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Case Report

Compound Analysis of Jing Liqueur and nrf2 Activation by Jing Liqueur—One of the Most Popular Beverages in China

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Abstract: The aim of this study is to identify the minor compounds in Jing liqueur, determine the concentration of metals, amino acids, and polysaccharides, and evaluate their Nrf2 activity and cytotoxicity. Jing liqueur that contains Chinese medicine is one of the best-selling liqueurs in China, which is also marketed in the United States. Totally, we have isolated 189 minor compounds including one new molecule (**7**) from a concentrated Jing liqueur, with the concentrations of most isolated compounds at micromolar levels. The structures of all these compounds were determined by using MS and NMR (1D and 2D) or by comparison of their chemical and physical data with reported values in the literatures. Besides, the concentrations of iron (0.52 mg/L), zinc (0.21 mg/L), calcium (11.0 mg/L), *L*-proline (2.33 mg/L), *L*-arginine (1.73 mg/L), total amino acids (9.84 mg/L), and total polysaccharides (337.4 mg/L) were determined. Jing liqueur, the five fractions and most of the compounds isolated from Jing liqueur were screened for their activities in the Nrf2-ARE and MTT assays. At 5.2 mg/mL the crude enhanced the Nrf2 activity. At 80 μg/mL, fraction IV weakly but fraction V strongly activated Nrf2. Among the compounds screened in the Nrf2 assay, eighteen activated Nrf2 at 40 μg/mL and compounds **51** and **126** from fraction V were the most active. The crude, all the five fractions, and Nrf2 activators were not cytotoxic toward HepG2 cells. In conclusion, Jing liqueur contains different classes of compounds including flavonoids, terpenoids, alkaloids, coumarins, cinnamic acid or coumaric acid, and phenyl ethanol (or acetic acid) derivatives, benzoquinone, naphthoquinone, anthraquinones or phenanphrene derivatives, xanthones, chromone, and γ-pyrone derivatives, lignans, other aromatic compounds, and others. Jing liqueur and the eighteen compounds, which were isolated from Jing liqueur, could activate Nrf2 without any cytotoxicity.

Keywords: Jing liqueur; traditional Chinese medicine; compounds; NMR; MS; Nrf2-ARE

1. Introduction

Jing liqueur [1–3] is a popular health beverage in China, which contains biologically active components from several tonic traditional Chinese herbal medicines. About 30 years ago, Jing Brand Co., Ltd. (Daye City, Hubei Province, China) began to sell its products overseas. Nowadays, Jing liqueur is sold in more than 20 countries and districts, including Hong Kong, Macau, Japan,

South Korea, Australia, and the United States etc. The liqueur is manufactured using modern bioengineering technology to prepare extracts from Chinese herbal medicines such as *Astragalus membranaceus*, *Cistanche deserticola*, *Dioscorea opposita*, *Lycium barbarum*, *Epimedium brevicornum*, *Cinnamomum cassia*, *Syzygium aromaticum*, *Angelica sinensis*, and *Imperata cylindrica*, several of which are also used as foods or dietary supplements. These Chinese herbal medicines are carefully selected and prepared according to their applications.

A. membranaceus is a very common traditional Chinese medicine (TCM) widely used as an immunostimulant, cardiotonic, hepatoprotective, antidiabetic, antitumor, and antiviral drug [4]. *C. deserticola* is a famous Chinese Materia Medica (CMM) used for the treatment of kidney deficiency, infertility, and chronic constipation [5]. *D. opposita* is a famous tonic Chinese medicine with beneficial effects on spleen, lung, and kidney in addition to the antidiarrheal activity. The tuber is also a favorite food in China used in a stir-fry or in soups [6]. *L. barbarum* a traditional food and medicine in East Asia has become increasingly popular in Europe and North America in recent years. *L. barbarum* is used in folk medicine to increase longevity and is reported to have beneficial effects on blurry vision and diminished visual acuity, infertility, abdominal pain, dry cough, fatigue, and headache [6]. *L. barbarum* is also a very popular ingredient in Chinese cuisine, which is consumed in soups, as porridge with rice, and added to numerous meat and vegetable dishes [7]. *E. brevicornum* is one of the most commonly used traditional Chinese medicines, and has the reported benefits of reinforcing the "kidney yang," strengthening the tendons and bones, and relieving rheumatic conditions. It is also used to treat impotence, seminal emission, weakness of the limbs, rheumatoid arthralgia, and hypertension [8]. *C. cassia* is used in traditional Chinese medicine for various ailments including abdominal pain, vomiting, diarrhea, dysmenorrhea, blood stasis, bruises, and traumatic bleeding. It is also used as an appetite stimulant and a flavoring agent [9]. *S. aromaticum* is a Chinese medicine that is used as an aromatic stomachic agent, to relieve abdominal bloating, increase gastric secretions, aid in digestion, and reduce nausea and vomiting. It is also one of the most ancient and valuable spices of the Orient [10]. *A. sinensis* is one of the most important drugs in traditional Chinese medicine, and is commonly used for treating gynecopathias, including anemia, dysmenorrhea, amenorrhea, premenstrual, and menopausal syndromes. It is also used in the management of cancer, cardiovascular diseases, and Alzheimer's disease. Chicken soup made with Radix Angelica is a popular dish in China [11]. *I. cylindrica* is also a traditional Chinese medicine used in treating hot blood, blood vomiting, blood stasis, hematuria, fever, polydipsia, damp heat jaundice, edema, reduced urine output, and painful urination [12].

Nuclear factor erythroid 2-related factor 2 (Nrf2) is a transcription factor that controls the expression of antioxidant and phase II detoxifying enzymes. Nrf2 is widely recognized for its cytoprotective role, and has defensive properties against neurodegenerative, airway, and cardiac diseases [13]. Nrf2 is also targeted for the prevention of cancer and other chronic diseases such as diabetes, where oxidative stress and inflammation contribute to pathogenesis [14]. Also, Nrf2 activation through cell lineage-specific Keap1 disruption is important for the improvement of autoimmune diseases [15]. In these settings, transient activation of Nrf2 by compounds such as sulforaphane or curcumin can stimulate the expression of Nrf2 target genes to combat oxidative and electrophilic stress, reorganize cortical actin, reduce stress fibers formation, and improve the integrity of cell-cell junctions [16]. On one hand, Nrf2 activators could be used for the prevention of chemical carcinogenesis, whereas Nrf2 inhibitors could be used for cancer treatment [17].

Although the phytochemical composition and pharmacological properties of these nine Chinese herbal medicines have been independently evaluated, Jing liqueur containing extracts from these nine herbal medicines has not been previously investigated much. It has been reported that Jing liqueur showed anti-inflammatory [1], immune enhancement [3], anti-fatigue [2,3] properties, and enhancing Shen-Yang (kidney Yang) or invigorating the vital activities of kidney [3]. We argue that the anti-inflammation and immune enhancement of Jing liqueur may be due to or at least partially due to the Nrf2 activation by these traditional Chinese herbal medicines used in Jing liqueur. Hence, we also decided to evaluate Jing liqueur for its effect on Nrf2 besides the analysis of minor compounds. In this study, we isolated one hundred eighty nine (189) minor compounds from

Jing liqueur including a new flavonoid (**7**), and determined their structures based on the MS data and NMR spectra. In addition, we determined the concentrations of iron, zinc, calcium, *L*-proline, *L*-arginine, total amino acids, and total polysaccharides. We also evaluated the effects of the crude Jing liqueur, the five fractions, and majority of the isolated compounds on the Nrf2 activity in a cell-based assay, and investigated the cytotoxicity of the crude Jing liqueur, the five fractions and the identified Nrf2 activators in a MTT assay. At 40 μg/mL, eighteen compounds demonstrated Nrf2 activation without any cytotoxicity, and compound **51** was slightly less active while compound **126** was more active than the positive control SF (5 μM), indicating that compounds **51** and **126** might be responsible for or partially account for the Nrf2 activation.

2. Materials and Methods

2.1. Plant Materials

Plant materials were collected in 2017 by researchers at Jing Brand Research Institute. Voucher specimens (JP20170219, *A. membranaceus*, Min County, Dingxi City, Gansu Province, China; JP20170259, *C. deserticola*, Hetian City, Xinjiang Uygur Autonomous Region, China; JP20170014, *D. opposita*, Jiaozuo City, Henan Province, China; JP20170214, *L. barbarum*, Zhongning County, Ningxia Hui Autonomous Region, China; JP20170244, *E. brevicornum*, Min County, Dingxi City, Gansu Province, China; JP20170143, *C. cassia*, Fangchenggang City, Guangxi Zhuang Autonomous Region, China; JP20170265, *S. aromaticum*, Indonesia; JP20170133, *A. sinensis*, Min County, Dingxi City, Gansu Province, China; and JP20170000, *I. cylindrica*, Louzhou City, Sichuan Province, China) are deposited at the herbarium of Jing Brand Research Institute, Daye City, Hubei Province, People's Republic of China.

2.2. Preparation of Jing Liqueur

Jing liqueur was prepared at Jing Brand Co., Ltd., using the company's proprietary technology. Following is a basic description of the process: (a). The raw Chinese herbal medicine (*Astragalus membranaceus*, *Cistanche deserticola*, *Dioscorea opposita*, *Lycium barbarum*, *Epimedium brevicornum*, *Cinnamomum cassia*, *Syzygium aromaticum*, *Angelica sinensis*, *and Imperata cylindrica*) was washed, dried, and sliced into pieces according to the protocols as described in the Chinese Pharmacopoeia. (b). Pieces of each herbal medicine were added to the "Xiaoqu white liqueur" with an alcohol content of 35% according to the company's process recipe. After percolation, filtration, and evaporation, various concentrated mother liquids were obtained. (c). The concentrated mother liquids were added to the "Xiaoqu white liqueur" with an alcohol content of 35% for precise blending according to a standardized process recipe. (d). Certain amount of white sugar was added to adjust the taste. (e). The finished product was kept in a storage tank and stored for one year. After quality control, Jing liqueur was filled into small bottles and packaged for shipping to commission merchants.

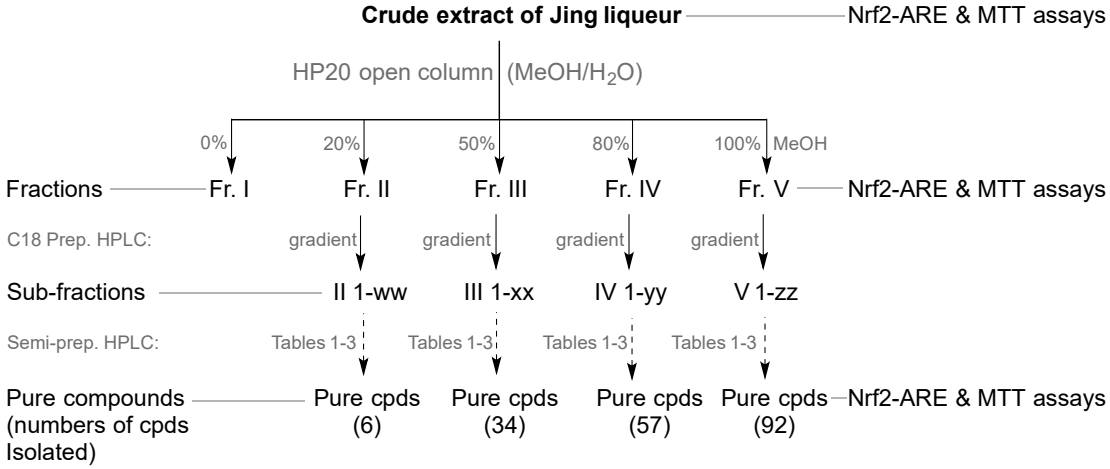
2.3. Concentration of Jing Liqueur

Before white sugar was added, one hundred sixty liters (160 L) of Jing Brand "Xiaoqu white liqueur" (a semi-finished product after step c in the Section 2.2) was concentrated under vacuum to yield a syrup-like liquid, which was about 232 g if completely dried and was used for the separation and purification of minor compounds.

2.4. HP20 Open Column, Preparative and Semi-Preparative HPLC

To generate five fractions for the Nrf2 and cytotoxicity assay, 20 mL Jing liqueur was dried to yield a sample (1.66 g) in a pilot study. The sample was dissolved in 10 mL water, and loaded onto an open column (HP20 6.6 g, 1.5 × 6.0 cm). HP20 is based on a unique rigid polystyrene/divinylbenzene matrix, in which a controlled pore size distribution and large surface area offer excellent resolution and the capacity for a wide range of molecules. The separation

mechanism of a HP20 column is very similar to that of the C18 reverse chromatography—the most polar compounds are eluted out of the column with water first, while the most non-polar compounds will be eluted out of the column with methanol. Hence, a gradient solvent system from 100% water to 100% methanol (0, 20, 50, 80, and 100% MeOH/H_2O) was used for the HP20 open column separation, and the eluents were dried using SpeedVac to yield five fractions (Fr. I: 1.5 g; Fr. II: 134 mg; Fr. III: 93.0 mg; Fr. IV: 8.0 mg; Fr. V: 1.3 mg). Separation of large amount of 160 L Jing Brand "Xiaoqu white liqueur" sample was scaled up accordingly. Fraction I was mainly composed of saccharides, which was not chemically investigated in this study. Fractions II, III, IV, and V each were first separated with a Thermo Scientific Ultimate 3000 preparative high performance liquid chromatography (HPLC) system (Column: Phenomenex Luna C18, 100 Å, 100 × 21.2 mm, 5 μm; Flow-rate: 10 mL/min) and then an Agilent 1100 semi-preparative HPLC system (Column: Phenomenex Luna C18 or C8, 100 Å, 250 × 10 mm, 5 μm; Flow-rate: 3 mL/min) to get pure compounds (Scheme 1).



Scheme 1. Flow chart of experimental design and numbers of pure compounds isolated from fractions II–V (See Tables 1–3 for retention times of the 189 pure compounds and high performance liquid chromatography (HPLC) conditions including columns, flow-rates, and solvent systems).

2.5. LC/MS Condition for the Analysis

System: Agilent 1260 HPLC coupled to 6120 quadrupole LC/MS or Agilent 1260 HPLC coupled to an Agilent 6530 Accurate-Mass Q-TOF LC/MS in positive or negative modes. Column: Phenomenex C18, 100 Å, 100 × 4.6 mm, 5 μm; Flow-rate: 0.2 mL/min; Solvent A: water 0.1% formic acid, Solvent B: acetonitrile 0.1% formic acid, loading at 10% B, increasing the solvent gradient to 100% B in 20 min, and then re-equilibrating the HPLC column over 7 min in 10% B. The molecular weights of all the isolated compounds were obtained through LC/MS analysis.

2.6. NMR Experiments

NMR spectra including 1D (one dimension) and 2D (two dimensions) experiments were recorded in acetone-d_6 or MeOH-d_4 or $CDCl_3$ or DMSO-d_6 on a Bruker 400 MHz NMR, which plays a major role in the structural determination of the isolated compounds.

2.7. Analysis of Metals, Amino Acids, and Total Polysaccharides

Iron (GB 5009, 90-2016), zinc (GB 5009, 14-2017), calcium (GB 5009, 92-2016), and amino acids (GB 5009, 124-2016) were analyzed according to the methods as described in the National Food Safety Standards, People's Republic of China. The concentration of total polysaccharides was measured according to the methods as published in the literatures [18].

2.8. Cell Culture and Condition

Nrf2 Antioxidant Pathway ARE Reporter—Hep G2 cell line was purchased from BPS Bioscience (San Diego, CA, USA). Cells were propagated at 37 °C in a humidified incubator with 5% CO_2, in Eagle's minimum essential medium (MEM, Corning, New York, NY, USA) with non-essential amino acids and supplemented with 10% fetal bovine serum (FBS, Invitrogen, Waltham, MA, USA), penicillin and streptomycin (Thermo Fisher, Waltham, MA, USA). Cells were trypsinized and split every 6 to 7 days.

2.9. Chemicals Exposure and Luciferase Assay to Measure the Nrf2 Activation

Sterile DMSO (dimethyl sulfoxide) stock solutions of crude, HP20 fractions and DL-sulforaphane (Sigma # S4441) were prepared in DMSO. The HepG2-Nrf2 stable cell line was seeded into 96-well plates at 4×10^4 per well in a final volume of 100 µL MEM. 24 h after seeding, media was replaced with fresh MEM and the cells were treated with the crude extract or fractions or pure compounds. Plates were incubated for 24 h, then 100 µL ONE-Step Luciferase reagent (BPS Bioscience) was added to each well and the assay was performed according to manufacturer's instructions. Luminescence was detected using a luminometer (LUMIstar Galaxy BMG, Offenburg, Germany) and data are expressed as relative luminescence units (RLU) emitted from total assays. DL-sulforaphane was used as a positive control at a concentration of 5 µM. All experiments were performed in triplicate.

2.10. Cell Viability Assay

Cell viability was assessed by methylthiazoltetrazolium (MTT) assay (Sigma-Aldrich, St Louis, MO, USA) according to the manufacturer's instruction. Briefly, cells (4×10^4) were seeded into a 96-well plate in 100 µL MEM and allowed to adhere overnight. Culture medium was replaced, and cells were treated with crude or fractionated samples (Fr. I-V) or pure compounds for 24 h treatments. The medium of each well was replaced by 200 µL fresh medium plus 50 µL of the MTT solution (5 mg/mL in PBS). The plates were incubated at 37 °C for 4 h. The absorbance being proportional to cell was subsequently measured at 570 nm in each well using a Bio-Rad 680 plate reader (Hercules, CA, USA). DL-sulforaphane was also used as a control at a concentration of 5 µM. All experiments were performed in triplicate.

2.11. Statistical Analysis

Values are expressed as the mean ± standard error of the mean p values < 0.05 were considered statistically significant. All analyses were performed with the Student t-test using GraphPad Prism 5.1 (GraphPad, La Jolla, CA, USA).

3. Results

3.1. Isolation and Structure Elucidation of Minor Compounds from Jing Liqueur

In order to analyze the minor compounds in Jing liqueur, we concentrated 160 L of Jing liqueur Brand "Xiaoqu white liqueur," and separated the extract with HP20 (See Section 2.4 and Scheme 1) into five fractions (Fraction I: 100% H_2O; Fraction II: 20% MeOH/H_2O; Fraction III: 50% MeOH/H_2O; Fraction IV: 80% MeOH/H_2O; Fraction V: 100% MeOH). Each of the fractions (II–V) was further separated with C18 preparative HPLC (Fraction II: 5–25% MeOH/H_2O in 42 min; Fraction III: 15–42% MeOH/H_2O in 42 min; Fraction IV: 23–80% MeOH/H_2O in 50 min; Fraction V: 50–10% MeOH/H_2O in 56 min), and subfractions (one subfraction per min) were collected. Then the subfractions were purified with semi-preparative HPLC to get the pure compounds (See Tables 1–3 for retention times of the 189 pure compounds and semi-preparative HPLC conditions including columns, flow-rates, and solvent systems). In total, one hundred eighty nine (189) compounds, including flavonoids, terpenoids, alkaloids, coumarins, cinnamic acid or coumaric acid, and phenyl ethanol (or acetic acid) derivatives, benzoquinone, naphthoquinone, anthraquinones or phenanphrene derivatives, xanthones, chromone, and γ-pyrone derivatives, lignans, other aromatic compounds, and others

were isolated and identified. The chemical structures were determined by using LC-MS and NMR as shown in Figures 1 and 2. The HPLC conditions, molecular formulas, sources of the corresponding plants, and references of the characterized compounds are summarized in Tables 1–3. All the MS, NMR spectra, and references for the 189 compounds isolated from Jing liqueur are listed in the Supplementary Material. These 189 compounds were simply categorized into nine classes.

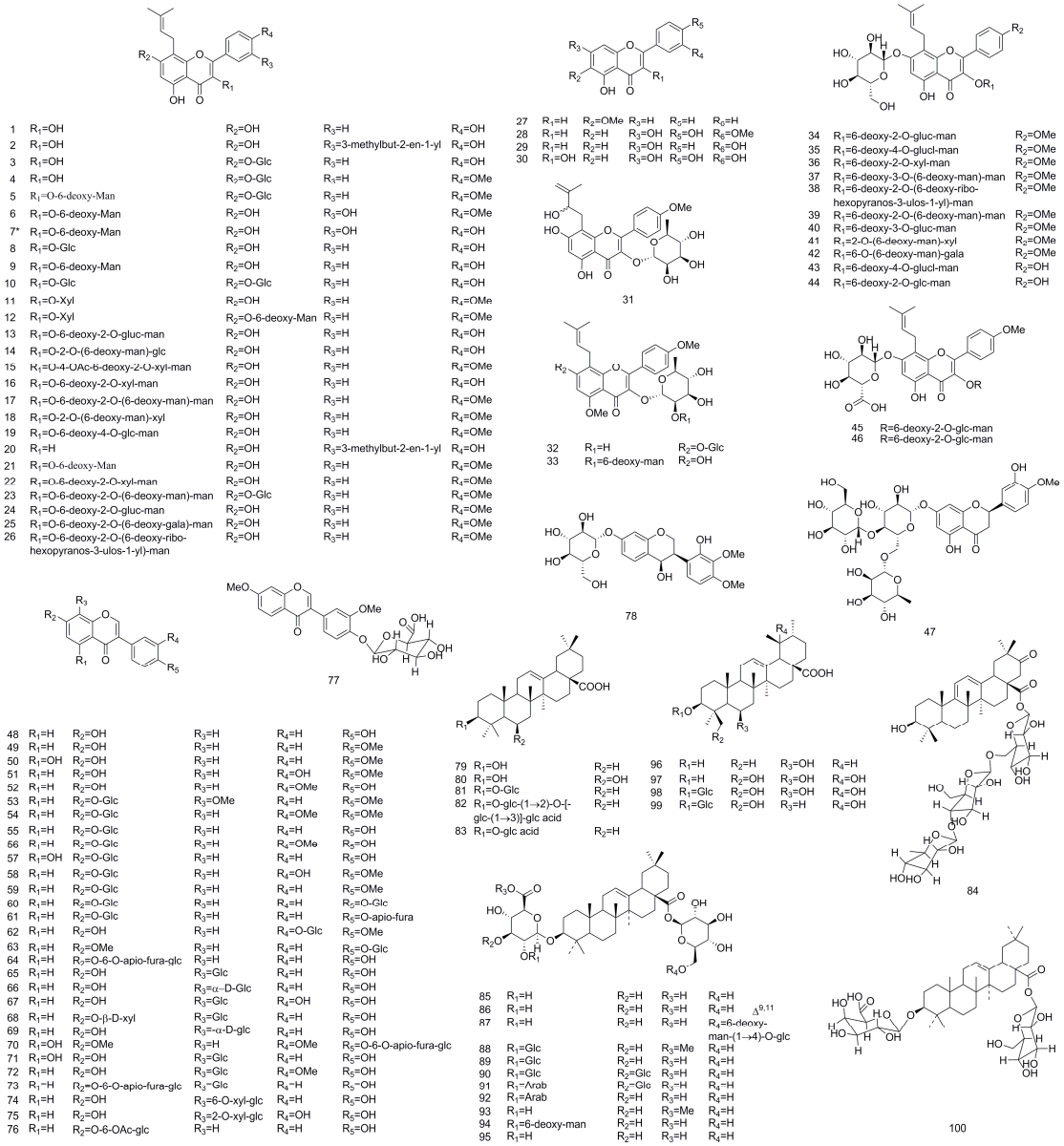


Figure 1. Structures of compounds **1–100** isolated from Jing liqueur.

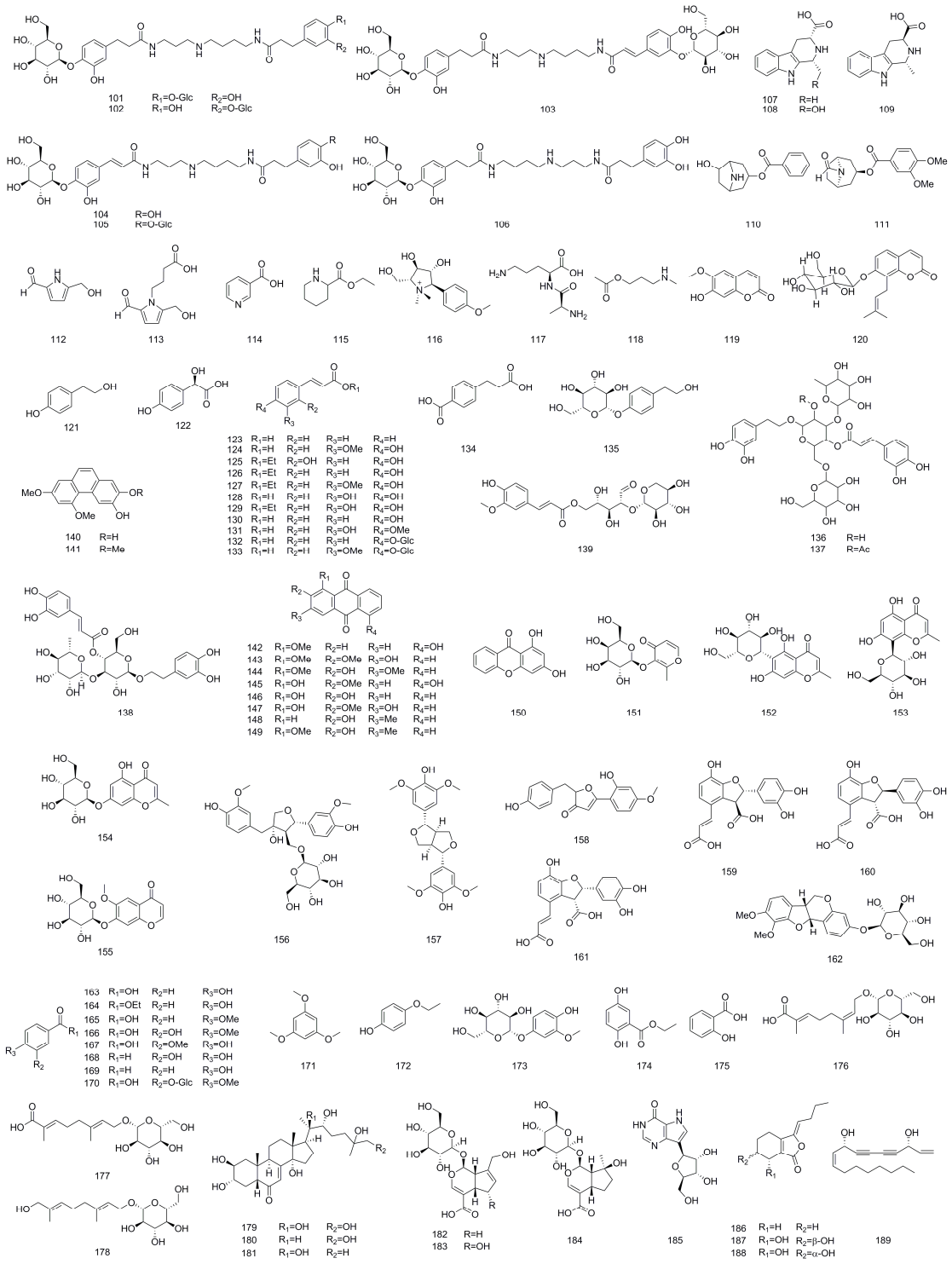


Figure 2. Structures of compounds **101–189** isolated from Jing liqueur.

(1). *Flavonoids*: Seventy-eight flavonoids (#1–78, Table 1, Figure 1) have been isolated. They are either aglycones or glycosides of flavone, flavonol, flavanone, isoflavone, and isoflavanone derivatives. The new compound (**7**) was obtained as a light yellowish powder. Its molecular formula was determined as $C_{26}H_{28}O_{11}$ based on NMR and high resolution ESI mass spectrometry (HRESIMS) data (*m*/*z* 517.1710 $[M + H]^+$, calcd for $C_{26}H_{29}O_{11}$, 517.1710). Its ^1H NMR spectrum in CD_3OD exhibited

signals similar to those of caohuoside C (**6**) [19]. The only difference between **6** and **7** was the presence of the ^{1}H NMR signal for a methoxy group at 4′-position in **6**, but absence in **7**. Hence, the new compound (**7**) was determined as 4′-*O*-demethyl caohuoside C (Tables 1 and S1, Figures 1 and S1). (2). *Terpenoids*: Twenty-two triterpenoids (#79–100) have been isolated from Jing liqueur (Table 2, Figure 1). These compounds are either simple oleanane or ursane derivatives, or glycosides of oleanolic acid. The sugars were connected to either 3-position, or 28-position, or both of the aglycones. Eighteen of these twenty-two triterpenoids are saponins, among which seventeen are oleanane glycosides and one is ursane glycoside [20]. (3). *Alkaloids*: Eighteen alkaloids including derivatives of spermidine, indole, tropane, pyrrole, piperidine, and alanyllysine (#101–118) have been isolated from Jing liqueur (Table 2, Figure 2). (4). *Coumarins, cinnamic acid or coumaric acid, and phenyl ethanol (or acetic acid) derivatives*: Twenty-one coumarins, cinnamic acid or coumaric acid, and phenyl ethanol (or acetic acid) derivatives (#119–139) were isolated from Jing liqueur (Table 2, Figure 2), including ten cinnamic acid analogs. (5). *Benzoquinone, naphthoquinone, anthraquinones, or phenanphrene derivatives*: Ten benzoquinone, naphthoquinone, anthraquinones, or phenanphrene derivatives (#140–149) were isolated from Jing liqueur (Table 3, Figure 2), eight of which were anthraquinones. (6). *Xanthones, chromone, and γ-pyrone derivatives*: Six xanthones, chromone, and γ-pyrone derivatives (#150–155) were isolated (Table 3, Figure 2). (7). *Lignans*: Seven lignans (#156–162) were isolated (Table 3, Figure 2). (8). *Other aromatic compounds*: Thirteen small aromatic compounds (#163–175) were isolated (Table 3, Figure 2). They were either benzoic acid or phenolic derivatives. (9). *Other compounds*: Fourteen other compounds (#176–189) were isolated, including three monoterpenoid glycosides, three cholesterol analogs, three iridoid glycosides, one nucleoside analog, three γ-lactone, and one alkyne derivatives (Table 3, Figure 2). In summary, one hundred eighty nine (189) compounds have been isolated, including ninety two (92) from fraction V, fifty seven (57) from fraction IV, thirty four (34) from fraction III, and six (6) from fraction II.

Table 1. Flavanoids (#1–78) isolated from Jing liqueur.

NO	Fr.	t_R, HPLC Condition	MF	Compound Name	Reference (See SM)
1	V	C.1, ACN, 33%	$C_{20}H_{18}O_6$	Noranhydroicaritin	Komatsu et al. 1970
2	V	Sepherdex LH20	$C_{25}H_{26}O_6$	Broussoflavonol F	Fang et al. 1995
3	IV	71 min, C.1, ACN, 18% 110 min	$C_{26}H_{28}O_{11}$	Epimedoside C	Li et al. 1990
4	IV	68 min, C.2, ACN, 30–35%, 80 min	$C_{27}H_{30}O_{11}$	Icariside I	Mizuno et al. 1987
5	V	56 min, C.1, ACN, 24%	$C_{33}H_{40}O_{15}$	Iearíline	Liang et al. 1988
6	V	C.1, ACN, 33%	$C_{27}H_{30}O_{11}$	Caohuoside C	Li et al. 1995
7*	V	C.1, ACN, 30%	$C_{26}H_{28}O_{11}$	4′-*O*-demethyl caohuoside C (New)	New
8	V	C.1, ACN, 35%	$C_{26}H_{28}O_{11}$	Phelodendrozide	Wang et al. 2010
9	V	C.1, ACN, 33%	$C_{26}H_{28}O_{10}$	baohuoside II	Dong et al. 1994
10	IV	53.5 min, C.2, ACN, 17% 100 min	$C_{32}H_{38}O_{16}$	Hexandraside E	Leu et al. 2006
11	V	C.2, ACN, 40–50%, 48 min	$C_{26}H_{28}O_{10}$		
12	V	49 min, C.2, ACN, 34%	$C_{32}H_{38}O_{14}$	Baohuoside IV	Li and Liu 1990
13	V	C.1, ACN, 35%	$C_{32}H_{38}O_{15}$	Icarisoside B	Fukai et al. 1988
14	V	C.1, ACN, 35%	$C_{32}H_{38}O_{15}$		Zhao et al. 2010
15	V	C.1, MeOH, 64%	$C_{34}H_{40}O_{15}$		Tu et al. 2011
16	V	C.1, ACN, 33%	$C_{31}H_{36}O_{14}$	Ikarisoside F	Fukai et al. 1988
17	IV	75 min, C.2, ACN, 30–35%, 80 min	$C_{33}H_{40}O_{14}$		Ueda et al. 1992
18	V	35.5 min, C.2 ACN, 36%	$C_{32}H_{38}O_{14}$		Zhao et al. 2010
19	V	39 min, C.2, ACN, 34%	$C_{33}H_{40}O_{15}$	Baohuoside VII	Li et al. 1988
20	V	Sepherdex LH20	$C_{25}H_{26}O_5$	5,7, 4′-trihydroxy-8, 3′-diprenylflavone	Guo et al. 2006
21	V	C.1, MeOH, 65%	$C_{27}H_{30}O_{10}$	Icariside II	Zhang et al. 2006
22	V	C.1, MeOH, 65%	$C_{32}H_{38}O_{14}$	Sagittatoside B	Mizuno et al. 1988
23	V	C.1, ACN, 24%	$C_{39}H_{50}O_{19}$	Epimedin C	Mizuno et al. 1988
24	V	C.1, ACN, 24%	$C_{33}H_{40}O_{15}$	Sagittatoside A	Mizuno et al. 1988
25	V	C.1, MeOH, 65%	$C_{33}H_{40}O_{14}$	2″-*O*-rhamnosyl icariside II	Zhao et al. 2016
26	V	C.1, MeOH, 64%	$C_{33}H_{38}O_{14}$	3‴-carbonyl-2″-β-L-quinovosyl icariside II	Zhang et al. 2006
27	V	C.1, ACN, 33%	$C_{16}H_{12}O_4$		Asahina et al. 1935
28	IV	85.5 min, C.1, ACN, 18% 110 min	$C_{39}H_{50}O_{20}$		Das and Tripathi 2002
29	V	Sepherdex LH20	$C_{15}H_{10}O_5$	Versulin	Geissman et al. 1946
30	V	Sepherdex LH20	$C_{15}H_{10}O_7$	Xanthaurine	Bao et al. 2004
31	V	C.1, ACN, 35%	$C_{27}H_{30}O_{11}$	Koreanoside E	Li et al. 2015
32	V	C.1, MeOH, 64%	$C_{34}H_{42}O_{15}$		Hu et al. 2010

33	V	C.1, MeOH, 64%	$C_{34}H_{42}O_{14}$		
34	V	C.1, ACN, 24%	$C_{39}H_{50}O_{20}$	Epimedin A	Han, Lee 2017
35	V	C.1, ACN, 24%	$C_{39}H_{50}O_{20}$	Maohuoside B	Li et al. 2006
36	V	C.1, ACN, 24%	$C_{38}H_{48}O_{19}$	Epimedin B	Guo and Xiao 2003
37	V	C.1, ACN, 24%	$C_{39}H_{50}O_{19}$	Hexandraside D	Mizuno et al. 1991
38	V	C.1, ACN, 28%	$C_{39}H_{48}O_{19}$		Zhao et al. 2008
39	V	C.1, ACN, 28%	$C_{39}H_{50}O_{19}$		Ueda et al. 1992
40	V	29 min, C.1, ACN, 22–33%, 60 min	$C_{39}H_{50}O_{20}$	Hexandraside F	Wang et al. 2007
41	V	33 min, C.1, ACN, 22–33%, 60 min	$C_{38}H_{48}O_{19}$		Zhao et al. 2010
42	IV	85.5 min, C.2, ACN, 18% 110 min	$C_{16}H_{12}O_6$	Diosmetin	Takeda et al. 2007
3	V	77.5 min, C.2, ACN, 16–20%, 100 min	$C_{38}H_{48}O_{20}$	Rouhuoside	Li et al. 1990
44	V	47 min, C.2, ACN, 22–33%, 60 min	$C_{38}H_{48}O_{20}$	Diphylloside A/Ikarisoside C	Jia et al. 1998
45	IV	39 min, C.2, MeOH, 50–60% 80 min	$C_{39}H_{48}O_{21}$		Jin et al. 2013
46	V	C.1, ACN, 28%	$C_{39}H_{48}O_{20}$		Jin et al. 2013
47	III	41.5 min, C.1, MeOH, 6–8.5%, 65 min	$C_{34}H_{44}O_{20}$		Li et al. 2012
48	V	38.5 min, C.2, ACN, 22–24%, 75 min	$C_{15}H_{10}O_4$	Isoaurostatin/4′,7-Dihydroxyisoflavone	Xu et al. 1979
49	V	Sepherdex LH20	$C_{16}H_{12}O_4$	Formononetin	Reiners 1966
50	V	47 min, C.2, ACN, 22–24%, 75 min	$C_{16}H_{12}O_5$	Biochanin A, Olmelin	Nilsson 1961
51	V	47 min, C.2, ACN, 22–24%, 75 min	$C_{16}H_{12}O_5$	Calycosin, Cyclosin	Markham et al. 1968
52	V	C.2, ACN, 20–22%, 75 min 55.5 min	$C_{16}H_{12}O_5$	7,4′-Dihydroxy-3′-methoxyisoflavone	Hirakura et al. 1997
53	V	C.2, ACN, 20–22%, 75 min 55.5 min	$C_{23}H_{24}O_{10}$	8-*O*-Methylretusin-7-*O*-β-D-glucopyranoside	Rukachaisirikul 2002
54	V	69 min, C.1, MeOH, 35%, 80 min	$C_{23}H_{24}O_{10}$		Clarke et al. 2004
55	IV	17 min, C.2, ACN, 15–18%, 80 min	$C_{21}H_{20}O_9$	Daidzoside	Xiao et al. 2016
56	IV	17min, C.2, ACN, 15–18%, 80 min	$C_{22}H_{22}O_{10}$	3′-Methoxydaidzin	Hirakura et al. 1997
57	IV	38 min, C.2, ACN, 20%, 90 min	$C_{21}H_{20}O_{10}$	Genistoside	Yuan et al. 2008
58	V	66.5 min, C.2, ACN, 22–24%, 75 min	$C_{22}H_{22}O_{10}$	Calycosin 7-glucoside	Markham et al. 1968
59	IV	59 min, C.1, ACN, 18% 110 min	$C_{22}H_{22}O_9$	Ononoside	Lebreton et al. 1967
60	IV	14.5 min, C.2, MeOH, 20%	$C_{27}H_{30}O_{14}$	Daidzein 7,4′-diglucoside	Li et al. 2014
61	IV	33.5 min, C.2, MeOH, 28%, 100 min	$C_{26}H_{28}O_{14}$	Neobacin	Breytenbach 1986
62	IV	19 min, C.2, ACN, 15–18%, 80 min	$C_{22}H_{22}O_{10}$	Caragiside B	Nisar et al. 2011
63	V	C.1, MeOH, 35%, 75 min	$C_{22}H_{22}O_9$	Isoononin	Liu et al. 2005
64	IV	24 min, C.2, MeOH, 20%	$C_{26}H_{28}O_{14}$	Ambocin	Breytenbach 1986
65	IV	C.2, ACN, 15–18%, 80 min	$C_{21}H_{20}O_9$	Neopuerarin	Zhang et al. 2009
66	IV	42 min, C.2, ACN, 20%, 90 min	$C_{21}H_{20}O_9$		Ma et al. 2017
67	IV	18 min, C.2, MeOH, 20%	$C_{21}H_{20}O_{10}$	8-C-Glucosyl-7,3′,4′-trihydroxy isoflavone	Wong et al. 2017
68	IV	63 min, C.2, MeOH, 20%, 56 min, 20–30% 40 min	$C_{26}H_{28}O_{13}$		Chen et al. 2009
69	IV	C.2, ACN, 15–18%, 80 min	$C_{21}H_{20}O_9$	Neopuerarin A	Zhang et al. 2009
70	III	39 min, C.2, MeOH, 6–8.5%, 65 min	$C_{28}H_{32}O_{15}$		Wang et al. 2006
71	III	26 min, C.2, MeOH, 28%, 50 min	$C_{21}H_{20}O_{10}$		Pistelli et al. 1998
72	IV	54 min, C.2, ACN, 20%, 90 min	$C_{22}H_{22}O_{10}$		Ohshima et al. 1988
73	IV	58.5 min, C.2, ACN, 20%, 90 min	$C_{26}H_{28}O_{13}$	Puerarin apioside	Ingham et al. 1986
74	IV	67.5 min, C.2, ACN, 20%, 90 min	$C_{26}H_{28}O_{13}$		Kinjo et al. 1987
75	III	45 min, C.2, MeOH, 6–8.5%, 65 min	$C_{26}H_{28}O_{14}$		Peng et al. 2011
76	IV	73 min, C.2, MeOH, 28% 100 min	$C_{23}H_{22}O_{10}$	Acetyldaidzin	Ohta et al. 1979
77	IV	24 min, C.2, ACN, 15–18%, 80 min	$C_{23}H_{22}O_{11}$		Zhou et al. 2013
78	IV	74 min, C.1, ACN, 18% 110 min	$C_{23}H_{28}O_{11}$	Astraganoside	Liu et al. 2007

C.1: C18, 5 μm, 250 × 10 mm, flow (3 mL/min); C.2: C8, 5 μ, 250 × 10 mm, flow (3 mL/min), see Section 2.4. **7**: new compound, MS and NMR data (Table S1 and Figures S2–S7).

Table 2. Terpenoids (#79–100), alkaloids (#101–118), coumarins, cinnamic acid or coumaric acid, and phenyl ethanol (or acetic acid) derivatives (#119–139) isolated from Jing liqueur.

NO	Fr.	t_R, HPLC Condition	MF	Compound	Reference (See SM)
79	V	58 min, C.2, ACN, 57%, 30 min, 57–62% 20 min, 62–70% 29 min	$C_{30}H_{48}O_3$	Oleanolic acid	Tan et al. 2002
80	V	26 min, C.2, ACN, 57%, 30 min, 57–62% 20 min, 62–70% 29 min	$C_{30}H_{48}O_4$	Sumaresinolic acid	Chan et al. 1992
81	V	C.1, MeOH, 72%	$C_{36}H_{58}O_8$	3-*O*-β-Glc-oleanolic acid	Dubois et al. 1990
82	IV	34 min, C.2, MeOH, 60–70%, 60 min	$C_{48}H_{76}O_{19}$	Calendulaglycoside B	Vidal-Ollivier et al. 1989
83	V	C.1, MeOH, 72%	$C_{36}H_{56}O_9$	Calenduloside E	Zhang et al. 2013
84	V	C.2, ACN, 30–35%, 60 min, 35–40% 20 min	$C_{48}H_{74}O_{18}$	Papyrioside LG	

85	V	27 min, C.1, ACN, 35–42%, 80 min, 42–100% 5 min	$C_{42}H_{66}O_{14}$	Chikusetsusaponin Iva	Yang et al. 1995
86	V	C.2, ACN, 30–35%, 60 min, 35–40% 20 min	$C_{42}H_{64}O_{14}$		Kuroda et al. 2006
87	IV	36.5 min, C.2, MeOH, 65–68%, 40 min	$C_{54}H_{86}O_{23}$	Scheffleraside II	Mshvildadze et al. 2001
88	V	C.2, ACN, 30–35%, 60 min, 35–40% 20 min	$C_{49}H_{78}O_{19}$	Chikusetsusaponin V methyl ester	Kondo et al. 1971
89	V	C.2, ACN, 30–35%, 60 min, 35–40% 20 min	$C_{48}H_{76}O_{19}$	Ginsenoside Ro	Matsuda et al. 1990
90	V	C.2, ACN, 30–35%, 60 min, 35–40% 20 min	$C_{54}H_{86}O_{24}$	Calendulaglycoside A	Vidal-Ollivier et al. 1989
91	V	C.2, ACN, 30–35%, 60 min, 35–40% 21 min	$C_{53}H_{84}O_{23}$	Elatoside C	Yoshikawa et al. 1993
92	V	24 min, C.1, ACN, 35–42%, 80 min, 42–100% 5 min	$C_{47}H_{74}O_{18}$	Pseudoginsenoside RT1	Morita et al.
93	V	55 min, C.1, ACN, 35–42%, 80 min, 42–100% 5 min	$C_{43}H_{68}O_{14}$	Silphioside A	Jiang et al. 1992
94	V	46 min, C.1, ACN, 35–42%, 80 min, 42–100% 5 min	$C_{48}H_{76}O_{18}$	Umbellatoside B	Sosa et al. 2011
95	V	20 min, C.2, ACN, 36%, 40 min	$C_{42}H_{66}O_{14}$	Wedelin	Matos et al. 1983
96	V	27.8 min, C.2, ACN, 57%, 30 min, 57–62% min, 62–70% 29 min	$C_{30}H_{48}O_4$	6β-Hydroxyursolic acid	Sakakibara et al. 1983
97	V	C.2, ACN, 35%	$C_{30}H_{48}O_6$	3β,6β,19α,24-tetrahydro xyurs-12-en-28-oic acid	Fang et al. 1996
98	IV	71 min, C.2, ACN, 18% 110 min	$C_{36}H_{58}O_{11}$		Abe et al. 1987
99	V	38 min, C.2, ACN, 20–22%, 75 min	$C_{36}H_{58}O_{11}$		Abe et al. 1987
100	V	20min, C.2, ACN, 36%, 40 min	$C_{42}H_{66}O_{14}$	Wedelin	Matos et al. 1983
101	IV	8 min, C.2, MeOH, 10–14%, 40 min	$C_{37}H_{55}N_3O_{16}$	Lycibarbarspermidine L	Zhou et al. 2016
102	IV	C.2, MeOH, 10–14%, 40 min	$C_{37}H_{55}N_3O_{16}$	Lycibarbarspermidine M	Zhou et al. 2016
103	IV	C.2, MeOH, 10–14%, 40 min	$C_{37}H_{53}N_3O_{16}$	Lycibarbarspermidine E	Zhou et al. 2016
104	IV	C.2, MeOH, 10–14%, 40 min	$C_{37}H_{53}N_3O_{16}$		Jin et al. 2015
105	IV	C.2, MeOH, 10–14%, 40 min	$C_{31}H_{43}N_3O_{11}$	Lycibarbarspermidine D	Zhou et al. 2016
106	IV	C.2, MeOH, 10–14%, 40 min	$C_{31}H_{43}N_3O_{11}$	Lycibarbarspermidine A	Zhou et al. 2016
107	IV	26.5min, C.2, ACN, 20%, 90 min	$C_{13}H_{14}N_2O_2$	Tetrahydroharman-3-carboxylic acid	Tsuchiya et al. 1999
108	III	23 min, C.2, MeOH, 15%, 95 min	$C_{13}H_{14}N_2O_3$		Herraiz et al. 2004
109	III	39 min, C.2, MeOH, 17%, 80 min	$C_{13}H_{14}N_2O_2$		Herraiz et al. 1993
110	III	36.5 min, C.2, MeOH, 15%, 95 min	$C_{14}H_{17}NO_3$	3α-Benzoyloxynortropan-6β-ol	Al-Said et al. 1986
111	IV	12 min, C.2, ACN, 20%, 90 min	$C_{17}H_{21}NO_5$	Confoline	Aripova et al. 1996
112	II	25 min, C.2, MeOH, 5%, 40 min	$C_6H_7NO_2$		Hiermann et al. 2002
113	III	47.5 min, C.2, MeOH, 17–20%, 100 min	$C_{10}H_{13}NO_4$		Chin et al. 2003
114	III	7.5 min, C.2, MeOH, 5–15%, 60 min	$C_6H_5NO_2$	Nicotinic acid	Krehl et al. 1946
115	IV	18 min, C.2, MeOH, 20%	$C_8H_{15}NO_2$		Singer et al. 1935
116	IV	8 min, C.2, MeOH, 10–14%, 40 min	$C_{14}H_{22}NO_4^+$	Codonopyrrolidium B	He et al. 2014
117	III	17 min, C.2, MeOH, 10%, 80 min	$C_8H_{17}N_3O_3$		Gegauer et al. 2003
118	III	19 min, C.2, MeOH, 10%, 80 min	$C_6H_{13}NO_2$		Perrin et al. 2000
119	IV	20 min, C.2, ACN, 15–18%, 80 min	$C_{10}H_8O_4$	Scopoletin	Best 1948
120	IV	23 min, C.2, ACN, 15–18%, 80 min	$C_{20}H_{24}O_8$	Vellein	Maruyama et al. 2009
121	IV	33.5min, C.2, MeOH, 28%, 100 min	$C_8H_{10}O_2$	Phenethyl alcohol	Wang et al. 1982
122	IV	35.5 min, C.2, ACN, 20%, 90 min	$C_8H_8O_4$	Pisolithin B	Benecke et al. 1984
123	V	43.5 min, C.2, ACN, 22–33%, 60 min	$C_9H_8O_2$	trans-β-Carboxystyrene	Billmann et al. 1909
124	IV	21 min, C.2 ACN, 15–18%, 80 min	$C_{10}H_{10}O_4$	Ferulic acid	Henderson and Farmer 1955
125	V	60 min, C.2 ACN, 22–24%, 75 min	$C_{11}H_{12}O_4$		Oonuma et al. 1993
126	V	C.1, ACN, 28%	$C_{11}H_{12}O_3$		Newman et al. 1952
127	V	C.1, ACN, 28%	$C_{12}H_{14}O_4$	Ethyl ferulate	Nakayama et al. 1996
128	III	71 min, C.1, ACN, 12–25%, 43 min, 25–30% 12 min	$C_9H_8O_4$	Caffeic acid	Baerheim 1951
129	IV	55 min, C.1, MeOH, 45–48%, 80 min	$C_{11}H_{12}O_4$	Ethyl caffeoate	Mao et al. 2011
130	III	37min, C.2, MeOH, 28%, 50 min	$C_9H_8O_3$	trans-*p*-Hydroxycinnamic acid	King et al. 1952
131	III	40 min, C.2, MeOH, 28%, 50 min	$C_{10}H_{10}O_4$	Isoferulic acid	Qiao and Chen 1991
132	III	48.5 min, C.2, MeOH, 15%, 95 min	$C_{15}H_{18}O_8$	*p*-Coumaric acid β-glucoside	Runeckles, Woolrich 1963
133	III	48.5 min, C.2, MeOH, 15%, 95 min	$C_{16}H_{20}O_9$	Glucosidoferulic acid	Ibrahim et al. 1970
134	IV	22 min, C.2, MeOH, 20%	$C_{10}H_{10}O_4$		Muratake et al. 2013
135	III	30 min, C.2, MeOH, 6–8.5%, 65 min	$C_{14}H_{20}O_7$		Fujimatu et al. 2003
136	IV	73.5 min, C.2, MeOH, 20%, 56 min, 58.5 min20–30% 40 min	$C_{35}H_{46}O_{20}$	Echinacoside	Frezza et al. 2017
137	III	29.5 min, C.2, MeOH, 30–40%, 90 min	$C_{37}H_{48}O_{21}$	Tubuloside A	Kobayashi et al. 1987

138	IV	80 min, C.2, MeOH, 28% 100 min	$C_{29}H_{36}O_{15}$	Verbascoside	Pham et al. 1988
139	V	60.5 min, C.2, ACN, 16–20%, 100 min	$C_{20}H_{26}O_{12}$		Wende and Fry 1997

C.1: C18, 5 μm, 250 × 10 mm, flow (3 mL/min); C.2: C8, 5 μ, 250 × 10 mm, flow (3 mL/min), see Section 2.4.

Table 3. Benzoquinone, naphthoquinone, anthraquinones, or phenanphrene derivatives (#140–149), xanthones, chromone, and γ-pyrone derivatives (#150–155), lignans (#156–162), other aromatic compounds (#163–175), and other types of compounds (#176–189) isolated from Jing liqueur.

NO	Fr.	t_R, HPLC Condition	MF	Compound	Reference (See SM)
140	V	C.1, ACN, 60%	$C_{16}H_{14}O_4$		Letcher and Nhamo 1973
141	V	C.2, ACN, 40–50%, 48 min	$C_{17}H_{16}O_4$	Batatasin I	Gonnet et al. 1973
142	V	C.1, ACN, 30%	$C_{15}H_{10}O_4$		Morton et al. 1941
143	V	C.1, ACN, 30%	$C_{16}H_{12}O_5$		Wang et al. 2011
144	V	C.1, ACN, 33%	$C_{16}H_{12}O_5$		Wu et al. 2003
145	V	C.1, ACN, 30%	$C_{15}H_{10}O_5$		Lee et al. 1994
146	V	C.1, ACN, 30%	$C_{16}H_{12}O_4$	Digitolutein	Koumaglo et al. 1992
147	V	34 min, C.2, ACN, 40%	$C_{15}H_{10}O_3$		Bistrzycki and Zen-Ruffinen 1920
148	V	34 min, C.2, ACN, 40%	$C_{15}H_{10}O_5$		Yang et al. 1992
149	IV	19 min, C.2, MeOH, 20%, 56 min, 20–30% 40 min	$C_{14}H_8O_4$		Varbanov et al. 1986
150	V	29 min, C.2, ACN, 22–27%, 60 min	$C_{13}H_8O_4$	1,3-Dihydroxyxanthone	Liang et al. 1982
151	III	39 min, C.2, MeOH 6–8.5%, 65 min	$C_{12}H_{16}O_8$		Baba et al. 1995
152	IV	15 min, C.2, MeOH, 20%	$C_{16}H_{18}O_9$	Biflorin	Zhang et al. 1997
153	III	46 min, C.2, MeOH, 15%, 95 min	$C_{16}H_{18}O_9$	Isobiflorin	Tanaka et al. 1993
154	III	37.5 min, C.2, MeOH, 6–8.5%, 65 min	$C_{16}H_{18}O_9$	Undulatoside A	Itoh et al. 2003
155	III	37.5 min, C.2, MeOH, 6–8.5%, 65 min	$C_{16}H_{18}O_9$		Wang et al. 2011
156	III	55 min, C.2, MeOH, 17%, 80 min	$C_{26}H_{34}O_{12}$		Yan et al. 2008
157	V	89 min, C.2, ACN, 16–20%, 100 min	$C_{22}H_{26}O_8$	Syringaresinol	Abu Zarga 1986
158	V	54 min, C.2, ACN, 22–33%, 60 min	$C_{18}H_{16}O_5$		Shi et al. 2007
159	IV	25.5 min, C.2, ACN, 15–20%, 50 min	$C_{18}H_{14}O_8$	Prolithospermic acid	Dai et al. 2010
160	III	29 min, C.2, MeOH, 28%, 50 min	$C_{18}H_{14}O_8$	7-Epiblechnic acid	Wang et al. 2010
161	III	29 min, C.2, MeOH, 28%, 50 min	$C_{18}H_{14}O_8$	Blechnic acid	Wada et al. 1992
162	V	69 min, C.2, MeOH, 35%, 80 min	$C_{23}H_{26}O_{10}$		Guo et al. 2014
163	V	36 min, C.2, ACN, 22–24%, 75 min	$C_7H_6O_3$	*p*-Salicylic acid	Sager and Schooley 1945
164	V	63.5 min, C.2, ACN, 22–24%, 75 min	$C_9H_{10}O_3$		Heim et al. 1957
165	V	41 min, C.2, ACN, 20–22%, 75 min	$C_8H_8O_3$	*p*-Methoxy benzoic acid	Reitberg and Schentag 1983
166	III	67 min, C.2, MeOH, 12–25%, 43 min, 25–30% 12 min	$C_8H_8O_4$		Parham et al. 1954
167	III	49.5 min, C.2, ACN, 13–15%, 90 min	$C_8H_8O_4$	Vanillic acid	Sammons and Williams 1946
168	IV	20min, C.2, MeOH, 20%	$C_7H_6O_3$	Rancinamycin IV	Li et al. 2004
169	IV	44 min, C.2, MeOH, 20%, 56 min, 20–30% 40 min	C7H6O2		Rivers 1947
170	II	39.5 min, C.2, MeOH, 7–9%, 70 min	$C_{14}H_{18}O_9$		Yang et al. 2013
171	V	57 min, C.2, ACN, 20–22%, 75 min	$C_9H_{12}O_3$	1,3,5-Trimethoxybenzene	Allain et al. 1980
172	IV	24min, C.2, MeOH, 20%	$C_8H_{10}O_2$	*p*-Hydroxyphenetole	Rosenwald 1951
173	II	22 min, C.2, MeOH, 2%, 60 min	$C_{13}H_{18}O_8$	Tachioside	Sano et al. 1991
174	IV	73 min, C.2, MeOH, 28% 100 min	$C_9H_{10}O_4$		
175	III	77 min, C.2, MeOH, 12–25%, 43 min, 25–30% 12 min	$C_7H_6O_3$	Salicylic acid	Ichniowski and Hueper 1946
176	III	78 min, C.2, MeOH, 25–30%, 60 min, 30–35% 40 min	$C_{16}H_{26}O_8$	Bodinierin	Xie et al. 2006
177	III	80 min, C.2, MeOH, 25–30%, 60 min, 30–35% 40 min	$C_{16}H_{26}O_8$	Kankanoside O	Liu et al. 2016
178	III	75 min, C.2, MeOH, 20–25%, 80 min	$C_{16}H_{28}O_7$		Fan et al. 2011
179	V	21 min, C.2, ACN, 16–20%, 100 min	$C_{27}H_{44}O_8$	(25R)-20,26-Dihydroxyecdysone	Suksamrarn 1998
180	III	43min, C.2, MeOH, 30–40%, 90 min	$C_{27}H_{44}O_7$	26-Hydroxyecdysone	Li et al. 2006
181	IV	C.2, ACN, 15–18%, 80 min	$C_{27}H_{44}O_7$	3-epi-20-Hydroxyecdysone	Thompson et al. 1974
182	III	47.5 min, C.2, MeOH, 6–8.5%, 65 min	$C_{16}H_{22}O_{10}$		Li et al. 1999
183	II	51 min, C.2, MeOH, 2%, 60 min	$C_{16}H_{22}O_{11}$	Desacetylasperulosidic acid	Inouye et al. 1974
184	II	47.5 min, C.2, MeOH, 7–9%, 70 min	$C_{16}H_{24}O_{10}$	Mussaenosidic acid	Kohda et al. 1989
185	III	9.5 min, C.2, MeOH, 5–15%, 60 min	$C_{11}H_{13}N_3O_5$	9-Deazainosine	Liu et al. 2005
186	V	38 min, C.2, MeOH, 35%, 80 min	$C_{12}H_{16}O_2$	Sedanonic acid lactone	Mitsuhashi and Nomura 1966
187	V	46 min, C.2, ACN, 20–22%, 75 min	$C_{12}H_{16}O_4$	Senkyunolide I	Huang et al. 2013
188	IV	65.5 min, C.2, ACN, 17% 100 min	$C_{12}H_{16}O_4$	Senkyunolide H	Huang et al. 2013
189	V	C.2, ACN, 60%	$C_{17}H_{24}O_2$	Falcarindiol	Lechner et al. 2004

C.1: C18, 5 μm, 250 × 10 mm, flow (3 mL/min); C.2: C8, 5 μ, 250 × 10 mm, flow (3 mL/min), see Section 2.4.

3.2. Analysis of Metals, Amino Acids, and Total Polysaccharides

Besides the isolation and structure determination of the above 189 compounds from Chinese herbal medicines, we determined the concentrations of two amino acids (*L*-proline, 2.33 mg/L; and *L*-arginine, 1.73 mg/L), total amino acids (9.84 mg/L), and three metals (iron, 0.52 mg/L; zinc, 0.21 mg/L; and calcium, 11.0 mg/L). The total amount of polysaccharides, the main component in fraction I was also determined (337.4 mg/L).

3.3. Nrf2 Activation

Results showed that a 5.2 mg/mL crude extract of Jing liqueur increased Nrf2 activity by approximately 7–8-fold (Figure 3). We also tested the five fractions from Jing liqueur. Nrf2 was strongly activated by fraction V at 80 μg/mL, weakly activated by fraction V and IV at 40 and 80 μg/mL, respectively (Figure 4), indicating that fraction V contains most of the Nrf2 activators in Jing liqueur. Fraction V at 20 μg/mL, fraction IV at 40 μg/mL, and both fractions III and II at 80 μg/mL marginally activated Nrf2, while fraction I was inactive. Next, we screened almost all the 189 isolated molecules except twenty one (21) because of their insufficiency. Eighteen (18) compounds showed Nrf2 activation when compared with the negative control (Figure 5). Among these eighteen Nrf2 activators (Figures 1, 2, and 5), four (**55**, **78**, **129**, and **168**) from fraction IV, and the other fourteen (**50**, **51**, **53**, **58**, **125**, **126**, **127**, **128**, **143**, **144**, **145**, **146**, **171**, and **186**) from fraction V (Table 4). Compounds **55**, **78**, **129**, and **168** from fraction IV and **50**, **53**, **58**, **128**, **143**, **144**, **145**, **146**, **171**, and **186** from fraction V were weakly active. When comparing the activity of the active compounds from fraction V, compounds **51**, **125**, **126**, and **127** showed much stronger activity than the other Nrf2 activators, which robustly enhanced the Nrf2 expression at 40 μg/mL (Table 4).

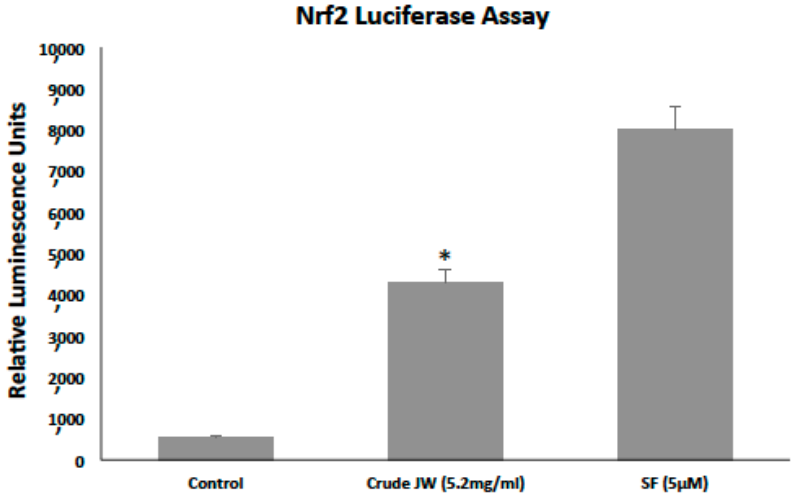


Figure 3. The effect of the crude on Nrf2 in ARE reporter-Hep G2 cells. Cells were seeded in 96-well plates at a density of 4×10^4 cells/well and incubated for 24 h. The cells were further treated with 5.2 mg/mL concentration of crude Jing liqueur for additional 24 h. The negative control cells were treated with 0.2% DMSO (dimethyl sulfoxide), and positive control cells were treated with 5 μM DL-sulforaphane (SF). Luciferase activity was determined. * $p < 0.05$.

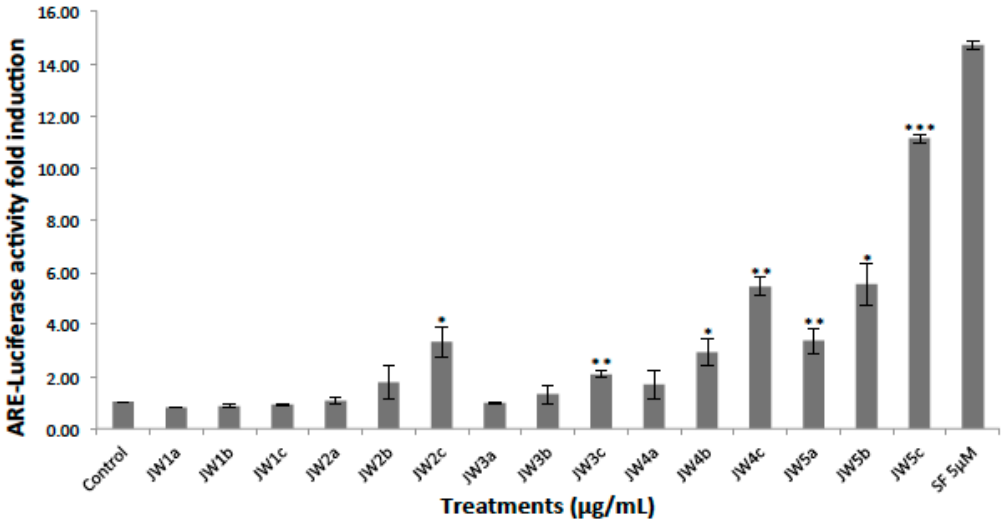


Figure 4. The effect of five fractions on ARE-luciferase reporter activity in ARE reporter-HepG2 cells. Cells were seeded in 96-well plates at a density of 4×10^4 cells/well and incubated for 24 h. The cells were further treated with 20, 40, 80 μg/mL concentrations of each fraction for additional 24 h. The negative control cells were treated with 0.2% DMSO, and positive control cells were treated with 5 μM DL-sulforaphane (SF). Luciferase activity was determined. Mean ARE-luciferase reporter activity represents the average of three-independent experiments ± S.E.M. * $p < 0.05$; ** $p < 0.01$, *** $p < 0.001$. (**a**: 20 μg/mL; **b**: 40 μg/mL; **c**: 80 μg/mL).

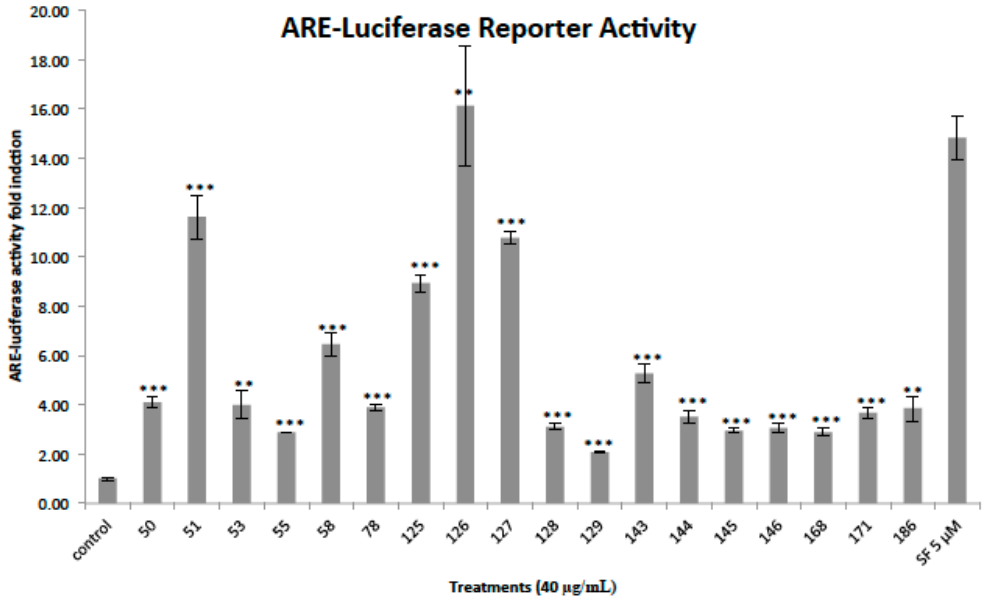


Figure 5. The effect of pure compounds on ARE-luciferase reporter activity in ARE repoter-HepG2 cells. Cells were seeded in 96-well plates at a density of 4×10^4 cells/well and incubated for 24 h. The cells were further treated with compounds (40 μg/mL each) for additional 24 h. The negative control cells were treated with 0.2% DMSO, and positive control cells were treated with 5 μM DL-sulforaphane (SF). Luciferase activity were determined. Mean ARE-luciferase reporter activity represents the average of three-independent experiments ± S.E.M. ** $p < 0.01$, *** $p < 0.001$.

Table 4. Relative Nrf2 activity induced by compounds (40 μg/mL) isolated from Jing liqueur.

Cpd	Fr.	Relative Nrf2 Activity (Fold Induction)	Cpd	Fr.	Relative Nrf2 Activity (Fold Induction)	Cpd	Fr.	Relative Nrf2 Activity (Fold Induction)
Control	-	1.0	**125**	V	9.5	**145**	V	3.0
50	V	4.0	**126**	V	16.0	**146**	V	3.0
51	V	11.5	**127**	V	10.5	**168**	IV	3.0
53	V	4.0	**128**	V	3.0	**171**	V	3.5
55	IV	3.0	**129**	IV	2.2	**186**	V	3.5
58	V	6.5	**143**	V	6.0	SF (5 μM)	-	15.0
78	IV	4.0	**144**	V	3.5			

3.4. Cytotoxicity Evaluation

In order to evaluate the cytotoxicity of Jing liqueur, we used the MTT assay to measure the activity of the crude and the five fractions. We tested the five fractions (I–V) at 20, 40, and 80 μg/mL, and none exhibited cytotoxicity as shown by the MTT results (Figure 6). The Nrf2 activators identified in this study were also evaluated for the activity against HepG2 in our MTT assay, and none of them showed any cytotoxicity at 40 μg/mL.

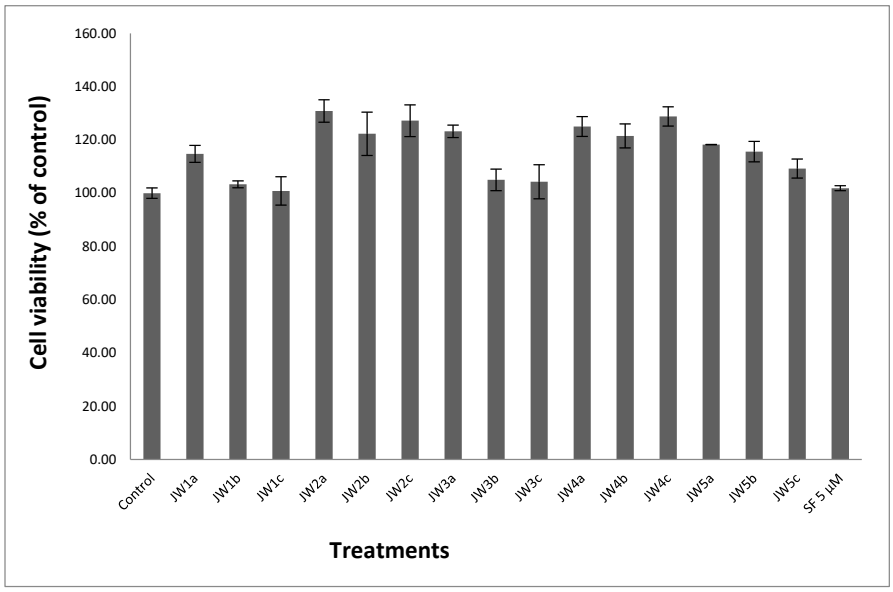


Figure 6. Evaluation of cytotoxicity activity of five fractions in ARE reporter-HepG2 cells. Cells were seeded in 96-well plates at a density of 4×10^4 cells/well and incubated for 24 h. The cells were further treated with 20, 40, 80 μg/mL concentrations of each fraction for additional 24 h. The negative control cells were treated with 0.2% DMSO cells. Cell viability was estimated with the methylthiazoltetrazolium (MTT) assay. Mean cytotoxicity activity represents the average of three-independent experiments ± S.E.M. (**a**: 20 μg/mL; **b**: 40 μg/mL; **c**: 80 μg/mL).

4. Discussion

One hundred eighty nine compounds have been isolated from Jing liqueur. Most of them are aromatic compounds including 78 flavonoids, 21 coumarins, cinnamic acid, or coumaric acid, and phenyl ethanol (or acetic acid) derivatives, 10 benzoquinone, naphthoquinone, anthraquinones, or phenanphrene derivatives, 6 xanthones, chromone, and γ-pyrone derivatives, 7 lignans, and 13 small aromatic compounds. The three major types of compounds are flavonoids, terpenoids, and alkaloids, and they have a broad range of biological activities including anti-oxidant, anti-inflammatory, antibacterial, and anticancer properties etc. These aromatic compounds, especially flavonoids, anthraquinones, cinnamic acid derivatives, lignans, and some other small molecule aromatic compounds, are probably the main anti-oxidant components in Jing liqueur.

L-proline and *L*-arginine are two of the six conditionally essential amino acids [21,22]. These amino acids and elements are important for heart muscle, immune function, blood production,

blood pressure regulation, and prevention of osteoporosis etc. Iron is an essential element for blood production [23]. Zinc is extremely important for the body's defense (immune) system to work properly, and plays a role in cell division, cell growth, wound healing, and the breakdown of carbohydrates [24]. Calcium helps to form and maintain healthy teeth and bones, which is important for the prevention of osteoporosis [25,26]. Olysaccharides are the most abundant type of compounds in Jing liqueur. We have a good reason to argue that olysaccharides together with flavonoids and terpenoids may account for some other biological activities besides the anti-oxidant property of Jing liqueur.

Fractions II–V were active in the Nrf2 assay at 80 μg/mL, fraction V was much more active than fractions II–IV, and fraction IV was more active than fractions II and III. Since most of the 189 compounds were isolated from fractions IV and V, majority of which are flavonoids, cinnamic acid derivatives, lignans, and other aromatic compounds, we argued that it is likely that Nrf2 activators in Jing liqueur are aromatic molecules. We evaluated the 168 compounds that were enough for the screening, and eighteen compounds activated Nrf2. Among these eighteen Nrf2 activators four were isolated from fraction IV, and the other fourteen were separated from fraction V. Clearly, most of the active compounds and the four most active Nrf2 activators (**51** and **125**–**127**) identified in this study were isolated from fraction V, which was consistent with our screening result of the five fractions with fraction V being the most active fraction. Almost all these eighteen Nrf2 activators are aromatic molecules except **186**, including six flavonoids (**50**, **51**, **53**, **55**, **58**, and **78**), five cinnamic acid derivatives (**125**–**129**), four anthraquinones (**143**–**146**), 3,4-dihydroxybenzaldehyde (**168**), 1,3,5-trimethoxybenzene (**171**), and (*E*)-3-butylidene-4,5,6,7-tetrahydroisobenzofuran-1(3*H*)-one (**186**). Jing liqueur, the five fractions at 80 μg/mL and the eighteen Nrf2 activators at 40 μg/mL were evaluated for their antiproliferative activity against HepG2, and none showed any cytotoxicity.

This study is significant because it was the first time to extensively investigate Jing liqueur chemically that has not been previously interrogated although the phytochemical components and biological activities of these nine Chinese plants as a single herbal medicine have been investigated. The Nrf2 activators especially compounds **51**, **125**, **126**, and **127** identified in this study could be used as biomarkers for quality control. Jing liqueur was reported to exhibit anti-inflammatory [1], immune enhancement [3], anti-fatigue [2,3] properties, and invigorating the vital activities of kidney [3]. Our study showed that the Nrf2 activation by Jing liqueur may account for the observed anti-inflammatory activity and immune enhancement of Jing liqueur. These experiments also demonstrated that to drink certain volume of Jing liqueur equivalent to the highest concentration tested in these experiments should be safe regarding the cytotoxicity of the metabolites of the herbal medicine in Jing liqueur. Hence, adequate consumption of Jing liqueur may offer health benefits mainly or partially because of the transient activation of Nrf2 considerably by the above-mentioned eighteen Nrf2 activators present in Jing liqueur. Of course, excessive drinking is not encouraged.

5. Conclusions

We isolated 189 compounds from fractions II–V of Jing liqueur, one of which (**7**) was a minor new flavonoid. Out of these 189 compounds, 78 are flavonoids, revealing the Jing liqueur is rich in phenolic compounds. The concentrations of most compounds were at micromolar levels (corresponding to μg/L levels). Fraction I was mainly composed of polysaccharides. The concentration of total polysaccharides was very high (337.4 mg/L), which may be worthy of further study for the components and functions. Both iron and zinc were less than 1 mg/L while the concentration of calcium was much higher (11 mg/L). *L*-proline and *L*-arginine were at mg/L levels. We also demonstrated that the crude extract of Jing liqueur, fractions II–V activated the Nrf2 transcription factor pathway, and fraction V was much more active than fractions II–IV, indicating that fraction V contains more Nrf2 activators than fractions II–IV. Screening of compounds demonstrated that most (14) of the eighteen active compounds including the two most potent Nrf2

activators (**51** and **126**) were isolated from fraction V. Nrf2 is an important defense mechanism for mitigating oxidative and electrophilic stress. Despite the "dark side" [27], Nrf2 activation is believed to have many beneficial effects on human health including inhibition of systemic inflammation, cancer prevention, relief of diabetes-induced cardiac oxidative stress, and neuroprotection. The activation of Nrf2 is highly consistent with the traditional use of the herbal medicines present in Jing liqueur, which itself is known for its tonic effects including general health and well-being promotion. Many of the reported beneficial properties of Jing liqueur including anti-inflammatory [1], immune enhancement [2], and anti-fatigue [2,3] properties could at least partially be justified by the presence of different types of compounds including Nrf2 activators. The crude extract and the five fractions were not cytotoxic against HepG2 cells at 80 μg/mL, and the compounds that activated Nrf2 were also not active in our MTT cytotoxicity assay at 40 μg/mL, the highest concentration of compounds tested in the Nrf2 assay. Further investigation of Jing liqueur on the Nrf2 pathway is warranted.

Abbreviations: Traditional Chinese medicine (TCM); Chinese Materia Medica (CMM); nuclear factor erythroid 2-related factor 2 (Nrf2); antioxidant responsive element (ARE); high performance liquid chromatography (HPLC); nuclear magnetic resonance (NMR); liquid chromatography–mass spectrometry (LC-MS); methylthiazoltetrazolium (MTT)

Supplementary Materials: The following are available online at www.mdpi.com/link. Figure S1: Structure of **7**. Table S1: ^{1}H and ^{13}C NMR data of **7** (400 MHz, CD_3OD). Figure S2: HR-ESI-MS spectrum of **7**. Figure S3: ^{1}H-NMR spectrum of **7** (400 MHz, CD_3OD). Figure S4: COSY (Correlation Spectroscopy) spectrum of **7** (400 MHz, CD_3OD). Figure S5: HSQC (Heteronuclear Single Quantum Coherence) spectrum of **7** (400 MHz, CD_3OD). Figure S6: HMBC (Heteronuclear Multiple Bond Correlation) spectrum of **7** (400 MHz, CD_3OD). MS, NMR, and references of the other known compounds.

Author contributions: Y.-S.C., J.X., M.C., Y.L. and S.C. designed research; Y.-S.C., J.X., M.C., Y.Y., A.M., D.W. and X.W. performed research; Y.-S.C. and S.C. analyzed data; and Y.-S.C. and S.C. wrote the paper. All authors have read and agreed to the published version of the manuscript.

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