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Research Article
Comparison of the Cytotoxicity and Mitochondrial Effects of *Gelsemium elegans* Extracts on Human Hepatocellular Carcinoma Cells

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Abstract

Background and Objective: *Gelsemium elegans* (*G. elegans*) has been used in Chinese folk medicine for the treatment of rheumatoid pain, skin ulcers and malignant tumors. The aims of the study were to compare cytotoxicity and mitochondrial effects between *G. elegans* alcohol extract and *G. elegans* acid water extract. Moreover, the difference on chemical components between *Gelsemium elegans* alcohol extract and acid water extract was analyzed and compared in order to explore which components were responsible for the activity. **Materials and Methods:** The dried root of *Gelsemium elegans* was extracted with 95% alcohol and 0.5% sulphuric acid to get *Gelsemium elegans* alcohol extract and *Gelsemium elegans* acid water extract, respectively. In cytotoxic experiments, cell viability, apoptosis and production of reactive oxygen species (ROS) were measured. The extent of mitochondrial damage was assessed by measuring the mitochondrial membrane potential and intracellular Ca^{2+}. The chemical components of the two different *Gelsemium elegans* extracts were characterized using LC-QqTOF MS method. **Results:** Viability experiments showed that *G. elegans* alcohol extract was more cytotoxic than the *G. elegans* acid water extract one. Both *G. elegans* alcohol extract and acid water extract induced apoptosis in HepG2 cells. The production of ROS was increased with increasing concentration of *G. elegans* extracts. Loss of mitochondrial membrane potential was detected after treatment with *G. elegans* extracts. The concentration of intracellular Ca^{2+} was significantly elevated at the concentration of 240 μg mL^{-1}. Furthermore, a total of 29 and 21 components have been tentatively identified from the *G. elegans* alcohol extract and acid water extract, respectively. **Conclusion:** The present results demonstrated that oxidative stress and mitochondrial dysfunction induced by *G. elegans* extracts were the possible mechanism of cytotoxicity. Alkaloids and iridoids were the major constituents and possibly the cytotoxicity of the active fractions of *G. elegans* extracts.

Key words: *G. elegans*, Oxidative stress, Cytotoxicity, Alkaloids, HepG2 cells, Mass spectrometry, reactive oxygen species

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Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

The genus *Gelsemium* includes three species: The Asian *G. Elegans* and two North American species, *Gelsemium sempervirens J.St.-Hil.* and *Gelsemium rankinii* Small[1]. *G. elegans* is distributed in southern China and southeaster Asia. The species is also known as Gou Wen, Da Cha Yao or Duan Chang Cao in China[2]. It has been used in traditional Chinese medicine to treat certain types of skin ulcers, neuropathic pain and cancer pain[3]. Previous pharmacological studies demonstrated that the *Gelsemium* possesses anti-tumor[4,5], antianxiety[6], analgesic[7], anti-inflammatory[8,9], immunomodulating[10] and anxiolytic activities[7]. However, *Gelsemium* is a known toxic plant and its toxicity limits its clinical use.

Previous several studies have demonstrated that the *Gelsemium* extracts and monomeric compounds possessed anti-tumor effects[4,5,11,12]. To date, a total of 121 alkaloids, 25 iridoids and a number of other compounds from a wide spectrum of secondary metabolite classes have been found in *Gelsemium*. Of these compounds alkaloids and iridoids, which almost discovered from genus *Gelsemium*, were regarded as the bioactive components of *Gelsemium* that were most likely to be responsible for the observed pharmacological effects. Their phytochemistry, pharmacology and toxicology of genus *Gelsemium* have been summarized in recent review[13]. Form these literatures, the *Gelsemium* extracts were more highly cytotoxicity to cancer cell lines compared to monomeric compounds. The detailed chemical components of the *Gelsemium* extracts have not been investigated in the pharmacological researches. Moreover, what plays the important factor in the *Gelsemium*-induced anti-tumor are still unknown.

An alkaloid extract of *G. elegans* (10 μg mL^{-1}) has inhibitory effect on hepatic carcinoma HepG2 cells and the mechanism of this anti-tumor action might be related to their apoptosis-inducing activities[14]. Common cellular mechanisms of apoptosis in HepG2 cells involved oxidative stress, calcium imbalance and inflammatory process[15]. Therefore, in present study the *Gelsemium* extracts-induced cytotoxicity might be related to oxidative stress. Some oxidative stress parameters and mitochondrial effects between *G. elegans* alcohol extract and *G. elegans* acid water extract in HepG2 cells were investigated and compared. Moreover, the difference on chemical components between *G. elegans* alcohol extract and acid water extract was analyzed and characterized in order to explore which components were responsible for the cytotoxicity.

MATERIALS AND METHODS

Reagents: Dimethyl sulfoxide (DMSO), Penicillin Streptomycin (100 U mL^{-1} penicillin and 100 mg mL^{-1} streptomycin), fetal bovine serum (FBS), DMEM culture medium, 0.25% Trypsin, 0.25% Trypsin-EDTA were purchased from GIBCO (Gibco, UK). Cell Counting Kit-8 (CCK8) assay kit was obtained from Wuhan Boster Biological Engineering Co. Ltd (Wuhan, China). 2′,7′-Dichlorofluorescein diacetate (DCFH-DA) assay kit, Fluo-3 AM, Rhodamine 123 assay were obtained from Beyotime Institute of Biotechnology (Shanghai, China). Annexin V-FITC apoptosis detection Kit was from KeyGEN Biotech (Nanjing, China). HPLC-grade acetonitrile and formic acid were purchased from Fisher Chemicals Co. (New Jersey, USA). Water was freshly prepared with the Millipore water purification system (MA, USA). Flasks and plates from cell culture were obtained from Corning Incorporated (Corning, NY, USA). All other chemicals were reagent grade and commercially available.

Plant materials and sample preparation of *G. elegans* extracts: The roots of *G. elegans* were collected from Longyan, Guangxi Province, China, in May, 2013 and authenticated by associate Professor Ming-Jun Zhang at Hunan Agriculture University. The air-dried root of *G. elegans* was ground to powder. Then, 10 Kg powder was put into extraction tank and procolated with 80 L 95% alcohol for 1.5 h two times at room temperature. The combined extract was filtered by 200 mesh filter. The filtrates were concentrated to *G. elegans* alcohol extract by vacuum drying method. The powder was obtained (630 g) and called as *G. elegans* alcohol extract in this study. The extract yield (w/w) was 6.3%. The content of total alkaloids of *G. elegans* alcohol extract was 6.4% using acid dye colorimetric method.

About 50 Kg powder was put into extraction tank and percolated with 400 L 0.5% sulphuric acid for 2 h two times at room temperature. The combined extract was filtered by 200 mesh filter. The filtrates were adjusted pH to neutral with 8 mol L^{-1} sodium hydroxide and concentrated to *G. elegans* acid water extract by vacuum drying method. The powder was obtained (9750 g) and called as *G. elegans* acid water extract in this study. The extract yield (w/w) was 19.5%. The content of total alkaloids of *G. elegans* acid water extract was 2.2%.

Before cell experiment, *G. elegans* alcohol extract and acid water extract were prepared by dissolving in alcohol and water, respectively. They were filtered through a 0.45 μm membrane filter and were diluted to the desired concentration with DMEM (alcohol is less than 0.1%), then stored at -80°C.

Cell cultures: HepG2 cells were obtained from Cell Resource Center of the Chinese Academy of Sciences (Shanghai, China). Cells were cultured in DMEM culture medium supplemented with 10% FBS, 100 U mL^{-1} penicillin and 100 μg mL^{-1} streptomycin in a humidified atmosphere of 5% CO_2, 95% air at 37°C. Cells were seeded at a density of 1×10^6 cells/dish on 60 mm culture dishes. The culture medium was changed fresh medium at intervals of 48-72 h. When cellular confluence was reached 90%, the cells were washed with phosphate buffered saline (PBS) and digested by 0.25% Trypsin-EDTA solution. The dissociated cells were used for experiments.

Cell viability assay: The cell viability was assessed by reduction of water soluble tetrazolium-8 to formazan. Briefly, cells were suspended at a density of 1×10^4 cells mL^{-1} in a volume of 100 μL per well on 96-well culture plates and allowed to attach overnight. The cells were incubated for 24 h with increasing concentrations of different extracts ranging from 15-240 μg mL^{-1} at 37°C. After incubation of cells for the designated time with different concentrations of *G. elegans* alcohol extract or water extract, medium was removed and cells of each well received 10 μL CCK-8 solution. After 4 h incubation, the optical density (OD) was then measured at 450 nm using a microplate reader (Infinite F50, TECAN Group LTD, Switzerland). Cell viability was calculated by dividing the OD of the treated-samples by those of control and the data were expressed as percentages of control.

Flow cytometric assay: Apoptosis and necrosis were determined by double-staining cells with Annexin V-fluorescein isothiocyanate (FITC) and propidium iodide (PI) labeling as our previous report[16]. HepG2 cells were seeded at 2×10^6 cells/well in 6-well plates for 24 h, then the cells were exposed to of *G. elegans* extracts for 24 h. After treatments, cells digested by 0.25% Trypsin-EDTA solution and harvested by centrifugation at 1000 g for 10 min. Then cells were washed twice with PBS which contained 2% BSA. The following assay was performed according to the protocol of Annexin V-FITC Apoptosis Detection Kit. Briefly, cells were suspended in 500 μL of binding buffer and then 5 μL of Annexin V-FITC and 5 μL of PI solution were added to each cell suspension and incubated for 15 min at room temperature in darkness. The samples were analyzed directly using a flow cytometer with an excitation wavelength of 488 nm and an emission wavelength of 530 nm.

Measurement of intracellular ROS: The two extracts-induced intracellular ROS were measured by using the fluorescent probe DCFH-DA as a part of previous report[16]. In brief, HepG2 cells were suspended at a density of 1×10^4 cells mL^{-1} in a volume of 100 μL per well on 96-well culture plates and allowed to attach overnight. The cells were incubated with alcohol extract or acid water extract of *G. elegans* for 24 h. Following treatment, cells were rinsed with PBS and incubated with 10 μM DCFH-DA for 30 min at 37°C. The cells were harvested and washed with DMEM. The ROS production was measured by the fluorescence intensity of cells at an excitation wavelength of 500 nm and an emission wavelength of 525 nm using a fluorescence microplate reader (SpectraMax M5e, Molecular Devices, USA). The fluorescence intensity of cell suspensions relative ROS production was expressed as a percentage of DCF fluorescence of control.

Mitochondrial membrane potential loss: The mitochondrial membrane potential loss was assessed using Rhodamine 123 assay Kit. Briefly, cells were seeded in 6-well plates at a density of 10^5 cells mL^{-1} and cultured overnight, then treated with different concentrations of *G. elegans* alcohol extract or acid water extract for 24 h. After treatments, the cells were washed twice with PBS and digested by 0.25% Trypsin-EDTA solution and harvested by centrifugation at 1000 g for 10 min. Then the cells were mixed with 1 μM of Rhodamine 123 for 10 min and analyzed at an excitation wavelength of 500 nm and an emission wavelength of 530 nm using a fluorescence microplate reader.

Measurement of concentrations of the intracellular Ca^{2+}: The concentration of intracellular Ca^{2+} was detected by using the fluorescent probe Fluo-3AM. In brief, HepG2 cells were suspended at a density of 1×10^4 cells mL^{-1} in a volume of 100 μL per well on 96-well culture plates and allowed to attach overnight. The cells were incubated with alcohol extract or acid water extract of *G. elegans* for 24 h. Following treatment, cells were rinsed with PBS and incubated with 5 μM Fluo-3AM for 50 min at 37°C. The cells were harvested and washed twice with PBS. Intracellular Ca^{2+} was measured by the fluorescence intensity of cells at an excitation wavelength of 488 nm and an emission wavelength of 530 nm.

LC-QqTOF MS analysis: The components extracts were analyzed by Agilent series 1290 Infinity liquid chromatography instrument coupled with an Agilent 6530 quadrupole time-of-flight mass spectrometry (LC-QqTOF MS, Agilent Technologies, Santa Clara, CA, USA) equipped with an electrospray interface. The work solution of crude extract was prepared by dissolving in methanol at the concentration of 0.1 mg mL^{-1} and filtered through a 0.45 μm membrane filter, 4 μL of which was subjected to LC-QqTOF MS analysis.

The HPLC system was equipped with a binary pump, an online degasser, an autosampler and a thermostatically controlled column compartment. The separation was performed on a ODS-C18 column (150×2.0 mm I.D. particle size 5 μm) using a gradient elution consisting of mobile phase A (0.1% formic acid) and mobile phase B (acetonitrile). The gradient was as follows: 0-5 min, an isocratic elution of 10% B. 5-20 min, a linear gradient to 90% B. 20-25 min, an isocratic elution of 90% B. 25.1 min, a gradient back to 10% B. The whole analysis took 30 min. The injection volume was 5 μL, the flow rate was 0.3 mL min^{-1}. The sample chamber in the auto sampler was maintained at 4°C, while the column was set at 30°C.

Mass spectrometric detection was operated in positive ESI mode. The MS parameters were as following: Capillary voltage, 4000 V; nebulizer pressure, 40 psig; drying gas flow rate, 9 L min^{-1}; gas temperature, 350°C. skimmer voltage, 60 V; octapole dc 1, 37.5 V; octapole rf, 250 V; fragmentor voltage, 175 V. Mass spectra were recorded across the range of 50-1000 m/z. Accurate mass measurements of each peak from the total ion chromatograms were obtained using an automated calibrate delivery system to provide the correction of the masses. The instrument performed the internal mass calibration automatically, using a dual-nebulizer electrospray source with an automated calibrant delivery system, which introduces the flow from the outlet of the chromatograph together with a low flow (approximately 10 μL min^{-1}) of a calibrating solution which contains the internal reference masses at m/z 121.0508 and 922.0098 in positive ion mode, whereas at m/z 112.9856 and 1033.9881 in negative ion mode. All the acquisition of data was controlled by Agilent Mass Hunter software (version B.01.03 Build 1.3.157.0 2).

Statistical analysis: All data were expressed as Mean±standard deviation (SD) of at least three independent experiments, except apoptosis and necrosis experiment that were performed in two duplicates. Statistical analysis was performed by using SPSS 17 (SPSS Inc., Chicago, IL). The differences between different groups were analyzed using one-way analysis of variance (ANOVA) followed by Least Significant Difference (LSD) *post hoc* test. A p-value of $p<0.05$ was considered significant.

RESULTS

Cytotoxicity of *G. elegans* alcohol extract and acid water extract on HepG2 cells: As shown in Fig. 1, when the concentration of alcohol extract was 60 μg mL^{-1}, cell viability was not significantly decreased. After treatment of the cells with 120 and 240 μg mL^{-1} of the alcohol extract, the cell viability was decreased by 34.4 and 62.5% of that in the control, respectively. However, *G. elegans* acid water extract was not cytotoxic when the cells were incubated with lower than 240 μg mL^{-1}. These data suggested that acid *G. elegans* water extract had lowest cytotoxicity and the alcohol extract showed strongest cytotoxicity on HepG2 cells. Finally, 60, 120 and 240 μg mL^{-1} of the two extracts for further study was chosen.

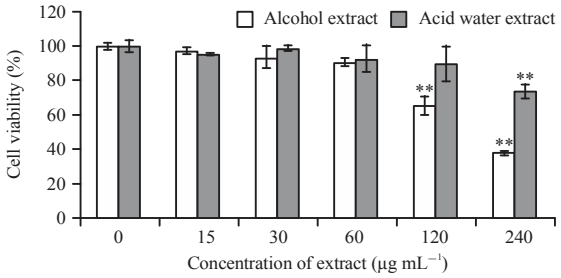

Fig. 1: Effect of *G. elegans* alcohol and acid water extracts on viability in HepG2 cells. Data are expressed as percentage of controls and represent means with Mean±SD (n = 9) of three independent experiments. **Significant different from control group at $p<0.01$

***G. elegans* alcohol extract and acid water extract induced apoptosis or necrosis:** To examine whether the cytotoxic effects of the two extracts involved apoptosis and/or necrosis, the percentage of apoptotic and necrotic cells with flow cytometry was analyzed. Cells in the lower left quadrant that were Annexin V^-/PI^- were viable cells. The Annexin V^+/PI^- cell population in the lower right quadrant were apoptotic cells. The Annexin V^+/PI^+ cell population in the upper right quadrant were late apoptotic or necrotic cells. As shown in Fig. 2, the two extracts could significantly increase the proportion of apoptotic and necrotic cells with respect to control. The percentage of apoptotic cells in lower right quadrant, 60, 120 and 240 μg mL^{-1} *G. elegans* alcohol extract was 33.7, 34.4 and 83.3%, respectively and the percentage of necrotic or late apoptotic in upper right quadrant, 60, 120 and 240 μg mL^{-1} alcohol extract was 27.3, 37.4 and 14.7%, respectively (Fig. 2a). However, The percentage of apoptotic cells in lower right quadrant, 60, 120 and 240 μg mL^{-1} *G. elegans* acid water extract was 20.7, 22.1 and 64.8%, respectively and the percentage of necrotic or late apoptotic in upper right quadrant, 60, 120 and 240 μg mL^{-1} alcohol extract was 2.6, 5.3 and 30.2%, respectively (Fig. 2b). The results revealed that *G. elegans* alcohol extract and

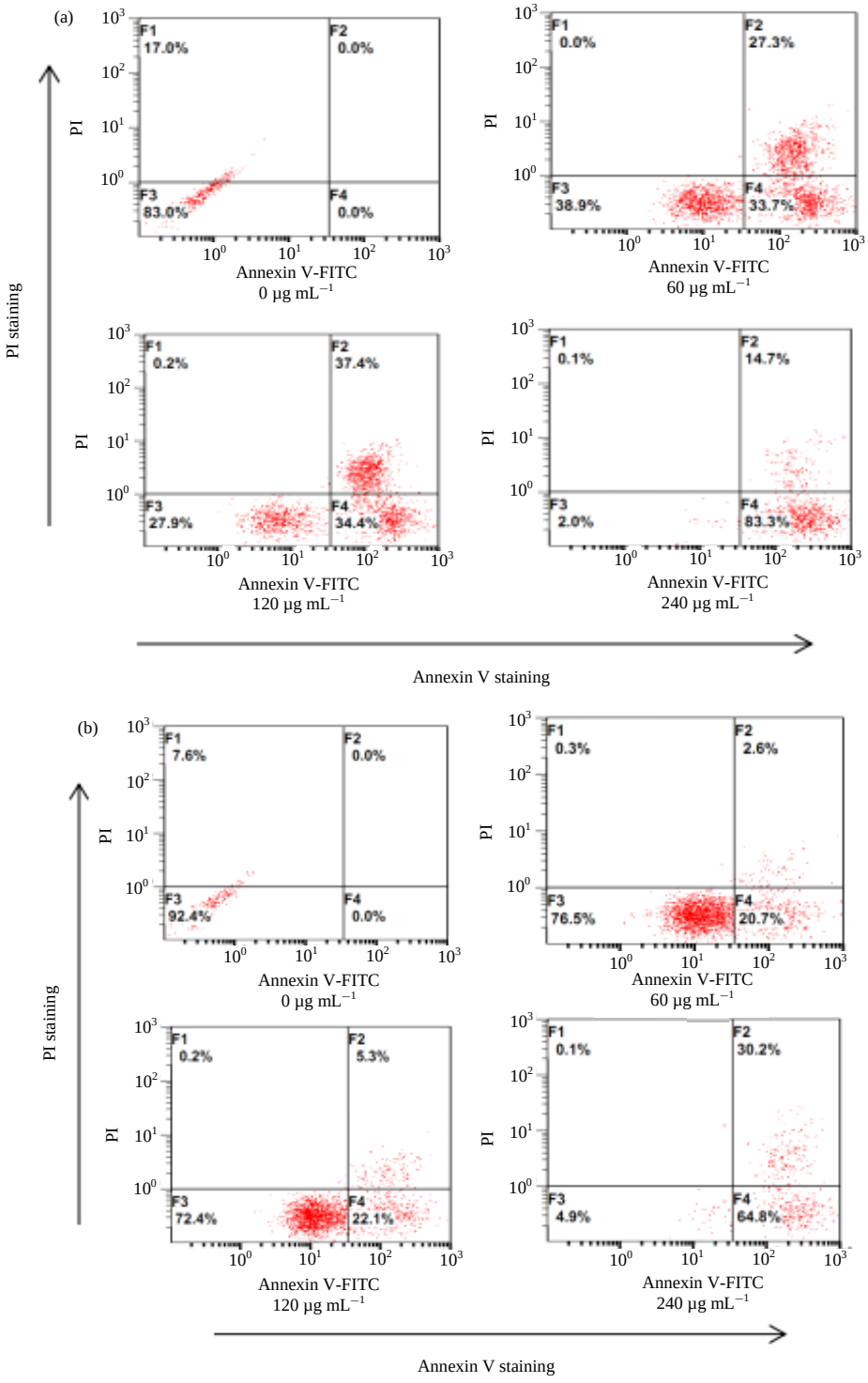

Fig. 2(a-b): Flow cytometric assay of HepG2 cells after treatment of *G. elegans* (a) Alcohol extract and (b) Acid water extract, dyed with Annexin V-FITC and PI double-staining

The lower left: viable cells (Annexin V^-/PI^-). Lower right: Early apoptotic cells (Annexin V^+/PI^-), Upper right: Late apoptotic or necrotic cells (Annexin V^+/PI^+), Upper left: Late necrotic cells or cellular debris (Annexin V^-/PI^+)

acid water extract *of G. elegans* induced apoptosis in HepG2 cells in a dose-dependent manner. Moreover, the *G. elegans* alcohol extract showed stronger cytotoxicity than the acid extract one.

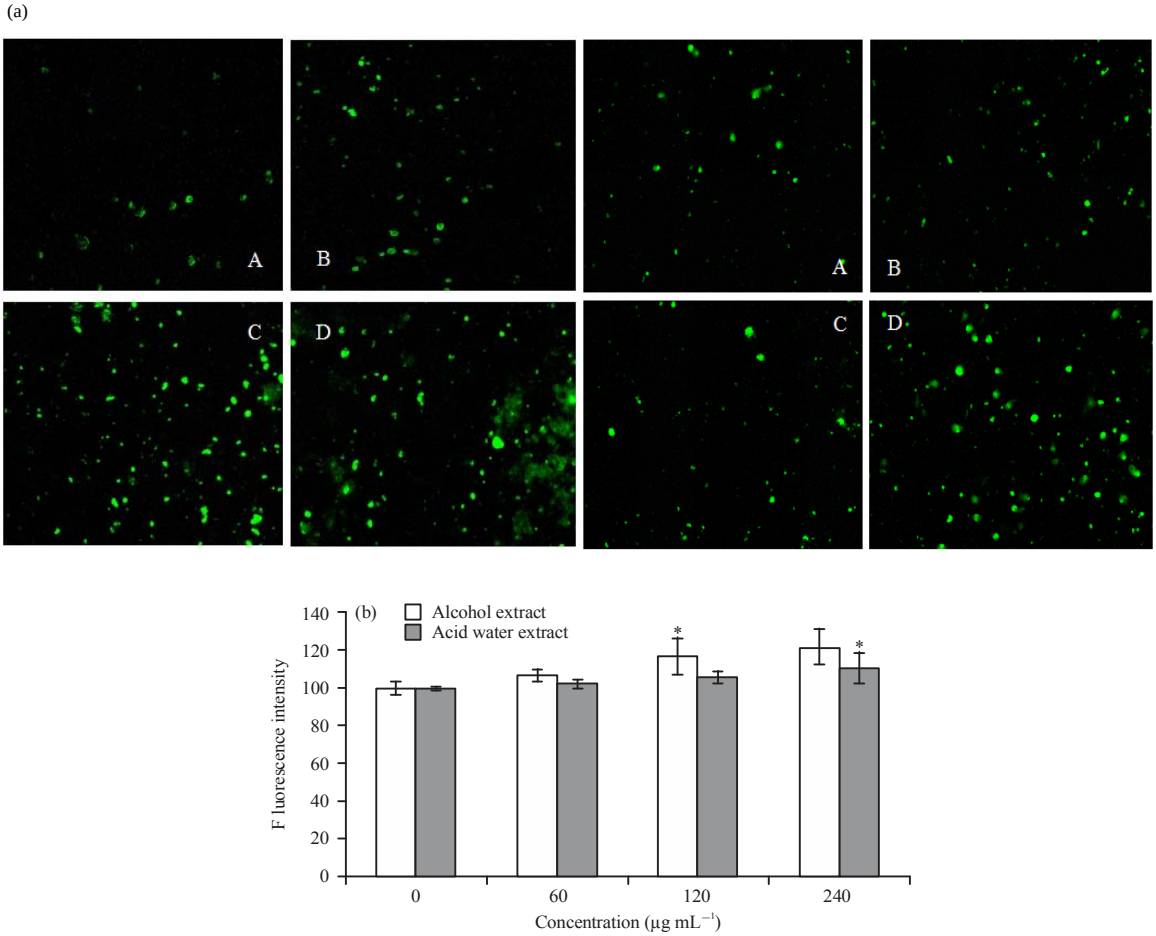

Fig. 3(a-b): Intracellular ROS were determined by the fluorescent probe DCFH-DA. Upper left panel and upper right panel show fluorescence microscopy image induced by *G. elegans* alcohol extract and acid water extract, respectively. A, B, C and D represent extract of 0 60 120 and 240 μg mL^{-1} was treated with HepG2 cells for 24 h, respectively. Lower panel show concentration-course analysis of ROS production by the fluorescence intensity and the results were expressed as fluorescence percentage of controls. *p<0.05, compared to control group

ROS production by *G. elegans* alcohol extract and acid water extract on HepG2 cells: The increase in green fluorescence caused by *G. elegans* alcohol extract was dose-dependent (Fig. 3, upper left panel). At low concentration of alcohol extract (<60 μg mL^{-1}), ROS production was not significantly different from the control, whereas significant ($p<0.05$) overproduction occurred with all other concentrations (Fig. 3 and lower panel). However, the increase in green fluorescence caused by *G. elegans* acid water extract was observed with the acid water extract greater than 240 μg mL^{-1} (Fig. 3, upper right panel).

Effect of *G. elegans* alcohol extract and acid water extract on mitochondrial membrane potential (Δψm): One of the widely used indices of apoptosis is the increased the loss of Δψm. As shown in Fig. 4, *G. elegans* alcohol extract in 120 and 240 μg mL^{-1} decreased significantly in Δψm. At all concentration of *G. elegans* acid water extract, mitochondrial membrane potential loss was not significantly different from the control.

Effect of *G. elegans* alcohol extract and acid water extract on intracellular Ca^{2+} concentration: At low concentration (<120 μg mL^{-1}), *G. elegans* alcohol extract and acid water extract have no difference compared with control. However, the two extracts increased significantly the Fluo-3AM intensity with concentration of 240 μg mL^{-1} (Fig. 5).

Comparison on main components of *G. elegans* alcohol extract and acid water extract: In order to reveal the cytotoxic differences, the main components in

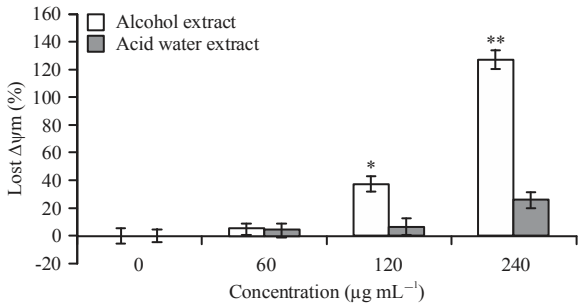

Fig. 4: Effect of *G. elegans* alcohol and acid water extracts on mitochondrial membrane potential loss in HepG2 cells. **p<0.01, compared to control group

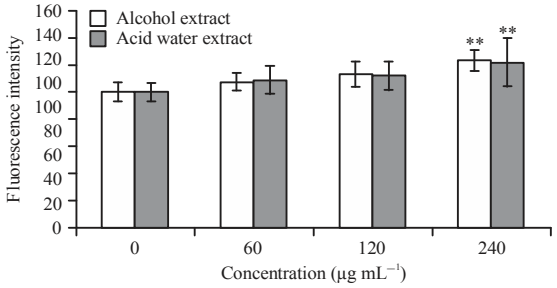

Fig. 5: Effect of *G. elegans* alcohol and acid water extracts on Fluo-3AM fluorescence intensities in HepG2 cells. The resulting fluorescence of Fluo-3AM indicated the Ca^{2+} level. *p<0.05, **p<0.01, compared to control group

G. elegans alcohol extract and acid water extract were analyzed by LC-QqTOF MS method. The characterization of components of three extracts was based on recently published personal analytical strategy[17,18]. In order to provide more convincing evidence for further explanation of the different cytotoxcity, the components between the two extracts were characterized. Combined with analysis results, a total of 29 components, which included 23 alkaloids and 4 iridoids and one phenolic acid and one coumarine were identified in the *G. elegans* alcohol extract. However, only 21 components which included 17 alkaloids, 3 iridoids and one phenolic acid, were identified in the *G. elegans* acid water extract (Fig. 6). As depicted in Table. 1, alcohol extract contains most ingredients between the two extracts, though 18 components were observed in the two extracts. Only three components (23, 31 and 32) only detected in *G. elegans* acid water extract. The results revealed that relatives of alkaloids and iridoids components in the *G. elegans* alcohol extract were more abundant than the acid water extract one based on the relative peak area.

DISCUSSION

Previous studies have demonstrated that the alkaloid extract of *G. elegans* possessed cytotoxic effects on HepG2 cells [5] . The mechanism of cytotoxic action can be attributed to the inducing cell apoptosis by influencing the cell cycle distribution and activation of cysteine protease caspase8 and caspase9. However, what plays the important factor in the *G. elegans*-induced cell apoptosis is still unknown. In the present study, the effect of *G. elegans* alcohol extract and acid water extract on ROS and mitochondrial damage in HepG2 cells were examined. To our knowledge, this is the first study showing the effects of two different *G. elegans* extracts in the HepG2 cells.

In the present study, viability assay showed that *G. elegans* acid water extract did not exhibit cytotoxic effects at the range from 15 and 120 μg mL^{-1} following 24 h of exposure. However, cytotoxic effects were observed at *G. elegans* alcohol extract concentration of >60 μg mL^{-1}. The results indicate that alcohol extract was more cytotoxic than the acid water extract one. The previous results showed that one of the mechanisms of anti-tumor of *Gelsemium* is to induce cell apoptosis[14]. Then using flow cytometry instrument to detect influence on HepG2 apoptosis, the both kinds of *G. elegans* extracts effect on rate of apoptosis were also increased with the increasing of concentration. The results also illustrated the *G. elegans* alcohol extract's toxicity was stronger than acid water extract.

Oxidative stress that leads to an increase in the production of reactive oxygen species (ROS) has been found to play an important role in the development of anti-tumor mechanism[15]. In present study, a significant increase in green fluorescence intensity after the two *G. elegans* extracts treatment already at cytotoxic concentration was observed. However, the increased level of ROS induced by *G. elegans* extracts was lower than those expected results. There was a report about gelsemine involved suppression of ROS and against cisplatin-mediated toxicity[19]. This finding suggested that moderately oxidative stress induced by *G. elegans* extracts would be partially overcome.

When ROS produces too much that could not be able to maintain balance, mitochondrial membrane channels open, lead to the change of the mitochondrial permeability and calcium imbalance. These changes were the starting events during the intrinsic mitochondrial apoptotic pathways in cells[20]. In order to further illustrate the two kinds of *G. elegans* extracts to induce the apoptosis of HepG2 cells, mitochondrial membrane potential was examined. The results showed that the downtrend of mitochondrial membrane potential with

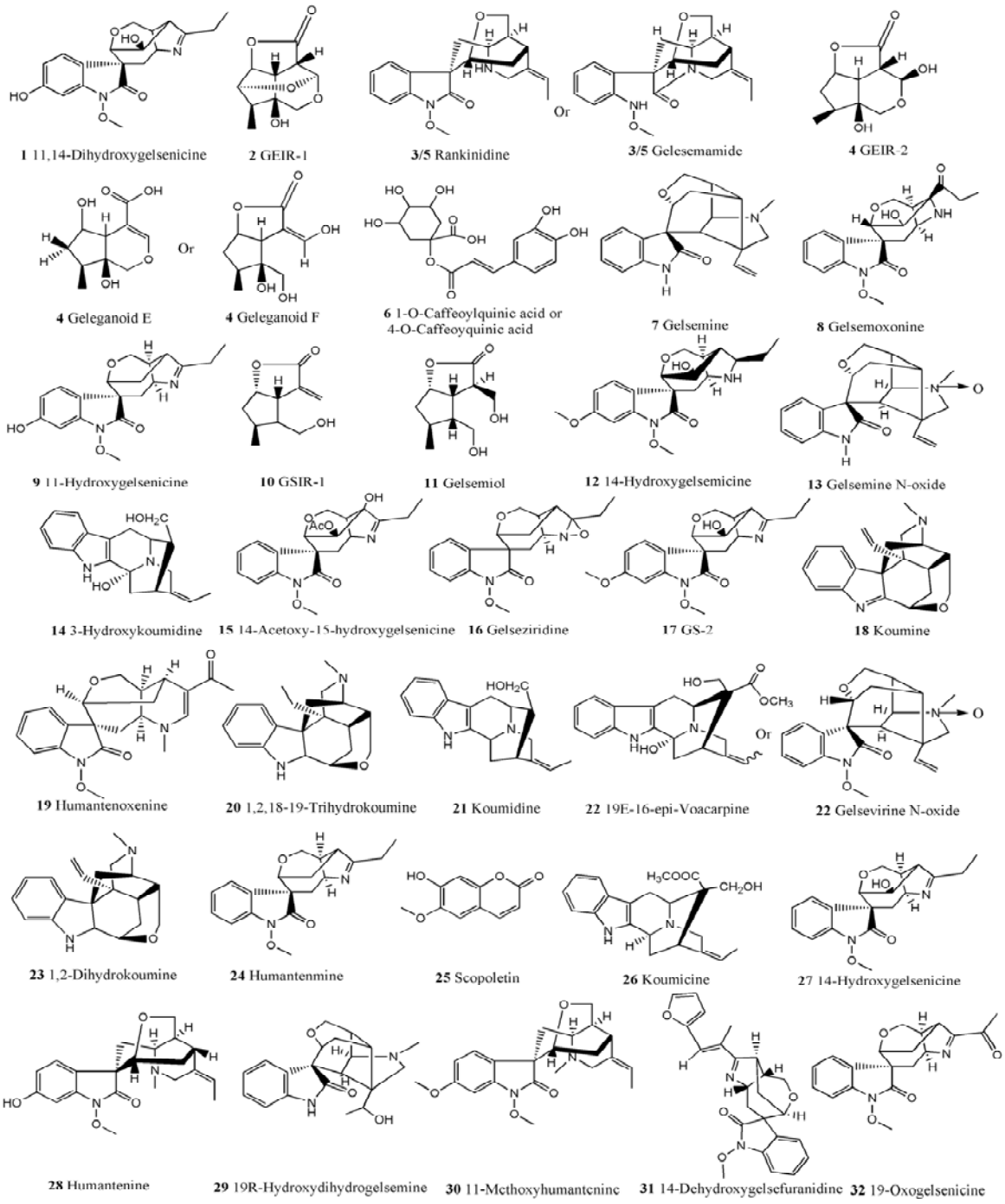

Fig. 6: Chemical structures identified in *G. elegans* alcohol and acid water extracts using LC-QqTOF MS

the increasing of concentration of *G. elegans* alcohol extract. *G. elegans* alcohol extract and acid water extract treatment exerted a considerable rise of Ca^{2+} level in HepG2 cells, suggesting that two extracts disturbed calcium imbalance in exposure time. So, ROS and mitochondrial damage would play an important role in the two extracts induced apoptosis.

Table 1: Characterization of chemical components of *G. elegans* alcohol extract and acid water extract byLC-QqTOF MS

Rt (min)	Molecular formula (Protonated)	m/z measured	Characterization	Peak area of alcohol extract	Peak area of acid water extract	Class
2.13	$C_{19}H_{23}N_2O_5$	359.1594	11,14-Dihydroxygelsenicine	485271.7	-	Gelsedine-type
2.33	$C_{10}H_{13}O^5$	213.0755	GEIR-1	1144740.7	255301	Iridoids
3.24	$C^{20}H^{25}N_2O^3$	341.1857	Rankinidineor gelesemamide	516785.5	-	Humantenine-type
4.05	$C_{10}H_{15}O_5$	215.0912	GEIR-2 or geleganoid E or geleganoid F	706711.0	-	Iridoids
4.05	$C_{20}H_{25}N_2O_3$	341.1860	Rankinidine or gelesemamide	495240.0	154136.7	Humantenine-type
4.46	$C_{16}H_{19}O_9$	355.1021	1-O-Caffeoylquinic acid or 4-O-Caffeoyquinic acid	1542846.7	149279.7	Phenolic acids
4.66	$C_{20}H_{23}N_2O_2$	323.1754	Gelsemine	31706029.0	14148835	Gelsemine-type
4.96	$C_{19}H_{23}N_2O_5$	359.1594	Gelsemoxonine	217153.3	29646.67	Gelsedine-type
5.16	$C_{19}H_{24}N_2O_4$	343.1653	11-Hydroxygelsenicine	2315920.7	-	Gelsedine-type
5.47	$C_{10}H_{15}O_3$	183.1015	GSIR-1	389920.3	131908.3	Iridoids
5.47	$C_{10}H_{17}O_4$	201.1123	Gelsemiol	653143.0	232419	Iridoids
5.47	$C_{20}H_{27}N_2O_5$	375.1919	14-Hydroxygelsemicine	1166746.7	244552.7	Gelsedine-type
5.57	$C_{20}H_{23}N_2O_3$	339.1707	Gelsemine N-oxide	40598.0	62526	Gelsemine-type
5.87	$C_{19}H_{23}N_2O_2$	311.1757	3-Hydroxykoumidine	328143.7	52242.33	Sarpagine-type
6.48	$C_{21}H_{25}N_2O_6$	401.1712	14-Acetoxy-15-hydroxygelsenicine	273740.3	-	Gelsedine-type
6.68	$C_{19}H_{24}N_2O_4$	343.1653	Gelseziridine	245572.7	-	Gelsedine-type
7.39	$C_{20}H_{25}N_2O_5$	373.1763	GS-2	254742.3	-	Gelsedine-type
7.99	$C_{20}H_{23}N_2O$	307.1806	Koumine	20938465.3	8341906	Koumine-type
8.10	$C_{21}H_{25}N_2O_4$	369.1814	Humantenoxenine	122163.3	70564.33	Humantenine-type
8.20	$C_{19}H_{23}N_2O_2$	311.1705	1,2,18-19-Trihydrokoumine	283619.0	-	Koumine-type
8.60	$C_{19}H_{23}N_2O$	295.1809	Koumidine	1444443.0	441086.3	Sarpagine-type
8.70	$C_{21}H_{25}N_2O_4$	369.1814	19E-16-epi-Voacarpine or Gelsevirine N-oxide	1349105.3	359460.7	Gelsemine-type
8.81	$C_{20}H_{25}N_2O$	309.1964	1,2-Dihydrokoumine	-	405435	Koumine-type
8.90	$C_{19}H_{23}N_2O_3$	327.1704	Humantenmine	11221137.3	217100.3	Gelsedine-type
9.11	$C_{10}H_9O_4$	193.0495	Scopletin	185216.0	-	Coumarin
9.11	$C_{21}H_{25}N_2O_3$	353.1859	Koumicine	10975761.0	3864205	Sarpagine-type
9.41	$C_{19}H_{24}N_2O_4$	343.1653	14-Hydroxygelsenicine	377434.7	-	Gelsedine-type
10.22	$C_{21}H_{27}N_2O_3$	355.2029	Humantenine	22454262.3	5692683	Humantenine-type
10.32	$C_{20}H_{25}N_2O_3$	341.1875	19R-Hydroxydihydrogelsemine	1136187.0	-	Gelsemine-type
10.62	$C_{22}H_{29}N_2O_4$	385.2129	11-Methoxyhumantenine	258338.0	103374.7	Humantenine-type
11.84	$C_{24}H_{24}N_2O_4$	405.1809	14-Dehydrxoygelsefuranidine	-	576169	Gelsedine-type
12.04	$C_{19}H_{21}N_2O_4$	341.1500	19-Oxogelsenicine	-	106098.5	Gelsedine-type

The different components of *G. elegans* alcohol extract and acid water extract were responsible for the different cytotoxicity. As it was known, that alkaloids and iridoids were regarded as the bioactive components of Gelsemium that are most likely to be responsible for the observed pharmacological effects. Recent study measured that the total alkaloids contents of *G. elegans* alcohol extract and acid extract were 6.44 and 2.15%, respectively[21]. In this study, the chemical components of *G. elegans* alcohol extract and acid water extract were further investigated and compared using our recently published LC-QqTOF method[17,18]. Current results showed that *G. elegans* alcohol extract contained some components of alkaloids and iridoids which were not existed in the *G. elegans* acid water extract one. Moreover, relatives of alkaloids and iridoids components in the *G. elegans* alcohol extract were more abundant than the acid water extract one based on the relative peak area. Therefore, the results obtained would give useful explanation of the toxicity difference between *G. elegans* alcohol extract and acid water extract based on how much alkaloid contents contained. However, the different toxicity of *G. elegans* alcohol extract and acid water extract will be further investigated in animals in the future. The active components of alkaloids and iridoid in the two extracts will require characterizing "What are absorbed" *in vivo.*

CONCLUSION

The present data demonstrated that *G. elegans* alcohol extract and acid water extract can induce cytotoxicity in HepG2 cells by increasing ROS production. The *G. elegans* alcohol extract and acid water extract induced disturbance of calcium imbalance and turbulence of mitochondrial membrane potential might play an important role in its cytotoxicity. The *G. elegans* alcohol extract exhibited stronger cytotoxicity than the *G. elegans* acid water extract one. This difference in effects might be related to the components of alkaloids and iridoids in the two extracts. The present results obtained would give useful data for the explanation of the cytotoxicity of *G. elegans* extracts and moreover would provide some advice for reducing the adverse reaction of *G. elegans* in clinical practice.

SIGNIFICANCE STATEMENT

This study firstly revealed that oxidative stress and mitochondrial dysfunction induced by *G. elegans* extracts were the possible mechanism of cytotoxicity. The chemical components of the *G. elegans* alcohol extract and acid water extract were characterized using LC-QqTOF MS method. This difference in cytotoxicity of the two extracts might be related to the components of alkaloids and iridoids.

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