

BASIC RESEARCH

Hot water extract of *Chlorella vulgaris* induced DNA damage and apoptosis

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OBJECTIVES: The aim of this study was to determine the antiproliferative and apoptotic effects of hot water extracts of *Chlorella vulgaris* on hepatoma cell line HepG2.

INTRODUCTION: The search for food and spices that can induce apoptosis in cancer cells has been a major study interest in the last decade. *Chlorella vulgaris*, a unicellular green algae, has been reported to have antioxidant and anti-cancer properties. However, its chemopreventive effects in inhibiting the growth of cancer cells have not been studied in great detail.

METHODS: HepG2 liver cancer cells and WRL68 normal liver cells were treated with various concentrations (0-4 mg/ml) of hot water extract of *C. vulgaris* after 24 hours incubation. Apoptosis rate was evaluated by TUNEL assay while DNA damage was assessed by Comet assay. Apoptosis proteins were evaluated by Western blot analysis.

RESULTS: *Chlorella vulgaris* decreased the number of viable HepG2 cells in a dose dependent manner ($p < 0.05$), with an IC_{50} of 1.6 mg/ml. DNA damage as measured by Comet assay was increased in HepG2 cells at all concentrations of *Chlorella vulgaris* tested. Evaluation of apoptosis by TUNEL assay showed that *Chlorella vulgaris* induced a higher apoptotic rate (70%) in HepG2 cells compared to normal liver cells, WRL68 (15%). Western blot analysis showed increased expression of pro-apoptotic proteins P53, Bax and caspase-3 in the HepG2 cells compared to normal liver cells WRL68, and decreased expression of the anti-apoptotic protein Bcl-2.

CONCLUSIONS: *Chlorella vulgaris* may have anti-cancer effects by inducing apoptosis signaling cascades via an increased expression of P53, Bax and caspase-3 proteins and through a reduction of Bcl-2 protein, which subsequently lead to increased DNA damage and apoptosis.

KEYWORDS: HepG2; *Chlorella vulgaris*; DNA damage; chemopreventive; apoptosis.

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INTRODUCTION

Hepatocellular carcinoma (HCC) is the fifth most common malignancy worldwide.¹ In Malaysia, HCC is the thirteenth most common cancer affecting the population.²

The etiology of liver cancer is multi factorial; some of the well known risk factors include hepatitis B and C viral infection, exposure to chemicals such as aromatic hydrocarbons, and the ingestion of aflatoxin B1.³⁻⁵

The process of carcinogenesis involves an initiating event which induces genetic damage, followed by survival and

progression of selected clones of the transformed cells to form tumors. Apoptosis, a form of programmed cell death, has been associated with delaying or inhibiting cancer growth.^{6,7} Much of cancer research over the past two decades has focused on genes that, when mutated, act in either a dominant or recessive manner to enhance proliferation with dysregulation of apoptosis that is responsible for initiating cancer and its progression.^{8,9} Many emerging data in recent years have shown that dietary chemopreventive agents preferentially inhibit growth of cancer cells by targeting signaling molecules, such as caspases, that subsequently lead to the induction of apoptosis.⁷ Some of these dietary agents include epigallocatechin-3-gallate (EGCG), found in green tea,¹⁰ curcumin in turmeric,¹¹ genistein in soybeans,¹² lycopene in tomatoes,¹³ anthocyanins in pomegranates¹⁴ and isothiocyanates in broccoli.¹⁵

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There is a large body of evidence that links DNA damage and apoptosis.^{16,17} The tumor suppressor protein p53 provides an important link between DNA damage and apoptosis as it has been shown to mediate the upregulation of apoptotic proteins such as Bax, caspase-3 and -8, Noxa, PUMA and p53AIP.¹⁸

Chlorella vulgaris, a unicellular green algae, has been widely used as a food supplement and credited with high antioxidant and therapeutic abilities.^{19,20} The supplement can be taken in the form of tablets, capsules, as a food additive or extracted as a liquid. Some health claims and benefits include improvement in the control of hypertension, fibromyalgia and ulcerative colitis.²¹ *In vivo* studies have revealed the significant antitumor and antigenotoxic efficacy of *C. vulgaris*.²⁰ A study by Hasegawa *et. al* showed that a glycoprotein, designated ARS-2, found in *C. vulgaris* extract has an anti-cancer effect on mice-induced fibrosarcoma.²²

To the best of our knowledge, the effect of *C. vulgaris* on hepatoma cells has not been studied in great detail. In this study, we investigated the anti-cancer effect of *C. vulgaris* extract on the hepatoma cell line HepG2 by evaluating changes in proliferation, DNA damage and apoptosis.

MATERIALS AND METHODS

Culturing and Extraction of *Chlorella vulgaris*

Stock of *C. vulgaris* Beijerinck (strain 072) was obtained from the University of Malaya Algae Culture Collection (UMACC, Malaysia) and grown in Bold's basal media (BBM) with a 12 hours dark 12 hours light cycle. Cells were harvested by centrifugation at 1000 rpm. The algae were dried using a freeze dryer and then mixed in distilled water at a concentration of 10% (w/v). The algal suspension was then boiled at 100°C for 20 minutes using reflux method followed by centrifugation at 10 000 rpm for 20 min. The supernatant was lyophilized using a freeze dryer to obtain the powdered form of *C. vulgaris*

Cell Culture and Treatment

Liver cancer cell line HepG2 and normal liver cell line WRL68 were maintained in Eagle's minimal essential medium (EMEM; Flow Laboratories, Sydney Australia) supplemented with 1mM sodium pyruvate (SIGMA, St Louis, MO, USA), 2mM glutamine, 10% fetal calf serum and 100 U/ml penicillin and streptomycin at 37°C in humidified 5% CO₂ incubator. Cell proliferation, apoptosis and collection of protein were performed when cells reached 70% confluence density. *C. vulgaris* extract at various concentrations (0-4 mg/ml) was added to cell lines 24 hours after incubation.

Cell Proliferation Assay

A 96-well plate was seeded with HepG2 and WRL68 cells at a uniform density of 2×10^4 cells per well. Twenty-four hours after incubation, cells were treated with the hot water extract of *C. vulgaris* and incubated for a further 24 hours. Cells were labeled with bromodeoxyuridine (BrdU) during the last 3 hours of *C. vulgaris* extract treatment. The cells were fixed with denaturing solution and the incorporation of BrdU was detected by immunoreaction. After substrate solution was added to each well, the amount of BrdU incorporated was determined by measuring the absorbance

at dual wavelengths (450/690 nm) using a scanning multi-well spectrophotometer [enzyme-linked immunosorbent assay (ELISA) reader]

Apoptosis by TUNEL Assay

Apoptotic cell death was determined by Dead End™ Colorimetric TUNEL System (Promega, Madison, WI, USA). After 24 hours of treatment with *C. vulgaris* extract, floating cells and adherent cells in culture were collected in a tube, trypsinized, centrifuged and washed in phosphate buffered saline (PBS). Cells were resuspended and applied to poly-L-lysine-coated slides and air dried. Cells were fixed by immersing slides in 4% formalin in PBS for 25 minutes at room temperature. After washing with PBS, cells were permeabilized by immersing the slides in 0.2% Triton × 100 solution in PBS for 5 minutes at room temperature. Cells were then equilibrated with 100 µl of equilibration buffer (2.5 mM Tris-HCl pH 6.6, 0.2 M potassium cacodylate, 2.5 mM CoCl₂, 0.25 mg/mL BSA.) for 7 minutes. The equilibrated areas were blotted with tissue paper before adding biotinylated nucleotide and TdT reaction mix (100 µl) to the slides. The slides were then covered with coverslips to ensure an even distribution of the reagent before incubating at 37°C for 60 minutes in a humidified chamber. Coverslips were removed and the slides were immersed in 2× saline-sodium citrate (SSC; sodium chloride 0.15M, trisodium citrate 0.015M) buffer for 15 minutes at room temperature, and washed twice with PBS. Endogenous peroxidases were blocked by immersing the slides in 0.3% hydrogen peroxide for 4 minutes at room temperature and washed again in PBS. Streptavidin horseradish peroxidase (HRP) solution (1:500 PBS) was added to each slide and incubated for 30 minutes at room temperature. After final washing with PBS, diaminobenzidine (DAB) solution was added to the slides for 20 minutes until light brown staining was observed. Finally, each slide was mounted with DPX (BDH, England) to be examined under light microscope.

DNA Damage Measurement by Comet Assay

The assay was performed as described by Singh *et al.*²³ Thirty microlitres of the HepG2 cell suspension (<400,000 cells/ml) was mixed with 80 µl of 1% low melting point (LMP) agarose and added to fully frosted slides that had been covered with a layer of 1% LMP agarose. Subsequently, the slides were immersed in lysis solution [2.5M NaOH, pH10; 0.1M ethylenediaminetetraacetic acid (EDTA); 0.01M Tris; and 1% Triton × 100] for 1 h at 4°C, followed by electrophoresis solution (300mM NaOH; and 1mM EDTA, pH13) for 20 min to allow DNA unwinding, and electrophoresed for 20 min at 25 V and 300 mA. Finally, the slides were neutralized with 0.4M Tris buffer (pH 7.5), stained with ethidium bromide (5 mg/ml) and analyzed using a fluorescence microscope (Carl Zeiss, Göttingen, Germany). Images of 50 randomly selected cells per experimental point were visually analyzed under the microscope.

Measurement of p53, Bax, Bcl-2, Caspase-3 and -8 by Western blotting

HepG2 cells (1×10^7 /dish) were seeded into a 9 cm dish and treated with various concentrations of hot water extract of *C. vulgaris* for 2, 6, 12, 18 and 24 hours. Subsequently, cells were incubated in RIPA lysis buffer [50 mM Tris-HCl, 150 mM NaCl, 1 mM ethylene glycol tetraacetic acid (EGTA),

1 mM EDTA, 20 mM NaF, 100 mM Na₃VO₄, 1% NP-40, 1% Triton X-100, 1 mM phenylmethylsulfonyl fluoride (PMSF), 10 µg/ml Aprotinin and 10 µg/ml Leupeptin] (SIGMA, St Louis, MO, USA) on ice for 30 min to lyse the cells. After centrifugation, total protein was determined using a Bio-Rad protein assay kit (Bio-Rad, Hercules, CA, USA). Protein was resolved (50µg) by 10–15% SDS-PAGE and transferred to polyvinyl difluoride (PVDF) membranes. The membrane was blocked with blocking buffer (5% skim milk in 1% Tween 20 in 20 mM Tris-buffered saline, pH7.5) by incubating for 1 h at room temperature followed by incubation with the appropriate primary antibody (p53, Bax, Bcl-2, caspase-3 and -8; Chemicon, Billerica, MA, USA) at dilutions of 1:1000 in blocking buffer for 2 h at room temperature. The membranes were then incubated with the respective secondary antibodies for 1 h and antigens were detected by using the enhanced chemiluminescence blotting detection system (GE Healthcare, Piscataway, NJ, USA).

Statistical Analysis

Results are expressed as mean ± SD with the experiment repeated at least 3 times. Statistical evaluation was done using the Student's t-test. A *p* value of <0.05 was considered significant.

RESULTS

Chlorella vulgaris inhibited the proliferation of human liver cancer cells (HepG2) in a concentration-dependent manner, ranging from 0–4 mg/ml as shown by BrdU proliferation assay with a 50% reduction at 1.6 mg/ml, (IC₅₀) (Fig. 1). The high IC₅₀ is expected as *C. vulgaris* is classified as a food and not a drug. Proliferation of normal liver cells (WRL68) was decreased when treated with *C. vulgaris* extract, resulting in a 50% reduction at 1.7 mg/ml. One hundred percent inhibition of proliferation of HepG2 cells and WRL68 cells was achieved at approximately 3 mg/ml and 4mg/ml, respectively.

It was clearly seen that *C. vulgaris* induced apoptosis in HepG2 cells with distinct nuclear condensation and

blebbing of the plasma membrane, a typical characteristic of apoptotic bodies (Fig. 2).²⁴

The percentage of apoptosis of HepG2 and WRL68 cells as induced by *C. vulgaris* extract at 2mg/ml is demonstrated in Figure 3; the rate of apoptosis was significantly higher (70%) in HepG2 cells compared to normal WRL68 cells (15%). WRL68 cells also underwent apoptosis but at a higher concentration of *C. vulgaris* (3–4 mg/ml), perhaps as a result of the cytotoxicity effects seen at higher concentrations (Fig. 1)

The involvement of pro- and antiapoptotic proteins is demonstrated in Figure 4. Figure 4a shows that the level of p53 increased in a dose-dependent manner and reached maximum induction at 2 h with 2 mg/ml treatment of *C. vulgaris*. Interestingly, treatment with *C. vulgaris* resulted in increased expression of Bax (Fig. 4c), and a decreased expression of Bcl-2 (Fig. 4b) in a time-dependent manner. However, the activation of the mitochondrial apoptotic pathway in downstream caspase-8 was not significant (Fig. 4e), but a significant increase in the active form of caspase-3 was observed after 24 h of treatment with *C. vulgaris* in a time-dependent manner (Fig. 4d). These results demonstrate that the mitochondrial signaling pathway is involved in *C. vulgaris*-induced apoptosis of HepG2 cells.

DNA damage in HepG2 cells was increased by *C. vulgaris* treatments at all concentrations (Fig. 5). At low doses of *C. vulgaris* extract, HepG2 and WRL68 cells generated smaller comets that were not significant, whilst at 2 mg/ml, *C. vulgaris* induced more damage in HepG2 cells (80%) compared to WRL68 (50%).

DISCUSSION

Many chemopreventive agents have been associated with antiproliferative and apoptotic effects on cancer cells because of their high antioxidant activity, targeting signaling molecules, and preventing or protecting cells from further damage or transformation into cancer cells.⁷ *Chlorella vulgaris* has been shown to exhibit high antioxidant activity compared to other microalgae.²⁵ The antiproliferative effect of *C. vulgaris* (Fig. 1)

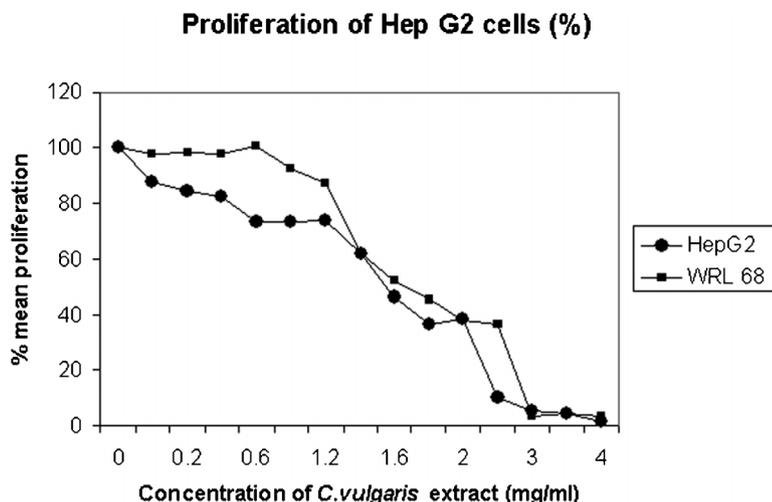


Figure 1 - The effect of *Chlorella vulgaris* extract on proliferation of HepG2 and WRL68 cell lines treated with different concentrations of *C. vulgaris* extract. Data are represented as mean ± SD. * Showed significant changes (*p*<0.05) for *n*=3 triplicate after 24 hours of treatment with different concentrations of *C. vulgaris* extract using bromodeoxyuridine (BrdU) assay with 50% reduction (IC₅₀) seen at 1.6 mg/ml for HepG2 cells and 1.7 mg/ml for WRL68 cells.

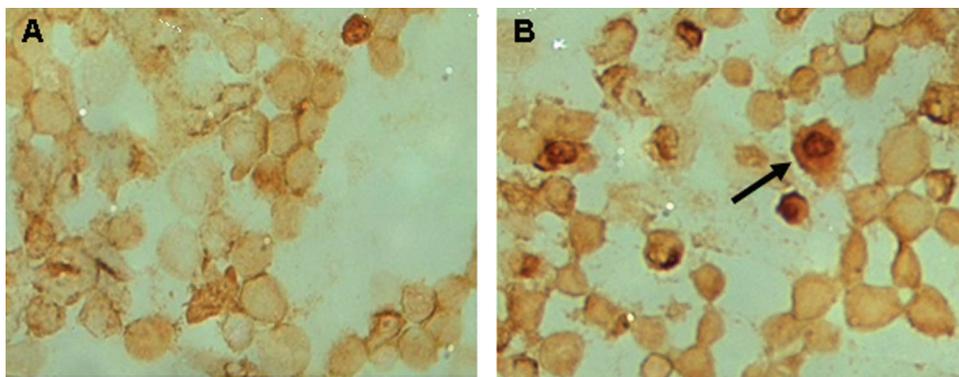


Figure 2 - Comparison of the morphology of WRL68 and HepG2 cells before and after treatment with 2 mg/ml *Chlorella vulgaris* extract. Apoptotic cells are absent in untreated WRL68 cells (a) and in treated (2 mg/ml *C. vulgaris*) WRL68 cells (b). Apoptotic cells are absent in untreated HepG2 cells (c), but present in treated (2 mg/ml *C. vulgaris*) HepG2 cells (d). Arrow shows condensation of chromatin and cell blebbing on HepG2 cells.

could be caused by an acidic glycoprotein found to have antitumour and antioxidant properties in tumour-bearing mice.²²

DNA damage is a common event in life following which, repair mechanisms and apoptosis will be activated to maintain genome integrity. The tumor-suppressor protein p53 provides an important link between DNA damage and apoptosis. DNA damage results in cell cycle arrest at checkpoints or at G1 or G2 stage to inhibit cell cycle progression and to induce apoptosis, thus protecting cells against further damage.²⁴ Antioxidants are important inhibitors of lipid peroxidation as a defence mechanism against oxidative damage.²⁶ Plants and microalgae have developed a protective mechanism by possessing antioxidant compounds mainly with phenolic moieties to eradicate the accumulation of reactive oxygen species (ROS) produced by ultraviolet (UV) radiation or heat from sunlight.²⁶ Our study showed that *C. vulgaris*, with its high antioxidant activity,

protected normal WRL68 cells against severe DNA damage but induced severe DNA damage in HepG2 cells.

The induction of apoptosis is known to be an efficient strategy for cancer therapy. Many Chinese herbal remedies

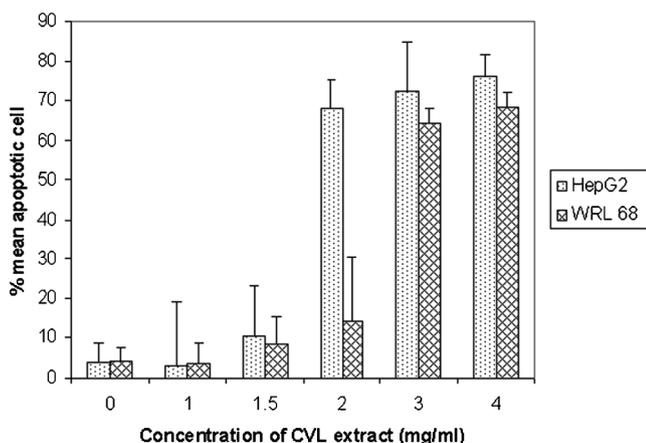


Figure 3 - The effect of *Chlorella vulgaris* extract on apoptosis of HepG2 and WRL68 cell lines. Data are represented as mean \pm SD. * showed significant changes ($p < 0.05$) for $n = 3$ triplicate after 24 hours of treatment with different concentrations of *C. vulgaris* extract using TUNEL assay. Per cent apoptotic cells are scored by taking an average of 1000 cells in 10 random fields. Apoptotic cells are almost absent in untreated and at 1 mg/ml of *C. vulgaris* but present quantitatively in HepG2 cells at higher concentrations of *C. vulgaris*.

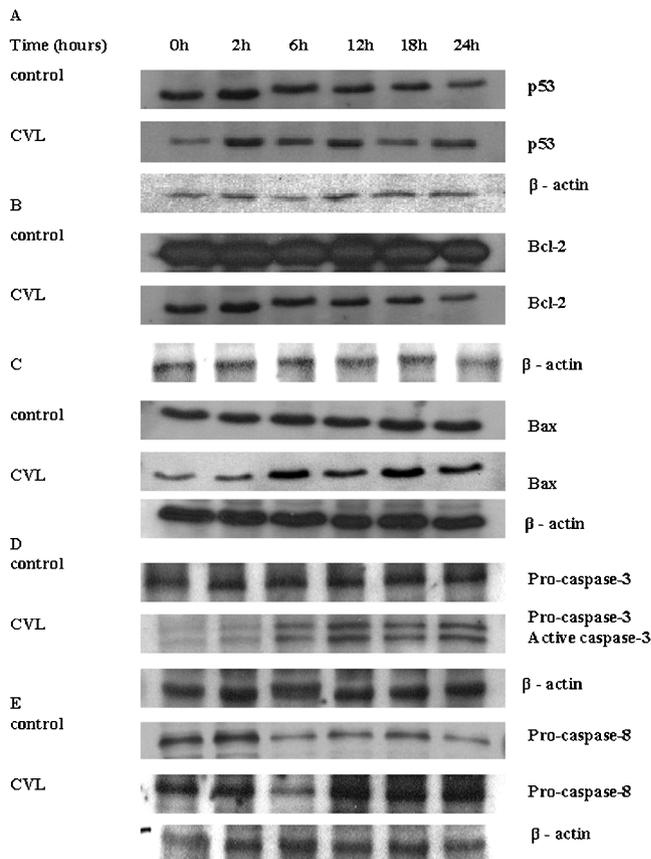


Figure 4 - Effect of *Chlorella vulgaris* on the protein expression of P53, Bax, caspase-3 and Bcl-2. *Chlorella vulgaris* at 2 mg/ml (optimal dose) induced higher expressions of P53 (a), Bax (c) (proapoptotic) and caspase-3 (d) proteins in HepG2 cells compared to the control cells after 24 hours treatment. Meanwhile, Bcl-2 (b) (antiapoptotic) protein expression was reduced compared to the control cells. No significant changes in the expressions of caspase-8 (e) proteins in hepatoma cells treated with *C. vulgaris* (2 mg/ml) were observed after 24 hours of treatment. CVL = *Chlorella vulgaris*.

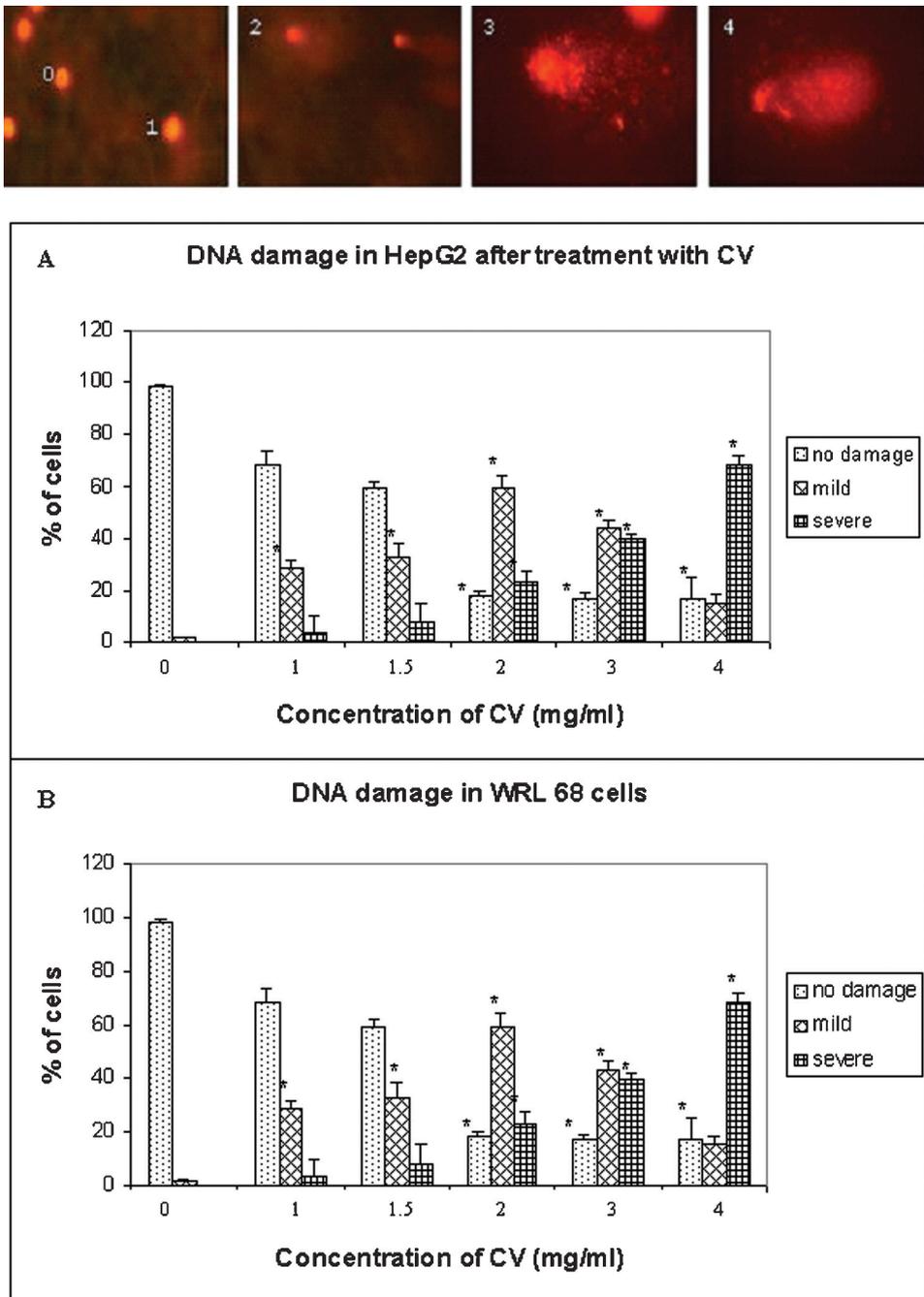


Figure 5 - Detection of DNA damage in HepG2 and WRL68 cells as measured by Comet assay after 24 hours treatment with *Chlorella vulgaris*. Data are represented as mean \pm SD for n=3 triplicate after 24 hours of treatment with *C. vulgaris* extract. (a) HepG2 cells treated with hot water extract of local *C. vulgaris*. (b) WRL68 cells treated with hot water extract of local *C. vulgaris*. Inset above the diagram shows representative image of a Comet generated in the single cell electrophoresis assay (Comet assay). 0 – cell without DNA damage. 1 & 2 – mild damage. 3 & 4 – severe damage.

such as *Ganoderma lucidum*, *Rubus coreanum*, *Paeoniae Radix* and *Phyllanthus urinaria* have been shown to trigger the apoptotic pathway in MCF-7 human breast cancer cells, HT-29 human colon cancer cells, HepG2 human hepatoma cells and Lewis lung carcinoma cells, respectively.²⁷⁻³⁰ Apoptosis is modulated by antiapoptotic and proapoptotic effectors, which involve a large number of proteins. The proapoptotic and antiapoptotic members of the Bcl-2 family act as a rheostat in regulating programmed cell death and as a target of anticancer therapy.³¹ The ratio of

death antagonists (Bcl2, Bcl-xL) to agonists (Bax, Bcl-xs, Bad, Bid) determines whether a cell will respond to an apoptotic stimulus. Down-regulation of the death suppressor Bcl-2 protein could repress tumor growth by promoting programmed cell death.³²⁻³⁴

To investigate the mechanisms of *C. vulgaris*-induced apoptosis, we assessed the expression and activity of tumor suppressor protein P53 and a host of pro- and antiapoptotic proteins: Bcl-2, Bax, and caspases-3 and -8. Our results showed that *C. vulgaris* mediated apoptosis in a P53-dependent

manner with increased expression of Bax and decreased expression of Bcl-2 proteins in a time-dependent manner. The activation of p53 and related family members can either enforce cell cycle arrest or induce apoptosis. The p53-dependent responses are directed against the damaged cell to protect the organism. The rules that govern the choice between growth, arrest and apoptosis are likely to be enforced by other proteins that can antagonize or synergize with p53 to regulate apoptosis.³⁵ The maximal increase of Bax expression in HepG2 cells after 18 h incubation with *C. vulgaris* may be the result of the increase of P53 accumulation that peaked after 2 h.

As a herbal extract, *C. vulgaris* contains a variety of compounds including antioxidants and a glycoprotein that may act on different pathways of tumor cell growth and survival, triggering an antagonistic effect of Bax and Bcl-2.

Apoptotic signals are generally believed to be mediated through a hierarchy of caspase activation controlled by one of two distinct pathways that are associated with either mitochondrial caspase-8 or -9. The initiating caspases then converge on the central effector caspases, caspases-3 and -7. Although we did not find significant activation of downstream caspase-8 there was, however, a significant increase of the active form of caspase-3 after 24 h of treatment with *C. vulgaris*, in a time-dependent manner. These results demonstrated that the mitochondrial signaling pathway is involved in *C. vulgaris*-induced apoptosis of HepG2 cells. It could be postulated that *C. vulgaris* did not induce the initiator caspase-8 but it may have activated the other initiator caspase-9. Among the ten different members of caspases identified in mammalian cells, caspase-3 may serve as a general mediator of apoptosis. When cells are undergoing apoptosis, executioner or effector caspase-3 triggers cellular proteins such as poly (ADP-ribose) polymerase and DNA fragmentation factor, resulting in the characteristic changes of apoptosis.³⁶ Caspase-3 is synthesized as a 33 kDa inactive proenzyme that requires proteolytic activation. Our results showed that a high level of proenzyme of caspase-3 was present in untreated tumor cells, and active caspase-3 gradually increased after *C. vulgaris* treatment, suggesting that *C. vulgaris* induced apoptosis through a caspase-3-dependent mechanism.

CONCLUSION

The results of the present study suggest that the anticancer mechanism of *C. vulgaris* in hepatoma cells (HepG2) is by inhibiting DNA synthesis, triggering DNA damage and inducing apoptosis. This is shown by the reduced incorporation of BrdU into replicating DNA of HepG2 cells and an increase in the number of DNA damage-inducing and apoptotic proteins, Bax and caspase-3 in HepG2 cells treated with *C. vulgaris*. The possible mechanism is speculated to be an increase in P53, Bax and caspase-3 expression that would subsequently lead to apoptosis.

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