Antioxidant and antitumor activities of *Capparis spinosa* L. and the related mechanisms

LEI YU[1-3], JINGHUI YANG[1-3], XIN WANG[1-3], BO JIANG[1-3], YONGXUE SUN[1-3] and YUBIN JI[1-3]

[1]Center of Research and Development on Life Sciences and Environmental Sciences, Harbin University of Commerce, [2]Institute of Materia Medica and Postdoctoral Programme of Harbin University of Commerce, Harbin 150076; [3]Engineering Research Center of Natural Anticancer Drugs, Ministry of Education, Harbin 150076, P.R. China

Received May 18, 2016; Accepted June 25, 2016

DOI: 10.3892/or.2016.5249

Abstract. The 'ethnodrug' *Capparis spinosa* L. has several pharmacological activities. First, it was found in previous experiments that an ethyl acetate extract of *Capparis spinosa* L. (CSE) exhibited antioxidant activity. In order to further research this finding, the present study investigate the blood biochemical indices, injury, energy metabolism, oxidative damage and mitochondrial membrane potential (Δψm) level of cardiac cells to study the effect of CSE on doxorubicin-induced cardiac toxicity. CSE had protective effects on the cardiac toxic effect of doxorubicin, and decreased the activity of lactic dehydrogenase (LDH) and creatine kinase (CK). CSE increased the ability of myocardial tissue to scavenge free radicals, inhibited lipid peroxidation, increased recovery activity of antioxidant enzymes, adjusted the energy metabolism of myocardial tissue, inhibited the generation of a large number of ROS in the cells, raised the level of Δψm, and improved the metabolism of free radicals. CSE demonstrated protective effects on doxorubicin-induced myocardial damage. Second, the quaternary ammonium hydroxide of *Capparis spinosa* L. (CSQAH) was found to possess antitumor activity, such as antiproliferative and apoptosis-induced effects on HepG2 cells. We investigated the regulatory mechanism of HepG2 apoptosis induced by CSQAH. Laser scanning confocal microscope and Fluo-3/AM staining were utilized to detect the Ca^{2+} concentration in the HepG2 cells. A microplate reader was used to measure the changes in Ca^{2+}-Mg^{2+}-ATP enzyme. Then, flow cytometry was applied to analyze the activity of ROS and the expression levels of Bcl-2 and Bax. As a result, different concentrations of CSQAH increased the concentration of Ca^{2+} in the cytoplasm in a dosage-dependent manner. CSQAH decreased the Ca^{2+}-Mg^{2+}-ATPase activity in the HepG2 cells. The levels of ROS in the CSQAH groups were significantly higher than the level in the control group. Flow cytometric analysis showed that the Bcl-2 expression levels in the CSQAH-treated groups were downregulated, while Bax expression levels were upregulated, and the effects were dosage-dependent. The regulatory mechanism of HepG2 cell apoptosis induced by CSQAH involved the increase in Ca^{2+} concentration and ROS levels, a decrease in Ca^{2+}-Mg^{2+}-ATPase activity in the HepG2 cells, and downregulation of anti-apoptotic Bcl-2 expression, and upregulation of apoptotic Bax expression. In summary, the present study demonstrated the antioxidant and antitumor activities of CSE which may suppress tumor growth and alleviate the side-effects of DOX, which may facilitate tumor treatment in a dual manner.

Correspondence to: Dr Lei Yu, Center of Research and Development on Life Sciences and Environmental Sciences, Harbin University of Commerce, 138 Tongda Street, Daoli Region, Harbin 150076, P.R. China
E-mail: yulei912@163.com

Key words: Capparis spinosa L., CSE, antioxidant activity, CSQAH, antitumor activity

Introduction

Capers are a common ingredient in Mediterranean cuisine, particularly Cypriot, Italian and Maltese recipes. *Capparis spinosa* L. (CSE), the caper bush, also called cishangan, *Euphorbia lathyris* L. and *Capparis masaikai* Levl., is a perennial plant that bears round, fleshy leaves and large white to pinkish-white flowers. CSE is found in the wild in the Mediterranean, East Africa, Madagascar, Southwestern and Central Asia, Himalayas, the Pacific Islands, Indomalaya and Australia. The plant is best known for the edible flower buds (capers), often used as a seasoning, and the fruit (caper berries), both of which are usually consumed pickled. The salted and pickled caper bud (called simply a caper) is often used as a seasoning or garnish. The mature fruit of the caper shrub are prepared similarly and marketed as caper berries. The buds are picked, then pickled in salt, or a salt and vinegar solution, and drained. Other parts of the Capparis plants are used in the manufacture of medicines and cosmetics. The shrubby plant is many-branched, with alternate leaves, thick and shiny, round to ovate. The flowers are complete, sweetly fragrant and showy, with four sepals and four white to pinkish-white petals. Intense flavor is developed as mustard oil is released from each caper bud. This enzymatic reaction leads to the formation of rutin, often seen as crystallized white spots on the surfaces of individual caper buds. In Greek popular

medicine, a herbal tea made of caper root and young shoots is considered beneficial against rheumatism (1-6).

CSE contains glucosinolates (glucocapparin, glucocleomin, glucoiberin, glucopangulin and singrin), flavonoids and choline, coumarins, saponins and tannins. Instruction exists on the use of sprouts, roots, leaves and seeds in the treatment of strangury and inflammation. Different flavonoids have been identified in the caper bush and capers: rutin (quercetin 3-rutinoside), quercetin 7-rutinoside, quercetin 3-glucoside-7-rhamnoside, kaempferol 3-rutinoside, astragalin and kaempferol 3-rhamnorutinoside. Capers contain more quercetin/weight than any other plant (7). Selenium is present in capers at high concentrations in comparison with other vegetable products. Furthermore, CSE extract was reported to be rich in flavonoids such as kaempferol, rutin, quercetin and quercetin derivatives, which are known to have anti-allergic, anti-inflammatory, antioxidant, anti-fungal, anti-bacterial anti-hepatotoxic, anti-diabetic, antiproliferative and antitumor properties. Recently, the effect of plant extracts on melanogenesis has been reported and compounds, such as glycyrrhizin, quercetin and scoparone, were found to stimulate melanogenesis (8-19).

Doxorubicin (DOX) is a potent broad-spectrum chemotherapeutic agent that is highly effective in treating patients with acute lymphoblastic leukemia, Hodgkin's lymphoma, aggressive non-Hodgkin's lymphomas, breast and ovarian carcinoma, and many solid tumors. The therapeutic activity of DOX is achieved through the processes of intercalating into DNA, inhibiting topoisomerase II, and preventing DNA and RNA synthesis. Unfortunately, its clinical chemotherapeutic use is limited by its severe toxicity on the heart when the accumulative dose reaches a threshold. The cardiotoxicity particularly subchronic and delayed cardiotoxicity is manifested by dose-dependent cardiomyopathy and refractory congestive heart failure with the unique pathological changes being distention of the endoplasmic reticulum, swelling of mitochondria, cytoplasmic vacuolization and myofibrillar disarray, and loss (sarcopenia) in cardiomyocytes as well as apoptosis. A great deal of research has been carried out to investigate the molecular mechanisms by which DOX selectively impairs the heart. As a result, a number of mechanisms were proposed although most of them are attributable to the basis that DOX increases the production of ROS in cardiomyocytes (20-25). It was found in previous experiments that ethyl acetate extract of CSE has antioxidant activity and improves the oxidative damage symptoms observed by morphological changes of pathology and hematoxylin and eosin (H&E) staining. The major mechanism of heart dysfunction induced by oxidative stress (free radical damage) that is responsible for DOX cardiotoxicity is the formation of ROS, which can harm membrane lipids and other cellular components, leading to cardiomyocyte apoptosis and death. In order to further research this issue, the present study investigated blood biochemical indices, injury, energy metabolism, oxidative damage and mitochondrial membrane potential ($\Delta\psi m$) level of cardiac cells to study the effect of CSE on DOX-induced cardiac toxicity. Thus, the first part of the study was designed to investigate whether CSE has any protective effect on oxidative damage induced by DOX and to explore whether or not CSE can be used as an adjuvant therapy for the long-term clinical use of DOX. Our theory basis can make a foundation for the following research.

In a previous study, we demonstrated that CSE inhibited tumor cell growth and the main constituent responsible for the antitumor activity is alkaloid (26). The quaternary ammonium hydroxide of *Capparis spinosa* L. (CSQAH) is one of the water-soluble alkaloids which is obtained by means of ammonium reineckate. It also inhibited tumor cell growth and induced cell apoptosis. However, the molecular mechanisms associated with the apoptosis of human hepatocellular carcinoma HepG2 cells by CSQAH is not clear and systematically understood. In the second part of the study, we therefore utilized flow cytometry (FCM) and laser scanning confocal microscopy (LSCM) to detect Ca^{2+} concentrations, Ca^{2+}-Mg^{2+}-ATP enzyme activity and ROS, Bcl-2 and Bax levels to investigate the regulatory mechanism of HepG2 cell apoptosis induced by CSQAH.

In summary, the present study demonstrated the antioxidant and antitumor activities of CSE which may suppress tumor growth and alleviate the side-effects of DOX, which may facilitate tumor treatment in a dual manner.

Materials and methods

Antioxidant activity of CSE and its protective effect on oxidative damage induced by DOX

Animals and treatment. The present study was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The protocol was approved by the Ethics Committee of the Animal Experiments of the Heilongjiang University of Chinese Medicine (permit no. 2013-004). All surgery was performed under sodium pentobarbital anesthesia, and all efforts were made to minimize suffering of the animals.

Kunming mice (animal certificate no. POO101009; provided by the Animal Experiment of Heilongjiang University of Chinese Medicine), half male and female, were randomly divided into five groups (n=12/group): normal, DOX and CSE groups; CSE-L, CSE-M and CSE-H (40, 80 and 120 mg/kg). The numbers of each group fit the statistical requirements. Food and water were provided freely and the mice were sacrificed by cervical dislocation in accordance with ethical requirements. Each mouse was weighed before administration. The normal group was orally administered normal saline (0.01 ml/g, daily), and the DOX groups were intraperitoneal injected with DOX hydrochloride (15 mg/kg) once on day 5. The CSE groups received different doses of CSE extract orally for five consecutive days and were intraperitoneally injected with a single dose of DOX hydrochloridex (15 mg/kg) 2 h after the CSE extract treatment on day 5, and then CSE was continued to be administered for 2 days.

Screening of antioxidant fraction by EPPH. The fruits of CSE (5 kg) were extracted with 95% ethanol. Evaporation of the solvent under reduced pressure provided the condensed ethanol extract, which was then extracted by petroleum ether, chloroform, ethyl acetate and *n*-butyl alcohol in turn. The five factions were assessed for their antioxidant activities by EPPH method (27).

Effect of CSE on injury and energy metabolism induced by DOX. The serum of all test groups was prepared. The levels

of lactic dehydrogenase (LDH) and creatine kinase (CK) were measured by ultraviolet spectrophotometric method according to the instruction manual of the reagent (28). Then, the cardiac tissue of all test groups was prepared. The ATPase activities were measured by ultraviolet spectrophotometric method according to the instruction manual of the reagent (29).

Effect of CSE on oxidative damage induced by DOX. The cardiac tissues of all test groups were prepared. The level of malondialdehyde (MDA) was determinated by ultraviolet spectrophotometric method according to the instruction manual of the reagent (30). Secondly, determination of intracellular ROS levels was performed by measuring a fluorescent product formed by DCFHDA (10 μmol/l). The samples were incubated in an incubator at 37˚C for 40 min, and then washed with phosphate-buffered saline (PBS) three times. The cells were made into a suspension using 300 ml PBS and put through a 200 mesh screen. The relative amount of fluorescent product was monitored by FCM at 48 and 535 nm (31). Finally, antioxidation enzyme indicators in the serum were measured. The mice were sacrificed under the influence of anesthesia. The heart was excised immediately, rinsed in ice-cold normal saline, blotted between two filter papers, and weighed. Heart tissue (0.5 g) was triturated and dropped into 10% tissue homogenate according to the volume and weight ratio. The cell debris was removed using centrifugation at 3,000 rpm for 15 min. The cell supernatants were collected, and then diluted with normal saline in proportion to the required concentration of tissue homogenate. The protein content of the cell lysates was determined using the Bradford assay. T-AOC of blood measurement, superoxide dismutase (SOD), glutathione (GSH) and catalase (CAT) of heart tissue homogenate assay were carried out according to the kit illustrations using ultraviolet spectrophotometer.

Effect of CSE on Δψm levels of cardiac cells induced by DOX. The purification of cardiac cells was carried out by counting with a hemocytometer. Cardiac cells (lx10^6) were placed in dimethyl sulfoxide (DMSO), and incubated in an incubator at 37˚C for 40 min with Rh123 for testing Δψm, and then washed with PBS three times. The 300 ml PBS suspension was filtrated through 200 mesh size screen. Cells (10^4) were collected and measured with FCM, excitation and maximum absorption at 488 and 530 nm (32).

Antitumor activity of CSQAH and its apoptosis induction on HepG2 cells

Cell culture. Human gastric carcinoma cell line SGC-7901 was cultured in RPMI-1640 medium with 10% calf serum at 37˚C in a 5% CO_2 incubator.

Measurement of Ca^{2+} by LSCM. HepG2 cells in a logarithmic growth phase were inoculated in 6-well plates. After 24 h, 1 ml of drugs was added to each hole. CSQAH was added at concentrations of 100, 200 and 400 μg/ml, respectively. The final concentration of the positive control group (HCPT) was 5 μg/ml. The control group received the same volume of RPMI-1640 culture media. After 48 h, the cell suspension was collected. The cells were washed with PBS and fixed with 4 μg/ml Fluo-3/AM 200 ml. Then, the cells were incubated for 1 h at 37˚C in the dark. The cells were washed one time and suspended with 400 μl PBS, and then the cells were detected with LSCM (33).

Analysis of Ca^{2+}-Mg^{2+}-ATP enzyme by adenosine triphosphatase assay kit. HepG2 cells in the logarithmic growth phase were inoculated in a 5-ml culture bottle. After incubating for 24 h, the cells were treated with 1 ml of drugs to each bottle (the doses same as above). After 24 h, the cells were digested with trypsin and washed with PBS two times. This operation is carried out according to the adenosine triphosphatase assay kit manual. Absorbance was measured with a microplate reader at 636 nm, zeroed with distilled water (34).

Detection of ROS using FCM. HepG2 cells in the logarithmic growth phase were inoculated in 6-well plates. After 24 h, 1 ml of the drugs was added to each hole (the doses same as above). After 48 h, the cells were collected and suspended in DCFH-DA (diluted 1:1,000 with RPMI-1640 without serum, the final concentration was 10 μmol/l). Then, the cells were incubated for 1 h at 37˚C and the cells were washed three times with RPMI-1640 without serum. After filtration with 300 mesh strainer, the cells were analyzed by FCM (35).

Analysis of Bcl-2 and Bax protein by FCM. HepG2 cells in the logarithmic growth phase were inoculated in a culture bottle. After incubating for 24 h, the cells were treated with 1 ml of drugs in each bottle (the doses same as above). After 24 h, the cells were collected and fixed with 40 g/l paraformaldehyde 2 ml for 40 min. Then, the cells were washed twice with PBS and treated with 0.1% Triton X-100 1 ml for 15 min, washed twice with PBS again, closed with 1% of BSA 1 ml for 1 h, and then centrifuged. The cells were treated with primary antibodies specific for Bcl-2 or Bax (diluted 1:200), respectively. Then, incubation was carried out for 1 h at 37˚C. The mixture was centrifuged and the supernatant was removed and washed with PBS, and then treated with the secondary antibodies (diluted 1:50). The cells were incubated at room temperature for 30 min in the dark and centrifuged. The supernatant was removed, while the cells were suspended in 800 μl of PBS. After being filtrated with 300 mesh strainer, the cells were analyzed by FCM (36-38).

Statistical analysis. Differences in proliferation between different groups were analyzed using one-way ANOVA. Statistical analysis was performed using SPSS 19.0 software. $P<0.01$ was considered to indicate a statistically significant difference. The results are expressed as mean ± SD.

Results

Antioxidant activity of CSE and its protective effect on oxidative damage induced by DOX

The antioxidant fraction of CSE. From the EPPH method, the most antioxidant activity of CSE was the ethyl acetate fraction shown in Table I.

Effect of CSE on injury and energy metabolism induced by DOX. The DOX group exhibited significantly an increased LDH value ($P<0.01$). Compared with the DOX group, CSE

Table I. Effect of the extracts on DPPH-free radical scavenging.

Extracts	50% Effective concentration	Antiradical efficiency/ (ml/mg)
Petroleum ether	0.99	1.98
Chloroform	1.06	2.13
Ethyl acetate	3.13	6.25
N-butyl alcohol	1.17	2.34
Water	1.51	3.02

The samples were assessed by the EPPH method. This method can be used for the quantitative analysis of samples (water, soil, sediment and sludge) for their antioxidant activities.

Table II. Effect of CSE on LDH and CK values in blood (n=10, mean ± SD).

Group	Dose (mg/kg)	LDH (U/l)	CK (U/ml)
Control	0	1,887.82±147.65	419.13±32.43
DOX	15	3,898.03±128.72[a]	809.04±34.33[a]
CSE-L	40	3,374.61±116.66[a,b]	574.65±30.75[a,b]
CSE-M	80	2,754.62±103.81[a,b]	496.80±22.95[a,b]
CSE-H	120	2,187.48±132.51[a,b]	504.30±28.91[a,b]

The serum of all test groups was measured by ultraviolet spectrophotometric method to obtain the values of LDH and CK. Compared with control, [a]P<0.01; compared with the DOX group [b]P<0.01. CSE, *Capparis spinosa* L.; LDH, lactic dehydrogenase; CK, creatine kinase.

Table III. Effect of CSE on ATP activities in cardiac tissue (n=10) mean ± SD.

Group	Dose (mg/kg)	Na^+K^+-ATPase (μmolPi/mgprot/h)	Ca^{2+}-TPase (μmolPi/mgprot/h)
Control	0	6.92±0.51	5.66±0.39
DOX	15	5.88±0.60[a]	4.68±0.52[a]
CSE-L	40	6.25±0.63[a]	5.12±0.42[a,b]
CSE-M	80	6.41±0.46[b]	5.38±0.44[c]
CSE-H	120	6.49±0.63[b]	5.49±0.41[c]

The ATPase activities in the cardiac tissue were measured by ultraviolet spectrophotometric method according to the instruction manual of reagent. Compared with control group, [a]P<0.01; compared with DOX group [b]P<0.05, [c]P<0.01. CSE, *Capparis spinosa* L.; DOX, doxorubicin.

Table IV. Effect of CSE on MDA levels in cardiac tissue (n=10, mean ± SD).

Group	Dose (mg/kg)	MDA (nmol/mgprot)
Control	0	4.82±0.19
DOX	15	9.19±0.37[a]
CSE-L	40	7.34±0.50[a,b]
CSE-M	80	6.67±0.20[a,b]
CSE-H	120	6.10±0.23[a,b]

The level of MDA in the cardiac tissues was determined by ultraviolet spectrophotometric method according to the instruction manual of the reagent. Compared with the control group, [a]P<0.01; compared with the DOX group, [b]P<0.01. CSE, *Capparis spinosa* L.; MDA, malondialdehyde; DOX, doxorubicin.

groups exhibited decreased levels of LDH (P<0.01). Compared with the normal group, the CSE groups had increased levels of LDH (P<0.01) (Table II). The DOX group exhibited a significantly increased CK value compared with the normal group (P<0.01). Compared with the DOX group, the CSE groups exhibited decreased levels of CK (P<0.01). The CSE groups exhibited increased levels of CK, compared with the normal group (P<0.01).

The DOX group exhibited decreased levels of Na^+K^+-ATPase and Ca^{2+}-ATPase compared with the normal group (P<0.01). The CSE groups showed an increase in the level of Na^+K^+-ATPase when compared with the DOX alone treated mice. No significant difference in Na^+K^+-ATPase was observed between the CSE-L group and the DOX group (P>0.05), while the levels of Na^+K^+-ATPase in the CSE-M and CSE-M groups were significantly increased compared with the DOX group (P<0.05). The CSE groups showed an increase in the level of Ca^{2+}-ATPase when compared with the DOX group. A significant difference was observed in the CSE-L group when compared to the DOX group (P<0.05), and significant differences were also observed in the CSE-M and CSE-H groups (P<0.01). The level of Ca^{2+}-ATPase in the CSE-L group was significantly lower compared with the normal group (P<0.01). No significant difference was observed in CSE-M and CSE-H groups when compared with the normal group (P>0.01; Table III).

Effect of CSE on oxidative damage induced by DOX. MDA levels in the cardiac tissues showed that the MDA level in the DOX group was increased, compared with the normal group. There was a significant differences(P<0.01). The CSE groups showed a significant decrease in the level of MDA (P<0.01) when compared with the DOX alone treated mice. The levels of MDA in the CSE groups were significantly increased compared with the normal group (P<0.01; Table IV).

The results showed that the DOX group, compared with the normal group, exhibited decreased levels of ROS. The CSE groups showed a decrease in the levels of ROS when compared with the DOX group. The CSE groups showed an increase in the level of ROS when compared with the normal group (Fig. 1).

In these tests, compared with normal group, the DOX group exhibited a significantly decreased T-AOC value (P<0.01). CSE groups, compared with the DOX group, exhibited significantly increased T-AOC values (P<0.01). T-AOC values in the CSE groups were significantly decreased compared with

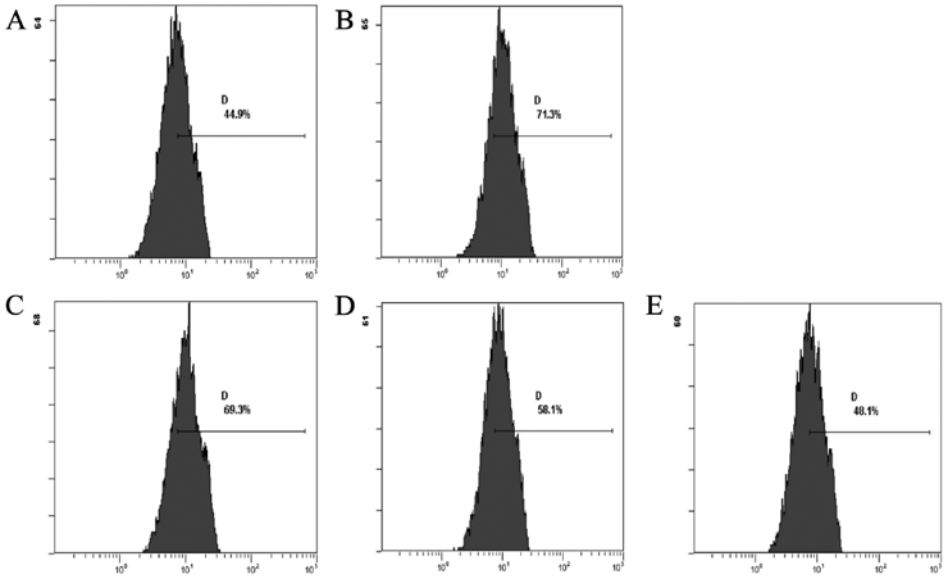

Figure 1. Effect of CSE on ROS levels in cardiac cells. HepG2 cells were inoculated with CSE for 48 h. The ROS levels were analyzed by flow cytometry. (A) Control. (B) Positive group, DOX, 15 mg/kg. (C) Low-dose group of CSE, 40 mg/kg. (D) Middle-dose group of CSE, 80 mg/kg. (E) High-dose group of CSE, 120 mg/kg.

Table V. Effect of CSE on T-AOC, SOD, CAT and GSH-Px (n=10, mean ± SD).

Group	Dose (mg/kg)	T-AOC	SOD (U/mg prot)	CAT (U/mg prot)	GSH-Px (mg/g prot)
Control	0	294.17±10.97	181.20±13.88	381.64±23.46	11.88±1.26
DOX	15	200.75±6.67[b]	162.20±14.54[b]	317.71±23.38[b]	8.31±0.99[b]
CSE-L	40	239.56±7.91[b,d]	176.91±11.96[c]	351.31±28.66[b,d]	9.58±0.62[b,d]
CSE-M	80	253.31±6.46[b,d]	178.53±13.51[c]	360.67±24.55[d]	10.16±0.63[b,d]
CSE-H	120	250.60±6.84[b,d]	166.25±13.62[c,a]	369.82±23.58[d]	9.50±0.68[b,d]

T-AOC of blood measurement, SOD, GSH and CAT of heart tissue homogenate assay were determined by ultraviolet spectrophotometer. Compared with the control, [b]P<0.01; compared with DOX group, [d]P<0.01. CSE, *Capparis spinosa* L.; T-AOC, total antioxidation ability; SOD, superoxide dismutase; CAT, catalase; GSH, glutathione; GSH-Px, GSH-peroxidase.

the normal group (P<0.01). Mice treated with DOX alone showed a significant (P<0.01) decrease in the activity of SOD in the heart as compared to the normal control mice. The CSE groups showed a significant (P<0.05) increase in the activity of SOD when compared with the DOX alone treated mouse. The SOD level in the CSE-H group was significantly decreased compared with the normal group (P<0.05). No significant difference was observed in the CSE-L and CSE-M groups when compared with the normal group (P>0.05). GSH levels in the cardiac tissues showed that the DOX group, compared with the normal group, exhibited a decreased level of GSH. There was a significant difference (P<0.01). The CSE groups showed a significant (P<0.01) increase in the levels of GSH when compared with the DOX alone treated mouse. The CSE groups compared with normal group had a significant difference (P<0.01). Mice treated with DOX alone showed a significant (P<0.01) decrease in the activity of CAT in the heart as compared to the normal control mice. CSE groups showed a significant (P<0.01) increase in the activity of CAT when compared with the DOX alone treated mouse. The CSE-L group showed a significant difference when compared with the normal group (P<0.01). No significant differences were observed in the CSE-M and CSE-H groups when compared with the normal group (P>0.05; Table V).

Effect of CSE on the Δψm levels in cardiac cells induced by DOX. The results showed that the DOX group, compared with the normal group, exhibited a decreased level of Δψm. The CSE groups showed an increase in the levels of Δψm when compared with the DOX group. The CSE groups showed an increase in the level of Δψm when compared with the normal group (Fig. 2).

Antitumor activity of CSQAH and its apoptosis induction on HepG2 cells

Observation of CSQAH-induced change in [Ca^{2+}] in the HepG2 cells. The brightness of the green fluorescence was greater with increasing concentrations of CSQAH. Different concentrations of CSQAH increased the concentration of Ca^{2+} in the cytoplasm in a dosage-dependent manner. HepG2 cells exhibited an increase in Ca^{2+} levels compared with the blank control group (P<0.01). As shown in Table VI and Fig. 3, the

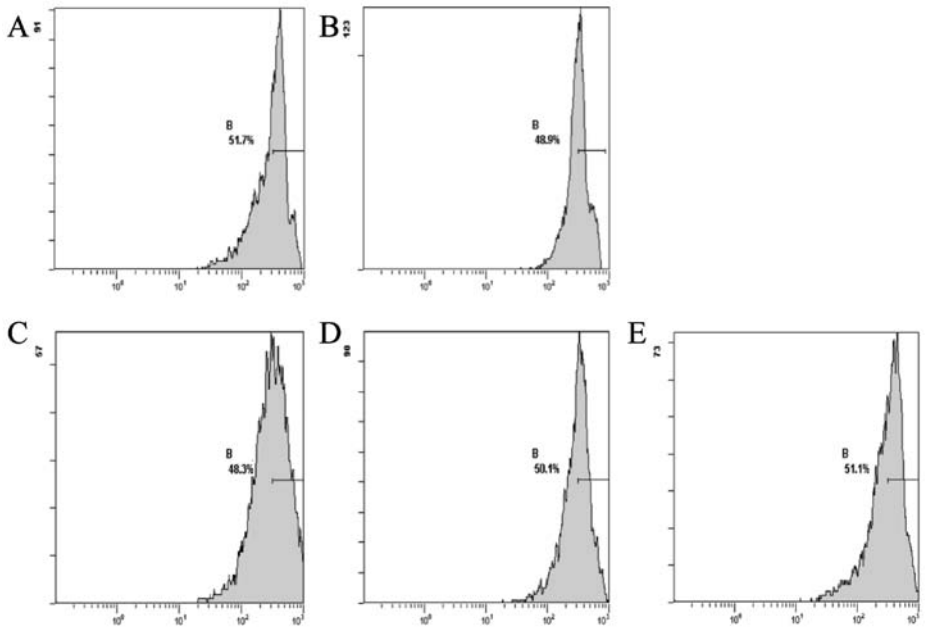

Figure 2. Effect of CSE on Δψm levels in cardiac cells. The cardiac cells of each group were purified and dyed by Rh123. The fluorescence intensity of each group was measured and analyzed by flow cytometry. (A) Control. (B) Positive group, DOX, 15 mg/kg. (C) Low-dose group of CSE, 40 mg/kg. (D) Middle dose group of CSE, 80 mg/kg. (E) High-dose group of CSE, 120 mg/kg.

Table VI. Effect of CSQAH on variation of $[Ca^{2+}]$ in the HepG-2 cells.

Group	Dose (μg/ml)	FI of $[Ca^{2+}]$
Control	0	39.31±2.16
HCPT	5	49.84±2.89[a]
CSQAH-L	100	60.37±3.91[b]
CSQAH-M	200	79.84±5.54[b]
CSQAH-H	400	108.52±5.69[b]

HepG2 cells were inoculated with 100, 200 and 400 μg/ml CSQAH for 24 h. Then, the cells were dyed using Fluo-3/AM and detected with a laser scanning confocal microscope. FI of $[Ca^{2+}]$ represents the fluorescence intensity of 50 cells for each figure. Compared with the control group, [a]P<0.05, [b]P<0.01. CSQAH, quaternary ammonium hydroxide of *Capparis spinosa* L.; HCPT, positive control group.

Table VII. Effect of CSQAH on activity of Ca^{2+}-Mg^{2+}-ATPase.

Group	Dose (μg/ml)	Ca^{2+}-Mg^{2+}-ATPase
Control	0	0.104±0.020
HCPT	5	0.067±0.024[b]
CSQAH-L	100	0.079±0.003[a]
CSQAH-M	200	0.065±0.004[b]
CSQAH-H	400	0.037±0.002[b]

After incubation with 100, 200 and 400 μg/ml CSQAH for 24 h, the activity of Ca^{2+}-Mg^{2+}-ATPase in the cells was measured with a microplate reader at 636 nm. Compared with the control group, [a]P<0.05, [b]P<0.01. CSQAH, quaternary ammonium hydroxide of *Capparis spinosa* L.; HCPT, positive control group.

result showed that the concentration of Ca^{2+} increased with the increase in the concentration of CSQAH. After 48 h, the green fluorescence of HCPT was significantly enhanced.

Effect of CSQAH on activity of Ca^{2+}-Mg^{2+}-ATPase. With the increasing concentration of CSQAH, the optical density (OD) was decreased. Thus, the Ca^{2+}-Mg^{2+}-ATPase enzyme activity in the HepG2 cells was decreased. The result is shown in Table VII. Each increasing concentration of CSQAH decreased the activity of Ca^{2+}-Mg^{2+}-ATPase enzyme. When the CSQAH groups were compared with the control group, there was a significantly difference (P<0.05 and P<0.01). The Ca^{2+}-Mg^{2+}-ATPase enzyme activity of the HCPT group was lower than that noted in the control group (P<0.01).

Effect of CSQAH on the level of ROS. Flow cytometric analysis showed that CSQAH increased the levels of ROS in te HepG2 cells. The production of ROS was decreased in a dose-dependent manner (Fig. 4).

Effect of CSQAH on the expression levels of Bcl-2 and Bax. When HepG2 cells were treated with CSQAH for 48 h, Bcl-2 and Bax were detected by FCM. The results are shown in Figs. 5 and 6. Significant changes in Bax and Bcl-2 expression (an increase in Bax and a decrease in Bcl-2) were observed in the CSQAH-treated HepG2 cells in a dose-dependent manner.

Discussion

Antioxidant activity of CSE and its protective effect on oxidative damage induced by DOX. After determination of the

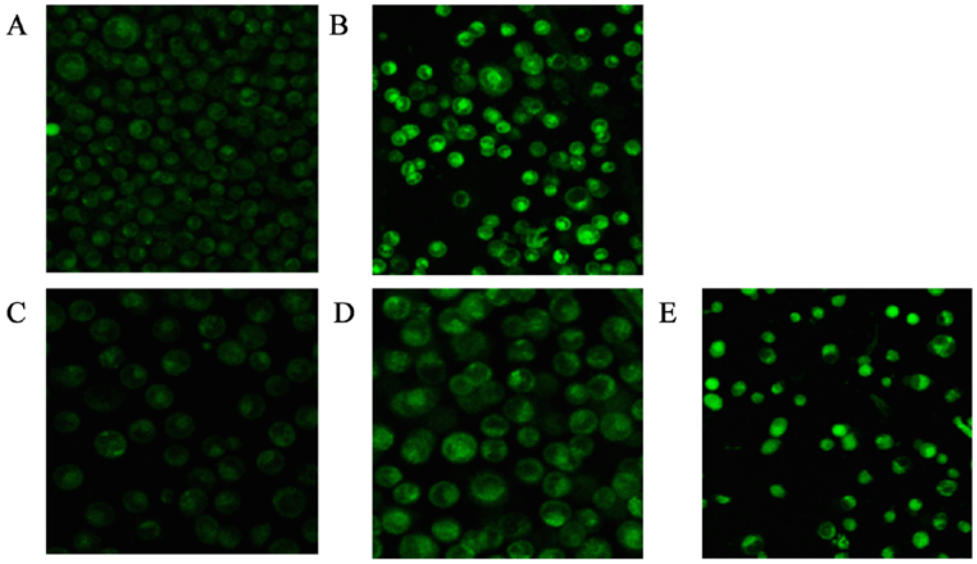

Figure 3. Fluorescence intensity of [Ca^{2+}] in the HepG2 cells following treatment with CSQAH. After HepG2 cells were inoculated with CSQAH for 24 h, the cells were dyed using Fluo-3/AM and [Ca^{2+}] was detected with a laser scanning confocal microscope. (A) HepG2 cells treated with RPMI-1640. (B) HepG2 cells treated with 5 μg/ml HCPT. (C) HepG2 cells treated with 100 μg/ml CSQAH. (D) HepG2 cells treated with 200 μg/ml CSQAH. (E) HepG2 cells treated with 400 μg/ml CSQAH. CSQAH, quaternary ammonium hydroxide of *Capparis spinosa* L.

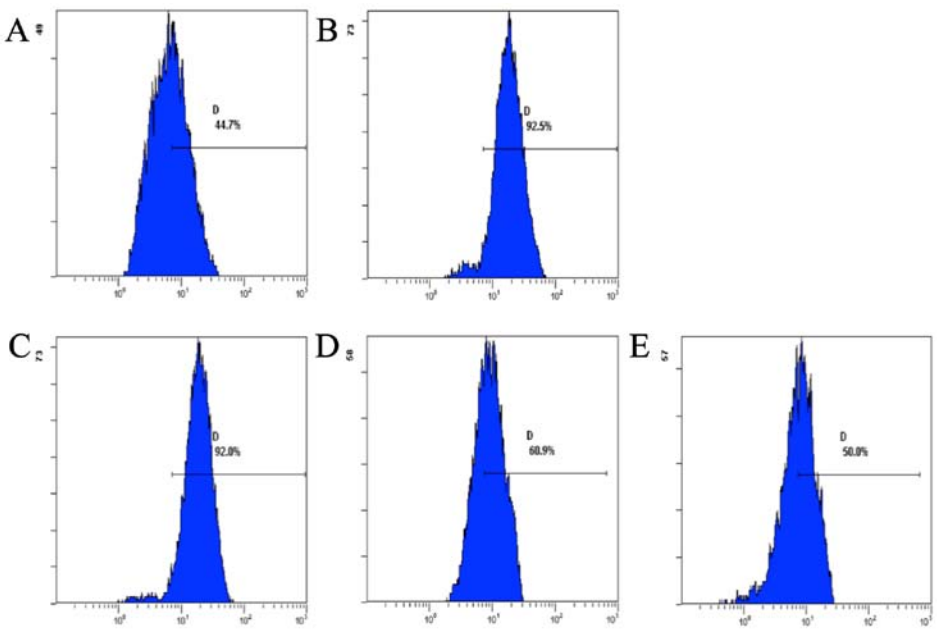

Figure 4. Effect of CSQAH on ROS in the HepG2 cells. HepG2 cells were inoculated with CSQAH for 48 h. The cells were suspended in DCFH-DA and analyzed by flow cytometry. (A) HepG2 cells treated with RPMI-1640. (B) HepG2 cells treated with 5 μg/ml HCPT. (C) HepG2 cells treated with 100 μg/ml CSQAH. (D) HepG2 cells treated with 200 μg/ml CSQAH. (E) HepG2 cells treated with 400 μg/ml CSQAH. CSQAH, quaternary ammonium hydroxide of *Capparis spinosa* L.

activity of lactic dehydrogenase (LDH) and creatine kinase (CK) in serum, the doxorubicin (DOX) group exhibited significantly increased LDH and CK values compared with the normal group (P<0.01). In the *Capparis spinosa* L. (CSE) groups, the level of LDH and CK were lower than the levels in the DOX group (P<0.01). These results indicated that DOX decreased the level of myocardial injury (LDH and CK); thus, CSE protected against DOX-induced myocardial injury. Previous pathology experimental observation found that DOX causes serious tissue and cell damage. After tissues and cells are damaged, enzymes are released into the bloodstream (39,40). DOX can alter the norms of blood biochemistry, and CSE can significantly inhibit DOX-induced increases in serum activity of LDH and CK. According to the results of the present study, it can be concluded that CSE protects myocardial tissue and cells in mice.

The damage of free radicals can reduce the activity of Na^+K^+-ATpase and Ca^{2+}-ATPase leading to abnormal energy metabolism. The activity of Na^+K^+-ATPase and Ca^{2+}-ATPase was reduced in the DOX group, while DOX initiated

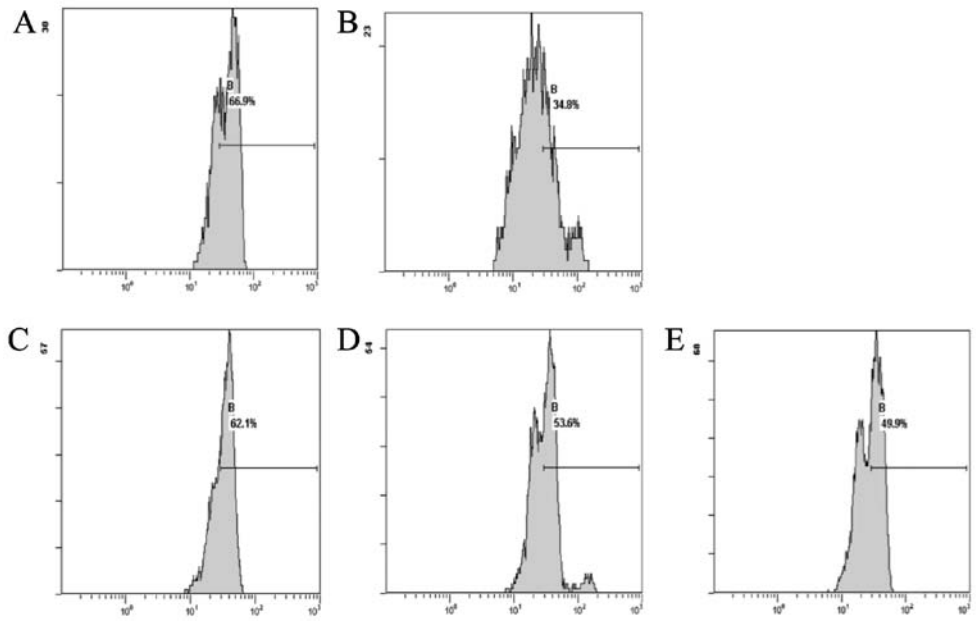

Figure 5. Effect of CSQAH on Bcl-2 expression in HepG2 cells. HepG2 cells were treated with CSQAH for 24 h. Then, the cells were inoculated with primary antibodies specific for Bcl-2 and epipolic secondary antibodies, respectively. The fluorescence intensity of each group was analyzed by flow cytometry. (A) HepG2 cells treated with RPMI-1640. (B) HepG2 cells treated with 5 μg/ml HCPT. (C) HepG2 cells treated with 100 μg/ml CSQAH. (D) HepG2 cells treated with 200 μg/ml CSQAH. (E) HepG-2 cells treated with 400 μg/ml CSQAH. CSQAH, quaternary ammonium hydroxide of *Capparis spinosa* L.

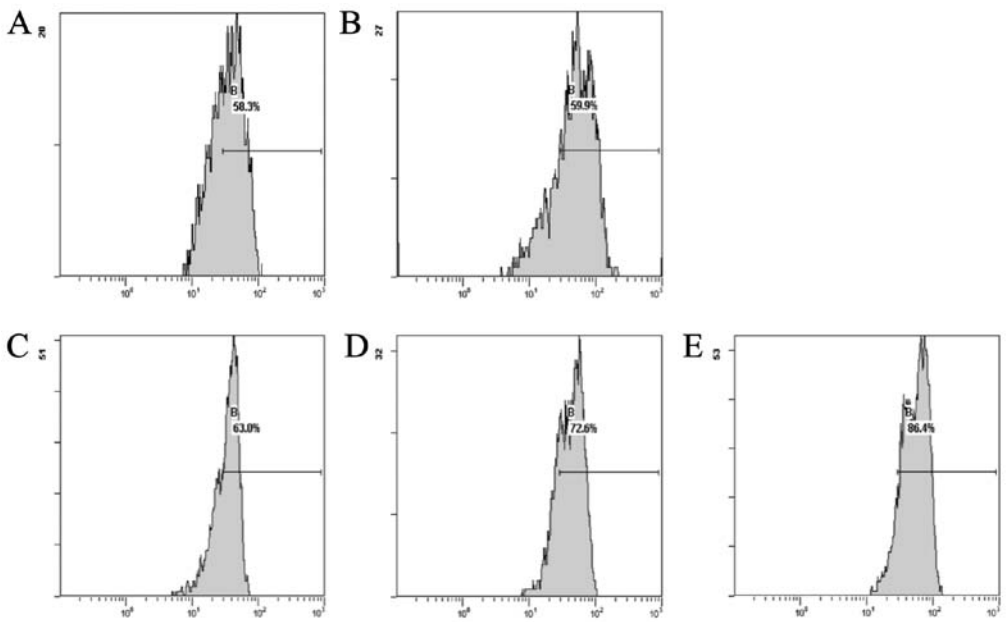

Figure 6. Effect of CSQAH on Bax expression in HepG2 cells. After HepG2 cells were inoculated with CSQAH for 24 h, the primary antibodies specific for Bcl-2 were added. Then the cells were washed and epipolic secondary antibodies were mixed. The fluorescence intensity, indicating the expression level of Bax, was analyzed by flow cytometry. (A) HepG2 cells treated with RPMI-1640. (B) HepG2 cells treated with 5 μg/ml HCPT. (C) HepG2 cells treated with 100 μg/ml CSQAH. (D) HepG2 cells treated with 200 μg/ml CSQAH. (E) HepG2 cells treated with 400 μg/ml CSQAH. CSQAH, quaternary ammonium hydroxide of *Capparis spinosa* L.

membrane lipid peroxidation (41). MDA is the metabolite of lipid peroxidation products, which could interfere with metabolic to cellular acidosis. An increase in Na^+-Ca^{2+} exchange, also accelerates the Ca^{2+} internal flow, then induces intracellular calcium overload. DOX can induce calcium overload, so the mitochondria need to consume a large amount of ATP to intake excessive-free Ca^{2+} to reduce the levels of ATP in the cell. Reduction of ATP also causes sarcoplasmic reticulum Ca^{2+}ATPase-power shortage, which reduces the ability to absorb Ca^{2+}. After further aggravation of calcium overload, a vicious circle may occur in the cell. CSE can increase the activity of ATPase and maintain the stability of the biofilm structure, protecting the biological membrane from oxidative damage. MDA is a sign of lipid peroxidation. MDA not only

reflects the degree of free radicals produced, but also reflects the degree of lipid peroxidation (42,43). The MDA levels in the CSE groups were significantly lower than the corresponding values in the DOX group, which indicated that CSE could significantly guard against lipid peroxidation. CSE has a protective effect on rat myocardial tissue.

In the present study, the level of intracellular ROS production was monitored by flow cytometry (FCM) (44,45). The results showed that the DOX group exhibited increased production of ROS compared with the normal group, whereas treatment with CSE significantly decreased the generation of ROS. These results suggested that CSE can balance ROS production and neutralization and inhibit DOX-induced intracellular ROS generation and protect cells from damage.

Through determination of the full blood total antioxidation ability (T-AOC), the DOX group exhibited significantly decreased T-AOC values ($P<0.01$). The CSE groups exhibited significantly increased T-AOC values compared with the DOX group ($P<0.01$). The results indicated that administration of CSE can improve body total antioxidation ability. Administration of DOX to rats significantly altered the cardiac activities of CAT, SOD and GSH-peroxidase (GSH-Px), which reflect the changes in free radicals in myocardial tissues. The mechanism of DOX-induced cardiotoxicity involves the generation of ROS (46,47). For increasing production of ROS by oxidative stress, the activity of the cardiac antioxidant enzyme SOD is significantly reduced. The main cellular damages caused by ROS included lipid peroxidation and MDA were formed in hearts. CSE has obvious ability to scavenge free radicals, which can be attributed to the enhancment of the activity of antioxidant enzymes (CAT, SOD and GSH-Px). DOX-induced cardiotoxicity caused the activity of cardiac antioxidant enzymes CAT, SOD and GSH-Px which were significantly reduced. Administration of CSE to the mice significantly increased the cardiac activities of CAT, SOD, and GSH-Px as compared to the DOX group, indicating the protective effect of CSE. The antioxidant effect of CSE has been shown to decrease DOX-induced cardiotoxicity as indicated by the ability to inhibit the production of free radicals, thus accelerating free radical consumption and reducing the consumption of antioxidant enzymes.

The formation of ROS which could harm membrane lipids, reduces the fluidity of membrane and increases the cell membrane permeability, causing $\Delta\psi m$ decrease (48-50). This could cause oxidative damage to proteins, resulting in protein denaturation and crosslink, and a change in enzyme activity (51). The level of $\Delta\psi m$ was monitored by FCM. The results showed that the DOX group exhibited a decreased level of $\Delta\psi m$ compared with the normal group. The CSE groups exhibited decreased levels of $\Delta\psi m$ compared with the DOX group. CSE prevented ROS-mediated peroxidative damage to the mitochondrial membrane and opened MPTP, and increased mitochondrial membrane fluidity.

Antitumor activity of CSQAH and its apoptosis induction in HepG2 cells. Ca^{2+} plays a pivotal role in the physiology and biochemistry of organisms and cells. They play an important role in signal transduction pathways, where they act as a secondary messenger. Ca^{2+} make their entrance into the cytoplasm either from outside the cell through the cell membrane via calcium channels or from some internal calcium storages. Ca^{2+} could damage cells when they enter in excessive numbers. Excessive entry of calcium into a cell may damage it or even cause it to undergo apoptosis, or death by necrosis (52,53). In this experiment, the Ca^{2+}-specific molecular probe Fluo-3/AM was used to carry CSQAH at different concentrations to treat HepG2 cells, and LCSM was used to observe changes in $[Ca^{2+}]$ in the cells. The result of the research showed that the strength of green fluorescence increased with the increase in the concentration of CSQAH. The HepG2 cells exhibited increase Ca^{2+} levels compared with the control group, significantly ($P<0.01$). This shows that the concentration of Ca^{2+} may increase with the increase in the concentration of CSQAH. On the one hand, this suggests that CSQAH can effect the calcium channels in HepG2 cells, which in turn leads to the rise of the concentration of Ca^{2+} in the cell. On the other hand, it could be that CSQAH activates endoplasmic reticulum, which is due to the stimulation of a specific Ca^{2+} release (54,55). When the concentration of Ca^{2+} is increased, endogenous nuclease is activated, cutting the DNA chain, inducing apoptosis of cells. Thereby, we indicate that the mechanism of apoptosis which was induced by CSQAH is possibly related to Ca^{2+} release. The result of the microplate reader showed that the OD value was decreased with the increasing dose, which indicated that the amount of inorganic phosphorus declined. It also demonstrated that the activity of Ca^{2+}-Mg^{2+}-ATPase enzyme was indirectly decreased. Experimental data showed that each group of CSQAH could decrease the activity of Ca^{2+}-Mg^{2+}-ATPase enzyme. There was a significantly difference in the CSQAH groups compared with the control group ($P<0.05$). The decrease in Ca^{2+}-Mg^{2+}-ATPase enzyme activity confirmed that the increase in Ca^{2+} concentration was inevitable, while Ca^{2+} overload induced apoptosis.

ROS are chemically reactive molecules containing oxygen. Examples include oxygen ions and peroxides. ROS are formed as a natural byproduct of the normal metabolism of oxygen and have important roles in cell signaling and homeostasis. ROS are constantly generated and eliminated in the biological system and are required to drive regulatory pathways. Under normal physiologic conditions, cells control ROS levels by balancing the generation of ROS with their elimination by a scavenging system. However, under oxidative stress conditions, excessive ROS may damage cellular proteins, lipids and DNA, leading to fatal lesions in cell that contribute to carcinogenesis. Ultra-ROS could damage DNA, RNA, and proteins. This may result in significant damage to cell structures. Cumulatively, this is known as oxidative stress (56-58). From the results obtained, with the increase in CSQAH concentration, the level of ROS was increased when compared with the control group ($P<0.01$). This illustrates that CSQAH could increase the level of ROS in HepG2 cells. The result indicates the accumulation of ROS, which play an important role in the mitochondrial control of apoptosis induced by CSQAH.

Apoptosis regulator Bcl-2 is a family of evolutionarily related proteins. These proteins govern mitochondrial outer membrane permeabilization (MOMP) and could be either pro-apoptotic (Bax, BAD, Bak and and Bok) or anti-apoptotic (including Bcl-2, Bcl-xL and Bcl-w). There are a number of theories concerning how the Bcl-2 gene family exerts their pro-apoptotic or anti-apoptotic effect. An important theory

states that this is achieved by activation or inactivation of an inner mitochondrial permeability transition pore, which is involved in the regulation of matrix Ca^{2+}, pH and voltage. It is also believed that certain Bcl-2 family proteins induce or inhibit the release of Cyt-c into the cytosol, which activate caspase-9 and -3, leading to apoptosis (59,60). When HepG2 cells were treated with CSQAH for 48 h, FCM analysis showed that Bcl-2 expression levels in the CSQAH-treated groups were downregulated, while Bax expression levels were upregulated, and the effects were dosage-dependent. The result showed that CSQAH could downregulate anti-apoptotic proteins.

In conclusion, the present study revealed the antioxidant and antitumor activities of CSE. On the one hand, the ethyl acetate extract of CSE showed antioxidant activity using the DPPH method to determine free radical elimination ability. CSE had protective effects on cardiac toxicity of DOX, decreasing the activity of LDH and CK. CSE improved the ability of myocardial tissue to scavenge free radicals, inhibited the lipid peroxidation, recovered activity of antioxidant enzymes, adjusted the energy metabolism of myocardial tissue, inhibited the generation of a large number of ROS in the cells, raised the level of $\Delta\psi m$, and improved the metabolism of free radicals. CSE had protective effects on DOX-induced myocardial damage. Moreover, the quaternary ammonium hydroxide of *Capparis spinosa* L. (CSQAH) induced HepG2 cells apoptosis by increasing Ca^{2+} concentrations and ROS levels, decreasing the Ca^{2+}-Mg^{2+}-ATPase activity in HepG2 cells, and downregulating anti-apoptotic Bcl-2 expression while upregulating apoptotic Bax expression. In summary, the present study demonstrated the antioxidant and antitumor activities of CSE which may suppress tumor growth and alleviate the side-effects of DOX, which may facilitate tumor treatment in a dual manner.

Acknowledgements

The present study was supported in part by the Open Research Program for Key Laboratory of College of Heilongjiang Province (China) (CPAT-2012003), the Natural Science Project of Department of Education of Heilongjiang Province (China) (12541205), the Innovation Talents Project of Science and Technology of Harbin City (China) (2014RFQXJ154), the Doctoral Research Project of Harbin University of Commerce (12DL008), and the Graduate Students Innovative Research Project of Harbin University of Commerce (YJSCX2015-390HSD).

References

1. Germanò MP, De Pasquale R, D'Angelo V, Catania S, Silvari V and Costa C: Evaluation of extracts and isolated fraction from *Capparis spinosa* L. buds as an antioxidant source. J Agric Food Chem 50: 1168-1171, 2002.
2. Orphanos PI: Germination of caper (*Capparis spinosa* L.) seeds. J Hortic Sci 58: 267-270, 1983.
3. Higton RN and Akeroyd JR: Variation in *Capparis spinosa* L. in Europe. Bot J Linn Soc 106: 104-112, 1991.
4. Castro Ramos RD and Nosti Vega M: The caper (*Capparis spinosa* L.). Grasas Aceites 38: 183-186, 1987.
5. Aytaç Z and Kınaci Ceylan GA: Yield and some morphological characteristics of caper (*Capparis spinosa* L.) population cultivated at various slopes in Aegean ecological conditions. Pak J Bot 2: 591-596, 2009.
6. Kulisic-Bilusic T, Schmöller I, Schnäbele K, Siracusa L and Ruberto G: The anticancerogenic potential of essential oil and aqueous infusion from caper (*Capparis spinosa* L.). Food Chem 1: 261-267, 2012.
7. Tlili N, Khaldi A, Triki S and Munné-Bosch S: Phenolic compounds and vitamin antioxidants of caper (*Capparis spinosa*). Plant Foods Hum Nutr 65: 260-265, 2010.
8. Bonina F, Puglia C, Ventura D, Aquino R, Tortora S, Sacchi A, Saija A, Tomaino A, Pellegrino ML and de Caprariis P: In vitro antioxidant and in vivo photoprotective effects of a lyophilized extract of *Capparis spinosa* L buds. J Cosmet Sci 53: 321-335, 2002.
9. Siracusa L, Kulisic-Bilusic T, Politeo O, Krause I, Dejanovic B and Ruberto G: Phenolic composition and antioxidant activity of aqueous infusions from *Capparis spinosa* L. and *Crithmum maritimum* L. before and after submission to a two-step in vitro digestion model. J Agric Food Chem 59: 12453-12459, 2011.
10. Issac Abraham SV, Palani A, Ramaswamy BR, Shunmugiah KP and Arumugam VR: Antiquorum sensing and antibiofilm potential of *Capparis spinosa*. Arch Med Res 42: 658-668, 2011.
11. al-Said MS, Abdelsattar EA, Khalifa SI and el-Feraly FS: Isolation and identification of an anti-inflammatory principle from *Capparis spinosa*. Pharmazie 43: 640-641, 1988.
12. Boga C, Forlani L, Calienni R, Hindley T, Hochkoeppler A, Tozzi S and Zanna N: On the antibacterial activity of roots of *Capparis spinosa* L. Nat Prod Res 25: 417-421, 2011.
13. Gadgoli C and Mishra SH: Antihepatotoxic activity of *p*-methoxy benzoic acid from *Capparis spinosa*. J Ethnopharmacol 66: 187-192, 1999.
14. Huseini HF, Hasani-Rnjbar S, Nayebi N, Heshmat R, Sigaroodi FK, Ahvazi M, Alaei BA and Kianbakht S: *Capparis spinosa* L. (Caper) fruit extract in treatment of type 2 diabetic patients: A randomized double-blind placebo-controlled clinical trial. Complement Ther Med 21: 447-452, 2013.
15. Lam SK and Ng TB: A protein with antiproliferative, antifungal and HIV-1 reverse transcriptase inhibitory activities from caper (*Capparis spinosa*) seeds. Phytomedicine 16: 444-450, 2009.
16. Eddouks M, Lemhadri A and Michel JB: Hypolipidemic activity of aqueous extract of *Capparis spinosa* L. in normal and diabetic rats. J Ethnopharmacol 98: 345-350, 2005.
17. Ali-Shtayeh MS and Abu Ghdeib SI: Antifungal activity of plant extracts against dermatophytes. Mycoses 42: 665-672, 1999.
18. Zhou HF, Xie C, Jian R, Kang J, Li Y, Zhuang CL, Yang F, Zhang LL, Lai L, Wu T, *et al*: Biflavonoids from caper (*Capparis spinosa* L.) fruits and their effects in inhibiting NF-kappa B activation. J Agric Food Chem 59: 3060-3065, 2011.
19. Ziyyat A, Legssyer A, Mekhfi H, Dassouli A, Serhrouchni M and Benjelloun W: Phytotherapy of hypertension and diabetes in oriental Morocco. J Ethnopharmacol 58: 45-54, 1997.
20. Houghton PJ, Hylands PJ, Mensah AY, Hensel A and Deters AM: In vitro tests and ethnopharmacological investigations: Wound healing as an example. J Ethnopharmacol 100: 100-107, 2005.
21. Wu JB, Ye SF, Liang CL, Li YC, Yu YJ, Lai JM, Lin H, Zheng J and Zhou JY: Qi-Dan Fang ameliorates adriamycin-induced nephrotic syndrome rat model by enhancing renal function and inhibiting podocyte injury. J Ethnopharmacol 151: 1124-1132, 2014.
22. Unverferth DV, Magorien RD, Leier CV and Balcerzak SP: Doxorubicin cardiotoxicity. Cancer Treat Rev 9: 149-164, 1982.
23. Alexander J, Dainiak N, Berger HJ, Goldman L, Johnstone D, Reduto L, Duffy T, Schwartz P, Gottschalk A, Zaret BL, *et al*: Serial assessment of doxorubicin cardiotoxicity with quantitative radionuclide angiocardiography. N Engl J Med 300: 278-283, 1979.
24. Legha SS, Benjamin RS, Mackay B, Ewer M, Wallace S, Valdivieso M, Rasmussen SL, Blumenschein GR and Freireich EJ: Reduction of doxorubicin cardiotoxicity by prolonged continuous intravenous infusion. Ann Intern Med 96: 133-139, 1982.
25. Arola OJ, Saraste A, Pulkki K, Kallajoki M, Parvinen M and Voipio-Pulkki LM: Acute doxorubicin cardiotoxicity involves cardiomyocyte apoptosis. Cancer Res 60: 1789-1792, 2000.
26. Ji YB and Yu L: N-butanol extract of *Capparis spinosa* L. induces apoptosis primarily through a mitochondrial pathway involving mPTP open, cytochrome C release and caspase activation. Asian Pac J Cancer Prev 15: 9153-9157, 2014.
27. Hossain MA and Rahma SMM: Total phenolics flavonoids and antioxidant activity of tropical fruit pineapple. Food Res Int 3: 672-676, 2011.

28. Wallimann T, Wyss M, Brdiczka D, Nicolay K and Eppenberger HM: Intracellular compartmentation, structure and function of creatine kinase isoenzymes in tissues with high and fluctuating energy demands: The 'phosphocreatine circuit' for cellular energy homeostasis. Biochem J 281: 21-40, 1992.
29. Obradovic M, Bjelogrlic P, Rizzo M, Katsiki N, Haidara M, Stewart AJ, Jovanovic A and Isenovic ER: Effects of obesity and estradiol on Na^+/K^+-ATPase and their relevance to cardiovascular diseases. J Endocrinol 218: R13-R23, 2013.
30. Pryor WA and Stanley JP: Letter: A suggested mechanism for the production of malonaldehyde during the autoxidation of polyunsaturated fatty acids. Nonenzymatic production of prostaglandin endoperoxides during autoxidation. J Org Chem 40: 3615-3617, 1975.
31. Bani S, Gautam M, Sheikh FA, Khan B, Satti NK, Suri KA, Qazi GN and Patwardhan B: Selective Th1 up-regulating activity of *Withania somnifera* aqueous extract in an experimental system using flow cytometry. J Ethnopharmacol 107: 107-115, 2006.
32. Jaiswal PK, Gupta J, Shahni S and Thakur IS: NADPH oxidase-mediated superoxide production by intermediary bacterial metabolites of dibenzofuran: A potential cause for trans-mitochondrial membrane potential ($\Delta\Psi m$) collapse in human hepatoma cells. Toxicol Sci 147: 17-27, 2015.
33. Hoffman A, Goetz M, Vieth M, Galle PR, Neurath MF and Kiesslich R: Confocal laser endomicroscopy: Technical status and current indications. Endoscopy 38: 1275-1283, 2006.
34. Fernando KC and Barritt GJ: Pinocytosis in 2,5-di-*tert*-butylhydroquinone-stimulated hepatocytes and evaluation of its role in Ca^{2+} inflow. Mol Cell Biochem 162: 23-29, 1996.
35. Devasagayam TPA, Tilak JC, Boloor KK, Sane KS, Ghaskadbi SS and Lele RD: Free radicals and antioxidants in human health: Current status and future prospects. J Assoc Physicians India 52: 794-804, 2004.
36. Lo K, Brinkman RR and Gottardo R: Automated gating of flow cytometry data via robust model-based clustering. Cytometry A 73: 321-332, 2008.
37. Julius MH, Masuda T and Herzenberg LA: Demonstration that antigen-binding cells are precursors of antibody-producing cells after purification with a fluorescence-activated cell sorter. Proc Natl Acad Sci USA 69: 1934-1938, 1972.
38. Basiji DA, Ortyn WE, Liang L, Venkatachalam V and Morrissey P: Cellular image analysis and imaging by flow cytometry. Clin Lab Med 27: 653-670, viii, 2007.
39. Schlattner U, Tokarska-Schlattner M and Wallimann T: Mitochondrial creatine kinase in human health and disease. Biochim Biophys Acta 1762: 164-180, 2006.
40. Vlkovicová J, Javorková V, Mézesová L, Pechánová O, Andriantsitohaina R and Vrbjar N: Dual effect of polyphenolic compounds on cardiac Na^+/K^+-ATPase during development and persistence of hypertension in rats. Can J Physiol Pharmacol 87: 1046-1054, 2009.
41. Ketzer LA, Arruda AP, Carvalho DP and de Meis L: Cardiac sarcoplasmic reticulum Ca^{2+}-ATPase: Heat production and phospholamban alterations promoted by cold exposure and thyroid hormone. Am J Physiol Heart Circ Physiol 297: H556-H563, 2009.
42. Del Rio D, Stewart AJ and Pellegrini N: A review of recent studies on malondialdehyde as toxic molecule and biological marker of oxidative stress. Nutr Metab Cardiovasc Dis 15: 316-328, 2005.
43. Marnett LJ: Lipid peroxidation-DNA damage by malondialdehyde. Mutat Res 424: 83-95, 1999.
44. Buranrat B and Connor JR: Cytoprotective effects of ferritin on doxorubicin-induced breast cancer cell death. Oncol Rep 34: 2790-2796, 2015.
45. Han D, Williams E and Cadenas E: Mitochondrial respiratory chain-dependent generation of superoxide anion and its release into the intermembrane space. Biochem J 353: 411-416, 2001.
46. Sheng B, Xu G, Chen D, Chen J, Gao W, Gao W, Li X, Yang J, Liu F, Gao Y, *et al*: TT-AOC, MDA, SOD, CAT and IL-6 levels in rat pulmonary edema induced by hypobaric hypoxia. J Third Mil Med Univ 23: 2364-2367, 2012.
47. Kirby AJ and Schmidt RJ: The antioxidant activity of Chinese herbs for eczema and of placebo herbs - I. J Ethnopharmacol 56: 103-108, 1997.
48. Szliszka E and Krol W: Soy isoflavones augment the effect of TRAIL-mediated apoptotic death in prostate cancer cells. Oncol Rep 26: 533-541, 2011.
49. Fernandez-Sanz C, Ruiz-Meana M, Castellano J, Miro-Casas E, Nuñez E, Inserte J, Vázquez J and Garcia-Dorado D: Altered FoF1 ATP synthase and susceptibility to mitochondrial permeability transition pore during ischaemia and reperfusion in aging cardiomyocytes. Thromb Haemost 113: 441-451, 2015.
50. Watanabe T, Saotome M, Nobuhara M, Sakamoto A, Urushida T, Katoh H, Satoh H, Funaki M and Hayashi H: Roles of mitochondrial fragmentation and reactive oxygen species in mitochondrial dysfunction and myocardial insulin resistance. Exp Cell Res 323: 314-325, 2014.
51. Gauthier LD, Greenstein JL, Cortassa S, O'Rourke B and Winslow RL: A computational model of reactive oxygen species and redox balance in cardiac mitochondria. Biophys J 105: 1045-1056, 2013.
52. Slinchenko NM: Effect of fluoroaluminate on the ATP-hydrolysing and Ca^{2+}-transporting activity of the purified Ca^{2+}, Mg^{2+}-ATPase of myometrium cell plasma membranes. Ukr Biokhim Zh 75: 95-98, 2003 (In Ukrainian).
53. Toledo-Maciel A, Gonçalves-Gomes S, de Gouveia Castex M and Vieyra A: Progressive inactivation of plasma membrane (Ca^{2+}+Mg^{2+})ATPase by Cd^{2+} in the absence of ATP and reversible inhibition during catalysis. Biochemistry 37: 15261-15265, 1998.
54. Monaco G, Decrock E, Arbel N, van Vliet AR, La Rovere RM, De Smedt H, Parys JB, Agostinis P, Leybaert L, Shoshan-Barmatz V, *et al*: The BH4 domain of anti-apoptotic Bcl-XL, but not that of the related Bcl-2, limits the voltage-dependent anion channel 1 (VDAC1)-mediated transfer of pro-apoptotic Ca^{2+} signals to mitochondria. J Biol Chem 290: 9150-9161, 2015.
55. Barrezueta LF, Oshima CT, Lima FO, De Oliveira Costa H, Gomes TS, Neto RA and De Franco MF: The intrinsic apoptotic signaling pathway in gastric adenocarcinomas of Brazilian patients: Immunoexpression of the Bcl-2 family (Bcl-2, Bcl-x, Bak, Bax, Bad) determined by tissue microarray analysis. Mol Med Rep 3: 261-267, 2010.
56. Rada B and Leto TL: Oxidative innate immune defenses by Nox/Duox family NADPH oxidases. Contrib Microbiol 15: 164-187, 2008.
57. Sumiyoshi H, Matsushita A, Nakamura Y, Matsuda Y, Ishiwata T, Naito Z and Uchida E: Suppression of STAT5b in pancreatic cancer cells leads to attenuated gemcitabine chemoresistance, adhesion and invasion. Oncol Rep 35: 3216-3226, 2016.
58. Park SY, Kim JY, Lee SM, Jun CH, Cho SB, Park CH, Joo YE, Kim HS, Choi SK and Rew JS: Capsaicin induces apoptosis and modulates MAPK signaling in human gastric cancer cells. Mol Med Rep 9: 499-502, 2014.
59. Muchmore SW, Sattler M, Liang H, Meadows RP, Harlan JE, Yoon HS, Nettesheim D, Chang BS, Thompson CB, Wong SL, *et al*: X-ray and NMR structure of human Bcl-x_L, an inhibitor of programmed cell death. Nature 381: 335-341, 1996.
60. Sucha L, Hroch M, Rezacova M, Rudolf E, Havelek R, Sispera L, Cmielova J, Kohlerova R, Bezrouk A and Tomsik P: The cytotoxic effect of α-tomatine in MCF-7 human adenocarcinoma breast cancer cells depends on its interaction with cholesterol in incubation media and does not involve apoptosis induction. Oncol Rep 30: 2593-2602, 2013.