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Sulfated heterorhamnans from the green seaweed *Gayralia oxysperma*: partial depolymerization, chemical structure and antitumor activity

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ABSTRACT

Sulfated heterorhamnans produced by *Gayralia oxysperma* were utilized for the preparation of two homogeneous and highly sulfated Smith-degraded products (M_w of 109 and 251 kDa), which were constituted principally by 3-linked α-L-rhamnosyl units 2- or 4-sulfate and 2-linked α-L-rhamnosyl units 4- or 3,4-sulfate, in different percentages. The homogeneous products and the crude extracts containing the sulfated heterorhamnans showed cytotoxic effect against U87MG cells. These sulfated polysaccharides induced an increase in the number of cells in G1 phase with concomitant increase of the mRNA levels of p53 and p21. The presence of 2-linked disulfated rhamnose residues together with the molecular weight could be important factors to be correlated with the inhibitory effect on human glioblastoma cells.

1. Introduction

The sulfated polysaccharides biosynthesized by seaweeds are build up of a variety of monosaccharide units, presenting different degrees of sulfation, sulfate groups positioning and molecular weight. Due to their pharmacological properties, these polymers represent an important source of natural bioactive compounds (Araújo et al., 2013; Ciancia, Quintana, & Cerezo, 2010; Damonte, Matulewicz, & Cerezo, 2004; Ghosh et al., 2009; Usov, 2011).

Sulfated polysaccharides produced by green seaweeds, particularly those ones belonging to the genus *Monostroma*, are known by their anticoagulant and/or antiherpetic activities. They are constituted by different proportions of 2-linked rhamnosyl units partially sulfated at C-3 and/or C-3/C-4 and 3-linked rhamnose residues sulfated at C-2 or C-4 (Harada & Maeda, 1998; Lee, Koizumi, Hayashi, & Hayashi, 2010; Lee, Yamagaki, Maeda, & Nakanishi, 1998; Li et al., 2011; Mao et al., 2008, 2009). Our research group has previously reported that sulfated heterorhamnans extracted from *Gayralia oxysperma* presented high anti-HSV activity. These polysaccharides were constituted by 3- and 2-linked α-L-rhamnose residues, being partially sulfated and branched by side chains containing uronic acids and xylose residues. This material rendered a purified fraction (M_w 1519 kDa) that presented the 3-linked rhamnosyl units mainly sulfated at C-4 or C-2, plus minor amounts of disulfated and nonsulfated rhamnose residues, with the 2-linked rhamnosyl units being sulfated on C-4, disulfated or unsulfated. In this study, a homogeneous heterorhamnan was submitted to a controlled Smith degradation process and its product was used to help in the chemical structure determination of the native polysaccharide (Cassolato et al., 2008).

Now, we have applied the controlled Smith degradation with the objective of producing partially degraded sulfated heterorhamnans with lower molecular weight and different structural characteristics when compared to the native heterorhamnans. These

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partially depolymerized heterorhamnans were evaluated as antitumor compounds and represent important tools for chemical structure-biological activity correlations.

Although the anticoagulant and antiviral activities of sulfated polysaccharides obtained from seaweeds are so far the most investigated ones, some of these polymers have shown to be promising chemotherapeutic agents against different types of tumors (Vishchuk, Ermakova, & Zvyagintseva, 2011; Vishchuk, Ermakova, & Zvyagintseva, 2013; Xue et al., 2012). In this context, glioblastoma multiforme is the most aggressive subtype of glioma and it is associated with a poor prognosis (Parsons et al., 2008). The current care for glioblastoma multiforme patients is surgical resection followed by adjuvant radiation therapy and chemotherapy, which promotes an average survival of only 15 months (Parsons et al., 2008). In this way, it is necessary to search for novel bioactive compounds capable of inhibiting glioblastoma development. As far as we know, there are no studies devoted to the investigation of the effects of sulfated polysaccharides obtained from seaweeds on human glioblastoma cell lines. Here, we report the structure of two homogeneous sulfated Smith-degraded products obtained from *G. oxysperma* sulfated heterorhamnans, as well as the antitumor activity of both crude extracts and Smith-degraded products against U87MG cells.

2. Material and methods

2.1. Collection of specimens

Specimens of *G. oxysperma* (Kützing) K.L. Vinogradova ex Scagel et al. were collected at Bahia de Paranaguá, Paraná State (southern coast of Brazil). A voucher specimen was deposited in the herbarium of the Department of Botany, Federal University of Paraná, Curitiba, Brazil (UPCB-58059). The material was cleaned, sun-dried, and milled.

2.2. Extraction of polysaccharides

The milled seaweed was extracted with water (5% w/v) at 80 °C with mechanical stirring for 4 h. The residue was removed by centrifugation and EtOH (3 vols.) was added to the supernatant. The precipitate was dissolved in water, dialyzed, concentrated and freeze-dried yielding the crude extract OX-1. Sequentially, the algal residue was re-extracted 4× as described above to give the crude extracts OX-2–OX-5. The crude extract OX-1 was reserved for future work whereas OX-2–OX-5 were combined yielding the polysaccharidic fraction named OX.

2.3. Analytical methods

Total carbohydrate was estimated as described by Dubois, Gilles, Hamilton, Rebers, and Smith (1956), using rhamnose as standard. Sulfate, uronic acid and protein contents were determined according to Dodgson and Price (1962), Filisetti-Cozzi and Carpita (1991) and Lowry, Rosebrough, Farr, and Randall (1951), respectively. Specific rotation was measured (20 °C) using a 10-cm cell and the sodium D line (589.3 nm) with a Rudolph Autopol III automatic polarimeter. For monosaccharide composition, the polysaccharides were hydrolyzed (M TFA, 100 °C, 4 h) reduced ($NaBD_4$) and acetylated (acetic anhydride:pyridine 1:1, 12 h, 25 °C). GC–MS analyses of the alditol acetates derivatives were performed with a Varian 3800 chromatograph, equipped with a fused silica capillary column (30 m × 0.25 mm) coated with DB-225MS (Durabond), and a Varian Saturn 2000R ITD spectrometer and were identified by their typical electron-impact fragmentation profiles and GC retention times (Jansson, Kenne, Liedgren, Lindberg, & Lonngren, 1976). The chromatograph was programed to run at 50 °C for 1 min, then 50–215 °C at 40 °C/min, using helium as carrier gas (1 mL/min).

2.4. Controlled Smith degradation

OX (4 g) was oxidized with 0.05 M aq. $NaIO_4$ (600 mL) under mechanical stirring (72 h, 25 °C, in the dark), and then treated with ethyleneglycol (40 mL) and dialyzed. After treatment with $NaBH_4$ (pH 9–10, 20 h), neutralization (acetic acid), dialysis and freeze-drying (70% yield), the material was submitted to partial acid hydrolysis (M TFA, 20 h, 25 °C) (Furneaux & Stevenson, 1990; Goldstein, Hay, Lewis, & Smith, 1965). The Smith-degraded products were dialyzed and freeze-dried to afford OXS (65% yield).

2.5. Fractionation of the Smith-degraded products

OXS fraction was submitted to anion-exchange chromatography (DEAE-Sephacel). The elution was carried out with water and sequentially with aq. NaCl solutions. The column eluents were analyzed for carbohydrate content (Dubois et al., 1956). The eluents were concentrated, dialyzed and freeze-dried, yielding a fraction eluted with water (OXSw) and five fractions (OXSa–OXSe) eluted with 0.5, 1.0, 1.5, 2.0 and 3.0 M NaCl, respectively (total yield 87%).

2.6. Carboxyl-reduction

OXSb and OXSc were submitted to carboxyl-reduction (Anderson & Stone, 1985; Taylor & Conrad, 1972). The uronic acid present in the OXSb and OXSc fractions was submitted to esterification with 1-cyclohexyl-3-(2-morpholinoethyl) carbodiimide metho-*p*-toluenesulfonate (pH 4.75, MES buffer). The products were reduced with $NaBD_4$ (pH 7.0, TES buffer,) to give OXSb-R and OXSc-R, with 82 and 87% yield, respectively.

2.7. Desulfation

Partial solvolytic desulfation of OXSb-R and OXSc-R in the pyridinium salt form Stevenson and Furneaux (1991) was carried out by Nagasawa, Inoue, and Tokuyasu (1979) method, yielding OXSb-RD and OXSc-RD, respectively.

2.8. Methylation analysis

The carboxyl-reduced Smith-degraded products (OXSb-R and OXSc-R) and desulfated products (OXSb-RD and OXSc-RD) were per-*O*-methylated using Ciucanu and Kerek (1984) method as previously reported (Cassolato et al., 2008). Briefly, the Smith-degraded products (30 mg) were first treated with triethylamine hydrochloride (Stevenson & Furneaux, 1991). The corresponding polysaccharides in the triethylamonium salt form were redissolved in Me_2SO (2 mL), and then NaOH (60 mg) was added. After 30 min at 25 °C under magnetic stirring, CH_3I (0.2 mL) was added and the reaction stirred for 30 min at 25 °C. The addition of NaOH and CH_3I was repeated twice. After addition of water (4 mL) and neutralization (50% aqueous AcOH), the products were dialyzed (water) and freeze-dried. The products were submitted to two more steps of methylation as described above. The per-*O*-methylated polysaccharides were hydrolyzed (formic acid 45%, for 16 h at 100 °C), reduced using $NaBD_4$ and acetylated. The partially methylated alditol acetates were analyzed by GC-MS and identified by their typical electron-impact breakdown profiles and retention times (Jansson et al., 1976). Additionally, the per-*O*-methylated OXSb-R and OXSc-R were submitted to solvolytic desulfation and sequentially to trideuteromethylation as described above, but using CD_3I.

Partially methylated and trideuteromethylated alditol acetates were generated and analyzed as described above.

2.9. High-pressure size-exclusion chromatography (HPSEC) analysis

HPSEC was carried out at 25 °C with1 mg/mL solutions of polysaccharide, using a multidetection equipment with a Waters 2410 differential refractometer and a Wyatt Technology Dawn-F multi-angle laser light scattering detector adapted on-line. The eluent was 0.1 M $NaNO_2$ soln., containing NaN_3 (0.2 g/L). d*n*/d*c* values were determined using five concentrations (0.2–1.0 mg/mL). HPSEC data were collected and analyzed by the Wyatt Technology ASTRA program.

2.10. Nuclear magnetic resonance (NMR)

NMR analyses were recorded at 70 °C using a Bruker Advance DRX400 NMR spectrometer equipped with a 5-mm multinuclear inverse detection probe. The base frequency was 400.13 and 100.63 MHz for ^1H and ^{13}C nuclei, respectively. For ^{13}C NMR spectra, samples were dissolved in D_2O (40 mg/mL). For ^1H and 2D NMR experiments, samples were deuterium exchanged with D_2O 99.9% (3×) and then dissolved in D_2O (20–25 mg/mL). ^1H, ^{13}C and ^{13}C-DEPT acquisition parameters were as previously reported (Ascêncio, Orsato, França, Duarte, & Noseda, 2006). 2D ^1H,^1H COSY, and ^1H,^{13}C HSQC experiments were carried out using the pulse programs supplied with the Bruker manual. The 2D TOCSY spectra were recorded with a 150-ms duration of MLEV-17 spin-lock. Chemical shifts are expressed relative to acetone (internal standard) at 31.45 and 2.225 ppm for ^{13}C and ^1H NMR spectra, respectively.

2.11. Cell culture and polysaccharidic fractions treatment

U87MG Cells were grown in DMEM high-glucose (Cultilab) supplemented with 10% fetal bovine serum and 50 μg/mL gentamicin (37 °C, 5% CO_2 atmosphere). For the following experiments the polysaccharidic fractions were, separately, dissolved in ultrapure water (5 mg/mL) and stored at −20 °C until utilized. The concentrations of the polysaccharides for subsequent assays were adjusted by total carbohydrate content (Dubois et al., 1956). Control conditions consisted of glioma cells maintained in culture medium.

2.12. Cytotoxicity assay

Cytotoxicity was evaluated by MTT assay. Briefly, cells (1×10^4 cells/well) were seeded in 96-well plates and incubated during 24 h (37 °C). Sequentially, 200 μL of polysaccharidic solutions (corresponding to 10, 100 and 1000 μg/mL of total carbohydrate) were added and incubated for 48 and 72 h (37 °C). Subsequently, the medium was removed, wells were filled with HBSS (180 μL) and MTT (20 μL, 5 mg/mL). After 3 h (37 °C), unreacted MTT was removed, and formazan crystals were solubilized in DMSO (200 μL/well). Optical density was evaluated at 550 nm.

2.13. Cell cycle analysis

Cell-cycle distribution of U87MG cells after treatment with polysaccharidic fractions was performed by flow cytometry analysis. First, cells (5×10^4 cells/plate) were incubated in 12-well plates (24 h, 37 °C). Sequentially were treated with 2 mL of OX, OXS, OXSb and OXSc solutions (100 μg/mL, 48 h). The treatment with OXSb was conducted also for 72 h. After centrifugation, the pellet was washed twice with PBSA and incubated with a cold staining solution of propidium iodide 50 μg/mL, 0.1% Triton X-100, 0.1% sodium citrate and 0.2 mg/mL RNAse A (30 min, in the dark). Samples were run in a FACSCalibur System and analyzed using WinMDI 2.9 software (developed by Joseph Trotter).

2.14. Real-time PCR analysis

U87MG cells (1×10^6 cells/plate, 100-mm cell culture plates) were treated with 10 mL of the polysaccharidic solutions (100 μg/mL, 48 h, 37 °C). Total RNA was extracted (Illustra RNAspin Mini kit, GE Healthcare) and quantified (Nanodrop 1000, Thermo Scientific). The cDNA was synthesized (Superscript III Reverse Transcriptase kit, Invitrogen), and the expression levels of p14/ARF, p21 and p53 were quantified by real-time PCR analysis using Rotor-Gene SYBR Green PCR Kit (Qiagen) in a Rotor-Gene™ equipment (Corbett Life Science). Primers used: p14/ARF (forward: 5′-CTGCAGGTCATGATGTTTGGC-3′, reverse: 5′-CGTCATGGACTGGACTGGTAC-3′); p21 (forward: 5′-ACTCTCAGGGTCGAAAACGG-3′, reverse: 5′-GATTAGGGCTTCCTCTTGGAGA-3′); p53 (forward: 5′-TGGTAATCTACTGGGACGGA-3′, reverse: 5′-TTGCGGAGATTCTCTTCCTCT-3′). As housekeeping control was used GAPDH (forward: 5′-ACCCACTCCTCCACCTTTGA-3′, reverse: 5′-CTGTTGCTGTAGCCAAATTCGT-3′).

2.15. Statistical analysis

Statistical analysis was performed using One-Way ANOVA followed by Tukey's Multiple Comparison Test. Comparisons between two groups were analyzed using Mann Whitney test. A p value of less than 0.05 was considered to be statistically significant. All data are presented as the mean ± SD of at least three independent experiments. All statistical analyses were performed using GraphPad Prism 5.0 software (GraphPad Software Inc., USA).

3. Results and discussion

3.1. Extraction of the polysaccharides, controlled Smith degradation and fractionation of the Smith-degraded products

The dried and milled green seaweed *G. oxysperma* was sequentially extracted five times with water at 80 °C, giving rise to OX-1–OX-5. The first extract was reserved for future studies. The polysaccharidic fractions OX-2–OX-5 were then combined, yielding the crude extract named OX (19%, w/w), which was submitted to controlled Smith degradation (Goldstein et al., 1965; Furneaux & Stevenson, 1990), to give Smith-degraded fraction OXS (65% yield). Table 1 shows yields, analyses, specific rotation and monosaccharide composition of OX and OXS. When compared to OX, the Smith-degraded product OXS showed an increase of the sulfate groups (26 to 34.5%) and rhamnosyl units (76 to 89%), concomitantly, a decrease of xylose (17.0 to 3.5 mol%) and uronic acid (17.5 to 7.5%) was also noted. These results are in accordance with our previous study (Cassolato et al., 2008), which reported the partial removal of xylose and uronic acids when the carboxyl-reduced heterorhamnan Go3r from *G. oxysperma* was submitted to controlled Smith degradation.

OXS was fractionated on a DEAE-Sephacel column to give the subfractions OXSw (eluted with water) and OXSa–OXSe (eluted with aq. NaCl solutions of increasing concentrations). The subfractions presented rhamnose (52–95 mol%) as the main neutral monosaccharide, followed by glucose (2.9–37.9 mol%) and xylose (2–10.8 mol%). Arabinose plus mannose (5.1 mol%) and galactose (8.8 mol%) were present only in OXSa and OXSe, respectively. All the subfractions were sulfated (22–41%) and contained uronic acids (5–23%) (Table 1). The subfractions OXSb and OXSc (77% total yield) showed symmetric HPSEC-MALLS-RI elution profiles (Fig. S1), with M_w of 109.3 and 251.1 kDa, respectively (d*n*/d*c* of 0.150

Table 1
Yield, chemical analyses, specific rotation, and monosaccharide composition of polysaccharides obtained from *Gayralia oxysperma* and its Smith-degraded products.

Fractions[a]	Yield (%)	Carbohydrate (%)	SO_3Na (%)	Uronic acid (%)	Protein (%)	$[\alpha]_D^{25}$ (°)	Monosaccharide (mol%)		
							Rha	Xyl	Glc
OX[b]	19.0	64.0	26.0	17.5	1.0	−34.0	76.0	17.3	4.4
OXS	61.5[c]	50.0	34.5	7.5	0.5	−39.5	89.0	3.5	7.5
OXSa	7.0[d]	29.0	24.5	23.0	2.0	+57.0	52.0	5.0	37.9
OXSb	26.0[d]	66.5	33.5	5.0	–	−57.5	93.3	3.8	2.9
OXSb-R	81.5[e]	n.d.	n.d.	–	n.d.	n.d.	89.6	2.0	2.9 (5.5)[f]
OXSb-RD	58.0[g]	n.d.	2.5	–	n.d.	n.d.	88.4	2.0	4.2 (5.4)[f]
OXSc	51.0[d]	60.0	41.0	7.0	–	−57.5	95.0	2.0	3.0
OXSc-R	87.0[e]	n.d.	n.d.	–	n.d.	n.d.	87.8	2.8	3.2 (6.2)[f]
OXSc-RD	62.0[g]	n.d.	2.5	–	n.d.	n.d.	87.8	2.7	3.3 (6.2)[f]
OXSd	2.5[d]	n.d.	22.0	5.5	2.5	n.d.	81.5	9.9	8.6
OXSe[b]	0.5[d]	n.d.	26.5	5.0	14.0	n.d.	70.6	10.8	9.8

– = Not detected.
[a] Fractions are defined in the text.
[b] OX and OXSe present galactose (2.3 and 8.8 mol%, respectively). OXSa presents arabinose and mannose (2.4 and 2.7 mol%, respectively).
[c] Percentage based on material recovered after controlled Smith degradation.
[d] Percentages based on material recovered from anion-exchange chromatography.
[e] Percentages based on material recovered after carboxyl-reduction.
[f] In parentheses mol % corresponding to glucuronic acid, analyzed as 1,6,6-trideutero glucitol hexaacetate in the carboxyl-reduced polymer.
[g] Percentages based on carboxyl-reduced material recovered after desulfation.

and 0.137, respectively). Considering their homogeneous characteristics, OXSb and OXSc were selected for structural studies performed herein.

3.2. Carboxyl-reduction and desulfation of the carboxyl-reduced Smith-degraded products

OXSb and OXSc were carboxyl-reduced to afford OXSb-R and OXSc-R, respectively. GC–MS analyses showed that, among all acetylated alditols, 65.5 and 66.0% of the glucitol hexaacetate found in OXSb-R and OXSc-R, respectively, corresponded to 1,6,6-trideutero glucitol hexaacetate. Therefore, OXSb and OXSc contained 5.5 and 6.2 mol%, respectively, of glucuronic acid (Table 1).

The desulfation procedure of OXSb-R and OXSc-R originated the partially desulfated fractions OXSb-RD (58% yield) and OXSc-RD (62% yield), with removal of 92 and 94% of sulfate groups, respectively. The monosaccharide compositions of the desulfated heterorhamnans were very similar to those found for their parent polysaccharides, indicating that no extensive degradation occurred during the solvolytic process (Table 1).

3.3. Methylation analyses

Per-*O*-methylation of the Smith-degraded products was carried out with the carboxyl-reduced (OXSb-R and OXSc-R) and their corresponding desulfated polymers (OXSb-RD and OXSc-RD). The comparative methylation analyses of the sulfated and desulfated products were used to partially determine the glycosyl linkages and sulfate groups positions (Table 2).

Among the methylation products of the sulfated fractions (OXSb-R and OXSc-R) the presence of di-*O*-methylated rhamnose residues (2,4-Rha and 3,4-Rha) indicated that rhamnosyl units are 3- and 2-linked, respectively. The monomethylated rhamnose (3-Rha, 2-Rha, and 4-Rha) and nonmethyated rhamnose residues (Rha) were attributed to 2,4-, 3,4-, 2,3-di-*O*-substituted and 2,3,4-tri-*O*-substituted rhamnosyl units. Additionally rhamnosyl units were present as nonreducing terminal (2,3,4-Rha) and as 4-*O*-substituted residues (2,3-Rha). Reduced uronic acid observed as 2,3,4,6-Glc, 2,3,6-Glc and 3,6-Glc correspond to nonreducing, 4-linked and 2,4-substituted glucuronic acid residues, respectively, in the parent polysaccharide. Furthermore, low amounts of glucosyl units 4-linked were detected among the methylation products.

Methylation analyses of the desulfated polymers, OXSb-RD and OXSc-RD showed substantial increase of 3-linked rhamnosyl (19.4 to 55.4 and 12.4 to 51.5 mol %, respectively) and 2-linked rhamnosyl units (3.5 to 27.1 and 2.1 to 29.5 mol %, respectively), indicating a substantial degree of substitution by sulfate groups in the parent fractions. Moreover, the disappearance of 2-Rha and 3-Rha together with the increase of 2,4-Rha and 3,4-Rha derivatives are in agreement with sulfation on C-4 of the 3-linked (10.4 and 18.4 mol%) and 2-linked rhamnosyl units (19.2 and 12.1 mol%), respectively. Furthermore the increase of nonreducing terminal rhamnose residues (2,3,4-Rha) in the methylation products of OXSb-RD (1.5 to 3.5 mol%) and OXSc-RD (2.0 to 3.8 mol%) with the disappearance of the dimethylated rhamnose residues (2,3-Rha) are in agreement with the later being related to nonreducing terminal 4-sulfated rhamnose residues in the original polysaccharides. Low amounts of nonreducing glucuronic acid (1 mol%) were also detected. Additionally, the disappearance of 2,4-substituted glucuronic acid together with an increase of 4-substituted glucuronic acid showed that OXSb and OXSc contain the acidic monosaccharide partially sulfated on C-2 (2.8 and 3.1 mol%, respectively). The 2,3-di-*O*-substituted rhamnose (4-Rha derivative) among the partially *O*-methylated derivatives of OXSb-RD and OXSc-RD (4.5 and 5.3 mol%, respectively) was attributed to branching points. After desulfation, the decrease of 4-Rha and the disappearance of Rha derivatives indicated the presence of monosulfated rhamnosyl units (3-linked 2-sulfate and/or 2-linked 3-sulfate) and disulfated rhamnosyl units (3-linked 2,4-sulfated and/or 2-linked 3,4-sulfated), respectively, in the parent polysaccharides. Moreover, the Rha disappearance could also suggest the presence of sulfated disubstituted rhamnosyl units.

In order to further elucidate the sulfate positioning related to the fractions OXSb-R and OXSc-R, sequential methylation, desulfation and trideuteromethylation analysis was employed. Partially methylated and/or trideuteromethylated 2,3,4-, 2,4-, 3,4- and 4-Rha derivatives were found, being the position of the sulfate groups in the original polysaccharides interpreted as the interchange by CD_3 groups as shown in Table 3.

The 4-Rha derivative yielded fragment-ions at *m*/*z* 131 and 134, corresponding to CH_3 and CD_3, respectively, at C-4. For OXSb and OXSc, the proportions of the mentioned fragments were 0.8:1 and 1:0.6, respectively. These findings are consistent with the 4-Rha derivative of OXSb-RD (4.5 mol%) and OXSc-RD (5.3 mol%) (Table 2) as being generated from 2-linked 3-substituted and/or to 3-linked

Table 2
Methylation analysis of the Smith-degraded and carboxyl-reduced fractions OXSb-R and OXSc-R and their partially desulfated products OXSb-RD and OXSc-RD.

Derivative[a]	Deduced linkage	Fractions[b]			
		OXSb-R	OXSb-RD	OXSc-R	OXSc-RD
2,3,4-Rha[c]	Rha*p*-(1→	1.5	3.5	2.0	3.8
2,3-Rha	→4-Rha*p*-(1→	2.0	–	2.0	–
2,4-Rha	→3-Rha*p*-(1→	19.4	55.4	12.4	51.5
3,4-Rha	→2-Rha*p*-(1→	3.5	27.1	2.1	29.5
3-Rha	→2,4-Rha*p*-(1→	19.2	tr.	12.1	tr.
2-Rha	→3,4-Rha*p*-(1→	10.4	tr.	18.4	tr.
4-Rha	→2,3-Rha*p*-(1→	23.5	4.5	18.0	5.3
Rha	→2,3,4-Rha*p*-(1→	11.6	–	23.3	–
2,3,4,6-Glc	Glc*p*A-(1→[d]	1.0	1.0	1.0	1.0
2,3,6-Glc	→4)-Glc*p*A-(1→[d]	3.0	5.7	4.6	7.9
3,6-Glc	→2,4)-Glc*p*A-(1→[d]	2.8	–	3.1	–
2,3,6-Glc	→4)-Glc*p*-(1→[d]	2.1	2.8	1.0	1.0

– = Not detected.
tr. = Percentages lower than 1.0% are considered as traces.
[a] Mol% of monosaccharide bearing methyl groups at the positions indicated.
[b] Fractions are defined in the text.
[c] 2,3,4-Rha analyzed as 1,5-di-*O*-acetyl-2,3,4-*O*-methylrhamnitol, etc.
[d] Determined after carboxyl-reduction using $NaBD_4$.

2-substituted rhamnosyl units (2.0 and 3.3 mol%, respectively), and also from those rhamnosyl units sulfated at C-4 (2.5 and 2.0 mol%, respectively).These results demonstrated that OXSb and OXSc contained unsulfated and 4-sulfated rhamnosyl units as branching points, which are present at every twenty two and nineteen rhamnosyl units, respectively (Table 3).

The characteristic fragment-ions at *m*/*z* 234, 237 and 240, which were produced from the 2,4-Rha derivative, are related to CH_3 groups at both C-2 and C-4 of the 3-linked rhamnosyl units; to CD_3 at C-2 (CH_3 at C-4) plus CD_3 at C-4 (CH_3 at C-2) of the 3-linked rhamnosyl units 2-sulfate plus 3-linked rhamnosyl units 4-sulfate; and CD_3 at both C-2 and C-4 of the 3-linked rhamnosyl units 2,4-sulfate, respectively. For OXSb, these fragment-ions showed a ratio of 0.6:1:0.14. These results, together with those of the methylation analyses (Table 2), showed that 55.4 mol% of 2,4-Rha detected in the methylation analyses of OXSb-RD (Table 2) was derived from 3-linked rhamnosyl (19.4 mol%), 3-linked rhamnosyl 2-sulfate (21.2 mol%), 3-linked rhamnosyl 4-sulfate (10.4 mol%) and 3-linked rhamnosyl 2,4-sulfate (4.4 mol%). For OXSc, the ratio of the above-mentioned fragment ions was 0.37:1:0.18, indicating that from 51.5 mol% of 2,4-Rha observed in the methylation analyses of OXSc-RD (Table 2), 12.4 mol% was derived from 3-linked rhamnosyl, 14.7 mol% from 3-linked rhamnosyl 2-sulfate, 18.4 mol% from 3-linked rhamnosyl 4-sulfate and 6.0 mol% from 3-linked rhamnosyl 2,4-sulfate.

The 3,4-Rha derivative generated characteristic ions at *m*/*z* 130 and 133, which corresponded to C-3 bearing CH_3 and CD_3 groups, respectively (2-linked-rhamnosyl units plus 2-linked-rhamnosyl units 4-sulfate and 2-linked-rhamnosyl units 3,4-sulfate, respectively). Fragment-ions at *m*/*z* 131 and 134 were correlated to C-4 bearing CH_3 and CD_3 groups, respectively, of the 2-linked-rhamnosyl units and 2-linked-rhamnosyl units 4-sulfate plus 2-linked-rhamnosyl units 3,4-sulfate, respectively. For OXSb these fragment-ions showed a ratio of 1:0.23 and 0.16:1, respectively, with OXSc presenting 0.89:1 and 0.11:1, respectively. Fragment-ions ratio analysis, together with the methylation analysis (Table 2), allowed us to determine that the 27.1 (OXSb-RD) and 29.5 mol% (OXSc-RD) of 3,4-Rha found within the methylation products were derived from 2-linked rhamnosyl units (3.5 and 2.1 mol%, respectively), 2-linked rhamnosyl units 4-sulfated (19.2 and 12.1 mol%, respectively) and 2-linked rhamnosyl units 3,4-sulfated (4.7% and 15.3 mol%, respectively).

Therefore, methylation analyses showed that the Smith-degraded heterorhamnans OXSb and OXSc present important

Table 3
Analysis of rhamnose derivatives from OXSb-R and OXSc-R after successive methylation, desulfation, and trideuteromethylation.

Derivative[a]	Composed by[b]	Corresponding units[c]	Fractions (mol %)		Mass fragments (*m*/*z*)[d]
			OXSb-R	OXSc-R	
2,3,4-Rha	2,3,4-Me_3-Rha	TNR	1.5	2.0	131, 175
	2,3-Me_2-4-CD_3-Rha	TNR4S	2.0	2.0	134,178
2,4-Rha	2,4-Me_2-Rha	3L	19.4	12.4	118, 131, 187, 234
	2-Me-4-CD_3-Rha	3L4S	10.4	18.4	118, 134, 190, 237
	2-CD_3-4-Me-Rha	3L2S	21.5	14.7	121, 131, 190, 237
	2,4-CD_3-Rha	3L2,4S	4.4	6.0	121, 134, 193, 240
3,4-Rha	3,4-Me_2-Rha	2L	3.5	2.1	115, 130, 131, 190
	3-Me-4-CD_3	2L4S	19.2	12.1	118, 130, 134, 190
	3,4-CD_3-Rha	2L3,4S	4.7	15.3	121, 133, 134, 193
4-Rha	4-Me-Rha	2L3R/3L2R	2.0	3.3	131, 203, 262, 290
	4-CD_3-Rha	2L3R4S/3L2R4S	2.5	2.0	134, 206, 265, 293

/ = and/or.
[a] Mol% of monosaccharide bearing methyl or trideuteromethyl groups at the positions indicated.
[b] Me and CD_3 represent methyl and trideuteromethyl groups, respectively.
[c] TNR, L, S and R correspond to nonreducing units, glycosidic linkage, sulfate and side chain at the position indicated, respectively.
[d] Fragment ions observed in the profile of the partially trideuteromethylated alditol acetates.

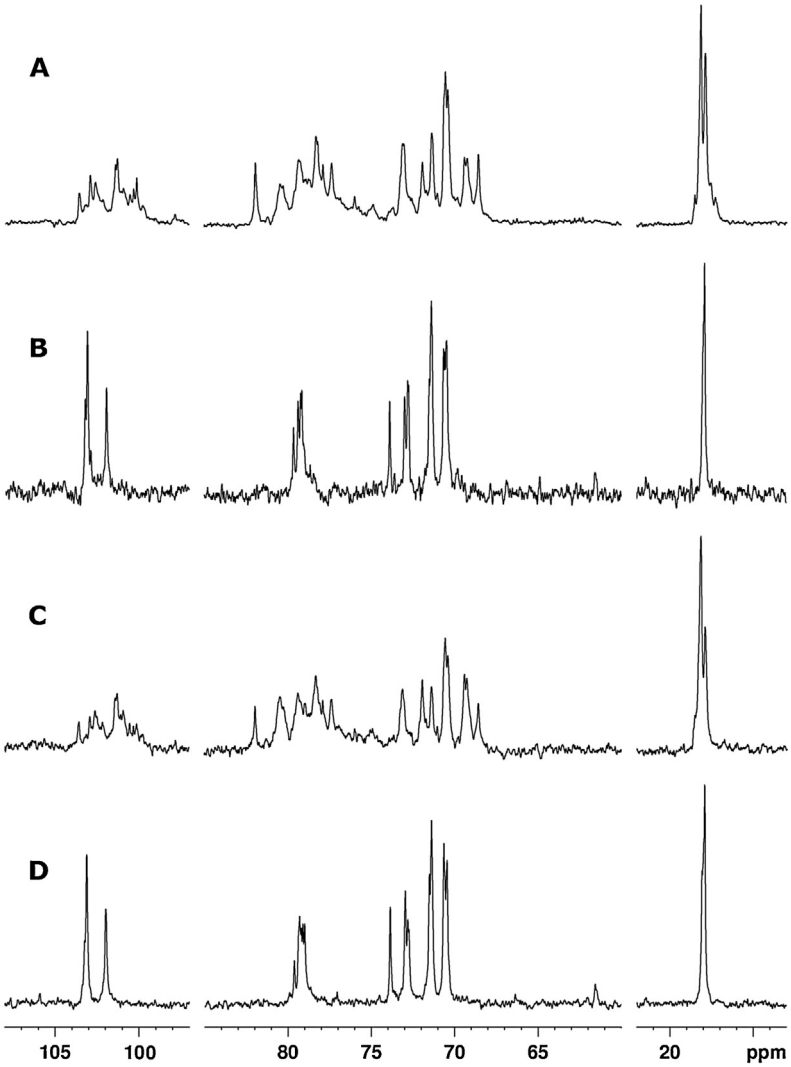

Fig. 1. ^{13}C NMR spectra of the Smith-degraded heterorhamnans OXSb (A) and OXSc (C) and their corresponding carboxyl-reduced desulfated fractions OXSb-RD (B) and OXSc-RD (D).

differences in terms of their structural features. These polysaccharides contain 3- and 2-linked monosulfated, disulfated and nonsulfated rhamnosyl units in a ratio of 1:0.18:0.45 (OXSb) and 1:0.47:0.32 (OXSc). It is noteworthy the higher content of 2-linked rhamnosyl 3,4-sulfate (3.2 fold) for OXSc in comparison to OXSb. Additionally, the Smith-degraded products are constituted by low percentages of 4-linked glucuronic acid (nonsubstituted or 2-sulfated) and they are also partially branched. Their side chains contain non-reducing glucuronosyl units and unsulfated and C-4 sulfated rhamnosyl units.

3.4. NMR analyses

The ^{13}C and ^{1}H NMR spectra of OXSb and OXSc were considered qualitatively similar, but with signals of different intensities (Fig. 1A and C, Figs. S2 and S3). The spectra assignments (Table 4) were carried out with the support of HSQC (Fig. 2, Fig. S4), DEPT (data not shown), COSY (Fig. S5) and TOCSY (Fig. S6) experiments, and literature data. The ^{13}C NMR spectra of OXSb and OXSc showed anomeric signals at 97.9–103.6 ppm attributed principally to rhamnosyl units. In the high-field region, signals at 17.9–18.4 ppm were attributed to C-6 of rhamnosyl units. The low intensity anomeric signals at 105.5 and 104.8 ppm were assigned to β-D-xylose and/or β-D-glucuronic acid units (Fig. 1A and C).

In the HSQC spectra of OXSb and OXSc (Fig. 2), the anomeric correlations at 103.6/5.06 and 102.9/5.03, 102.6/5.09, 102.2/5.12 ppm were attributed to 3-linked rhamnosyl and 3-linked 4-sulfate units, respectively. The upfield-shifted signals of C-3 and C-5 (76.0, 75.7 and 68.6–69.4 ppm, respectively) of the 4-sulfated 3-linked rhamnosyl units, when compared with the corresponding signals of 3-linked rhamnosyl units (C-3 and C-5 at 79.0–79.4 and 70.4 ppm, respectively) are in agreement with the β-effect of sulfation on C-4. The C-4 signal of 3-linked rhamnosyl 4-sulfate units is downfield-shifted (7.5–8.4 ppm) when compared with those of 3-linked rhamnosyl units present in the spectra of the sulfated (OXSb and OXSc) and carboxyl-reduced desulfated (OXSb-RD and OXSc-RD) polymers (Fig. 1). A similar effect was reported for the oversulfated α-D-mannan and sulfated xylomannan obtained from the red seaweed *Nemalion helminthoides* (Recalde, Carlucci, Noseda, & Matulewicz, 2012), for the 4-sulfated and 4-xylosylated 3-linked rhamnose residues present in the sulfated heterorhamnans from *G. oxysperma* and in the *O*-polysaccharide from *Xanthomonas campestris*, respectively (Cassolato et al., 2008; Senchenkova, Shashkov, Laux, Knirel, & Rudolph, 1999). Moreover, in the HSQC spectra, the C-1/H-1 correlations at 100.1/5.49 and 100.5/5.34 ppm

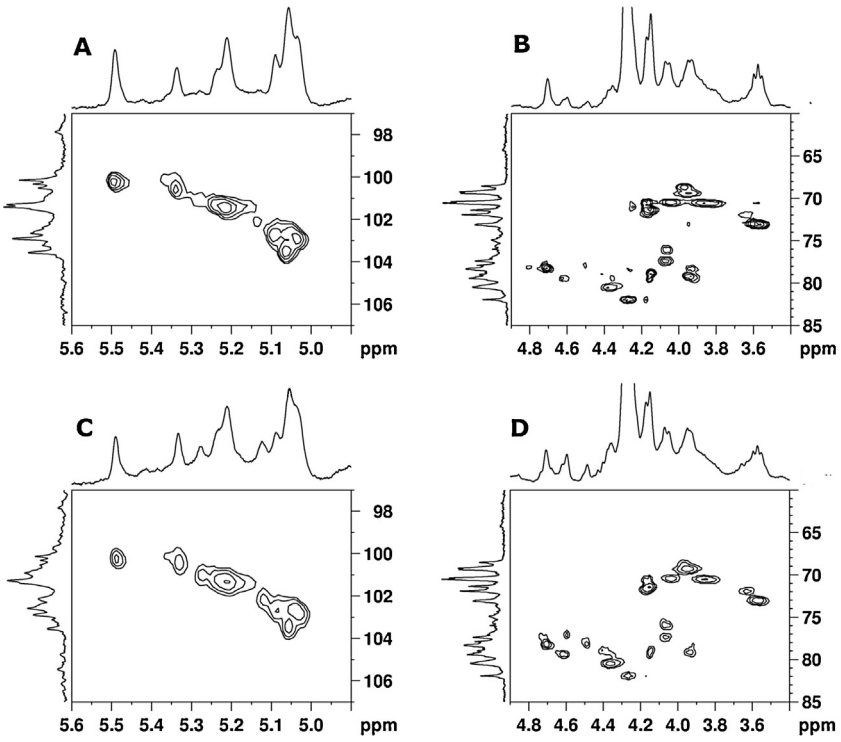

Fig. 2. HSQC NMR spectra of the Smith-degraded heterorhamnans OXSb (A and B) and OXSc (C and D).

were attributed to 3-linked rhamnosyl units monosulfated at C-2 and disulfated at C-2 and C-4 (Cassolato et al., 2008). From the HSQC and COSY experiments, the C-1/H-1–C-6/H-6 resonances for the 3-linked rhamnosyl 2- and 2,4-sulfated units could be assigned (Table 4). In the case of the 3-linked rhamnosyl 2-sulfated units, the C-2/H-2 and C-3/H-3 resonances at 78.3/4.70 and 77.3/4.07 ppm, respectively, are in agreement with the presence of sulfate groups at C-2. These signals are downfield-shifted (6.9 ppm) and upfield-shifted (1.7 ppm), respectively, in comparison with the corresponding signals of the non-substituted 3-linked rhamnose residues (Table 4, Fig. 1). Additionally, the assignments for 3-linked rhamnose 2-sulfate residues are in good agreement with those reported for 3-linked rhamnose 2-substituted (Knirel, Ovod, Paramonov, & Krohn, 1998; Senchenkova et al., 1999) and for the α-D-mannose 2-sulfate (Kolender, Pujol, Damonte, Matulewicz, & Cerezo, 1997). The assignments for the 3-linked rhamnosyl 2,4-sulfated units in comparison to those of the 3-linked rhamnosyl 2-sulfated units (Table 4) are in accordance with the additional sulfate on C-4, and with the resonances reported for 3-linked 2,4-substituted rhamnosyl units (Winn & Wilkinson, 2001).

From the HSQC spectra of OXSb and OXSc, the anomeric signals at 101.3–101.4/5.21, 5.24 ppm were attributed to 2-linked rhamnosyl units, which are sulfated on C-4 and at both C-3, C-4. The COSY spectra of these fractions showed H-1–H-2 cross-peaks at 5.21/4.15, 5.24/4.15 and 5.21/4.60, 5.24/4.60 ppm, which were assigned to 2-linked 4-sulfated and 3,4-disulfated rhamnose residues, respectively. In addition, from the COSY spectra, it was possible to determine the H-3 (4.49 ppm) and H-4 (4.36–4.62 ppm) of the 2-linked 3,4-disulfated rhamnose residues. In agreement with the presence of sulfate groups on C-3 and C-4, the values for C-2 and C-3, C-4 signals (77.0 and 78.1, 79.4 ppm, respectively) are upfield-shifted (2.6 ppm) and downfield-shifted (6.8, 6.6 ppm, respectively) in comparison to the corresponding resonances for the 2-linked rhamnose residues (Table 4, Fig. 1). Furthermore, in the HSQC spectra of OXSb and OXSc, the C-4/H-4 signals at 81.9/4.26 ppm of the 2-linked rhamnosyl 4-sulfate residues are consistent with sulfation on C-4 and absence of substitution on C-3. The C-1/H-1 correlations at 100.9–101.1/5.28, 5.24 ppm were attributed to the 2,3-substituted rhamnosyl residues, corresponding to the branching points of the Smith-degraded products.

The anomeric region of the ^{13}C NMR spectra of the carboxyl-reduced and desulfated fractions OXSb-RD and OXSc-RD (Fig. 1B and D) showed two major resonances at 103.1 ppm (with shoulders at 103.2 and 102.9 ppm) and at 102.0 ppm, which were assigned to 3-linked and 2-linked (plus 2,3-linked) rhamnosyl units, respectively.

From the HSQC spectra of OXSb-RD and OXSc-RD (Fig. S4), the C-1/H-1–C-6/H-6 signals for the 3-linked rhamnose-(1 → 3)-rhamnose-(1 → 2)-rhamnose residues (C-1/H-1 of 3-linked rhamnosyl units at 103.1/4.99 ppm) and 2-linked rhamnosyl residues (C-1/H-1 at 102.0/5.20 ppm) were determined (Table 4), and they are in accordance with those previously reported by Cassolato et al. (2008).

In the ^{1}H NMR spectra of OXSb-RD and OXSc-RD (Figs. S2 and S3) the low intensity H-1 signal at 5.28 ppm was attributed to 2,3-substituted rhamnose residues. Additionally, the anomeric signals at 5.06, 4.99, 5.21 and 5.28 ppm presented relative integrals of 1:0.55:0.61:0.05 for OXSb-RD and of 1:1:1:0.09 for OXSc-RD. These results show that, in terms of sequence of the rhamnosyl units along the polysaccharidic chain, OXSc present higher content of 3-linked rhamnose-(1 → 2)-rhamnose residues when compared to OXSb. Notwithstanding this observation, methylation analyses of OXSb-RD and OXSc-RD (Table 3) showed similar percentages of 3-linked (55.4 and 51.5 mol%, respectively) and 2-linked rhamnosyl units (27.1 and 29.5%, respectively) the Smith degraded products OXSb and OXSc contain ~10 and ~26 mol%, respectively, of 3-linked rhamnose-(1 → 2)-rhamnose residues.

3.5. Antitumor activity of the sulfated heterorhamnans and their Smith-degraded products

The effect of the sulfated polysaccharides from *G. oxysperma* in the human glioblastoma model was evaluated by MTT assay. U87MG cells were treated with OX, OXS, OXSb and OXSc (10, 100

Table 4
Chemical shift assignments of NMR spectra of the Smith degraded fractions OXSb and OXSc (I) and their carboxyl-reduced and desulfated products OXSb-RD and OXSc-RD (II).

Rhamnosyl residues	Chemical shift (ppm)						Chemical shift (ppm)					
	C-1	C-2	C-3	C-4	C-5	C-6	H-1	H-2	H-3	H-4	H-5	H-6
I												
[→3)-α-L-Rhap-(1→]	103.6	71.4	79.0–79.4	72.0	70.4	17.9–18.2	5.06	4.17	3.94	3.57–3.60	4.05	1.31–1.45
[→3)-α-L-Rhap-4S-(1→]	102.9	71.4	76.0	80.4	68.6–69.4	17.9–18.2	5.03	4.17	4.07	4.36	3.93–3.96	1.31–1.45
	102.6	71.4	76.0	80.4	68.6–69.4	17.9–18.2	5.09	4.17	4.07	4.36	3.93–3.96	1.31–1.45
	102.2	71.6	75.7	80.4	68.6–69.4	17.9–18.2	5.12	4.19	4.09	4.36	3.93–3.96	1.31–1.45
[→3)-α-L-Rhap-2S-(1→]	100.1	78.3	77.3	73.1	70.4,70.5	17.9–18.2	5.49	4.70	4.07	3.57	4.05, 3.86	1.31–1.45
	100.5	78.3	77.3	73.1	70.4,70.5	17.9–18.2	5.34	4.70	4.07	3.57	4.05, 3.86	1.31–1.45
[→3)-α-L-Rhap-2,4S-(1→]	100.1	78.3	76.0	80.4	68.6–69.4	17.9–18.2	5.49	4.70	4.07	4.36	3.93–3.96	1.31–1.45
	100.5	78.3	76.0	80.4	68.6–69.4	17.9–18.2	5.34	4.70	4.07	4.36	3.93–3.96	1.31–1.45
[→2)-α-L-Rhap-4S-(1→]	101.3–101.4	79.0–79.4	70.4	81.9	68.6–69.4	17.9–18.4	5.21, 5.24	4.15	4.05	4.26	3.93–3.96	1.31–1.45
[→2)-α-L-Rhap-3,4S-(1→]	101.3–101.4	77.0	78.1	79.4	68.6–69.4	17.9–18.4	5.21, 5.24	4.60	4.49	4.62	3.93–3.96	1.31–1.45
[→2,3)-α-L-Rhap-(1→]	100.9–101.1	78.1	79.4	72.0	70.5	17.9–18.4	5.24, 5.28	4.49	4.62	3.65	3.86	1.31–1.37
II												
[→3)-α-L-Rhap-(1→3)]	102.9–103.2	71.4	79.3	72.7–72.9	70.4–70.6	17.9–18.1	5.06	4.15	3.90–3.94	3.57–3.61	3.76–3.88	1.28–1.33
[→3)-α-L-Rhap-(1→2)]	103.1	71.4	79.0–79.1	72.7–72.9	70.4–70.6	17.9–18.1	4.99	4.15	3.85	3.57–3.61	3.76–3.88	1.28–1.33
[→2)-α-L-Rhap-(1→]	102.0	79.6	71.5	73.8	70.4–70.6	17.9–18.1	5.20	4.08	3.94–3.97	3.48–3.53	3.76–3.88	1.28–1.33
[→2,3)-α-L-Rhap-(1→3)]	102.0					17.9–18.1	5.28					1.28–1.33

Fig. 3. Viability of U87MG cells after treatment with OX, OXS, OXSb and OXSc fractions (10, 100 and 1000 μg/mL) for 48 h (A) and 72 h (B). Data are shown as percentage relative to control (100%) ± SD of four independent experiments each one in triplicate (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$). Control conditions consisted of glioma cells maintained in culture medium.

and 1000 μg/mL) for 48 and 72 h and cell viability was then determined (Fig. 3). All evaluated polysaccharidic fractions were able to reduce the number of U87MG viable cells with a dose-dependent profile. Notably, OXSb, in general, presented lower cytotoxic effect in comparison to the other evaluated fractions. The treatment with OX, OXS, OXSb and OXSc for 48 h at 100 μg/mL promoted 48.4, 46.1, 26.6 and 28% of reduction of cell viability, respectively, and these conditions were selected to evaluate the mechanisms of action performed by these polysaccharides.

Different studies report that sulfated polysaccharides with antitumor activity present low or absence of cytotoxicity to normal cells (Vishchuk et al., 2013). The sulfated heterorhamnans from *G. oxysperma* showed no cytotoxicity for Vero cells (Cassolato et al., 2008). In this context, the sulfated polysaccharides obtained from seaweeds could be a good antitumor candidate, since that the majority of chemotherapeutics agents cytotoxic to cancer cells are also cytotoxic to normal cells.

With the objective of investigating the mechanisms of cell growth inhibition induced by sulfated heterorhamnans from *G. oxysperma*, U87MG cells treated with polysaccharidic fractions were submitted to cell-cycle analysis (Fig. 4A). Treatment of U87MG cells with OX, OXS and OXSc (48 h at 100 μg/mL) promoted a significant increase in the percentage of cells in the G1 phase and a decrease in the percentage of cells in S and G2-M phases. For OXSb, the effects on cell cycle progression were only observed after treatment for 72 h at 100 μg/mL (Fig. S7). All evaluated fractions did not induce DNA fragmentation in U87MG cells (Fig. 4B). These results suggest that sulfated heterorhamnans from *G. oxysperma* and their Smith-degraded products could inhibit the growth of glioma cells and alter the cell cycle progression.

To further elucidate the mechanism of cell cycle regulation mediated by the polysaccharides herein studied, the mRNA expression levels of the main cell-cycle markers p14/ARF, p21 and p53 were evaluated (Fig. 4C). Except for OXSb, all evaluated polysaccharidic fractions promoted a significant increase in the p53 and

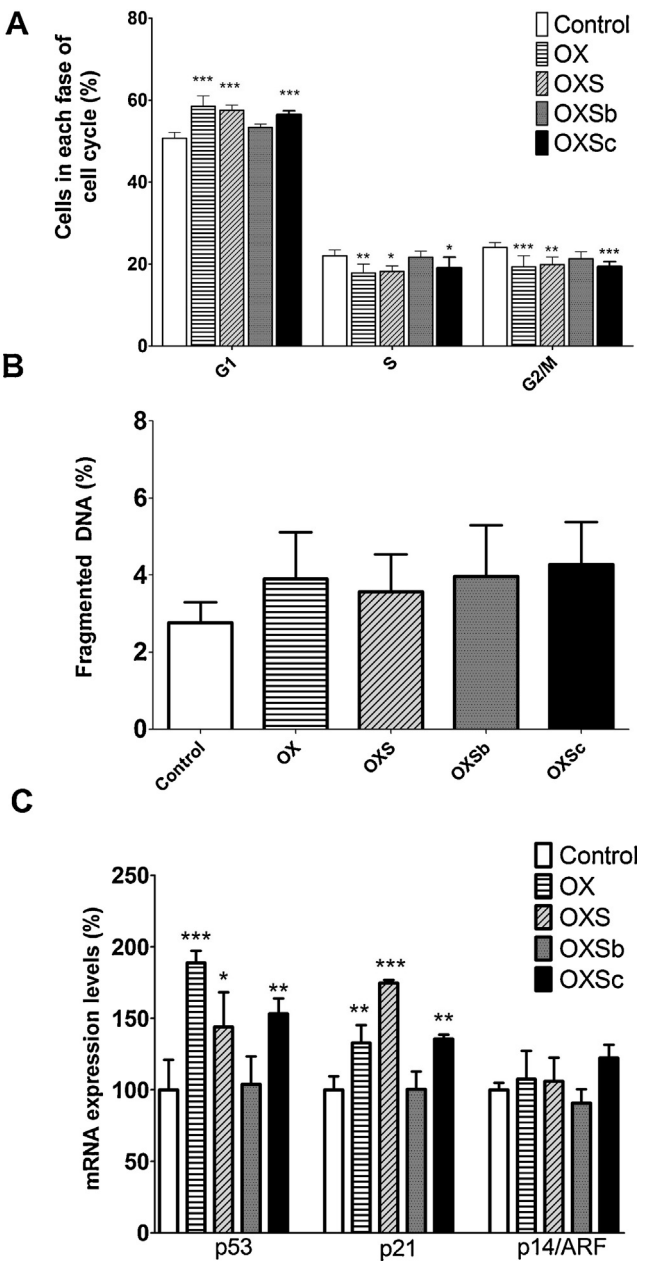

Fig. 4. Effects of OX, OXS, OXSb and OXSc fractions at 100 μg/mL for 48 h on cell cycle progression (A), in the percentage of fragmented DNA (B) and on cell cycle markers mRNA expression levels (C). (A) And (B) graphs represent mean ± SD of four independent experiments, each one performed in duplicate (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$). C graph represents the percentage relative to control (100%) ± SD (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$) of five independent experiments, each one performed in duplicate. Control conditions consisted of glioma cells maintained in culture medium.

p21 mRNA levels in the U87MG cells. There were no alterations in p14/ARF mRNA levels.

The p21 marker preferentially inhibits the cyclin-dependent kinase of the G1/S phase. The transcriptional regulation of p21 is mediated by p53, which in turn regulates target proteins that are involved in cell cycle arrest and DNA repair. Alterations on p53 via frequently have been described for glioblastoma cases (Parsons et al., 2008).

The antitumor activity displayed by sulfated polysaccharides has currently been associated with the degree of sulfation of the polymer (Cho, Lee, & You, 2011). However, in the present study, OX and OXSc (26 and 41% of sulfate groups, respectively) showed similar cytotoxic profiles, suggesting that the percentage of sulfate groups was not the determinant factor for the antitumor activity in this case.

The cytotoxic activity of OXSb was clearly lower than that of OXSc, taking in account that the amount of OXSb in terms of μmols was ~2.5 higher than that of OXSc. Considering that OXSb and OXSc are homogeneous polysaccharides, with molecular weight and chemical structure determined, we are proposing some factors that could be correlated with the antitumor activity of these compounds.

OXSb and OXSc present molecular weight of 109 and 251 kDa, respectively, suggesting that the significant decrease of the molecular weight promotes a decrease in the cytotoxic activity, even with a substantial retention of sulfate groups, as observed for OXSb (33.5% of sulfate groups). Together with molecular weight and content of sulfate groups, structural features such as monosaccharide composition, glycosidic linkage types and sulfate positions are important factors for the biological activity displayed by sulfated polysaccharides.

The presence of disulfated units in polysaccharides obtained from seaweeds was correlated with a high anticoagulant activity. Specifically, in sulfated polysaccharides isolated from green seaweeds, the presence of 2-linked rhamnosyl units 3,4-sulfated could promote an ordered binding zone in the polysaccharidic chain required to interact with target proteins (Ciancia et al., 2010).

Therefore, the sulfate positioning and the higher content of 2-linked disulfated rhamnose residues of OXSc, when compared to OXSb, are probably important requisites for its higher antitumor activity. Additionally, an adequate balance between the molecular weight and sulfate group positions seems to be essential for the sulfated heterorhamnans and their Smith-degraded polymers display the antitumor activity against U87MG cells.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.carbpol.2014.09.089.

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