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C-Phycocyanin isolated from *Microcystis aeruginosa* Kützing mitigates renal injury induced by potassium dichromate via toll-like receptor-4 down regulation in rats

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Abstract

C-Phycocyanin is among the most promising microalgal derived molecules with an array of pharmacological activities. Toll-like receptors (TLRs) have a vital role in regulating inflammatory response in renal injury. The aim of this study was to evaluate the possible renal therapeutic effect of phycocyanin isolated from *Microcystis aeruginosa* Kützing harvested from high-rate algal pond on potassium dichromate (PD)-induced renal injury in rats. Rats were assigned randomly into: Group I: Normal control group. Group II: Rats injected with a single injection of PD (15 mg/kg; s.c) and served as renal injury group. Group III and IV: Rats received C-phycocyanin (25 & 50 mg/kg body weight; orally), after PD injection, daily for 7 days. Injection of PD induced a marked renal injury, evidenced by a significant increase in kidney functions and renal contents of malondialdehyde (MDA), tumor necrosis factor–alpha (TNF-α), toll-like receptor 4 (TLR4), heat shock protein 70 (HSP70) and insulin growth factor-1 (IGF-1) In addition PD reduced renal content of reduced glutathione (GSH) and exhibited tubular degeneration and necrosis. C-phycocyanin has renal therapeutic effect against renal injury induced by PD through improving kidney function, down regulation of oxidative stress and TLR4/TNF-α/HSP70 inflammatory pathway as well as modulation of IGF-1.

Keywords: Renal injury; phycocyanin; TLR 4; HSP70; IGF-1

1. Introduction

C-Phycocyanin is a non-toxic, water-soluble pigment protein separated from microalgae. It exhibits a multitude of pharmacological activities; antioxidant, anti-inflammatory, hepatoprotective, and neuroprotective effects [1]. Among phycocyanin pigments; C-phycocyanin (C-PC), a photosynthetic pigment is largely found in cyanobacteria, rhodophytes, cryptophytes, and glaucophytes. C-PC is classified as a phycobiliprotein (PBP), as other pigments such as allophycocyanin and phycoerythrin. C-PC is a highly fluorescent protein with linear prosthetic groups (bilins) that are linked to specific cysteine residues [2]. C-PC is widely studied and has several applications in the food, cosmetic, drug, medicine, and biotechnology industries. In the food industry, due to its blueness and functional properties, C-PC has been mainly used as a natural dye and a good alternative to highly toxic and carcinogenic artificial colorings. [3]. Thus, phycocyanin has become a new hot spot in the field of drug research.

Engineered algae-based wastewater treatment systems, in other words namely high rate algal ponds (HRAPs) have been recently well established to reduce the footprint and improve nutrient removal efficiency and effluent specifications. Production of algal biomass is considered an added benefit for HRAPs which can be further utilized for bioenergy production; biofuels, and the supply of algal bioactive compounds [4]. In our previous work, microalgal community predominated by *Microcystis aeruginosa* growing in high rate algal pond showed prominent activities in antioxidant, anti-

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inflammatory, anti-diabetic and wound healing assays [5].

Potassium dichromate (PD) is a highly toxic form of chromium (VI) and induces acute renal injury in humans and experimental animals [6]. PD exposure not only elevates reactive oxygen species concentration inducing oxidative damage in kidney [7], but also mediates inflammatory process through which increased pro-inflammatory cytokine as renal tumor necrosis factor-alpha (TNF-α) content expressed [8].

Toll-like receptors (TLRs) are a family of pattern - recognition receptors and important driver in regulating inflammatory response [9]. TLRs activated innate immune and involved in the pathogenesis of renal injury, endothelial dysfunction, and vascular remodeling [10]. Activation of TLR4 initiates the release of cytokines and chemokines (TNF-α, CXCL1, CCL2 and CCL5) and results in kidney inflammatory response [11]. TLR4 stimulates cardiorenal dysfunction [12], renal inflammation and fibrosis [13]. In addition, in acute and chronic kidney disease, heat shock protein 70 (Hsp70) expression was elevated. In experimental models of kidney diseases, Hsp70 serum levels were increased, and curcumin treatment reduced its production [14]. Heat shock proteins elevated following heat-associated stress of cells then they modify other proteins structure. They are considered as diagnostic and prognostic biomarkers in human. Hsp70 is the most conserved protein across species [15].

In this context, C-phycocyanin was isolated and purified from the dried algal biomass predominated with *Microcystis aeruginosa* Kützing and was further investigated for therapeutic effect against PD-induced renal injury in rats via suppression of TLR4/TNF-α/HSP70 in rats.

2. Materials and Methods:

2.1. Chemicals and Kits

Standard proteins for molecular weights, and electrophoresis chemicals were purchased from Sigma-Aldrich Chemical Co. Ammonium sulphate was purchased from Bio Xtra, 99 %; Sigma Aldrich. Potassium dichromate (PD) was obtained from National Research Centre (Dokki, Cairo, Egypt). Creatinine, blood urea nitrogen (BUN), malondialdehyde (MDA) and reduced glutathione (GSH) kits were purchased from Biodiagnostic, Egypt. Tumor necrosis factor –alpha (TNF-α), Toll-like receptor 4 (TLR4), heat shock protein70 (HSP70) and insulin growth factor-1 (IGF-1) were purchased from NOVA, Beijing, China, ELISA kits.

2.2. Algal biomass production system

A semi-pilot system is constructed for treating wastewater. It includes primary facultative pond followed by high rate algal pond (HRAP) with dimensions of 7.5×2.4×0.3m (L×W×H) and active volume of 6 m^3, it then followed by tube settler/rock filter [5]. Algal biomass was collected from HRAP along the operating period June 2017 to May 2018. Continuous microscopically investigation for algal community was carried out three times a week. Algal biomass is harvested biweekly from high rate algal pond (6m^3) and precipitated by cationic starch[16]. The collected biomass is dried using sun drier and grinded to fine particles (0.1mm)

2.3. Extraction and estimation of C-Phycocyanin

A 500 g of dried algal biomass was suspended in 1000 ml of 20 mM acetate buffer containing 50 mM sodium chloride and 0.002 M sodium azide (pH-5.10). C-Phycocyanin was extracted by repeated freezing (-20^o C) and thawing at room temperature until the blue color becomes in acetate buffer (Step I). Cell debris was removed by centrifugation at 5,000 rpm for 10 min and the extract thus obtained was termed as crude extract. Amount of protein in C-PC was measured and purity was determined by using the formulae: Purity= A620/A280 [17].

2.4. Purification

The crude extract was subjected to a single step precipitation using 65 % $(NH_4)_2SO_4$ and kept overnight at 4^{o}C. The pellet was recovered by centrifugation at 27,000 rpm for 15 min at 4^{o}C and dissolved in 10 ml of the same extraction buffer and termed as ammonium sulfate extract (ASE). Ten ml of ASE was dialyzed against the extraction buffer using dialyses membrane (Dialyses membrane-70, MWCO; 12–14 kDa) procured from Hi-Media. Dialyses was performed twice against 1,000 ml extraction buffer, first at room temperature and again dialysed against 1,000 ml of extraction buffer at 4^{o}C overnight. The resultant extract was recovered from the dialyses membrane and filtered through 0.45 µm filter. Phycocyanin having an A_{618}/ A_{280} above 4.0 was considered pure [17].

2.5. Protein determination

Proteins content was detected by Bradford method utilizing bovine serum albumin as a standard protein [18].

2.6. Electrophoretic analysis

Electrophoretic analysis of the purified phycocyanin was carried out on 12% non-denaturing SDS-PAGE [19], while its molecular mass was detected[20]. The proteins were visualized by Silver stain [21].

2.7. Molecular Docking Study

Docking calculations were carried out using DockingServer [22]. The MMFF94 force field [23] was used for energy minimization of ligand molecule (phycocyanin) using DockingServer. Gasteiger partial charges were added to the ligand atoms. Non-polar hydrogen atoms were merged, and rotatable bonds were defined.
Docking calculations were carried out on 3fxi-immune system (Toll-like receptor 4) protein model. Essential hydrogen atoms, Kollman united atom type charges, and solvation parameters were added with the aid of AutoDock tools [24]. Affinity (grid) maps of 20×20×20 Å grid points and 0.375 Å spacing were generated using the Autogrid program. AutoDock parameter set- and distance-dependent dielectric functions were used in the calculation of the van der Waals and the electrostatic terms, respectively. Docking simulations were performed using the Lamarckian genetic algorithm (LGA) and the Solis & Wets local search method [25]. Initial position, orientation, and torsions of the ligand molecules were set randomly. Each docking experiment was derived from 10 different runs that were set to terminate after a maximum of 250000 energy evaluations. The population size was set to 150. During the search, a translational step of 0.2 Å, and quaternion and torsion steps of 5 were applied [26].

2.8. Experimental design

Adult male Wister albino rats weighing 120 – 140g purchased from the animal house colony of the National Research Centre (Dokki, Cairo, Egypt) and were kept in the animal house under conventional laboratory conditions. Experiments were performed according to the National Regulations of Animal Welfare and Institutional Animal Ethical Committee (IAEC) and in compliance with the guiding principles for animal experimentation as enunciated by the US guidelines (NIH publication #85-23, revised in 1985).

Renal injury was induced by a single injection of PD (15 mg/kg, subcutaneously) [27]. Rats were assigned randomly into 4 groups (n=8) as follow: Group I: Normal control group were injected subcutaneously with saline. Group II: Rats were injected with PD and served as renal injury group. Group III-IV: Rats received C-phycocyanin daily (25 & 50 mg/kg; orally) [28], after 24 h of PD injection for 7 days.

2.8.1. Estimation of kidney function

Blood samples were taken, 24 h following the last treatment, from the abdominal aorta under light anesthesia with pentobarbital sodium. Collected blood samples were allowed to stand for 10 min at room temperature then centrifuged at 4 ^{o}C using cooling centrifuge (Laborezentrifugen, 2k15, Sigma, Germany) at 3000 r.p.m for 10 min [29] and used for determination of creatinine and BUN. Creatinine and BUN serum levels were estimated according to the method by Bartles et al. (1972) and Fawcett, and Soctt (1960), respectively [30, 31].

2.8.2. Estimation of oxidative stress

The animals were sacrificed by cervical dislocation, and one kidney from each rat was immediately dissected out, washed with ice-cooled physiological saline and homogenized in 0.15M KCl solution [32] using a tissue homogenizer (MPW−120, Bit-Lab Medical instruments, Poland) to prepare the 20% homogenate. Homogenized tissues were centrifuged at 4000 rpm/min for 10 min at 4^{o}C using a cooling centrifuge (Laboratory Centrifuge, 2 K15, Sigma Co., Germany). The supernatant was collected and stored at −80 °C for determination of kidney contents of MDA and GSH.

2.8.3. Estimation of inflammatory mediators

TNF-α, TLR 4, HSP70 and IGF-1 renal contents were determined using ELISA kit (NOVA kit, Beijing, China). Standards and samples were pipetted into wells with immobilized antibodies specific for rat TNF-α, TLR 4, HSP70 and IGF-1, then were incubated for 30 min at 37C. After incubation and washing, horseradish peroxidase-conjugated streptavidin was pipetted into the wells and incubated for 30 min at 37C, which were washed once again. Tetramethylbenzidine (TMB) substrate solution was added to the wells and incubated for 15 min at 37C; a color was developed proportionally to the amount of TNF-α, TLR 4, HSP70 or IGF-1 bound. Color development was discontinued (stop solution) and after 10 min color intensity was measured at 450 nm[33].

2.9. Histopathological examination of kidney

Kidney was immediately removed and washed in saline solution. The kidney was fixed in 10% phosphate buffered formalin. Following an overnight fixation, slices (3–4mm) of kidney tissue were dehydrated in ascending grades of alcohol, cleared in xylene and embedded in paraffin wax (58-60°C). Blocks were made and sectioned of 5 μm thickness with a microtome. The tissue sections were stained with hematoxylin and eosin and observed under the light microscope. The slides were observed for histopathological changes and microphotographs were taken using a microscope system (Olympus, Japan)[34].

2.10. Data analysis

All values are presented as means ± standard error of the means (SE). Comparisons between different groups were carried out using one-way analysis of variance (ANOVA) followed by least significant difference test (LSD). Difference was considered significant when $P<0.05$. GraphPad prism® software (version 5) was used to carry out these statistical tests.

3. Results

3.1. Protein content and molecular weight

The isolated phycocyanin showed protein content of 100 µg/mg. The SDS-PAGE showed that C-phycocyanin molecular weight was 14 kDa (Figure 1&2).

3.2. Molecular docking study

The virtual docking of the 3D structure of C-phycocyanin on toll-like receptor 4 active site showed high affinity for binding as indicated by the estimated free energy of binding of -1.76 kcal/mol. The total intermolecular energy was calculated as -4.72 kcal/mol, which is largely due to the hydrogen bonding between the ligand molecule and the Lys363 moiety. Additionally, there were pi-pi interaction with Tyr292 and polar interaction with Arg264 (Figure 3).

3.3. Effect of C-phycocyanin on serum creatinine and blood urea nitrogen (BUN)

Serum creatinine and BUN levels were elevated after induction of renal injury by PD by 70% and 81%, respectively, as compared with normal control group. Whereas treatment with low dose of phycocyanin significantly reduced serum creatinine and BUN levels by 18%, moreover, treatment with high dose of phycocyanin significantly reduced serum creatinine and BUN levels by 27% and 33%, respectively, as compared with PD group (Table 1).

Table 1: Effect of C-phycocyanin on serum creatinine and blood urea nitrogen (BUN)

	Normal Control	Potassium dichromate (15 mg/kg)	C-Phycocyanin (25 mg/Kg)	C-Phycocyanin (50 mg/Kg)
Creatinine (mg/dl)	1.52±0.08	2.59±0.08 [a]	2.11±0.04 [ab]	1.88±0.01 [ab]
BUN(mg/dl)	2.55±0.07	4.61±0.08 [a]	3.76±0.02 [ab]	3.08±0.10 [ab]

Data were expressed as mean ± SE.
Statistical analysis was carried out by one-way ANOVA followed by LSD test.
[a] Significantly different from normal control at $P<0.05$.
[b] Significantly different from renal injury group (PD) at $P<0.05$.

3.4. Effect of C-phycocyanin on renal contents of MDA and GSH

Induction of renal injury by PD significantly elevated MDA renal contents by 81% and reduced GSH renal content by 54%, as compared with normal control group. Treatment with low dose of phycocyanin significantly reduced renal MDA contents by 26% and elevated GSH renal content by 70%, also, treatment with high dose of phycocyanin significantly reduced MDA contents by 36% and elevated GSH renal content by 93%, as compared with PD group (Figure 4).

3.5. Effect of C-phycocyanin on renal TNF-α, TLR4 and HSP70 contents

PD injection produced significant elevation of TNF-α, TLR4 and HSP70 renal contents by 161 %, 183% and 173%, as compared with normal control group. Treatment with low dose of phycocyanin significantly reduced only renal TNF-α and TLR4 contents by 16% and 34%, respectively, while, treatment with high dose of phycocyanin significantly reduced renal TNF-α , TLR4 and HSP70 contents by 42%, 45% and 23%, respectively, as compared with PD group (Figure 5).

3.6. Effect of C-phycocyanin on insulin growth factor-1(IGF-1)

IGF-1 renal content was elevated after injection of PD by 5 fold, as compared with normal control group. Whereas phycocyanin low dose significantly reduced IGF-1 renal content by 29%, moreover, phycocyanin high dose significantly reduced its content by 70%, as compared with PD group (Figure 6).

3.7. Histopathological Examination

Photomicrograph of the kidney showed normal architecture of the renal tissue in the normal control group while in PD group: the renal corpuscle showing hyper cellularity, pyknotic and accumulation of hyaline materials in the Bowman´s space were observed (yellow thick arrows), moreover, the proximal convoluted tubules showing dilatation, degeneration and necrosis of the epithelial lining cells and proteinaceous casts in tubular lumen were detected (yellow arrows). In Phycocyanin low dose group: the renal corpuscle showed atrophy and in others accumulation of hyaline materials in the Bowman's space (arrows), also, Phycocyanin high dose group showed normal renal corpuscle (H&E.X 100) (Figure 7).

4. Discussion

The pursuit for novel therapeutic agents has urged the search in the secondary metabolites of various organisms for any possible pharmacological activities. Microalgae presented themselves strongly in this field providing various classes of chemical compounds with proved beneficial medicinal values. Nevertheless, the bottle neck for the extended use of

microalgae in the pharmaceutical and nutritional fields exists in their cultivation techniques; for maximal biomass production, which vary from different types of open ponds to closed systems [35]. High rate algal ponds are functional units in waste-water treatment techniques utilizing the microalgae in the elimination of organic load. These ponds provide typical systems for the massive production of microalgae with minimal cost [5].

Phycocyanin is a pigment-binding protein isolated from different algal species. C-PC is the phycocyanin isolated from blue-green algae, as *Spirulina platensis*, *Anabaena*, and *Microcystis aeruginosa*. C-PC has been widely used as medicine and food and cosmetic colorant. Several studies have demonstrated that C-PC functions in antioxidation, inflammation, antitumor, and immunity enhancement. In addition, C-PC can be processed into a fluorescent reagent, fluorescent probe, and fluorescent tracer, which are used in medical diagnosis, immunology, biological engineering, and other research fields because of its intense fluorescence. C-PC is also a nontoxic photosensitizer that can be used in adjuvant therapy in the photodynamic therapy (PDT) of tumors [36].

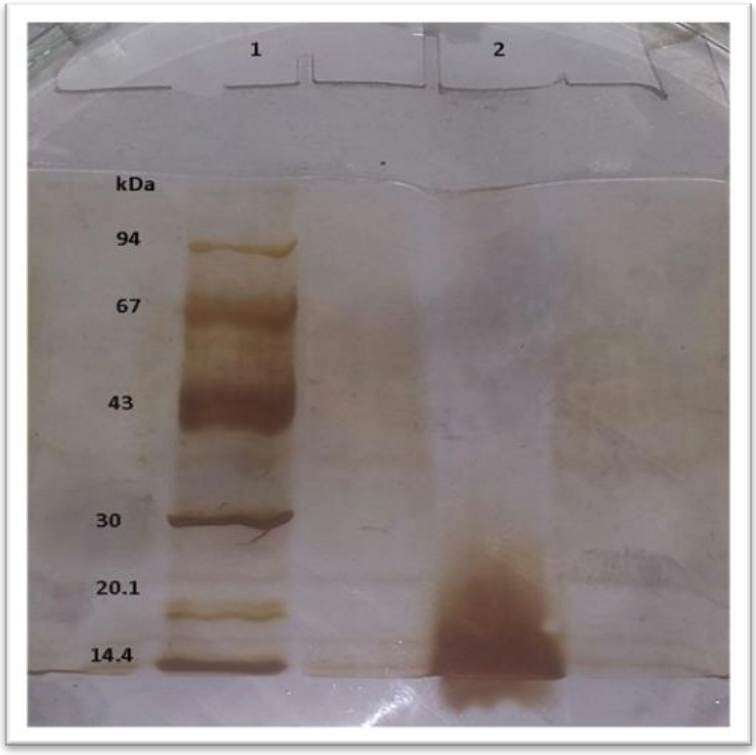

Figure 1: Molecular weight determination of purified phycocyanin by Electrophoretic analysis on 12 % SDS-PAGE (1) Low molecular weight standard marker proteins and (2) purified PC phycocyanin

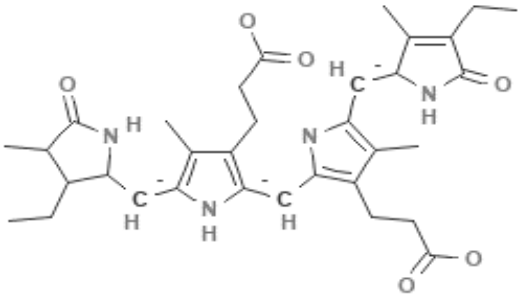

Figure 2: Chemical structure of C-phycocyanin

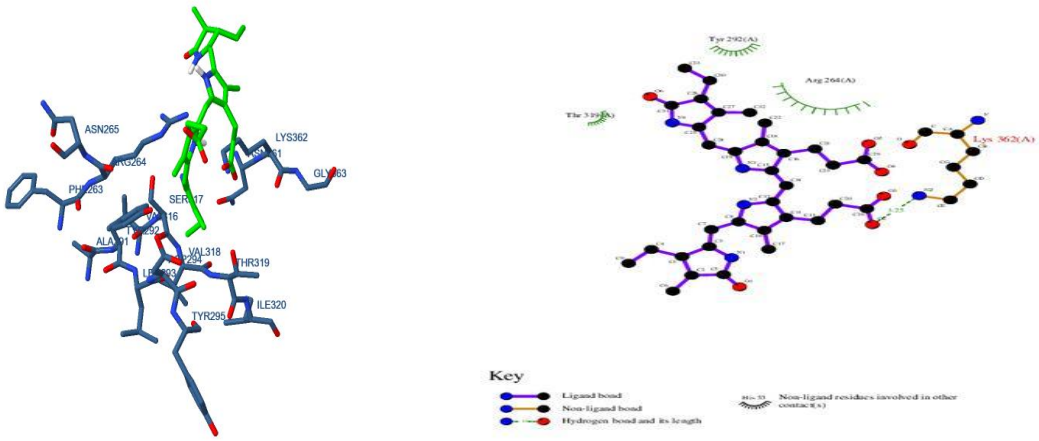

Figure 3: Docking of C-phycocyanin on the active site of toll-like receptor-4

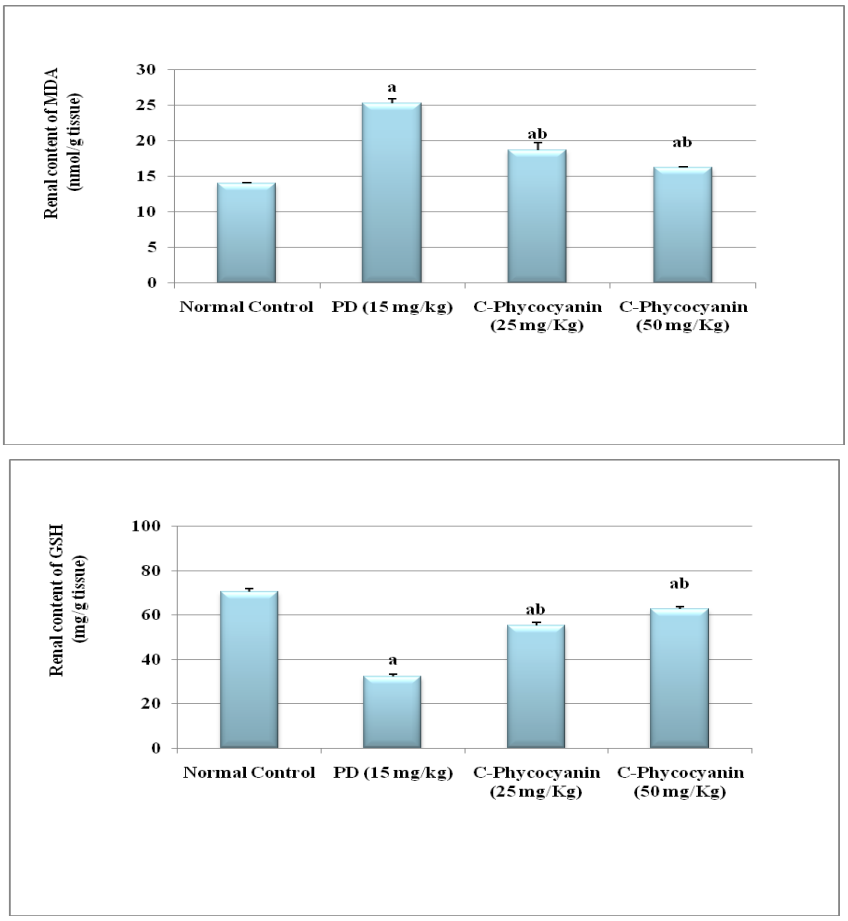

Figure 4: Effect of C-phycocyanin on renal MDA and GSH contents

Data were expressed as mean ± SE.
Statistical analysis was carried out by one-way ANOVA followed by LSD test.
[a] Significantly different from normal control at $P<0.05$.
[b] Significantly different from renal injury group (PD) at $P<0.05$.

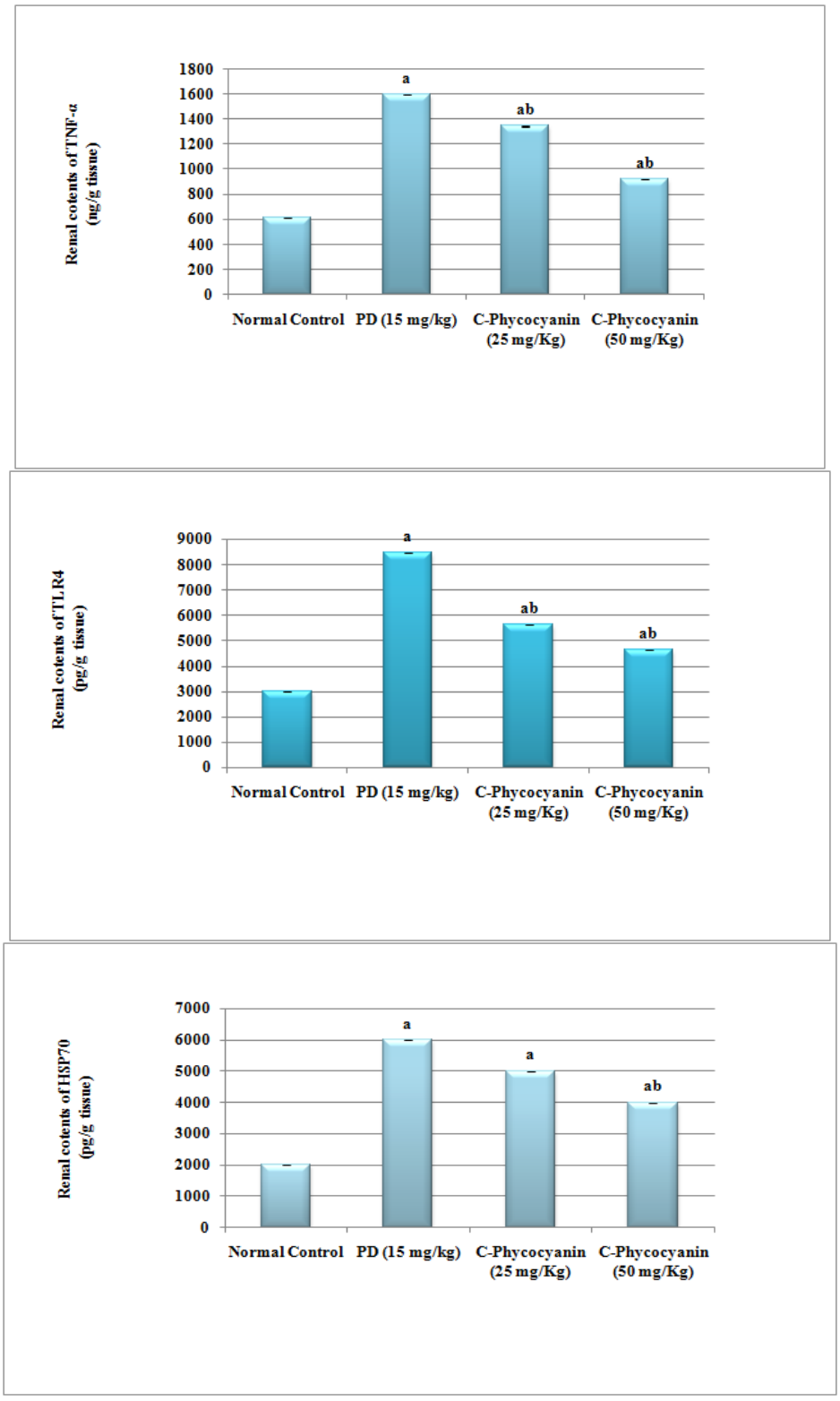

Figure 5: Effect of C-phycocyanin on renal TNF-α, TLR4 and HSP70 contents
Data were expressed as mean ± SE.
Statistical analysis was carried out by one-way ANOVA followed by LSD test.
[a] Significantly different from normal control at $P<0.05$.
[b] Significantly different from renal injury group (PD) at $P<0.05$.

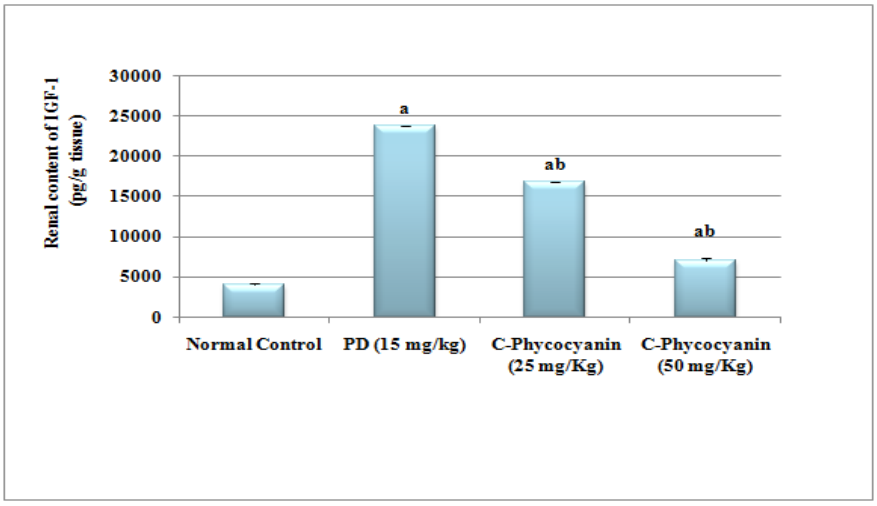

Figure 6: Effect of C-phycocyanin on renal IGF-1 content
Data were expressed as mean ± SE.
Statistical analysis was carried out by one-way ANOVA followed by LSD test.
[a] Significantly different from normal control at $P<0.05$.
[b] Significantly different from renal injury group (PD) at $P<0.05$.

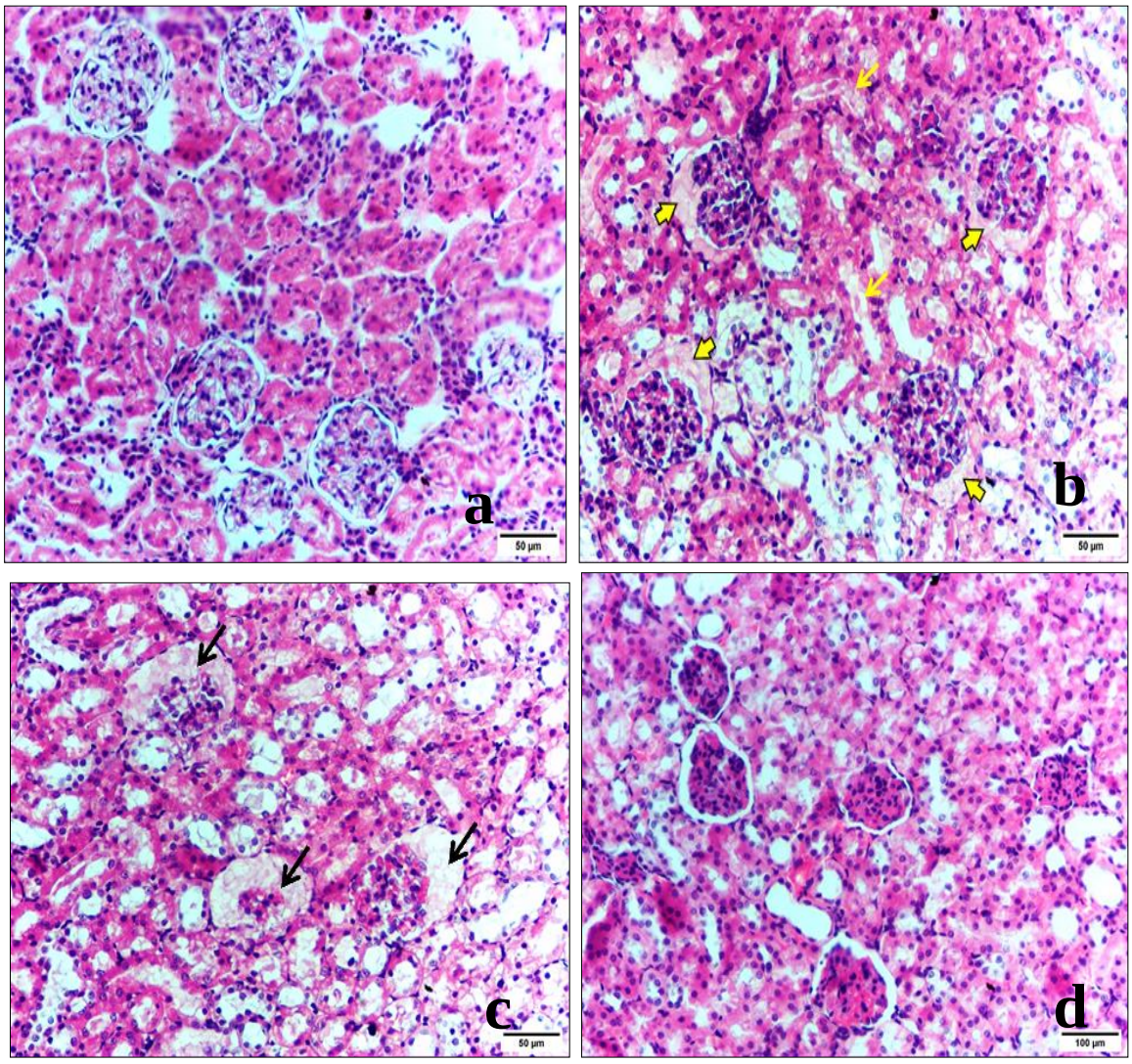

Figure 7: Photomicrograph of the kidney
a) The normal control group: the main architecture of the renal tissue are within normal limit (H&E.X 200). b) PD group: the renal corpuscle showing hyper cellularity, pyknotic and accumulation of hyaline materials in the Bowman´s space (yellow thick arrows), the proximal convoluted tubules showing dilatation, degeneration and necrosis of the epithelial lining cells and proteinaceous casts in tubular lumen were detected (yellow arrows) (H&E.X 40). c) C-Phycocyanin low dose group: the renal corpuscle showed atrophy and in others accumulation of hyaline materials in the Bowman's space (arrows) (H&E.X 200). d) C-Phycocyanin high dose group: the renal corpuscle are within normal (H&E.X 100).

In the present study, C-phycocyanin was isolated from the algal biomass predominated with *Microcystis aeruginosa* collected from the waste-water treatment unit in Zenin- Cairo, Egypt high rate algal pond. It was purified and electrophoretic analysis (12% SDS-PAGE) was carried out under non-denaturing conditions. The protein content of the purified phycocyanin was determined as 100 μg/mg and its molecular weight was determined as 14 KDa. The purified C-phycocyanin was investigated for its pharmacological activity as renal protective and therapeutic effect. Injection of a single dose of PD, in the present study, demonstrated higher kidney functions than in normal rats and supported by the histopathological damages, as hyaline materials in the Bowman's space, dilatation, degeneration and necrosis of the proximal convoluted tubules and proteinaceous casts in tubular lumen were detected. These results showed severe nephrotoxicity and renal failure by PD and are in an accordance with our previous study [27]. Treatment with phycocyanin significantly reduced serum creatinine and BUN levels, especially, treatment with high dose of phycocyanin showed improvement in the renal histopathological picture of rats and the renal corpuscle and tubules are normal. These results explain the therapeutic effect of C-phycocyanin. Previous literature showed that oral administration of phycocyanin (300 mg/kg) for 10 wk protected against albuminuria and renal mesangial expansion in db/db mice, and normalized tumor growth factor-β and fibronectin expression. Phycocyanin also normalized urinary and renal oxidative stress markers and the expression of NAD(P)H oxidase components in diabetic nephropathy [37]. So, oral administration of phycocyanin, in this study, may offer a novel and feasible therapeutic approach for treating acute renal injury.

The pathological pathways of AKI involved production of prooxidants; free radicals and nonradical oxidants. The generation of these prooxidants alters lipids, proteins, and nucleic acids, inducing cell death. Renal tubules are vulnerable to damage with oxidative stress as it rich with mitochondria which are the main sites of free radical production [38]. In the current study, PD injection induced a state of imbalance between prooxidants and antioxidants generation while treatment with phycocyanin scavenged prooxidants and increased antioxidant levels as it significantly reduced MDA and elevated GSH renal contents, especially, treatment with high dose of phycocyanin.

Renal injury, also, is found to be associated with inflammation in which macrophages activated and secreted TNF-α producing kidney damage [39]. Moreover, TLRs are contributing to inflammation inducing acute kidney injury and immune-mediated glomerulonephritis and solid organ transplant rejection [40]. They are highly expressed in innate immune cells due to pathogens and environmental stressors [41] and are found in renal tubular epithelial cells [42]. Furthermore, overexpression of TLR4 stimulates not only inflammation, but also vascular remodelling and endothelial dysfunction [10]. In current work, induction of renal injury by PD caused inflammation that evidenced by elevated renal contents of TNF-α and TLR4, as compared with the normal control group. PD provokes acute renal failure through increasing kidney TNF-α content [32] and caused, also, an immediate inflammatory reaction in brain and lungs tissues [43]. post-treatment with phycocyanin reduced renal TNF-α and TLR4 contents, as compared with PD group. Molecular docking study asserted the interaction between C-phycocyanin and TLR 4 active site with estimated free energy of binding of -1.76 kcal/mol. C-phycocyanins induced the inhibition of NO and prostaglandin E (2) over-production through suppressing iNOS and COX-2 induction and attenuation of TNF-α formation and neutrophil infiltration into inflammatory sites [36].

HSPs are proteins that interact with TLRs during the immune cells maturation and cytokine secretion [44] activating NF-κβ [45]. Previous study showed, in acute interstitial glomerulonephritis and interstitial nephritis, a significant elevation in the HSP70 expression by tubular cells [46]. In present study, HSP70 binds to TLR4 stimulating NF-κβ activation which in turn released TNF-α, in inflamed tissue induced by PD. While, phycocyanin post-treatment exerted anti-inflammatory effect through decreasing HSP70 kidney content and its binding to TLR4 then reducing TNF-α release due to its high affinity to

bind with TLR4, as compared to PD group. Phycocyanin, in another disease acts in the same mechanism as it reduced the expression of TLR-NF-κB pathway and regulating inflammatory immune responses to treat pulmonary inflammatory fibrosis [47].

IGF-1activation is another pathological pathway that was observed in this model of acute kidney injury that induced by PD. This result is in a line with previous study [48, 49]. IGF-1 has role in cellular hypertrophy, differentiation and apoptosis of renal tubular epithelial cells [50]. On the other hand, phycocyanin post-treatment reduced IGF-1 kidney contents, for the first time, as compared to PD group.

Conclusion

The findings of the current study revealed that C-phycocyanin isolated from the algal biomass predominated with *Microcystis aeruginosa* Kützing (50 mg/kg, p.o) post-treatment produced marked renal therapeutic effects as evidenced by lowering kidney functions, oxidative stress, inflammatory pathway and histopathological injury induced by PD via suppression of TLR4/TNF-α/HSP70 pathway and modulating IGF-1 that played main role in PD-induced renal injury.

Author Contributions

Abeer Salama: Conceptualization, Methodology, Formal analysis, Resources, Writing - Review & Editing. Rehab A. Hussein: Conceptualization, Methodology, Formal analysis, Resources, Writing - Review & Editing. Walaa S. A. Mettwally: Methodology, Formal analysis. Mohamed S. Helmy: Methodology, Formal analysis. Gamila H. Ali: Conceptualization, Resources, Writing - Review & Editing, Project administration, funding acquisition.

Conflicts of Interest

The authors declare no conflict of interest.

Ethical considerations

The ethical issues (including plagiarism, misbehavior, data provision, forgery, duplicate release or submission, redundancy) were fully observed by the author.

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