**Centranthera grandiflora alleviates alcohol-induced oxidative stress and cell apoptosis**

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**[ABSTRACT]** Alcohol liver disease (ALD) has become a global threat to human health. It is associated with a wide range of liver diseases including alcohol fatty liver, steatosis, fibrosis and cirrhosis, and finally leads to liver cancer and even death. *Centranthera grandiflora* is a traditional Chinese medicinal herb commonly used to treat ALD, but no research about its mechanism is available. This study evaluated the hepatoprotective effect and mechanism of *C. grandiflora* against alcohol-induced liver injury in mice. We found that the ethanol extracts of *C. grandiflora* (CgW) alleviated the alcohol-induced liver injury, enhanced the levels of antioxidant enzymes, and reduced the amount of lipid peroxides. CgW also affected cell apoptosis by inhibiting the activity of Bax, cleaved-caspase 3 and cleaved-caspase 9, and increasing the activity of Bcl-2. In conclusion, the results showed that CgW can effectively improve ALD through alleviating oxidative stress and inhibiting cell apoptosis for the first time. This study suggested that *C. grandiflora* is a promising herbal medicine for ALD treatment.

**[KEY WORDS]** *Centranthera grandiflora*; Alcohol liver diseases; Oxidative stress; Apoptosis; Hepatoprotective effect

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**Introduction**

Alcohol is a worldwide consumed beverage and food additive [1]. Chronic and excessive alcohol consumption can induce alcohol liver disease (ALD), which is a major cause of morbidity and mortality worldwide [2]. ALD is associated with a broad spectrum of liver disorders, such as alcohol fatty liver, steatosis, fibrosis and cirrhosis. Without effective interventions, ALD may eventually lead to liver cancer and even death [3,4].

The pathogenesis of ALD is very complicated, and the underlying mechanisms have not been well understood. According to previous studies, ROS plays an important role in the development of ALD [5]. A large amount of ROS damaged the liver which is the main organ of alcohol metabolism by inducing oxidative stress and pro-inflammatory cytokines [6,7]. ROS inflicted cell death and tissue damage by targeting vital cellular components such as DNA, lipids, and proteins, thus arising as key players in disease pathogenesis [8]. Furthermore, overproduction of ROS destroyed antioxidant defense system, which involved enzymes that eliminated ROS, such as superoxide dismutase (SOD) and glutathione peroxidase (GSH-Px). In contrast, excessive ROS induced apoptosis by activating proteins in the caspase family and other signaling cascades. Hence, inhibiting the ROS-triggered oxidative stress and apoptosis is crucial in regulating the progression of ALD [9,10].

Natural plants have been widely used in China for nutritional supplementation and medical treatment over thousands of years. The abundant resource of Chinese herbs has a promising application prospect for some miscellaneous diseases [11]. *C. grandiflora* is a traditional Chinese medicinal herb mainly...
distributed in Yunnan and Guizhou, which has various pharmacological functions, such as anticoagulation, anti-myocardial ischemia, anti-inflammatory and preventing cardiovascular disease \[^{12, 13}\]. It is commonly used to treat rheumatism, arthralgia, gastroenteritis, laryngitis, alcoholic liver disease, liver cancer and other diseases. Recently, iridoid glycosides were separated from *C. grandiflora* and exerted a wide range of activities, such as anti-cancer effects, regulating blood sugar levels and improving liver injury \[^{14, 15}\]. However, the mechanisms underlying the effects of *C. grandiflora* on ALD are still unclear. Therefore, this study is aimed to explore the protective effect of *C. grandiflora* on ALD and explain the corresponding mechanism of action.

**Material and Methods**

**Extract preparation**

*C. grandiflora* was provided and identified by DONG Wen-Han from the Science and Technology Administration Department of Yunnan Agricultural University (Yunnan, China). The dry whole plant (40 g) was cut into small fragments, and extracted with 95% ethanol at room temperature for seven days. The solution was filtered and concentrated to dryness (2.1 g). Finally, the resultant dried extract (CgW) was stored at −20 °C for later use. To control the quality of CgW, its fingerprints were analyzed (Fig. 1) by UFLC-MS on a UFLC system (LC-20AD, SHIMADZU, Kyoto, Japan) using Shim-pack VP-ODS (150 mm × 2.0 mm, 4.6 μm). The separation was performed in gradient mode using mobile phases consisting of 0.1% trifluoroacetic acid–water (A) and 0.1% trifluoroacetic acid–water–acetonitrile (B) (0 min 5% B and 30 min 50% B). The flow rate was 0.2 mL·min\(^{-1}\). The column temperature was maintained at 40 °C and the injection volume was 1 μL.

![Fig. 1 Analysis of CgW by ultra fast liquid chromatography (UFLC)](image)

**Antibodies and reagents**

Primary antibodies against Bcl-2, Bax, caspase 3, cleaved-caspase 3, caspase 9, and cleaved-caspase 9 were purchased from Wanleibio (Shenyang, China). Secondary antibodies were bought from Hangzhou Xianzhi Biotechnology Co., Ltd. (Hangzhou, China). The Reactive Oxygen Species Assay Kit was obtained from Beyotime Biotechnology (Nanjing, China). The Dulbecco’s Modified Eagle’s Medium (DMEM) was from Gibco (Carlsbad, CA, USA). Fetal bovine serum (FBS) was purchased from Biological Industries (Kibbutz Beit Haemek, Israel). GSH, SOD, and MDA biochemical kits were from Jiancheng Bioengineering Institute (Nanjing, China). Annexin V-FITC/PI double stains apoptosis assay kit, RIPA lysis buffer, MTT and PMSF were bought from KeyGen Biotechnology (Nanjing, China).

**Cell culture**

Human normal hepatic cells L02 (human liver-7702) provided by China Pharmaceutical University were preserved in our laboratory and used for in vitro assays. The cells were cultured in DMEM supplemented with 10% fetal bovine serum, 100 U·mL\(^{-1}\) of penicillin and 100 μg·mL\(^{-1}\) of streptomycin in 5% CO\(_2\) atmosphere at 37 °C.

**MTT assay**

MTT assay was conducted to evaluate cell viability \[^{19}\]. L02 cells were seeded in 96-well culture plates (5 × 10\(^4\)/wells) in triplicate and cultivated at 37 °C under 5% CO\(_2\) in a humidified chamber overnight. Then, the cells were treated with various concentrations (0, 6.25, 12.5, 25, 50, 100, 200, and 400 μg·mL\(^{-1}\)) of CgW for 24 h. Then, 20 μL of MTT was added to each well and the plate was incubated for another 4 h \[^{17}\]. The supernatant was removed and 200 μL DMSO was added to each well. The absorbance was read at the absorbance of 570 nm, and the cell viability was calculated. The same method was used to determine the effect of H\(_2\)O\(_2\) (20–5000 μmol·L\(^{-1}\)) on the viability of L02 cells.

**Measurement of ROS**

The production of intracellular ROS was determined by ROS assay kit. ROS can oxidize non-fluorescent DCFH to produce fluorescent DCF. Therefore, the level of ROS in the cells can be detected through assessment of the fluorescence intensity of DCF. L02 cells were pre-treated with 60 μmol·L\(^{-1}\) H\(_2\)O\(_2\) for 24 h, and then treated with CgW at different concentrations for 24 h. Then the cells were incubated with 10 μmol·L\(^{-1}\) DCFH-DA at 37 °C for 30 min. The level of ROS was determined by flow cytometry and fluorescence microscopy \[^{4}\].

**Detection of cell oxidative stress**

The contents of malonaldehyde (MDA), antioxidant (GSH) and antioxidant enzyme (SOD) are important parameters reflecting the potential antioxidant capacity \[^{19}\]. L02 cells were treated with 60 μmol·L\(^{-1}\) H\(_2\)O\(_2\) for 24 h, and then treated with CgW at different concentrations for 24 h. Then the cells were incubated with 10 μmol·L\(^{-1}\) DCFH-DA at 37 °C for 30 min. The level of ROS was determined by flow cytometry and fluorescence microscopy.

**Apoptosis analyses by flow cytometry**

Cell apoptosis was measured by annexin V-FITC/PI double staining and flow cytometry analysis. L02 cells were cultured in 6 well plates for 12 h, pre-treated with 60 μmol·L\(^{-1}\) H\(_2\)O\(_2\) for 24 h, and then treated with different concentrations of CgW for 24 h. An annexin V-FITC/PI assay kit was used to assess the effect of CgW on cell apoptosis \[^{19}\].

**Western blot**

Total proteins from cells were extracted on ice with RIPA lysis buffer containing PMSF (1 : 100) for 30 min. Then, about 30 μg protein was loaded on 12% polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto polyvinylidene fluoride (PVDF) membrane. The mem-
branched was then blocked in 5% BSA and incubated in appropriate primary antibodies at 4 °C for overnight. The relative amounts of bands were detected by enhanced chemiluminescence using the Bio-Red Laboratories Quantity One Software [20].

**Animal experiment**

Male ICR mice (aged 7–8 weeks, and weighting 17–19 g) were purchased from Shanghai Sippe-BK Lab Animal Co., Ltd. (Shanghai, China). ICR mice were selected by Sauschka from Swiss mice. The study was conducted according to the guidelines of the Declaration of Helsinki and the Guidelines of China Pharmaceutical University, and approved by Department of Science and Technology (SYXX (SU) 2016-0011). The mice were maintained in an environmentally-controlled room on a 12 h:12 h light:dark cycle under a temperature of (23 ± 2 °C) and humidity of (45% ± 5%). All the mice were fed with standard diet and water. After acclimation, the mice were randomly divided into five groups (n = 10): a normal group, an ethanol stimulation group (30%, W/V, 2.4 g kg⁻¹), a low-dose CgW group (50 mg kg⁻¹), a medium-dose CgW group (100 mg kg⁻¹) and a high-dose CgW group (200 mg kg⁻¹).

Mice in the normal and alcohol groups were treated by oral gavage with distilled water, while the others were given different doses of CgW once a day for 30 days. On the 16th day of the experiment, all the groups except the normal group were challenged with alcohol (30%, W/V, 2.4 g kg⁻¹) for 15 days. After the final dosing, the mice were sacrificed and blood and liver samples were collected.

**Measurement of serum biochemical indicators**

Blood samples of the mice were collected via the orbital venous plexus and centrifuged at 5000 r min⁻¹ at 4 °C for 10 min. Then, serum aspartate aminotransferase (ALT), alanine aminotransferase (AST), total cholesterol (TCHO), low density lipoprotein cholesterol (LDL-C), uric acid (UA) and total bilirubin (TBIL) activity were measured using available assay kits.

**Histopathological analysis**

Liver samples of the mice were immediately fixed with 10% formalin solution for 48 h. Then, the fixed tissues were embedded in paraffin, sliced into 5 μm thick sections, and stained with hematoxylin and eosin (H&E). The stained sections were observed under a fluorescent microscope and the images of liver tissues were captured using a Olympus CKX41 microscopic camera [21].

**Measurement of liver MDA, ADH and ALDH**

Liver tissues were homogenized in an ice-cold saline solution using a Tissue Tearor (Shanghai Jingxin Industrial Development Co., Ltd., Shanghai, China) and then centrifuged at 5000 r min⁻¹ at 4 °C for 10 min. The supernatants were collected for measurement of MDA, ADH and ALDH, according to the manufacturer’s instruction from Jiancheng Bioengineering Institute (Nanjing, China).

**Statistical analysis**

Data are presented as mean ± SD. Statistical analysis was performed by GraphPad Prism 5.0 software using one way ANOVA with Turkey’s e method for multiple comparisons. The differences between groups were considered to be statistically significant at P < 0.05.

**Results**

**The quality control of CgW**

The UPLC results of CgW were shown in Fig. 1. Quantitative analysis indicated that the contents of aucubin, geniposidic acid, luteolin-3′-O-L-rhamnoside, and luteolin-7-O-D-glucoside in CgW were 5.11%, 6.45%, 0.10% and 15.9%, respectively.

**Effects of CgW on alcohol-induced liver injury in mice**

Alcohol-induced hepatic damage is characterized by release of aspartate transaminase (ALT) and alanine aminotransferase (AST) into the circulatory system. The increase of serum ALT and AST indicates cell membrane damage and mitochondrial damage in the liver, respectively. As shown in Fig. 1, alcohol consumption significantly increased the activity of serum ALT (Fig. 2A) and AST (Fig. 2B) (P < 0.05), compared with the normal group. The low dose of CgW (50 mg kg⁻¹) largely reduced the levels of serum ALT (P < 0.01) and AST (P < 0.05), indicating restored liver function and ameliorated liver injury. In contrast, the medium and high concentrations of CgW exhibited slight effects. Meanwhile, the contents of UA (Fig. 2C) and TBIL (Fig. 2D) in serum were measured to assess the condition of the liver. Alcohol-consumption seriously increased UA and TBIL contents by 36.7% (P < 0.05) and 52.9% (P < 0.01) compared with the control group. CgW largely reduced UA content by 32.0% (P < 0.01) at the low dose and decreased TBIL content by 26.9% (P < 0.05) at the medium dose.

As alcohol can cause lipid metabolic disorder [22], the levels of LDL-C and TCHO in different groups were determined. CgW reduced the effect of ethanol on the amount of LDL-C and TCHO concentration by 33.9%–50.1% and 13%–24%, respectively (Figs. 2E and 2F). These results suggested that CgW has an excellent function in improving alcohol-induced liver lipid metabolism disorder.

H&E staining was used to evaluate the pathological changes in the liver. In the normal group, the liver showed a normal structure with tight cell arrangement, clear hepatic cord structure, without necrosis. The alcohol-treated liver presented serious swelling, loose cytoplasm, ballooning, degeneration along with necrosis; some of the tissue was infiltrated by inflammatory cells. However, CgW treatment resulted in largely reduced alcohol-induced liver degeneration and necrosis, indicating that CgW has a significant effect on improving alcohol-induced liver injury (Fig. 3).

**Effects of CgW on alcohol-induced oxidative stress in mice**

Alcohol-induced liver injury is associated with increased oxidation and free radical mediated tissue damage, which has been widely investigated in animals and humans [22]. MDA is one of the most important products of membrane lipid peroxidation. As shown in Fig. 4A, alcohol administration caused a great elevation of MDA in the liver by 33.3% compared with the control. Furthermore, the low and high doses of CgW decreased the elevated MDA by 25.0% and 33.3%, respectively, compared with the alcohol group. Antioxidant enzymes GSH...
Effects of CgW on oxidative stress in L02 cells

According to the results mentioned above, animal experiments showed that CgW treated ALD by alleviating oxidative stress. ROS consists of superoxide anion (O_2^-), hydrogen peroxide (H_2O_2), hydroxyl radicals (·OH) and nitric oxide (NO), and H_2O_2 was used to induce oxidative stress in vitro. In order to investigate the protective effect of CgW on oxidative stress induced cell damage, different concentrations of H_2O_2 were used to induce L02 cell injury in MTT assay. As shown in Fig. 5A, when H_2O_2 concentrations ≥ 60 mmol·L^{-1}, the cell viability significantly decreased. Therefore, culture media containing 100 mmol·L^{-1} H_2O_2 was used to induce L02 cell injury. CgW (100, 200, and 400 μg·mL^{-1}) pretreatment for 24 h prevented cell apoptosis against H_2O_2-stimulated oxidative stress (Fig. 5B). The concentration of CgW showed no significant toxic effect on L02 cells (Fig. 5C).

To further assess whether CgW can alleviate liver damage through alleviating oxidative stress, ROS, MDA, SOD and GSH were estimated. As shown in Fig. 5, ROS production remarkably increased by three folds in L02 cells treated with H_2O_2 alone, compared with the control. However, pretreatment with CgW significantly attenuated the generation of ROS. ROS production was reduced by 36.7% (P < 0.001) and 60.8% (P < 0.001) after treatment with 100 and 200 μg·mL^{-1} of CgW, respectively (Figs. 5D and 5E). Similarly, the content of MDA significantly decreased after treatment with medium and high doses of CgW (100 and 200 μg·mL^{-1}) (Fig. 5F).

SOD and GSH are the most important anti-oxidants, so we investigated the inhibitory effect of CgW on oxidative stress through SOD and GSH evaluation. Treatment with H_2O_2 significantly decreased SOD by 37.5% (P < 0.05) and GSH by 61.6% (P < 0.01) (Figs. 5G and 5H). However, CgW increased the levels of SOD and GSH at the dose of 200 μg·mL^{-1} (P < 0.05 and 0.001, respectively), without signific-
Fig. 4 Effect of CgW on liver oxidative stress and injury in alcohol-induced mice. (A) MDA content, (B) GSH content, (C) SOD activity, (D) ADH activity, and (E) ALDH activity in alcohol-induced liver were measured. Data are expressed as mean ± SD (n = 10). *P < 0.05, **P < 0.01, ***P < 0.001 vs the control group; #P < 0.05, ##P < 0.01, ###P < 0.001 vs the alcohol-induced group.

Fig. 5 Effect of CgW on H2O2-induced cell injury and oxidative stress. (A) Effect of H2O2 on L02 cell viability; (B) effect of CgW on H2O2-treated L02 cell viability; (C) effect of CgW on L02 cell viability; (D) and (E) intracellular ROS production; (F) MDA content; (G) SOD activity, and (H) GSH content. Data are expressed as mean ± SD (n = 3). *P < 0.05, **P < 0.01, ***P < 0.001 vs the control group; #P < 0.05, ##P < 0.01, ###P < 0.001 vs the H2O2-induced group.

As long-term excessive alcohol intake leads to hepatic damage, the protective effects of CgW against liver injury and oxidative stress induced by alcohol were investigated. CgW attenuated the increase in MDA and ADH activities in alcohol-induced liver, while it decreased the decrease in SOD activity, and GSH content. These results indicate that CgW plays a protective role in alcohol-induced liver injury by modulating mitochondrial apoptosis signaling pathway.

Effects of CgW on mitochondrial apoptosis signaling pathway

As long-term excessive alcohol intake leads to hepato-
cyte apoptosis, annexin V-FITC/PI double staining and flow cytometry were used to evaluate the protective effect of CgW on apoptosis. As shown in Fig. 6A, the percentage of early and late apoptosis in the H2O2 group largely increased by 30.05%. In contrast, when L02 cells were pre-treated with...
Fig. 6  Effect of CgW on H<sub>2</sub>O<sub>2</sub>-induced cell apoptosis. (A) Cell apoptosis was analyzed by flow cytometry and annexin V-FITC/PI double staining; (B) Hochest 33258 staining L02 cells; (C) the expression of protein related to apoptosis including Bcl-2, Bax, caspase 3, cleaved-caspase 3, caspase 9, and cleaved-caspase 9 in L02 cells; (D) the quantitative analysis of the gray value based on (C). Data are expressed as mean ± SD (n = 3). *P < 0.05, **P < 0.01 vs the control group; *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001 vs the H<sub>2</sub>O<sub>2</sub>-induced group.
different concentrations of CgW, the percentage of apoptotic cells decreased. According to the results, CgW has a considerable effect on decreasing cell apoptosis.

To analyze liver cell apoptosis more clearly, we used the Hochest 33258 dye to observe cell apoptosis. Compared with the control group, the number of fluorescently labeled cells treated with H$_2$O$_2$ significantly increased, the fluorescence intensity was deeper, and the nucleus was densely stained. However, CgW significantly reduced the number of fluorescently labeled cells, and the fluorescence intensity was gradually reduced in a dose-dependent manner (Fig. 6B).

The levels of Bcl-2, Bax, cleaved-caspase 3, cleaved-caspase 9 and other proteins which play a key role in cell apoptosis were examined using Western blot. Compared with the control group, the levels of Bax, cleaved-caspase 3, and cleaved-caspase 9 treated with H$_2$O$_2$ were significantly increased. However, CgW inhibited the expression of these apoptosis-promoting proteins and enhanced the expression of the apoptosis suppression protein Bcl-2 (Figs. 6C and 6D), indicating that CgW can improve mitochondrial apoptosis by inhibiting the expression of related proteins.

**Discussion**

The incidence of ALD is steadily rising in the world. Due to the lack of suitable diagnostic techniques, patients are often diagnosed at an advanced stage, leading to a high mortality. Nowadays, many drugs have been developed for the treatment of ALD, targeting oxidative stress, inflammation and the imbalance of flora [26]. But there are still no effective treatments for alcoholic hepatitis. *C. grandiflora* contains complex ingredients, and exerts anti-oxidative, anti-inflammatory and antibacterial effects. CgW is a low toxic substance, as compared with the control group; CgW showed no remarkable effect on decreasing cell apoptosis.

Increased MDA in the liver can cause tissue damage and interfere with the antioxidant system. SOD can eliminate harmful substances produced after the metabolism in the body, protect cells from oxidative damage of superoxide and hydrogen peroxide free radicals and play a vital role in maintaining the balance between oxidation and anti-oxidation. The level of SOD directly reflects the body’s ability to scavenge free radicals and is considered as an important ROS scavenger. In the current study, compared with the alcohol consumption group, exposure to CgW significantly reduced MDA levels and increased the antioxidant SOD and GSH activity in vivo and in vitro. CgW ameliorated alcohol and H$_2$O$_2$-induced oxidative stress injury. CgW supplementation improved the body’s condition and movements of mice with the injury induced by alcohol. At the same time, CgW significantly reduced the levels of serum AST and ALT and improved liver swelling, inflammation and necrosis. The levels of serum TCHO and LDL-C decreased, which indicated that CgW can improve the disorder of lipid metabolism in mice.

One of the most important findings in our study was that CgW inhibited H$_2$O$_2$-induced apoptosis by inhibiting the mitochondrial apoptosis signaling pathway. Bax and Bcl-2 play important roles in regulating cell survival and apoptosis. Elavation of the ratio of Bax/Bcl-2 can promote the formation of Bax/Bcl-2 homodimers, resulting in an increase of the permeability of mitochondrial outer membrane and loss of mitochondrial membrane potential [25]. Our results showed that CgW reversed the increased ratio of Bax/Bcl-2 after H$_2$O$_2$ stimulation, and then inhibited the activation of caspase 3 and caspase 9, and L02 cell apoptosis (Fig. 7).

Fig. 7 Mechanisms of CgW in alleviating the alcohol-induced liver injury

Overall, our research demonstrated for the first time that CgW can effectively improve ALD through attenuating oxidative stress. Moreover, the protective effect of CgW on alcohol-induced liver injury may be related to the mitochondrial apoptosis signaling pathway. The results suggested that *C. grandiflora* may be a good candidate for the prevention of alcohol liver injury.

**References**