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Anti-HIV activity of fucoidans from three brown seaweed species

Thanh Thi Thu Thuy[a,*], Bui Minh Ly[b], Tran Thi Thanh Van[b], Ngo Van Quang[a], Ho Cam Tu[c], Yue Zheng[c], Carole Seguin-Devaux[c], Bilan Mi[d], Usov Ai[d]

[a] Institute of Chemistry, Vietnam Academy of Science and Technology, Viet Nam
[b] Nha Trang Institute of Technology Research and Application, Vietnam Academy of Science and Technology, Viet Nam
[c] Laboratory of Retrovirology, CRP-Santé, Luxembourg
[d] N.D.Zelinsky Institute of Organic Chemistry, Russian Academy of Sciences, Leninskii Prospect 47, Moscow 119991, Russian Federation

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ABSTRACT

Fucoidans are sulfated polysaccharides derived from marine brown algae. In the current work the anti-HIV activity of three fucoidans, extracted from three brown seaweeds *Sargassum mcclurei*, *Sargassum polycystum* and *Turbinara ornata* and collected from Nha Trang bay, Vietnam was investigated. Fucoidans extracted from the three species displayed similar antiviral activities with a mean IC_{50} ranging from 0.33 to 0.7 μg/ml while displaying no cell toxicity. Our results showed that the anti-HIV activity of fucoidans is not primarily linked to the sulfate content and the appropriate position of sulfate groups in the fucoidan backbones was also not associated with the antiviral activity. Fucoidans inhibited HIV-1 infection when they were pre-incubated with the virus but not with the cells, and not after infection, blocking the early steps of HIV entry into target cells. These data contribute to a better understanding of the influence of fucoidans structural characteristics on their biological activity.

1. Introduction

Fucoidans are sulfated polysaccharides derived from marine brown algae. A common structural feature of fucoidans is that they contain a large amount of α-L-fucose residues and sulfate groups, together with a minor amount of other monosaccharide residues including galactose, mannose, xylose, rhamnose, and glucuronic acid (Li, Lu, Wei, & Zhao, 2008; Usov & Bilan, 2009). Fucoidans were reported to exhibit a wide range of physiological and biological activities (Berteau & Mulloy, 2003; Kusaykin et al., 2008; Wijesinghe & Jeon, 2012), such as anti-inflammatory, anticoagulant, antithrombotic (Cumashi et al., 2007; Ustyuzhanina et al., 2013), antiviral including anti-HIV (Ghosh et al., 2009; Lee, Hayashi, Hashimoto, Nakano, & Hayashi, 2004; Trinchero et al., 2009), immunomodulatory (Raghavendran, Srinivasan, & Rekha, 2011), antioxidant (Wang, Zhang, Zhang, Song, & Li, 2010), and antitumor (Senthilkumar, Manivasagan, Venkatesan, & Kim, 2013; Synytsya et al., 2010). A first report on the HIV inhibitory property of sulfated polysaccharides from sea algae was published in 1987 (Nakashima et al., 1987). Queiroz et al. (2008) further reported that sulfated fucans from the seaweed species *Dictyota mertensii*, *Lobophora variegata*, *Spatoglossum schroederi* and *Fucus vesiculosus* inhibit HIV reverse transcriptase.

Despite an extensive investigation of the biological activity of fucoidans from marine seaweeds during the last decade, the overall relationship between the activity of polysaccharides and their structure remains unclear (Berteau & Mulloy, 2003). It is usually believed that biological activity of fucoidans is related to the content and position of sulfate groups, monosaccharide composition, molecular weight, and structure of the backbone and branches (Ale, Mikkelsen, & Meyer, 2011; Patankar, Oehninger, Barnett, Williams, & Clark, 1993; Pomin, 2009).

The current challenges of the current HIV therapy prompt the worldwide researchers to search for other natural and affordable anti-HIV compounds. Based on preliminary promising results, the structure–activity relationship of the SPs structural characters on HIV-1 inhibition need to be elucidated for a deeper understanding of their binding structure and their antiviral mechanisms.

The present paper is devoted to the investigation of the anti-HIV activity of fucoidans extracted from three brown seaweed species *Sargassum polycystum*, *Sargassum mcclurei* and *Turbinara ornata* collected at Nha Trang bay, Vietnam. Native polysaccharides and several polysaccharide fractions differing in sulfate content were tested to unravel the role of sulfate groups on the anti HIV-activity of fucoidans. Similarly to other fucoidans present

* Corresponding author at: 18 Hoang Quoc Viet, Cau giay, Hanoi, Viet Nam. Tel.: +84 4 37563788; fax: +84 4 38361283.
E-mail address: thuyttt@ich.vast.ac.vn (T.T.T. Thuy).

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in algae belonging to the family Sargassaceae, these polysaccharides are mainly constituted by the highly sulfated galactofucans. Structural characteristics and fractionation of a fucoidan from *S. mcclurei* were described recently (Pham et al., 2013). The polysaccharide was shown to have complex monosaccharide composition. Its highly sulfated main fraction is a galactofucan containing a backbone built up of 3-linked α-L-fucose 2,4-disulfate residues. Structurally related, but less sulfated galactofucan was obtained from *T. ornata* (Thanh, Tran, Yuguchi, Bui, & Nguen, 2013). Fractionation of fucoidan from *S. polycystum* and structural analysis of its highly sulfated galactofucan fraction was also described recently in the literature (Bilan et al., 2013). Additional information on the position of sulfate groups in the polysaccharide samples used in this work was obtained by partial acid hydrolysis followed by ESI–MS of the derived fucose monosulfates.

2. Experimental

2.1. Seaweed collection

The algae *S. polycystum*, *S. mcclurei* and *T. ornata* were harvested from Nha Trang bay, Vietnam in June, 2010. A voucher specimen of each species is deposited in Nha Trang Institute of Technology Research and Application. The collected seaweeds were washed with tap water in order to remove salt, epiphytes, and sand attached to the surface of the samples and then dried by air in the shade. The dried seaweeds were crushed, grounded into a powder form, passed through a 40-mesh sieve and then stored at room temperature.

2.2. Extraction of fucoidan

The extraction was followed by the method described previously (Bilan et al., 2002). 200 g of dried seaweed was treated at room temperature with a 4:2:1 MeOH–$CHCl_3$–water mixture to remove colored matter, filtered and vacuum dried to get defatted algal biomass. This material was extracted with 2% aqueous $CaCl_2$ solution under mechanical stirring at 85 °C for 8 h. An aqueous hexadecyltrimethylammonium bromide solution (10%) was added to the extract. The precipitate formed was centrifuged, washed with water, stirred with 20% ethanolic NaI solution for 2–3 days at room temperature, washed with ethanol, and dissolved in water. The solution was dialyzed, concentrated and lyophilized to give fucoidan as sodium salt. Fucoidans obtained from *S. mcclurei*, *S. polycystum* and *T. ornata* were named F_{SM}, F_{SP} and F_{TO}, respectively. The yields of fucoidans were calculated based on dried seaweed weight.

2.3. Chemical analyses

Neutral monosaccharide compositions were elucidated by GLC (Bilan et al., 2002). Alditol acetates were prepared by hydrolysis of fucoidan samples in 2 M CF_3COOH (TFA), 8 h at 100 °C, followed by sodium borohydride reduction and acetylation, and analyzed by 17AAFW Shimadzu GC-FID.

Sulfate content was estimated using gelatin/$BaCl_2$ method (Dodgson, 1961) after hydrolysis of fucoidan in 2 M TFA as described above.

2.4. Partial acid hydrolysis

Mild acid hydrolysis of fucoidans was carried out using trifluoroacetic acid (0.75 M, 1 h, 60 °C), the resulting solutions were evaporated several times with methanol.

2.5. ESI–MS spectra

ESI–MS experiments were performed on a Xevo TQ MS, Waters-USA. The analyses were carried out in negative mode. Dried partial acid hydrolysis products of fucoidans were dissolved in 1:1 MeOH–water and introduced into the mass spectrometer.

2.6. Anti-HIV activity assays

2.6.1. Cytotoxicity and viral infection assays

Protection of fucoidans on U373-CD4-CXCR4 cells infected by luciferase-tagged viral X4 pseudotype particles was evaluated. Pseudotype particles were produced by co-transfecting a luciferase-tagged pNL4-3-derived vector deleted of the env ectodomain, pNL4-3Δenv (Baatz et al., 2011), and a pCMV-derived HXB2 full env plasmid using lipofectamine (Invitrogen) into HEK293T cells. Viral supernatant produced by HEK293T cells was collected 48 h after transfection. P24 antigen concentration was measured using P24 ELISA kit (Perkin Elmer). U373-CD4-CXCR4 cells were infected with 200 pg of pNL4-3Δenv-HXB2 pseudotype particles. All infections were conducted in triplicate wells. Infection was synchronized by spinoculation at 1200 × *g* for 2 h at 25 °C and incubation for 1 h at 37 °C before supernatants were replaced with fresh medium. Serial dilutions of fucoidans were added during and after spinoculation. Infection was monitored after 48 h by measuring bioluminescence activity in the cell lysates (Promega luciferase assay system) using a Tecan Genios Pro Luminometer. Percentage of protection was expressed as the percentage of inhibition of the relative light units (RLUs) of U373-CD4-CXCR4 cells infected without fucoidans (100%) and in the presence of increased concentrations of fucoidans. IC_{50} values were calculated by non-linear regression using GraphPad Prism v5.01 (GraphPad Software). Statistical analyses were performed using GraphPad Prism v 5.01. Mean IC_{50} of fucoidans were compared using *t*-tests. A p value was considered significant when lower than 0.05.

Cell viability was assessed by flow cytometry using the LIVE/DEAD Fixable Dead Cell Stain Kit (Invitrogen) in U373-CD4-CXCR4 cells incubated with serial dilutions of fucoidans for 48 h according to manufacturer's protocol. Percentage of cell viability was expressed as the percentage of viable U373-CD4-CXCR4 cells without fucoidans (100%) and in the presence of increased concentrations of fucoidans.

To decipher if the antiviral activity was due to direct interactions of the compounds with either the virus, the cell membrane surface or the viral life cycle, pre-incubation of crude extracts with U373-CD4-CXCR4 cells or with the pNL4-3Δenv-HXB2 pseudotype particles as well as post-incubation of crude extracts with infected U373-CD4-CXCR4 cells were tested.

2.6.2. Cell lines

HEK293T (ATCC) cells were maintained in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% FBS, L-glutamine (2 mM), penicillin (50 U/mL) and streptomycin (50 μg/mL) (all from Gibco). U373-CD4-CXCR4 cells (AIDS Research and Reference Reagent Program, NIAID) were maintained in DMEM supplemented with 15% FBS, L-glutamine (2 mM), penicillin (100 U/mL), streptomycin (100 μg/mL), geneticin (300 ng/mL) (Gibco) and puromycin (1 ng/mL) (Sigma–Aldrich).

3. Results and discussion

Three fucoidans were extracted from three brown seaweeds *S. mcclurei*, *S. polycystum* and *T. ornate*. The yields, sulfate content and sugar composition of three crude fucoidans are given in Table 1. Like other marine fucoidans, these polysaccharides contain several neutral monosaccharide components, namely, fucose, mannose,

Table 1
Yield and chemical analysis of crude fucoidans.

Crude fucoidan	Yield (% dried seaweed)	Neutral sugar composition (mol %)					Sulfate content (mass %)
		Fuc	Man	Gal	Xyl	Glc	
F_{SP}	2.75	20.30	1.90	13.70	2.60	1.10	23.40
F_{TO}	2.50	30.30	Trace	25.60	Trace	Trace	25.60
F_{SM}	2.10	40.00	10.70	20.10	6.20	10.04	30.50

galactose, xylose and glucose with different molar ratios. F_{SP} and F_{TO} were mainly composed of fucose, a high proportion of galactose and very small amount of glucose, xylose and mannose. The highest fucose content was detected in F_{SM}.

Fucoidans were subjected to mild hydrolysis as described in experimental part to obtain fragments suitable for mass spectrometric investigation. Mass spectra may be used for determination of position of sulfate groups in fucose monosulfates (Tissot, Salpin, Martinez, Gaigeot, & Daniel, 2006) according to specific fragmentation patterns of molecular anions $[FucSO_3]^-$. Signal at *m*/*z* 183 indicates sulfate group at C-4, signal at *m*/*z* 139 corresponds to sulfate group at C-2, and signal at *m*/*z* 169 was assigned to sulfate group at C-3 of fucose monosulfates.

Mass spectra of partial hydrolysis products of all three fucoidans (data not shown) contain a signal at *m*/*z* 243 corresponding to the deprotonated molecule of monosulfated fucose $[FucSO_3]^-$. The MS^2 spectra of this daughter ion at *m*/*z* 243 of three fucoidans F_{SM}, F_{SP} and F_{TO} were shown in Figs. 1–3, respectively.

In Fig. 1, ESI–MS/MS of monosulfated fucose $[FucSO_3]^-$ at *m*/*z* 243 was similar to that observed for *Fucus evanescens* reported by Anastyuk, Shevchenko, Nazarenko, Dmitrenok, and Zvyagintseva (2009), where signals from fucose 4-sulfate (*m*/*z* 183) and fucose 2-sulfate (*m*/*z* 139) have approximately equal intensity. No evidence on sulfation at C-3 was obtained, since the corresponding fragment ion at *m*/*z* 169 was not observed. Hence, the partial hydrolyzate of

Table 2
Position and content of sulfate groups and anti-HIV activity of three crude fucoidans.

Crude fucoidan	Sulfate group position	Sulfate content (mass %)	Mean IC_{50}, μg/ml (SD)[a]
F_{SP}	C2 < C4	23.40	0.34 ± 0.12
F_{TO}	C2 > C4	25.60	0.39 ± 0.18
F_{SM}	C2 = C4	30.50	0.96 ± 0.59

[a] Mean IC_{50} was calculated from three independent experiments. SD, standard deviation.

F_{SM} contained approximately equal amounts of 2- and 4-sulfated fucose. In contrast, Fig. 2 shows that the ion at *m*/*z* 183 was the major fragment, and the high abundance of this fragment indicates that the fucosyl units of F_{SP} are mainly sulfated at position 4. In Fig. 3, signals from fucose 4-sulfate (*m*/*z* 183) and fucose 2-sulfate (*m*/*z* 139) were detected, but the ion at *m*/*z* 139 was the major fragment indicating that fucosyl units of F_{TO} are mainly sulfated at position 2. Similar result was reported for a fucoidan from *Laminaria cichorioides* (Anastyuk et al., 2010). Thus, according to mass-spectrometric evidence, all three fucoidans have sulfate groups attached to C-2 and C-4 of fucose residues, but in different proportions.

These data on the position of sulfate groups in three crude fucoidans were summarized in Table 2 together with their sulfate content and their anti-HIV activity (mean of IC_{50}).

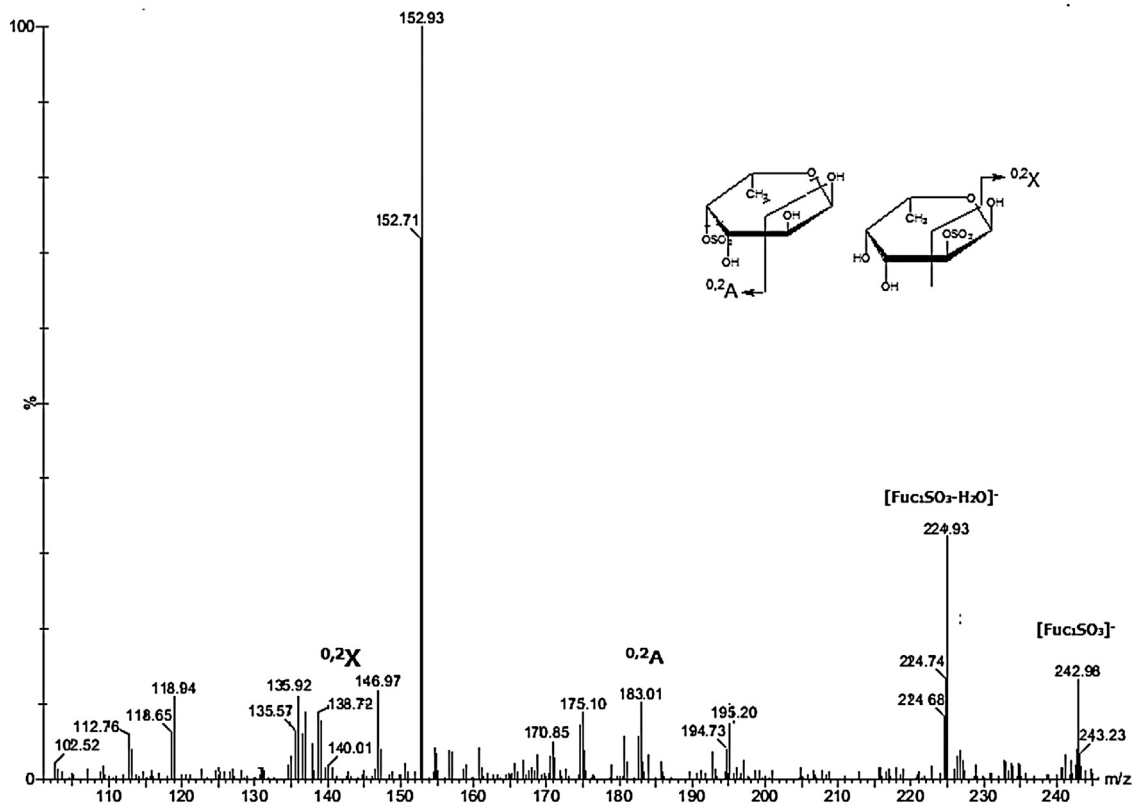

Fig. 1. Negative ESI–MS/MS spectra of the monosulfated fucose ion $[FucSO_3]^-$ at *m*/*z* 243 of F_{SM}.

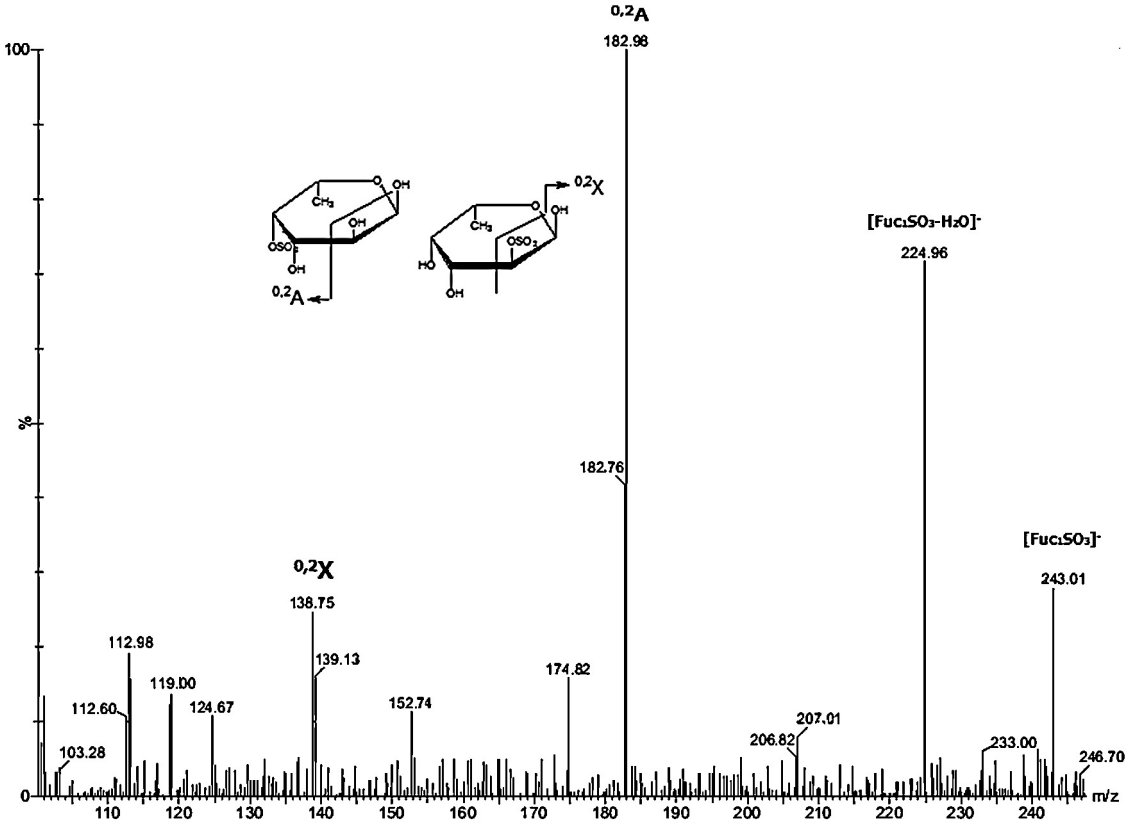

Fig. 2. Negative ESI–MS/MS spectra of the monosulfated fucose ion $[FucSO_3]^-$ at m/z 243 of F_{SP}.

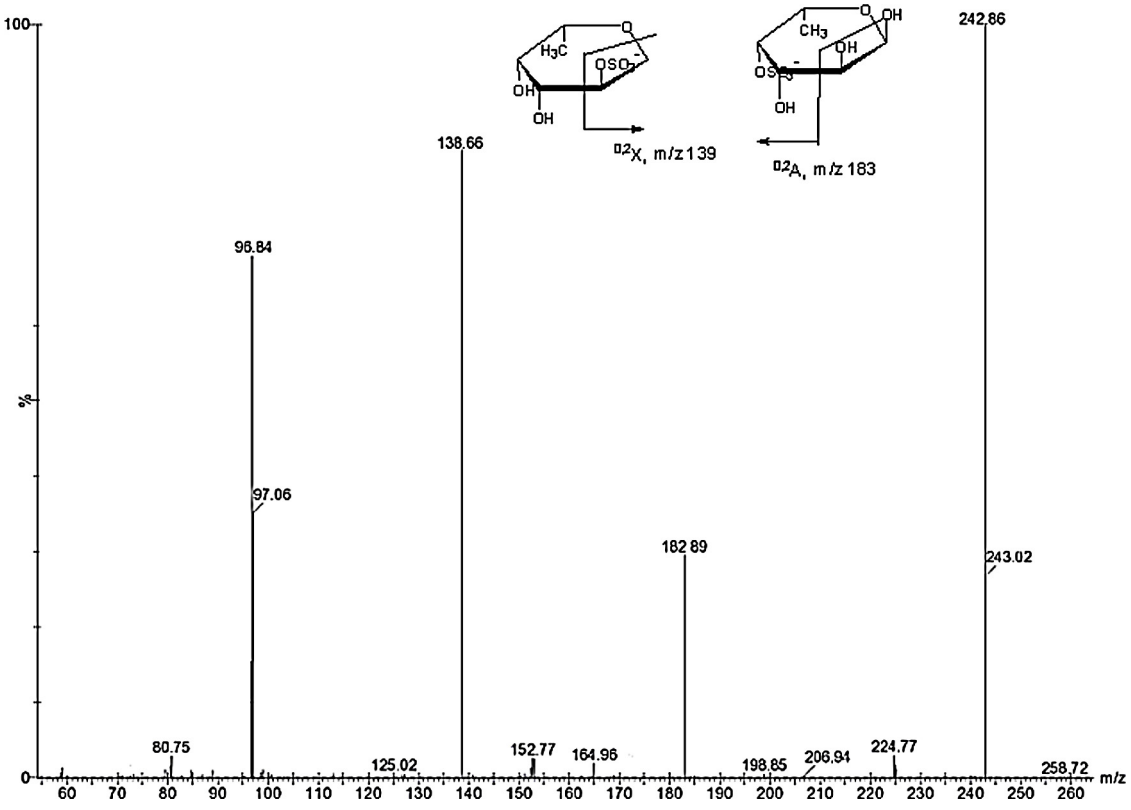

Fig. 3. Negative ESI–MS/MS spectra of the monosulfated fucose ion $[FucSO_3]^-$ at m/z 243 of F_{TO}.

Table 3
Yield and chemical analysis of fractions from F_{SM}.

Fraction (M NaCl)	Yield (% of F_{SM})	Monosaccharide composition (mol %)					SO_3Na (mass %)
		Fuc	Man	Gal	Xyl	Glc	
F_{SM1} (0.5)	8.5	30.4	21.7	20.7	9.20	18.0	17.60
F_{SM2} (1.0)	19.2	46.2	8.1	30.11	6.31	12.90	24.71
F_{SM3} (1.5)	28.9	60.5	Trace	38.40	Trace	0.70	33.03

Table 4
Yield and chemical analysis of fractions from F_{SP}.

Fraction (M NaCl)	Yield (% of F_{SP})	Monosaccharide composition, mol %					SO_3Na (mass %)
		Fuc	Man	Gal	Xyl	Glc	
F_{SP1} (0.5)	6.0	8.8	5.8	12.8	8.7	3.6	4.4
F_{SP2} (1.0)	18.0	12.7	3.3	20.3	5.8	1.5	19.3
F_{SP3} (1.5)	17.0	29.0	1.0	12.1	2.4	Trace	26.6
F_{SP4} (2.0)	17.0	34.4	–	21.1	Trace	–	27.4

The protection against HIV-1 was further estimated on U373-CD4-CXCR4 cells infected with pseudotype viral particles tagged with luciferase to measure HIV infection (Table 2; Fig. 4A) as well as cell viability in presence of fucoidans by flow cytometry (Fig. 4B). All fucoidans exhibited similar anti-HIV activity with a mean IC_{50} ranging from 0.33 to 0.7 μg/ml while displaying no cytotoxicity up to 2 μg/ml for F_{TO} and F_{SP} and 20 μg/ml for F_{SM}, respectively. The specific CXCR4 inhibitor AMD3100 showed an IC_{50} of 21.6 nM whereas the non-sulfated polysaccharide hydroxyethil starch had no antiviral activity.

Several authors reported that the sulfate content of sulfated polysaccharides is one of the most important factors for its biological effects (Haroun-Bouhedja, Ellouali, Sinquin, & Boisson-Vidal, 2000; Nishino & Nagumo, 1991). As shown in Table 2, the sulfate content was the highest in F_{SM} as compared to F_{SP} and F_{TO}, however their antiviral activity was not significantly different. In addition, the amount of 2- and 4-sulfated fucose observed in these fucoidans were all different suggesting that they do not play a role in the anti-HIV activity.

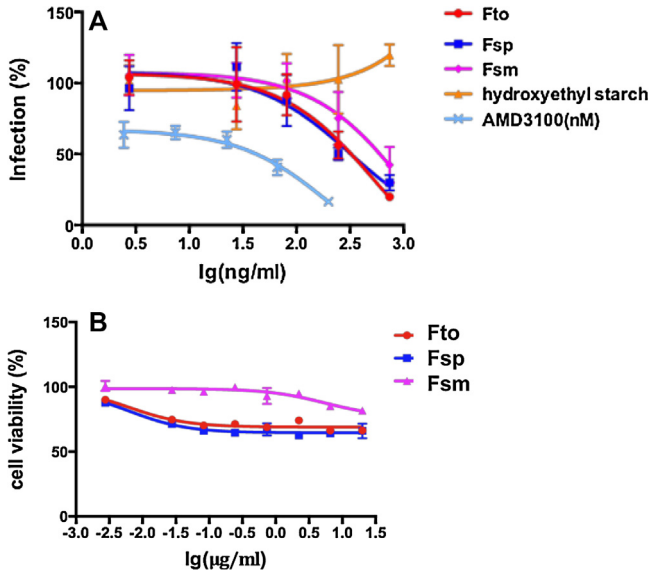

Fig. 4. (A) Infection of U373-CD4-CXCR4 cells by X4 pseudotype viral particles in presence of the fucoidans F_{SP}, F_{TO} and F_{SM}. One representative experiment of three is shown. Each concentration of the three crude fucoidans were tested in triplicates in each independent experiment. The CXCR4 inhibitor AMD3100 was used as a positive control and hydroxyethyl starch was used as a negative control as a non-sulfated polysaccharide. (B) Viability of U373-CD4-CXCR4 cells in the presence of the fucoidans F_{SP}, F_{TO} and F_{SM}. One representative experiment of two is shown. Each concentration of the three crude fucoidans were tested in duplicates in each independent experiment.

To further investigate this relationship crude F_{SM} and F_{SP} fucoidans (containing respectively the highest and lowest content of sulfate) were fractionated into three (F_{SM1}, F_{SM2}, F_{SM3}) and four fractions (F_{SP1}, F_{SP2}, F_{SP3}, F_{SP4}) by ion-exchange chromatography on DEAE-Sephadex column using aqueous sodium chloride of increasing concentration as eluant. The chemical analysis of the fractions was reported in Tables 3 and 4. All fractions were examined for their anti-HIV activity, and the content and position of sulfate groups were determined. The results were summarized in Tables 5 and 6.

Although the fraction F_{SM3} was containing the highest content of sulfate (comparable to the sulfate content of F_{SM}), they did not exhibit a significantly higher antiviral activity as compared to F_{SM1} or F_{SM2}.

The fraction F_{SP1}, having a very low sulfate content, did not show any antiviral activity. Nevertheless, the derived fractions F_{SP2}, F_{SP3} and F_{SP4} showed a similar antiviral activity comparable to F_{SP}. Despite the purification of fucoidans by ion-exchange chromatography, the antiviral activity was not improved and remains similar between all fractions. These data do not support any role of the sulfate content on the anti-HIV activity of fucoidans but rather the role of a specific fucoidan that might have been lost during the purification of F_{SP1} and shared between all other fractions. The direct dependence of biological activity on the position of sulfate groups was also not observed.

Table 5
Position and content of sulfate groups and anti-HIV activity of three fractions of F_{SM}.

Fractions	Sulfate group position	Sulfate content (mass %)	Mean IC_{50}, μg/ml (SD)[a]
F_{SM1}	Mainly on C4	17.60	0.75 (±0.6)
F_{SM2}	Mainly on C4	24.71	0.39 (±0.27)
F_{SM3}	C2 > > C4	33.03	0.18 (±0.16)

[a] Mean IC_{50} was calculated from three independent experiments. SD, standard deviation.

Table 6
Position and content of sulfate groups and anti-HIV activity of four fractions of F_{SP}.

Fractions	Sulfate group position	Sulfate content (mass %)	Mean IC_{50} (μg/ml) (SD)[a]
F_{SP1}	Mainly on C4	4.4	NA
F_{SP2}	Mainly on C4	19.3	0.37 (±0.17)
F_{SP3}	C2 > C4	26.6	0.23 (±0.4)
F_{SP4}	C2 > C4	27.4	0.15 (±0.12)

[a] Mean IC_{50} was calculated from three independent experiments. SD, standard deviation.

Finally, to investigate the antiviral mechanisms of fucoidans on HIV-1, F_{SP} were incubated 2 h before infection either with U373-CD4-CXCR4 cells or with viral pseudotype particles and 2 h post-infection. Fucoidans inhibited HIV-1 infection when they were pre-incubated with the virus (mean $IC_{50} = 0.38 \pm 0.14$ μg/ml) but not with the cells and not at the time of post-infection. These data indicate that fucoidans bind to the virus and inhibit HIV entry into target cells.

4. Conclusion

We have demonstrated that fucoidans isolated from the three species *S. mcclurei*, *S. polycystum* and *T. ornata* have significant anti-HIV activity. Although highly sulfated preparation of crude extracts was obtained by anion-exchange chromatography, they did not demonstrate any increased anti-HIV activity. Neither sulfate content nor position of sulfate groups was related to the anti-HIV activity of fucoidans suggesting the involvement of other structural parameters like molecular weight, the type of glycosidic linkage or even a unique fucoidan sequence. Although the presence of sulfo groups seems to be necessary for the anti-HIV activity (Schaeffer & Krylov, 2000), our data do not support random sulfation as the main antiviral factor. Fucoidans are structurally complex, known for their antiviral activities due to their direct interactions with either enveloped viruses or with cell membrane surface (Ghosh et al., 2009). In this study we showed that the antiviral activity of these fucoidans is due to their binding with HIV-1 blocking the early steps of HIV entry. These macromolecules might exert their anti-HIV-1 activity by shielding off the positively charged amino acids present in the viral envelope glycoprotein gp120 (Moulard et al., 2000) or by the strong binding with a specific sulfation motif (de Parseval et al., 2005). This former hypothesis is in agreement with the specific interactions of sulfated polysaccharides with proteins leading to their biological activities such as the binding of heparin with antithrombin III or chondroitin 4 sulfate with plasmodium falciparum or the lyme disease spirochete (Andrews, Klatt, Adams, Mischnick, & Schwartz-Albiez, 2005; Mulloy, 2005).

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