Original Article

# *Chlorella sp.* Protective Effect on Acetaminophen-Induced Liver Toxicity in ICR Mice


## Abstract

**Background:** A *Chlorella* sp. (CLC) has a health supplement in health effects including an ability to treat cancer. The *Chlorella* sp. Ability to reduce acetaminophen-induced liver injury is still unknown. The hepatoprotective function of CLC was determined in an APAP-induced liver injury mouse model. **Methods:** Male ICR mice were randomly divided into normal control, APAP, APAP + Sm (silymarin) and APAP + CLC (0.2%, 0.5% and 1%) groups. The glutamic oxaloacetic transaminase (GOT), glutamic pyruvic transaminase (GPT), Albumin, and BUN plasma activities were detected using blood biochemistry assay. The hepatic tissue GOT, GPT, superoxide dismutase (SOD) and catalase (CAT) activity were also detected. Lipid peroxidation, MDA, protein expression levels were examined. **Results:** The results showed that the 1% CLC supplementation group and Silymarin (Sm) could significantly alleviate increased serum GOT, GPT and BUN, and the decreased serum Albumin. At the same time, the increased hepatic tissue GOT and GPT activities were alleviated as well as MDA. Enhanced SOD and CAT protein expression levels were increased in APAP-induced liver injury. Lipofuscin and hepatic veins cups disappeared in the Sm and 1% CLC supplementation groups shown with H&E staining. **Conclusions:** Therefore, CLC probably could develop hepatoprotective products against chemical-induced liver damage.


**Keywords:** *Acetaminophen, catalase, Chlorella sp. crude lysate, glutamic oxaloacetic transaminase, glutamic pyruvic transaminase, hepatoprotective function, superoxide dismutase*


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## Introduction

Microalgae are usually used as additives in food to enhance the nutritional value and improve the health of humans and animals because of their chemical composition and bioactive molecules.[1,2] The high-protein content of microalgae is the major reason for the non-traditional source of protein. The microalgae amino acids synthesize essential amino acids in humans and animals and compare favorably with that of other food proteins. In addition, many valuable antioxidants are present in microalgae, e.g., chlorophyll, carotenoids, astaxanthin, lutein, and phycobiliproteins. A *Chlorella sp.* is a type of unicellular green algae, which is a popular food supplement or health food worldwide.[3-5] Taiwan is one of the major producers of *Chlorella*-related products. Chlorella sp. has been reported to have certain beneficial physiological effects, such as antihypertensive, antioxidative, hypocholesterolemic, and antitumor activities in animal and human studies.[6-9] However, the hepatoprotective activity of the extract derived from *Chlorella sp.* has not been extensively studied. Some reports indicated that *Chlorella sp.* is a good prophylactic-therapeutic agent against obesity-related complications.[10] Acetaminophen, or N-acetyl-para-amino-phenol (APAP), is widely used analgesic-antipyretic drugs. Although they are considered safe drugs,[11] they cause hepatic necrosis and renal failure when given in high doses. Increasing acute liver failure cases attributed to APAP use during the last two decades have been reported. Oxidative stress is reported to play a fundamental role in APAP-induced liver damage pathogenesis.[12] The search for new bioactive products with antioxidant activities is necessary to overcome APAP-induced hepatic oxidative damage.[13] Therefore, the present study was undertaken to evaluate the protective effect of the protein and antioxidant-enriched crude lysate from *Chlorella sp.* against APAP-induced liver injury in mice.


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## Materials and Methods

### Microalgal strain, *Chlorella* sp., cultivation

Freshwater microalgae *Chlorella* sp. was originally obtained from the Taiwan Fisheries Research Institute collection (Pingtung, Taiwan).

### Animals

Male ICR mice (4 weeks old; 20 ± 2 g) were obtained from the National Laboratory Animal Center (NLAC, Taipei, Taiwan). Mice were quarantined and acclimated for 1 week prior to experimentation. The animals were handled under standard laboratory conditions including a 12-h light/dark cycle in a temperature and humidity controlled room (at 22 ± 2°C and 60 ± 5% relatively humidity). Food (normal chow diet) and water were available *ad libitum*. Our Institutional Animal Care and Use Committee approved the protocols for this animal study. The animals were cared for in accordance with the institutional ethical guideline.

### Experiments

The animals were randomly divided into 6 groups with each consisting of 6 mice. Mice groups and treatments are listed in Table 1. The group control served as the normal control and was given PBS buffer as a placebo by intraperitoneal injection (IP). The group control, APAP (Sigma-Aldrich), 0.1% silymarin (SM) (Sigma-Aldrich), 0.2%, 0.5%, and 1% CLC (*Chlorella sp.*), and animals were administered 200 mg/kg body weight of APAP dissolved in PBS buffer by IP twice a week for a period of 4 weeks. After APAP intoxication, group APAP served as the APAP negative control.

### Blood biochemistry assay

The glutamic oxaloacetic transaminase (GOT), glutamic pyruvic transaminase (GPT), blood urea nitrogen (BUN), and albumin activities in serum were measured to evaluate hepatoxicity and renal toxicity. An autoanalyzer (DRI-CHEM 3500s; FUJIFILM, Kanagawa, Japan) was used in these experiments.

### Hepatic tissue GOT, GPT, SOD, and CAT activity determination

GOT and GPT activities were measured according to the ELISA protocol of a commercially available kit (Cayman Chemical Company). The SOD activity was evaluated by using a tetrazolium salt for detection of superoxide radicals generated by xanthine oxidase and hypoxanthine. One unit of SOD is defined as the amount of enzyme needed to exhibit 50% dismutation of the superoxide radical. The SOD activity is standardized using the cytochrome c and xanthine oxidase coupled assay. The CAT activity was measured using $H_2O_2$ decomposition according to the Aebi. The final reaction mixture comprised 10 mM $H_2O_2$ in 50 mM phosphate buffer at pH 7.0. The decomposition of $H_2O_2$ was followed by spectrophotometry at 240 nm.

**Table 1: Short names of experimental ICR mice groups and treatments**

| Group | Treatment | Diet | Short name |
|---|---|---|---|
| **Normal control** | PBS IP inject | Chow diet | **control** |
| **APAP** (negative control) | APAP IP inject | Chow diet | **APAP** |
| **0.1% silymarin** (positive control) | APAP IP inject | Chow diet containing 0.1% silymarin (w/w) | **Sm+APAP** |
| **0.2% CLC** | APAP IP inject | Chow diet containing 0.2% CLC (w/w) | **0.2% CLC+APAP** |
| **0.5% CLC** | APAP IP inject | Chow diet containing 0.5% CLC (w/w) | **0.5% CLC+APAP** |
| **1% CLC** | APAP IP inject | Chow diet containing 1% CLC (w/w) | **1% CLC+APAP** |

**Control=**Group normal served as normal control with feeding chow diet and was given PBS buffer as placebo by IP twice a week for a period of 4 weeks. **APAP=**Group APAP served as negative control. APAP with feeding chow diet and was given 200 mg/kg body weight of APAP dissolved in PBS buffer by IP twice a week for a period of 4 weeks. **SM+APAP=**Group Sm+APAP served as positive control 0.1% silymarin with feeding chow diet containing 0.1% silymarin (w/w) and was given 200 mg/kg body weight of APAP dissolved in PBS buffer by IP twice a week for a period of 4 weeks. **0.2% CLC+APAP, 0.5% CLC+APAP, and 1% CLC+APAP=**Group 0.2% CLC, 0.5% CLC, and 1% CLC of mice were fed chow diet containing 0.2%, 0.5%, and 1% CLC (w/w) individually and given 200 mg/kg body weight of APAP dissolved in PBS buffer by IP twice a week for a period of 4 weeks

### Hepatic tissue malondialdehyde, lipid peroxidation, and intermediate determination

The malondialdehyde (MDA) content (Ann Arbor, MI, USA), a measure of polyunsaturated fatty acids peroxidation, was assayed in the form of thiobarbituric acid-reactive substances. Briefly, a volume of 0.5 ml of liver homogenate was mixed with 3 ml of 1% $H_3PO_4$ (v/v) and 1 ml of 0.6% thiobarbituric acid (TBA, w/v), and then, heated to and maintained at 100°C for 40 min. The samples were allowed to reach room temperature, and 2 ml of butanol was added. After shaking vigorously with the vortex, the butanolic phase was obtained by centrifugation at 3,000 ×*g* for 10 min to determinate the absorbance at 535 nm.

### Histopathology hematoxylin and eosin staining examination

Liver samples were dissected out and excised from experimental animals from each group, then washed with normal saline, fixed in 10% formalin, and processed for paraffin embedding following the microtome technique.

The sections were taken at 6 μ thickness, processed in alcohol-xylene series, and stained with hematoxylin and eosin (H and E stain). The sections were examined microscopically for the evaluation of histopathological changes.

### Statistics

Data were compared with one-way analysis of variance test to evaluate differences among multiple groups. All results are expressed as the mean ± standard deviation (SD). $^{*}P < 0.05$ or $^{\#}P < 0.05$ differences were considered statistically significant. A statistical analysis was performed using statistical software (SPSS, Chicago, IL, USA).

## Results

### Preparation of the crude lysate of a *Chlorella sp.* (CLC).

*Chlorella* sp. biomass chemical composition is shown and compared in Table 2. The main feature is the abundant protein content (50–55%). The crude protein, soluble carbohydrates, total fiber, fat, ashes, and nucleic acids obtained in the CLC biomass were 63.7 ± 2.1, 17.3 ± 1.1, 4.5 ± 0.6, 3.4 ± 0.6, 5.6 ± 0.7, and 1.4 ± 0.2 (g/100 g), respectively individually after ultrasonic extraction [Table 2]. The ultrasonic-extracted CLC biomass crude protein was 1.2 times higher than that of non-extracted biomass ($P < 0.05$). The CLC also contains a number of biologically active compounds such as chlorophylls (2093.7 ± 166.3 mg/100 g) and carotenoids (211.3 ± 19.7 mg/100 g) ($P < 0.05$) compared with non-extracted biomass. These compounds have free radical scavenging potential, i.e., antioxidative activity. The chlorophylls and carotenoids of CLC biomass both were increased 1.5 to 2.0-fold compared with that of non-extracted biomass ($P < 0.05$).

**Table 2: Chemical composition of a *Chlorella* sp. (CLC) biomass and its crude lysate**

| Constituent (g/100 g) | Non-extracted biomass[a] | Ultrasonic-extracted biomass[b] |
|---|---|---|
| Crude protein (N×6.25) | 53.6 ± 2.3 | 63.7 ± 2.1* |
| Soluble carbohydrates | 16.7 ± 1.0 | 17.3 ± 1.1 |
| **Total fiber** | 8.5 ± 1.0 | 4.5 ± 0.6 |
| Fat | 6.6 ± 0.8 | 3.4 ± 0.6 |
| Ash | 6.5 ± 0.7 | 5.6 ± 0.7 |
| Nucleic acids | 5.5 ± 0.6 | 1.4 ± 0.2 |
| **Pigments (mg/100 g)** | | |
| Chlorophylls | 1393.7 ± 147.9 | 2093.7 ± 166.3* |
| Carotenoid | 130.3 ± 15.6 | 211.3 ± 19.7* |

Each data indicates the mean SD from three independent determinations. *$P$<0.05 significant difference compared to non-extracted biomass. The *Chlorella* sp. biomass was harvested and spray-dried without extraction. The CLC was harvested and spray-dried with ultrasonic extraction

### *Chlorella sp.* (CLC) supplementation effect on liver marker enzymes in serum after APAP-induced hepatotoxicity

The ICR mice were fed with a chow diet containing 0.2%, 0.5%, and 1% (w/w) of CLC and 0.1% SM to test feeding APAP toxicity. After 28 days, the ICR mice were sacrificed to determine the serum GOT, GPT, albumin, and BUN activities for evaluating the liver function [Figure 1]. As shown in Figure 1a, the average serum GOT level results in group 1% (w/w) of CLC and SM had significant differences compared between the group APAP-induced the hepatotoxicity ($P < 0.05$). At the same time, both of SM administration and 1% CLC supplementation could reduce the increases in serum GTP-induced by APAP treatment [Figure 1b]. The mice challenged with APAP for 4 weeks showed significant increases in serum GOT and GPT activities compared with those in the control indicating that APAP-induced the hepatotoxicity ($P < 0.01$) [Figure 1a and b]. However, APAP-induced the hepatotoxicity led serum albumin decreased compared with control ($P < 0.01$) [Figure 1c], once SM and 1% CLC supplementation had increased serum albumin ($P < 0.01$) compared with APAP-induced injury. The serum BUN levels after APAP-induced increased hepatotoxicity when compared with control ($P < 0.01$) [Figure 1c]. SM and 1% CLC among these groups were significantly reduced BUN activity compared with APAP-induced [Figure 1d]. Hence, 1% CLC in feed was selected to evaluate the *Chlorella* sp. supplementation effect against APAP-induced liver injury in the study.

### *Chlorella sp.* (CLC) supplementation effect on hepatic tissue function markers, GOT, GPT, SOD, and CAT activity after APAP-induced hepatotoxicity

Overdosed APAP administration would induce oxidative stress in the liver because of hepatic function markers, GOT, and GPT, increased ($P < 0.05$) [Figure 2a and b], but decreased hepatic tissue, SOD, and CAT activities ($P < 0.05$) compared with control [Figure 2c and d]. In APAP-treated and SM administration, hepatic GOT and GPT activities were significantly reduced ($P < 0.05$) compared with APAP-treated. We also observed 1% CLC administration for APAP-induced hepatotoxicity was significantly reduced ($P < 0.05$). By contrast, hepatic tissue antioxidative enzymes and CAT activities were increased in SM and 1% CLC ($P < 0.05$), when compared with APAP-induced hepatotoxicity group [Figure 2d]. However, hepatic tissue antioxidative enzymes and SOD activities displayed no significant differences after CLC supplementation in APAP-induced oxidative stress [Figure 2c]. It is indicated that the low degree of

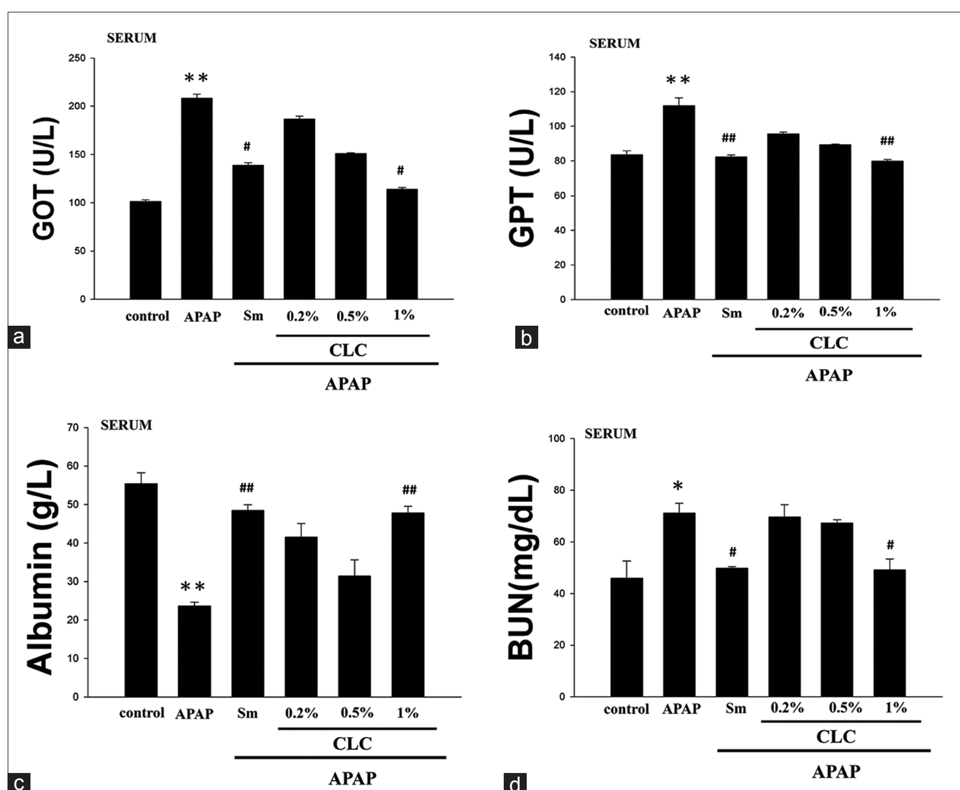


**Figure 1: Liver function of the ICR mice supplement with a *Chlorella sp.* (CLC) for hepatoprotective test in serum. The APAP, SM, 0.2% CLC, 0.5% CLC, and 1% CLC groups were administrated with 200 mg/kg APAP, and the normal control was administrated with PBS as placebo twice a week for 4 weeks. The Sm was fed with the chow diet containing 0.1% Silymarin (w/w). The group 0.2% CLC, 0.5% CLC, and 1% CLC were fed with the chow diet containing 0.2, 0.5, and 1% of CLC (w/w), respectively. The ICR mice were sacrificed after 4 weeks to determine the (a) GOT, (b) GPT, (c) albumin, and (d) BUN activity in serum. All of the values were expressed as the mean ± SD from 6 mice of each group. *$P < 0.05$, significant difference compared with control. #$P < 0.05$, significant difference compared with APAP-induced liver injury**

CLC concentration not reflected on serum and tissue. On the contrast, the high degree levels defense for the APAP-induced oxidative stress by CLC were close with that by SM administration.

### *Chlorella sp.* (CLC) supplementation effect on hepatic lipid peroxidation, malondialdehyde, after APAP-induced hepatotoxicity

Furthermore, we wanted to know the CLC supplementation effect on lipid peroxidation against APAP-induced oxidative stress in ICR mice [Figure 2e]. Experimental animals treated with APAP caused a significant increase in the primary lipid peroxidation and MDA product levels in comparison to the normal control ($P < 0.05$). ICR mice supplemented with SM and 1% CLC showed significantly inhibited hepatic APAP-induced hepatotoxicity elevation ($P < 0.05$). These eliminated MDA levels by 1% CLC supplementation were statistically similar to the level in the SM treatment group.

### Histopathological changes in *Chlorella sp.* (CLC) supplementation on liver tissue after APAP-induced hepatotoxicity

Histopathological examinations showed that the APAP administration section observed collagen released (black arrows); the liver tissue cross-sections revealed severe hepatocyte necrosis with inflammatory cell infiltrate (brown arrows) [Figure 2f]. Mice livers simultaneously administered with APAP after supplementation with 1% CLC revealed improved liver condition not showed the inflammatory cell infiltration. The minimal hepatocyte necrosis damage shown in 0.5% CLC and the situation was comparable to that of mice administrated with SM and 1% CLC. According to histopathological examinations, severe hepatic lesions induced by APAP were remarkably reduced by 1% CLC supplementation, which was in good consistency with the results found in liver marker enzymes in serum and hepatic oxidative stress and lipid peroxidation.

## Discussion

The *Chlorella* sp. (CLC) is a protein-enriched powder containing around 65% of microalgae proteins. Marine macro- and microalgae-derived bioactive peptides have been widely applied for human nutrition and health. Marine-derived bioactive peptides have been identified as having antioxidant, antihypertensive, anticoagulant, or antimicrobial activities.[14-16] In the present study, the crude lysate of *Chlorella* sp. was prepared and used as a diet supplement to investigate the bioactivity functions on hepatoprotection. Significantly, different liver injury

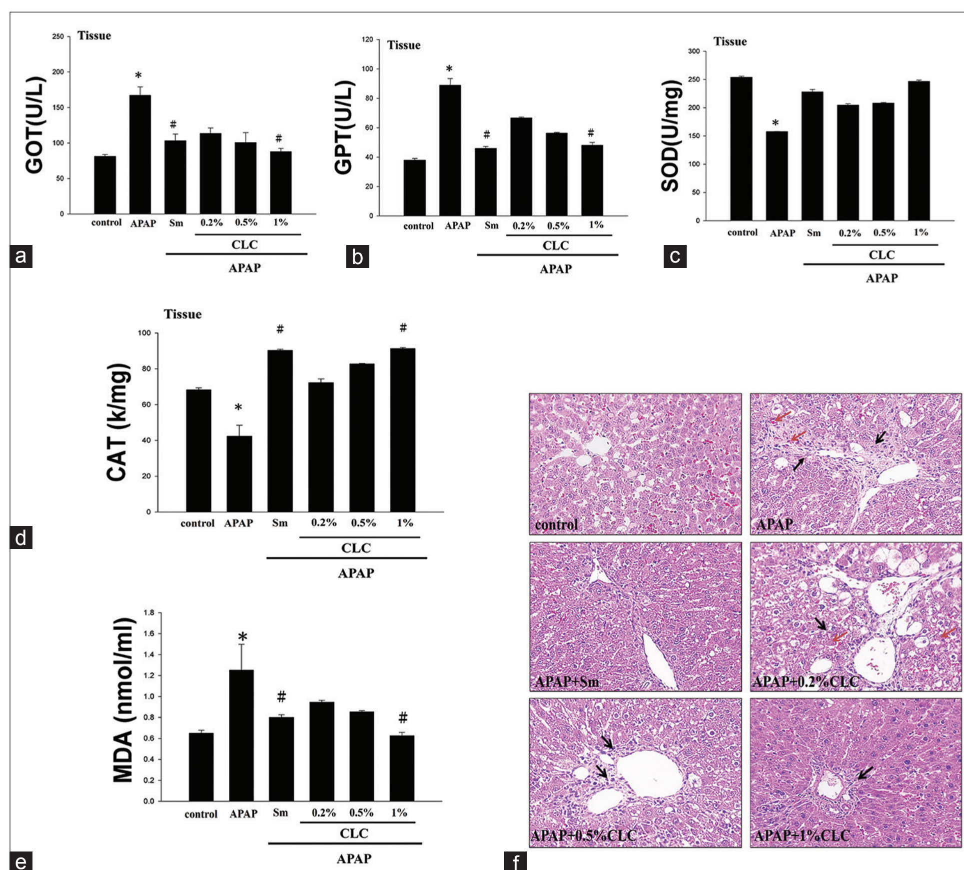


**Figure 2: *Chlorella sp.* (CLC) supplementation effect on liver function marker enzymes in the mice hepatic tissue of administrated with APAP. GOT and GPT activities were measured according to the ELISA protocol of commercially available kit. All the groups were sacrificed after 4 weeks to liver sample to determine (a) GOT and (b) GPT activities in hepatic tissue. All of the values were expressed as the mean ± SD from 6 mice of each group. *$P < 0.05$, significant difference compared with control. #$P < 0.05$, significant difference compared with APAP-induced liver injury. All mice were sacrificed after 4 weeks and their livers isolated and homogenated for hepatic (c) SOD and (d) CAT activities determination. The SOD activity is standardized using the cytochrome c and xanthine oxidase coupled assay. The CAT activity is expressed in the equation showed as follows: $k/\text{mg protein} = 2.3/(t_2-t_1) \times \log (A_1/A_2)$, where k is the first-order reaction rate constant, t is the time over which the decrease of $H_2O_2$ due to CAT activity was measured 30 s, and $A_1/A_2$ is the optical density at times 0 and 30 s, respectively. All of the values were expressed as the mean ± SD from 6 mice of each group. *$P < 0.05$, significant difference compared with control. #$P < 0.05$, significant difference compared with APAP-induced liver injury.(e) MDA is a marker for oxidative stress results from lipid peroxidation of polyunsaturated fatty acids. The determination of hepatic lipid peroxidation at the absorbance at 532 nm. All of the values were expressed as the mean ± SD from 6 mice of each group.*$P < 0.05$, significant difference compared with control. #$P < 0.05$, significant difference compared with APAP-induced liver injury. (f). The APAP, SM, 0.2% CLC, 0.5% CLC, and 1% CLC groups were administrated with 200 mg/kg APAP, and the normal control was administrated with PBS as placebo twice a week for 4 weeks. The Sm was fed with the chow diet containing 0.1% Silymarin (w/w). The group 0.2% CLC, 0.5% CLC, and 1% CLC were fed with the chow diet containing 0.2%, 0.5%, and 1% of CLC (w/w), respectively. All mice were sacrificed and the liver was removed, fixed, and embedded in paraffin. Histopathological photomicrographs of mouse livers of 6 groups stained with hematoxylin and eosin (H and E,200×). Collagen release is expressed by black arrows. Inflammatory cell infiltration is expressed by brown arrows**

in the mice administrated with APAP was found. However, the liver injury could be recovered by CLC supplementation added in the diet for mice. According to our knowledge, the *Chlorella* sp. crude extracts against the drug APAP-induced hepatotoxicity. The similar result in a rat model test has also been reported from the hot-water-extraction from *C. vulgaris* supplementation.[17] There is a lack of study reports on the effects of *Chlorella* sp. supplementation on APAP-induced liver injury. Our current results showed that CLC supplementation is responsible for the increased resistance to oxidative stress induced by APAP. CLC supplementation could significantly alleviate the increased SOD and CAT activities in the APAP-treated mice. Oxidative stress was reported to play a fundamental role in the pathogenesis of APAP-induced liver damage.[18-20] Lipid peroxidation, MDA, is one of the major outcomes of free radical-mediated injury that directly damages membranes and generates a number of secondary products.[21] Antioxidant function and protection against lipid peroxidation are probably the most invoked mechanisms of protection by *Chlorella* sp. extracts.[22] The antioxidant enzymes such as SOD and CAT are related to direct elimination of ROS to prevent and neutralize the free radical-induced damage.[23-25] In APAP-induced hepatotoxicity, the balance between ROS production and these antioxidant defenses may be lost; oxidative stress results through a series of events deregulates the cellular functions leading to hepatic necrosis. According to the results, we proposed such oxidative liver injury induced by APAP could be reduced by CLC supplementation.

The potential antioxidant capacity of *Chlorella* sp. has been attributed to the effect of specific ingredients in *Chlorella* sp., such as chlorophylls and carotenoids compounds. The chlorophylls and carotene effect on ROS scavenging ability has been reported, with the theory that a few water-soluble or lipid-soluble ingredients of *Chlorella* sp. can decrease oxidative stress *in vivo* and *ex vivo*.[26] Chlorophyll is not only a benefit in treating liver recovery and ulcers but is also predicted to stimulate tissue growth because the chemical structure of chlorophyll is similar to that of hemoglobin.[27-30] The present study demonstrated the *Chlorella* sp. may have protective functions such as ROS scavenger, meaning that *Chlorella* sp. could be used as a therapeutic treatment for oxidative stress-induced liver diseases such as APAP hepatotoxicity. Therefore, *Chlorella* sp. lysate may be considered as a potential source of natural antioxidant with hepatoprotective activity. Further, detailed investigations on this microalgae strain are needed to identify and isolate the hepatoprotective components in the extract and to justify its use in the treatment of liver disorders.


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### Conflicts of interest

There are no conflicts of interest.

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