International Journal of Biological Macromolecules 114 (2018) 565–571

Contents lists available at ScienceDirect

International Journal of Biological Macromolecules

journal homepage: http://www.elsevier.com/locate/ijbiomac

Genotoxicity and osteogenic potential of sulfated polysaccharides from *Caulerpa prolifera* seaweed

Gildácio Pereira Chaves Filho [a], Angélica Fernandes Gurgel de Sousa [a], Rafael Barros Gomes Câmara [b], Hugo Alexandre Oliveira Rocha [b], Silvia Regina Batistuzzo de Medeiros [a], Susana Margarida Gomes Moreira [a,*]

[a] Departamento de Biologia Celular e Genética, CB, UFRN, Brazil
[b] Laboratório de Biotecnologia de Polímeros Naturais-BIOPOL, Departamento de Bioquímica, CB, UFRN, Brazil

ARTICLE INFO

Article history:
Received 17 November 2017
Received in revised form 6 March 2018
Accepted 19 March 2018
Available online 22 March 2018

Keywords:
Bone regeneration
Osteoinductive effect
Green macroalgae

ABSTRACT

Marine algae are sources of novel bioactive molecules and present a great potential for biotechnological and biomedical applications. Although green algae are the least studied type of seaweed, several of their biological activities have already been described. Here, we investigated the osteogenic potential of Sulfated Polysaccharide (SP)-enriched samples extracted from the green seaweed *Caulerpa prolifera* on human mesenchymal stem cells isolated from Wharton jelly (hMSC-WJ). In addition, the potential genotoxicity of these SPs was determined by cytokinesis-block micronucleus (CBMN) assay. SP-enriched samples did not show significant cytotoxicity towards hMSCs-WJ at a concentration of up to 10 μg/mL, and after 72 h of exposure. SP enrichment also significantly increased alkaline phosphatase (ALP) activity, promoting calcium accumulation in the extracellular matrix. Among the SP-enriched samples, the CP0.5 subfraction (at 5 μg/mL) presented the most promising results. In this sample, ALP activity was increased approximately by 60%, and calcium accumulation was approximately 6-fold above the negative control, indicating high osteogenic potential. This subfraction also proved to be non-genotoxic, according to the CBMN assay, as it did not induce micronuclei. The results of this study highlight, for the first time, the potential of these SPs for the development of new therapies for bone regeneration.

1. Introduction

Sulfated polysaccharides (SPs) obtained from marine algae have attracted the attention of researchers because of their anticoagulant, antioxidant, antiproliferative, antitumoral, anti-inflammatory, antiviral activities, among others [1–3]. Recent studies linked marine algae SPs to the osteogenic differentiation of stem cells, through the regulation of growth factors binding and signaling pathways [4–7]. Kim and colleagues reported that SPs extracted from brown seaweeds promote osteoblast differentiation via JNK- and ERK-dependent BMP2-Smad 1/5/8 signaling in human mesenchymal stem cells [1–3]. However, to date, only few studies have explored the biological activities of SPs from green macroalgae (Chlorophyta division) species, such as *Caulerpa prolifera*, whose SPs extracts have presented antibacterial, antitumor and anticoagulant activities [8–10]. In addition, *C. prolifera*, similar to green algae in general, has a broad distribution along tropical waters [11,12], including the Rio Grande do Norte coastline, making it a good source for the extraction of compounds for biotechnological applications [8]. Despite its biotechnological potential, there are no studies on the application of SPs from *C. prolifera* in regenerative medicine. Indeed, there are only a few studies on functionalization of biomaterials for bone tissue engineering using ulvans, which are SPs isolated from green algae belonging to genus of *Ulva* and *Entermorpha* [13–15]. In recent years, some studies have also described the use of SPs from seaweeds, such as fucoidans and ulvans, for the development of nanomaterials (nanoparticles and nanofibers) for drug delivery systems. Along with their biological activities, these SP-based nanomaterials also avoid aggregation during blood circulation due to their physicochemical properties, indicating their potential application in approaches to regenerative medicine [10,16,17]. Since each SP has a unique structure, each of them is regarded as a novel compound with potentially new properties and distinct pharmacological activities. Furthermore, it is well accepted that several factors, including seasonality and extraction method, can influence the composition of SPs and consequently their biological effect [18–20]. Therefore, it is clear that new studies must be focused on the elucidation of the biological activity of SPs from different algal sources. In this context, this study aimed to investigate the osteogenic potential of the extract enriched in SPs from *C. prolifera* on human mesenchymal stem cells (hMSC).

The hMSC can be isolated from various tissues; however, MSCs isolated from human umbilical cord present unique advantages, such as a

* Corresponding author at: Universidade Federal do Rio Grande do Norte, Departamento de Biologia Celular e Genética, CB – UFRN, Campus Universitário, Lagoa Nova, 59072-970 Natal, RN, Brazil.
E-mail address: ssmargarida@gmail.com. (S.M.G. Moreira).

https://doi.org/10.1016/j.ijbiomac.2018.03.132
0141-8130/

non-invasive extraction protocol. In particular, hMSC-from Wharton jelly (hMSC-WJ) have showed to possess appealing characteristics for clinical applications than stem cells from other umbilical cord compartments, advocating their use as a model for the evaluation of inductive differentiation molecules [21].

Another important aspect concerning biomedical applications of new molecules or new materials is their biological safety. This includes an evaluation of cytotoxicity and genotoxicity. However, the latter is often neglected. An evaluation of genotoxicity is a crucial parameter for technological regulation and risk-assessment for several types of compounds, including SPs. For this reason, genotoxicity tests for drugs-validation are required by regulatory agencies, such as Food and Drug administration (FDA), European Medicines Agency, and Agência Nacional de Vigilância Sanitária (ANVISA, Brazil). Among the available genotoxicity tests, the Cytokinesis-Block Micronucleus (CBMN) assay has become one of the standard cytogenetic tests for genetic toxicology testing in human and mammalian cells due to its good reproducibility and reliability [22].

Despite the fact that green marine algae are accepted as an important source of novel bioactive molecules, only few studies have focused on their potential on regenerative medicine. This includes their SPs, whose biological activity and genotoxicity also remain quite unexplored. Therefore, we investigated the osteogenic potential of the crude extracts enriched in SPs extracted from the *C. prolifera*, as well as several subfractions obtained from its precipitation with acetone. In addition, the potential genotoxicity of these extracts was determined by the CBMN assay.

2. Methodology

2.1. Preparation and analysis of SP-enriched samples

Caulerpa prolifera (P. Forsskal, Lamouroux) was collected along the coast of Natal – RN, Brazil and SP-enriched samples were obtained following the method proposed by Costa and collaborators [8]. After collection and identification, the alga was dried in an oven, at 50 °C, under ventilation, ground in a blender, and macerated with acetone, for 24 h. Approximately 50 g of dry powdered algae were suspended in two volumes of 0.25 M NaCl (200 mL) and incubated with maxataze (a mix of proteases from *Esporobacillus*; BioBra's, Montes Claros, MG, Brazil), for 16 h, 60 °C, with a pH 8.0, under agitation. The mixture was filtered through cheesecloth and precipitated with 2.0 volumes of ice-cold methanol, under gentle agitation. The material was kept at 4 °C for 12 h. The precipitate (crude extract) was collected, by centrifugation (10,000 *g*, 20 min), and dried under vacuum. To prepare the CP subfractions, the crude extract was solubilized in two volumes of NaCl (0.25 M) and ice-cold acetone was added to the solution until it appeared turbid. After incubation at 4 °C for 16–18 h, the sample was centrifuged (10,000 *g*, 4 °C, 15 min) and the pellet dried under vacuum. This procedure was repeated to obtain the following subfractions: CP0.3, CP0.5, CP0.7, CP0.9, CP1.1, CP1.5 and CP2.0. These subfractions and the crude extract were resuspended in distilled water and stored to posterior analysis.

The total sugar concentration was estimated by the phenol-H_2SO_4 reaction, as proposed by Dubois [23], using D-galactose as a standard as described by Nader and Dietrich [24]. After acid hydrolysis of the polysaccharides (6 N HCl, 100 °C, 4 h) sulfate content was measured by the barium/gelatin method, as previously described [25].

The analysis of SP-enriched samples were performed in triplicate in two independent experiment.

2.2. MSC-WJ isolation and MTT assay

This study was approved by the National and Local Ethics Committees (number 508.459) and followed the criteria of the Helsinki convention. All umbilical cords specimens were obtained after a written informed consent signed by mothers. The hMSCs were isolated by treating the Wharton jelly's (WJ) from umbilical cordon with trypsin and collagenase [26], and characterized by their differentiation capacity and flow cytometry as described previously [27]. Cells, at passages 4–7, were cultured in growth medium composed of α-Minimum Essential Medium (α-MEM, Gibco, Gaithersbug, MD, USA), supplemented with 10% fetal bovine serum (FBS; Gibco), antibiotics (10,000 U/mL penicillin G and 25 μg/mL streptomycin, Gibco), and incubated in a humidified atmosphere containing 5% CO_2 at 37 °C.

The effect of SP-enriched samples on hMSC proliferation was assessed by 3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay (MTT; Invitrogen, Oregon, USA). Cells seeded in a 96-well plate (2×10^3 cells/well) were incubated in growth medium supplemented with SP-containing samples at different concentrations (0.1; 0.5; 1; 5 and 10 μg/mL), for 24, 48 and 72 h. MTT solution was added at a final concentration of 1 mg/mL, and incubated for 4 h. Subsequently, the medium was carefully removed, the formazan crystals were solubilized with dimethyl sulfoxide (DMSO), and the absorbance was measured at 570 nm using a microplate reader (μQuant™; Biotek).

The analyses of MTT reduction were performed in triplicate in three independent experiments.

2.3. Alkaline phosphatase activity assay

Alkaline phosphate (ALP) activity was measured for screening the osteogenic effect of each SP-enriched sample (crud extract and subfractions) on hMSC-WJ. Cells seeded in a 24-well plate (2×10^4 cells per well) were treated with SP-containing samples (0.5 and 5 μg/mL), and after 5 days of treatment, the ALP activity was accessed using *p*-nitrophenyl phosphate (Sigma-Aldrich Co.) as described by Cho et al. [28]. Cells were washed thrice with phosphate buffer solution (PBS), lysed, and incubated in the reaction medium (0.1 M $NaHCO_3$–Na_2CO_3 buffer, pH 10, containing 0.1% Triton X-100, 1.5 mM $MgCl_2$ and 3 mM *p*-nitrophenyl phosphate) for 1 h, at 37 °C, and the absorbance was measured at 405 nm. ALP activity was normalized by total cellular protein that was quantified by BCA protein assay (Thermo Scientific Pierce) according to the manufacturer protocol. Cells maintained in osteogenic medium, and basal medium without SP-enriched samples were used as positive and negative controls, respectively. The ALP activity assay was performed in triplicate in three independent experiments.

2.4. Mineralization of extracellular matrix

Mineralization was assessed by Alizarin Red-Sulfate (AR-S; Sigma Chemical, St. Louis, MO, USA) staining, using two different approaches: (1) cells cultured for 21 days with continuous exposure to each SP-enriched sample and (2) cells exposed for 72 h to SP-enriched samples and maintained in growth medium without SP-containing samples, for 18 days. Cells seeded in a 96-well plate (2×10^3 cells/well), and treated with SP-containing samples at concentration of 0.5 and 5 μg/mL, were fixed with 70% (v/v) ice-cold ethanol for at least 1 h, at 8–4 °C. Cells were stained using 40 mM AR-S in deionized water (pH 4.2) for 1 h at room temperature, and washed thrice with PBS before imaged (Olympus CKX41 inverted microscopy). For a quantitative analysis, 10% (v/v) of acetic acid was used for the solubilization. Subsequently, 100 μL of this solution was transferred to a 96-well plate, and the absorbance was measured at 405 nm. Results were normalized using the total cellular protein. This analysis was performed in triplicate in three independent experiments.

2.5. Cytokinesis block micronucleus assay

Chinese hamster ovarian cell line (CHO-K1 cells) is an established cell line for CBMN assays [32]. These cells were cultured in Dulbecco's modified eagle's medium (DMEM; Gibco, Gaithersbug, MD, USA), supplemented with 10% FBS and antibiotics (10,000 U/mL penicillin G and

25 μg/mL streptomycin). CBMN assay was carried out following the guidelines for the *in vitro* mammalian cell micronucleus test [29], and developed according to the Fenech protocol [22], with some modifications described below. The CHO-K1 cells were seeded in a 6-well plate (9.6×10^4 cells/well) and incubated for 24 h before the treatment with 5 μg/mL of SP-enriched samples that showed positive osteogenic results (crude extract and CP0.5 subfraction). Cytochalasin B (Cyt B; Sigma) was added to a final concentration of 5 μg/mL complete fresh medium, and incubated for another 24 h. Thereafter, cells were washed with PBS, detached using 0.25% trypsin, and 0.05% EDTA suspended in cultured medium, and fixed by three treatments with methanol:glacial acetic acid (9:1, v/v). Air-dried slides, stained with an aqueous solution of 4% Giemsa for 5 min, were analyzed by optical microscope, following the criteria for counting and characterizing micronuclei (MN), nuclear buds (NBUDs), nucleoplasmatic bridges (NPBs) and mononuclear and multi-nuclear cells, as proposed by Fenech [22]. The nuclear division index (NDI) was determined for each condition. Treatment with mitomycin C (0.1 μg/mL) (simultaneously added with cyt-B) [30] for 24 h was used as positive control, and cells cultured in DMEM were utilized as negative control. Approximately 2000 binuclear cells were analyzed per treatment in triplicate in three independent experiments, and results were expressed interms of the number of occurrences of alterations per 1000 binucleated cells.

2.6. Statistical analysis

The values are expressed as the mean ± standard deviation. Statistical analysis was performed by two-way analysis of variance (ANOVA), followed by Tukey's test for comparisons between the means of the control and test groups for MTT reduction test. ALP activity, degree of mineralization, and CBMN assay were analyzed by one-ANOVA followed by Tukey's test.

3. Results

3.1. Preparation and characterization of SP-enriched samples from C. prolifera

The chemical analysis of the SP-enriched samples obtained from *C. prolifera* is summarized on Table 1. CP0.5 and CP0.7 subfractions presented the highest yield. The crude extract displayed the highest sulfate/total sugar ratio (0.557 ± 0.019) followed by the CP0.5 subfraction (0.440 ± 0.011). Crude extract also showed 6.75 ± 1.70% proteins content. However, in CP0.5, no proteins were detected. The order of sulfate/total sugar ratio was: crude extract > CP0.5 > CP1.5 = CP0.7 = CP1.1 > CP2.0 > CP0.9 > CP0.3.

3.2. The effect of SPs isolated from C. prolifera on hMSC-WJ capacity for MTT reduction depended on sample, concentration, and time taken for testing

SP-containing samples showed no effect on cell capacity to reduce MTT at 24 h of exposure (Fig. 1). However, after 72 h, the effect on MTT reduction depended on both, sample and concentration used. Thus, the MTT reduction increased by 10–15% ($p \leq 0.05$) for CP0.5, CP0.7, and CP2.0 subfractions at 1 μg/mL, whereas CP0.5 and CP0.7 subfractions (Fig. 1), when used at 10 μg/mL, inhibited MTT reduction by 20–30% ($p \leq 0.05$).

Table 1
Chemical composition of SPs samples from *C. prolifera* seaweed.

Sample	Yield (%)	S/TS	Prot. (%)
Crude extract	–	0.56 ± 0.02	6.75 ± 1.70
CP0.3	7.20 ± 0.28	0.03 ± 0.01	n.d.
CP0.5	27.25 ± 1.77	0.44 ± 0.01	n.d.
CP0.7	23.30 ± 2.12	0.25 ± 0.04	n.d.
CP0.9	13.35 ± 0.63	0.09 ± 0.02	n.d.
CP1.1	6.60 ± 0.14	0.26 ± 0.02	n.d.
CP1.5	5.20 ± 0.28	0.27 ± 0.02	n.d.
CP2.0	7.60 ± 0.42	0.14 ± 0.03	0.3 ± 0.0

S = Sulfate; TS = Total sugar; Prot = Protein; nd = not detected.

3.3. SPs isolated from C. prolifera promoted osteogenic differentiation of hMSC-WJ

Alkaline phosphatase (ALP) activity and the degree of mineralization were measured as early and late marker of osteogenic differentiation, respectively. SP-containing samples significantly ($p < 0.05$) induced ALP activity in hMSC-WJ, and this effect depended on both the sample and concentration used. The highest ALP activity was 79.78 ± 4.80% in the crude extract, and 60.00 ± 5.00% in the CP0.5 subfraction, using 5 μg/mL of sample, in relation to the negative control (Fig. 2). In fact, when compared to the positive control, the crude extract and the CP0.5 subfraction showed similar results, with no significant differences between the groups.

To examine whether SP-enriched samples from *C. prolifera* promote calcium accumulation in the extracellular matrix, hMSC-WJ were treated with the SP-containing samples. After 21 days of continuous treatment, results showed that CP0.5 subfraction at 5 μg/mL significantly increased the mineralization of the extracellular matrix (5.91 ± 0.21×, $^*p < 0.001$) when compared with the negative control (Fig. 3). Moreover, treatment with the CP0.5 subfraction (5 μg/mL) for only 72 h also increased mineralization of extracellular matrix by 1.18 ± 0.11× ($p < 0.001$) (Fig. 3a and b).

3.4. SPs from C. prolifera do not induce genotoxicity

After the treatments with the crude extract and CP0.5 subfraction (at 5 μg/mL), CHO-K1 binucleated cells were counted, and the frequency of CBMN and other nuclear alterations such as MN, NPBs, and NBUDs resulting from DNA damages were analyzed. Under the tested conditions, the crude extract and CP0.5 subfraction did not induce micronuclei in CHO-K1 cells, as compared to the positive control (mitomycin C) (Table 2). In addition, the NDI results showed that these samples did not affect the cell proliferation.

4. Discussion

Marine algae are a source of molecules with multiple interesting proven biological activities. However, they remain quite unexplored. In particular, bioactivities of SPs isolated from green algae have not been elucidated despite their potential for biomedical applications. Nevertheless, they present a more complex and diverse chemistry than other algae, and they are broadly distributed. Taking into account the biological properties and availability of green algae, they make an attractive source for the development of pharmacological and medical applications [10]. In this context, the evaluation of the osteogenic effect of water soluble SPs isolated from the green alga *C. prolifera* was the objective of this study.

The results of MTT test showed no significant effect of SPs from *C. prolifera* on the proliferation of hMSC-WJ. However, when samples were used at concentrations of up to 1 μg/mL, a trend toward increased cell proliferation was observed, while concentrations above 5 μg/mL resulted in the opposite effect (Fig. 1). It is noteworthy that the effect of different SPs concentrations on MTT reduction varied according to cell type. For instance, the concentrations reported by Costa et al. [8] are highly toxic to hMSC-WJ used in this study (results not shown). This previous study examined the effect of SP-containing samples on the proliferation of HeLa cells. The results from this study showed that at 0.1 mg/mL, after 72 h of incubation, 50% of HeLa cells were viable. However, under this conditions, we observed that hMSC-WJ were not viable at all, reinforcing the idea that the effect of these SP-enriched samples are dependent on cell type.

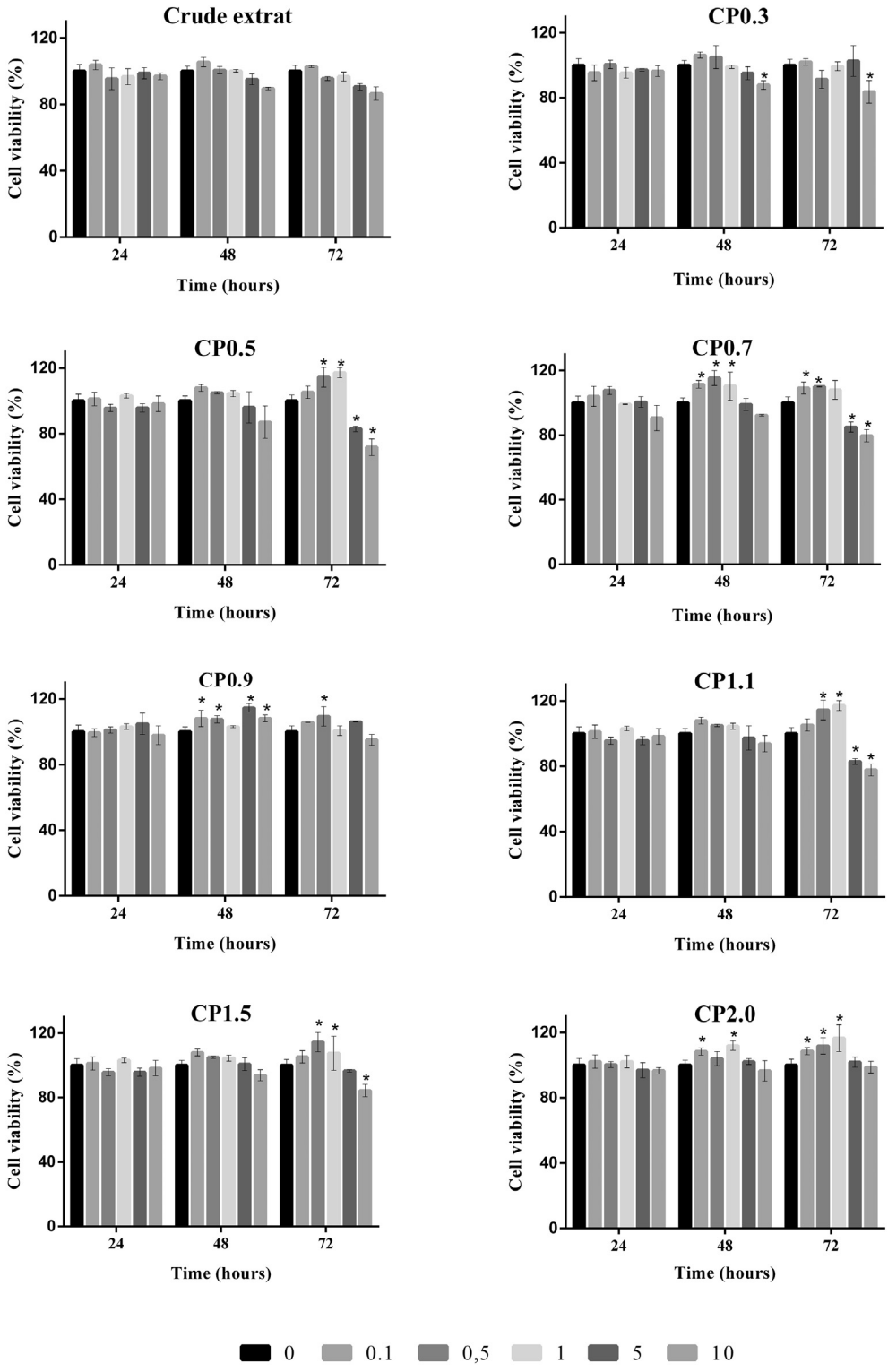

Fig. 1. Evaluation of the cytotoxicity of SP-enriched samples extracted from *C. prolifera* on hMSC-WJ, by MTT assay. hMSC-WJ were treated with SP-containing samples (Crude extract, CP0.3, CP0.5, CP0.7, CP0.9, CP1.1, CP1.5 and CP2.0 subfractions) at different concentrations (0,1–10 μg/mL), along 24, 48 and 72 h. Data represent the mean ± standard deviation of three independent experiments (*$p < 0.05$).

As shown in Table 1, the subfractions CP0.5 and CP0.7 presented the best yield (27.25 ± 1.767% and 23.30 ± 2.12, respectively) However, the CP0.5 subfraction also has the highest sulfate/total sugar ratio (0.440 ± 0.011), representing a 150% increase in relation to the CP0.7 subfraction, which had the second highest sulfate/sugar ratio (0.254 ± 0.038). It is well accepted that amount of sulfate directly influences the biological activity. For example, Qi et al. [28] showed that ulvans (SPs of green algae) containing higher sulfate amount exhibited increased antioxidant activity. The positive effect of sulfated groups on SPs from green algae has also been observed in osteogenic differentiation combined with biomaterials or biomolecules [31,32], such as angiogenic factors, in a synergistic approach. Taking into account the high yield and sulfate content, we choose to proceed with CP0.5 sample for further assays.

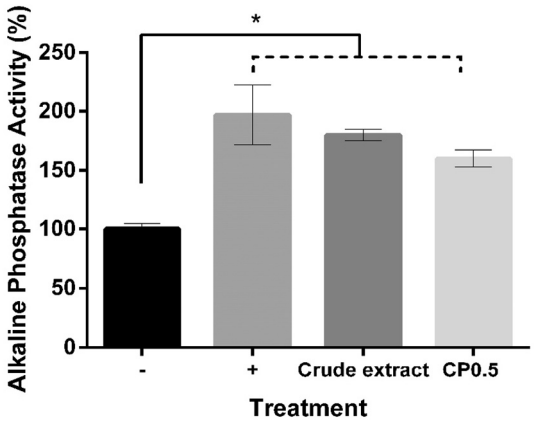

Fig. 2. Evaluation of osteogenic activity of SP-containing samples extracted from *C. prolifera*. ALP activity was assessed by the conversation of *p*-nitrophenyl. Results were normalized by total protein content as describe in methods. Data represent the mean ± standard deviation of three independent experiments (*$p < 0.05$). − = Negative control; + = Positive control.

In the present study, we report the increase of ALP activity in hMSC-WJ, treated with SP-enriched samples of *C. prolifera*, and show that the effect on ALP activity is dependent on sample used. Thus, the crude extract and CP0.5 subfraction, at 5 μg/mL promoted an increase in ALP activity by 79.78 ± 4.80% and 60.0 ± 5.0%, respectively, compared to negative control, which is a result similar to that obtained to the positive control.

Some researchers have investigated the effect of fucoidan (sulfated L-fucose-rich polysaccharide) isolated from different brown seaweeds on the osteogenic differentiation of stem cells (from bone marrow and adipose tissue) and preosteoblastic cells [5,28,33]. Changotade et al. [33] demonstrated the synergistic effect of low molecular weight fucoidan of unspecified origin, on the osteogenic differentiation of stem cells derived from adipocyte tissue, co-treated with known osteogenic differentiation stimulators. In another study, Cho and coworkers [28] revealed that fucoidan (in the absence of other osteogenic stimulators) increased ALP activity in MG-63 cells (a preosteoblastic cells). These results were confirmed by Kim and colleagues on MSCs isolated from the bone marrow. Furthermore, these results elucidated the molecular basis of the osteogenic effects of fucoidan in MSCs, showing

Table 2
Effect of the exposure of CHO-K1 cells to crude extract and CP0.5 subfraction (5 μg/mL) on the frequencies of MN, NBUDs, and NPBs in 1000 binucleate cells.

Treatment	MN	NBUDs	NPBs	NDI
NC	9.75 ± 1.76	28 ± 7.45	23.3 ± 1.4	1.99 ± 0.005
Crude extract	8 ± 2.12	29.25 ± 1.06	31 ± 5.65	1.99 ± 0.05
CP0.5	7 ± 2.12	28.25 ± 4.95	24 ± 1.06	1.98 ± 0.07
PC	46.2 ± 3.18*	71.75 ± 3.9*	74 ± 4.95*	1.99 ± 0.002

Cells maintained in growth medium and growth medium containing mitomycin C were used as negative (NC) and positive control (PC), respectively. Data represent the mean ± standard deviation of three independent experiments (*$p < 0.05$).

that osteogenic differentiation is induced through BMP2-Smad 1/5/8 signaling by activating ERK and JNK [5]. However, it is important to reinforce that in the present study used no osteogenic inductors, such as dexamethasone and beta glycerol phosphate, with SP-enriched samples from *C. prolifera*, and the ALP activity was still significantly increased in MSC-WJ, which are not preosteoblastic cells. This pointing highlights the tremendous biotechnological potential of this green alga species.

The CP0.5 subfraction, at 5 μg/mL, also induced extracellular matrix mineralization, which increased to about 5.91 ± 0.21× at the end of 21 days, and to 1.18 ± 0.11× after only 72 h of treatment in comparison with negative control. These results correspond to a similar result obtained to the positive control. Although the results of ALP activity assay were similar for the crude extract and the CP0.5 subfraction, the former did not induce extracellular matrix mineralization under the conditions used in the assay (data not shown). Our finding strongly suggest that the CP0.5 subfraction holds SPs with greater osteogenic effect on hMSC-WJ, by inducting and enhancing the mineralization of extracellular matrix, a phenotypic marker of the final stages of osteogenic differentiation [5]. According to their composition, the crude extract has the major content of sulfate, although it is contaminated with proteins. Thus, this sample should contain small peptides resulting from protease treatment instead intact proteins. However, the CP0.5 subfraction contained almost the same sulfate amount (0.440 ± 0.011), and no proteins were detected in this sample. It is well accepted that molecules with the presence of polyanionic disaccharide repeating structures, such as heparan sulfate (negatively charged), in the extracellular matrix, could interact with many proteins (including structural proteins present in the extracellular matrix, growth factors and their receptors, among others), thereby regulating cellular behavior

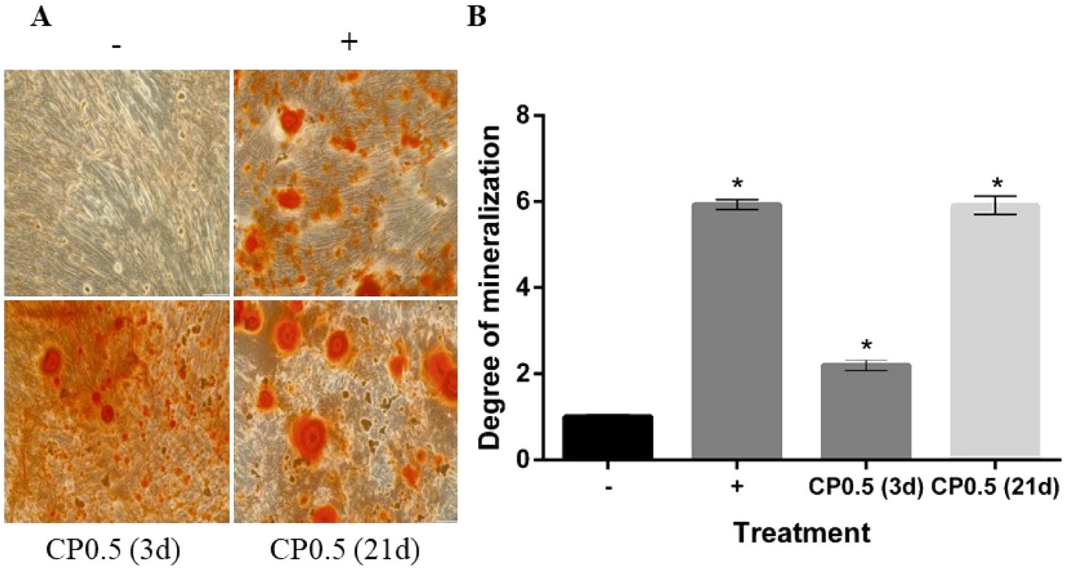

Fig. 3. Osteogenic activity of SP-enriched samples extracted from *C. prolifera*. (a) Determination of calcium accumulation using AR-S staining: (3d) hMSC-WJ treated with CP0.5 subfraction (5 μg/mL) for 3 days and maintained in growth medium for up to 21 days of culture; or (21d) cells maintained in growth medium supplemented with SP-containing samples along 21 days. (b) Quantification of the calcium accumulation at 405 nm. Scale bar = 1 mm. Data represent the mean ± standard deviation of three independent experiments (*$p < 0.05$).

characteristics, such as stem cell differentiation [34]. The crude extract had 6.75 ± 1.70% of contaminant proteins (Table 1), and we hypothesized that these proteins could interact with the sulfate present on the SPs of the sample, altering their activity/bioavailability. Alternatively, they could interact with cell receptors, modulating the cell response. In addition, the crude extract is a complex mixture of molecules that may affect cellular behavior, whereas the CP0.5 subfraction, being a more refined sample, may result in less biological interference. Therefore, the subfractioning of the crude extract acted to purify and concentrated the SPs in CP0.5 subfraction, resulting in improved osteoinductive activity and low toxicity for long exposure periods. Nevertheless, our findings suggest that the high sulfate content of our samples, especially on subfraction CP0.5, may play a role in the hMSC differentiation, and have potential for bone regeneration approaches.

Since potential for biomedical application is being explored, the genotoxicity of crude extract and CP0.5 subfraction was accessed using CBMN assay on CHO cells. We chose this cell model because it is an established cell line for genotoxicity assays, including on the CBMN assay [22,35]. Samples enriched in SPs from *C. prolifera* did not present genotoxic effects under the conditions of the CBMN assay. Nevertheless, the negative results in the micronucleus tests were also reported with fucoidan [36,37]. Importantly, this is the first study demonstrating the lack of genotoxicity of SPs isolated from green seaweed. Thus far, such evaluation has only been carried out for carotenoids extracted from green seaweed [38].

These results represent a step toward the development of alternative therapies for several bone diseases as well as the improvement of existing therapies. We demonstrated the osteogenic potential of these SPs and their non-genotoxicity. Importantly, the water solubility of the samples represents a great advantage for industrial and biomedical applications. Another advantage that highlight the potential application of these products is the ability to achieve the desired effect with lower doses (5 μg/mL), even using perinatal MSCs, without genotoxic effect. Thus, more studies are needed in order to determine and isolate the bioactive molecules responsible for these biological effects from the CP0.5 subfraction, and propose correlations to the structural activity and molecular mechanism involved in the osteogenic activity of these SP-enriched samples.

5. Conclusion

In this study, we demonstrated the osteogenic potential of sulfated polysaccharides extracted from *C. prolifera.* Human MSCs were induced to differentiate into osteoblasts using low amounts of water-soluble CP0.5 sample, which is a subfraction with a higher SPs content, indicating a role for these molecules in MSC differentiation. In addition, we also showed the non-genotoxicity of these SPs, by CBMN assay. Taken together, these results highlight the potential of SPs from *C. prolifera* for the development of alternative therapies for bone regeneration. To our knowledge, this is one of the only few studies demonstrating the osteogenic potential of isolated compounds in this type of green seadweed, thus contributing to information regarding the therapeutic and pharmacological potential of marine products.

Acknowledgements

Funding

This work was supported by Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq) process n° 401600/2016-5, INCT in Regenerative Medicine (CNPq process n. 465656/2014-5). Coordenação de Aperfeiçoamento Pessoal de Nível Superior-CAPES - Edital Ciências do Mar. Gildácio received a grant from Pró-Reitoria de Pesquisa (Propesq, UFRN). Hugo Rocha and Silvia Medeiros are CNPq fellowship honored researchers.

Authors' contributions

GPCF and AFGS developed the experimental assays and data analysis; RBGC extracted, prepared and characterized the SP-enriched samples; GPCF, HAOR, SRBM, and SMGM conceived the study, participated in its design and data analysis, and helped to draft the manuscript. All authors read and approved the final manuscript.

References

[1] M.T. Ale, J.D. Mikkelsen, A.S. Meyer, Important determinants for fucoidan bioactivity: a critical review of structure-function relations and extraction methods for fucose-containing sulfated polysaccharides from brown seaweeds, Mar. Drugs 9 (10) (2011) 2106–2130.
[2] G. Jiao, G. Yu, J. Zhang, H.S. Ewart, Chemical structures and bioactivities of sulfated polysaccharides from marine algae, Mar Drugs 9 (2) (2011) 196–223.
[3] L. Wang, X. Wang, H. Wu, R. Liu, Overview on biological activities and molecular characteristics of sulfated polysaccharides from marine green algae in recent years, Mar Drugs 12 (9) (2014) 4984–5020.
[4] P. Garg, M.M. Mazur, A.C. Buck, M.E. Wandtke, J. Liu, N.A. Ebraheim, Prospective review of mesenchymal stem cells differentiation into osteoblasts, Orthop. Surg. 1 (2017) 13–19.
[5] B.S. Kim, H.J. Kang, J.Y. Park, J. Lee, Fucoidan promotes osteoblast differentiation via JNK- and ERK-dependent BMP2-Smad 1/5/8 signaling in human mesenchymal stem cells, Exp. Mol. Med. 47 (2015), e128.
[6] S.J. Park, K.W. Lee, D.S. Lim, S. Lee, The sulfated polysaccharide fucoidan stimulates osteogenic differentiation of human adipose-derived stem cells, Stem Cells Dev. 21 (12) (2012) 2204–2211.
[7] J. Pereira, S. Portron, B. Dizier, C. Vinatier, M. Masson, S. Sourice, I. Galy-Fauroux, P. Corre, P. Weiss, A.M. Fischer, J. Guicheux, D. Helley, The in vitro and in vivo effects of a low-molecular-weight fucoidan on the osteogenic capacity of human adipose-derived stromal cells, Tissue Eng Part A 20 (1–2) (2014) 275–284.
[8] L.S. Costa, G.P. Fidelis, S.L. Cordeiro, R.M. Oliveira, D.A. Sabry, R.B. Camara, L.T. Nobre, M.S. Costa, J. Almeida-Lima, E.H. Farias, E.L. Leite, H.A. Rocha, Biological activities of sulfated polysaccharides from tropical seaweeds, Biomed Pharmacother 64 (1) (2010) 21–28.
[9] S. Selim, A. Amin, S. Hassan, M. Hagazey, Antibacterial, cytotoxicity and anticoagulant activities from Hypnea esperi and Caulerpa prolifera marine algae, Pak. J. Pharm. Sci. 28 (2) (2015) 525–530.
[10] T.H. Silva, A. Alves, E.G. Popa, L.L. Reys, M.E. Gomes, R.A. Sousa, S.S. Silva, J.F. Mano, R.L. Reis, Marine algae sulfated polysaccharides for tissue engineering and drug delivery approaches, Biomatter 2 (4) (2012) 278–289.
[11] F. Tuya, L. Png-Gonzalez, R. Riera, R. Haroun, F. Espino, Ecological structure and function differs between habitats dominated by seagrasses and green seaweeds, Mar. Environ. Res. 98 (2014) 1–13.
[12] M. Dolbeth, P. Stalnacke, F.L. Alves, L.P. Sousa, G.D. Gooch, V. Khokhlov, Y. Tuchkovenko, J. Lloret, M. Bielecka, G. Rozynski, J.A. Soares, S. Baggett, P. Margonski, B.V. Chubarenko, A.I. Lillebo, An integrated Pan-European perspective on coastal lagoons management through a mosaic-DPSIR approach, Sci. Rep. 6 (2016), 19400.
[13] A.A. Barros, A. Alves, C. Nunes, M.A. Coimbra, R.A. Pires, R.L. Reis, Carboxymethylation of ulvan and chitosan and their use as polymeric components of bone cements, Acta Biomater. 9 (11) (2013) 9086–9097.
[14] G. Toskas, S. Heinemann, C. Heinemann, C. Cherif, R.D. Hund, V. Roussis, T. Hanke, Ulvan and ulvan/chitosan polyelectrolyte nanofibrous membranes as a potential substrate material for the cultivation of osteoblasts, Carbohydr. Polym. 89 (3) (2012) 997–1002.
[15] M. Dash, S.K. Samal, C. Bartoli, A. Morelli, P.F. Smet, P. Dubruel, F. Chiellini, Biofunctionalization of ulvan scaffolds for bone tissue engineering, ACS Appl. Mater. Interfaces 6 (5) (2014) 3211–3218.
[16] G. Toskas, R.-D. Hund, E. Laourine, C. Cherif, V. Smyrniotopoulos, V. Roussis, Nanofibers based on polysaccharides from the green seaweed Ulva Rigida, Carbohydr. Polym. 84 (3) (2011) 1093–1102.
[17] J. Venkatesan, S. Anil, S.-K. Kim, M. Shim, Seaweed polysaccharide-based nanoparticles: preparation and applications for drug delivery, Polymers 8 (2) (2016) 30.
[18] V. Grauffel, B. Kloareg, S. Mabeau, P. Durand, J. Jozefonvicz, New natural polysaccharides with potent antithrombic activity: fucans from brown algae, Biomaterials 10 (6) (1989) 363–368.
[19] H.A. Rocha, C.R. Franco, E.S. Trindade, L.C. Carvalho, S.S. Veiga, E.L. Leite, C.P. Dietrich, H.B. Nader, A fucan from the brown seaweed Spatoglossum schroederi inhibits Chinese hamster ovary cell adhesion to several extracellular matrix proteins, Braz. J. Med. Biol. Res. 34 (5) (2001) 621–626.
[20] K. Ehrig, S. Alban, Sulfated galactofucan from the brown alga Saccharina latissima–variability of yield, structural composition and bioactivity, Mar Drugs 13 (1) (2014) 76–101.
[21] A. Subramanian, C.Y. Fong, A. Biswas, A. Bongso, Comparative characterization of cells from the various compartments of the human umbilical cord shows that the Wharton's jelly compartment provides the best source of clinically utilizable mesenchymal stem cells, PLoS One 10 (6) (2015), e0127992.
[22] M. Fenech, Cytokinesis-block micronucleus cytome assay, Nat. Protoc. 2 (5) (2007) 1084–1104.
[23] M. Dubois, K. Gilles, J.K. Hamilton, P.A. Rebers, F. Smith, A colorimetric method for the determination of sugars, Nature 168 (4265) (1951) 167.

[24] H.B. Nader, C.P. Dietrich, Determination of sulfate after chromatography and toluidine blue complex formation, Anal. Biochem. 78 (1) (1977) 112–118.
[25] K.S. Dodgson, R.G. Price, A note on the determination of the ester sulphate content of sulphated polysaccharides, Biochem. J. 84 (1962) 106–110.
[26] P. Salehinejad, N.B. Alitheen, A.M. Ali, A.R. Omar, M. Mohit, E. Janzamin, F.S. Samani, Z. Torshizi, S.N. Nematollahi-Mahani, Comparison of different methods for the isolation of mesenchymal stem cells from human umbilical cord Wharton's jelly, In Vitro Cell. Dev. Biol. Anim. 48 (2) (2012) 75–83.
[27] C.K. Rebelatto, A.M. Aguiar, M.P. Moretao, A.C. Senegaglia, P. Hansen, F. Barchiki, J. Oliveira, J. Martins, C. Kuligovski, F. Mansur, A. Christofis, V.F. Amaral, P.S. Brofman, S. Goldenberg, L.S. Nakao, A. Correa, Dissimilar differentiation of mesenchymal stem cells from bone marrow, umbilical cord blood, and adipose tissue, Exp. Biol. Med. (Maywood) 233 (7) (2008) 901–913.
[28] Y.-S. Cho, W.-K. Jung, J.-A. Kim, I.L.W. Choi, S.-K. Kim, Beneficial effects of fucoidan on osteoblastic MG-63 cell differentiation, Food Chem. 116 (4) (2009) 990–994.
[29] OECD, Test No. 487: In Vitro Mammalian Cell Micronucleus Test, OECD Publishing.
[30] M.J. Aardema, R.D. Snyder, C. Spicer, K. Divi, T. Morita, R.J. Mauthe, D.P. Gibson, S. Soelter, P.T. Curry, V. Thybaud, G. Lorenzon, D. Marzin, E. Lorge, SFTG international collaborative study on in vitro micronucleus test III. Using CHO cells, Mutat. Res. 607 (1) (2006) 61–87.
[31] X. Yuan, R.J. Smith Jr., H. Guan, C.N. Ionita, P. Khobragade, R. Dziak, Z. Liu, M. Pang, C. Wang, G. Guan, S. Andreadis, S. Yang, Hybrid biomaterial with conjugated growth factors and mesenchymal stem cells for ectopic bone formation, Tissue Eng. A 22 (13–14) (2016) 928–939.
[32] B. Labat, S. Morin-Grognet, F. Gaudiere, L. Bertolini-Forno, O. Thoumire, J.P. Vannier, G. Ladam, H. Atmani, Synergistic influence of topomimetic and chondroitin sulfate-based treatments on osteogenic potential of Ti-6Al-4V, J. Biomed. Mater. Res. A 104 (8) (2016) 1988–2000.
[33] S.I. Changotade, G. Korb, J. Bassil, B. Barroukh, C. Willig, S. Colliec-Jouault, P. Durand, G. Godeau, K. Senni, Potential effects of a low-molecular-weight fucoidan extracted from brown algae on bone biomaterial osteoconductive properties, J. Biomed. Mater. Res. A 87((3) (2008) 666–675.
[34] S. Zhao, C. Deng, Z. Wang, L. Teng, J. Chen, Heparan sulfate 6-O-sulfotransferase 3 is involved in bone marrow mesenchymal stromal cell osteogenic differentiation, Biochemistry (Mosc) 80 (3) (2015) 379–389.
[35] X. Xu, H. Nagarajan, N.E. Lewis, S. Pan, Z. Cai, X. Liu, W. Chen, M. Xie, W. Wang, S. Hammond, M.R. Andersen, N. Neff, B. Passarelli, W. Koh, H.C. Fan, J. Wang, Y. Gui, K.H. Lee, M.J. Betenbaugh, S.R. Quake, I. Famili, B.O. Palsson, J. Wang, The genomic sequence of the Chinese hamster ovary (CHO)-K1 cell line, Nat. Biotechnol. 29 (8) (2011) 735–741.
[36] K.J. Kim, O.H. Lee, B.Y. Lee, Genotoxicity studies on fucoidan from Sporophyll of Undaria pinnatifida, Food Chem. Toxicol. 48 (4) (2010) 1101–1104.
[37] M.Y. Song, S.K. Ku, J.S. Han, Genotoxicity testing of low molecular weight fucoidan from brown seaweeds, Food Chem. Toxicol. 50 (3–4) (2012) 790–796.
[38] S. Bhagavathy, P. Sumathi, Evaluation of antigenotoxic effects of carotenoids from green algae Chlorococcum humicola using human lymphocytes, Asian Pac J Trop Biomed 2 (2) (2012) 109–117.