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Exploring the neuroprotective potential of an *iota-carrageenan* in *in vitro* Parkinson's disease model: evaluation of pharmacological safety, antioxidant properties, and mitochondrial function

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Abstract

Background: Parkinson's disease (PD) is a neurodegenerative disorder with a worldwide health impact, characterized by well-established roles of reactive oxygen species, mitochondrial dysfunction, and apoptotic biomarkers. Although various treatments are available for PD patients, they often come with adverse effects, and pharmacological efficacy decreases over time. Sulphated polysaccharides are a class of diverse anionic biopolymers reported to have several pharmacological activities. The present study aimed to assess the *in vitro* neuroprotective potential of the iota-carrageenan (*CSf*) isolated from the red alga *Solieria filiformis*.
Methods: After purification process by precipitation method with cetylpyridinium chloride (CPC), *CSf* was characterized by yield, free-sulphate content, and gel permeation chromatography analysis. The antioxidant potential was determined by 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging activity, hydrogen peroxide (H_2O_2) radical scavenging activity, Reducing power method, and oxygen radical absorbance capacity (ORAC). Cytotoxicity was evaluated using human neuroblastoma (SH-SY5Y) and Balb/c (3T3) mouse fibroblasts cells. The neuroprotection potential was analyzed by 6-hydroxydopamine (6-OHDA)-induced neurotoxicity model on SH-SY5Y cells.
Results: As expected, *CSf* revealed about 28% of free-sulphate content and an estimated molar mass of 425 kDa. Despite the low antioxidant capacity exhibited by *CSf*, it showed the ability to scavenge H_2O_2. Furthermore, CSf protected SH-SY5Y cells against 6-OHDA induced damage by modulating mitochondrial membrane potential, reducing H_2O_2 generation, and regulating caspase-3 activity. In addition, no cytotoxic effects were recorded on SH-SY5Y and 3T3 cells, in presence of *CSf*.
Conclusion: The neuropharmacological effects and safety of *CSf* suggest its potential for the development of novel therapeutic strategies against PD.
Keywords: 6-OHDA, 3T3, biopolymer, caspase-3, H_2O_2, SH-SY5Y, *Solieria filiformis*, sulphated polysaccharide

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Introduction

Parkinson's disease (PD) is a second most frequent neurodegenerative disorder, which is characterized mainly by a progressive loss of catecholaminergic neurons [1]. Because of its multifactorial pathogenesis, PD origin is

still unclear, no effective cure is currently available, and its treatment remains a challenge as well [2]. However, *in vitro* neurodegenerative cellular models have shown the involvement of oxidative stress, mitochondrial dysfunction and apoptotic pathway activation in PD pathogenesis [3, 4]. Among those experimental models, researchers have frequently used the neurotoxin 6-hydroxydopamine (6-OHDA), which was initially identified in PD patients, along with the human neuroblastoma SH-SY5Y cell-line, to assess neuroprotective potential of drugs [5-8].

The search for natural active polymers may provide new therapeutic alternatives for the treatment of neurodegenerative diseases, such as PD [9, 10]. Among those promising biopolymers, a group of highly complex and heterogeneous polymers present in the extracellular matrix of marine algae, named sulphated polysaccharides (SPs), has been accumulating evidences supporting their neuroprotective activity [11-14]. Carrageenans represent a generic name of a family of SPs found in marine algae, and these molecules have been reported to have a range of uses in the food, cosmetics, and pharmaceutical industries [15-17]. Based on chemical composition, carrageenans are divided into six basic forms: Iota (ι)-, Kappa (κ)-, Lambda (λ)-, Mu (μ)-, Nu (ν)- and Theta (θ) [18]. The red marine alga *Solieria filiformis* (Kützing) P. W. Gabrielson (Gigartinales, Solieraceae) represents a source of ι-carrageenan (*CSf*) [19]. Its chemical structure has been described in the literature and consists essentially an *iota*(ι)-type composed of a 3-linked β-*D*-galactopyranose-4-sulphate (G4S-units) connected to 4-linked 3,6-anhydro-α-*D*-galactopyranose-2-sulphate (DA2S-units) or 3,6-anhydro-α-*D*-galactopyranose (DA-units) [20-23]. Moreover, *CSf* has been reported to possess anti-inflammatory, antiviral, vasorelaxant, antinociceptive, and gastroprotective activities [19, 21-25] along with the absence of *in vivo* toxicity [21]. Nonetheless, the neurological impact of the *CSf* and its pharmacological potential is not clear yet. Therefore, this study aimed to evaluate the neuroprotective potential of the *CSf* against 6-OHDA-induced neurotoxicity on SH-SY5Y cells.

Methods

Materials

SH-SY5Y and Balb/c 3T3 mouse fibroblast (3T3) cell-lines were obtained from the DSMZ Human and Animal Cell Lines Bank. The cell culture was performed according to the supplier's handling information. Fetal bovine serum (FBS) was purchased from Gibco (Gaithersburg, MD, USA). JC-1 dye (T3168) was obtained from Molecular Probes (Eugene, OR, USA). Caspase-3 fluorimetric assay kit (Casp3f) was purchased from BioVision (Milpitas, CA, USA). Hydrogen peroxide assay kit (Amplex™ Red, A22188) was purchased from Life Technologies (Carlsbad, CA, USA). The absorbances of antioxidant and cellular assays were measured in Synergy H1 Multi-Mode Microplate Reader (BioTek® Instruments, Winooski, VT, USA). All solutions used in the cellular assays were previously diluted in culture medium without FBS, and sterile filtered (0.2 μm, Whatman™, Little Chalfont, UK). 96-well plates and other chemicals and reagents were obtained from Sigma-Aldrich (Carlsbad, CA, USA).

CSf

Specimen of the red seaweed *S. filiformis* were collected during winter (August) at the beach of Trairí city (Ceará, Brazil), followed by cleaning process and storage at –20 °C until further use. A voucher specimen (number 35682) was deposited at the Herbarium Prisco Bezerra, Department of Biological Sciences, Federal University of Ceará, Brazil. The isolation of *CSf* was carried out as previously described by Coura *et al*. [26]. Briefly, the total extract was submitted to protease digestion by papain (60°C, 6 h) in 100 mM sodium acetate buffer (pH 5.0) containing EDTA and cysteine (both 5 mM), followed by method of purification through precipitation with cetylpyridinium chloride. After, the following chemical analysis were performed: the yield of carrageenan per gram of alga tissue (dry amount of 5 g) [27], the percentage of free-sulphate [28], and the molecular mass by gel permeation chromatography (GPC) [29]. Additionally, potential presence of protein contaminants was also assessed by Bradford method [30].

Antioxidant potential

The evaluation of the antioxidant potential of the *CSf* (at 0.1, 0.5, 1.0, and 2.0 mg/mL) was performed and calculated as described previously by Souza *et al*. [29], by four different methods: DPPH (1,1-diphenyl-2-picrylhydrazyl) assay, hydrogen peroxide (H_2O_2) radical scavenging activity, reducing power method (RP), and oxygen radical absorbance capacity (ORAC). Ascorbic acid was used as the standard (positive control) in the first three methods. The data were expressed as percentage. Trolox standard (6-hydroxychromane substituted with a carboxy group at position 2 and methyl groups at positions 2, 5, 7, and 8) was used to calculate the equivalence in the ORAC assay, where the oxygen radical absorbance capacity of the *CSf* was expressed as μmol Trolox equivalents per gram of the sample. Distilled water was used as a negative control in the assays conducted.

Cytotoxic assay

The cytotoxicity of *CSf* was evaluated on SH-SY5Y and 3T3 cell lines by MTT (3-(3,4-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay, as described in Souza *et al*. [29]. Briefly, the cells were culturing in complete growth medium (CGM): DMEM Han's F-12 (Biochrom, T481-01) medium with addition of HEPES (3.2%-Panreac, A3268.0100), sodium carbonate (2.2%-Panreac, 131638.1211), FBS (10%, fetal bovine serum-Alfagene, LTID 10270 -106), penicillin G (100 U/mL), amphotericin B (0.25 μg/mL), and streptomycin (100 μg/mL-Sigma, A5955). Every three days, the complete growth medium was refreshed. The cells were subcultured in T25-flasks and maintained in controlled conditions (95% humidity, 5% CO_2, 37 °C). The cell suspension was seeded into 96-well plates and incubated until total monolayer was achieved. Then, 1 mg/mL of *CSf* was added and the

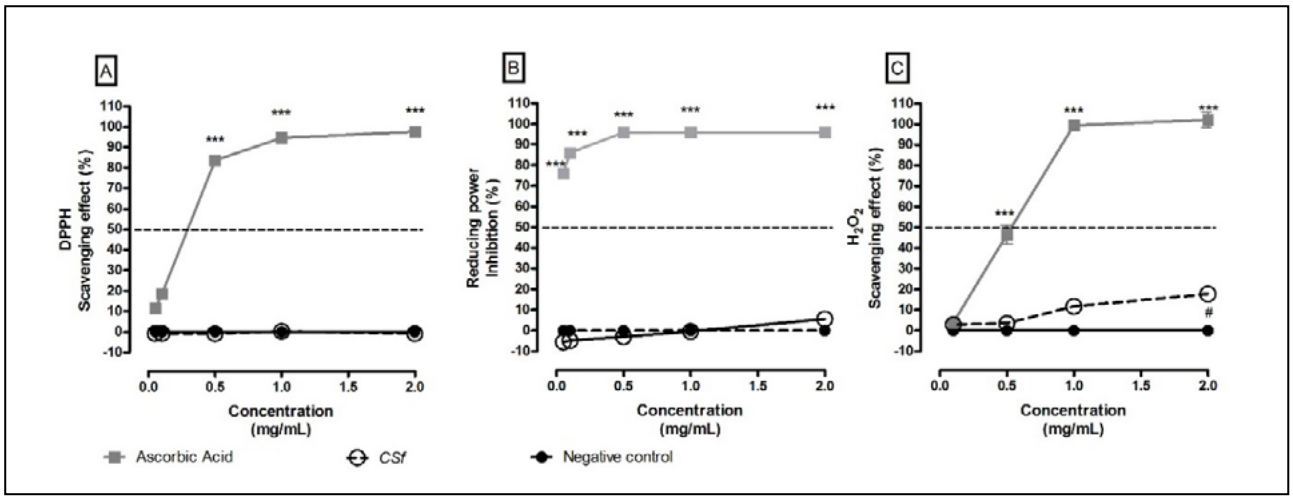


Figure 1. Antioxidant potential of *CSf*. (**A**) DPPH, (**B**) reducing power (RP), and (**C**) H_2O_2 assays, respectively. Figure 1A and 1C illustrate the scavenging activity of *CSf* at varying concentrations, while Figure 1B the inhibition of reducing power by CSf compared to ascorbic acid. Notably, in these assays, *CSf* demonstrates less than 50% of the antioxidant potential observed for ascorbic acid. The values correspond to mean ± SEM at least three independent experiments carried out in triplicate. Two-way ANOVA, Bonferroni test. ***$P < 0.001$, in comparison with the positive control (Ascorbic acid), and $^{\#}P < 0.05$, in comparison with the negative control..

plates were incubated for 24 hours. After, the intracellular metabolic activity was assessed with MTT assay (1.2 mM MTT, during 4 h at 37 °C). The formazan products were dissolved in isopropanol (Panreac, 131090.1611), contained HC (0.04 M). The absorbance was measured at 570 nm and reported as a percentage of the non-treated cells (negative control).

Analysis of potential *in vitro* neuroprotection

The neuroprotective potential of *CSf* was evaluated according to Souza *et al*. [29] by 6-OHDA-induced neurotoxic cellular model on the SH-SY5Y cell line.

6-OHDA-induced cytotoxic model on the SH-SY5Y cell line

Briefly, cells at full confluence were transferred into a 96-well plate and incubated either under exposure to 6-OHDA (100 µM) alone or followed by the addition of *CSf* (1 to 0.01 mg/mL) for 24 hours. As a negative control, cells were incubated only with culture medium. The neurotoxic effects were assessed using the MTT assay, with the results reported as a percentage relative to the negative control.

Mitochondrial membrane potential (MMP) depolarization assay

To evaluate the neuroprotective effect of *CSf* on the MMP, SH-SY5Y cells at total confluence were transferred to a 96-wells plate and incubated for 6 hours either in the presence of 6-OHDA (100 µM) alone or followed by the addition of *CSf* (1 and 0.6 mg/mL). As a negative control, cells were incubated only with culture medium. MMP was then analyzed through JC-1 MMP assay, according to the supplier's information. The absorbance measurements of JC-1 aggregates (490 nm/590 nm) and its monomeric form (490 nm/530 nm) were conducted in real-time over 30 minutes. Results were reported as a percentage of the ratio of JC-1 monomers to aggregates relative to the negative control.

Caspase-3 assay

To determine the effect of *CSf* in the caspase-3 activity, cells at full confluence were transferred to a 96-wells plate and incubated for 6 hours either in the presence of 6-OHDA (100 µM) alone or followed by the addition of *CSf* (1 and 0.6 mg/mL). As a negative control, cells were incubated only with culture medium. Then, caspase-3 activity was measured, according to the supplier's information. The absorbance measurements (496 nm/520 nm) were performed in real-time for 60 min. The results were obtained using a linear regression model of the fluorescence spectral data and expressed in arbitrary units (ΔUA) of fluorescence/milligrams of protein/time (min).

H_2O_2 generation

To verify the effect of *CSf* in generation of H_2O_2, cells at full confluence, were transferred into microplates and incubated under exposure of 6-OHDA (100 µM) and/or only *CSf* (1 and 0.6 mg/mL) for 12 hours. As a negative control, cells were incubated only with culture medium. Afterward, quantification of H_2O_2 levels was performed using the hydrogen peroxide assay kit (Amplex™ Red), according to the supplier's information. The absorbance measurements (590 nm/530 nm) were performed in real-time during 60 min. The results were obtained using a linear regression model of the fluorescence spectral data and expressed as a percentage relative to the negative control.

Data and statistical analyses

Antioxidant and cellular assays results are presented as the SEM (mean ± standard error of mean). Paired Student's t-test was used to compare two groups. For comparisons involving three or more groups, One- or Two-way ANOVA (Analysis of Variance) was performed, and Bonferroni's post hoc test. Statistically significant differences were considered when *p*-value < 0.05. GraphPad Prism® 5.01 (GraphPad Software, San Diego, CA; www.graphpad.com) was used to perform the statistical analyses. All data

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were obtained of at least three independent experiments, carried out in triplicate and at different times.

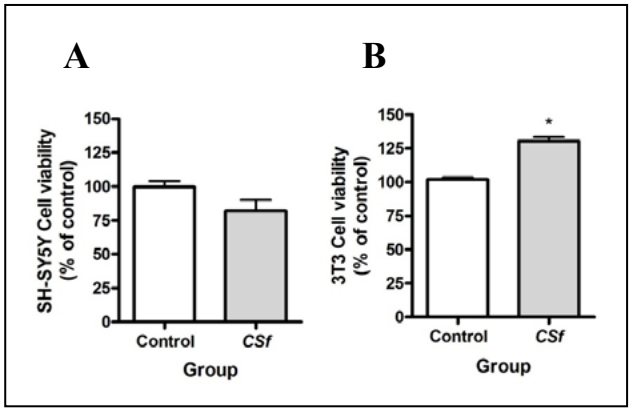


Figure 2. Cytotoxicity of *CSf* (1 mg/mL) on SH-SY5Y (**A**), and 3T3 cells (**B**), respectively. In Figure 2A, cellular viability does not show significant difference when compared to the control group, whereas in Figure 2B, an opposite trend is observed, indicating enhanced viability. Paired Student's t-test, $^{*}P < 0.05$, when compared with control group.

Results

Chemical analysis and antioxidant potential

The extraction yield of *CSf* was approximately 20% per gram of dry alga, with a free-sulfate content of about 28%. No protein contaminants were detected. The molar mass of *CSf* was estimated at 425 kDa. A single broad peak observed in the GPC analysis indicated a highly polydisperse molar mass. The antioxidant activity analysis of *CSf* revealed low antioxidant properties in the ORAC assay (28.2 ± 2 µmol Eq. Trolox per gram of *CSf*) and no activity in the DPPH and RP methods (Figures 1A and 1B). Although CSf has exhibited low antioxidant potential (less than 50%) compared to ascorbic acid, it showed a tendency toward H_2O_2 scavenging activity at a concentration of 1 mg/mL compared to the negative control. This effect became significant ($P < 0.05$) at a concentration of 2 mg/mL (Figure 1C).

Cytotoxicity and mitochondrial assessment

CSf (1 mg/mL) showed no cytotoxicity in the tested cell lines compared to their respective control groups (Figure 2). Furthermore, *CSf* (1 mg/mL) exhibited a capacity to increase the mitochondrial activity of 3T3 cells in relation to the control group (Figure 3A). Regarding the evaluation of neuroprotective activity on SH-SY5Y cells, it was possible to observe that the presence of *CSf* (1 and 0.6 mg/mL) protected the cells against mitochondrial activity changes ($P < 0.001$, 96.2 ± 0.05 and 62.4 ± 0.05, respectively), compared to 6-OHDA- treated group (42.7 ± 0.02). Moreover, this effect was also observed in the MMP depolarization assay (Figure 3B), where *CSf* (1 and 6 mg/mL) reduced the MMP depolarization ($P < 0.001$, 34.1 ± 0.02 and 54.6 ± 0.03, respectively) induced by 6-OHDA (99.8 ± 0.02). However, *CSf* was not able to return the MMP to basal levels (1.7 ± 0.001).

Analysis of caspase-3 activity and H_2O_2 generation

Concerning the caspase-3 assay, the SH-SY5Y cells cultivated in presence of 6-OHDA exhibited a reduction of this enzyme activity when treated with *CSf* (1 mg/mL: $P < 0.001$, 196.3 ± 0.55; and 0.6 mg/mL: $P < 0.01$, 233.1 ± 0.68, respectively), in relation to the non-treated group (505 ± 0.67) (Figure 3C). Furthermore, *CSf* (1 and 0.6 mg/mL) did not promote significant changes in caspase-3 activity in non-treated cells with the neurotoxin. Concomitantly, the analysis of H_2O_2 generation revealed high levels of H_2O_2 in cells exposed to 6-OHDA ($P < 0.05$, 215.6 ± 0.18), in relation to the control group (100.1 ± 0.27) (Figure 3D). On the other hand, SH-SY5Y cells treated with the highest concentration of *CSf* (1 mg/mL), maintained H_2O_2 generation at basal levels (119.1 ± 0.31). Additionally, *CSf* (1 and 0.6 mg/mL) did not stimulate significant changes in the H_2O_2 generation in non-treated cells with the neurotoxin, when compared to the control group.

Discussion

Because of structural heterogeneity and composition of polymers from marine algae, a broad range of bioactivities has been found. Furthermore, these polymers have shown potential for various pharmacological and biotechnological applications. Among them, a chemical and structural diversity of SPs have been investigated and classified [29, 31, 32]. Despite being relatively new to the scientific literature, neuroprotective activities from marine algae polymers are increasingly reported. These studies have been investigated and collaborated to a possible development of new therapeutic strategies and pharmacological applications [29, 33-35]. In the present study, the chemical characteristics, antioxidant potential, and cytotoxic and neuroprotective effects of the SP isolated from *S. filiformis* are reported. Indeed, the chemical features of the *CSf* have been previously well-reported in the literature [20-23]. Evidently, the analysis performed in this study revealed yield and sulphate content similar to the ι-carrageenan described by Araújo *et al*. [19]. Furthermore, a higher value of molecular mass with a polydispersive characteristic was identified. These findings are commonly exhibited by SPs from marine algae, due to the grouping of polysaccharide chains [11].

Among the bioactivities, the antioxidant action can serve as an indicator of a potential neuroprotective activity [36]. Antioxidants are compounds capable of either delay or inhibiting oxidation processes and belong to the defense mechanism of an organism against the development of pathologies associated with the attack of free radicals [37]. The antioxidant potential of the SP isolated from *S. filiformis* has been previously reported [22, 25]. Nevertheless, the results shown here revealed that *CSf* possesses weak antioxidant properties in general. The differences observed in the antioxidant capacity of a SP can be influenced by various factors, such as concentration, the antioxidant assay as well as by the extraction method chosen to analysis. Indeed, Peñuela *et al*. [25] reported a positive antioxidant

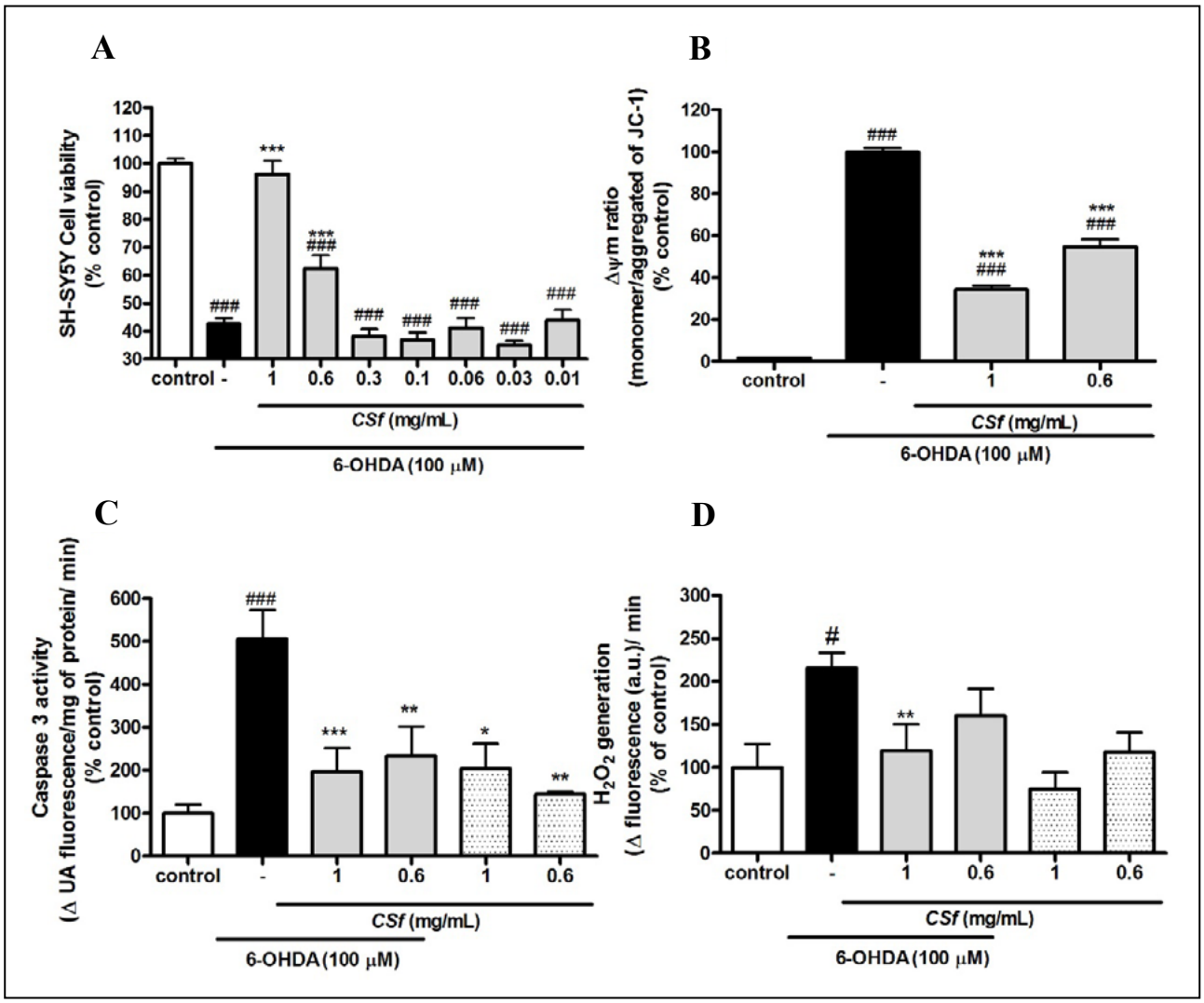


Figure 3. Neuroprotective effects of *CSf* in 6-OHDA-induced neurotoxicity model. (**A**) MTT assay, (**B**) MMP depolarization assay, (**C**) caspase-3 assay, and (**D**) H_2O_2 generation, respectively. In Figure 3A, seven concentrations of *CSf* were evaluated in the presence of 100 μM of the neurotoxin 6-OHDA. The most effective concentrations identified were subsequently analyzed further and are presented in Figure 3B, C, and D. Figure 3C and D also include results obtained in the absence of the neurotoxin. The values correspond to mean ± SEM. One-way ANOVA, Bonferroni test. $^{\#}P < 0.05$ and $^{\#\#\#}P < 0.001$, respectively, in relation with control group. $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$, respectively, in relation with 6-OHDA group.

action of a SP from *S. filiformis* (5 mg/mL) when tested in ABTS (2,2′-azino-bis-3-ethylbenzo thiazoline-6-sulfonic acid) and FRAP (ferric reducing power) assays. However, its activity was absent in the DPPH assay. According to the authors, due to the limitations of each antioxidant assay, the extraction method chosen might lead to under-estimation or undetectability of the radical scavenging activity of SPs. Considering these limitations, it is worth mentioning that four different assays were performed in the present study. Moreover, and interestingly, the *CSf* (1 mg/mL) showed potential for H_2O_2 scavenging capacity.

Cellular models have been proportioned to investigate molecular and physiologic findings related to several pathogeneses, including those in PD studies. In previous studies, SPs showed no toxic effects when analyzed in both *in vitro* and *in vivo* models [38-44]. Mehrban *et al*. [45] reported non-cytotoxic effects induced by *ι*-carrageenan on 3T3 cells. Therefore, it was also decided to include the 3T3 cell line as a toxicological control. In the present study, our data suggest that *CSf* does not induce a cytotoxic effect, corroborating with previous *in vitro* cytotoxic studies performed on 3T3 and other cell lines, such as colon epithelial cells derived from HT-29 (colorectal adenocarcinoma), HCT-8 (human ileocecal colorectal adenocarcinoma), Caco-2 (human colorectal adenocarcinoma), and HepG2 (human hepatoma) [45, 46]. Moreover, the *CSf* seems to stimulate the viability of 3T3 cells in the present study. According to Sun *et al*. [47], SPs (such as heparin, chondroitin sulphate, *λ*-carrageenan, and dextran sulphate) act on fibroblast growth factors and protect them from denaturation. In addition, the *CSf* did not promote toxic effects on SH-SY5Y cells. Thereby, the findings shown here align with previous studies, which suggest that the use of *CSf* is pharmacologically safe on the cells analyzed, and its presence in the cell culture can promote an increase in the viability of fibroblast cells.

Recently, neuroprotective activities of SPs isolated from seaweeds have been reported in the literature [44, 48-50]. However, few studies have focused on the application of carrageenans in neurodegeneration models. Equally

to the SH-SY5Y cell-line, the neurotoxin 6-OHDA has been useful to carrying out a widely recognized model for experimental PD scientific studies [3, 4, 7, 51]. Mitochondrial dysfunction has been shown to be related to the development of PD pathogenesis [52]. Actually, the mitochondria are the main target of 6-OHDA, which leads to the membrane permeabilization of this organelle and consequently to an apoptotic cascade in neuronal cells [53]. Our findings agree with previously reported studies in the literature showing the mitochondrial protective action stimulated by SPs [16, 29, 54]. Furthermore, our data suggest that *ι*-carrageenan used in this study has a superior effect on mitochondrial protection, when compared with *κ*-carrageenan isolated from red marine alga *Hypnea musciformis* in study by Souza *et al*. [29]. According to Ma *et al*. [55], the sulphate content in SPs is directly related to the improvement of mitochondrial protection. Consequently, the higher sulphate content in *ι*-carrageenan, in relation to the *κ*-carrageenan, was possibly responsible for the superior effect observed. Hence, our data suggest that ι-carrageenan not only shown a protective activity on mitochondria but also has a superior effect compared to *κ*-carrageenan.

The endoprotease caspase-3 has an apoptotic function that contributes to cell death by degrading proteins and it has been associated to neurodegenerative diseases, such as Alzheimer's disease and PD [49, 56]. Sato *et al*. [12] reported that SPs possess a capacity to modulate caspase-3 activity. Therefore, the neuroprotective effect of the *CSf* on caspase-3 activity was investigated. In the last year, SPs from seaweeds have been reported to regulate caspase-3 activity in *in vitro* models [16, 29, 49, 57]. For instance, Wei *et al*. [49] showed that *k*-carrageenan found in marine red algae can modulate the caspase-3 pathway and decrease cellular apoptosis induced by fragment of beta-amyloid peptide. Similarly, a *k*-carrageenan (*H. Musciformis*) has shown an antiapoptotic activity in SH-SY5Y cells treated with 6-OHDA [29]. Corroborating these previous studies, the present findings suggest that the *CSf* mediates antiapoptotic activity against 6-OHDA-induced cell death through caspase-3 modulation.

The neurotoxic effect of 6-OHDA results from oxidative stress induced by the production of reactive oxygen species (ROS) through its auto-oxidation after being taken up by the neuron via the dopamine transporter [58]. According to Kick *et al*. [59], a surplus of endogenous ROS such as H_2O_2 is associated with mitochondrial disturbances, leading to apoptotic factors release, caspase cascade activation, and finally, cellular death. Therefore, the balance between the generation of H_2O_2 and its neutralization by endogenous cellular defense mechanisms is one important factor in cellular homeostasis [60]. In the present study, the *CSf* attenuated H_2O_2 generation induced by 6-OHDA exposure without inducing cytotoxicity on normal cells. Corroborating with the present data from the H_2O_2 radical scavenging assay, these findings suggest that the *CSf* exhibits a homeostatic capacity, downmodulating the endogenous H_2O_2 generation induced by neurotoxin to basal levels in SH-SY5Y cells.

Conclusions

The current investigation has provided insight into the pharmacological potential of *CSf* demonstrating neuroprotective effects in a neurotoxic model through the modulation of H_2O_2 generation and caspase-3 activity to basal levels, as well as protection of the mitochondria. Additionally, *CSf* exhibited no cytotoxicity in the tested cells, suggesting its potential pharmacological safety for use in the development of novel treatments for neurodegenerative disorders. Although, the presented results are promising, additional more complex model studies are necessary to provide deeper insights into the mechanisms of action and potential translational applicability of *CSf*.

Declarations

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Conflict of interest: The authors have declared that no competing interests exist.

Ethical approval and informed consent: Not applicable.

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