160E+03	4.980E+03	82.0	73.70	77.40	52.60
Shandongjinan	199.0	186.0	23.90	19.80	268.0	245.0	1.040E+03	1.250E+03	3.730E+03	4.920E+03	3.600E+03	4.250E+03	87.0	76.10	78.70	63.80
Hebeianguo	62.70	75.40	26.70	17.80	162.0	152.0	7.520E+02	1.060E+03	1.920E+03	3.240E+03	1.910E+03	3.050E+03	79.40	70.80	62.90	42.60
Anhuibozhou	192.0	178.0	34.40	35.70	385.0	373.0	1.160E+03	1.390E+03	2.930E+03	3.390E+03	3.230E+03	4.840E+03	81.0	73.40	134.0	95.0
Shan-Xi	290.0	269.0	42.10	58.90	274.0	251.0	1.150E+03	1.460E+03	4.380E+03	5.120E+03	4.420E+03	5.310E+03	92.0	82.0	76.70	59.60
Shandonglinyi	271.0	283.0	89.0	62.70	296.0	287.0	1.120E+03	1.380E+03	4.410E+03	5.180E+03	4.380E+03	5.260E+03	95.0	83.0	103.0	72.80
Sichuanguangyuan	272.0	281.0	39.30	41.20	251.0	263.0	1.080E+03	1.300E+03	4.260E+03	5.090E+03	4.130E+03	5.420E+03	97.0	79.00	72.50	56.70
Hebei	57.50	62.30	26.30	38.40	142.0	155.0	721.0	9.40E+02	1.860E+03	3.020E+03	1.840E+03	3.930E+03	81.0	70.80	68.40	48.20

(1) syringaresinol, (2) medioresinol, (3) matairesinol, (4) apigenin, (5) genkwanin, (6) hydroxygenkwanin, (7) genkwadaphnin, (8) yuanhuacine.

* vinegar processed Genkwa Flos

Table 2. Calibration curve, LOD and LOQ of eight compounds

Analyte	Regression equation	linear range (μg/mL)	*R*	LOD (ng/mL)	LOQ (ng/mL)
Syringaresinol	$Y = 1.390\,X + 9.29 \times 10^{-3}$	0.2120 - 4.240	0.9994	3	10
Medioresinol	$Y = 0.3517\,X - 3.419 \times 10^{-3}$	5.568×10^{-2} - 1.114	0.9992	3	10
Matairesinol	$Y = 0.5017\,X - 1.202 \times 10^{-2}$	0.4624 - 9.250	0.9996	3	10
Apigenin	$Y = 0.1008\,X + 4.295 \times 10^{-2}$	2.260 - 45.20	0.9995	5	20
Genkwanin	$Y = 0.1507\,X + 2.397 \times 10^{-2}$	3.174 - 63.49	0.9997	5	20
Hydroxygenkwanin	$Y = 4.646 \times 10^{-2}\,X - 3.951 \times 10^{-2}$	3.296 - 65.92	0.9992	5	20
Genkwadaphnin	$Y = 0.3075\,X + 1.224 \times 10^{-3}$	8.90×10^{-2} - 1.779	0.9997	1	5
Yuanhuacine	$Y = 6.959 \times 10^{-2}\,X - 3.077 \times 10^{-3}$	0.1872 - 3.744	0.9995	1	5

447 **Table 3**. Precision, repeatability, and recovery of eight compounds

Compounds	Precision			Repeatability RSD (%)	Recovery	
	Concentration (μg/g)	Intra-day RSD (%)	Inter-day RSD (%)		Average (%)	RSD (%)
syringaresinol	60 120 180	2.5	3.1	3.0	97.8	2.1
medioresinol	10 20 30	3.2	2.6	3.7	97.7	3.1
matairesinol	110 220 330	2.6	4.2	2.9	99.1	2.6
apigenin	350 700 1050	2.7	3.7	3.4	96.0	2.0
genkwanin	1000 2000 3000	2.4	3.2	2.6	99.3	3.1
hydroxygenkwanin	1000 2000 3000	1.9	2.6	2.7	96.7	3.1
genkwadaphnin	30 60 90	2.8	3.1	4.5	96.2	3.3
yuanhuacine	50 100 150	3.4	3.8	4.6	97.0	3.3

448 **Table 4.** The maximum inhibition rates (MIR) and *IC*50 of HL-7702 cell following incubation for 24, 48 and 72 h, respectively.(n = 5, mean ± SD)

Compound	MIR			*IC*50		
	24 h	48 h	72 h	24 h	48 h	72 h
Genkwadaphnin	39.20%	56.13%	63.71%	177.5 ± 7.5	112..5 ± 9.6	45.82 ± 4.26
Yuanhuacine	69.24%	76.11%	84.4%	45.82 ± 4.96	15.24 ±7.32	10.35 ± 5.43
Diosbulbin-B	75.46%	82.8%	85.2%	20.17 ±1.98	13.69 ±1.85	8.60 ± 1.21

Figure Captions

Figure 1. Structures of the investigated components and IS.

Figure 2. Typical SIM chromatograms of mixed standards (A) and crude Genkwa Flos sample (B): (1) syringaresinol, (2) medioresinol, (3) matairesinol, (4) apigenin, (5) genkwanin, (6) hydroxygenkwanin, (7) genkwadaphnin, (8) yuanhuacine.

Figure 3. Typical morphology of HL-7702 cells (200×) following various treatment with diosbulbin-B for 24 h. (A) blank control, (B) 5 μg/mL, (C) 10 μg/mL, (D) 20μg/mL, (E) 50 μg/mL, (F) 100 μg/mL; (1) diosbulbin-B, (2) genkwadaphnin, (3) yuanhuacine.

Figure 4. The inhibition rate after incubated with genkwadaphnin (A), yuanhuacine (B) and diosbulbin-B (C) in different concentrations on HL-7702 cell in 24, 48, and 72 h, respectively.

Figure 5. AST and ALT values in HL-7702 following the treatment of genkwadaphnin, yuanhuacine and diosbulbin-B for 72 h. (*, $p < 0.05$, compared with blank control).

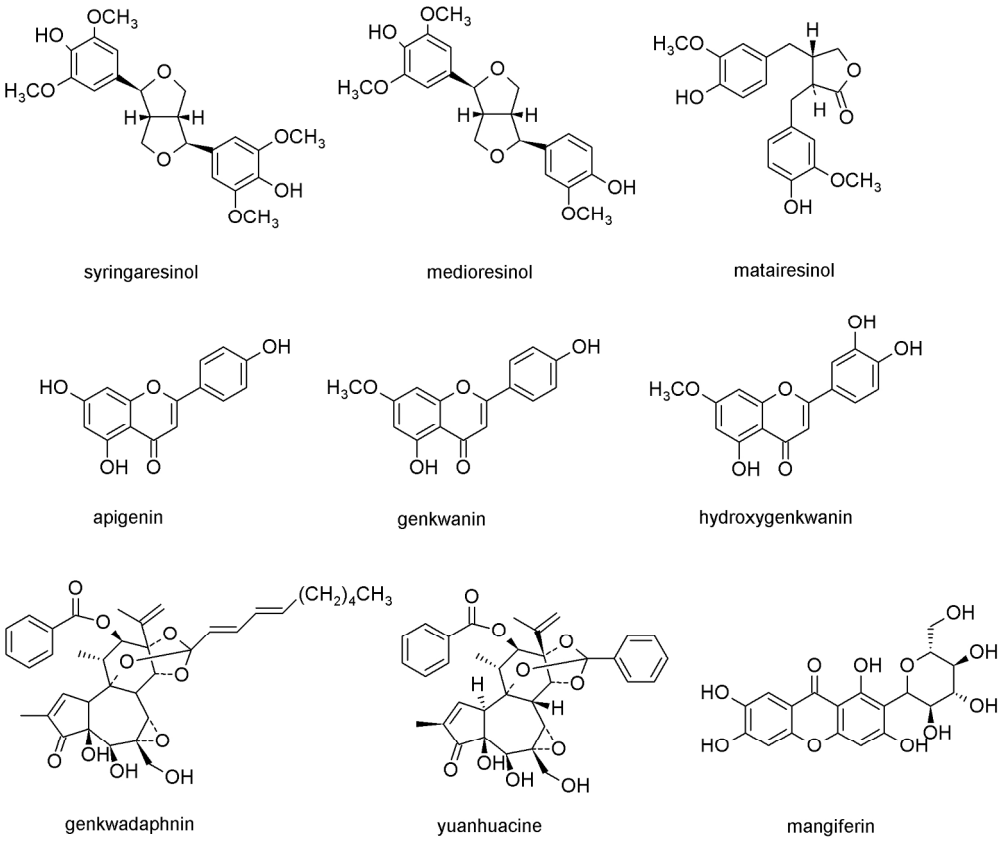

Figure 1. Structures of the investigated components and IS.
190x159mm (300 x 300 DPI)

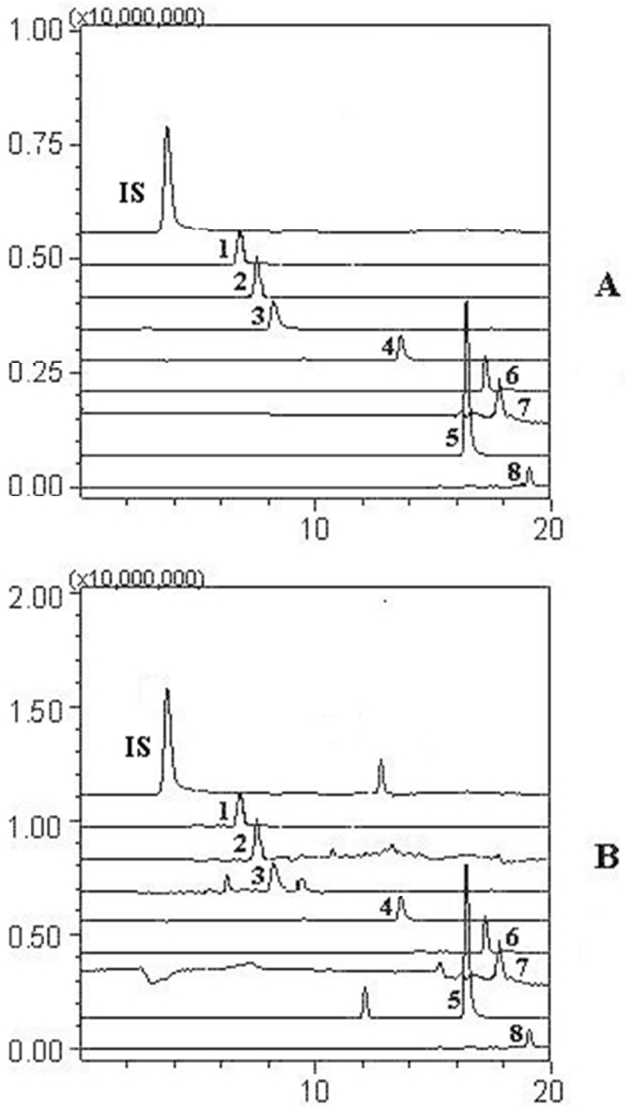

Figure 2. Typical SIM chromatograms of mixed standards (A) and crude Genkwa Flos sample (B): (1) syringaresinol, (2) medioresinol, (3) matairesinol, (4) apigenin, (5) genkwanin, (6) hydroxygenkwanin, (7) genkwadaphnin, (8) yuanhuacine.
33x50mm (300 x 300 DPI)

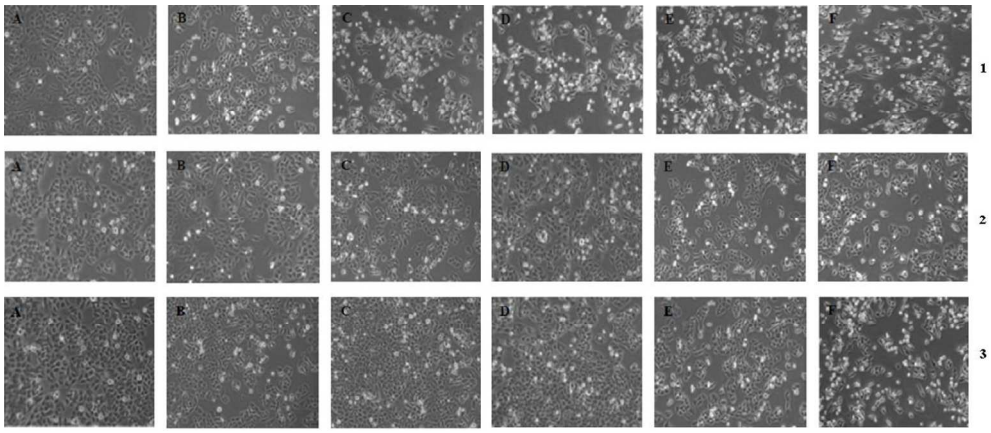

Figure 3. Typical morphology of HL-7702 cells (200×) following various treatment with diosbulbin-B for 24 h. (A) blank control, (B) 5 μg/mL, (C) 10 μg/mL, (D) 20μg/mL, (E) 50 μg/mL, (F) 100 μg/mL; (1) diosbulbin-B, (2) genkwadaphnin, (3) yuanhuacine.
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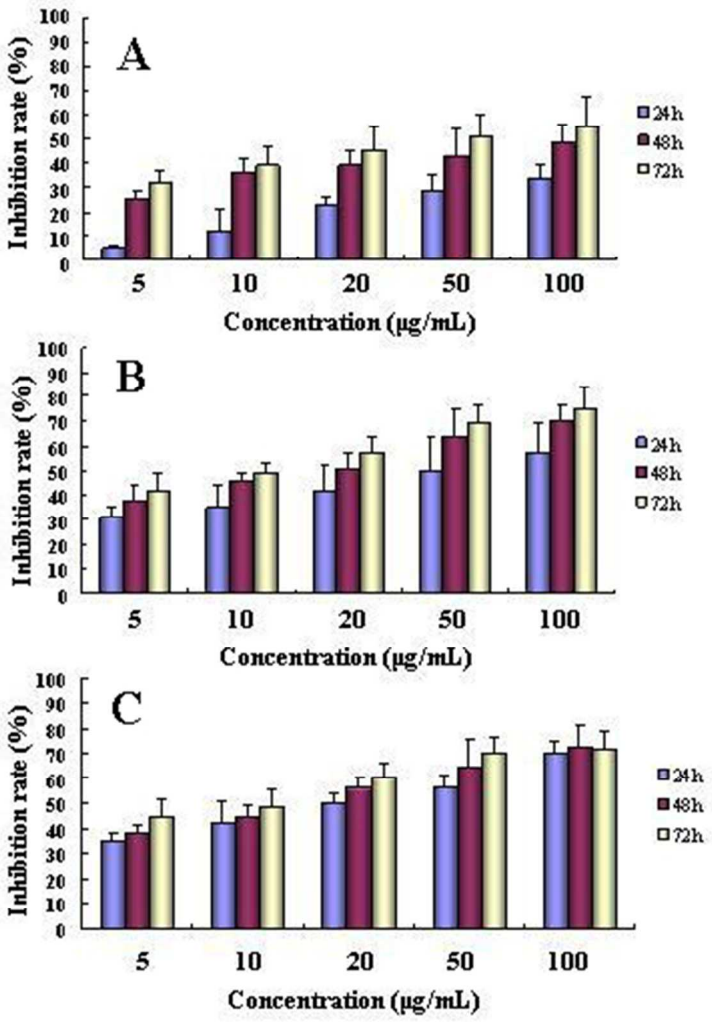

Figure 4. The inhibition rate after incubated with genkwadaphnin (A), yuanhuacine (B) and diosbulbin-B (C) in different concentrations on HL-7702 cell in 24, 48, and 72 h, respectively.
31x44mm (300 x 300 DPI)

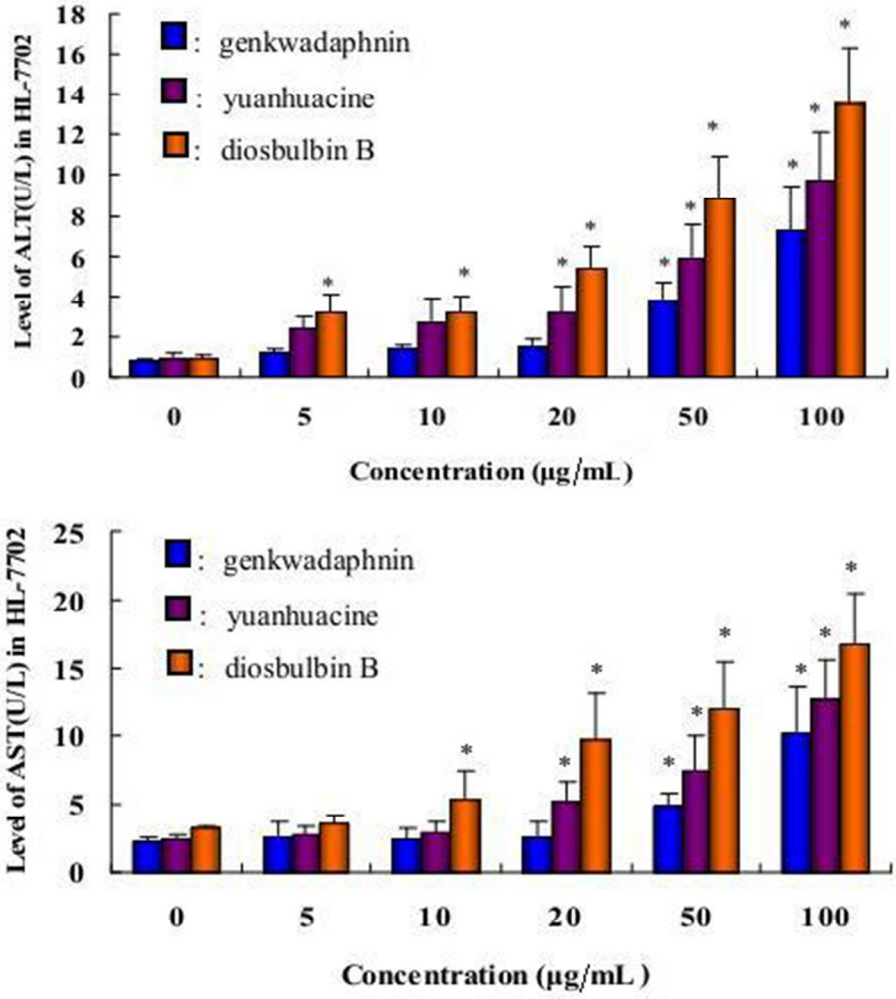

Figure 5. AST and ALT values in HL-7702 following the treatment of genkwadaphnin, yuanhuacine and diosbulbin-B for 72 h. (*, $p < 0.05$, compared with blank control).
40x43mm (300 x 300 DPI)