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Ultrastructural Studies on The Protective Effect of The Blue Green Alga (*Aphanizomenon flos-aquae*) Against Carbon Tetrachloride-Induced Hepatotoxicity in Mice

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ABSTRACT

The present study was designed to detect the possible potential hepatoprotective effect of the aquatic blue-green alga *Aphanizomenon flos-aquae* (AFA) against hepatic intoxication induced by carbon tetrachloride (CCl4) in adult male mice. A total of 28 Swiss adult albino mice (*Mus musculus*) of CD1 strain, were allotted to four groups, each group of seven mice. Group (I) negative control group: contains normal healthy mice; group (II) positive control group: are mice who received a dose of CCl_4 (0.5 mg/kg body weight), dissolved in olive oil, through intraperitoneal injections twice a week for six weeks; group (III) AFA control group: received a daily oral dose of AFA (72.8 mg/kg body weight) for 10 consecutive days; group (IV): they are mice that were given a daily oral prophylactic dose of AFA (72.8 mg/kg body weight) for a period of 10 continuous days prior to treatment with CCl4. Carbon tetrachloride caused liver damage as evidenced by marked changes in the normal cellular structure of the hepatocytes such as swelling of the mitochondria, loss of glycogen rosettes, condensation of chromatin, and loss of definitive shape of the nucleus. However, the protection offered by *Aphanizomenon flos-aquae* suppressed significantly cellular damage caused by CCl_4; as indicated by the normal nucleus and nuclear chromatin distribution, increase in glycogen content, and normal mitochondrial structure. These results substantiated the potential hepatoprotective activity of AFA.

INTRODUCTION

Chronic liver diseases (CLDs) are a serious health problem in the recent decades **(Moon *et al.*, 2020; Cheemerla and Balakrishnan, 2021)**. Despite the etiology, CLDs follow the same common sequence. The first step is incidence of injury which initiates the inflammatory response followed by activation of a group of inflammatory cells and cytokines. Fibrosis is the next step which is very critical. It is known as healing process in which activation of hepatic stellate cells (HSCs) occurs. The hepatic stellate cells are in rest state in case of the normal liver; but once activated, they are transformed into active myofibroblasts. Myofibroblasts are responsible for production of components of

extracellular matrix (ECM) such as collagen type ı and collagen type ıı, and growth factor-β1(GF-β1). Deposition of components of ECM aids in replacing the damaged tissue and accelerates healing process. Growth factor-β1 helps in activation of more hepatic stellate cells (HSCs) **(Kisseleva and Brenner, 2021)**. In case of healthy liver, fibrogenesis process is a balanced process; but in case of incidence of continuous injuries, more HSCs are activated, and as a result more ECM components are secreted. Overproduction of collagen leads to formation of fibrous scar. These sequences lead to destruction of the hepatic architecture as the fibrous tissue replaces almost all the normal tissue **(Roehlen *et al.,* 2020)**. Preventing progression of fibrosis is a very important step, as progression of fibrosis ends up to the next severe stage which is known as cirrhosis. Cirrhosis is followed by many complications as hepatocarcinoma, liver dysfunction and eventually death. Treating fibrosis is a pivotal step. In case of early stages etiological treatment by removing causative agent is the best option **(Tan *et al.,* 2021)**. In case of late stage; there are many strategies such as pharmaceutical medicines, herbal medications, medication with natural products, and advanced genetic therapy **(Zhang *et al.,* 2023)**.

Recently, there is an growing interest in the natural products as a remedy, due to their benefits and low risk or side effects **(Shan *et al.,* 2019)**. The most common natural products used nowadays are the blue green algae (BGA) **(Vu *et al.,* 2020)**. The distribution of BGA is very wide as they can be aquatic found in both fresh and marine water or terrestrial **(Dodds *et al.,* 1995; Nuzzo *et al.,* 2018)**. One of the most unique feature of BGA is their high ability to endure the extremes.

One of the most promising blue green alga is *Aphanizomenon flos-aquae* (AFA). The rich profile of AFA makes it one of the most powerful members of the BGA. It possesses various properties as it is considered as anti-inflammatory **(Cavalchini and Scoglio, 2009; Abdelhafez *et al.,* 2018)**, anti-oxidant **(Benedetti *et al.,* 2010; Nuzzo *et al.,* 2018)**, protective agent and immune-modulatory agent **(Babusyte *et al.,* 2013)**. Anti-oxidant properties of AFA is because of its concentrated content of pigments among all BGA especially phycocyanin that is powerful anti-oxidant **(Benedetti *et al.,* 2006; Rinalducci *et al.,* 2009; Nuzzo *et al.,* 2018)**. Thanks to presence of prosthetic groups of protein phycocyanibilins (PCB), the AFA shows significant anti-oxidant and anti-inflammatory properties **(Zizzo *et al.,* 2020)**.

According to all these properties, we aim to evaluate the ameliorative effect of AFA on cellular structure of hepatocyte against induced-hepatotoxicity in the mice.

MATERIALS AND METHODS

The present study comprised a total of 28 Swiss adult albino mice (*Mus musculus*) of CD1 strain that were purchased from Theodore Bilharz Research institute (TBRI), Imbaba, Giza, Egypt. Each mouse was weighing about 24 g. The mice were housed in an animal house with a natural 12 hr light/dark cycle at 27°C. They had free access to pellet

diet and tap water. All animals were healthy and allowed to adapt to laboratory conditions for at least one week beforehand any treatment or tests.

Drug preparation and administration regimes:

AFA (*Aphanizomenon flos-aquae*) is a blue green alga; purchased from RAAD ALGEN, Hamburg, Germany. AFA (72.8 mg/kg body weight) was prepared according to **Paget and Barnes** equation **1964** for oral administration in distilled water, one dose daily for 10 consecutive days. The calculation of the mice dose was as follows: human body weight × human dose × 0.0026

The mice used in this study were randomly allotted to 4 groups, each group of 7 mice:

Group (1): Negative control group:

The mice received only pellet diet and water *ad libitum* and did not receive any treatment.

Group (2): Positive control group:

The mice injected with a dose of carbon tetrachloride (0.5 mg/kg body weight) dissolved in olive oil through intraperitoneal injection (i.p.) twice-a-week for six weeks.

Group (3): AFA control group:

The mice treated with a daily oral dose of AFA (72.8 mg/kg body weight) for 10 consecutive days.

Group (4): AFA 'prophylactic dose' + CCl4 group:

The mice pretreated a daily oral prophylactic dose of AFA (72.8 mg/kg body weight) for 10 continuous days before the treatment with CCl4 as the mice were injected with a dose of carbon tetrachloride (0.5 mg/kg body weight) dissolved in olive oil through intraperitoneal injection (i.p.) twice-a-week for six weeks.

The preparation of the semithin and ultrathin sections:

At the end of the experiment, the mice were sacrificed; and the livers were immediately dissected out. The tissue specimens were immediately fixed in cold (4℃) glutaraldehyde solution in phosphate buffer (pH 7.2) for two hours, and then were washed thoroughly in cold phosphate buffer for two hours. The specimens were then post-fixed in cold 1% osmium tetroxide (OsO_4) in 0.2 M phosphate buffer for two hours at 4℃. The post-fixed materials were washed two times (eash for 30 minutes) in cold phosphate buffer. Then, they were dehydrated through ascending series of ethyl alcohol (50%, 70%, 80%, 90%, 95%) each for 15 minutes, followed by two changes in absolute alcohol (15 minutes each).

For resin infiltration, the specimens were treated with pure acetone for three times for 10 minutes each, then in mixture of acetone and resin as follow: resin and acetone in ratio of 1:1 for 1 hour, then in resin and in concentration of 2:1 of acetone for one hour. Finally, the tissue specimens were transferred to rubber molds, which were filled with pure resin. The samples were then kept for 48 hours in an oven adjusted at 60℃.

The polymerized resin blocks were trimmed and sectioned at 0.5 µm thickness to get semithin sections on Lecia EMUC6 ultra-microtome using glass knives. The sections were stained with 1% toluidine blue for a few seconds on a hot plate, and then examined to select the suitable areas for ultrathin sectioning.

Silver-gold ultrathin sections (70-80 nm in thickness) were cut and mounted on uncoated copper grids. Then the grids were stained firstly with uranyl acetate followed by lead citrate, examined and photographed using a JEOL-JEM 1010 at 80 kV transmission electron microscope (Tokyo, Japan) at the Regional Mycology and Biotechnology Centre at Al-Azhar University, Egypt.

RESULTS

Ultrastructural investigations of livers of the negative control (untreated) and AFA-treated groups showed normal nuclear and cytoplasmic structures of the hepatocytes with normal organelles. The nucleus of the liver cells was surrounded by nuclear envelope with nuclear pores. There were two types of normally distributed chromatin: heterochromatin and euchromatin. Each nucleus contained one nucleolus, and some nuclei contained two nucleoli. Numerous cytoplasmic organelles were observed such as: mitochondria, rough endoplasmic reticulum, smooth endoplasmic reticulum, and Golgi complex. Glycogen was normally distributed in the form of rosette-shaped deposits. There was a bile canaliculus between the adjacent cells (Fig. 1A and B). Cell junctions were observed between the two adjacent hepatocytes and consisted of tight junction and desmosomes (Fig. 1B). Worthy to mention that treatment with AFA did not change any subcellular feature of the hepatocytes.

Liver sections of positive control group (Fig. 1C and D) revealed marked organelle injury. Numerous damages were observed such as swollen mitochondria, marked depletion in glycogen content, deposition of numerous intense electron-dense bodies, condensation of nuclear chromatin and irregular nuclear envelope. Also, sinusoidal area of the positive control group (Fig. 1E) revealed obvious damage in Kupffer cell represented in highly degenerated cytoplasm and pyknotic nucleus. Furthermore, the endothelial cell showed marked damages such as deposition of electron dense bodies, disorganization of the rough endoplasmic reticulum and vacuolization of the cytoplasm. Also, the smooth endoplasmic reticulum was highly-dilated; and cristolysis was observed in the swollen mitochondria.

On the other hand, electron-micrographs from AFA-prophylactic group (Fig. 1F and G) revealed improved cellular structures with mild pathological signs compared with the positive control group. The nucleus of the hepatocyte was normal with intact membranes. Also, the nuclear chromatin was normally-distributed and not clumped, and the nucleolus was normal. The cytoplasmic organelles of the hepatocyte appeared almost normal with

less mitochondrial swelling compared with the positive control group. Lipid droplets and large deposites of rosette-shaped glycogen were clearly found.

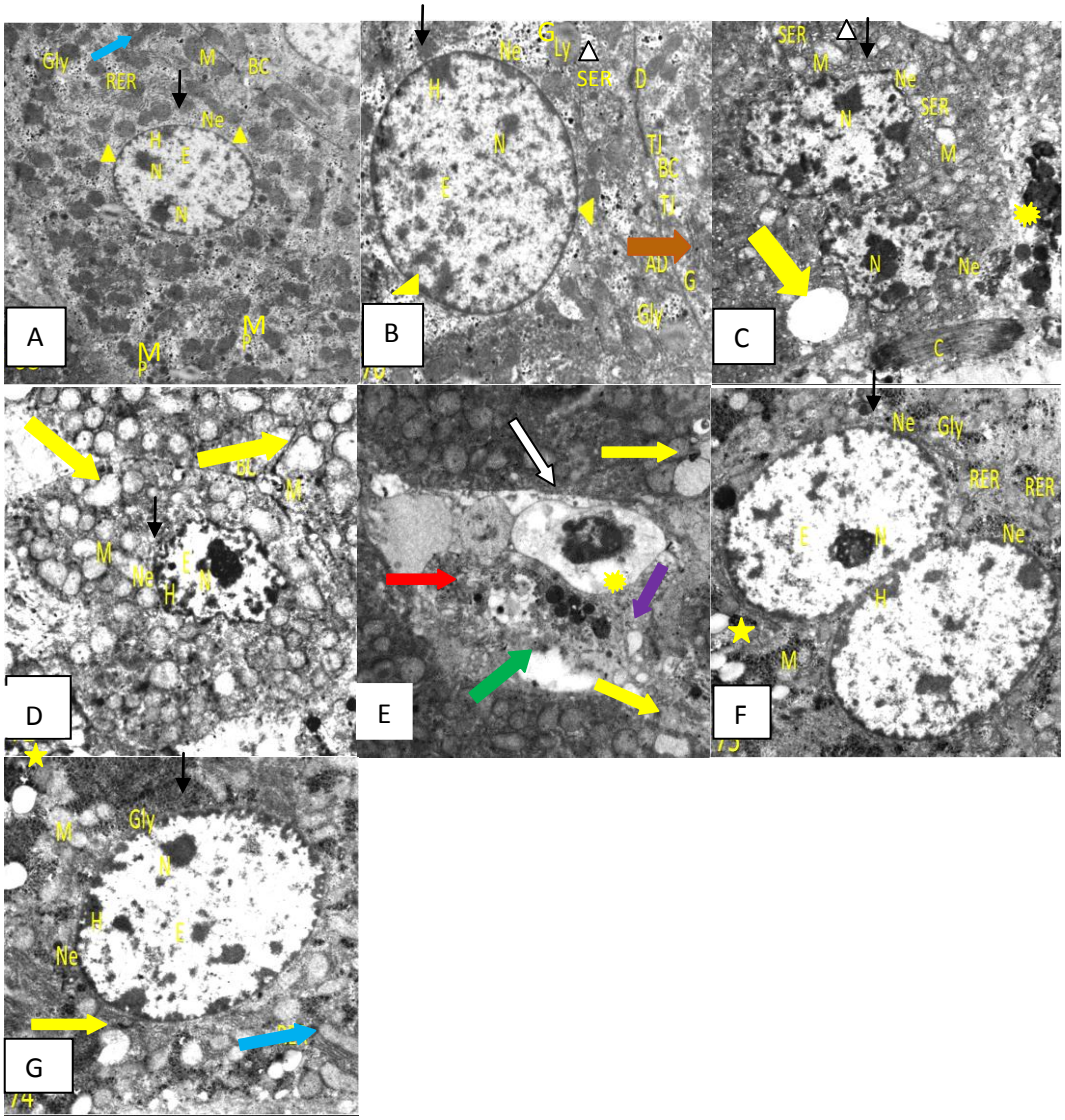

Figure (1): Transmission electron micrographs of liver sections: **(A)** Negative control group showing a hepatocyte which contains central normal nucleus surrounded by the nuclear envelope (Ne) (black arrow) with nuclear pores (yellow arrow heads), the nucleus contains normally-distributed chromatin as there are two types of chromatin: heterochromatin (H) and euchromatin (E). The nucleus contains normal nucleolus (N) which sometimes be more than one. Cytoplasmic organelles such as: mitochondria (M) and rough endoplasmic reticulum (RER) are intact. Glycogen (Gly) is normally-distributed in the cytoplasm. A bile canaliculus (BC) lies between two adjacent hepatocytes. **(B)** *Aphanizomenon flos aquae* control group: the hepatocyte contains central nucleus surrounded by nuclear envelope (Ne) (black arrow) with nuclear pores (yellow arrow head). The nucleus contains heterochromatin (H) and euchromatin (E). The hepatocyte contains cytoplasmic organelles such as: Mitochondria (M), rough endoplasmic reticulum (RER)(blue arrow), Golgi complex (G) (brown arrow) and smooth endoplasmic reticulum (SER) (white head arrow). There is normal distribution of glycogen (Gly) in the cytoplasm. Note the presence

of bile canaliculus (BC) and the cell junctions such as tight junction (TJ) and desmosome (D). **(C and D)** Positive control group revealed some cellular damages caused by CCl_4 . The nucleus is with irregular nuclear envelope (Ne) (black arrow), and abnormal distribution of heterochromatin (H) and euchromatin (E). Notice severe intense electron-dense bodies in the cytoplasm (asterisk), decrease in the glycogen content, deposition of collagen (C) at periphery, and swollen mitochondria (yellow arrow). **(E)** positive control group of sinusoidal area reveals higly-degenerated cytoplasm of Kupffer cell with pyknotic nucleus (white arrow), highly-vacuolated endothelial cell (green arrow) with disorganization of the RER (red arrow) and electron-dense bodies (asterisk). Notice the highly-dilated SER (violet arrow) and swollen mitochondria with cristolysis (yellow arrow). **(F and G)** *Aphanizomenon flos-aquae* prophylactic group shows almost normal hepatocytes with normal nuclei each is surrounded by intact nuclear envelope (Ne) (black arrow), and contains normal nucleolus and normal distribution of heterochromatin (H) and euchromatin (E). Glycogen (Gly) content and rough endoplasmic reticulum (RER) (blue arrow) are normal. Notice that there are some lipid droplets (star) and swollen mitochondria (yellow arrow).

DISCUSSION

The current study was performed to assesses the ameliorative potency of the aquatic blue green alga (AFA) against hepatotoxicity in CCl_4 -treated mice model. It is a trial to find a natural substitute for synthetic drugs with good pharmaceutical properties to protect from hepatotoxicity.

Administration of a dose of CCl_4 (0.5 mg/kg body weight) through intraperitoneal injection in this study showed severe cellular damages in the hepatocytes such as: mitochondrial swelling, decrease in glycogen content and abnormalities in the nucleus and the nuclear content. **Üstüner *et al.* (2021)** reported that injection with 0.2 CCl4 for 10 days showed similar features such as swelling in the mitochondria and abnormal condensation of the nuclear chromatin. Also, **Li *et al.* (2021)** and **Unsal *et al.* (2021)** demonstrated that carbon tetrachloride induced hepatic injury by producing highly-toxic free radicals of trichloromethyl radical ($\bullet CCl_3$) and trichloromethyl peroxyl radical ($\bullet CCl_3\ O_2$) through metabolization of CCl_4 *via* cytochrome P450. The free radical contains more than one unpaired electrons in the atomic orbitals; which are responsible for induction of a chain reaction, and attack unsaturated fatty acids in the membranes of cytoplasmic organelles of high phospholipid content such as mitochondria and endoplasmic reticulum, resulting in lipid peroxidation **(Üstüner *et al.*, 2021).**

In the current study, CCl_4 treatment caused severe swelling in the mitochondria. **Zhao *et al.* (2021)** showed that the mitochondria contain phospholipid bilayers, and especially in the inner membrane; as it is the major site of cellular energy production and elimination of free radicals. The excessive free radicals produced by CCl_4 through its metabolization in SER by cytochrome P450 cause mitochondrial stress. According to **Tang *et al.* (2006)**, lipid peroxidation of the membranes of the mitochondria results in the decline in the performance of the ATP-dependent sodium pumps accompanied by increasing in the cytoplasmic $Ca^{+\ +}$. Increment of hepatocellular $Ca^{+\ +}$ is followed by

the excessive deposition of the sodium in mitochondria which leads to the swelling of the mitochondria.

Our data manifested that using AFA-prophylactic dose (72.8 mg/kg body weight/day) caused obvious improvement in ultrastructural features of the hepatocytes. One of the most important constituents of AFA is phycocyanin. **Vadiraja *et al*. (1998)** demonstrated that phycocyanin has powerful anti-oxidant properties by inhibiting cytochrome p450-mediated reactions which aid in metabolization of CCl_4 into toxic metabolities. As well, a study performed by **Bhat and Madyastha (2000)** showed that phycocyanin reduced CCl4-induced lipid peroxidation *in vivo*. Intraperitoneal injection of phycocyanin (50-200 mg/kg body weight) 3 hr prior to CCl4 treatment led to significant decline in production of malondialdehyde compared with rats injected only with CCl4. In case of CCl4 intoxication, metabolization of CCl_4 resulted in toxic free radicals which aid in promoting oxidative stress, which induces lipid peroxidation. It was observed that protection by phycocyanin against CCl4-induced lipid peroxidation may be due the capability of phycocyanin to scavenge peroxyl free radicals **(Bhat and Madyastha, 2000)**.

In the current study, oral administration of prophylactic dose of AFA protected the mitochondria against mitochondrial damages caused by oxidative stress produced by metabolities of CCl_4 ; and as a result AFA-ameliorated group showed no mitochondrial swelling compared with the group treated with CCl_4 only. These results are in agreement with **Khalil *et al.* (2020)** who reported that pre-treatment using blue green alga (spirulina) 300 mg/kg body weight once daily for four weeks suppressed the lipid peroxidation which is responsible for mitochondrial damage and endoplasmic reticulum stress due to presence of phycocyanin. **Niu *et al.* (2017)** who performed a study in which different concentrations of phycocyanin (2, 5, 8, 10 μg/ml) were added to porcine zygote medium 5 during *in vitro* culture showed similar results as he demonstrated that administration of phycocyanin improved the mitochondrial structure and membranes and showed decline in the number of the swollen mitochondria against H_2O_2 -induced oxidative stress.

In the current study, the treatment with CCl_4 showed high dilatation in RER of the hepatocyte, while the nucleus possessed irregular envelope and clumped chromatin. **Tasci *et al.* (2008)** reported that rats treated with CCl4 for 12 weeks (twice-a-week) showed disorganization of content of nucleus as margination and clumping of the chromatin. **Batool *et al*. (2018)** showed that treating rats with CCl_4 for four weeks on alternate days induced disturbance of endoplasmic reticulum, known as ER stress due to CCl_4 metabolites.

The peripheral margination and clumping of chromatin noticed in this work CCl_4 -treated groups are hallmarks of dying and dead cells. Also, other nuclear changes such as irregularity of nuclear envelope was found to be associated with some insults such as ischemia, X-ray irradiation, viral infection and after certain chemotherapeutic agents

(Underwood, 1990). Changing nucleocytoplasmic ratio can be postulated to be a compensatory change reflecting the enhanced cellular metabolism to combat the injurious effect of CCl_4 on the hepatocytes.

Carbon tetrachloride showed a rapid and prolonged depletion of liver glycogen in the mice. In glycogenesis, glycogen is synthesized from glucose monomers. Extracellular glucose enters the cell through a glucose transporter on the hepatocyte membrane **(Feng *et al.*, 2020)**. Depletion of glycogen may be due to decrease in glucose-6-phosphatase as a result of oxidative stress induced by CCl_4 ; decreasing of this enzyme leads to decline in the content of glucose, and eventually depletion of glycogen **(Jiang *et al.*, 2012)**. Also, **Semenovich *et al.* (2023)** reported that treating rat with oral dose of CCl4 (1.0 ml/kg body weight), dissolved in petrolatum oil, 3 times a week for 6 weeks showed marked depletion in glycogen, and that may be due to the demand of hepatocytes for glucose to synthesize UDP-glucuronate which is required for glucuronidation reactions to detoxify liver toxins, which in this case is CCl_4 .

The depletion in hepatic glycogen may be due to abnormal change in glycogen metabolism produced by CCl_4 . I-form of glycogen transferase is the promotor of activation of glycogen phosphorylase; which is important enzyme in glycogenesis process. Oxidative stress caused by toxic free radicals of CCl_4 leads to a decrease in the activity of I-form of glycogen transferase, as a result, activity of glycogen phosphorylase declines and leads to decrease in the formation of glycogen, and eventually depletion in the cytoplasm as shown by **Hickenbottom and Hornbrook (1971)**. Also, a previous report of **Tang *et al*. (2006)** showed that the maintenance of the mitochondrial membranes is important for mitochondria to perform their functions. The depletion of content of rosette-shapd glycogen in the liver cells can be due to the decline in the ATP production because of the damage in the mitochondrial membranes.

In present study administration of AFA (72.8 mg/kg body weight/day) daily for ten days prior to treatment with CCl_4 showed a marked increase in glycogen content unlike when mice were treated with CCl_4 only. **Ren *et al.* (2018)** reported that administration of phycocyanin showed increased in content of glycogen and excessive activation of glycogen synthase (GS) by suppressing the reduction of phosphorylation of glycogen synthase kinase 3 (GSK-3). Phycocyanin induces phosphorylation of GSK-3, which is responsible for activation of GS. Activation of GS leads to the conversion of glucose to glycogen which eventually results in increasing in glycogen content.

AFA showed ameliorative properties thanks to its concentrated content of β-carotene, phycocyanin and vitamins; these are in agreement with **Alam *et al.* (2013)** who showed that the presence of β-carotene, enzyme superoxide dismutase, vitamins or selenium in blue green algae produces a protective effects against paracetamol-induced liver damage. As well, **Abdel-Daim *et al.* (2013)** who performed an experiment on spirulina observed that sirulina at doses of 500 and 1000 mg /kg respectively, 1.0 hr before induction of hepatotoxicity showed hepatoprotective effect. That hepato-protective effect of the blue

green algae may be due to their rich profile of bioactive components such as: β-carotene, blue pigment phycocyanin, linolenic acid, sulphated polysaccharide, and vitamins, which induce the activity of free radical scavenging enzyme system that stimulates hepatic protection. According to **Pleonsil *et al.* (2013) –** who performed a study on c-phycocyanin of *Spirulina* **–** he confirmed that c-phycocyanins can act as radical scavenger in oxidative stress-induced diseases; and it has strong antioxidant and anti-inflammatory proprieties.

CONCLUSION

In conclusion, ultrastructural results included in this work demonstrate that the blue green alga *Aphanizomenon flos-aquae* (AFA) has a pharmacologically-promising positive ameliorative activity on CCl_4 -induced hepatotoxicity.

For better understanding of how AFA could protect the cellular organelles from hepatotoxins, further studies on antioxidant capacity, lipid peroxidation protection and prophylaxis of liver function should be performed.

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