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Anti-invasion and apoptosis induction of chlorella (*Chlorella sorokiniana*) in Hep G2 human hepatocellular carcinoma cells

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ABSTRACT

The effects of 80% ethanolic extract derived from commercial granule chlorella (GPE) on cell viability, invasion capacity and apoptosis in human hepatoma cell line (Hep G2 cells) were investigated. The results demonstrated that GPE decreased cell viability, induced apoptosis and showed invasion inhibitory effects in the Hep G2 cells. GPE-triggered apoptosis was confirmed by 4′-6-diamidino-2-phenyindole (DAPI) staining and comet assay. GPE promoted an increase of reactive oxygen species (ROS) and Ca^{2+}, and loss of mitochondrial membrane potential ($\Delta\Psi m$) accompanied by cytochrome *c* release that was due to the decrease of Bcl-2 in the Hep G2 cells. GPE also induced the protein levels of apoptosis-inducing factor (AIF), increased the levels of caspase-3, -8 and -9, and stimulated the levels of fatty acid synthase (Fas) and Fas ligand (FasL) in the Hep G2 cells. Additionally GPE inhibited invasion of Hep G2 cells by down-regulation of the expression of matrix metalloproteinase (MMP)-2 and -9. Furthermore, cellular glutathione content and superoxide dismutases (SOD) activities were significantly reduced and thiobarbituric acid-reactive substances (TBARS) levels were significantly increased after GPE treatment. These results suggest that GPE can induce cytotoxicity on Hep G2 cells and inhibit the invasive capacity of malignant cells.

1. Introduction

Hepatocellular carcinoma is one of the most common cancers in the world and it is a multifactorial disease, caused by smoking, alcohol, toxins and the human hepatitis virus. Inducing cell apoptosis is an important strategy for killing cancer cells. Apoptosis is a programmed cell death that leads to elimination of unwanted, damaged or infected cells. Cells undergo apoptosis through distinct pathways including fatty acid synthase (Fas) and Fas ligand (FasL) which results in the activation of the caspase-8, mitochondria-dependent pathway and the caspase-3-dependent pathway triggering the cytoplasmic release of pro-apoptotic mitochondrial proteins before leading to apoptosis (Eva, Una, & Afshin, 2003). Chlorella, unicellular green algae, has been found to contain highly nutritious substances with various biological effects. Glycoproteins derived from *Chlorella vulgaris* exhibited antitumour (Kuniaki et al., 1998) and immunomodulatory effects (Takashi et al., 2000). (Sulaiman, Shamaan, Ngah, & Yusof, 2006) showed that *C. vulgaris* has a chemopreventive effect

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on liver cancer induced by ethionine in rats. Several reports supported that the bioactive component polysaccharides from *Chlorella pyrenoidosa* were responsible for antitumor potential and immunomodulatory activities (Jianchun et al., 2007; Yang, Shi, Sheng, & Hu, 2006). Furthermore, chlorella contains various pigments such as carotenoids and chlorophylls which have been attributed to the biological properties including antioxidative, antilipidemic, antiatherosclerotic and antitumour activities (Cha et al., 2010). Researches have demonstrated that the bioactive compounds and biological activities of chlorella depend on the extraction solvents and extraction techniques used (Cha et al., 2010; Plaza et al., in press). Plaza et al. (in press) pointed out that ethanol as the most appropriate solvent to extract compounds with biological activities from chlorella not only for the higher yields and chemical composition obtained but also for the GRAS (generally recognized as safe) consideration. A recent study has shown that water extract of chlorella inhibits growth of human hepatoma Hep G2 cells (Wu, Ho, Shieh, & Lu, 2005), information on its antitumor properties and cellular mechanisms remain limited. Because it was observed in our pre-study that 80% ethanolic extract (GPE) showed a higher growth inhibitory effect on Hep G2 cells than aqueous or 50% ethanolic extract derived from commercial granule chlorella. Therefore, in this study, we investigated the effects of GPE on cell viability, invasion capacity and apoptosis in human hepatoma cell line (Hep G2 cells). The pigments, β-carotene, chlorophyll a, chlorophyll b, and lutein will also be determined by using HPLC equipped with a photodiode array detector.

2. Materials and methods

2.1. Preparation of 80% ethanolic extract of chlorella (GPE)

Dried, powdered chlorella (*Chlorella sorokiniana*) was purchased from Taiwan Chlorella Manufacturing Co. Ltd. (Taipei, Taiwan, ROC). Chlorella was extracted with 80% ethanol (50-fold) by stirring for 2 h. The decoction was filtered, evaporated and dried by a vacuum freeze-dryer. The yield of the dried GPE was 14.0%. The extract was sealed in plastic bottles and stored at −70 °C. For the present experiments, GPE was dissolved in DMSO before adding it to cell cultures.

2.2. Chemical and reagents

β-Carotene, chlorophyll a, chlorophyll b, 4′-6-diamidino-2-phenyindole (DAPI), dimethyl sulphoxide (DMSO), ethidium bromide (EtBr), lutein, monobromobimane (MbBr), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), phenylmethanesulphonyl fluoride (PMSF), 1,1,3,3-tetraethoxypropane (TEP), triton X-100, tris–HCl and ribonuclease-A (RNase A) were obtained from the Sigma Chemical Co. (St. Louis, MO, USA). Dulbecco's modified eagle medium (DMEM), fetal bovine serum (FBS), penicillin–streptomycin and trypsin–ethylenediaminetetraacetic acid were obtained from Invitrogen Life Technologies (Carlsbad, CA, USA). All other chemicals were at least reagent grade.

2.3. HPLC analysis

The pigments composition including β-carotene, chlorophyll a, chlorophyll b and lutein of the GPE were analyzed according to a method described by Gouveia et al. (2006). The GPE was dissolved in the mobile phase and filtered through a 0.45 μm filter before HPLC analysis. Reversed-phase analysis of pigments in GPE were performed on a HPLC (LCP 4100, ECOM, Praha 2, Czech Republic) with a Polaris C18 cartridge column (Varian, CA, USA, 250 mm × 4.6 mm i.d., 5 μm particle size) and a photodiode array detector (DAD 230, ChromTech, Apple valley, MN, USA) with acetonitrile:methanol:water (65:35:2) as eluent and with a flow rate 1.0 ml/min. Injection volume was 20 μl. Finally, chromatographic data were analyzed using WorkDAD HPLC system manager software (Chrom Tech, MN, USA).

2.4. Human hepatoma cell line and culture condition

Human hepatoma cell line (Hep G2) was obtained from the Food Industry Research and Development Institution (Shinchu, Taiwan). The Hep G2 cells were cultured in DMEM supplemented with 10% FBS, 2 mM L-glutamine, and 1% penicillin–streptomycin (100 U/ml penicillin and 100 μg/ml streptomycin). The cells were maintained in a humidified 5% CO_2 incubator at 37 °C and the cells were sub-cultured every 3–4 days to maintain logarithmic growth and were allowed to grow for 24 h before treatments were applied.

2.5. Morphological changes and cell viability analysis

The Hep G2 cells were plated in 12-well plates at a density of 1×10^5 cells/ml. The cells were treated with different concentrations (31.2, 62.5, 125.0, 250.0 and 500.0 μg/ml) of GPE or PBS as a solvent control and grown at 37 °C, 5% CO_2 and 95% air for 24 or 48 h. To determine morphological changes, the cells were photographed by a phase-contrast microscope and the MTT assay (Dariusz, Sarah, Richard, & Michael, 1993) was used to determine cell viability.

2.6. DAPI staining

The cells were incubated with different concentrations (31.2, 62.5, 125.0, 250.0 and 500.0 μg/ml) of GPE for 48 h. The cells were washed by PBS and stained by DAPI staining before being photographed (Su, Chen, Lin, Wu, & Chung, 2006).

2.7. Single cell gel electrophoresis (Comet assay)

The cells were incubated with different concentrations (31.2, 62.5, 125.0, 250.0 and 500.0 μg/ml) of GPE for 48 h. The cells were then harvested by centrifugation, the DNA was isolated and was then gel electrophoresis according to the method described by Su et al. (2006). The DNA damage in the GPE-treated cells was quantified as comet moment (the DNA product in the tail and the mean migration distance in the tail) were compared with untreated cells.

2.8. Measurement of reactive oxygen species (ROS) and superoxide anion production, mitochondrial membrane potential ($\Delta\Psi m$) and Ca^{2+} release

The Hep G2 cells were incubated with 500.0 μg/ml of GPE for 1, 3, 6 or 24 h. The cells were harvested and washed twice with PBS then re-suspended with specific fluorochromes. For ROS analysis, the cells were re-suspended in 500 μl 10 μM of 2,7-dichlorodihydrofluorescein diacetate (DCFH-DA); for $\Delta\Psi m$ analysis, the cells were re-suspended in 500 μl 1 μM of 3,3′-dihexyloxcarbocyanine (DiOC6), and for Ca^{2+} detection, the cells were re-suspended in 3 μg/ml Indo 1/AM, respectively and analyzed by flow cytometry as previously described (Wu et al., 2006). The superoxide anion measurement, the cells were incubated with 500.0 μg/ml of GPE for 2, 4, 6, 12, 24 and 48 h, based on the NBT assays, was carried out according to the method of Freire et al. (2003).

2.9. In vitro invasion assay

Cell invasion was determined by using Matrigel-coated transwell cell culture chambers (8 m pore size; Millipore Corp., Billerica, MA, USA) according to the method of Chen et al. (2010).

2.10. Western blotting

The total proteins were collected from Hep G2 cells after treatment with 500.0 μg/ml of GPE over 0, 6, 12, 24 and 48 h, then each protein was determined individually such as Bcl-2, cytochrome c, AIF, Fas, FasL, caspase-3, -8, -9, MMP-2, -9 and β-actin were measured by sodium dodecylsulphate polyacrylamide gel electrophoresis (SDS-PAGE) and Western blotting as described by Su et al. (2006).

2.11. Measurement of lipid peroxide levels, superoxide dismutase (SOD) activity and glutathione (GSH) levels in Hep G2 cells

The Hep G2 cells after treatment with 500.0 μg/ml of GPE over 48 h and harvested, and then sonicated with 1 mM PMSF buffer in order to obtain the cell homogenate. The thiobarbituric acid reactive substances (TBARS) method was used to estimate cellular TBARS level spectrophotometrically at 535 nm (Botsoglou et al., 1994). TEP was used as a standard. Cell GSH was reduced by dithiothreitol/phosphate solution and derivatized with MbBr prior to HPLC analysis (Yanga, Chou, Liu, Tsaia, & Kuo, 1995). SOD activity was determined spectrophotometrically at 325 nm based on the SOD-mediated decrease in the rate of pyrogallol autoxidation under alkali conditions (Stefan & Gudrun, 1974).

The protein content of cell homogenate was determined based on the Biuret reaction (Smith et al., 1985) of the BCA kit using BSA standards. The TBARS and GSH levels in cells were expressed as nanomoles/mg protein and the specific activity of the SOD was expressed as unit/mg protein.

2.12. Statistical analysis

The data were analyzed using Student's *t*-test to make a statistical comparison between control and GPE-treated cells. The results were expressed as mean ± SD of three independent experiments in replicate for both control and GPE-treated groups. Values of $P < 0.05$ were taken as being significant.

3. Results

3.1. The carotenoids and chlorophylls in GPE

Identification of carotenoids and chlorophylls in GPE by HPLC-DAD. Fig. 1 showed the HPLC chromatogram of the lutein, chlorophyll b and chlorophyll a in GPE. The identification of carotenoids and chlorophylls were primarily based on retention time and absorption spectra by comparison with four known standards. The retention time of lutein, chlorophyll b, chlorophyll a and β-carotene were 7.41, 15.72, 24.58 and 41.48 min, respectively and the absorption maximum wavelength (λ_{max}) was 442.5, 464.7, 430.2 and 449.1 nm, respectively. The regression equations based on the standard curves for lutein, chlorophyll b, chlorophyll a and β-carotene were $y = 165.27x + 159.46$, $y = 41.03x + 87.78$, $y = 46.72x + 41.95$, and $y = 4.04x + 23.95$, respectively, with correlation coefficients all being higher than 0.99. Based on HPLC analysis, the contents of lutein, chlorophyll a, and chlorophyll b were 3.8, 42.9 and 15.1 mg/g in GPE. However, there was no observed amount of β-carotene in GPE.

3.2. The GPE on morphological changes, cell viability and apoptosis of Hep G2 cells

After the Hep G2 cells were exposed to different concentrations of GPE over 48 h, the results from phase-contrast microscope examination indicated that the cells were morphologically-changed by GPE treatment (data not shown). Effects of GPE on the growth of Hep G2 cells were investigated by the MTT method. As shown in Fig. 2A, the growth inhibitory effect of GPE was observed in a dose and time-dependent manner. At 48 h, the maximal effect on proliferation inhibition was observed with 500 μg/ml GPE, which inhibited proliferation in 36.2% of Hep G2 cells.

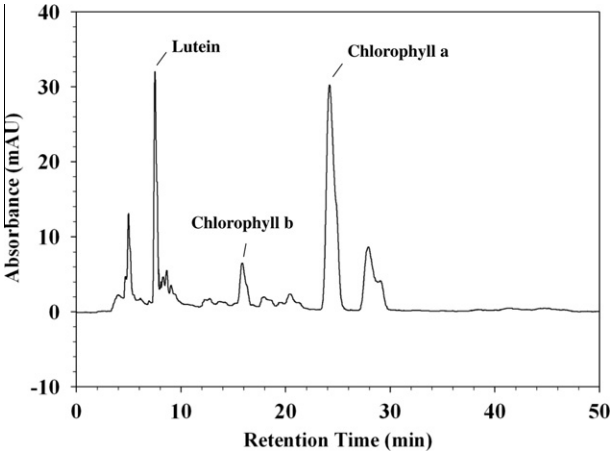

Fig. 1 – HPLC-DAD chromatogram of carotenoids and chlorophylls in GPE. HPLC-DAD conditions were described in the text.

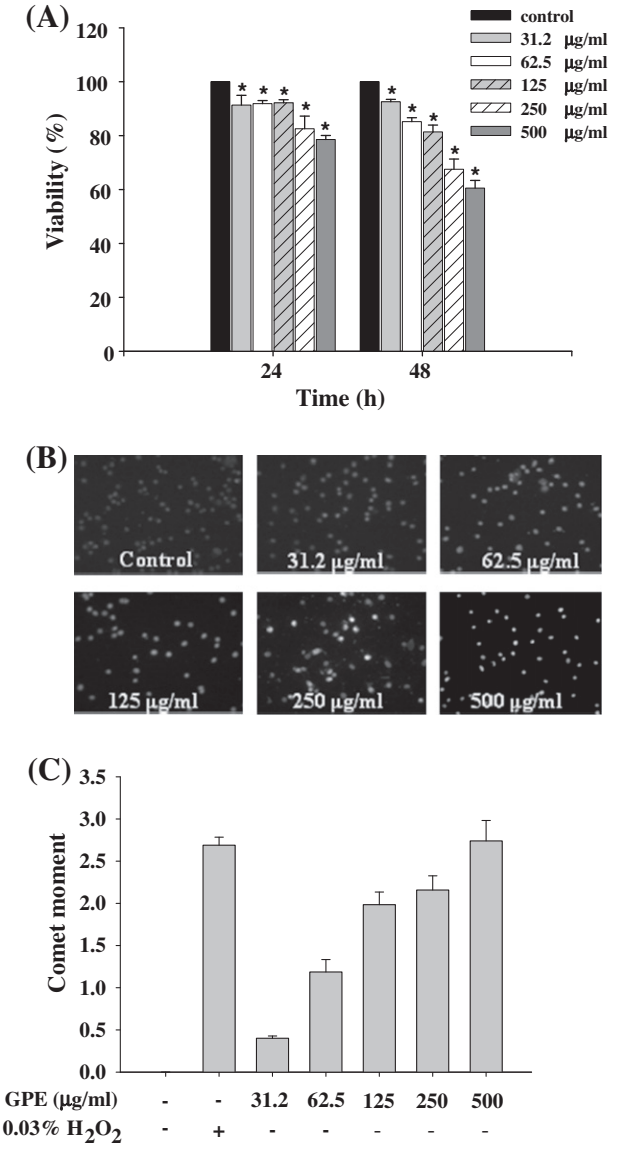

Fig. 2 – Effect of GPE on Hep G2 cells survival determined by MTT assay (A), apoptosis examined by DAPI staining (B) and comet assay (C). Each value represents the mean ± S.D. of three experiments. An asterisk indicates significant difference ($P < 0.05$) between GPE-treated cells and control as analyzed by Student's t-test.

To investigate the effect of GPE on the occurrence of apoptosis from Hep G2 cells, we isolated cells after 48 h then stained using DAPI and photographed by fluorescence microscope. As shown in Fig. 2B, the percentage of cells stained by DAPI was significantly different between GPE- treated and untreated cells. Apparently, the effects of GPE induced apoptosis were in a concentration-dependent manner. A separate experiment was conducted to re-confirm the influence of GPE on DNA damage. As shown in Fig. 2C, treatment of Hep G2 cells with GPE caused elevation in the DNA damage in a linear dose dependent manner ($R^2 = 0.977$) measured as comet moment.

3.3. *The GPE on the levels of ROS, superoxide anion, Ca^{2+} and mitochondrial membrane potential ($\Delta\Psi m$) in Hep G2 cells*

The ROS levels were stimulated quite early and time-dependently when the Hep G2 cells were incubated with 500.0 μg/ml GPE (Fig. 3A). The percentage of ROS was significantly different between GPE treated and untreated cells. As shown in Fig. 3B, superoxide anion was induced in Hep G2 cells at 12 h after GPE treatment and was of a time-dependent manner. Furthermore, the addition of GPE caused significant increase levels of superoxide anion in Hep G2 cells over 24 and 48 h. Flow cytometric analysis indicated that the $\Delta\Psi m$ levels significantly decreased in the GPE-treated cells as compared with the control cells (Fig. 3C). Also, it can be seen in Fig. 3D that the cytoplasmic Ca^{2+} were significantly increased as compared with the control cells and were in a time-dependently manner.

3.4. *The GPE on the expressions of apoptosis-associated specific proteins from Hep G2 cells*

The data demonstrated that 500.0 μg/ml GPE for 24 and 48 h can significantly decrease the expression of Bcl-2 (Fig. 4A) and increase the expressions of cytochrome c, AIF (Fig. 4A), caspases-3, -9 (Fig. 4B), Fas and FasL and caspase-8 (Fig. 4C) which may contribute to the occurrence of apoptosis in the examined cells.

3.5. *Anti-invasion effect of GPE in Hep G2 cells*

Effects of GPE on cell invasion were investigated using a reconstructed basement membrane and results were shown in Fig. 5A. After 48 h incubation, significantly fewer cells had invaded and migrated through the artificial membrane in the GPE-treated cells than in the control cells. The quantification of cells in the lower chamber from Fig. 5B indicated that GPE significantly inhibited Hep G2 cell invasion, the percentage of inhibition ratio is 24–75% and this effect was in a concentration-dependent manner. Furthermore, protein levels of MMP-2 and -9 significantly decreased following 500.0 μg/ml of GPE treatment in the Hep G2 cells (Fig. 5C).

3.6. *The GPE on the redox status of HepG2 cells*

Treatment with GPE (500 μg/ml) for 48 h was associated with a significant decrease in cellular GSH levels and SOD activities by 46.2% and 75.9% respectively as compared with the control cells (Table 1). Meanwhile, TBARS levels in Hep G2 cells increased significantly after GPE treatment by 66.8% when compared with the control cells.

4. Discussion

It is well known that hepatocellular carcinoma is one of the major diseases causing death throughout the world and many investigators are working to discover a new agent for hepatoma therapy. Many studies have reported that natural plants and/or their naturally occurring compounds, acacetin (Hsu, Kuo, & Lin, 2004) and apigenin (Chianga, Ng, Lin, Kuo, & Lin,

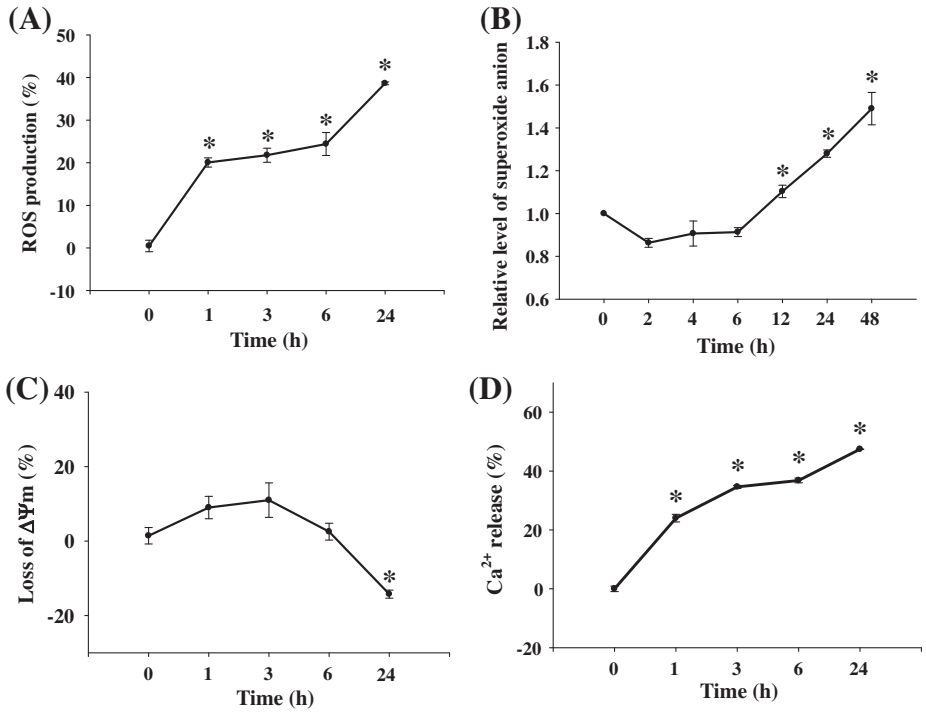

Fig. 3 – Effect of GPE on intracellular ROS (A), superoxide anion production (B), mitochondria membrane potential (C) and intracellular calcium levels (D) in Hep G2 cells. The result is expressed as the mean ± S.D. of three experiments. An asterisk indicates significance ($P < 0.05$) from the 0 h.

2006), common flavonoids in fruits and vegetables; curcumin, a natural product present in turmeric (López-Lázaro, 2008); *Euchresta formosana* Radix (Hsu et al., 2007), *Piper methysticum* Forster (Lude et al., 2008), *Physalis angulata* and *Physalis peruviana* (Wu et al., 2004), traditional herbal medicines; are potential inhibitors of tumor cell proliferation and apoptotic inducers in hepatoma cells. In fact, many plant derivatives have been used as anticancer agents in clinical patients (Katz, 2001). Chlorella has been found to contain a great variety of nutrients that are essential for human health and it is also widely used as a food supplement (Jianchun et al., 2007). The results of this study demonstrated that *in vitro* treatment of Hep G2 cells with GPE over 48 h induced morphological changes associated with the decrease of the percentage of cell viability in dose- and time-dependent manners. Our DAPI staining and comet assay resulted also indicated that GPE induced DNA damage in the Hep G2 cells. The anti-apoptotic protein, Bcl-2, play an important role in the induction of apoptosis (Willis, Day, Hinds, & Huang, 2003). Our data showed that GPE treatment decreased the levels of Bcl-2 and mitochondrial membrane potential ($\Delta\Psi m$) and promoted the levels of cytochrome c release from mitochondria in Hep G2 cells. The data also showed that GPE induced AIF expression before leading to apoptosis in Hep G2 cells. AIF is another possible protein that can be liberated from mitochondria to nuclei to trigger DNA fragmentation and participates in the induction of apoptosis in a caspase-independent manner (Susin et al., 1999). We also demonstrated a significant induction of the execution protease of apoptosis, caspase-3 and caspase-9. This is in agreement with many reports that released cytochrome c from mitochondria activates initiator caspase-9, in turn, activates a sequential cascade of caspases,

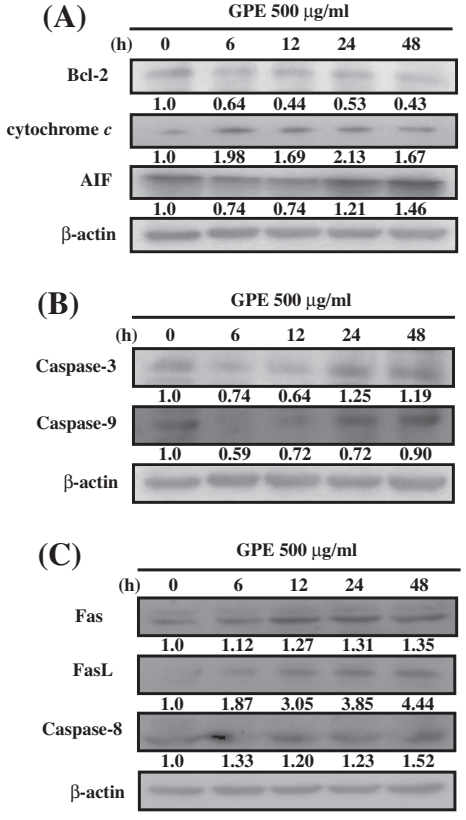

Fig. 4 – The expression of Bcl-2, cytochrome c and AIF (A), caspase-3 and caspase-9 (B), and Fas, FasL and caspase-8 (C) in Hep G2 cells treated with GPE (500 μg/ml). The protein levels were determined by Western blot analysis. β-Actin was used as the protein loading control.

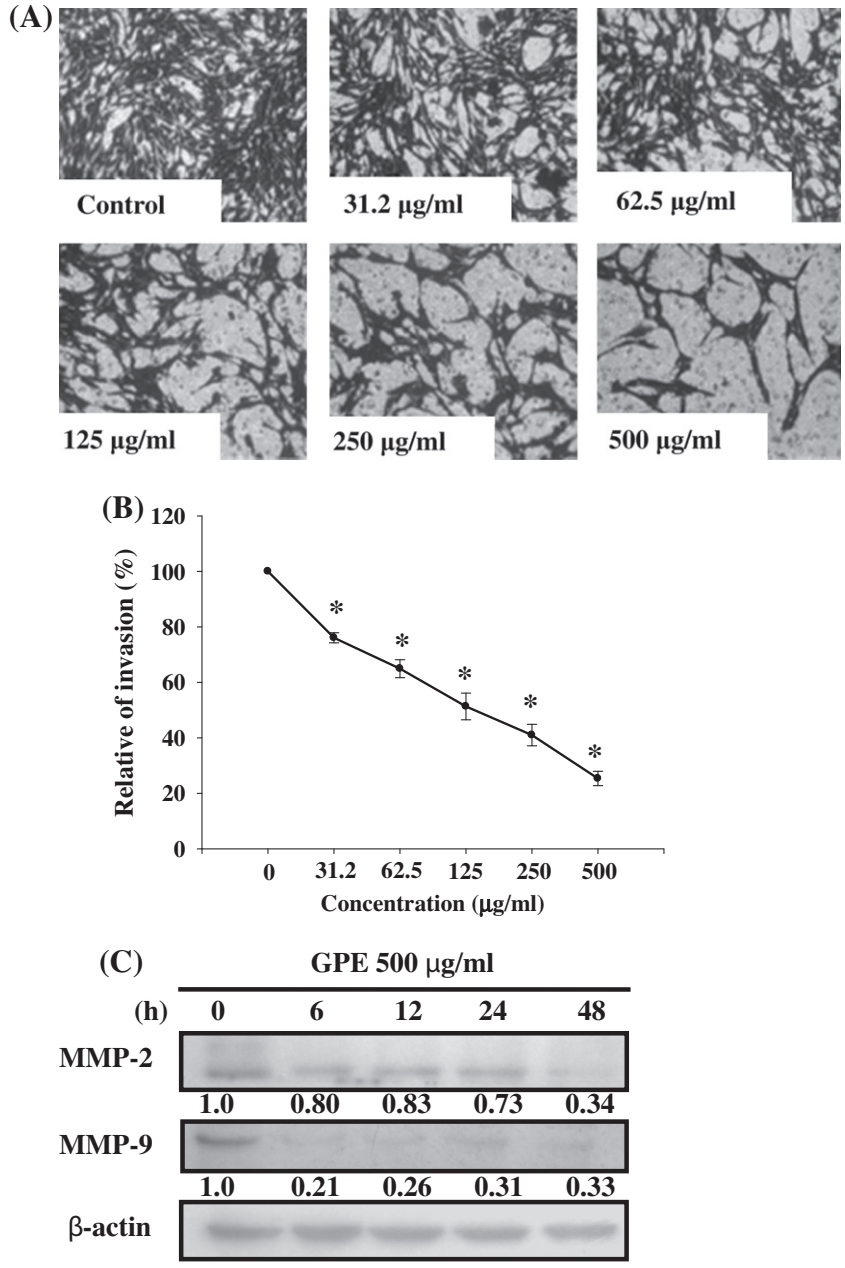

Fig. 5 – Effects of GPE on the invasion of Hep G2 cells *in vitro*. (A) Cells that penetrated through the matrigel to the lower surface of the filter were stained with crystal violet and shown under a light microscope at 100×. (B) Quantification of cells in the lower chambers, which was done by counting at 100×. (C) The expression of MMP-2 and MMP-9 in Hep G2 cells treated with GPE (500 μg/ml). An asterisk indicates significant difference ($P < 0.05$) between GPE-treated cells and the control group as analyzed by Student's *t*-test.

Table 1 – Effect of GPE on redox system of Hep G2 cells over 48 h incubation. The result is expressed as the mean ± S.D. of three experiments. An asterisk indicates significance ($P < 0.05$) from the control group.

	Control group	GPE-treated group
TBARS (nmole/mg protein)	75.52 ± 3.92	125.98 ± 13.13*
SOD (unit/mg protein)	7.17 ± 0.80	1.73 ± 0.60*
GSH (nmole/mg protein)	315.76 ± 7.51	170.03 ± 13.17*

The result is expressed as the mean ± S.D. of three experiments. An asterisk indicates significance ($P < 0.05$) from the control group.

especially caspase-3, resulting in the proteolysis of death substrates and subsequent DNA degradation and apoptotic death (Cory, Vaux, Strasser, Harris, & Adams, 1999; Wang, 2001).

ROS are recognized as mediators of the apoptotic signaling pathway (Li et al., 2003). Higher levels of ROS are known to induce not only cell death (Wulf, 2002), but also DNA damage and genomic instability (Cerutti, 1994). The redox state of the cell is also known to regulate its growth behavior (El-Missiry & El-Aziz, 2000). Other studies have indicated that cancer chemo-preventive agents induce apoptosis in part with the generation of ROS and the disruption of redox homeostasis (Lin, Fujii, & Hou, 2003; Xia, Lundgren, Bergstrand, DePierre, & Nässberger, 1999). In this study, we found that GPE increased the production of ROS, especially superoxide anion and H_2O_2, in short periods of time. Also, the treatment with GPE over 48 h was associated with a significant decrease in cellular GSH levels and SOD activities, and with an increase in TBARS levels in Hep G2 cells. Cellular glutathione is a major component of the intracellular reducing factor and a critical determinant for proper apoptotic signaling cascade and for the correct cell dismantling during apoptosis (Armstrong et al., 2002). Evidence suggests that severe intracellular GSH depletion can impair cell's defense against toxic compounds and may result in cell injury and death (Reed, 1990). Recently, it has been suggested that lipid peroxidation, determined by TBARS levels may impair a variety of intra- and extra-mitochondrial membrane transport systems that contribute to apoptosis (Kristal, Park, & Yu, 1996). The present data indicated that GPE may be a modulator of the cellular redox status and exert pro-oxidant effects in Hep G2 cells exposed to oxidative stress. It is well known that ROS may lead to endoplasmic reticulum stress and in turn lead to Ca^{2+} release. Our experiment demonstrated that cells treated with GPE for as little as one hour increased the levels of Ca^{2+} in Hep G2 cells. However, the detailed mechanisms of GPE on endoplasmic reticulum stress remain to be deciphered. The Fas/FasL system is another major mechanism for apoptosis. When Fas receptor proteins are activated by binding to FasL, the activation of caspase 8 occurs, in turn, execute cell apoptotic death (Rao et al., 2004). We observed the enhanced expression of Fas, FasL and caspase 8 suggesting that at least part of GPE-induced Hep G2 cells' apoptosis might be due to Fas-mediated death.

Cancer development involves multi-step process as which cancer eventually spreads from one area of the body to other organs or tissues during the late metastasic stage. It is well known that gelatinases such as MMP-2 and MMP-9 are directly involved in metastasis and that the suppression of gelatinase and will greatly contribute to the control of metastasis (Zeng, Cohen, & Guillem, 1999). Also, MMP-2 and MMP-9 are considered to be particularly important targets for the development of anticancer drugs because they are associated with aggressive, advanced, invasive or metastatic tumor phenotype (Birkedal-Hansen et al., 1993). In the present study, we demonstrated that GPE treatment can inhibit the levels of MMP-2 and MMP-9 coinciding with the inhibition of invasion of the Hep G2 cells after treatment with GPE. Our study provides additional information on the antimetastatic potential of GPE beyond its antitumor activity.

Recently, the field of available natural sources has been further increased by also including some algae and, even more interestingly, microalgae. These microorganisms are a potentially great source of natural compounds that could be used as functional ingredients (Plaza, Herrero, Cifuentes, & Iban~ez, 2009). Chlorophylls and carotenoids are present abundantly in green plants and possess important biological activities including antioxidant (Tsai, Wu, & Chen, 2010), antitumour (Tsai et al., 2010) and anti-invasion (Kozuki, Miura, & Yagasaki, 2000). According to Wu, Wu, and Shi (2007) Chlorella contained 2–4 mg/g dry cell weight of lutein. Lutein is not only an important natural food dye and additive but also a strong antioxidant that may be useful in reducing the incidence of cancer (Park, Chew, & Wong, 1998). Cha, Koo, and Lee (2008) reported that the bioactive carotenoid, xanthophylls of *Chlorella ellipsoidea* exerted strong antiproliferative effects on human colon cancer cells. Tsai et al. (2010) reported that carotenoids and chlorophylls isolated from *Gynostemma pentaphyllum* had antiproliferative effect on hepatoma cells. In this study, GPE contain lutein, chlorophyll a, and chlorophyll b and they may have contributed, in a substantial part at least, to the antitumour and anti-invasion activities of GPE.

In conclusion, for the first time, we found that the molecular mechanism of GPE induced cytotoxicity on Hep G2 cells. GPE may be a modulator of the cellular redox status and can exhibit pro-oxidant activity in Hep G2 cells. In addition, GPE induced ROS and Ca^{2+} production, decreased the Bcl-2 levels, changed the $\Delta\Psi m$ before it triggered the release of cytochrome *c* and subsequently induced the processing of procaspase-9 and procaspase-3 which led to the cleavage of DNA fragmentation. GPE also induced AIF and Fas/FasL pathways before leading to apoptosis in Hep G2 cells. Furthermore, we show for the first time that GPE can inhibit the invasion of Hep G2 cells *in vitro*. Chlorophylls and lutein may be responsible for the functional ingredients in GPE. Taken together these findings provide new insights into the possible pathways of chlorella-induced apoptosis and anti-invasion potential in human hepatoma G2 cells.

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