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Chemodiversity of Galactans from Different Red Seaweed Species with Emphasis on their Antioxidant and Antiproliferative Activities *in vitro*

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HIGHLIGHTS

- Extraction and characterization of galactan from different five red species.
- The biological activities of galactans were correlated to their uronic acid and sulphate content.
- *Galaxaulra rugosa* galactan had the highest antioxidant and antiproliferative activity.
- The extracted galactans can be used to improve the human health.

Abstract: Crude galactans isolated from five different seaweed species collected from the Lebanese coast were chemically characterized and then evaluated for their antioxidant and antiproliferative properties *in vitro*. The yield of extracted sulfated galactans varied in the range 4.03%-14.32% dry weight in *Tricleocarpa fragilis* and *Osmande dechybrida*, respectively. The chemical composition of all extracted galactans exhibited high levels of carbohydrate, uronic acid and sulfate contents with trace amounts of protein. FTIR analysis showed that all the extracted galactans were agar except for *Galaxaulra rugosa* galactan (GRG) and *O. dechybrida* galactan (ODG). The antioxidant activity of all extracted galactans using multifunctional antioxidant ability was assayed by total antioxidant capacity, DPPH radical-scavenging activity, hydrogen peroxide scavenging assay and reducing power**.** Galactans from *G. rugosa* exhibited higher antioxidant activity compared with the standard antioxidant (α-tocopherol). Whereas *T. fragilis* galactan (TFG) showed the lowest antioxidant activity. The maximum antiproliferative potency was detected in GRG and *Ligora viscida* galactan (LVG) at 1.5%. The tested biological activity of the isolated galactans increased with increasing concentrations, indicating the dose-dependency of their chemical properties. The antiproliferative activity showed a significant positive correlation with antioxidant activities. Moreover, these activities were correlated with the combined effects of uronic acid and sulfate content. Identification of these sulfated polysaccharides in algal species is chemotaxonomic significance in view of their potential economical applications as a natural product. The most activated galactans have been selected for further fractionation and characterization and the necessary *in vivo* experiments are already in progress.

Keywords: DPPH; FTIR; Hell cell; Reducing Power; Macroalgae; Polysaccharides; Rhodophyta.

INTRODUCTION

Marine macroalgae “seaweed” represent a potentially renewable source of various bioactive compounds such as polysaccharides, carotenoids, proteins, fatty acids, vitamins and minerals [1-4]. Rhodophyta are the largest group of eukaryotic algae containing about 5000-6000 species. Sulfated galactans are the main polysaccharides in the red macroalgae cell wall matrix and intercellular space [5] and represent 50%-60% of their total dry weight; thus they are mainly composed of galactans consisting entirely of galactose or modified galactose units [6] besides cellulose and hemicellulose-like polysaccharides [7]. Red seaweed, especially those in the orders "Gelidiales and Gigartinales" are a major source of marine sulfated galactans, with at least 70-80 of these species being industrially exploited for galactan production [8]. Galactan sulfates are linear polysaccharides with alternating 3-linked β-D-galactopyranose units and 4-linked 3,6-anhydro-α-galactopyranose or α-galactopyranose units, with different positions and degrees of sulfation. Methyl ethers, pyruvic acid ketals, and single stubs of β-D-xylopyranose and/or other monosaccharide substituent are sometimes present and characterized by high molecular weight (>100 kDa).

On the basis of stereochemistry, sulfated galactans are classified into agars, carrageenans and D/L hybrids (also known as nonideal or complex sulfated galactans) [8]. Generally, most red macroalgal polysaccharides possess various biological activities and industrial applications [9-10]. Since ancient times, sulfated galactans such as agar and carrageenan have been consumed by humans and later utilized in traditional medicine while exhibiting a broad spectrum of biological abilities regarding human health [6-11-12]. Carrageenans are used as a thickening agent in food as well as in pharmaceutical compounds and are generally regarded as safe. Agars are utilized in biotechnology research as media for microbial growth or for separation matrixes. The macroalgal galactans are natural antioxidants and replace the synthetic commercial antioxidants e.g., butylated hydroxyanisole and butylated hydroxyl toluene which may be toxic and cause liver damage [13]. Numerous researchers have reported on the biological activities of sulfated galactans from red seaweed including antioxidant, anti-inflammatory, anticoagulant [5], antiviral, antiproliferative and antitumor properties [10-14].

The aim of the present study was to extract and characterize crude galactans from different red seaweed species collected from the Lebanese coast and to evaluate their antioxidant and antiproliferative activities against HeLa cells *in vitro*.

MATERIAL AND METHODS

Collection and identification of the tested seaweed

The five red seaweed collected in this study were *Galaxaulra rugosa, Tricleocarpa fragilis* (order: [1]Nemaliales, family: [1]Galaxauraceae), *Ligora viscida* (family: [2]Ligoracea), *Osmande dechybrida* and *Palisada perfosata* (order: [2]Ceramiales, family: [1]Rhodomelaceae). Seaweed samples were hand-picked from Rawché beach, Mediterranean Sea, Lebanese during December 2019. The samples were brought to the laboratory in an ice tank to avoid evaporation then washed with tap water to remove dirt, and salt particles. A portion of the algal samples was preserved in formalin (4%) for taxonomical identification and the remaining samples were shade dried (35±3°C) for 72 h. Samples were then powdered and stored in plastic bags at 2°C for further tests. All the algal samples were identified following the description of Aleem [15]; Kanaan & Belous [16]. The species names applied were according to Guiry and Guiry [17].

Extraction and preparation of sulfated galactans

The sulfated galactans were extracted according to Farias and coauthors [18] method. The extracted galactans were designated as, GRG=*Galaxaulra rugosa*, LVG=*Ligora viscida*, ODG=*Osmande dechybrida*, PPG=*Palisada perfosata*, TFG=*Tricleocarpa fragilis*. The ratio of extracted galactan yield was calculated basis on the algal dry weight.

Chemical properties of the extracted galactans

The moisture and ash contents of the isolated galactans were determined after drying at 120 C for 2 h and igniting at 550°C for 6 h. Total sugar and protein were estimated according to Dubois and coauthors [19]; Lowery and coauthors [20] methods, respectively. Uronic acid was determined colorimetrically by a UV-Vis spectrophotometer, glucuronic acid was used as a standard [21]. Sulfate content was detected

turbidimetrically (Hach 2100A) after acid hydrolysis of the polysaccharides (HCl 6 mol/L, 100ºC, 4 h) as indicated by the gelatin–barium method [22].

Fourier transform infrared (FTIR) spectra

FTIR spectrophotometer scanning between 4000 and 400 cm^{-1}. The dried sulfated galactans (10 mg) were mixed with KBr (100 mg) and compressed to prepare as a salt disc. The frequencies of different components present in each sample were analyzed.

Biological activities

Antioxidant activity

Total antioxidant capacity assay "TAC"

The antioxidant capacity of different crude extracts was estimated at 695 nm as previously described Smirnoff and Cumbes [23] method [19] and expressed as ascorbic acid equivalent (mg/ASA equivalent).

Determination the DPPH radical-scavenging activity

The scavenging effects of the extracted galactans were determined by the method of Choi and coauthors [24] at 492 nm and calculated using previously described formulas [25]. α tocopherol was used as a standard.

$$\text{DPPH scavenging activity } (\%) = \frac{(\text{Ac} - \text{As})}{Ac} \times 100$$

Where, Ac= Absorbance of control
As= Absorbance of sample

Hydrogen peroxide scavenging assay

The hydrogen peroxide scavenging activity of the samples was quantified at 230 nm using the method of Gülcin and coauthors [26] and calculated using the following formula:

$$\%\text{ of scavenging} = \frac{(Ac - As)}{Ac} \times 100$$

Reducing power (RP)

The RP of the tested polysaccharides was determined by the method of Wang and coauthors [27] at 700 nm. Ascorbic acid was used as a standard antioxidant.

Cell proliferation studies

The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay was performed to measure the antiproliferative of the extracted sulfated galactans on Hela cervical cancer cell lines which were procured from the American University of Beirut. These cells are an aneuploid tumorigenic cells obtained from a malignant human cervical carcinoma, express E6 and E7 proteins from integrated HPV18 DNA and show aberrant checkpoint control [28]. Briefly, cells were seeded into 96-well plates at a density of 5 $\times 10^3$ cell/well and allowed to attach overnight in 300 mL Dulbecco's modified Eagle medium incubated at 37°C and 5% CO_2 before to galactan treatment. Subsequently, cells were treated with galactans at different concentrations "0.05; 0.1; 1.0 and 1.5 mg/mL" for 72 h at 37ºC and 5% CO_2.After incubation, traces of the extracted galactans were removed by washing the cells twice with 200 mL PBS followed by the addition of 100 mL of fresh medium containing 10 mL of 12 mM MTT dissolved in PBS, and the cells were then incubated for 4 h at 37ºC with 5% CO_2. To solubilize the product of MTT cleavage, 100 mL of isopropanol containing 0.04 N HCl was added to each well and thoroughly mixed using a multichannel pipettor. Within 1 h of HCl-isopropanol addition, the absorbance at 570 nm was read using a Multiskan Ascent Microplate Reader (Thermo Labsystems, Franklin, MA, USA). The ratio of cell proliferation inhibition was calculated as follows:

$$\text{Inhibition } \% = \left[\frac{\text{Ac} - \text{As}}{\text{Ac}}\right] \times 100$$

Each concentration of the respective galactans was repeated at least five times

Statistical analyses

All the data are expressed as mean ± standard deviation (SD) of five replications. The two-way ANOVA test with replication was used to determine significant differences among the extracted galactans from different species. The Pearson correlation coefficient (r) was calculated ($P < 0.05$) to assess the strength of the linear relationship between both biological activities. IC50 values were estimated using non-linear regression analysis.

RESULTS AND DISCUSSION

Galactan contents

Polysaccharides derived from red seaweed play a vital role as food and pharmaceutical additives due to their specific chemical components [9]. The highest and lowest yields of galactan were obtained from *O. dechybrida* (14.32% DW) and *T. fragilis* (4.03% DW), respectively. Variation in the red seaweed galactan content depends on species and time of harvest [29]. The amounts of the extracted galactans shown relatively low compared with a previous report in which ~16%–18% galactans were detected from *Laurenica* spp. [30] but were higher than those detected in other studies conducted on the Lebanese coast: e.g., 2.5% in *Corallina* spp. [31] and 1.7% in *T. fragilis* [10].

Chemical analysis of the extracted galactans

The chemical compositions of all the extracted crude galactans are tabulated in Table 1. The maximum ash (7.85%), sulfate (28.59%) and uronic acid (5.49%) contents were detected in GRG. The estimated sulfate contents were similar to the sulfate content of red alga galactans (15%-40%) [32]. Uronic acids representing a class of acids polysaccharides had been detected in a few species. A similar ratio of uronic acids was reported in galactans of *Laurencia* (4%-9%) [30] and *Palmaria decipiens* (4.8%) [33]. GRG contained the least moisture content (0.81%). Protein concentrations were not detected in LVG, GRG and ODG. The carbohydrate content of the extracted galactan ranged from 34.81% to 76% DW in GRG and ODG, respectively.

Table 1. Analytical data and molecular weight of the extracted galactans

Algal spp.	LVG	PPG	GRG	ODG	TFG
Yield (% algal DW)	10.93^{a}±1.02	7.59^{b}±0.58	5.15^{c}±085	14.32^{d}±1.85	4.03^{c}±0.87
Moisture (% galactan)	2.29^{a}±0.41	2.85^{a}±0.25	0.81^{b}±0.02	3.48^{c}±0.52	0.95^{b}±0.02
Ash (% galactans)	6.89^{a}±0.89	5.12^{b}±0.35	7.85^{a}±1.01	7.54^{a}±1.25	2.13^{c}±0.01
Total carbohydrate (% galactan)	43.34^{a} ±2.12	64.2^{b} ±2.15	34.81^{c} ±2.32	76.34^{d}±3.25	47.183^{a}±1.40
Sulfate (% galactan)	25.66^{a} ±1.54	20.39^{b} ±2.12	28.59^{c}±2.1	14.96^{d}±1.85	8.25^{e}±1.12
Protein (% galactan)	ND	0.0002±0.0	ND	ND	0.0001±0.0
Uronic acid (%galactans)	4.15^{a} ±0.85	3.19^{b}±0.25	5.49^{c} ±1.1	3.04^{b}±0.04	2.87^{b}±0.58
M.W. (kDa)	153^{a}±3.5	149^{b}±2.5	146^{b}±2.85	152^{a}±3.5	155^{a}±3.54

Data are expressed as mean±standard deviation (SD), n=5. Superscripts Letters indicate significant differences ($P < 0.05$).
Abbreviations: GRG=*Galaxaulra rugosa*; LVG=*Ligora viscida*; ODG=*Osmande dechybrida*; PPG= *Palisada perfosata*; TFG=*Tricleocarpa fragilis*

FTIR spectroscopic analysis of the extracted galactans

FTIR is an additional tool for the identification of the main phycocolloids structure. The FTIR spectra of the extracted crude galactans from all selected red seaweed were similar, although some minor peak patterns were detected (Figure 1). The strong band between 3380.77 and 3388.23 cm^{-1} demonstrated the sulfated polygalactans in the tested crude galactans. The strong signal at 1640.01–1640.81 cm^{-1} in all the samples corresponds to the carboxyl group of uronic acid [34]. Minor bands at 1374.6–1377.27 cm^{-1} were also indicative of the sulfate ester substitution [35]. An intense band at 1209.89–1242.5 cm^{-1} representing the sulfate ester (S=O) was detected in all the samples and was much stronger in the carrageenan standards than in agar [36]. The IR signals between 1000 and 1200 cm^{-1} corresponded to the sugar ring and glycosidic bond C–O stretching vibrations. The low intensity signals at 932.99 cm^{-1} in the GRG and ODG samples were attributable to 3,6-anhydrogalactose residues and were common to agar and carrageenans. The strong signal

between 817.49 and 818.58 cm^{-1} belonged to the 6-sulfate group of D-galactose units [37]. The bands between 700-800 cm^{-1} particularly at ~782 and ~715 cm^{-1} were attributed to the region of agar–type polysaccharides. Thus, the various peaks mentioned above indicate that all the extracted galactans were of the agar type except GRG and ODG which may be carrageenan. These results are in agreement with Gómez-Ordóñez and Rupérez [38] who reported that 930 cm^{-1} band is characteristic of carrageenan standards which detecting in the GRG and ODG samples.

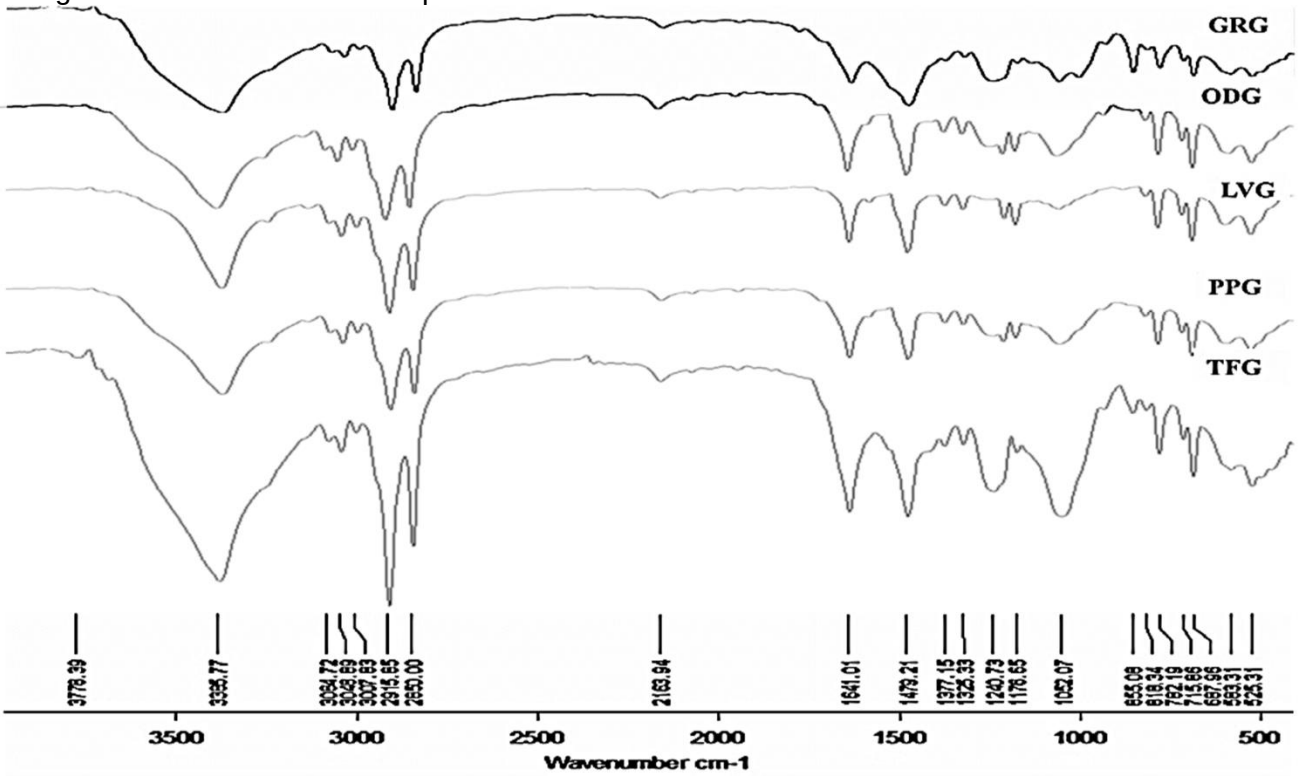

Figure 1. FTIR spectra of GRG, LVG, ODG, PPG and TFG.

Biological activities

Evaluation of antioxidant activity

Antioxidant activity was estimated by different methods, including TAC, DPPH, H_2O_2 scavenging (HP) and RP assays (Figure 2). The five extracted galactans from different algae species exhibited antioxidant activities with significant differences in the following order, GGR > GLV > GPP > GOD > GTF based on the varying methods and their concentrations ($P < 0.05$). The obtained results for the scavenging activity of DPPH were > 90% at 1 and 1.5 mg/g for ODG and GRG, and these activities were more than that of the standard antioxidant (α-tocopherol). The antioxidant activities of seaweed polysaccharides are closely related to their physicochemical properties such as molecular weight, sulfate content, and polyphenol content [39-40]. The antioxidant activity of the extracted galactans was positively correlated with the sulfate content ($r = 0.71$) and uronic acid ($r = 0.84$). The sulfated polysaccharides had an unremarkable impact on inhibition of the formation of the radicals in relation to their dose and uronic acid concentration [41]. Moreover, uronic acid is an important component that can decrease the generation of hydroxyl radicals by chelating Fe2+, and increasing their RP; in addition, a relatively high uronic acid content can promote antioxidant ability [42]. There was a significant relationship between the antioxidant properties of galactans and their protein content ($r = 0.94$) although proteins were only present in trace amounts. This result was similar to that of Yang and coauthors [43] who documented the antioxidant effects of polysaccharides linked to their bound-proteins since there is electrostatic attraction between anionic polysaccharide and cationic protein [44].

There exists a negative correlation between antioxidant activity and galactan molecular weight "r=-0.94" that may be related to the non-compact structure of low-MW polysaccharides and consequently the more potentially available hydroxyl and amine groups reacting with free radicals [45].

Antiproliferative activity

The antiproliferative activity of the extracted galactans on HeLa cells was significantly different between the tested galactans ($P < 0.05$), and is described here in descending order; GRG > ODG > PPG > LVG > TFG depending on their concentrations (Figure 3). Hence, HeLa cells viability decreased with increasing galactan concentration from 0.05 to 1.5 mg/mL. In this connection, Zein and coauthors [10] recorded the anti-proliferative efficiency of red alga *T. fragilis* polysaccharides from the Lebanese coast against the colorectal human cancer cell lines (HT-29 and HCT-116). Sulfated galactans from *Gracilaria fisheri* exhibited antiproliferative action on the cholangiocarcinoma cell line [46].

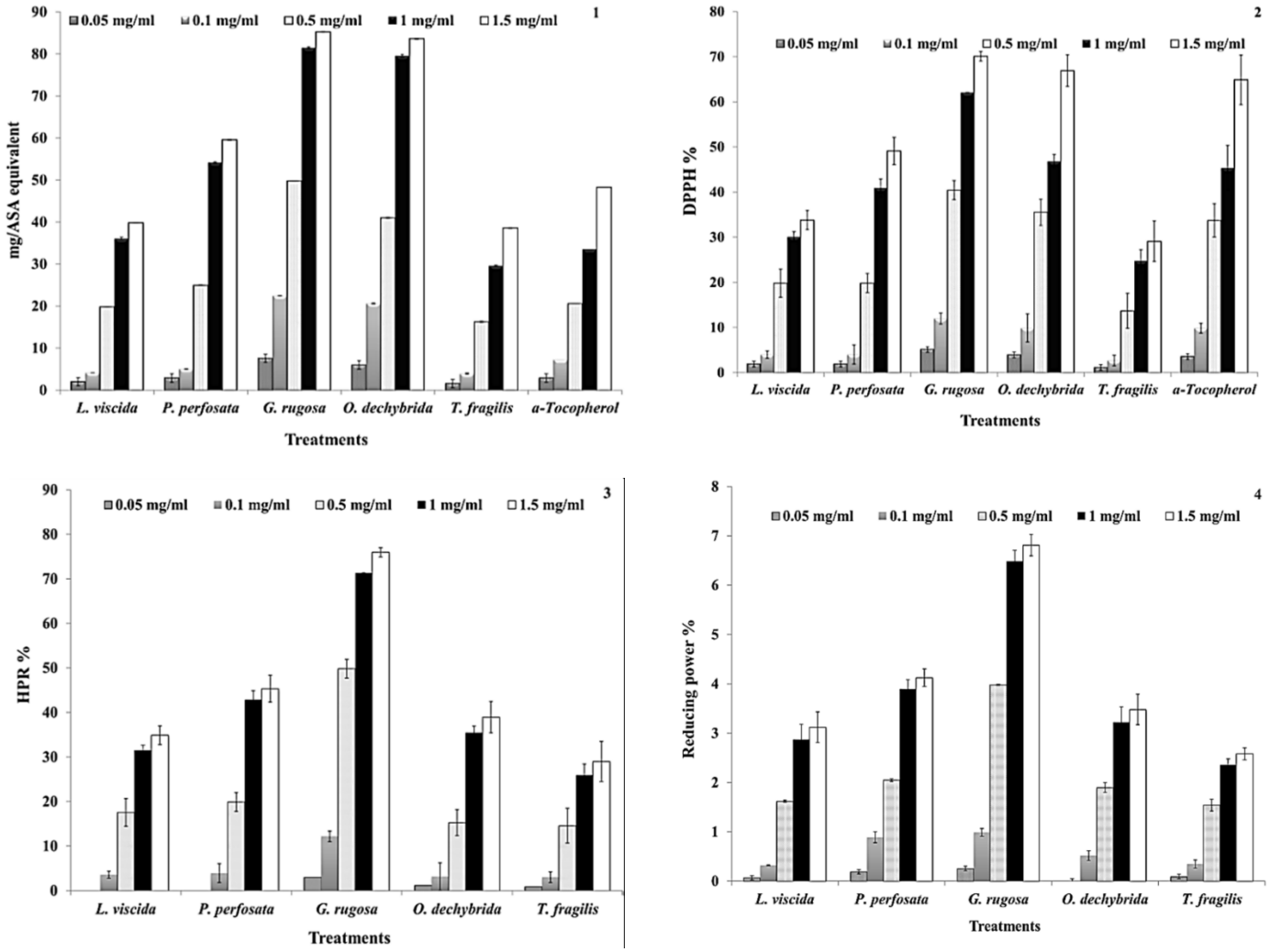

Figure 2. Antioxidant activity "[1]total capacity antioxidant (TCA), [2]DPPH, [3]H_2O_2 scavenging (HP) and [4]reducing power (RP)" of the extracted galactans from the tested seaweed.

The antiproliferative activity of the tested galactans is positively dependent on their sulfate content "r = 0.99" as well as their uronic acid concentration "r=0.85", similar to their antioxidant activity. Furthermore, the biochemical composition of the polysaccharide is very important for its antiproliferative efficiency [14]. These results were in agreement with those of Shao and coauthors [41] who indicated that the antitumor and antioxidant activities of the polysaccharides *in vitro* may be related to combined effects of sulfate and uronic acid content. Costa and coauthors [14] recorded a positive link between inhibition of HeLa cell proliferation and sulfate content.

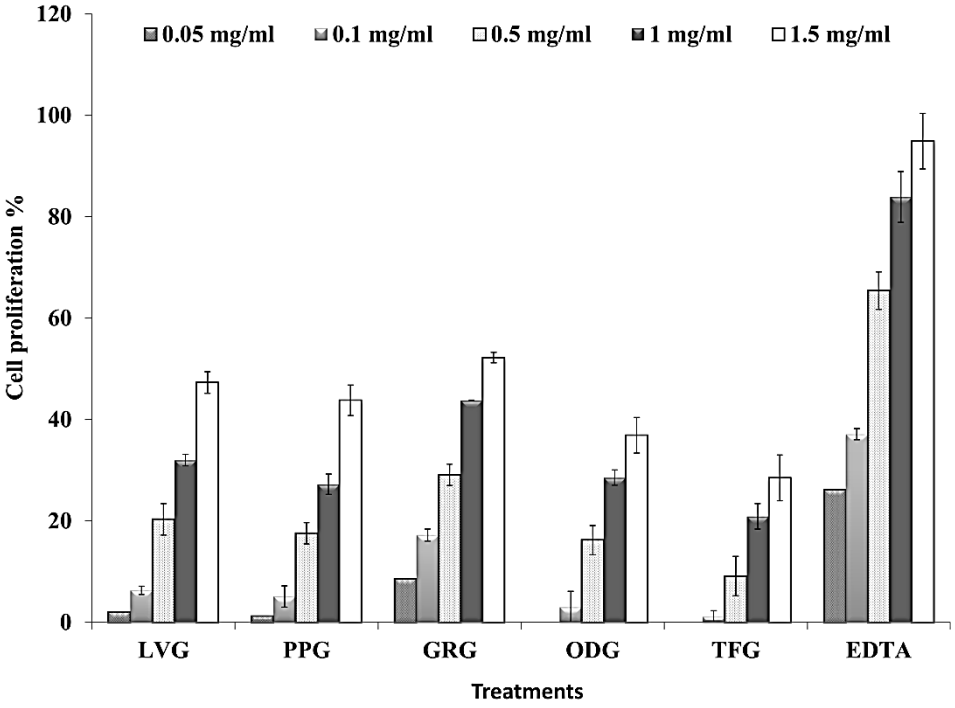

Figure 3. Influence of GRG, LVG, ODG, PPG and TFG on inhibition of cell proliferation of HeLa cells after 72 h incubation.

Correlation coefficient analyses indicated that there was a relationship between antiproliferative and DPPH "r = 0.77" and HPR "r = 0.71". A similar observation was reported by Jing and coauthors [46] who demonstrated the ability of polysaccharides to fight cancers related to their antioxidant activities.

CONCLUSION

It can be concluded from the present study that the galactans derived from red seaweed can be utilized as a natural and renewable source of antioxidant substances for the prevention of the oxidative deterioration of functional food or in pharmaceutical applications. Generally, the biological activities of the extracted galactans were dependent on their species and fine structural features. Differences in the chemical composition of red seaweed galactans require additional studies to elucidate their value in the aforementioned applications. Therefore, the extracted galactans from red seaweed can be made available in powder form which can play a non-negligible role in pharmaceutical and food uses**.**

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Conflicts of Interest: The authors declare that they have no conflict of interest.

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