Cytotoxicity effect of *Capparis spinosa* L. on the HepG2 human hepatocellular carcinoma cell line

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Hepatocellular carcinoma (HCC) is a complex condition associated with a poor prognosis. HCC is the third most common cause of cancer mortality which is continuously increasing. *Capparis spinosa* is used by traditional medical physicians in Iran to treat liver disorders and has been shown to prevent HCC. Treatment of HepG2 cells with *Capparis spinosa* ethanolic extract (CSE) inhibited cell proliferation in a dose dependent manner, and enhanced apoptosis of HepG2 cells by activation of caspase-8 and caspase-9 and down regulation of Bcl-2. Treatment of HepG2 cells with CSE also increased expression of the Cdk inhibitory protein (Cip1/p21). This study is a new design to investigate the *in vitro* effect of CSE on anti-HCC property.

**Keywords:** Hepatocellular carcinoma, *Capparis spinosa* L., Cell proliferation, Apoptosis, Cell cycle

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Hepatocellular carcinoma is a major health problem which is increasing in the world and it is the third highest cause of cancer-related death¹,². The incidence of HCC is expected to continue to increase until 2030, and it is cancer with the second highest increase in all death rates³.

Many plants produce have been used in the treatment of the most frequent diseases in that it has been estimated that about 50% of the approval drugs in developing countries are from natural produces⁴. High mortality of HCC, the problems encountered in their treatment and the toxic effects of cancer chemotherapy drugs motivate researchers to investigate the anti-HCC effects of safe herbs. The use of medicinal plant is one of the important approaches in hepatocellular carcinoma treatment and prevention.

*Capparis spinosa* L. (Capparidaceae) is a medicinal plant indigenous to central and West Asia. Various parts of this plant including its root bark, fruit, leaf, seeds and stem have been used as traditional medicine for several properties, such as anti-oxidant, anti-carcinogenic, hepatoprotective, diuretic, anti-rheumatism, anti-arthritis, anti-haemorrhoidal, anti-anemia, anti-mutagenic and anti-microbial effects⁵,⁶,⁷. *Capparis spinosa* is a very good source of glucosinolates (glucocapparin, glucoiberin and sinigrin), flavonoids (rutin, kaempferol), phenolic acids and alkaloids⁵,⁸.

Hydroalcoholic extract of *Capparis spinosa* root bark showed hepatoprotective effects against CCl₄ induced liver damage in mice⁹. In the last few years *Capparis spinosa* is known for its anti-cancer properties, and its cytotoxic effects has been studied in HepG2 hepatocellular carcinoma, HT-29 colon carcinoma, MCF-7 breast adenocarcinoma, Hep-2 larynx carcinoma and Hela cervix adenocarcinoma cell lines⁵,¹⁰,¹¹. Despite these Investigations, the mechanisms of *Capparis spinosa*-induced toxicity are still largely unclear.

However, there is lack of study on the anti-HCC effect of *Capparis spinosa*. In this study, we investigated the effect of CSE on reduction of proliferation, the induction of apoptosis and the arrest of cell cycle in HepG2 cell line.

**Methodology**

**Chemicals and reagents**

RPMI-1640 medium, L-glutamine, fetal bovine serum (FBS), penicillin-streptomycin and trypsin-EDTA were obtained from Gibco BRL (Grand Island, NY, USA). 3-(4, 5-Dimethylthiazol-2-yl)-2, 5-
diphenyltetrazolium bromide (MTT) were purchased from Sigma (St. Louis, MO, USA). The Caspase-8/9 colorimetric assay kits were bought from biovision (Milpitas, CA, USA). Bcl-2 and p21 colorimetric assay kits were obtained from Assay biotech (Sunnyvale, CA, USA)

Collection of plant material

*Capparis spinosa* was collected in July 2014 from Gonbad Qabus, province of Golestan, North-East of Iran and was dried at room temperature for 5 days.

Preparation of plant extract

The powder of different parts (root bark, fruits, leaves and stems) of *Capparis spinosa* (400 gm) was extracted three times with 80 % ethanol (1:10, w/v). The extract was then filtered through Whatman No. 4 paper, concentrated, lyophilized and stored at -20 °C until required. When required for experimental purposes, the freeze dried extract was reconstituted with methanol. The concentration of methanol used to dilute the lyophilized powder was 0.4%, a concentration that did not affect cell growth.

Cell culture

HepG2 human hepatocellular carcinoma cell line was purchased from Pasteur Institute Collection of Cell Cultures, Tehran, Iran. The cells were cultured in RPMI-1640 medium with 4.5 gm/l glucose, L-glutamine and 10% FBS in the presence of 100 U/ml penicillin and 0.1 gm/l streptomycin. Cells were incubated at 37 °C with 5% CO₂. The culture medium was replaced at least every two days for all experiments.

Cell proliferation assay

Cell proliferation inhibition was assessed with a 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) assay. The cells were plated in 96-well plates at a density of 1.3×10³ cells/well and incubated for 24 hrs. The cells were treated with different concentrations of CSE (150, 310, 620, 1250, 2500 and 5000 µg/mL) for 48 hrs. Then, cancer cells were incubated with fresh medium containing 1 gm MTT/L at 37 °C for 3-4 hrs. Then, almost all old medium containing MTT was gently replaced by 200 µL of DMSO and then pipetted to dissolve any formed formazan crystals. Finally, the microplates were incubated at room temperature for 30 min. The degree of formazan formation, an indicator of living and metabolically active cells, was measured by an ELISA reader at a wavelength of 570 nm.

Microscopic cell morphology

After cell treated with CSE at IC₅₀ for 48 hrs, cell morphological changes were examined under inverted microscope and micrographs were taken with camera attached (Nikon Eclipse TS100, Japan).

Caspase 8 and 9 activity assay

The cancer cells were treated with different doses of CSE for 48 hrs, then harvested and lysed with chilled cell lysis buffer and incubated on ice for 10 min. Cell lysates were clarified by centrifugation at 10,000 x gm for 1 min at 4 °C. The protein concentration in the supernatant was quantified, and 200 µg of protein was added to 50 µl of lysis buffer for each assay. 50 µl of 2X reaction buffer (containing 10 mM dithiothreitol, DTT) was added to each sample. Samples were incubated with 5 µl of the IETD-pNA (caspase-8) and LEHD-pNA (caspase-9) substrate for 2 hrs at 37 °C. Optical density reading was taken at a wavelength of 405 nm on a microplate reader.

Bcl-2 and p21 protein levels assay

The cells were treated with different concentration of the CSE for 48 hrs. The cells were fixed by 100 µl of Fixing Solution for 20 min at room temperature. To each sample, 100 µl Quenching Buffer was added and incubated for 20 min at room temperature. The cells were incubated with 200 µl of blocking buffer for 1 hr. To each sample, 50 µl of 1x Anti Bcl-2 antibody and anti- GAPDH antibody/ anti-p21 antibody and anti-GAPDH was added for Bcl-2 and p21, respectively. After incubating for 16 hrs at 4 °C, 50 µl of 1x HRP-conjugated anti- rabbit IgG antibody and HRP-conjugated anti-mouse IgG antibody was added and incubated for 1.5 hrs. Samples were mixed with 50 µl of Substrate and incubated for 30 min in the dark. Finally, 50 µl of stop solution was added and read OD at 450 nm immediately using the microplate reader.

Statistical analysis

All data were expressed as mean ± standard deviation. The statistical differences were analyzed using Student’s t-test. Values of *p*<0.05 were considered significant.

Results

Anti-Proliferative Activity

As shown in Fig. 1 the growth of HepG2 cells was significantly inhibited in a dose dependent manner. CSE treatment of HepG2 cells resulted in 31 and 61% decrease in cell viability at the doses of 310-1250 µg/mL at 48 hrs as compared with control.
The highest growth inhibitory effect of CSE was observed at 5000 µg/mL concentration and the low CSE concentration (150 µg/mL) did not show a significant cytotoxic effect on HepG2 cells. The concentration of Capparis spinosa on the inhibition of 50% of HepG2 cells viability (IC$_{50}$) was 621±3.94 µg/mL.

**Morphological changes**

HepG2 cells treated with CSE for 48 hrs (Fig. 2) showed hallmark features of apoptosis which were manifested as cell shrinkage with irregular shape, reduction of cell volume, plasma membrane blebbing, nuclear condensation and apoptotic bodies.

**Caspase 8 and 9 Activity**

To assess the potential involvement of caspases in HepG2 cell apoptosis, we detected the activity of caspase-8 and caspase-9 (Fig. 3). CSE treatment resulted in a significant increase in caspase-8/9 activity in a dose-dependent manner although increase in caspase-8 compared with caspase-9 activity was noted.

**Bcl-2 protein level**

We found out that the Bcl-2 protein level was reduced in HepG2 cells after 48 hrs of treatment with CSE compared with untreated cells (Fig. 4). However, the level of Bcl-2 protein decreased with the increase in CSE concentration, indicating that CSE could down regulate Bcl-2 protein level. These results demonstrated that the level of anti-apoptotic protein Bcl-2 was decreased by CSE in a dose-dependent manner.

**p21 protein level**

As shown in (Fig. 5), our results showed that p21 protein level was significantly increased in CSE treated groups comparing with the control group. In fact, the increase in p21 protein level seems to parallel the increase in CSE concentration. The highest level was observed in cells treated with 1250 µg/mL of CSE.
Discussion

*Capparis spinosa* is a medicinal plant which has been recommended in traditional medicine for the treatment of hepatic disorders. Ethanol root bark extract of *Capparis spinosa* showed significant dose-dependent protection against CCl4 induced hepatocellular injury. P-Methoxy benzoic acid isolated from the methanolic soluble fraction of the aqueous extract of *Capparis spinosa* was found to possess significant anti-hepatotoxic activity against CCl4 and paracetamol induced hepatotoxicity in vivo and thioacetamide and galactosamine induced hepatotoxicity in isolated rat hepatocytes, using in vitro technique. In this study we showed the potential use of *Capparis spinosa* as a source of anticancer drug. CSE tested for its cytotoxicity, apoptotic effect and cell cycle arrest against the HepG2 hepatocellular carcinoma cell line. The concentrations ranging from 310 µg/mL to 5000 µg/mL were effective in inducing cytotoxicity in the cancer cells and IC50 value was calculated 621±3.94 µg/mL.

*Capparis spinosa* exhibit potent anti-cancer activity in several types of tumor cells, including human colon carcinoma, human larynx epidermoid carcinoma and human cervix adenocarcinoma. Studies on an isolated protein from *Capparis spinosa* showed potent cytotoxicity against HepG2 cell line in a dose dependant manner. The anti-proliferative potency of the isolated protein toward HepG2 cells seems to be relatively high while the potency toward HT29 and MCF-7 and cells is lower. Our findings were consistent with this data which demonstrated a similar effect of *Capparis spinosa* on HepG2 HepG2 hepatocellular carcinoma cell line.

In order to understand the mechanism of the CSE on HCC, we investigated the effect of CSE on the induction apoptosis and the arrest of cell cycle progression in HepG2 cell line. Apoptosis is a normal physiological process that plays an important role in homeostasis and growth of the normal and cancer cells. Dysregulation of apoptosis is usually considered as a major cancer property. In our study, the morphologies of CSE treated cells revealed the apoptosis. The mechanism by which CSE induces apoptosis in HepG2 cells is not understood. For this reason, in this study we demonstrated effect of CSE on caspase 8/9 activity and Bcl-2 protein level. Previous studies have showed that the caspase family proteases play important roles in the process of apoptosis. Caspase 8 initiates disassembly in response to extracellular apoptotic ligands and caspase 9 activates disassembly in response to agents or insults that trigger the release of cytochrome c from mitochondria. The caspase-8/9 activity was increased significantly in treated HepG2 cells as compared to control and these results demonstrated that the activation of caspase-8/9 is involved in CSE-induced cell apoptosis.

Apoptosis is also tightly regulated by anti-apoptotic effector molecules such as Bcl-2. Over expression of Bcl-2 protein has been reported in many types of cancers, including hepatocellular carcinoma, leukemia and lymphomas. Additionally, our results also show that Bcl-2 protein level is significantly decreased in CSE-induced cell. This result is similar to previous studies that indicated that the level of Bcl-2 expression decreases with apoptosis in HepG2 cells. In general, according to the increased activity of caspase 8/9, and reduced levels of Bcl-2 could be one of the important ways in CSE action against HepG2 cell that considered apoptosis and proved our hypothesis about the role of CSE in the induction of apoptosis in these cells. According to results of this study it seems that CSE induced apoptosis in HepG2 cells either in intrinsic and extrinsic pathways.

The cell division relies on the activation of cyclins, which bind to cyclin-dependent kinases (CDKs) to induce cell cycle progression towards S phase and later to initiate mitosis. Uncontrolled cyclin-dependent kinase activity is often the cause of human cancer and their function is regulated by cell-cycle inhibitors such as the p21 Cip/Kip protein. In our study the cell cycle regulatory protein p21 (Cip1/p21) were assessed to evaluate the effect of CSE on cell cycle regulation. Analysis of the Cip1/p21 protein levels in HepG2 cells indicated that CSE in a dose-dependent manner enhancement of Cip1/p21 protein level. Different roles have been considered for p21 which the most obvious role is in arresting cell cycle. Different roles such as induction apoptosis and prevention of cell proliferation have been confirmed in several studies. In this study, we noted its role in relation to arresting cell cycle but it’s not a reason for denying other roles of p21 that induced by CSE in HepG2 cells.

Conclusion

In conclusion, we demonstrated that CSE inhibited the proliferation of HepG2 hepatocellular carcinoma. CSE also induced cell apoptosis by up regulation of
caspase8/9 activity and down regulation of Bcl-2 level which are involved in extrinsic and intrinsic pathway of apoptosis. Furthermore, CSE induced cell cycle arrest by Cip1/p21 expression. Further investigations are currently underway to investigate in more detail the mechanism of action of Capparis spinosa.

References