MANSOURA JOURNAL OF CHEMISTRY
Official Journal of Faculty of Science, Mansoura University, Egypt
E-mail: scimag@mans.edu.eg ISSN: 1687-5060

Ameliorative Effect of Brown Algae Polysaccharides Loaded on Chitosan Nanoparticles against Cisplatin Nephrotoxicity: In Vivo Study

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Received:12/2/2023
Accepted: 22/2/2023

Abstract: Cisplatin is recognized as active anticancer drug for treatment of various human cancers; however, it exhibits numerous undesirable side effects as nephrotoxicity resulting in a significant treatment complication. Brown algae polysaccharides are widely used for pharmaceutical industries due to their antioxidant and anti-inflammatory properties. Chitosan nanoparticles (CSNPs) have demonstrated significant promise for use in the delivery of drugs. Nanoparticles were prepared using the ionic gelation technique using chitosan (CS) and sodium tripolyphosphate (STPP). All nanoparticles revealed spherical shape. The average particle sizes of the fabricated nanoparticles were between 270 ± 82 nm for CSNPs and 148 ± 35 nm for polysaccharides loaded on CSNPs (PS-CSNPs). The main purpose of this study was to detect the effect of brown alga (*Turbinaria triquetra*) polysaccharides loaded and unloaded on chitosan nanoparticles against Cisplatin-induced nephrotoxicity in rats. Forty-two male Wistar rats were divided into 7 equal groups. Control untreated group, CSNPs group, polysaccharide (PS) group, PS loaded on chitosan nanoparticles (PS-CSNPs) group, cisplatin (Cis) group, Cis + PS group, and Cis + PS-CSNPs group. Serum urea and creatinine, creatinine clearance, renal malondialdehyde (MDA), nitric oxide (NO), and paraoxonase 1 (PON 1) were determined. **Results:** Brown alga PS either loaded or unloaded on CSNPs efficiently attenuated Cis-induced nephrotoxicity evidenced by significant reduction in serum urea and creatinine, renal MDA and NO along with significant elevation in creatinine clearance and renal PON 1 compared to the Cis-treated group. However, the improvement was higher in the nephrotoxic group treated with the loaded PS. **Conclusion:** The current study revealed that the nanoencapsulation of brown alga PS using CSNPs has marked ameliorative effects against Cis nephrotoxicity more better than PS do alone. The observed results offer a new therapeutic approach in attenuating Cis-induced nephrotoxicity.

keywords: Algae, Chitosan nanoparticles, Cisplatin, Nephrotoxicity, Polysaccharides.

1. Introduction

Cisplatin (Cis) is considered as one of the best anticancer medications frequently used for the treatment of various cancers even with its side effects [1]. The nephrotoxicity which can be induced in cancer patients receiving Cis, seems to be due to mitochondrial dysfunction, cell membrane peroxidation, inhibition of protein synthesis, DNA damage and oxidative stress [2]. Oxidative stress is defined as any condition in which there is a significant imbalance between the generation of reactive oxygen species (ROS) and the antioxidants in the body [2]. Although the kidney plays a vital role in removal of waste products, it accumulates some of these substances, including Cis, in the proximal tubular portion to

a higher level than other tissues and organs do. Aggregation of Cis leads to strong toxicity in renal proximal tubule cells and eventually causes tissue destruction and renal failure [3]. There are many compounds with antioxidant and anti-inflammatory properties such as the natural polysaccharides which are considered as the main bioactive compounds in natural medicine due to their antitumor, immunomodulatory, kidney protection, and anti-inflammatory properties. The polysaccharides have expanded the scientific research, especially in combination with Cis [4].

In the last few years, a great interest has been developed to extract bioactive compounds especially polysaccharides from algae because of their numerous health beneficial effects [5]. Like all the brown algae, the species *Turbinaria triquetra* (*T. triquetra)* comprises primary algal metabolites like proteins, lipids, and polysaccharides such as alginate, fucoidan, laminarin and mannitol [6]. Nanoparticles (NPs) are employed in biomedical applications because of their unique features. Targeting a drug delivery system to the specified organs or cells without harming the healthy surrounding cells is one of the main biological applications of NPs [7]. Chitosan nanoparticles (CSNPs) have been thoroughly researched for the delivery of different antioxidant and anti-cancer agents due to the beneficial biological characteristics of chitosan such as its safety, biodegradability, biocompatibility bioadhesive, permeability-enhancing and cationic properties [8]. Thus, the aim of the current study was to detect the effect of brown alga (*T. triquetra*) polysaccharides loaded and unloaded on chitosan nanoparticles against Cis-induced nephrotoxicity in rats.

2. Materials and methods

2.1 Chemicals

Cisplatin (Cis), 2,2-Diphenyl-1-picrylhydrazyl (DPPH), Ascorbic acid, Chitosan (CS) (Mw: 190-310 KD, DD: 75–85%), and Sodium tripolyphosphate (STPP) were purchased from Sigma-Aldrich Chemical Co (St. Louis, MO, USA). Other chemicals used in the study were of analytical grade.

2.2 Brown Alga Collection and Cleaning

Fresh samples of *T. triquetra* were collected from Hurghada, Red Sea, Egypt. Samples were thoroughly washed with seawater and then with tap water, immediately after collection. The epiphytes were fairly removed using a fine brush, then the cleaned samples were air-dried in the shade at room temperature and then milled to a fine powder. 450g was obtained and kept for further analysis.

2.3 Extraction of Polysaccharides

Semi-purified polysaccharides (PS) was extracted and deproteinized as previously mentioned by ***Mettwally et al.*** [9]. Fresh *T. triquetra* (450 g) was successively extracted till exhaustion by gentle stirring using methanol (1:5 w/v). After filtration, the remaining powder of *T. triquetra* was air dried, and then re-extracted with boiling distilled water (1:5 w/v) for 4 hr., followed by filtration using muslin, yielding a mucilage-like extract. These procedures were repeated many times till the filtrate became clear and not viscous. The filtrates were collected, concentrated, and then precipitated using cold ethanol (1:4 v/v) and kept at 4 °C overnight to increase precipitation. The solution was then centrifuged at 9300 rpm and 4 °C for 7 min, yielding a dark brown precipitate. Finally, the protein- bound polysaccharide obtained was washed using ethanol and then dried (PP, 44g).

40 g of PP was further semi-purified by deproteinization using sevag method. Firstly, PP was re-dissolved in distilled water. Secondly, The Sevag reagent, chloroform and n-butyl alcohol (4:1) were added to the liquified polysaccharide (1:5, v/v), then centrifuged at 9300 rpm and 4 °C for 7 min. The denaturized protein appeared in the interface was removed with the organic layer. The same procedures were repeated many times till no protein seemed at interface. Aqueous layers were collected, then concentrated, and eventually precipitated using cold ethanol and kept at 4 °C overnight. Finally, deproteinized semi-purified polysaccharide was harvested by centrifugation at 4 ^{o}C, 9300 rpm for 5 min, and then dried, yielding a dark brown to black polymer (PS, 11.36 g)**.**

2.4 Determination of antioxidant activity

The antioxidant activities were determined using the DPPH assay method as previously

mentioned by ***Chang et al.*** [10]. A standard curve of ascorbic acid was prepared and the antioxidant activities of PS and PP were calculated and determined.

2.5 Encapsulation of PS using chitosan using ionic gelation

Fractions that showed higher antioxidant activity were directed for nanoencapsulation. The nanoparticles were prepared by ionic gelation of CS with STPP according to the methods described by ***Antoniou et al.*** [11], and ***Morales-Olán et al.*** [12]**,** with some modifications. In brief, 0.2 % (w/v) CS solution was prepared in 1% (v/v) aqueous glacial acetic acid. In parallel, STPP aqueous solution (0.1 % w/v) was prepared then added dropwise to CS solution by ratio (1:2). For synthesis of nanoparticles that incorporate PS, 0.7 mg/ml was dissolved in the STPP solution then added dropwise to the CS solution.

2.6 Microstructure Characterizations

The morphological characteristics of CSNPs and CSNPs loaded with PS (PS-CSNPs) were examined by a high-performance digital imaging transmission electron microscopy (TEM) machine (JEOLH-7650, Hitachi High-Technologies Corp., Tokyo, Japan) with an acceleration voltage operating at 200 kV. Additionally, hydrodynamic size and zeta-potential of these nanoparticles were performed at 25 ^{o}C on a Zetasizer Nano-ZS (Malvern Instruments, Worcestershire, UK) on the basis of dynamic light scattering (DLS)**.**

2.7 Animal Ethics

Male Wistar rats, (8–10 weeks old; weighing 130 ± 20 g), were purchased from the Animal House of National Research Centre (NRC), Dokki, Egypt. The rats were contained in clean cages of polypropylene under controlled room temperature (25 ± 2ºC) and humidity (55%) with a 12 h light/dark cycle. The rats were supplied by a standard diet in addition to water ad libitum throughout the experiment. The study was carried out according to protocols and guidelines approved previously by the Institutional Animal Ethics Committee (Code Number: Sci-Ch-M-2021-99: dated 24.05.2021), Mansoura University, Mansoura, Egypt.

2.8 In Vivo Experimental Design

After two weeks of acclimatization period, 42 rats were randomly and equally divided into seven groups (6 rats in each group). In the experiment, the dose of Cis was determined according to ***Mapuskar et al.*** [13], while the dose of CSNPs, PS and PS-CSNPs was determined according to ***Raghavendran et al.*** [14]. **Group 1 (Control untreated group):** Normal rats left without any treatment. **Group 2 (CSNPs group)**: Normal rats received CSNPs as a carrier orally (100 mg/kg bw, day after day for 4 weeks). **Group 3 (PS group)**: Normal rats received PS orally (100 mg/kg bw, day after day for 4 weeks). **Group 4 (PS-CSNPs group):** Normal rats received PS-CSNPs orally (100 mg/kg bw, day after day for 4 weeks). **Group 5 (Cis group):** Normal rats were injected intraperitoneally (i.p) with a single dose of Cis (10 mg/kg bw, two weeks before starting of the experiment to induce nephrotoxicity). **Group 6 (Cis + PS group):** Normal rats were injected i.p with a single dose of Cis (10 mg/kg bw) till reach nephrotoxicity, then received PS orally (100 mg/kg bw, day after day for 4 weeks). **Group 7 (Cis + PS-CSNPs group)*:*** Normal rats were injected i.p with a single dose of Cis (10 mg/kg bw), then received PS-CSNPs orally (100 mg/kg bw, day after day for 4 weeks).

2.9 Urine, blood and tissue sampling

The rats were fasted for 24 hr. at the end of the experimental period (4 weeks), and then urine samples were collected in sterile containers, centrifuged at 5000 x g for 10 min, and finally frozen at -20 °C. Blood samples were taken from the retro-orbital venous plexus, placed in plain tubes then centrifuged at 4000 x g for 10 min for serum separation. Kidneys were removed and frozen at -80°C until homogenization.

2.10 Investigations of the Biochemical Parameters

Serum urea [15] as well as creatinine in serum and urine [16] were measured spectrophotometrically using commercially available kits (Bio-diagnostic Co, Egypt). The formula below was used to determine creatinine clearance:

$$\text{Creatinine clearance (ml/min/24 hr.)} = \frac{\text{mg creatinine/dl urine} \times \text{ml urine 24 hr}}{\text{mg creatinine/dl serum} \times 1440}$$

The frozen kidney tissues were cut into small pieces, homogenized in phosphate-buffered saline (PBS) (1:5 w/v) and then centrifuged for 15 min. at 4 °C and 10,000 rpm. The supernatant was removed after centrifugation for measuring malondialdehyde (MDA) using the method of ***Islayem et al.*** [17], nitric oxide (NO) according to ***Palm et al.*** [18]**,** and paraoxonase 1 (PON 1) using the method of ***Richter et al.*** [19].

2.11 Statistical Analysis

Data were analyzed by one-way analysis of variance (ANOVA). In case of significance between groups, Tukey's multiple comparison test was used, and the data were expressed as mean ± standard error (SE). Statistical analysis was performed by SPSS software (version 22.0, IBM Corp., Chicago, USA, 2013). A statistical probability of P value < 0.05 was considered as statistically significant, while P value < 0.001 was considered as highly significant, otherwise is non-significant.

3. Results and Discussion

3.1Determination of antioxidant activity

The results of scavenger percentage (%) and half- maximal inhibitory concentration (IC50) of PS and PP compared to ascorbic acid are presented in table 1. The results revealed scavenger percentage as follow, PS > PP. The reduction in scavenger percentage for PP may be attributed to the antagonistic effect among the PP components [20].

Table (1): Scavenger percentage (%) and IC50 of the samples

Sample (1 mg/ml)	Scavenger percentage (%)	IC50 (mg/ml)
PS	24	2.08
PP	21	2.38
ascorbic acid	62	0.80

3.2 Microstructure Characterizations

PS was selected for nanoencapsulation because it showed higher antioxidant activity than PP. In figure 1: TEM micrographs of (A) CSNPs, and (B) PS-CSNPs are shown. All nanoparticles revealed spherical shape. The average particle sizes of the fabricated nanoparticles were between 270 ± 82 nm for CSNPs and 148 ± 35 nm for PS-CSNPs. This decrease in average particle size after incorporation of PS could be attributed to the strong electrostatic interaction between polysaccharides, chitosan polymer chains and STPP [21]. On the other hand, DLS results are recorded in table 2, which demonstrate larger particle sizes than that observed through TEM analysis. This may be interpreted by the hydration shell formed during DLS measurements [22]. In the present study, the formed nanoparticles revealed positively charged surface as follow: PS-CSNPs > CSNPs. Greater zeta potential value accompanied by less PDI were recorded for PS-CSNPs. Also, PS-CSNPs suspension showed more stability than CSNPs. Increasing in zeta potential value along with decreasing in PDI value increases suspension stability according to ***Kriegseis et al.*** [23]. Their results are in agreement with the results of the present study.

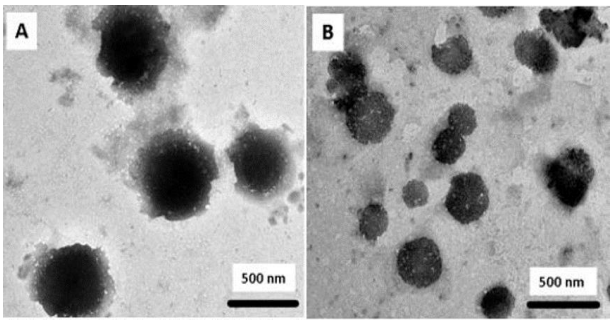

Figure (1): TEM micrographs of (A) CSNPs, (B) PS-CSNPs.

Table (2): Average particle size, zeta potential and polydispersity index (PDI) of CSNPs and PS-CSNPs based on DLS measurements are shown

Nanoparticles	Average particle size (nm)	Zeta potential (mV)	PDI
CSNPs	490.1 ± 31.4	22.2 ±1.2	0.319
PS-CSNPs	360.5 ± 33.7	33.4± 1.8	0.223

3.3 Biochemical Assays

Effect of treatments on serum urea and creatinine, creatinine clearance and oxidative stress markers (renal MDA, NO and PON 1) (Table 3)

When compared to the untreated control group, the administration of Cis to rats caused nephrotoxicity, as shown by highly significant increase in serum urea and creatinine, renal MDA and NO with highly significant reduction in creatinine clearance (P=0.000). These results were in agreement with those of ***Kabel et al.***

[24], and ***Gilani et al.*** [25], who attributed the significant elevation in serum urea and creatinine and the significant reduction in creatinine clearance after Cis administration to the reduction in glomerular filtration rate (GFR) and renal blood flow resulted from changes in renal hemodynamics and also vasoconstriction. The results of oxidative stress markers recorded in this study agreed with ***Gilani et al.*** [25], ***Soliman et al.*** [26], ***Zhou et al.*** [27], who reported that the rise in levels of oxidative stress markers and ROS in Cis group were due to the decrease in the activity of the renal antioxidant enzymes.

Cis nephrotoxicity may be due to several mechanisms such as inflammation, apoptosis and oxidative stress [2]. Meanwhile, cells of renal tubules are more prone to nephrotoxicity due to the high metabolic activity of these cells and their presence in a hypoxic microenvironment. Moreover, cytochrome p450 and other enzyme systems in the kidney oxidize drugs, forming toxic metabolites [28].

ROS that formed after Cis administration can alter mitochondrial membrane potential by opening the mitochondrial permeability transition pores, releasing cytochrome C, followed by activation of caspase 3, resulting in cell death [28]. In the current study, animals given orally with PS after induction of nephrotoxicity showed observable decrease in serum urea and creatinine, renal MDA and NO levels ($p<0.05$) and significant increase in creatinine clearance and renal PON 1 level ($p<0.05$) compared to Cis group. This significant improvement in kidney functions and the observable amelioration against ROS may be attributed to the antioxidant activity of PS, where PS exhibited its effect through increasing the activity of antioxidant enzymes as PON 1 that resulted in reduction in ROS level as MDA and NO and thus, inhibited the release of cytochrome C and the activation of caspase 3 that are implemented in Cis nephrotoxicity [27, 29]. The present results agreed with ***Deyab et al.*** [29]**,** who reported the strong relation between the increasing of brown algae consumption and human diseases prevention could be explained by their content of antioxidants which include phenolics, alkaloids, flavonoids, coumarins, steroids and polysaccharides such as alginic acid, fucoidan, laminarin, and mannitol. Meanwhile, nephrotoxic rats treated with PS-CSNPs demonstrated more observable decrease in serum urea and creatinine, renal MDA and NO levels ($p<0.001$) and highly significant elevation in creatinine clearance and renal PON 1 level ($p<0.001$) compared to Cis group, showing the intrinsic role of the positively charged nanoparticles of chitosan in achieving higher improvement in kidney functions and better amelioration against ROS in the kidney. Positively charged nanoparticles interact with the heparan sulfate proteoglycans that carry negative charge in glomerular basement membrane, enhancing drug specificity to the kidney [30].

In contrast, no significant changes were observed in the biochemical parameters between all groups not treated with Cis and the control group. Products extracted from algae as PS are safe to human, animals, and plants as reported by ***Michalak and Chojnacka.*** [31]

Table (3): Effect of administration of loaded and unloaded polysaccharides on serum urea and creatinine, creatinine clearance and oxidative stress markers (renal MDA, NO and PON 1) in rats of all studied groups

Groups	Serum urea (mg/dl)	Serumcreatinine(mg/dl)	Creatinineclearance (ml/min/kg b.w)	Renal MDA (nmol/g tissue)	Renal NO (nmol/g tissue)	Renal PON 1 (u/g tissue)
Control	30.3 ± 0.88	0.318 ± 0.02	1.7 ± 0.11	10.91 ± 0.35	19.49 ± 1.25	104.2 ± 1.78
CSNPs	34.5 ± 2.28^{a}	0.348 ± 0.02^{a}	1.63± 0.09^{a}	11.87 ± 0.23^{a}	20.19 ± 1.32^{a}	101 ± 1.69^{a}
PS	36.17 ± 2.60^{a}	0.360 ± 0.02^{a}	1.56 ± 0.06^{a}	12.05 ± 0.50^{a}	22.74 ± 2.0^{a}	97.2 ± 2.15^{a}
PS-CSNPs	34.0 ± 2.42^{a}	0.343 ± 0.06^{a}	1.61 ± 0.12^{a}	11.83 ± 0.40^{a}	20.64 ± 1.12^{a}	100 ± 1.63^{a}
Cis	109.5 ± 6.75^{b*}	1.04 ± 0.07^{b*}	0.14 ± 0.01^{b*}	33.29 ± 1.68^{b*}	47.65 ± 2.31^{b*}	35 ± 1.82^{b*}
Cis + PS	92.0 ± 3.97$^{c\#}$	0.868 ± 0.03$^{c\#}$	0.52 ± 0.01$^{c\#}$	28.26 ± 0.57$^{c\#}$	39.08 ± 1.16$^{c\#}$	46.2 ± 2.83$^{c\#}$
Cis + PSCSNPs	55.33 ± 2.06^{c*}	0.585 ± 0.02^{c*}	0.86 ± 0.04^{c*}	20.22 ± 0.70^{c*}	28.36 ± 1.26^{c*}	83 ± 1.57^{c*}

Values are represented as means ± SE. In the same column, superscript letters: (a) shows non-Significant difference compared to control group, (b) shows Significant difference compared to control group, while (c) shows Significan t difference compared to Cis group. Symbols: (#) designates a significant value at a P value of < 0.05, whereas (*) designates a highly significant value at a P value of < 0.001. (Tukey HSD post one way ANOVA test).

Ionic gelation method is one of several methods created to prepare CSNPs, and it has gained a lot of attention because it is safe, without utilization of organic solvents, convenient, and controllable [32]. What reported in the studies of ***Michalak and Chojnacka.*** [31], *and* ***Ahmed and Badr.*** [32], support that *T. triquetra* PS and CSNPs used in the present study are non-toxic and safe.

4. Conclusion

We concluded that PS-CSNPs has marked ameliorative effects against Cis nephrotoxicity than PS do alone, and this is evidenced by highly observable reduction in serum urea and creatinine, renal MDA and NO levels and highly significant elevation in creatinine clearance and renal PON 1 level compared to Cis group. These results detect PS-CSNPs as a promising nano form of PS to offer a new candidate in attenuating Cis- induced nephrotoxicity more better than PS do alone.

Funding

This study was funded by Academy of Scientific Research and Technology (ASRT), Cairo, Egypt**.**

5. References

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