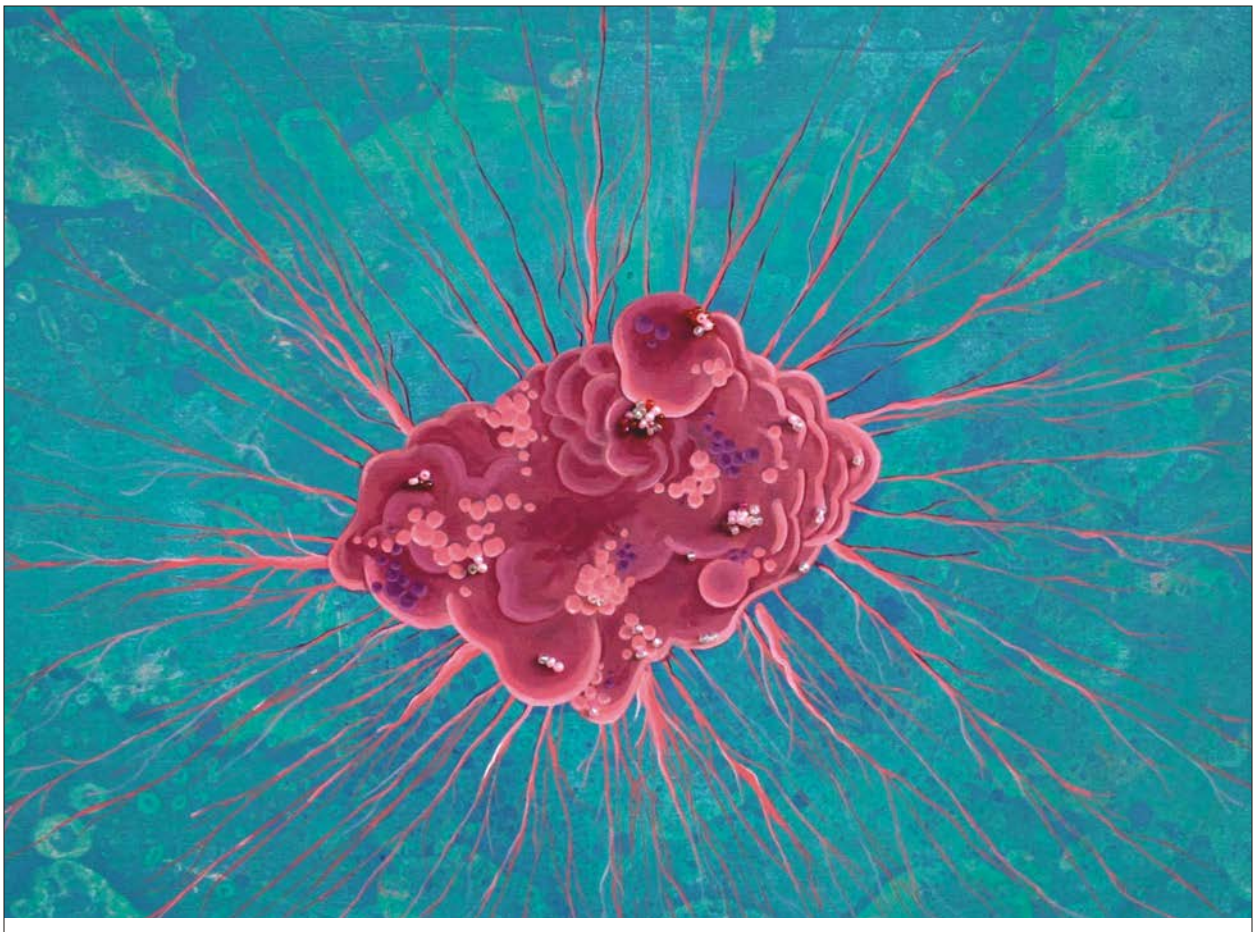

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Chemopreventive Effect of *Chlorella vulgaris* in Choline Deficient Diet and Ethionine Induced Liver Carcinogenesis in Rats

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Abstract: *Chlorella vulgaris* (CV), a unicellular green micro alga, has been widely used as a food supplement and credited with chemopreventive potential against several cancers. CV is reported to have a massive amount of antioxidant such as carotenoids, vitamin E, minerals and enzymes. However its antioxidant effect has not yet been explored in great detail. The aim of this study is to determine the effect of *Chlorella vulgaris* on the antioxidant enzyme status in liver cancer induced rats. Male Wistar rats (200-250 g) were divided into 8 groups in terms of diet given: control group (normal rat chow), liver cancer induced group (choline deficient diet + 0.1% ethionine in drinking water (CDE)), CV group with three different doses (50, 150 and 300 mg kg^{-1} body weight) and liver cancer group treated with CV at different concentrations (CDE + CV at 50, 150 and 300 mg kg^{-1} body weight). Blood sample was taken from rats via orbital sinus at 0, 4, 8 and 12 weeks for the determination of endogenous antioxidant enzymes [superoxide dismutase, (SOD), catalase and glutathione peroxidase, (GPx)] and lipid peroxidation active metabolite, malondialdehyde (MDA). Levels of SOD increased significantly ($p<0.05$) in CDE group when compared to the control group (normal rat chow) at 8 and 12 weeks of experiment. CV at all doses managed to reduce SOD activity at all weeks of experiment. There was no significant change of catalase level between the control and CDE group at the respective weeks of experiment but CV was able to reduce catalase activity ($p<0.05$) in CDE rats. There was a significant increase ($p<0.05$) of GPx activity in the CDE group compared to the control group at week 12. However, CV did not have the same effect on GPx as for SOD and catalase activities whereby GPx activity increased further ($p<0.05$) in CDE rats when treated with CV. The level of MDA increased significantly ($p<0.05$) in CDE rats when compared to the control group at week 12. CV at 150 and 300 mg kg^{-1} body weight managed to reduce MDA level in CDE rats. In conclusion, *Chlorella vulgaris* may have a protective role in liver cancer induced rats by replacing or compensating the activities of endogenous antioxidant enzymes and by reducing lipid peroxidation.

Key words: *Chlorella vulgaris*, antioxidant, anticancer, liver cancer induced rats

Introduction

Hepatocellular carcinoma (HCC) is one of the most frequent malignant neoplasms worldwide. The prevalence is between 250, 000 to 1,000,000 new cases per year, most of which occur in tropical

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Africa and Southeast Asia (Haydon and Hayes, 1995). In Malaysia, liver cancer is one of the major cancers affecting the population. In male population, the incidence of HCC has been ranked at the 10th place while in female it was ranked at the 15th place among the top 15 cancers in Malaysia (National Cancer Registry, Malaysia, 2004). Cirrhosis, radiation, free radicals, chronic infection of hepatitis B and C, genetic changes, metabolic disorder and exposure to certain chemical carcinogens such as aflatoxin, ethionine, diethylnitrosamine (DEN) and 2-acetylaminofluorene (AAF) have all been implicated in the pathogenesis of liver cancer. Choline deficient diet as a promoter and ethionine as the initiator has been used as a model to induce liver cancer in rats (Akhurst *et al*., 2001). Ethionine has been suggested to cause oxidative stress and cellular injury due to the enhanced formation of free radicals. This chemical carcinogen will be metabolized to its active ethyl radical metabolite and the reactive product interacts with DNA causing mutation which would lead to carcinogenesis (Farber, 1963).

Chlorella vulgaris (CV) has been studied in great detail for its nutritive values and other medicinal benefits. Tanaka *et al*. (1998) have found that a glycoprotein derived from this algae exhibited a pronounced antitumor effect against fibrosarcoma induced mouse. The glycoprotein extracts may have immunoprotection effect by enhancing the migration of T cells to tumor sites, promoting the development of T cells helper (Th) to produce interferon (IFN) through activation of macrophages (Hasegawa *et al*., 1990, 1997). Wang *et al*. (1979) showed that *Chlorella vulgaris* has a protective effect on the hepatic damage induced by ethionine in rats.

In recent years, the study on CV antioxidant capacities is of interest to many researchers. High levels of free radical or reactive oxygen species (ROS) can lead to a variety of biochemical and physiological disorders such as cardiovascular disease, aging, mutation and cancer (Fang *et al*., 2002; Singh *et al*., 2004). These free radicals will be scavenged by the antioxidant endogenous enzymes such as catalase (CAT), glutathione peroxidase (Gpx) and superoxide dismutase (SOD) to maintain the balance between oxidants and antioxidants in the organisms for their survival and health (Andersen *et al*., 1997; Tantry, 2003). Other than endogenous antioxidant enzymes, a non-enzymatic antioxidant defense system such as ascorbic acid (vitamin C), alpha-tocopherol (vitamin E) and β-carotene also exist as a safeguard against the accumulation of these free radicals (Matés *et al*., 1999). Estimation of endogenous antioxidant enzymes and circulatory lipid peroxidation end-products (MDA) as biochemical markers are reliable methods for evaluating the action of chemopreventive agents (Sundaresan and Subramanian, 2003). This study focused on the possible role of CV as antioxidant in protecting the damage caused by carcinogen ethionine in liver cells.

Materials and Methods

Animals and Diet

A total of 144 male Wistar rats (200-250 g) were supplied from Animal Care Unit, Universiti Kebangsaan Malaysia (UKM) (Kuala Lumpur, Malaysia) and were kept in polycarbonate cages in a room with controlled temperature, humidity and light-dark-cycle. They were divided into 8 groups according to diet given. Rats in the control group were given both normal diet and drinking water (normal rat chow from Gold Coin, Malaysia). Liver cancer induced rats were given choline deficient diet (ICN Biochemicals, USA) plus 0.1% ethionine (Sigma Chemical Co., USA) in drinking water (abbreviated as CDE group). The method of Akhurst *et al*. (2001) was followed for the induction of liver carcinogenesis. However, ethionine was given at 0.1% in drinking water instead of supplementing it in the pellet to reduce the risk of mortality upon administration to the rats and to minimize the exposure of the carcinogen. Three groups were given *Chlorella vulgaris* (CV) alone in three different doses (50, 150 and 300 mg kg^{-1} body weight). The treatment group consisted of three groups of CDE rats treated with three different doses of CV (50, 150 and 300 mg kg^{-1} body weight).

The rats were given 0.1 mL/100 g kg^{-1} body weight per day of CV via gavage (*force feeding*). The duration of the experiment was three months and the blood sample was taken from rats via orbital sinus at 0, 4, 8 and 12 weeks for endogenous antioxidant enzymes analysis (SOD, GPx and catalase) and for the measurement of lipid peroxidation active metabolite (MDA). The study was approved by the Animal Ethics Committee of Faculty of Medicine, UKM and was conducted at the Department of Biochemistry, Faculty of Medicine, UKM, (Kuala Lumpur, Malaysia) from the period of 2002-2005.

Chlorella Vulgaris

Stock of *Chlorella vulgaris* Beijerinck (strain 072) was obtained from University of Malaya Algae Culture Collection (UMACC, Malaysia) and grown in Bold Basal Media (BBM) (12 h dark:12 h light cycle). The algae were centrifuged three times at 3000 rpm for 10 min at 4°C to separate them from the media. The pelleted algae were then diluted in distilled water at three different doses (50, 150 and 300 mg kg^{-1} body weight) before being used throughout the experiment.

Biochemical Determinations

Superoxide dismutase activity was measured by the method of Beyer and Fridovich (1987) at 560 nm spectrophotometrically. One unit of enzymatic activity is defined as the amount of the enzyme that causes 50% inhibition of the reduction of formazan observed in the blank. Catalase activity was determined by the method of Aebi (1984) while glutathione peroxidase measurement was assayed using the method of Paglia and Valentine (1967) at 340 nm spectrophotometrically. Plasma lipid peroxidation (LPO) was determined according to the method of Ledwozyw *et al*. (1986) in which the released of malodialdehyde (MDA) served as the index of LPO. 1,1,1,3-tetraethoxypropane (Sigma, USA) was used as the standard.

Statistical Analysis

Data were analysed using the SPSS package. Results refer to mean±SEM with the experiment repeated at least three times. Statistical evaluations were assessed using the analysis of variance (ANOVA). A $p<0.05$ was considered significant.

Results

The protective role of endogenous antioxidant enzymes, SOD and GPx levels in rats treated with CDE to induce the formation of liver nodules could be seen by the increased levels of both enzymes in scavenging free radicals. Blood level of SOD increased significantly ($p<0.05$) in the CDE group when compared to the control group (normal rat chow) at 8 and 12 weeks of experiment (Fig. 1). Blood GPx level also increased significantly ($p<0.05$) in the CDE group when compared to the control group at 12 week of experiment (Fig. 2). Catalase level however did not show any significant changes between the control and CDE group at all weeks of experiment (Fig. 3). Lipid peroxidation (MDA levels) was increased in CDE rats as shown by a significant increase in MDA levels ($p<0.05$) when compared to the control group at 12 week of experiment (Fig. 4). Treatment with CV brought about changes in the endogenous antioxidants enzymes and lipid peroxidation that further protected the CDE treated rats. Both SOD and catalase levels decreased significantly ($p<0.05$) in the CDE group treated with CV at all doses when compared to the CDE group alone at 4, 8 and 12 weeks of experiment, respectively

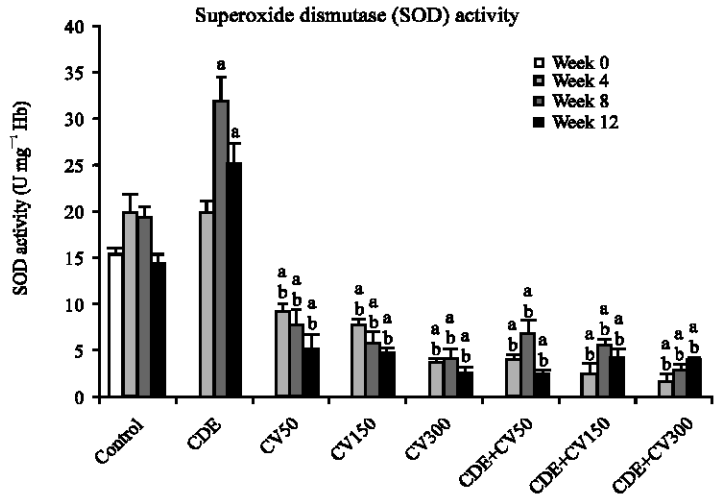

Fig. 1: Effect of *Chlorella vulgaris* (CV) on superoxide dismutase activity, (SOD) (U mg^{-1} Hb) in rats treated with choline deficient diet and 0.1% carcinogen ethionine in drinking water (CDE) to induce liver cancer. Data are expressed as mean±SEM [a] significantly different ($p \leq 0.05$) compared to the control group at the same week. [b] significantly different ($p \leq 0.05$) compared to the CDE group at the same week. Note: for week 4, 8 and 12 of experiments, a control is included and the values of SOD activity had no significant changes compared to control of week 0. For simplicity of presenting the results, control of week 4, 8 and 12 are not included in the results shown

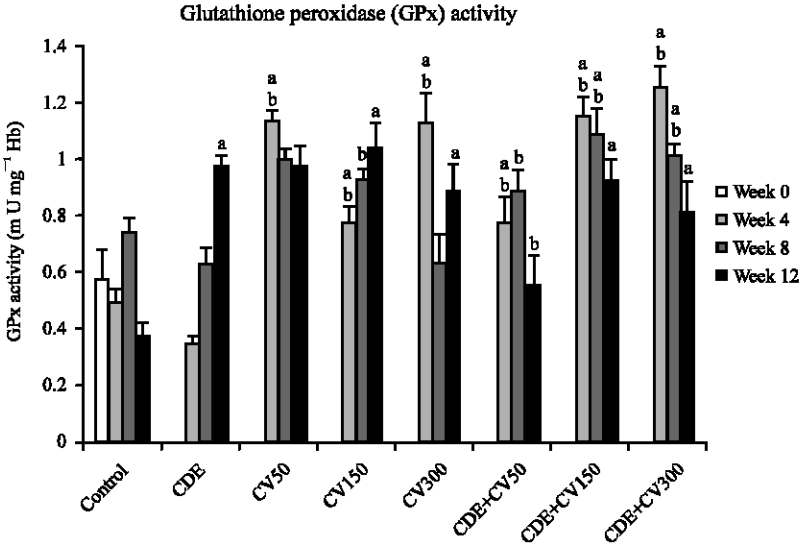

Fig. 2: Effect of CV on glutathione peroxidase activity, (GPx) (mU mg^{-1} Hb) in CDE rats at different weeks of experiment. Data are expressed as mean±SEM [a] significantly different ($p \leq 0.05$) compared to the control group at the same week. [b] significantly different ($p \leq 0.05$) compared to the CDE group at the same week

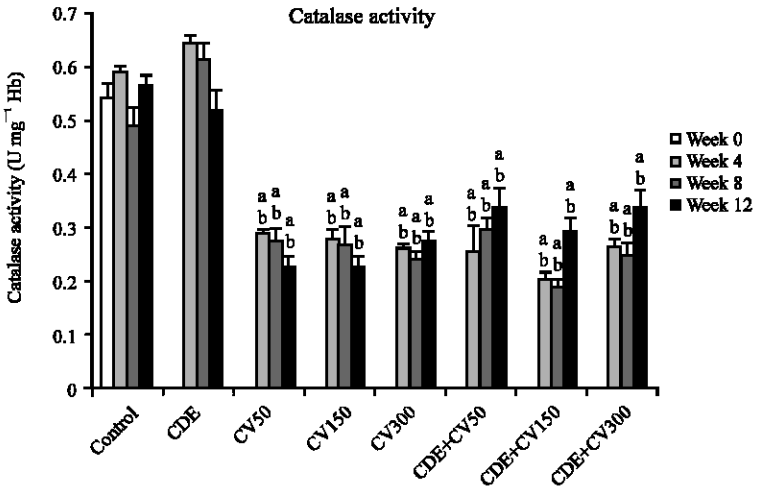

Fig. 3: Effect of CV on catalase activity, (CAT) (U mg^{-1} Hb) in CDE rats at different weeks of experiment. Data are expressed as mean± SEM [a] significantly different ($p \leq 0.05$) compared to the control group at the same week. [b] significantly different ($p \leq 0.05$) compared to the CDE group at the same week

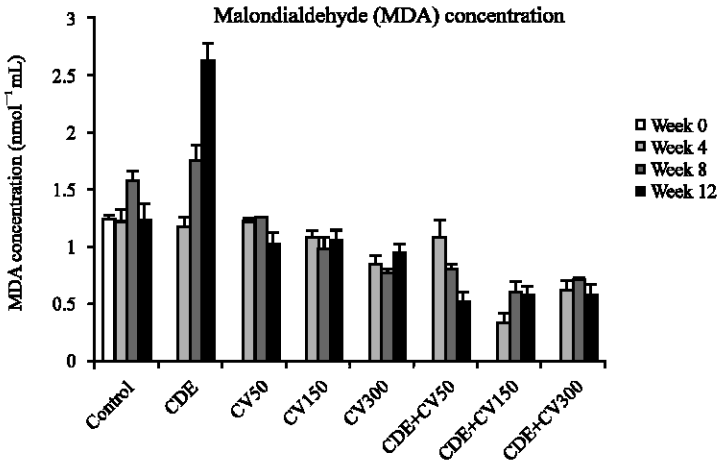

Fig. 4: Effect of CV on malondialdehyde level, (MDA) (nmol^{-1} mL) in CDE rats at different weeks of experiment. Data are expressed as mean±SEM [a] significantly different ($p \leq 0.05$) compared to the control group at the same week. [b] significantly different ($p \leq 0.05$) compared to the CDE group at the same week

(Fig. 1 and 3). SOD and catalase levels also decreased significantly ($p<0.05$) in the CV group alone at all doses compared to the control group at the respective weeks of experiment (Fig. 1 and 3). GPx level however, increased significantly ($p<0.05$) in CDE rats treated with CV when compared to control group at all weeks of experiment and to the CDE group at 4 and 8 weeks of experiment (Fig. 2), but it decreased significantly ($p<0.05$) at week 12 of experiment when treated with CV at all doses. Lipid

peroxidation decreased significantly in CDE rats when treated with high doses of CV (150 and 300 mg kg^{-1} body weight) at all weeks of experiment as shown by low levels of MDA ($p<0.05$) when compared to the CDE group at all weeks of experiment.

Discussion

Many epidemiological and animal studies have established unequivocally that diet can influence the incidence of diseases such as cancer by either modulating enzymes responsible for metabolic activation of carcinogen (Surh, 1999) or by scavenging superoxide and free radicals that are known to be increased in cancer cells (Shahidi, 1997). Our earlier work done in the laboratory has clearly shown that the algae extracts of *Chlorella vulgaris* (CV) have chemopreventive properties by reducing the number of preneoplastic liver nodules in choline deficient diet and ethionine (CDE) induced hepatocarcinogenesis in rats (Sulaiman *et al*., 2005). To our knowledge, this is the first report that documents the occurrence of a protective effect of CV in CDE induced hepatocarcinogenesis.

Free radical and non-oxidising species are regularly produced in our body during normal metabolism. However, an imbalance between oxidants and antioxidants will result into accumulation of free radicals causing oxidative stress, lipid peroxidation and tissue damage (Singh *et al*., 2004). As a safeguard against this damaging effect, these radicals will be scavenged by the endogenous antioxidant enzymes such as catalase (CAT), glutathione peroxidase (GPx) and superoxide dismutase (SOD) to maintain the balance between oxidants-antioxidants in organism for their survival and health (Andersen *et al*., 1997). The results of several investigations involving antioxidant enzymes during cancer were conflicting or equivocal. Increased GPx and catalase but decreased SOD activities were found in the liver of mice exposed to benzo(a)pyrene, a potent carcinogen to induce liver cancer (Abdel-Baky *et al*., 2002). Singh *et al*. (2004) and Sundaresan and Subramanian (2003) reported decreased activities of SOD, GPx and catalase in N-nitrosodiethylamine (DEN) induced hepatocellular carcinogenesis rats. Increased GPx but decreased catalase and SOD activities were found in dimethylnitrosamine-treated hepatectomized rats (Liotti *et al*., 1998). In this study, results of the experiment showed increased activities of SOD and GPx with no change in catalase activity and increased MDA level (lipid peroxidation end-product) in liver cancer induced rats treated with CDE diet. The increase in endogenous antioxidant enzymes (SOD and GPx) could be explained by the high concentration of free radical found during pathogenesis of cancer. The increase in SOD and GPx activities during carcinogenesis were usually compensated by the decrease of catalase activity as explained by Lee *et al*. (2005) in which a decrease in the capacity of some antioxidant enzymes may be compensated by an increase in the capacity of other antioxidant enzymes. Depletion in the activity of these antioxidant enzymes can be owed to an enhanced radical production during the ethionine metabolisms. The antioxidant effect of CV extracts could be clearly seen by the reduction of antioxidant enzymes (SOD and catalase) and MDA level in CDE rats. Failure in the reduction of GPx could be explained by the compensative effect of catalase for either SOD or GPx activies (Lee *et al*., 2005). The decrease in the level of these antioxidants after the administration of CV may be due to the direct reaction of CV which is rich in antioxidants such as beta carotene, vitamin E and C with reactive oxygen species (ROS). CV itself may scavenge free radicals thus replacing the effect of the endogenous antioxidant enzymes activity. An alternative explanation is that CV as an exogenous antioxidant, may work synergistically with the endogenous antioxidant enzymes to scavenge free radicals and to inhibit the production of lipid peroxidation end-product (MDA) (Abdel-Baky *et al*., 2004). A decrease in free radical generation and detoxification of free radical by CV led to a low level of oxidative stress and this will prevent the cell from becoming cancerous as observed in our earlier study whereby

hepatocarcinogenesis induced rats exhibited reduced preneoplastic lesions with CV treatment (Sulaiman *et al*., 2005). CV could also play a role in preventing oxidant induced liver injury by supplying antioxidants such as carotenoid, vitamin C and E, as well as minerals to the liver micro-circulation, thereby augmenting liver antioxidant capacity.

Lipid peroxidation (LPO) is regarded as one of the basic mechanisms of tissue damage caused by free radical (Esterbauer *et al*., 1991). Administration of ethionine has been reported to generate LPO products (MDA) and choline deficient diet enhanced the formation of the activated oxygen species in the preneoplastic nodules in rat liver (Farber, 1963; Allen and Poirier, 1997; Nakae, 1999). LPO generation can be prevented at the initiation stage of carcinogenesis by free radical scavengers and antioxidants (Torel *et al*., 1986). In our present study, the protective effect of CV extracts could be seen by the reduction of LPO (MDA level) in rats induced with liver carcinogenesis. This may represent the antioxidative potency of CV by inhibiting and quenching the LPO chain generation which would cause cell membrane and tissue injury (Singh *et al*., 1998).

The present study showed that *Chlorella vulgaris* (CV) has antioxidant capacities which can reduce the blood levels of SOD, catalase and GPx in liver cancer induced rats. The alga is also capable of reducing lipid peroxidation (low MDA level). Thus, *Chlorella vulgaris* may be involved in scavenging the free radicals (such as superoxide anions and hydrogen peroxide) that are widely generated during liver carcinogenesis. In conclusion, *Chlorella vulgaris* may have a protective role during liver carcinogenesis by replacing or compensating the activities of endogenous antioxidant enzymes and by reducing lipid peroxidation.

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