Sulfated polysaccharides of seagrass *Halophila ovalis* suppresses tumor necrosis factor-α-induced chemokine interleukin-8 secretion in HT-29 cell line

Neelakandan Yuvaraj, Venkatesan Arul

**Abstract:**

OBJECTIVES: The present study aims to investigate the anti-oxidant and anti-inflammatory properties of seagrass *Halophila ovalis* sulfated polysaccharide on HT-29 cell line.

SUBJECTS AND METHODS: Monosaccharides composition was identified using liquid chromatography-mass spectrometry (LC-MS) and the functional groups were analyzed using Fourier transform-infrared (FT-IR) spectroscopy. The antioxidant and anti-inflammatory potential of crude extract and purified fractions was investigated in vitro.

RESULTS: FT-IR spectra revealed that the presence of different functional groups and the presence of galactose (82.4%), xylose (7.6%), fructose (4.0%), mannose (2.0%), fucose (1.6%), glucose (1.2%), and arabinose (1.0%) was observed using LC-MS. Ho-SP and its fractions showed radical scavenging activity in hydroxyl, 2-azinobis-3-ethylbenzothiazoline-6-sulfonic acid, and ferric reducing antioxidant power assay in a dose-dependent manner. Noticeable anti-inflammatory activity of purified fraction Ho FrIV (IC_{50} = 43.85 µg/ml) was observed in a non-cytotoxic range of concentrations and inhibited the tumor necrosis factor-α (TNF-α)-induced interleukin-8 (IL-8) secretion (0.27 ng/ml) in HT-29 cell line.

CONCLUSION: Overall, the results presented in this study suggest that purified fraction Ho FrIV of Ho-SP could suppress the TNF-α-induced secretion of IL-8 in HT-29 and thus could be used as a promising antioxidant and anti-inflammatory candidate with potential benefits.

Keywords: Anti-inflammatory, antioxidant, *Halophila ovalis*, HT-29, liquid chromatography-mass spectrometry

**Introduction**

Reactive oxygen species (ROS) and free radicals have attracted increasing attention over the past decade. Under pathological conditions, excessive generation of ROS initiates biomolecular oxidations and creates oxidative stress. This linked with the pathogenesis of many diseases such as stroke, diabetes, cancer, myocardial infarction, septic and hemorrhagic shock, and Alzheimer’s and Parkinson’s diseases.[1] Anti-oxidant enzymes and supplements may mitigate the negative effects of oxidative damage in the major signaling pathways of cells.[2]

Aberrant production of pro-inflammatory factors such as chemokines often results in chronic inflammation, and one such chemokine is interleukin-8 (IL-8).[3] The release of these cytokines results in the development of many inflammatory diseases such as rheumatoid arthritis, and inflammatory bowel disease. In addition, IL-8 function as a significant regulatory factor within the tumor microenvironments.[4] Hence, abrogation of IL-8 activity represents...
Yuvaraj and Arul: Anti-oxidant and anti-inflammatory potential of Halophila ovalis

Seagrasses play an important role in marine ecosystems and human livelihoods. The global distributions of seagrasses have been explored in the past decade and are documented for a wide range of biological activities, such as antibacterial, antioxidant, and anti-inflammatory activities. Sulfated polysaccharides received extensive attention due to their intriguing bioactive properties. The high amount of sulfated polysaccharides in seagrasses documented for the first time in contrast with the terrestrial and freshwater species. Determination of monomeric constituents, molecular size, sulfation site, and structural motif has an impact on functional properties with special attention toward potential pharmacological applications. Till date, the sulfated polysaccharide of Ruppia maritima was purified, and its structure was characterized.

In our previous study, we reported the physicochemical characteristics and existence of the antinociceptive and anti-inflammatory activities of the sulfated polysaccharide fractions extracted from seagrass H. ovalis in vivo. The present study was undertaken to highlight for the first time the antioxidant, cytotoxic effects on peripheral blood mononuclear cells (PBMCs), and cytokine IL-8 secretion on HT-29 cell lines in vitro.

Subjects and Methods

Materials
Human colon cancer cell line HT-29 was procured from the National Centre for Cell Sciences, Pune, India. The Dulbecco’s modified Eagle’s medium, Roswell Park Memorial Institute 1640 medium (RPMI 1640 medium), trypan blue, fetal bovine serum (FBS), 3-(4, 5-Dimethylthiazol-2-thiazolyl)-2, and 5-diphenyl-2H-tetrazolium bromide (MTT) were purchased from Gibco BRL, CA, USA. Penicillin G (100 U/ml) plus streptomycin (100 µg/ml) was supplied by Invitrogen, CA, USA. 2, 2’azinobis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) diammonium salt, histopaque-1077, phytotrehemagglutinin (PHA), standard sugars, and Vitamin C were purchased from Sigma Chemical Co. IL-8-specific enzyme-linked immunosorbent assay (ELISA) kit was purchased from BioLegend’s (San Diego, USA).

Extraction of sulfated polysaccharide
Seagrass H. ovalis was collected at low tide from Chunnambur estuary (Puducherry), India, during September 2016. Plant specimen was authenticated by Prof. N. Parthasarathy, Salim Ali School of Ecology, Pondicherry University. The methodology used for the sequential extraction and purification of polysaccharide was adapted according to our previous study described earlier. The extraction mode includes three main steps – depigmentation, precipitation, and extraction of the polysaccharide with hot water. Purification was achieved in a gradient manner using sodium chloride as an eluant.

Monosaccharide composition analysis
The monosaccharide composition analysis was carried out using liquid chromatography-mass spectrometry (LC-MS) as described earlier. Briefly, an individual stock solution of each monosaccharide was prepared at a concentration of 1 mg/ml with deionized water. Working standards were prepared from the stock solution with acetonitrile/water (80:20 v/v) by appropriate dilution. For sugar analysis, 10 mg of crude sample was dissolved in 4 M trifluoroacetic acid and hydrolyzed at 120°C for 5 h. The resulting solution was concentrated in vacuo, and the excess acid was removed by repeated co-distillation with anhydrous ethanol. The solution was reconstituted in 1 ml of acetonitrile/water (80:20 v/v) for monosaccharide composition analysis.

LC separation was performed on an Agilent system using SeQuant ZIC-pHILIC column (4.6 mm × 150 mm, 5 µm, Merck, Germany) at room temperature. Sample and standards (5 µl) were injected with Agilent auto-sampler and analyzed with 3200 Trap MS/MS system with the flow rate of 400 µl/min. The mobile phase was composed of 0.1% formic acid in acetonitrile: Methanol (70:10:20, v/v). The infrared spectral analysis of crude galactan sulfate was carried on Fourier transform infrared (FT-IR) spectrophotometer (Thermo Nicolet Model: 6700). Briefly, the galactan sulfate was ground with potassium bromide powder and then pressed into a polymer film for FT-IR measurement in the frequency range of 4000-400/cm.

Antioxidant assays
Scavenging ability on hydroxyl radicals
The scavenging ability of purified galactan sulfate on hydroxyl radicals was determined as described earlier. The absorbance at 624 nm of aqueous solutions of 0.435-mM brilliant green (BG), 0.25-mM solution of FeSO₄, and varying concentrations of purified fractions (0.5–2.5 mg/ml) were measured as time function immediately after the addition of H₂O₂. The scavenging effect was expressed as ΔAbs/(ΔAbs)_{initial} × 100, where ΔAbs is the difference between the absorbance of BG in the presence and in the absence of polysaccharide,
measured at 30 min reaction time. \( (\text{Abs})_{\text{initial}} \) is the initial absorbance in the absence of the polysaccharide.

**Scavenging ability on 2-azinobis-3-ethylbenzothiazoline-6-sulfonic acid radicals**

The radical scavenging activity of purified fractions of *H. ovalis* was measured by ABTS radical cation (ABTS\(^+\)) test as described previously with slight modifications.\(^1\) ABTS\(^+\) was produced by reacting 7 mM of ABTS solution with 2.45 mM of potassium persulfate, and the mixture kept in the dark for 16 h at room temperature. In the moment of use, the ABTS\(^+\) solution was diluted with ethanol to an absorbance of 0.70 ± 0.02 at 734 nm. Each sample with various concentrations (0.5–2.5 mg/ml) was added to 2 ml of ABTS\(^+\) solution and mixed vigorously. After reaction at room temperature for 6 min, the absorbance at 734 nm was measured. The ABTS\(^+\) scavenging effect was calculated by the following formula: ABTS\(^+\) scavenging effect (\%) = \( A_{1} - A_{s}/A_{1} \times 100 \), where \( A_{1} \) was the absorbance of the control and \( A_{s} \) was the absorbance of the test sample.

**Ferric-reducing antioxidant power assay**

Total reducing capacity of purified fractions of *H. ovalis* was determined as described earlier.\(^2\) The polysaccharide solution of 1 ml at different concentrations (0.5–2.5 mg/ml) was mixed with 2.5 ml of sodium phosphate buffer (0.2 M, pH 6.6) and 2.5 ml of 1% potassium ferricyanide. The reaction mixtures were incubated at 50°C for 20 min followed by the addition of 2.5 ml of 10% trichloroacetic acid to stop the reaction. The reaction mixtures were then centrifuged at 1000 \( \times \) g for 10 min. The upper layer of solution (2.5 ml) was mixed with distilled water (2.5 ml), 0.1% FeCl\(_3\) (1.5 ml) and the absorbance was measured at 700 nm. An increase in absorbance results increasing in reducing power.

**Anti-inflammatory assay**

The anti-inflammatory assay on PBMCs was carried out as described earlier.\(^3\) Briefly, PBMCs (2 × 10\(^6\) cells/well) in a volume of 200 µl in RPMI-1640 medium containing 10% FBS and 1 µg/ml of PHA was seeded in a 96-well U bottom plate followed by the addition of purified fractions at various concentrations. The culture was incubated at 37°C for 24 h in a CO\(_2\) incubator containing 5% CO\(_2\) and 90% humidity. Assays were conducted in triplicate for each concentration. Experimental data represent the mean ± standard deviation (SD) of each compound unless otherwise stated.

**Cell viability assay**

The cytotoxicity of the purified fractions Ho FrIII and Ho FrIV to PBMCs was evaluated using calorimetric MTT assay with slight modifications. Briefly, the cells (1 × 10\(^5\) cells/ml) were seeded in a 24-well plate and incubated for 24 h. The cells were then treated with Ho FrIII and Ho FrIV (1, 10, 50, 100 µg/ml) for 24 h. Triton X was used as negative control and the deionized water as a solvent control. Following the removal of medium from the wells, 10 µl of MTT (5 mg/ml resuspended in PBS) was added to each well. The floating cells were removed after 4 h of incubation at 37°C. A volume of 50 µl of dimethyl sulfoxide was added to each well to lyse the cells and the absorbance was measured at 570 nm. Finally, the percentage of cell viability was calculated using the formula

\[
\text{Cell viability} (%) = \left( \frac{\text{Absorbance of test sample}}{\text{Absorbance of control}} \right) \times 100.
\]

**Measurement of interleukin-8 cytokine level**

Human colon carcinoma cell line HT-29 (1 × 10\(^6\) cells/well) was induced by tumor necrosis factor-\( \alpha \) (TNF-\( \alpha \)) (1 µg/ml). After 2 h of incubation, cells were treated with Ho FrIV (1, 10, 50, and 100 µg/ml) for 24 h. Determination of IL-8 release in the culture supernatant was performed in triplicate using an IL-8-specific ELISA system (Biolegend), according to the manufacturer’s instructions.

**Statistical analysis**

All data were expressed as mean ± SD. Statistical significant differences between the experimental groups were determined by one-way ANOVA, and differences were considered to be statistically significant if \( P < 0.05 \). Tukey’s post hoc test was performed for multiple group comparison between concentrations. All computations were done by employing statistical software (SPSS Version 7.5).

**Results**

**Chemical composition analysis**

The findings from the chemical characterization analysis presented in our earlier study revealed that Ho-SP consisted mainly of sulfate (21%), total sugars (74.9%), and protein (3.4%) on dry weight. The monomeric composition was demonstrated by (LC-MS) analysis. The result revealed that it is a heteropolysaccharide composed of relatively higher galactose content (82.4%) followed by xylose (7.6%), fructose (4.0%), mannose (2.0%), fucose (1.6%), glucose (1.2%), and arabinose (1.0%) at various retention times as compared with standard.

**Infrared spectra analysis**

The FT-IR spectrum of *H. ovalis* galactan sulfate is depicted in Figure 1. The broad stretching intense characteristic peak at 3420/cm indicated the presence of hydroxyl group and C–H stretching at 2920/cm, respectively. The presence of O-acetyl group was indicated by vibration at 1720/cm. A stronger signal at 1620/cm was attributed to the asymmetric stretch vibrations of COO – group and at
1420/cm was due to the symmetric stretch vibrations of COO − and the stretch vibration of C–O within—COOH. The absorption at 1254/cm common to all sulfate esters and an additional sulfate absorption band at 823/cm (C–O–S, secondary equatorial sulfate) indicated that majority of sulfate groups are located at positions 2 and/or 3. Signals at 1050/cm correspond to stretching vibrations of C–O, respectively.

**Antioxidant assays**

**Hydroxyl radical assay**

The hydroxyl radical generated through the Fenton reaction in this system was scavenged by *H. ovalis* polysaccharide. The scavenging ability of purified fractions Ho FrI, Ho FrII, Ho FrIII, and Ho FrIV was portrayed [Figure 2]. The results indicated that the scavenging ability of test samples and vitamin C increased in a concentration-dependent manner. As shown in Figure 2, the purified fraction Ho FrIV exhibited significant hydroxyl scavenging effect (*P* < 0.05) between concentrations and IC50 was observed at 1.04 mg/ml. In fact, the scavenging effect was superior to those of the commercial counterpart such as Vitamin C at every concentration point. However, the hydroxyl scavenging ability of other three fractions Ho FrI, Ho FrII, and Ho FrIII were statistically not significant (*P* > 0.05) and inferior to ascorbic acid at all concentration points.

**Scavenging ability on 2-azinobis-3-ethylbenzothiazoline-6-sulfonic acid radicals**

The anti-oxidant capacity of *H. ovalis* sulfated polysaccharides was measured using an assay based on electron transfer. In this study, ABTS+ cation radical was employed as an oxidant. The scavenging ability of purified fractions Ho FrI, Ho FrII, Ho FrIII, and Ho FrIV on ABTS radicals was portrayed [Figure 3]. Purified fraction Ho FrIV showed significant ABTS radicals scavenging activity (*P* < 0.05) and IC50 was observed at 0.55 mg/ml. Whereas, purified fraction Ho FrIII scavenged ABTS radicals at IC50 of 2 mg/ml, respectively. The results indicated that purified fraction Ho FrIV has stronger scavenging ability on ABTS radicals than the commercial counterpart. However, the other three purified fractions were statistically not significant (*P* > 0.05) and exhibited less scavenging effect than Vitamin C at every concentration point.

**Reducing power assay**

The reducing power of purified fractions Ho FrI, Ho FrII, Ho FrIII, and Ho FrIV was found to be increasing in concentration-dependent [Figure 4]. Indeed, the reducing power of purified fraction Ho FrIV was found to be close to the standard ascorbic acid at every concentration point and exceeded at 2.5 mg/ml than the commercial counterpart. However, the reducing power of other purified fractions HoFrI, Ho FrII, and Ho FrIII was
inferior to that of positive control at all the concentration point. The reducing capacity of purified fractions was not statistically significant \((P > 0.05)\) as compared to the commercial counterpart. However, both the test sample and standard showed the significant difference between the concentrations \((P < 0.05)\). This property was correlated with the presence of reductones, which were reported to be terminators of free radical chain reaction by donating a hydrogen atom.

**Anti-inflammatory assay**

**Mitogen-induced lymphocyte proliferation**

The anti-inflammatory effect of purified fractions Ho FrI, Ho FrII, Ho FrIII, and Ho FrIV on the inhibition of mitogen-induced PBMCs proliferation was studied preliminarily and the results were shown [Figure 5a]. Purified fractions Ho FrIV and Ho FrIII inhibited the proliferation of PBMCs \((IC_{50})\) at 43.85 and 61.28 \(\mu g/ml\). The trypan blue exclusion test revealed that purified fractions Ho FrIV and Ho FrIII have noncytotoxic effect at tested concentrations [Figure 5b]. No microscopically visible alteration of normal cells morphology and destruction of cell layer were observed (data not shown).

**Effect of galactan sulfate on interleukin-8 secretion**

IL-8 is a sensitive marker in the early stages of inflammation and infection. The effect of purified fraction Ho FrIV on TNF-\(\alpha\)-induced epithelial IL-8 secretion was analyzed in the HT-29 colonic epithelial cell line [Figure 6]. The increase in IL-8 secretion was observed in TNF-\(\alpha\) treated HT-29 cells. Whereas, purified fraction Ho FrIV significantly reduced the IL-8 secretion in a concentration-dependent manner.

**Discussion**

In recent decades, screening of marine resources has been the focus of natural-product chemists to develop new drugs and dietetic foods.[8] Several reports have shown that chemical components and molecular weight of biopolymer are important factors for its biological properties.[11] The presence of novel sulfated galactan from seagrass *R. maritima* L. (Ruppiaceae) has been documented recently.[12] Similarly, we reported the existence of low-molecular-weight sulfated galactan from seagrass *H. ovalis* and characterized its structural and functional properties.[13] The monosaccharide composition analysis of sulfated galactans revealed the presence of galactose, xylose, fructose, mannose, fucose, glucose, and arabinose. Similarly, in seagrass *Cymodocea nodosa*, galactose and mannose were found to be the major sugar components.[8] Whereas, *H. wrightii* contains glucose, galactose, and xylose as major sugars. The presence of individual monosaccharide varies in their composition according to the species that can be related to its biological and pharmaceutical potential. For the first time, we are reporting the presence of fucose in *H. ovalis* sulfated galactans, which is a unique characteristic feature of sulfated fucans.[8] A more plausible explanation for the presence of fucose in the sulfated galactans is that evolutionary distant organisms that share the marine environment is a convergent adaptation due to environmental selective pressure.[10]
The FT-IR spectrum showed that the major absorption bands were observed at 3429/cm (O–H stretching), 1049/cm (hemiacetal stretching), 1254/cm (S = O asymmetric stretching), and band at 823/cm could be assigned to the sulfate group in the axial position of the C-6 of galactose. In addition to that the presence of O–acetyl group was indicated by vibration at 1720/cm which is a characteristic feature of sulfated fucans. A stronger signal at 1620/cm was attributed to the asymmetric stretch vibrations of COO – group and at 1420/cm was due to the symmetric stretch vibrations of COO – and the stretch vibration of C–O within–COOH. The present result showed that algae and marine angiosperms have a tendency to present sulfate groups in the C-2 or C-6.

Seagrasses are considered as a new source of natural antioxidants and reported to be an effective, nontoxic antioxidant candidate in recent years. Therefore, the purified sulfated galactan fractions were evaluated in a comprehensive manner employing a variety of in vitro methods as free radical scavengers for the prevention of oxidative damage in living organisms. Among the most used antioxidant measurement activity of polysaccharides, hydroxyl radicals are those based on the competitive reaction with a probe, which scavenges these radicals. For hydroxyl radical, there were two types of anti-oxidation mechanism; one suppresses the generation of the hydroxyl radical, and the other scavenges the hydroxyl radicals generated. Metal ion-induced hydroxyl radical generation was monitored by the decrease of the probe absorbance due to the reaction with radicals. The present study revealed that purified fraction Ho FrIV exhibited stronger hydroxyl scavenging potential than commercial standard Vitamin C and other fractions. Whereas, the sulfated polysaccharides extracted from seagrass H. wrightii and brown seaweed Dictyopteris delicatula J. V. Lamour (Dictyotaceae) showed relatively less scavenging activity on hydroxyl radicals.

ABTS is an electron transfer based antioxidant assay. Therefore, change in the ABTS** absorbance at 1 min in the presence of seagrass sulfated galactan at different concentrations indicated the antioxidant potential. This finding suggests that the chemical structure of the polysaccharides plays some role in the H abstraction reaction by the ABTS** cation radical as reported earlier. The present findings showed that purified sulfated galactan Ho FrIV scavenged the ABTS radicals (IC50 0.55 mg/ml). Similarly, C. nodosa sulfated polysaccharides exhibited ABTS radical scavenging activity (IC50 1.14 mg/ml) than the commercial counterpart.

The reducing capacity of Ho-SP would result in the reduction of Fe3+/ferricyanide complex to the ferrous form (Fe2+). The reducing power of galactan sulfate is attributed to the yellow color of the test solution changes into various shades of green and blue colors. Several reports revealed that the structure of compounds containing more than one of the functional groups, OH,‑SH,‑COOH,‑PO...−S−, and −O−, is in favor of chelating ability.

PBMC is considered to be the best in vitro model for studying the immunomodulatory and anti-inflammatory properties. The present study revealed that sulfated galactans exert anti-inflammatory properties in human PBMC. It has been reported that biological activities are related to the molecular weight and sulfate content of the polysaccharides. Interestingly, we found higher sulfate content (15.15 µg/mg) of dried tissue. However, the sulfate content was found to be less in other seagrass species Halophila decipiens Ostenf (Hydrocharitaceae), R. maritima and H. wrightii. It has been reported that sulfated polysaccharide from brown seaweed Sargassum filipendula C. Agardh was most active with cell proliferation inhibition at 0.1 mg/ml. However, we reported that purified fractions Ho FrIII and Ho FrIV of H. ovalis sulfated polysaccharides inhibited the PBMC proliferation at IC50 of 0.06 mg/ml and 0.04 mg/ml. The bioactivities of seaweed polysaccharides are closely related to several structural parameters, such as sulfate groups, molecular weight, and types of sugar.

It is conceivable that the cytokines and chemokines act as promoters of carcinogenesis, for example, they may activate cancer cell proliferation, inhibit cancer cell apoptosis, or act as immunosuppressors, or angiogenic factor. IL-8 functions as an autocrine growth factor in hepatoma and that IL-8 is a well-known angiogenic factor.

Figure 6: Dose-dependent effect of purified fraction Ho FrIV on tumor necrosis factor-α-induced interleukin-8 secretion in colonic epithelial cell line HT-29. Each datum represents mean ± standard deviation of three determinations. *P < 0.05, significantly different from the control value. Blank, none (phosphate-buffered saline).

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It was reported that the expression of chemokines could be increased by cross-linking of Fas receptors in several cell types. The secretion of IL-8 found to be induced in the colon cancer cell line by Fas receptor ligation and increased expression of IL-8, macrophage inflammatory protein (MIP) MIP-1, and MIP-2. Recent studies revealed that mollugin, a Chinese herb inhibited TNF-α-induced inflammatory responses and chemotaxis in HT-29 cells through inhibition of nuclear factor-Kappa B (NF-Kb) activation and decreased monocyte chemotactic protein-1, IL-8, and ICAM-1 expression. The naturally occurring sulfated polysaccharides have the potential to bind to a variety of physiologically important proteins and thereby harbor an intrinsic risk of inducing off-target effects and cytotoxicity. The present study interestingly showed that galactan sulfate lacked cytotoxic effects and IL-8 secretion was greatly reduced in concentration-dependent manner in TNF-α-induced HT-29 cell line. Similarly, galactans from red seaweeds lacked cytotoxic effects and showed a broad spectrum of antiviral activity against herpes simplex virus-1 (HSV) and HSV-2. Further study is needed to find out whether the sulfated polysaccharides decreases IL-8 secretion through NF-kB activation and/or by blocking the cross-linking of chemokines with Fas receptors.

Conclusion

The present findings suggest that galactan sulfate extracted from seagrass Halophila ovalis, which has an apparent molecular weight of 20 KDa possessed good antioxidant and anti-inflammatory activity in vitro and decreased the chemokine IL-8 secretion. Accordingly, further work is currently underway in our laboratories to investigate its mode and mechanisms of action.

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Nil.

Conflicts of interest

There are no conflicts of interest.

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