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Sulfated Polysaccharide Extraction, Isolation, Characterisation, and Biological Activity of Ascidian Viscera Microcosmus exasperatus

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Abstract: The sea invertebrates known as ascidians produce sulfated glycosaminoglycans (GAGs) in their viscera.Ascidian GAGs are thought to be counterparts of mammalian GAGs and have a lot of promise as bioactive substances with anticoagulant and anticancer properties. Our primary goals are to investigate the composition, structure, and biological activity of MicrocosmusexasperatusGAGs because of their widespread distribution and suitability as an organism for large-scale mariculture in a variety of marine habitats.Additionally, we want to create effective procedures for the extraction and purification of sulfated polysaccharides for use in clinical settings and large-scale manufacturing. Agarose gel electrophoresis and enzymatic treatments were used to characterise the GAGs obtained from M. exasperatus viscera after they were extracted by proteolytic digestion and purified by ion-exchange liquid chromatography. APTT tests were used to assess anticoagulant activity. In an in vitro model of tumour cell culture, antitumoural efficacy was evaluated using wound healing, clonogenic, and MTT assays, respectively.Two of the three different polysaccharides that M. exasperatus presents—a dermatan sulphate and a fucosylated dermatan sulfate—were identified by our data.For total polysaccharides (TP), antitumoural efficacy was verified. Long-term TP incubation reduces LLC tumour cell growth/proliferation at various doses, whereas TP inhibits tumour cell migration at various concentrations. Short-term incubation has little effect on tumour cell survivability at low concentrations.Our conclusion is that M. exasperatus has a great deal of promise as a substitute source of GAG, generating antitumoural chemicals at low concentrations that do not have anticoagulant effect and do not improve other characteristics of malignancy, including tumour cell migration.In order to increase the therapeutic effectiveness of existing therapies, we envision using these compounds as antitumoural medicines in next preclinical research for the treatment of cancer.

Keywords: *Microcosmusexasperatus*;ascidians;sulfatedglycosaminoglycans;cancer

1. Introduction

There are over 2000 species of Ascidians, which are marine invertebrates [1–7].Aplousobranchia, Stolidobranchia, and Phlebobranchia are the three orders into which they are divided [3,8–13]. Ascidians can be found alone or in colonies and are filter feeders [14,15]. Being hermaphrodites, they have evolved amazing reproductive techniques that combine asexual and sexual reproduction, enabling rapid population growth for each individual [3,16–20].

The understudied species of ascidians Microcosmusexasperatus (Stolidobranchia; Pyuridae) is discovered off the coast of Rio de Janeiro, Brazil.However, this species is found all across the globe and is particularly prevalent in tropical and subtropical seas.Therefore, M. exasperatus has been characterised as a marine biological indicator for environmental pollution and stress monitoring [21–26].

M. exasperatus tunici is mostly constituted of sulfated polysaccharides, notably high-molecular-weight sulfated L-galactans, according to studies [27, 28]. To far, however, there has been no assessment of the kinds, structures, and biological activity of glycosaminoglycans (GAGs) found in the viscera of M. exasperatus.

It is well known and researched that certain species of ascidians, including Styelaplicata, Phallusigra, and Cionaintestinalis, produce sulfated GAGs in their viscera and have various forms of sulfated polysaccharides, including galactans and fucans, in their tunic [29, 30]. These ascidian-derived GAGs are thought to be comparable to mammalian GAGs and are crucial for a variety of biological functions, including cell proliferation, infection, inflammation, tissue integrity, and fertilisation [31–33]. As a result, ascidians are now regarded as a potential natural source for the synthesis of polysaccharides with medicinal qualities that include antitumoural, anticoagulant, antimicrobial, antioxidant, neuritogenic, neuroprotective, and anti-inflammatory effects [34–38].

A common sulfated GAG utilised in clinical settings as an efficient anticoagulant and antithrombotic agent is mammalian heparin.This chemical has also been investigated as a potentially effective therapy option for cancer and tumour progression [39, 40].However, the presence of molecules like heparin is not limited to mammals [41], and the hunt for comparable compounds from marine invertebrates, like ascidians, that have comparable biological activities but might not have any undesirable side effects, provides a promising alternative in this field [42]. Compared to mammalian heparin, the ascidian Styelaplicata has been reported to synthesise low-anticoagulant heparin.S. plicataheparin, on the other hand, activates heparin cofactor II with almost the same efficacy as reverse heparin. Studies comparing the hemorrhagic effects of mammalian heparin and marine invertebrates showed that, in contrast to the control, mammalian heparin increased blood loss by about two times [35,41]. Ascidians have also been reported to have dermatan sulphates in addition to heparin. Interestingly, the ascidian DS sulfation pattern varies by order, whereas the DS of Stolidobranchia and Phlebobranchia species are mostly constituted of IdoA2S-GalNAc4S and IdoA2S-GalNAc6S, respectively. Different anticoagulant effects are conferred by the variation in the N-acetyl-galactosamine sulfation site of several ascidiandermatansulfates. Whereas IdoA2S-GalNAc6S DS has no discernible anticoagulant action, IdoA2S-GalNAc4S DS is noticeably anticoagulant.The variation in these dermatansulfates' anticoagulant efficacy indicates that GAG binding to heparin cofactor II necessitates a particular sulphate pattern made up of enriched sequences of [α-L-IdoA(2SO4)-1→3β-D-GalNac(4SO4)]. [35, 41, 43].

As previously stated, it is crucial to develop a particular protocol for the collection, isolation, extraction, and purification of sulfated polysaccharides found in the viscera of these animals, particularly for this ascidian species, because of the clinical potential of GAGs derived from marine invertebrates and because they are comparable to mammalian heparin. The collection of bioactive compounds of marine origin is expanded by the identification of Microcosmus exasperatus sulfated GAGs. Assessing whether these compounds retain their bioactivity throughout the extraction and purification procedure is also crucial in order to thoroughly investigate their potential therapeutic uses.

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2. Results

MorphologyoftheAscidianMicrocosmusExasperatus

M. exasperatus ascidians present a densely inhabited tunic (Figure 1A), while the viscerapresentsanorangepigment(Figure1C)thatcanbeobservedafterdissection;finally, an intense purple color can be observed in the mantle (Figure 1B).

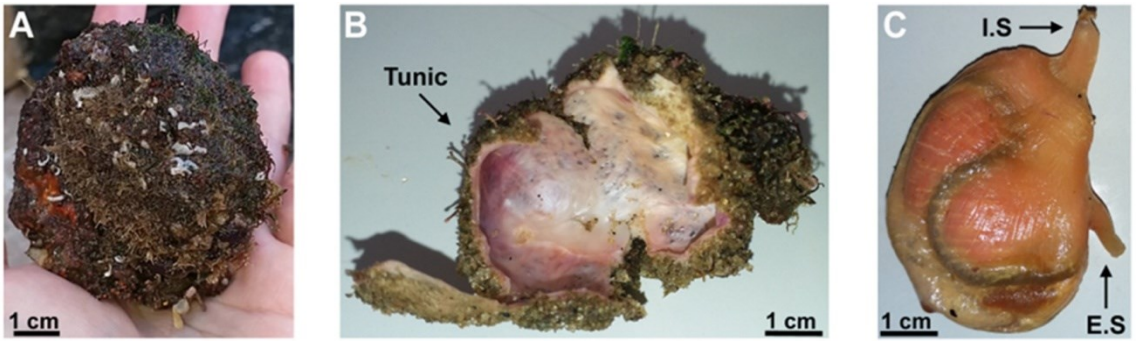

Figure1. Morphologyoftheascidian*Microcosmusexasperatus*.(**A**)*M.exasperatus*beforedissection;
(**B**) *M. exasperatus* after dissection—tunic; (**C**) *M. exasperatus* after dissection—viscera (I.S.: inhalantsiphon; E.S.: exhalant siphon). Scale bar: 1 cm.

MicrocosmusExasperatusViscera PresentsaDermatanSulfatean da Fucosylated-Dermatan Sulfate

Agarose gel electrophoresis was used to analyse M. exasperatus total polysaccharides (TP) in terms of polysaccharide pattern. This analysis shows three distinct bands. By comparing them with commercially available standard GAGs, we were able to identify one band that migrates with standard heparin (arrow C), another band that migrates with standard DS and above standard heparin (arrow B), and a third band that migrates with standard heparin (arrow A) (Figure 2A). TP was then separated using a DEAE cationic column and ion exchange liquid chromatography. Two peaks, identified as sulfated polysaccharides 1 (SP1) and 2 (SP2), were separated by a stepwise gradient (Figure 2B).Intriguingly, when compared to GAGs from other ascidian species, it was possible to see that the polysaccharides produced specifically by this species are shifted from the cationic column at lower NaCl concentrations [9,29,44]. This suggests that they present a lower negative charge density and, consequently, a lower sulfation degree. Agarose gel electrophoresis was used to characterise SP1 and SP2 independently. It was shown that SP1 has one isolated band that migrates with normal heparin, but SP2 has two bands (Figure 2C). Through particular enzymatic treatments of the SP1 and SP2 fractions with a combination of heparinases I and II, chondroitinases AC and ABC, and deamination by HNO2, the identification of the polysaccharides was further examined. SP1 is resistant to HNO2deamination and all enzymatic treatments, as shown in Figure 2D. However, chondroitinase ABC destroyed SP2, but not chondroitinase AC, indicating that SP2 is a dermatan sulphate (Figure 2E). We used nuclear magnetic resonance (NMR) spectroscopy to examine SP1 identification (Figure 3). The 1D and 1H NMR spectra of SP1 and Ludwigothurea grisea's fucosylated-chondroitin sulphate (FUCCS) are compared in blue and red, respectively, in Panel A of Figure 3. Important areas are indicated, including ring atoms and anomeric regions. Iduronic acid and related toucose were identified as the sources of the signals in the anomeric region. The existence of hydrogen atoms bound to carbon 6 in the CH3 region (designated as F6) may also be confirmed. Due to their polymer composition, the signal profile is comparable to that of FUCCS. Next, as seen in Figure 3B, we conducted a 2D1H-13CHSQC experiment to overcome 1D1HNMRoverlapping signals. Three significant spectral areas were identified. Data from the literature was used to assign the spectrum (Table 1).Using iduronic acid (I) units, 4-sulfated GalNAc (A), 6-sulfated GalNAc (A'), and glucuronic acid (U), we discovered distinctive signs of dermatan sulphate.We also discovered signals associated with fucose (F) in addition to these signals. These findings suggest a fucose-substituted dermatan sulphate polymer. Its identify as a fucosylated-dermatan sulphate is further supported by the combination of NMR evidence and SP1 resistance to chondroitinase treatment.

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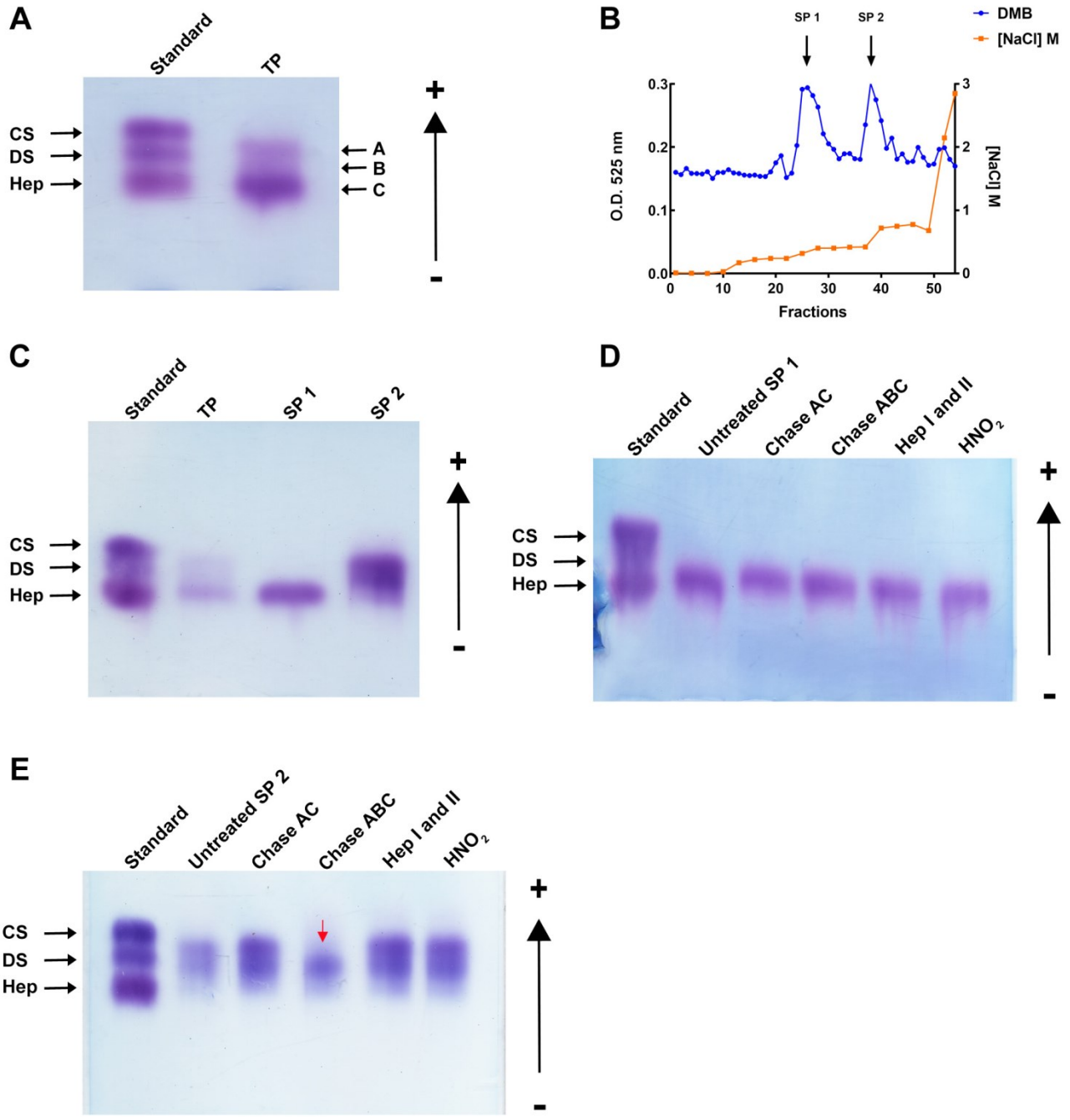

Figure2. *M.exasperatus*presents three polysaccharides in its viscera. (**A**) Total polysaccharides sepa- total polysaccharides (TP), M. exasperatus, arrow A: dermatan sulphate, arrow B: heteropolysaccharide, and arrow C: fucosylated-dermatan sulphate, as determined by agarose gel electrophoresis-GAGstandard (CS: sharpchondroit insulfate; DS: mammalian dermatansulfate; Hep: porcineheparin).(B) A stepwise gradient chromatogram using ion exchange chromatography for GAG separation. DMB (blue line) and NaCl gradient (orange line) detection of metachromatic GAG. (C) Agarose gel containing fractionated sul- fated polysaccharides (SP2-dermatan sulfate-containing fraction; SP1-fucosylated-dermatan sulphate). GAG identification requires specific degradation treatments. Following deamination with HNO2, SP1 and SP2 were subjected to lyases (Chase-chondroitinase-AC and ABC; Hep-heparinases-I and II). (D) SP1 and (E) SP2. The deteriorated band is shown by the red arrow. Heparinases I and II combination, HNO2, chondroitinase AC, chondroitinase ABC, and GAGs standard, untreated. Toluidine blue was used to dye every gel.

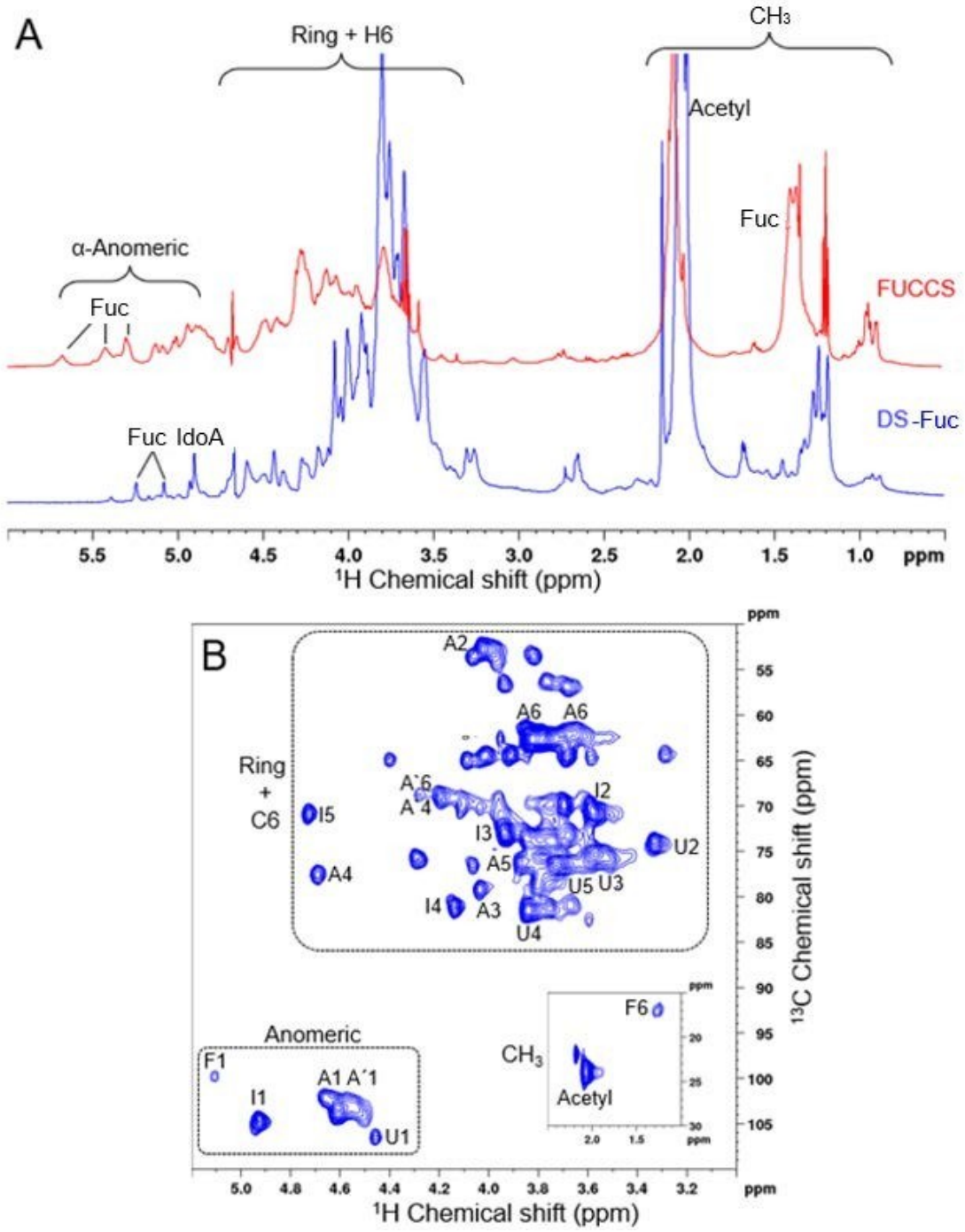

Figure3.SP1NMRspectrarevealthat*M.exasperatus viscera*presentsafucosylated-dermatansulfate.

(**A**)Comparisonof1Dand^{1}HNMRspectrafromSP1andfucosylated-chondroitinsulfate(FUCCS) from the sea cucumber *Ludwigothureagrisea*(in blue and red, respectively).(**B**) Two-dimensional ^{1}H-^{13}CHSQCexperiment. Importantregions,suchasanomericandringatoms,arehighlighted;the CH_3 region is presented as a small inset. Signals are assigned as iduronic acid (I) units, 4-sulfated GalNAc (A), 6-sulfated GalNAc (A'), glucuronic acid (U), and fucose (F).

We, therefore, conclude that *M.exasperatus*fraction SP1 presents a fucosylated-dermatan sulfate, and SP2 fraction presents a combination of a metachromatic uronic acid containing polysaccharide and a dermatan sulfate.

Table1. ChemicalshiftcomparisonofSP1andliteraturedata.

Units	C-H1	C-H2	C-H3	C-H4	C-H5	C-H6
GalNAC-4S#	4.58–103.6	4.02–54.0	4.00–78.5	4.76–79.2	3.83–77.4	3.77–63.8
GalNAC-4Sa	4.67/4.55–103.2	4.01–53.0	4.03–79.4	4.70–77.9	3.87–76.3	3.82/3.66–62.6
GalNAC-6S#	4.58–103.6	4.02–53.4	3.98–81.7	4.20–70.3	3.97–75.1	4.24–70.1
GalNAC-6Sa	4.67/4.55–103.2	3.98–53.0	3.98–81.7	4.19–69.1	-	4.19–69.1
GlcA#	4.46–106.5	3.37–75.2	3.57–76.2	3.77–83.8	3.66–79.5	-
GlcAa	4.46–106.6	3.34–74.3	3.57–75.3	3.82–82.0	3.72–76.5	-
IdoA*	4.90–102.8	3.53–71.6	3.95–73.2	4.10–82.3	4.72–72.4	-
IdoAa	4.92–104.0	3.57–71.1	3.94–72.9	4.13–81.1	4.73–71.2	-

*[44],#[29],aThiswork,seeHSQCFigure3.

MicrocosmusExasperatus-DerivedDermatanSulfate-ContainingFraction(SP2)Presents Anticoagulant Activity

In order to assess *M.exasperatus*GAGs anticoagulant potential, we investigated the SP1 andSP2fractions'performanceinanactivatedpartialthromboplastintime(APTT)assay. It was seen that the dermatan sulfate-containing fraction (SP2) presents mild anticoagulant activity, approximately one hundred times lower than the sixth International Standard for Unfractionated Heparin (Figure 4). On the other hand, the fucosylated dermatan sulfate (SP1) fraction did not present anticoagulant activity at the tested concentrations.

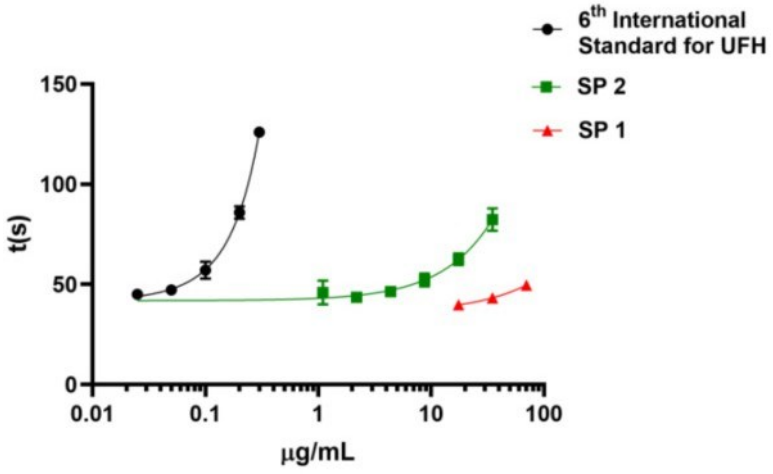

Figure4. Evaluation of activated partial thromboplastin time (APTT) of *M.exasperatus*dermatan sulfate-containing fraction and UFH (unfractionated heparin) The x-axis shows the concentrations of thepolysaccharides,andthey-axisshowscoagulationtimeinseconds.Thegreenlineshowsthe*M. exasperatus* dermatan sulfate-containing fraction (SP2), the red line shows the fucosylated-dermatan sulfate (SP1), and the black line shows the 6th International Standard for Unfractionated Heparin. The ascidian dermatan sulfate presented, approximately, one hundred times lower activity compared to the heparin standard. $n = 3$.

MicrocosmusExasperatusTotalPolysaccharides(TP)PresentsAntitumorActivity

We then looked at whether M. exasperatus TP might have anticancer properties. It has been shown that sulfated GAGs alter cell behaviour, and their effects may change depending on the concentration. Thus, using MTT, clonogenic, and wound healing tests, respectively, we examined how M. exasperatus TP affected tumour

cell viability, colony development, and migration.The mouse Lewis lung cancer cell line (LLC)'s colony expansion and cell viability are shown in Figure 5. Cells were treated with M. exasperatus TP for 24 hours in MTT tests, which were regarded as a short-term therapy, while the clonogenic

The experiment, which included incubating cells with TP for 72 hours and monitoring colony development for an additional 7 days, was regarded as a long-term therapy.

Figure5.
*M.exasperatus*totalpolysaccharides(TP)reducetumorcellviabilityinalong-term,low- concentration treatment.(**A**) MTT assays and (**B**,**C**) clonogenic assays were performed in order to evaluate mouse Lewis lung carcinoma cells' susceptibility to *M. exasperatus* TP. ANOVA with Bonferronipost-test. *p<0.05,**p<0.01,and***p<0.001,n=3.

We discovered that M. exasperatus TP has varying effects on LLC cell viability at lower doses (500 ng/mL) and higher values (50 µg/mL).The MTT test evaluation of the brief incubation period showed that the increased concentration seemed to improve cell viability. However, the long-term incubation assessed using a clonogenic growth test showed that colony development is enhanced by higher concentrations and adversely affected by lower and medium concentrations. Lastly, we used the wound healing experiment to examine the impact of TP on LCC cell migration (Figure 6).It's noteworthy to see that M.exasperatusTP decreased LLC cell movement at 500 ng/mL and 50 µg/m when cells were observed for wound closing activity. Lower TP concentrations, such 500 ng/mL, should be taken into consideration for anticancer efficacy, according to the combination of the obtained data. In short-term incubation, the lowest concentration tested had no effect on cell survival; in long-term incubation, however, it was able to decrease cell growth and migratory activity.

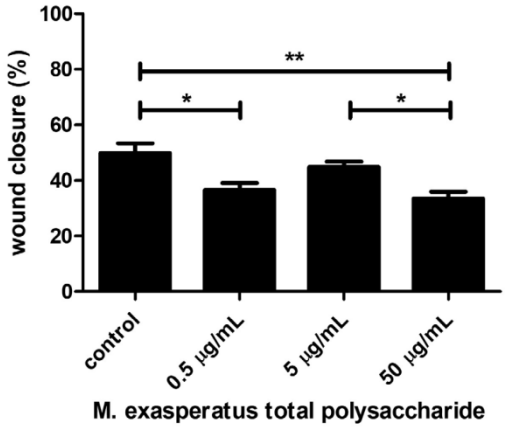

Figure 6. *M. exasperatus* total polysaccharides (TP) affect tumor cell migration. Mouse Lewis lungcarcinoma cells were evaluated regarding their migration behavior in a wound healing/scratch assay.ANOVA with a Bonferroni posttest.* $p < 0.05$ and ** $p < 0.01$.n = 3.

Discussion
The literature has previously documented the GAGs and related biological activities of several ascidian species [35].Nevertheless, every species is a unique organism that presents diversity in these molecules' structure, variety, and biological activity. In order to determine the variety of GAGs and sulfated polysaccharides that may be regarded as mammalian equivalents, as well as the unique biological activities that they show, it is crucial to investigate novel species. Therefore, Microcosmusexasperatus, a common but little-studied ascidian species, was looked at as a possible option for the large-scale, sustainable manufacture of mammalian heparin analogues. In order to rule out any seasonality in the species' glycan production based on reproductive cycles, ascidians were collected at several times throughout the year.These cycles take place throughout the warmer summer months, however as the temperature rises, water layer stratification takes place, which leads to the loss of food for ascidians.On the other hand, the thermocline decreases when seawater cools (fall), which leads to the revival of food sources that are abundant in the deep sea [45].

During the initial collection, which took place in the summer, a large number of M. exasperatus specimens were visible on Praia Vermelha, Rio de Janeiro.By collecting more specimens, we were able to increase the quantity of indry weight (81.98 g). These specimens were likewise very prevalent in the second collection, which was conducted in the winter. A dry weight of

61.87 g was recorded, which was still less than that of the summer collection.However, we were able to notice a more distinct presence of these specimens in the same location in the third collection during the fall, and as a result, we acquired the lowest value in dryweight (18.86g).The abundance of young and adult specimens discovered during these collections supports our hypothesis that M. exasperatus reproduces primarily in the summer, which is also consistent with the literature's description of the brief time required for ascidians to develop—the larval stage lasting only a few minutes or, at most, a few hours. Within a few days to a few weeks, an adult person with functioning organs will differentiate [46–48]. However, since tropical places often have a small temperature window, it was also feasible to detect a significant incidence of M. exasperatus specimens throughout the winter.

The ecological relationship between ascidians and epibiont organisms is always present, regardless of seasonality, and may act as a form of camouflage or barrier to predators throughout the non-larval phase of ascidians, as evidenced by the fact that there was no discernible difference in the number of organisms that inhabit the tunic of these ascidians in the three collections that were conducted [45,49].

A solution comprising total polysaccharides, or TP, was produced after the collection of specimens, viscera dissection, and sulfated polysaccharide extraction. Three different kinds of uronic acid-containing sulfated polysaccharides were identified in this TP using agarose gel electrophoresis. We then used ion exchange liquid chromatography to fractionate M. exasperatus TP. We found that these sulfated polysaccharides could be separated into two different parts, known as SP1 and SP2. In addition, we found that these sulfated polysaccharides elute at lower NaCl concentrations than sulfated polysaccharides from other ascidian species based on our analysis of the resultant chromatogram [9,35,43]. According to these results, sulfated polysaccharides from M. exasperatus seem to have a lower negative charge density and, as a result, a lower sulfation degree. When M. exasperatus GAGs species were identified, the presence of adermatan uronic acid-containing polysaccharide that is resistant to heparinases, chondroitinases, and HNO2 deamination, as well as a fucosylated dermatan sulphate. Remarkably, this is the first report of a fucosylated dermatan sulphate produced from ascidian. The SP1 and SP2 fractions exhibit modest (SP2) to nonexistent (SP1) anticoagulant activity, according to our analysis of their anticoagulant activity. As previously mentioned in the literature, there is a considerable correlation between the GAG sulfation pattern and the anticoagulant action of dermatan sulphates derived from ascidians. Significant anticoagulant action is seen in IdoA2S-GalNAc4S dermatan sulphates, but not in IdoA2S-GalNAc6S dermatan sulphates [9,35,41,43].Furthermore, M. exasperatus, a member of the Stolidobranchia order, should promote these sulfation patterns, which are order-specific.

Dermatan sulphate is an anticoagulant. Thus, a modest degree of sulfation ordered as IdoA2S-GalNAc4S may be the cause of M. exasperatus dermatan sulfate's weak anticoagulant action. The evaluation of the fucosylated dermatan sul- fate (SP1 portion) revealed that, at the studied quantities, this sulfated polysaccharide exhibited no anticoagulant effect. Given the description of fucosylated chondroit sulphate as having considerable anticoagulant action, it was surprising to find that M. exasperatus fucosylated dermatan sulphate lacked anticoagulant activity [50].

Given the increasing interest in using combination therapy to improve the effectiveness of cancer treatment, we wanted to find out whether M. exasperatus polysaccharides would be a good substitute for the usage of ammalheparin.As a marine invertebrate, M. exasperatus may be a suitable substitute for mammals since its large-scale production might be geared towards a more sustainable method, especially needing less freshwater and dryland. We believe that M. exasperatus is a particularly intriguing organism to be investigated for the manufacture of antitumor polysaccharides because of the evolutionary distance between ascidians and mammals, which also increases the safety against cross-species contamination. A mouse Lewis lung cancer cell line (LLC) was used in a cellular model to assess the antitumor activity utilising three distinct methods. We discovered that the effects of M. exasperatus total polysaccharides on tumour cells vary according on the length of treatment, from low (0.5μg/mL) to high (50μg/mL) concentrations.In contrast to controls, we discovered that the high-TP dosage decreased cell migration while acting as a tumour cell stimulant, increasing viability and colony formation.Conversely, the low-TP dosage showed antitumor effects with relation to colony formation and cell migration. As the

There were no differences between the short-term treatment and the control condition. In conclusion, our findings suggests that when contemplating combination treatment, long-term, extremely low dosages of M. exas-peratus TP should be used, preferably considerably below the concentration of anticoagulants. Furthermore, as these procedures may be carried out in bulk, M. exasperatus TP extraction and perhaps fractionation can be readily modified to a large-scale routine. Lastly, we point out that M. exasperatusa is a promising organism for the extraction of special sulfated polysaccharides, dermatan sulphate, and fucosylated-dermatan sulphate, with therapeutic properties as modulators of cell behaviour, given that this ascidian species is found throughout the world. Glycans found in marine invertebrates are a pertinent and expanding field of study in both the therapeutic and environmental sciences, and

further research in this field need to be encouraged.

3. MaterialsandMethods

SpecimensCollection andDissection

By freediving in Praia Vermelha and Rio de Janeiro, Microcosmus exasperatus ascidians species were gathered (SISBIO permit: 66457-1) and put in collecting buckets with saltwater before being brought to the lab.A scalpel and scissors were used in the lab to dissect the viscera, slicing the tunic to remove the viscera.Viscera were preserved in 100% ethanol, and unics were either frozen (for use in other studies) or thrown away.Removal of GAGs from the Microcosmus and Exasperatus Viscera

Following M. exasperatus visceradissection, GAG extraction was carried out as outlined in [43] to produce a final solution that included just the sulfated polysaccharides (referred to as total polysaccharides, or TP), and fractionation was then carried out.Depigmentation and Delipidation

The viscera were cultured in 92.8% ethanol at room temperature to guarantee that the extracted material was devoid of any fatty tissue and colours.For a total of five days, ethanol was substituted every day to ensure that lipids and pigments were effectively removed. The viscera were then let to dry for 24 hours at 60°C.The material was weighed after it had dried.Decomposition by Proteolysis

To liberate the polysaccharides from the tissue, the sample was digested using papain.In a digestion solution containing 100 mM sodium acetate, 5 mMEDTA, 5 mM cysteine, and pH 5.0, 5 mL of the dry material was rehydrated for two hours. Papain was added to initiate the digestion process at a final concentration of 0.5 mg/mL, and it was then incubated for 24 hours at 60°C. A spatula was then used to mechanically homogenise the mixture, more papain was added, and samples were incubated at 60°C.To fully homogenise the solid materials, this procedure was carried out three times.After centrifuging the final digested sample for 30 minutes at 3200 rpm, the pellet—the undigested fraction—was disposed of and the supernatant—the solubilised fraction containing the free polysaccharides—was recovered.GAGs Precipitation

After proteolytic digestion, polysaccharide precipitation in the supernatant was created by adding cetylpyridinium chloride (CPC) until the final concentration was 0.5% m/v. The resulting mixture was homogenised and incubated for 24 hours at room temperature after the addition of CPC. The suspension was then centrifuged for 30 minutes at 3200 rpm. The particle was fully dissolved in a 2M sodium chloride (NaCl) solution with 15% ethanol, and the supernatant was disposed of. Following that, the CPC-free polysaccharides were precipitated by addingethanol to 70% of the solution, then incubate for 24 hours at -20°C.The suspension was then centrifuged for 30 minutes at 3200 rpm, the supernatant was disposed of, the pellet was further re-examined in 80% ethanol, centrifuged for another 30 minutes at 3200 rpm, and the pellet was allowed to air dry.510 U Kunitz/mL of DNAse (Sigma, St. Louis, MO, USA) was added to the pelleted TP after it had been rehydrated in DNAse buffer (20 mM Tris-HCl pH 7.5; 5 mM NaCl; 3 mM MgCl2; 5 mM CaCl2) (1:1) for 24 hours at 37 °C.Analysis of Sulfated Polysaccharides Electrophoretic Profile

Following the acquisition of the TP, an agarose gel electrophoresis was carried out in order to determine the polysaccharide electrophoretic profile present in M. exasperatus viscera by comparing it to three commercially available GAG standards: shark chondroitin sulphate, pig heparin, and malamiandermatansulfate. Samples were run for around two hours at 100V in a 0.5% agarose gel in a 1.3-diaminopropane/50 mM acetic acid buffer, pH 9.0.For 24 hours, polysaccharides were precipitated by gel immersion in a solution of acetyltrimethylammonium bromide (cetavlon0.1%).The gel sheet was then dried under a light (Ourolux 250 watts infrared) and dyed with 0.1% toluidine blue in a solution of acetic acid, ethanol, and water (0.1:5:5, v/v).Purification and Fractionation of Sulfated Polysaccharides through Ion Exchange Liquid Chromatography

Ion-exchange liquid chromatography was used to separate TP.A diethylaminoethyl cellulose anion exchange column (DEAE sepharose) (GE Healthcare) connected to the FPLC (fast protein liquid chromatography) Äkta Prime apparatus (Amersham Biosciences,Amersham,UK,SN:1,102,380) was first filled with 200 µgof material and allowed to acclimatise to a pH 8.0 buffer of 0.02 M Tris-HCl. Using a 0.02M Tris-HCl buffer containing 3MNaCl, pH8.0, the GAGs were eluted from the column in a linear NaCl gradient (0–3M) at a flow rate of 3 mL per minute, with fractions of 3 mL each tube being collected.The concentration of NaCl was then determined by measuring conductivity.As a result, values of 240, 370, and 700 mM NaCl were identified as being used in a stepwise gradient of NaCl for bulk sulfated polysaccharidefraction.In a 96-well plate, the sulfated polysaccharide elution pattern was evaluated by metachromasia characteristics using 100µL of DMB and 20µL of sample.After that, the fractions containing metachromatic material associated to the graph's peaks (SP1 and SP2) were pooled and precipitated in 70% ethanol at -20°C for 24 hours. Then, the mixture was measured in a Versamax spectrophotometer using a microplate reader (Molecular Devices, San Jose, CA, USA) at 525 nm.The After centrifuging the suspension for 30 minutes at 3200 rpm, the pellet was washed with 80% ethanol, centrifuged once more for 30 minutes at 3200 rpm, and allowed to air dry.Specific Degradation

of Lyases and Nitrous Acid Deamination
In order to identify the sulfated polysaccharide species present in the samples, enzymatic treatments were carried out on SP1 and SP2 using chondroitinases AC and ABC (Seikagaku, Japan), a mixture containing heparinases I and II, and deamination with nitrous acid (HNO2).The sample, enzyme, and 2×buffer ratio of 1:1:1 were used to incubate each sample for 24 hours with 0.05 units of chondroitinases AC and ABC and 0.2 units of heparinases I and II in 2× digestion buffer (100 mM Tris-HCl, 30 mM sodium acetate, 1.0 mM EDTA, pH 8.0) for chondroitinases and (40 mM Tris-HCl, 100 mM NaCl, 8 mM CaCl2, pH 7.5) for heparinases at 37◦C. Each enzyme was added again after a 24-hour period.
Additionally, deamination was carried out, and the reaction of sulphuric acid (H2SO4) at a final concentration of 0.25 M with sodium nitrite (NaNO2) at a concentration of 0.25 M for 20 minutes generated nitrous acid. The samples were then combined with an equal amount of nitrous acid (HNO2) (1:1, v/v) and allowed to sit at room temperature for an hour. In addition to using commercial standards as a reference, agarose gel electrophoresis was used to assess enzymatic degradation and deamination in order to compare intact and digested material.Analysis of Uronic Acid
Using the carbazole technique, the uronic acid content of the TP solution, SP1 and SP2, was used to quantify the concentration of polysaccharides present [51]. The procedure is mixing 10µL of the polysaccharide solution with 190µL of distilled water and 1 mL of sulphuric acid with 0.1% borate, then letting it sit at 100 °C for 12 minutes. Following cooling, 40 µL of carbazole (0.2% in ethanol) is added to the solution, which is then homogenised and incubated for 10 minutes at 100◦C. The solution is then cooled again, and the absorbance at 525 nm is determined. A standard curve for glucuronolactone was used to assess the sample concentration.Analysis of Nuclear Magnetic Resonance (NMR)
The SP1 portion (2 mg) was subjected to nuclear magnetic resonance (NMR), 1D, and 2D spectra using a triple resonance probe (900MHz Bruker, Karlsruhe, Germany).
32 scans with a 1.5-s inter-scan delay were used to capture the 1HNMR1D spectrum at 35°C. Time proportion phase incrementation (TPPI) is used to identify quadrature in the indirect dimension of the 1H/13C HSQC (heteronuclear single quantum coherence) spectrum. A total of 1048 × 512 points and 64 scans were captured.ActivatedPartialThromboplastinTime (APTT)
As explained by Eggleton [52] and Thomas [53], the activated partial thromboplastin time (APTT) test was used to assess anticoagulant activity.
In short, 10 µL of solution containing various polysaccharide concentrations (SP1:17.5;35;70µg/mL;SP2:1.1;2.19;4.38;8.75;17.5;35µg/mL;UFH:0.025;0.05;0.1;0.2;0.3µg/mL) was incubated with 100 µL of human plasma mixture for 1 minute at 37◦C.Next, we added 100µL of cephalin (Biolab-MerieuxAS, Rio de Janeiro, Brazil).Following two minutes of incubation at 37°C, 100 µL of 25 mMCaCl2 was added to the mixture. Using a coagulometer (KC4 Delta), the formation time of a fastable clot capable of holding the metallic sphere in the well was used to determine the amount of time needed for coagulation. Polysaccharide microgrammes per millilitre (µg/mL) is the unit of measurement for activity. The gathered data led to an apolynomial function, as per the following equation:[UI]+b2. [T/T0]=a+b1.A standard curve based on the sixth International Standard for Unfractionated [UI]2

NIBSC (Potters Bar, UK) provided the heparin (6th UFH) (2145 units per vial), which was diluted to produce a UFH solution with anticoagulant activity equal to 10 IU/mL.The LLC (mouse Lewis Lung Carcinoma) cell line (murine lung carcinoma, ATCC, supplied by Prof. Dr. Lubor Borsig, University of Zürich) was used in this investigation. The cells were cultivated in Dulbecco's Modified Eagle Medium (DMEM), supplemented with 10% foetal bovine serum (Vitrocell, Waldkirch, Germany) and 4.5g/mL glucose (both Sigma, St. Louis, MO, USA). They were then incubated at 37 °C in a humid environment with 5% CO2. For further growth, cells were extracted from the culture flasks and plates using a 0.25% trypsin-EDTA solution (Sigma, St. Louis, MO, USA).Assays for Cell Viability (MTT)
The vitality of cells exposed to M. exasperatus TP was evaluated by plating 5 ×103 cells perwell in 96-well plates.Following 24 hours of plating, cells were treated with either M. exasperatus TP or vehicle (PBS) at several doses (0.5 µg/mL, 5 µg/mL, and 50 µg/mL) for a further 24 hours. After that, cells were cultured for two hours in a cell medium containing 0.5 mg/mL of tetrazolium salt (MTT, Roche, Basel, Switzerland). Following incubation, 200 µL of dimethyl sulfoxide (DMSO, Sigma-Aldrich, St. Louis, MO, USA) per well was used to dissolve the formazan crystals, and the cell media was disposed of. A SpectraMax Plus microplate reader (Molecular Devices, San Jose, CA, USA) was used to measure absorbance at 560 nm. Softmax Pro software 5.4.1 (Molecular Devices, San Jose, CA, USA) was used for analysis. Using control cells as a benchmark, viability was computed as a percentage of absorbance.The Clonogenic Assay
A clonogenic test was developed to assess LLC cells' capacity to form colonies.Trypsinization, quantification, and plating at 500 cells/well in 6-plate wells were performed using regularly grown cells.During the low-density plating, M. exasperatus TP was introduced to the culture medium at concentrations of 0.5 µg/mL, 5

µg/mL, and 50 µg/mL, or vehicle (control condition). Following 72 hours of usual culture conditions, the cells were cultivated for a further 7 days, with medium changes occurring every 48 hours, after which the culture medium (control and TP media) was swapped out for regular medium.Cells were washed with PBS after 10 days, fixed in 100% ethanol for 1 hour, then stained with 0.4% trypan blue for 15 minutes.For colony quantification, wells were photographed under a stereomicroscope and examined using ImageJ software.Assessment, Cell Migration, and Wound Healing

The cells were cultivated to 80% confluence after being plated at a density of 1.5 × 105 cells/well in 6-well plates. Once sufficient confluence was achieved, a sterile P1000 tip was used to manually remove the cells off the plate in a cross-shaped pattern. After washing the wells to get rid of any cell debris, they were incubated for 24 hours at 0.5 µg/mL, 5 µg/mL, and 50 µg/mL in either TP-medium or control (normal culture media).Cells were photographed at 0 and 24 hours using the cross-center as a reference to assess wound closure and cell migration.The percentage of migrated distance in relation to the initial wound width was used to quantify migration using ImageJ software.

Conclusion

The ascidian microcosmusex asperatus is a promising source of complex and long-sulfated polysaccharides with potential medicinal uses, according to this research.Since M. exasperatus is found all over the globe, sustainable methods might be used to increase its large-scale production while preserving a high glycosylation yield with little negative effects on the ecosystem.Although we have examined the anticancer effects of M. exasperatus on total polysaccharides, further fractionation is both feasible and scalable.In conclusion, we show that the glycans of marine invertebrates, particularly those generated by ascidians, should be investigated more as they provide a fascinating substitute for glycans found in mammals.

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