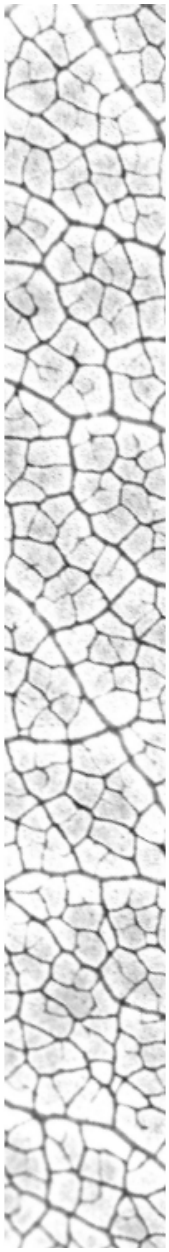

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Health Protective Actions of Phycocyanin Obtained from an Egyptian Isolate of *Spirulina platensis* on Albino Rats

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Abstract

Background: The objective of the present study was to investigate the potential hepatoprotective of phycocyanin as a natural source for antioxidant against oxidative stress induced artificially by CCl_4 liver injury in Wister rats.

Material and Methods: The antioxidant activity of phycocyanin isolated from *Spirulina platensis* was evaluated *in vitro* by using DPPH assay. Also, hepatoprotective effect of phycocyanin at different levels (100, 150 and 200 mg/kg body weight) was evaluated in Wistar Albino rats against carbon tetrachloride induced liver injury.

Results: The hepatoprotective effect was estimated using several biochemical parameters and histopathological examination. *Spirulina platensis* phycocyanin, applied at different levels, could counteract the major rises in the levels of serum ALT, AST, urea and creatinine, total lipid and triglycerides, induced by subjecting Albino rats to CCl4 intoxication. It could also moderate the reducing action of CCl_4 exerted on the levels of antioxidant enzymes (glutathione-S-transferase, superoxide dismutase and catalase). The negative control group showed a normal histological structure of the portal area, central veins and surrounding hepatocytes. Focal necrosis with inflammatory cells infiltration was detected in the hepatic parenchyma as associated with dilatation in the portal vein and inflammatory cells infiltration in the portal area of the positive control. The histopathological sections of the CCl_4-stressed animals receiving phycocyanin at different concentrations were approaching the status of the negative control.

Conclusions: Phycocyanin can be recommended for food and health applications aiming at lowering potential oxidative stress.

Keywords: *Spirulina platensis*, Phycocyanin, antioxidant, *Cyanobacteria,* CCl_4

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INTRODUCTION

Liver injury is caused by different agents, such as virus, chemicals, alcohol, and auto-immune diseases. Carbon tetrachloride (CCl_4), is an industrial hepatotoxic solvent that can artificially induce oxidative stress through its transformation into free radicals in animal tissue of different organs including liver, kidneys, heart, lung, testis, brain and blood (Jiang *et al.*, 2012). It induces acute hepatotoxicity through metabolic activation of reactive metabolites triggering lipid peroxidation, and leading finally to a number of pathological situations (Manibusan *et al.* 2007). Development of suitable antioxidant molecules is gaining much importance as it plays a key role, in preventing or delaying hepatotoxicity. Butylhydroxyanisole (BHA) and butylhydroxytoluene (BHT) are the generally used as artificial antioxidants but they may be associated with potential health risks and toxicity. The use of artificial oxidants has diminished for fear of any possible carcinogenic effects. So, there is crucial need to substitute them with new harmless natural antioxidants. Phycocyanins (a biliprotein pigment found in the blue-green algae) appear to be good candidates, since they can provide 20 times more antioxidant activity than ascorbic acid (Romay *et al.* 2003). The therapeutic activity of phycocyanin are largely attributed to its antioxidant potentials via its hydroxyl and peroxyl free radical-scavenging properties (Farooq *et al.* 2014). Phycocyanin is a phycobiliprotein which is composed of bilins and apoprotein, where bilins are the linear tetra pyrrole molecules (Dammeyer and Frankenberg-Dinkel 2006). The objective of the present study was to investigate the potential hepatoprotective of phycocyanin as a natural source for antioxidant against oxidative stress induced artificially by CCl_4 liver injury in Wister rats.

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MATERIALS AND METHODS

Micro-organism and Culture Preparation

The indigenous cyanobacterial strain (*Spirulina platensis*) used in the present study was previously obtained from Dr. Ali Salama Assistant Professor of Microbiology- Microbiology Department- Faculty of Agriculture- Zagazig University- Zagazig-Egypt. Zarrouk medium used to cultivate the pure culture of *Spirulina* strain containing the following constituents (all in g liter^{-1} $NaHCO_3$ (16.8 g); K_2HPO_4 (0.5 g); $NaNO_3$ (2.5 g); NaCl (1.0 g); $MgSO_4.7H_2O$ (0.2 g); $FeSO_4.7H_2O$ (0.01 g); K_2SO_4 (1.0 g); $CaCl_2$. $2H_2O$ (0.04 g); EDTA (0.08 g). The *Spirulina platensis* SOS11 culture was grown at 28±2 °C with an illumination of 2000 lux light intensity culture flasks of 250 mL volume were used and were each flask supplied with 300–400 mL of air:min^{-1}. The cultures were grown for a period of 10 days.

Phycocyanin Extraction and Purification

Phycocyanin was extracted from the fresh *Spirulina platensis* strain biomass using the modified methods of (Sarada *et al.* 1999) as previously described (Sitohy *et al.*, 2015). The levels of the phycocyanins (phycocyanin (C-PC), allophycocyanin(C-APC) andphycoerythrin(C-PE) concentrations were determined using the spectrophotometric method of (Boussiba and Richmond 1979). The contents of phycocyanin components were calculated following equations:

$$C-PC\ (mg/ml) = (OD620 - 0.7OD650)/7.38 \text{ (A)}$$

$$C-APC\ (mg/ml) = (OD650 - 0.19OD620)/5.65 \text{(B)}$$

$$C-PE\ (mg/ml) = (OD540 - 2.8[C-PC] - 1.34[C-APC]/12.7 \text{(C)}$$

Quantitative Determination of Total Amino Acids

Total amino acids composition of phycocyanin was determined by amino acid analyzer apparatus model “Eppendorf LC3000”. The peak area and percentage of each amino acid were calculated by computer software AXXIOM CHROMATOGRAPHY- 727**.**

Antioxidant Activity Evaluation (DPPH Radical-Scavenging Activity)

The antioxidant activity of phycocyanin at different concentrations (40, 80, 160, 320, 640, 1250 and 2500 µg/mL) isolated from *Spirulina platensis* was evaluated by using DPPH-assay according to the method described by (Hatano *et al.* 1988).

The radical scavenging capacity of the samples (0.5 mL sample + 3mL 0.1 mM DPPH dissolved in ethanol) was measured at 517 nm after incubation period of 30, min at room temperature and was calculated using the following equation.

$$Radical\ scavenging\ activity\ (\%) = \frac{Abs.control - Abs.sample}{Abs.control} x100$$

The SC_{50} (the concentration of the sample that scavenges 50% of the DPPH radicals) was calculated by linear regression of curves showing percentage scavenging versus sample concentration.

Anticancer Activity (MTT-Assay)

The cytotoxic activity of *S. platensis* phycocyanin was assessed in MCF-7 cell lines. The cells were plated in 96-well plates (1 × 10^5 cells per well) in triplicate and incubated overnight at 37 °C. After 24 h, the *Spirulina* phycocyanin was added from a stock diluted to concentrations ranging from 400 to 1600 µg/mL. A volume of 50 mL of each concentration of *Spirulina* phycocyanin was added in triplicate to selected wells. The cells were then incubated for 24 h. Following incubation, 15 mL of the MTT labeling reagent was added to each well and incubated in a humidified atmosphere at 37 °C for 4 h. Following incubation, 100 mL of the solubilizing reagent, sodium dodecyl sulfate (10%) was added to each well and mixed gently for 1 h at room temperature. The absorbance of each well was measured at 570 nm using a spectrophotometer and inhibition rate was calculated as follow:

$$Inhibition\ rate\ (\%) = \text{A570 nm of control} - \text{A570 nm of tested material} / \text{A570 nm of control}$$

Hepatoprotective Effect of Phycocyanin in Rats

In the present study, we report the effect of phycocyanin on carbon tetrachloride induced hepatotoxicity in rats.

Biological Experiment

The experiment was conducted on 25 male Wistar rats, they were housed in screen-bottomed aluminum cages in rooms maintained at 25 ±1 °C with alternating cycles of light and dark of 12 h duration (El-Saadany et al. 1991, Sitohy et al. 2013). The animals were fed on basal diet according to AIN-93 guidelines (Reeves *et al.,* 1993) and were provided with water ad libitum during the experimental period. The animals were randomly divided into five groups with five rats in each group. Group one was reserved as normal control (NC), groups two–four animals were administrated intraperitoneal (IP) injection with single dose of 0.5 mL/kg body weight (50 % CCl_4/corn oil). Group two kept as injury control (IC), groups three, four and five received phycocyanin at doses of 100, 150 and 200 mg/kg body weight/day, respectively for 28 days.

Biochemical Determination in Blood

Blood samples were collected at end of experiment obtained from the retro-orbital plexus veins from individual rats by means of fine capillary heparinized tubes, and were allowed to clot. Serum was separated by centrifugation at 3000 *xg* for 15 min. and was used to investigate the biochemical parameters including function tests of liver and kidney. Determinations were done on activities of liver enzymes of alanine transaminase (ALT) aspartate transaminase (AST), creatinine, urea, as well as serum total protein and

serum albumin (Bradford 1976, Doumas 1975, Reitman and Frankel 1957). Globulin was calculated by subtracting the albumin from serum total protein. Kidney function parameters of urea and creatinine were determined by (Tabacco *et al.* 1979).

Antioxidant Markers

Liver samples were washed immediately with ice-cold saline to remove excess blood. Liver tissue was homogenized in cold 0.1M potassium phosphate saline (pH =7.4) at extraction ratio of 1:9 w/v. The homogenate was centrifuged at 5000 *xg* for 10 min at 4 ˚C, and then the supernatant was analyzed for antioxidant markers. Determinations were done on lipid peroxides "malondialdehyde, MDA" (Uchiyama and Mihara 1978), glutathioneperoxidase(GPx), measured spectro-photometrically using Ellman's reagent [5,5'-dithiobis-(2-nitrobenzoic acid) "DTNB"] (Moron *et al.* 1979). Glutathione-S-transferase activity was determined using aromatic substrate by monitoring change in absorbance due to thioether formation (Habig *et al.* 1974).

Histopathological Examination

Small tissue specimens were collected from fresh liver and rapidly fixed in 10 % neutral buffered formalin. After proper fixation, thin paraffin sections were routinely prepared and stained with Hematoxylin and Eosin stain (H&E) for the histopathological lesions in hepatic and renal tissues. The liver sections were graded numerically to assess the degree of histopathological features of acute hepatic injury. Hepatocyte necrosis, fatty change, hyaline degeneration, ballooning degeneration, and infiltration of Kupffer cells and lymphocytes were prominent in the histological findings.

Statistical Analysis

The data were analyzed using the statistical package program SPSS version 23 Software for Windows. The results were expressed as mean ±SE of studied groups using the analysis of variance test (one-way ANOVA) followed by Post Hoc tests using LSD to make multiple comparisons between all studied treatments. Pearson correlation coefficient was done between all parameters to measure how strong a relationship is between them. IBM Corp. Released 2013. IBM SPSS Statistics for Windows, Version 23.0. Armonk, NY: IBM Corp.

RESULTS AND DISCUSSION

Chemical Characterization of Phycocyanin

Cyanobacterial phycobiliproteins are classified into three main groups: phycocyanin (C-PC), phycoerythrin (C-PE) and allophycocyanin (C-APC) depending on inherent colour and absorbance properties. The absorption maximam for C-PC, C-PE and C-APC are between 610 - 620, 540 - 570, and 650 - 655 nm, respectively (Bermejo *et al.* 2003, Reis *et al.* 1998). The obtained result showed its highest concentrations of the three phcocynin components in the following order; C-PC> C-APC > C-PE (98 ±9, 64 ±8, 14 ±6 µg/mL, respectively) (Data not shown). The result obtained by (Salama *et al.*, 2015) was 112.7, 70.9 and 16.9 µg/mL for C-PC > C-APC > C-PE, respectively. The amino acids composition of phycocyanin isolated from *Spirulina platensis* were listed in **Table 1**. The contents of the hydrophobic amino acids residues (Pro, Gly, Ala, Val, Ile, Leu, Phe) is around 41.28 % (16.02+ 3.08+ 2.84+ 3.14+ 3.2+ 9+ 4, respectively) of the total amino acids. The content of the acidic amino acid residues (asp + glu; 18.45 + 13.46, respectively) is higher than that of the basic amino acids (arg + lys + his; 7.8 + 5.4 + 1.5, respectively). In Gram-negative bacteria this adsorption is followed by insertion of the peptides into the outer membrane structure stimulated by hydrophobic interaction (Abdel-Hamid *et al.* 2016, Abdel-Shafi *et al.* 2016, Osman *et al.* 2016, 2018, Sitohy *et al.* 2013) the antibacterial peptides then cause destruction and permeabilisation of the cytoplasmic membrane.

Table 1. Amino acid composition (g amino acid / 100 g phycocyanin protein) of phycocyanin isolated from Spirulina

Amino acids	Concentration (g / 100 g phycocyanin)
Aspartic	18.45
Threonine	3.26
Serine	6.55
Glutamic	13.46
Proline	16.02
Glycine	3.08
Alanine	2.84
Cystine	0.52
Valine	3.14
Methionine	---
Isoleucine	3.2
Leucine	9
Tyrosine	0.65
Phenylalanine	4
Histidine	1.5
Lysine	5.4
Arginine	7.8

Antioxidant Activity of Phycocyanin

DPPH radical scavenging activity (RSA) of phycocyanin at different concentrations (40, 80, 160, 320, 640, 125 and 2500 µg/ mL) was observed in **Fig. 1A**. It can be noted that, the antioxidant activity of phycocyanin increased gradually with increasing concentration. The respective SC_{50} values were determined and the calculated SC_{50} value of the phycocyanin was 104 µg/mL. The SC_{50} of phycocyanin from *Limnothrix* was about 80 µg/mL (Gantar *et al.* 2012) but still comparable to antioxidant activity of rutin at a SC_{50} value of 55 µg/mL (Čanadanović-Brunet *et al.* 2009). Some amino acids, such as histidine, tyrosine, methionine, and cysteine, have been reported to show antioxidant activity (Abdel-Hamid *et al.* 2017, Osman *et al.* 2014). In particular, histidine exhibited strong radical scavenging activity due to the decomposition of its imidazole group (Xie *et al.* 2008). (Sarmadi and Ismail 2010) described the mechanisms of action of amino acids: and they showed that aromatic amino acids

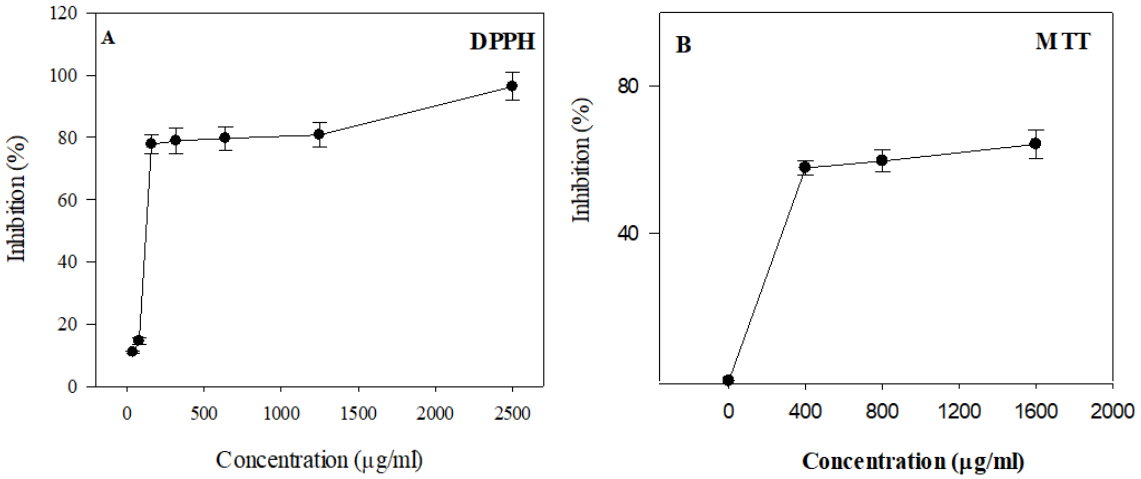

Fig. 1. DPPH radical scavenging activity (A) of phycocyanin at different concentrations (40, 80, 160, 320, 640, 125 and 2500 µg/ ml) and Anti-proliferative action (B) of phycocyanin at different concentrations (400, 800 and 1600 µg/ml) against MCF-7 cell line by MTT assay.

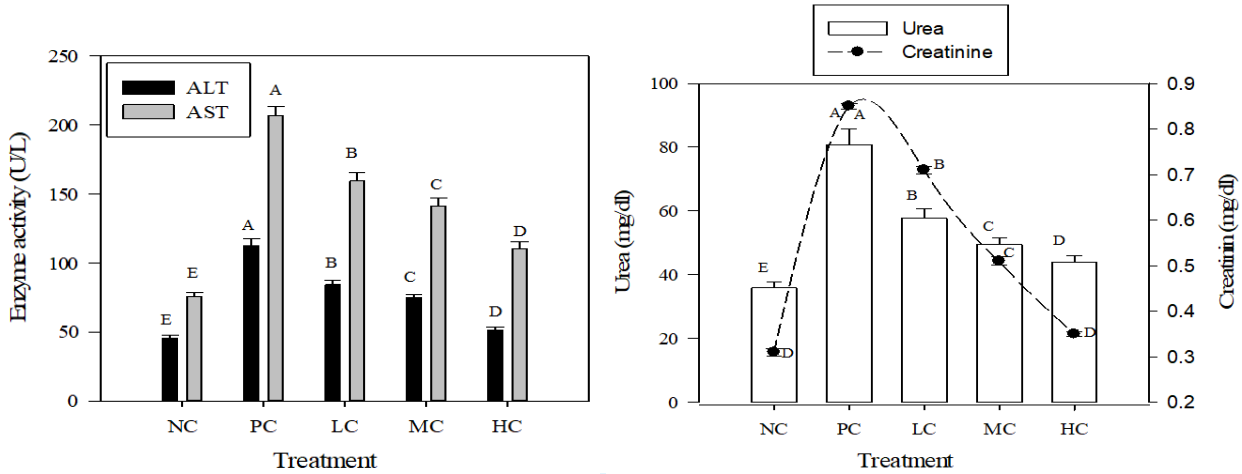

Fig. 2. Effect of phycocyanin treatments at different concentrations (100, 150 and 200 mg/ kg body weight) on the activities of ALT and AST (A) and on the serum urea and creatinine **(B)** in normal (NC) and CCl_4-treated rats (PC). NC: normal control; PC: positive control; LC: low concentration (100 µg/ml); MC: medium concentration (150 µg/ml); HC: high concentration (200 µg/ml)

convert radicals to stable molecules by donating electrons while keeping their own stability; Also the hydrophobic amino acids enhance the solubility of peptides in lipids, which facilitates accessibility to hydrophobic radical species; while the acidic and basic amino acids contain carboxyl and amino groups in their side chains, which act as chelators of metal ions and as hydrogen donors. Dietary use of antioxidants has been shown to promote health by increasing antioxidant capacity (Samaranayaka and Li-Chan 2011).

Anticancer Activity (MTT-assay)

The data depicted in **Fig. 1B** show clear concentration-dependent inhibition in the proliferation of MCF-7 cell line. This inhibition was 58% at the lowest used concentration of phycocyanin (400 µg/mL) and increased up to 60 % and 64 % when increasing the concentration up to 800 and 1600 µg/mL, respectively. In a previous study the maximum cellular proliferative inhibition of MCF-7 was only 20% (Li *et al.*, 2009) but at lower concentration of *Spirulina* phycocyanin (80 µg/mL). It can be noticed that the anti-proliferative activity of phycocyanin obtained for the Egyptian isolate may be comparable to the one obtained from *Spirulina platensis* isolated under Chinese conditions. As a consequence, it can be concluded that the biological activity of the phycocyanin is mainly governed by the genetic information of the biological source determined by the genus and species of the micro-organism, rather that the geographical habitat or the environmental conditions.

Hepatoprotective Effect of Phycocyanin in Rats

Rats subjected to CCl_4 developed significant hepatocellular damage as evident from the activities of ALT and AST compared to normal values, and which have been used as reliable markers of hepatotoxicity

Table 2. Effect of phycocyanin treatments at different concentration (100, 150 and 200 mg/ kg body weight) on the activity of antioxidant enzyme in normal (NC) and CCl_4-treated rats (PC)

Treatment	GST[1] mM/min/ mg protein	SOD[2] U/ mg protein	CAT[3] µmol/ mg protein
NC	3.59±0.04b	44.13±0.35a	15.9±0.32a
PC	2.14±0.02d	12.43±0.33e	4.57±0.3e
LC	3.28±0.01c	15.6±0.21d	5.7±0.15d
MC	3.54±0.02b	20.53±0.29c	7.3±0.12c
HC	3.79±0.01a	28.77±0.09b	9.37±0.07b
P value	0.000(***)	0.000 (***)	0.000(***)
LSD 0.05	0.0712	0.8546	0.679

Notes: SE, P and LSD refer to standard error, probability and last significance difference. If the p-value is less than 0.05, we reject the null hypothesis that there is no difference between the means and conclude that a significant difference does exist. Means with the same letter are not significantly different from each other.
[1]Glutathione-S-transferase [2] Superoxide dismutase [3]Catalase
NC: normal control; PC: positive control; LC: low concentration (100 µg/ml); MC: medium concentration (150 µg/ml); HC: high concentration (200 µg/ml)

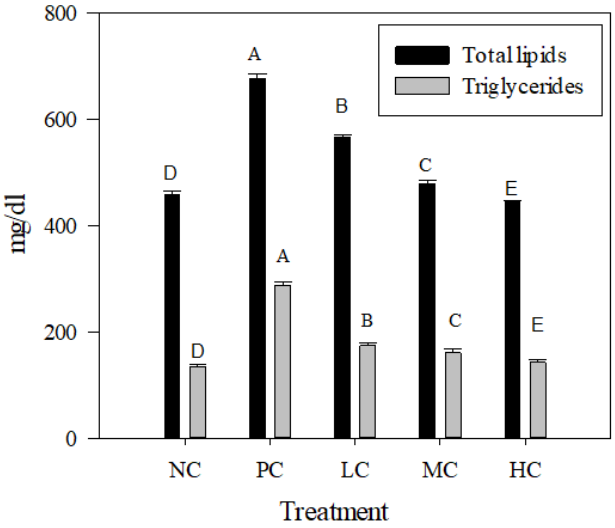

Fig. 3. Effect of phycocyanin treatments at different concentration (100, 150 and 200 mg/ kg body weight) on the serum total lipids and triglycerides in normal (NC) and CCl_4-treated rats (PC). NC: normal control; PC: positive control; LC: low concentration (100 µg/ml); MC: medium concentration (150 µg/ml); HC: high concentration (200 µg/ml)

(**Fig. 2A**). ALT enzyme is a relevant indicator of cell membrane damage whereas AST is an indicator of mitochondrial damage (Chaung *et al.* 2003). Rats were received phycocyanin at doses of 100, 150 and 200 mg/kg body weight/day, respectively for 28 days exhibited a significant reduction in the levels of ALT and AST as compared with liver injury control rats group. These results were agreement with those obtained by (Hamad et al. 2011, Soni *et al.* 2008, Taha and Osman 2015). Levels of urea and creatinine in the positive control were higher than those of the negative control (**Fig. 2B**) after 28 day of experiments. The phycocyanin treatments (100, 150 and 200 mg/kg body weight/day) showed levels lower than those of the positive control. This reducing effect of phycocyanin on the renal performance indicators (serum creatinine and serum urea) is in accordance with previously effects associated with *Spirulina platensis* spphycocyanine (Khan *et al.* 2005) who reported a anephroprotective role of *Spirulina platensis* phycocyanin (500 mg /kg BW) in rats with cyclosporine-induced nephrotoxicity with no significant differences in plasma urea, creatinine in the control and treatment. The data in **Fig. 3** represent the changes in serum lipids in Albino rats when subjected to a single dose of CCl_4 and then treated with phycocyanin at different levels (100, 150 and 200 mg/kg BW). It is observed that CCl_4 increased the levels of serum total lipid and serum triglycerides in Albino rats. These increases are in accordance with similar study (Soni et al. 2008). The highest level of phycocynin brought the level of total lipids to less than that of the negative control and the level of triglycerides was very close to the negative control. The results in **Table 2** show that subjecting Albino rats to CCl_4 drastically reduced the levels of serum antioxidant enzymes; glutathione-S-transferase, superoxide dismutase and Catalase; by about 42, 71 and 74% of their levels in the negative control, respectively. These CCl_4 –induced reductions in the activities of the antioxidant enzymes agree with the findings of (Nagaraj *et al.* 2012) and may be due to the CCl_4 oxidative stress triggered by its transformation into free radicals; trichloromethyl ($CCl_3^{\bullet}$) and peroxytrichloromethyl ($\bullet OOCCl_3$) (Weber *et al.* 2003) that can deplete the antioxidant capacity of the living cells. Treating with Phycocyanin at different levels could counteract this negative action in a concentration-dependent manner. Displaying rats to CCl_4 reduced the level of serum total protein and serum globulin but slightly increased the level of serum albumin as compared to the negative control (**Table 3**). Treating the rats with phycocyanin at different levels, corrected this reduction and brought the level total protein to the level of the negative control. Phycocyanin treatments depressed albumin into levels lower than that of the negative control and could increase serum globulin to levels higher even of the original control. These increases are in accordance with similar study (Soni et al. 2008). Hepatic injury through carbon tetrachloride (CCl_4) induced lipid peroxidation is well known and has been extensively used in the experimental models to understand the cellular mechanisms behind oxidative damage and further to evaluate the therapeutic potential

Table 3. Effect of phycocyanin treatments at different concentration (100, 150 and 200 mg/ kg body weight) on the serum proteins in normal (NC) and CCl_4-treated rats (PC)

Treatment	Total protein	Albumin	Globulin	A/G
NC	7.6±0.0b	3.03±0.0b	4.57±0.01c	0.66±0.b
PC	5.5±0.1b	3.67±0.a	1.83±0.1d	2.03±0.2a
LC	7.7±0.1c	2.6±0.23c	5.1±0.25b	0.51±0.0b
MC	8.13±0.0a	2.42±0.03c	5.71±0.1a	0.42±0.0b
HC	8.33±0.0a	2.76±0.04bc	5.57±0.06ab	0.49±0.01b
P value	0.000 ***	0.000 ***	0.000 ***	0.000 ***
LSD 0.05	0.33215	0.35887	0.47804	0.3266

Notes: SE, P and LSD refer to standard error, probability and last significance difference. If the p-value is less than 0.05, we reject the null hypothesis that there is no difference between the means and conclude that a significant difference does exist. Means with the same letter are not significantly different from each other.
NC: normal control; PC: positive control; LC: low concentration (100 µg/ml); MC: medium concentration (150 µg/ml); HC: high concentration (200 µg/ml)

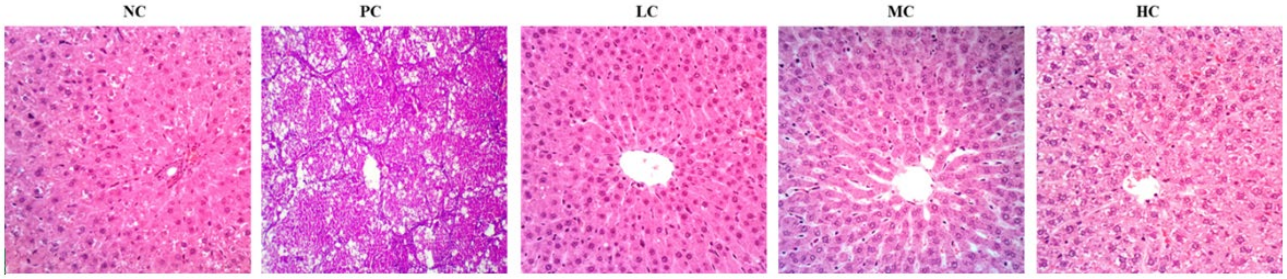

Fig. 4. Histopathological images of liver sections of rats intoxicated with single dose of CCl_4 and treated with different levels of phycocyanin (100, 150 and 200 mg/kg body weight).
NC: normal control; PC: positive control; LC: low concentration (100 µg/ml); MC: medium concentration (150 µg/ml); HC: high concentration (200 µg/ml).

of dietary antioxidants. Liver injury was evaluated by histopathological approach in **Fig. 4**. The negative control group (NC) showed a normal histological structure of the portal area, central veins and surrounding hepatocytes. Focal necrosis with inflammatory cells infiltration was detected in the hepatic parenchyma is associated with dilatation in the portal vein and inflammatory cells infiltration in the portal area (positive control). The sections of the animals receiving phycocyanin at different concentration were approaching the status of the negative control.

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