Polysaccharides from *Phormidium versicolor* (NCC466) protecting HepG2 human hepatocellular carcinoma cells and rat liver tissues from cadmium toxicity: evidence from *in vitro* and *in vivo* tests

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Abstract

The *in vitro* antioxidant, cytotoxic and cytoprotective properties and *in vivo* hepatoprotective activities of crude polysaccharides extracted from cyanobacteria *Phormidim versicolor* NCC466 (CFv-PS) were investigated. The CFv-PS, identified as heteropolysaccharides with molecular weight of 63.79 kDa, exhibited relatively strong antioxidant activity, in a concentration-depended manner, in *in vitro* assays. Additionally, CFv-PS did not induce cytotoxic effect on HepG2 human hepatocellular carcinoma cells within the range of tested concentrations (25-150 µg mL^{-1}) while preventing them against Cd. Moreover, in rats subjected to Cd-induced hepatotoxicity, CFv-PS pretreatment significantly ($P < 0.05$) reduced the level of ALAT, ASAT, biliburin, MDA, protein carbonyl and DNA damage, and markedly increased enzyme activities in liver tissues. These findings suggest that the cyanobacteria *Phormidium versicolor* is a potential source of natural products possessing antioxidant, cytoprotective and hepatoprotective properties.

Keywords: Antioxidant defense mechanism; Cadmium exposure; HepG2 cells; *P. versicolor* polysaccharides; Rat liver

1. Introduction

Cadmium (Cd) is one of the most widespread toxic metals in the aquatic and terrestrial environments [1, 2], and commonly detected in industrial and agriculture production and commodities [2]. Long term exposure to Cd is considered to be harmful to human health [3, 4]. Cd can be absorbed through food chain, and then accumulated in organs particularly in liver which is the metabolic dock of its entry and the major destination of its toxicity. The injury of liver induced by Cd depends on the dose and duration of Cd exposure, as previous research suggested that low Cd dose exposure led to pathological changes in the liver tissues [5]. With acute or chronic Cd, significant DNA damage was also observed [6]. Additionally, reactive oxygen species (ROS) also contributes to the liver damage induce by Cd, as emerging evidence regards oxidative stress as a major influencing factor in various development of diseases (please add a reference). Redox balance and homeostasis are disturbed under the excessive oxidative stress condition, which result in cell dysfunction, apoptosis and necrosis [6-8].

Different antioxidants were effective in reducing Cd-induced liver damage. Recently, many natural antioxidants have been used in the treatment of various diseases. Indeed, marine microalgae are considered to possess several antioxidants due to numerous bioactive substances such as phenolic compounds and fatty acids [9], carotenoids and proteins [10], and polysaccharides [11].

Algal polysaccharides have been demonstrated to play an important role as free radical scavengers *in vitro* and as antioxidants capable of preventing oxidative damage in living organisms [12, 13], and exhibit a wide array of therapeutic effects on cancer, atherosclerosis and hepatitis in different animals [14]. Polysaccharides extracted from *Phormidium versicolor*, isolated from Sfax solar saltern, are a group of heteropolysaccharides mainly composed of galactose, glucose, xylose, *N*-acetyl glucosamine, rhamnose and saccharose, plus small amounts of arabinose, ribose, mannose and glucuronic acid. Such polysaccharides have shown potent antioxidant and antimicrobial properties [11].

On the basis of such research findings, microalga polysaccharides are worth to be tested further as protective bioactive compounds and even applied in therapies against Cd-induced oxidative damages. Thus, the aim of this study was to explore the therapeutic role of crude polysaccharides extracted from *Phormidium versicolor* (CFv-PS) and the attainable mechanism against Cd-induced toxicity in the hepatic cell model (HepG2) and Wistar male rat liver.

2. Materials and methods

2.1. Chemicals and reagents

Cadmium chloride ($CdCl_2$, CAS Number 10043-52-4), nitroblue tetrazolium, *N*-(1–naphthyl) ethylene diamine and Tris-HCl were purchased from Sigma, St. Louis, MO, USA. All other chemicals and reagents used in this study were of analytical grade.

2.2. Microalga culture and polysaccharide extraction

Our study focused on a cyanophyceae (*Phormidium versicolor* NCC466) isolated from the Sfax solar saltern (Tunisia) which was growing in BG11 medium under axenic conditions. Crude polysaccharides were extracted from *Phormidium versicolor* according to the procedures described in our previous study [11] with minor modification. Briefly, 50 g of the dried microalgae powder was depigmented with acetone stirring overnight at room temperature, then with ethanol (80%) for 2 h at 80 °C, followed by hot water extraction (3 times) with an extraction ratio of deionized water to raw material of 21.52 mL g^{-1} for 4 h at 81 °C. After centrifugation, the supernatant was collected from the crude extract and concentrated to 1/4 of the original volume, deproteinized by sevag solution (chloroform : butyl alcohol = 4:1), dehydrated and lyophilized to obtain crude polysaccharides.

The extraction yield of crude polysaccharides was calculated as follows:

$$\text{Polysaccharide yield (\%)} = \left(\frac{\text{Dry weight of the crude polysaccharide}}{\text{Dry weight of microalgae}}\right) \times 100 \qquad (1)$$

2.3. CFv-PS physico-chemical characterization

2.3.1. Analysis of total carbohydrate, protein, uronic acid and sulfate

Total carbohydrate was determined by the phenol sulphuric acid method [15]. Protein content was estimated according to Bradford [16]. The content of uronic acid was estimated by the method of Blumenkrantz and Asbœ-Hansen [17] using glucuronic acid as reference. Sulfate content was determined according to method of Dodgson and Price [18] using potassium sulfated as a reference.

2.3.2. Determination of viscosity and molecular weight

The viscosity and molecular weight of CPv-PS were determined by Ubbelohde capillary viscometer using the Mark-Houwink relationship [19]:

$$[\eta] = K * (MW)^{\alpha} \qquad (2)$$

where K = 7.95 x 10^{-2}, α = 0.79 determined in 0.1 M sodium acetate and 0.3 M acetic acid solution at 25±0.02 °C [20]. The average of five replicates was taken for the viscosity measurements.

2.3.3. Monosaccharide composition

The CFv-PS sugar content was evaluated according to Belhaj et al. [11]. In brief, CFv-PS samples (2 mg) were hydrolyzed with 4 M trifluoroacetic acid in methanol (100 °C for 8 h) for measurement of individual sugars with D-myoinositol as the internal standard. Samples were methylsilylated in 100 µL of dry pyridine and 100 µL of *N*,*O*-bis(trimethylsilyl)-trifluoroacetamide at 4 °C overnight, and then analyzed by gas chromatography-mass spectrometry (GC-MS) on 5975C series GC-MSD with Triple-Axis Detector (Agilent technologies). After sample injection, compounds were separated on a HP-5 ((5% phenyl)-methylpolysiloxane) column, using helium as the carrier gas. All sugars were quantified in total ion current (TIC) mode using calibration curves. The standard curve of each monosaccharide using D-myoinositol as internal standard was determined by plotting concentration ratio against its peak area ratio.

2.3.4. FT-IR spectrum analysis

The FT-IR spectrum of CFv-PS was recorded between 400 and 4000 cm^{-1} in Nicolet 5700 FT-IR spectrometer (Thermo Fisher Scientific Inc., MA, USA). The transmission spectra of the samples were recorded by using the KBr pellet containing 0.1% of sample. The spectral data were analyzed by the OriginPro 2017 software program.

2.4. *In vitro* antioxidant assays

The antioxidant activities of the crude polysaccharides were estimated by five different methods as described by Ozgen et al. [21], Danis et al. [22], Packer [23], Gutteridge and Halliwell [24], and Vinson and Howard [25] for the radical cation decolorization (ABTS) assay, ferrous ion chelating activity, nitric oxide (NO) radical scavenging activity, lipid peroxidation (LPO) inhibition and protein glycation assays, respectively.

The inhibition of protein glycation was expressed as the emission and extinction wavelength ratio (350 nm/450 nm). The other antioxidant capacities were estimated according to the following formula:

$$\% \text{ Inhibition} = \left[\frac{(A_{control} - A_{test})}{A_{control}}\right] \times 100 \quad (3)$$

where $A_{control}$ is the absorbance of the control reaction and A_{test} is the absorbance of the extract reaction.

2.5. *In vitro* cytotoxicity and cytoprotective effects of CFv-PS

To evaluate the cytotoxic activity of CFv-PS, the half maximal inhibitory concentrations (IC_{50}) on human hepatocellular carcinoma (HepG2) cells were estimated through the MTT assay [26]. The MTT assay method is based on the fact that metabolically active cells can reduce the MTT by the mitochondrial enzyme succinate deshydrogenase to form insoluble purple formazan crystals that are solubilized subsequently, and thus the metabolic activity of cells can be measured by spectrophotometry.

HepG2 cells were kept in minimum essential medium (MEM), supplemented with 10% fetal calf serum, 1% L^{-1} glutamine and 50 µg mL^{-1} of gentamycin sulfate (37 °C, 5% CO_2 atmosphere). Cells from logarithmically growing cultures were seeded in 96-well plates at a density of 1 x 10^5 cells mL^{-1} (100 µL $well^{-1}$). After 24 h incubation, the cells were treated with various concentrations of CFv-PS (25, 50, 100 and 150 µg mL^{-1}) and $CdCl_2$ (25, 30, 40, 50 and 60 µM), respectively, for 24 h. Then, MTT reagent (5 mg mL^{-1}, 10 µL) was added 4 h before the end of the reaction. The supernatants were then replaced with 100 µL DMSO. Herein, negative control cells were incubated in the absence of CFv-PS and $CdCl_2$, respectively.

Absorbance was measured at 570 nm using an automatic plate reader, and the percentage of cell proliferation was calculated according to eq. (4):

$$\text{Viability (\%)} = \frac{\text{Averge of Test (OD)} - \text{Average of Blank (OD)}}{\text{Average of Control (OD)} - \text{Average of Blanc (OD)}} \times 100 \quad (4)$$

The ability of CFv-PS in preventing HepG2 cells against toxicity induced by Cd was assessed. Briefly, HepG2 cells were pre-treated with CFv-PS (25 µg mL^{-1}), diluted in MEM medium (dilute to what value?). After 24 h of incubation at 37 °C under 5% CO_2, the cells were exposed to $CdCl_2$ (30 µM, diluted in MEM medium). The wells containing cells without cadmium were served as negative control. The cells were divided into 3 groups: the 1st group served as normal cells; the 2nd group was treated with 25 µg mL^{-1} of CFv-PS; the 3rd group was exposed to 30 µM of $CdCl_2$; the 4th group was pre-treated with 25 µg mL^{-1} of CFv-PS for 24 h followed by exposure to 30 µM of $CdCl_2$. The results were expressed as percentage of cells

viability according to eq. (4). To determine morphological changes, the cells were photographed by a phase-contrast microscope, followed by examination of images.

2.6. *In vivo* hepatoprotective effect of CFv-PS

The experimental protocol was approved by the Local Ethics Committee for Animal Experiments in Tunisia, and performed according to the ethical principles and institutional guidelines and international Guide for the Use of Animals in Biomedical Research. Adult male Wistar rats (200 ± 30 g) purchased from the Tunisian Central Pharmacy were used for the study. After acclimatization, rats were grouped with six per cage and maintained at a temperature of 25±3 °C with a normal 12 h light/dark cycle. The animals were fed with a commercial pellet diet (Socco, Sfax, Tunisia) and water ad libitum.

CFv-PS (250 mg kg^{-1}) and Cd chloride (0.7 mg kg^{-1}) were dissolved in deionised water, and administrated to rats by gastric gavage and through intraperitoneal injection, respectively, for 1 month. These dosages were chosen based on previous reports (add a reference?) and pilot studies (data not shown).

The animals were randomly divided into four groups (n = 5): Group I, control; Group II, treated with CFv-PS (250 $mg.kg^{-1}.day^{-1}$); Group III, treated with Cd chloride (Cd; 0.7 $mg.kg^{-1}$ day^{-1}) and Group IV, treated by CFv-PS and $CdCl_2$ (250 and 0.7 $mg.kg^{-1}$ day^{-1}, respectively).

After 1 month of treatment, the animals were deprived of food overnight, anesthetized by exposure to diethyl ether, and then sacrificed by cervical decapitation. Blood was collected, serum was separated by centrifugation at 4000 rpm (4 °C) for 15 min, and used for liver marker assays. The liver tissue was dissected, washed in ice-cold saline, patted dry and weighed. A small portion of tissue was stored in 10% formalin for histopathological examination. From the remaining tissue, 500 mg was weighed and homogenized in 1 mL of PBS buffer (pH 7.4), and then centrifuged at 9000 rpm for 30 min. The supernatants were used for biochemical analyses.

2.6.1. Serum biomarkers analysis

Serum transaminases (ASAT and ALAT) and bilirubin levels were determined by colorimetric method using commercial reagent kits (Ref. 200094; 20102), which were purchased from Biomaghreb, Ariana, Tunisia.

2.6.2. Liver biomarkers analysis

The activities of hepatic superoxide-dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx), malondialdehyde (MDA), protein oxidation, ascorbic acid, metallothionein (MT), DNA damage were determined, as they are considered as indices of antioxidant status of liver tissues.

SOD activity was assessed by the nitro blue tetrazolium (NBT) reduction method [27]. Catalase activity was estimated by measuring the rate of decrease in H_2O_2 absorbance, as described by Aebi [28]. To determine GPx activity, the rate of NAD(P)H oxidation was measured at 340 nm in the presence of reduced glutathione [29].

Lipid peroxidation was estimated calorimetrically by measuring thiobarbituric acid reactive species (TBARS) as previously described by Niehaus and Samuelsson [30].

The formation of carbonyl groups in liver was used as an indicator for oxidative damage to proteins, based on the reaction with dinitrophenylhidrazine (DNPH), as previously described by Levine et al. [31].

Ascorbic acid levels were assessed spectrophotometrically based on the reaction with DNPH and sulfuric acid, as described by Omaye et al. [32].

Metallothionein levels were determined using a spectrophotometric assay using Ellman's reagent [0.4 mM DTNB in 100 mM KH_2PO_4] at pH 8.5 in a solution containing 2 M NaCl and 1 mM EDTA [33].

DNA damage was measured by a "DNA precipitation" assay, based on the K-SDS precipitation of DNA-protein cross link [34]. Fluorescence reading was taken at 260 nm (excitation) and 280 nm (emission).

2.6.3. Histopathological and morphological analysis

Parts of liver tissues obtained from each animal were fixed in 10% formalin solution, dehydrated in ascending grades of alcohol and embedded in paraffin. Sections of 4-µm thickness were taken, stained with hematoxylin and eosin (H&E) and examined under light microscope.

2.7. Statistical analysis

Data were evaluated with the SPSS 20.0 (IBM) and origin Pro (8.5.1) software. Statistical comparisons were made using one-way analysis of variance. A value of $P < 0.05$ was considered as statistically significant.

3. Results

3.1. Physico-chemical characterization of CFv-PS

CFv-PS were extracted from the cyanobacteria *P. versicolor* using hot water, with an extraction yield of 21.56% (Table 1). The total carbohydrate content was determined to be 79.37% by the phenol-H_2SO_4 assay, and its uronic acid content was 4.37% (Table 1). CFv-PS were also found to contain a small amount of sulfate (6.83%), due possibly to sulfate-bounded polysaccharides (Table 1). Additionally, protein content was insignificant (0.45%) since crude polysaccharides were deproteinized by sevag solution. The viscosity and molecular weight of CFv-PS were 630 mL g^{-1} and 63.79 kDa, respectively (Table 1).

Furthermore, CFv-PS were also estimated for monosaccharide composition by GC-MS. Fig. 1a shows the GC-MS chromatogram of monosaccharides separated from the crude polysaccharides, and the quantified constituents were also listed in Table 1. As shown in Fig. 1a, the peaks in the chromatogram were identified (in the order of retention time) as arabinose (Ara), xylose (Xyl), ribose (Rib), rhamnose (Rha), *N*-acetyl glucosamine (GclNAc), galactose (Gal), glucose (Glu), mannose (Man), glucuronic acid (GlcA) and saccharose (Sac), with Gal and Glu as the dominating sugar units.

The FT-IR spectra of CFv-PS are shown in Fig. 1b. The strong and broad adsorption peak at 3392 cm^{-1} was assigned for O-H stretching vibration. The characteristic bands of CFv-PS appearing at 2925 cm^{-1} and 1338 cm^{-1} were attributed to C-H asymetric vibrations, while the band at 1643 cm^{-1} was attributed to the vibration free carboxyl group due to the presence of uronic acid [35]. In addition, bands at 1078 cm^{-1} and 600 cm^{-1} were assigned to C-O stretching vibration and the presence of xylose in the structure, respectively [36, 37].

3.2. *In vitro* antioxidant activity of CFv-PS

Five parameters were monitored and the results are shown in Fig. 2 and Table 2. All studied activities increased dose-dependently and the maximum responses were obtained at CFv-PS concentration of 2 mg mL^{-1}.

CFv-PS exhibited potent ABTS radical and nitric oxide scavenging activities, with IC_{50} values of 0.76±0.04 and 0.67±0.08 mg mL^{-1}, respectively (Table 2). At 2 mg mL^{-1}, CFv-PS scavenged 74.32% and 75.62% of ABTS and NO radicals, respectively (Fig. 2a).

As shown in Figure 2a, the ferrous ion of the CFv-PS extract reached a chelating activity of 71.45% (IC_{50} = 0.93±0.07 units?) at 2 mg mL^{-1}.

The ability of the CFv-PS to inhibit the lipid peroxidation was measured and compared with that of the positive control (BHT at 100 µg mL^{-1}). In this investigation, the CFv-PS extract showed a strong ability to inhibit lipid peroxidation by 69.42% (Fig. 2a).

Finally, to estimate the effect of CFv-PS on protein glycation, an *in vitro* assay was performed according a glycation-inducing reaction system purified bovine albumin, fructose and glucose. CFv-PS was added to this incubation system, and a significant inhibition of protein glycation at 2 mg mL^{-1} concentation was observed (Fig. 2b). The low IC_{50} value (I cannot see this value in Table 2) confirmed the protective action of the CFv-PS against glycation.

3.3. Cytotoxicity and cytoprotective effects on hepato carcinoma cell line (HepG2)

The effects of CFv-PS and Cd on the viability of HepG2 cells were investigated by the MTT method. The viability of HepG2 cells treatment with CFv-PS at concentrations ranging from 0 to 150 µg mL^{-1} showed that CFv-PS concentrations < 100 µg mL^{-1} did not show any significant ($P < 0.05$) cytotoxicity effect (Fig. 3a).

The cell viability was significantly decreased ($P < 0.05$) in a dose-dependent manner during exposure to $CdCl_2$ (Fig. 3b). The concentration of 30 µM of Cd, which decreased cell viability by 38±1.5%, was used for further experiments. Interestingly, HepG2 cells treated with CFv-PS before Cd exposure could be protected from fatality. As shown in Fig. 3c, the cells were morphologically changed and cell viability remarkably increased to 87% after pretreatment with 25 µg mL^{-1} of CFv-PS. The results showed that CFv-PS effectively provided the cells with neuroprotection from damages induced by Cd.

3.4. Capacity of hepatoprotective activities *in vivo*

3.4.1. CFv-PS effects on Cd-induced hepatotoxicity

Several enzyme activities in serum were commonly used for the investigation of early liver damage. As displayed in Fig. 4a-b, serum ALAT, ASAT and bilirubin activities of Cd-treated rats were increased by about 1.9, 1.2 and 3.5 fold, respectively, when compared to those of normal control group (Group I). However, pretreatment with CFv-PS significantly ($P < 0.01$) decreased the ALAT, ASAT and bilirubin activities as compared to the Cd-treated group. Moreover, pretreatment with CFv-PS alone did not show any significant changes in these biomarkers as compared to the normal control group.

Light microscopic examination of liver tissues is shown in Fig. 4c. There was no abnormal appearance in the liver of rat in normal control group and CFv-PS group. Intoxication of Cd

caused severe hepatocyte necrosis, condensed nuclei and massive inflammatory cells infiltration. However, CFv-PS pretreatment ameliorated Cd-induced liver damage, as demonstrated by necrotic areas markedly decreased and slight inflammatory cells infiltrations.

3.4.2. CFv-PS inhibition on oxidative stress

Cd-induced oxidative stress in rat liver was evaluated by assessing several enzymatic and non enzymatic antioxidant activities. As shown in Table 3 and Fig. 5, Cd decreased the levels of SOD, CAT GPx ascorbic acid and DNA in liver by 2.2, 1.9, 1, 1.4 and 2.9 fold, respectively, and increased MDA, protein carbonyl, and metallothionein levels by 1.8, 2.8 and 3.6 fold, respectively, when compared to normal control group. In comparison, rats in CFv-PS pretreated group showed significant improvement ($P < 0.05$) compared to the control values. CFv-PS pretreatment increased SOD, CAT, GPx, Ascorbic acid and DNA in liver by 1.7, 1.4, 1, 1.2 and 2.2 fold, respectively, and decreased MDA, protein carbonyl, and metallothionein levels by 30.6%, 48.9%, and 33.4%, respectively, as compared with those values of Cd-treated group. Nevertheless, treatment with CFv-PS alone did not show any marked alterations in all biomarkers when compared to the normal control group.

4. Discussion

Cd is an environmental pollutant extremely toxic to human and animals. Owing to its long half-life, Cd tends to accumulate in different organs and tissues causing severe damage. It has been found that Cd administration led to over generation of reactive oxygen species (ROS) such as superoxide radical, hydrogen peroxide, nitric oxide and hydroxyl radical [5]. ROS have received considerable attention due to their involvement in a variety of physiological processes and pathological events such as oxidative damage of DNA, protein and lipid, inflammation, neurodegenerative and heart diseases, carcinogenesis diabetes and liver injury [5, 38, 39]. The delicate balance between ROS production and clearance is critical to maintain healthy cellular physiology [40]. Antioxidants can prevent or reduce substrate oxidation, and protect the organisms from oxidative damage induced by excessive free radicals [41, 42]. However, some commercial synthetic antioxidants like gallic acid, butylated hydroxytoluene (BHT) and propyl gallate (PG) have adverse effects [43]. Hence, there is increasing interest in searching for natural antioxidants. Microalgae, in particular cyanoprokaryotes, have been suggested as a very convenient source of natural products with high added value and high bioactive capacity as antioxidants [44, 45]. They protect organisms from the free radicals produced during

photosynthesis. Microalgae could be easily grown in a laboratory and used for large-scale cultivation in bioreactors with the ability to control the quality of the cultures by providing purified culture medium that is free of toxic substances. Therefore, microalgae provide a more accessible way to produce qualitative biomolecules of interest [9, 10, 12]. Among those biomolecules, polysaccharides are one of the best widespread free radical scavengers [12]. Recent research data reported antioxidative potential of polysaccarides derived from different microalgae [12, 13], as well as production of specific antioxidants [11]. To date, there are no reports published on *Phormidium versicolor* concerning antioxidative potential. The lack of data about this cyanoprokaryotic specie and the numerous reports on diverse biological activities of polysaccharides from many microalgae species motivated our investigations on the cytotoxic, cytoprotective and hepatoprotective effects of crude *Phormidium versicolor* NCC466 polysaccharides (CFv-PS), against Cd, under *in vitro* and *in vivo* conditions.

The antioxidant capacities of CFv-PS *in vitro* were assessed. The CFv-PS extract exhibited significant dose-dependent antioxidant capacity, consistent with our previously findings [11]. Antioxidant potential of the current polysaccharides could be attributed to their medium molecular weight (MW) and monosaccharides composition [46]. It is widely known that polysaccharides with low to medium molecular weight have the strongest reducing power, due to the polysaccharide chains. The medium MW polysaccharides present a higher content of reducing power to eliminate and accept the free radicals [46]. Although the role of monosaccharides in the antioxidant activity of polysaccharides remained unclear, there is evidence that the composition and ratios of monosaccharide type influence the antioxidant properties. The results confirm previous reports that galactose, uronic acid content and the complexity of monosaccharide composition in polysaccharides play key roles in their antioxidant activity [47, 48]. Additionally, the antioxidant capacity of the current CPv-PS could be attributed to the hydroxyl groups (-OH). In this regard, Zhang et al. [49] indicated that O-H bonds exhibited strong antioxidant properties, which could be attributed to the lower bond dissociation energies of -OH groups.

The antioxidants capacity and efficacy of polysaccharides can be assessed more accurately by the effect of these macromolecules on the level of oxidation in biological tissues of experimental animals. Prior to evaluating the protective effects of the studied polysaccharide extract, the effect of CFv-PS on viability was investigated to ensure non-cytotoxic to HepG2 cells using MTT assay. After being treated with various concentrations of CFv-PS (25, 50 100 and 150 µg mL^{-1}), it was confirmed that CFv-PS was non-cytotoxic to HepG2 cells up to the concentration of 100 µg mL^{-1}. Whereas, treatment with 30 µM of Cd induced damnification of

the HepG2 cells, and only 38% survived suggesting that HepG2 cells were sensitive to Cd pollution. However, it was obvious that pretreatment with 25 μg mL^{-1} of CFv-PS dramatically mitigated and prevented Cd-induced oxidative damage in HepG2 cells, as reflected by morphological change of necrosis and increase of cell viability. The protective effect of the current polysaccharides was attributed to their potential antioxidant capacity. Likewise, other studies also found that polysaccharides can inhibit apoptosis from Cd-induced injury in HepG2 cells [5]. Such evidence confirm that polysaccharides have a significant protection effect on Cd-induced reduction of cell viability and the damnification of HepG2 cells, although the mechanism requires further study.

Subsequently, hepatoprotective effect of CFv-PS was evaluated. ALAT, ASAT, as well as bilirubin activities, which could leach out of hepatocytes into blood circulation resulting the enhancement of atherosclerosis and blood viscosity [47], are widely used to evaluate liver damage. Herein, serum activities were significantly increased ($P < 0.05$) after Cd treatment, indicating Cd-induced hepatotoxicity. Increased ALAT, ASAT and biliburin activities might be attributed to hepatic structural damage, causing release of these enzymes into the circulation [5]. However, the liver ALAT, ASAT and bilirubin activities were markedly ($P < 0.05$) reduced in rats treated with CFv-PS, suggesting that CFv-PS effectively conditioned the hepatocytes, maintained hepatic structural integrity, decreased leakage of the enzymes into circulation and alleviated Cd-induced liver injury. This conclusion was further confirmed by histopathology, which showed that CFv-PS pretreatment obviously reduced the necrotic area caused by Cd.

In order to understand the hepatoprotective activity of CFv-PS *in vivo*, both antioxidant and oxidant systems were examined. Hepatocellular damage can be triggered by Cd through over generation of free radicals [5], which react with proteins or lipids leading to their peroxidation [50]. Therefore, Cd-induced liver injury is closely associated with the formation of free radicals and antioxidants can reduce the toxicity of Cd. A series of antioxidant enzymes (SOD, CAT and GPx) and non-enzymatic antioxidants (Metallothionein, Vitamin C) are regarded as important defense systems against oxidative stress [5].

SOD, CAT and GPx could convert superoxide to peroxide and then to H_2O and O_2, also could terminate the chain reaction of lipid peroxidation [51]. MDA and protein carbonyl provide an indicator of lipid peroxidation and protein oxidation, respectively. In the present study, Cd treatment led to significant decreases in antioxidant enzymes and non-enzymatic SOD, CAT, GPx activities and vitamin C concentration in liver tissues. The results are consistent with the findings of Navaneethan and Rasool [52]. Additionally, Elmallah et al. [53] reported that Cd stress significantly decreased antioxidant enzymes SOD and CAT in rat liver.

On the contrary, the metallothionein levels with subsequent increase in the level of lipid and protein peroxidation markers MDA and protein carbonyl, as well as DNA damage. Whereas, in rats pretreated with CFv-PS, these effects were attenuated, suggesting the hepatoprotective effects of CFv-PS were, at least in part, caused by their radical scavenging and antioxidant activities, suggesting that crude polysaccharide extracted from *P. versicolor* could be developed as potential natural antioxidant. The findings agree with other reports on polysaccharides inhibiting Cd-induced oxidative stress, which suggested a level of protection by polysaccharides against Cd-induced liver damage [5, 54-56]. However, the polysaccharide mechanism and signaling pathways involved remain to be further elucidated.

5. Conclusions

In this study, the *in vitro* antioxidant and the cytoprotective, and *in vivo* hepatoprotective activities of *Phormidum versicolor* polysaccharide (CFv-PS) were assessed in detail. The excellent antioxidant activities of CFv-PS might be correlated with its hydroxyl groups, medium molecular weight and uronic acid content. Furthermore, CFv-PS showed potential protective effects against Cd toxicity. These preventive effects may be mediated through the augmentation of apoptosis, supression of oxidative stress and enhancement of antioxidant defense system. The findings suggested that the cyanobacteria *Phormidium versicolor* is a potential excellent source of antioxidants, cytoprotective and hepatoprotective polysaccharides, which could be developed as a natural functional food ingredient and novel pharmaceutical to enhance human and animal health.

Conflict of interest

The authors declare that there are no conflicts of interest.

Author contributions

As principal investigator, Dr Dalel Belhaj and Dr Khaled Athmouni had full access to all the data and full responsibility for the integrity of the data and the accuracy of data analysis. They are responsible for experimental design, analysis and data interpretation, and drafting of the manuscript. All authors have read and approved the final manuscript to submit in its current form for consideration for publication.

Acknowledgments

The authors acknowledge the “Ministry of Higher Education, Scientific Research and Technology, Tunisia”, for the support of this study. The authors are grateful to Prof Sami Maalej, Head of Biodiversity and Aquatic Ecosystems Ecology and Planktonology Laboratory.

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Figure captions

Figure 1. Chromatogram (a) and FT-IR spectra (b) of CFv-PS.

Figure 2. Antioxidant activity of CFv-PS: (a) Scavenging of ABTS, Fe^{+} ion chelating activity, inhibitory effect of lipid peroxidation and nitric scavenging capacity; (b) protein glycation. The values are average of three separate experiments.

Figure 3. Cytotoxicity of CFv-PS (a) and Cd (b), and cytoprotective activity of CFv-PS against Cd induced toxicity in HepG2 cells (c). Group I: control; Group II: treated with CFv-PS (250 mg kg^{-1} day^{-1}); Group III: treated with $CdCl_2$ (0.7 mg kg^{-1} day^{-1}); Group IV: treated by CFv-PS and $CdCl_2$ (250 and 0.7 mg kg^{-1} day^{-1}, respectively). All data were expressed as mean ± SD for five rats in each group. Values with the same superscript letters were not significantly different from each other at $P < 0.05$.

Figure 4. Effects of CFv-PS on the levels of serum ALAT and ASAT (a), total, conjugated and unconjugated biliburin (b), and histopathological examination against Cd-induced injury in male Wistar rats. Group I: control; Group II: treated with CFv-PS (250 mg kg^{-1} day^{-1}); Group III: treated with $CdCl_2$ (0.7 mg kg^{-1} day^{-1}); Group IV: treated by CFv-PS and $CdCl_2$ (250 and 0.7 mg kg^{-1} day^{-1}, respectively). All data were expressed as mean ± SD for five rats in each group. Values with the same superscript letters were not significantly different from each other at $P < 0.05$.

Figure 5. Mean±SD effects (n = 3) of CFv-PS on DNA level and agarose gel electrophoresis of DNA fragmentation in the liver of Cd-treated rats. Group I: control; Group II: treated with CFv-PS (250 mg kg^{-1} day^{-1}); Group III: treated with $CdCl_2$ (0.7 mg kg^{-1} day^{-1}); Group IV: treated by CFv-PS and $CdCl_2$ (250 and 0.7 mg kg^{-1} day^{-1}, respectively). All data were expressed as mean±SD for five rats in each group. Values with the same superscript letters were not significantly different from each other at $P < 0.05$.