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Sulfated polysaccharides of Green Algae: Chemical Composition and Antioxidant Activity *in vitro* of Fractionated Ulvans from *Ulva lactuca* and *Ulva papenfussii*

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ABSTRACT

The study evaluated the chemical composition, molecular weight, and antioxidant activity of ulvan extracted from *Ulva papenfussii* and *Ulva lactuca*. The results revealed distinct differences in the monosaccharide composition, sulfate content, and uronic acid levels between ulvans from both species, with ulvan from *U. papenfussii* exhibiting higher levels of iduronic acid and sulfate (16.39 ± 0.45 %w/w and 28.46 + 3.63 %w/w). Ulvan was fractionated using anion exchange chromatography (DEAE) based on charge differences, yielding three main fractions from each species. This study is the first to analyze different ulvan fractions from Vietnamese green algae using the anion exchange chromatography method. The antioxidant activity of the ulvan fractions was determined by their ability to DPPH radical scavenging activity, reducing power activity, and antioxidant activity, with UL2 (29.5 ± 0.94 Sc%) and UP2 (25.9 ± 1.77 Sc%) demonstrating the most prominent results. These active fractions were characterized by higher sulfate and uronic acid content, suggesting a potential structure–activity relationship. These findings highlight the potential of ulvan in the food and pharmaceutical industries.

Key words: Fractionated ulvans, chemical ccomposition, antioxidant activity, *Ulva papenfussii*, *Ulva lactuca*.

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INTRODUCTION

Ulvan, a sulfated polysaccharide found abundantly in the cell walls of green algae, is composed primarily of monosaccharides such as rhamnose, iduronic acid, xylose, and glucuronic acid, with smaller amounts of galactose [1]. The monosaccharide composition profile and sulfation level of ulvan are influenced by various factors, including the type of algae species, the season during which it is harvested, environmental conditions, and the methods used for extraction [1].

Following extraction, ulvan is purified from the crude mixture using various methods. Techniques such as alcohol precipitation, salting, membrane filtration, and chromatography are common, offering unique benefits and limitations [2]. The choice of purification method depends on specific experimental requirements and the the researcher's expertise. Ensuring that the purified ulvan is devoid of contaminants is crucial, as confirmed by characteristic analytical results. High purity and accurate structural characterization of ulvan are essential to assessing its potential effects in bioactivity studies [2].

Ion exchange chromatography is a separation technique based on the differential affinity of ions and polar molecules for a charged ion exchange medium. There are two main types: anion exchange, which captures negatively charged molecules, and cation exchange, which binds positive ones. For ulvan, a negatively charged polysaccharide (sulfate groups), anion exchange chromatography (AEC) is preferred. This process employs a positively charged stationary phase that selectively attracts and binds ulvan molecules under conditions where the pH of the solution is greater than the isoelectric of the molecule point (pH > pI). Polysaccharides with more negatively charged groups typically exhibit greater binding to the ion exchange media [3]. Ion exchange chromatography is highly efficient for purifying polysaccharides, offering advantages in resolution, capacity, automation, and cost-effectiveness, making it a common choice in laboratory and industrial settings [3]. Given that many ulvan extracts contain substantial impurities, chromatography could not be more widely adopted as an initial purification step prior to an in-depth analysis of the structural and biological properties of ulvan. The polyanionic, heterogeneous characteristics of ulvan make it especially well-suited to AEC and size-exclusion chromatography (SEC). AEC is particularly effective in eliminating protein impurities and neutral polysaccharides. Both weak anion exchangers, such as diethylaminoethyl (DEAE) [4, 5], and strong exchangers, such as quaternary amine-functionalized resins [6], have been successfully utilized to achieve high-purity ulvan preparations.

The growing interest in natural bioactive compounds has led to a rising demand for antioxidants, particularly in the food and pharmaceutical industries, which prioritize renewable resources. Research indicates that sulfated polysaccharides extracted from green algae exhibit significant antioxidant activity in vitro, underscoring their potential as valuable natural antioxidants for diverse applications [7–9].

The antioxidant capacity of ulvan has been widely investigated using various in vitro assays, such as DPPH (1,1-diphenyl-2-picryl hydrazil) radical scavenging, superoxide anion scavenging, ferric reducing antioxidant power, hydroxyl radical scavenging, and lipid peroxidation inhibition [10]. These sulfated polysaccharides' sulfate content and molecular weight of significantly influence their antioxidant effectiveness [11, 12]. The study of Qi has suggested that ulvan extracted from *Ulva pertusa* with a high sulfate content (32.8% w/w) exhibited nearly double the hydroxyl radical scavenging activity compared to its lower-sulfated counterpart (19.5% w/w) at the same concentration [12]. Similarly, over-sulfated ulvan from *Enteromorpha linza* has demonstrated enhanced radical scavenging efficiency compared to its native form [13]. Additionally, fractions of ulvan with reduced molecular weights generally show greater hydroxyl radical scavenging activity, with one study observing an inverse correlation between molecular weight (ranging from 18.2 to 100.5 kDa) and antioxidant performance (50 to 90%) [14]. However, some research suggests that sulfated polysaccharides

with high molecular weights from *Enteromorpha prolifera* show stronger superoxide radical scavenging activity [8]. These findings underscore the importance of standardizing extraction, purification, and testing procedures to establish a reliable structure-activity relationship for the antioxidant effects of ulvan, as this relationship may not follow a universal pattern [15].

Exploring marine sulfated polysaccharides in Southeast Asia has received growing attention due to their diverse biological activities and rich algal biodiversity. Among them, ulvan extracted from green macroalgae of the *Ulva* and *Enteromorpha* genera has demonstrated promising antioxidant, anticoagulant, and anticancer potentials [16, 17]. Despite this, research on ulvan has remained relatively limited in Vietnam compared to fucoidan from brown algae. Recent studies have focused on optimizing extraction processes [18], structural elucidation, and preliminary biological evaluations, revealing significant variations in ulvan's composition and bioactivities depending on species and extraction methods. Given Vietnam's extensive coastline and the wide distribution of native *Ulva* species, systematic investigations into ulvan's structure-function relationship are crucial for advancing its potential applications in food, pharmaceutical, and cosmeceutical industries. Such studies also contribute to building a scientific foundation for the sustainable utilization of green algae biomass in developing antioxidant-enriched natural products.

U. lactuca and *U. papenfussii* are two primary species of green algae that grew in Vietnam, with *U. lactuca* being the most prevalent due to its rapid growth rate [19, 20]. This species has been extensively studied for its structural characteristics, biological functions, and potential pharmaceutical applications, making it a promising candidate for marine biotechnology [21]. In contrast, *U. papenfussii* has received less research attention, with only recent studies from our group exploring the structure and bioactivity of ulvan derived from this species [20]. There have been no published reports on the fractionation and purification of ulvan, specifically from *U. papenfussii*.

In previous work, we successfully extracted ulvan from *U. lactuca* and *U. papenfussii* using a hot water extraction method [19, 20]. Partial purification of ulvan was achieved through alcohol precipitation and cetavlon precipitation. However, to obtain a highly purified ulvan suitable for bioactivity assays, we further fractionated and refined the ulvan into specific fractions in this study. These ulvan fractions from the two algae species will undergo analysis to determine their chemical composition, molecular weight, and *in vitro* antioxidant activities, including total antioxidant capacity, DPPH radical scavenging ability, and reducing power. This study aims to fractionate and purify ulvan from *U. lactuca* and *U. papenfussii*, characterize their molecular composition, and assess their antioxidant capacities to uncover structure-activity relationships.

MATERIALS AND METHODS

U. lactuca and *U. papenfussii* were collected from the coastal waters of Nha Trang Bay in Khanh Hoa, Vietnam (June–July 2023). Dr. Vo Thanh Trung identified species at the Nha Trang Institute of Technology Research and Application. Upon collection, the seaweed samples were thoroughly rinsed with tap water to remove any attached debris, sand, and organic matter. The cleaned samples were then air-dried in the shade to preserve their bioactive compounds before being finely ground into a uniform powder for subsequent analysis.

Polysaccharide isolation

Ulvan extraction from *U. lactuca* and *U. papenfussii* was conducted following a modified version of the classical method described by Bilan, which remains a standard approach for purifying polysaccharides such as fucoidan from brown algae [22]. Extracting these green algae involved a hot water procedure with specific adjustments to optimize results.

Twenty grams of powdered algae were suspended in 400 mL of water, adjusted to pH 6, and heated to 80^{o}C for 2 hours. After this initial extraction, the mixture was filtered, and the

residual seaweed underwent a second extraction under identical conditions. The combined extracts from both rounds were centrifuged to separate a clarified solution. Cetavlon (hexadecyltrimethylammonium bromide) was added to the extract to initiate ulvan precipitation, which continued until precipitation was complete [20]. Following centrifugation, the precipitate was dissolved in a sodium iodide-ethanol solution to convert ulvan into its sodium salt form. This sodium ulvan was then re-precipitated by adding ethanol [22, 23].

The resulting precipitate was thoroughly rinsed with ethanol and redissolved in water, followed by the addition of 96% ethanol at a 4:1 (v/v) ratio. It was then stored overnight at 4^{o}C to ensure complete precipitation. After centrifugation and a wash with 70% ethanol, the purified ulvan was obtained as a fine powder by freeze-drying, ready for further characterization and analysis [23, 24].

Polysaccharide fractionation

Ulvan samples were dissolved in water and centrifuged. The samples were then loaded onto a column (2.6 cm × 40 cm) containing DEAE-Macroprep resin (Bio-Rad, CA, USA) at a 1 mL/min flow rate.

Initially, the column was washed with 0.1 M NaCl solution to remove unbound substances. Subsequently, ulvan was eluted using a gradient of NaCl elution solution ranging from 0.1 M to 2 M at a flow rate of 5 mL/min. The eluted fractions were analyzed for total carbohydrate content using the phenol-sulfuric acid [25]. Specifically, 20 µL of the sample was mixed with 20 µL of 5% phenol reagent and vortexed. Then, 100 µL of concentrated sulfuric acid was added, followed by thorough mixing. The mixture was heated in a water bath for 5 minutes and cooled to room temperature, and its absorbance was measured at a wavelength of λ = 490 nm.

The eluted fractions were combined into different ulvan fractions based on the total carbohydrate content. These fractions were passed through a 10 kDa membrane for concentration and salt removal, then freeze-dried until a constant weight was achieved.

Chemical Composition of polysaccharides

The chemical analysis of ulvan was conducted following the procedure outlined by Nguyen et al., (2020) [24]. The ulvan samples underwent a two-step acid hydrolysis, and the resulting hydrolysates were analyzed for monosaccharides using the Dionex ICS-5000 HPAEC-PAD system with pulsed amperometric detection (PAD). Standards for L-rhamnose, D-galactose, D-glucose, D-xylose, D-glucuronic acid, and L-iduronic acid were purchased from Sigma-Aldrich.

The sulfate content was determined using the turbidimetric method described by Jackson and McCandless (1978) [26]. After TFA hydrolysis, this method mixed 110 µL of hydrolysates with 120 µL of 8% TCA. This mixture added 60 µL of 2% $BaCl_2$ in 15% PEG6000 reagent, and the mixture was allowed to stand for 35 minutes. The suspension of $BaSO_4$ formed was then measured at 500 nm using a microplate reader (TECAN Infinite 200, Salzburg, Austria). A linear standard curve for sulfate response was generated using K_2SO_4.

Assay of Molecular weight

The molecular weight (MW) of the fucoidan fractions was determined using High-Performance Size Exclusion Chromatography (HP-SEC) with an Ultimate iso-3100SD pump and WPS-3000 sampler (Dionex, Sunnyvale, CA, USA), connected to an RID-A refractive index detector (Shodex, Showa Denko K.K., Tokyo, Japan). Separation was performed with a Shodex SB-806 HQ GPC column 300 mm × 8 mm and a Shodex SB-G guard column 50 mm × 6 mm (Showa Denko K.K., Tokyo, Japan). Elution occurred at a flow rate of 0.5 mL/min at 40^{o}C, and pullulans with molecular weights of 1, 5, 12, 110, 400, and 800 kDa were used as standards [20].

Antioxidant actititives

Total Antioxidant Capacity Measurement

The total antioxidant potential of the ulvan extracts was assessed using the

phosphomolybdenum method [27]. In this assay, 0.3 mL of the ulvan solution (5 mg extract per ml of distilled water) was combined with 3 mL of a reagent solution consisting of 0.6 M sulfuric acid, 28 mM sodium phosphate, and 4 mM ammonium molybdate. The mixture was incubated at 95°C for 90 minutes. Following incubation, absorbance was measured at 695 nm with a spectrophotometer, using a blank solution prepared with distilled water in place of the ulvan sample. The antioxidant capacity was quantified as ascorbic acid equivalents, expressed as mg of ascorbic acid per gram of sample.

DPPH Radical Scavenging Activity

A modified version of the method by Blois was employed [28]. This assay utilized DPPH as a stable synthetic free radical to evaluate the free radical scavenging ability of ulvan. Sample concentrations ranging from 400 µg/mL (dissolved in distilled water) were prepared, and 2.25 mL of each sample was mixed with 0.75 mL of a 75 µM DPPH solution in ethanol (99%). The mixture was allowed to stand in the dark at 25°C for 30 minutes. The absorbance was then recorded at 517 nm. The IC_{50} value, or the concentration required to reduce the DPPH radical concentration by 50%, was calculated. The scavenging activity percentage was derived using the formula: $\%Inhibition = [(A\_control - A\_sample)/A\_control] \times 100$, *where*: *A_control* is the absorbance of the control without the sample; and *A_sample* represents the absorbance of the ulvan solution.

Reducing Power Assay

The reducing power of the ulvan extracts was assessed according to the modified method of Zhu [29]. Various concentrations of the sample (4.0 mg/ml in distilled water) were prepared. Each sample (1 mL) was mixed with 2.5 mL of 0.2 M phosphate buffer (pH 6.6) and 2.5 mL of 1% potassium ferricyanide ($K_3Fe[CN]_6$), then incubated at 50°C for 20 minutes. The reaction was halted by adding 2.5 mL of 10% trichloroacetic acid. Subsequently, 2.5 mL of the solution was combined with 2.5 mL of distilled water and 0.5 mL of 0.1% ferric chloride ($FeCl_3$). Absorbance was immediately measured at 700 nm against a distilled water blank.

RESULTS

Fraction of the ulvans

In most extraction processes, crude polysaccharides are often accompanied by various impurities, including cellulose, proteins, and glucuronan, which are co-extracted with ulvan. These extraneous compounds complicate the isolation of pure ulvan, necessitating additional purification steps. As per established protocols, initial purification was achieved through ethanol precipitation and cetavlon treatment. However, to achieve higher purity and to separate ulvan based on its charge characteristics, we further fractionated it using anion exchange chromatography.

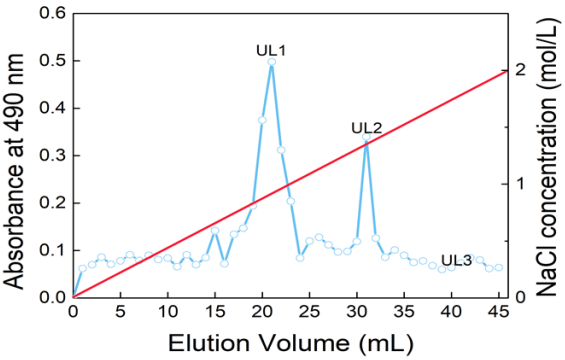

Figure 1. Anion exchange chromatography-based fractionation of ulvan extracted from *U. lactuca*. Ulvan fractions (UL1, UL2, UL3) were obtained using DEAE-macro prep column chromatography with stepwise NaCl elution. Fractionation was guided by total carbohydrate content measured by the phenol-sulfuric acid method. The fractions differ in monosaccharide composition and sulfate content

The crude ulvan extracted through hot water treatment was applied to a DEAE Macroprep ion exchange chromatography column, where it was selectively eluted according to charge as salt concentration increased. Each eluted fraction was analyzed

for its total carbohydrate content, allowing us to identify and isolate three distinct ulvan fractions, as illustrated in (Fig. 1). The distinct fractions were grouped based on the analysis of total carbohydrate content using the phenol-sulfuric acid method. As a result, three fractions were obtained and labeled sequentially in order of elution, from smallest to largest: the first fraction (UL1), the second fraction (UL2), and the final fraction (UL3).

Figure 1 presents a schematic overview of the ulvan fractionation process from *U. lactuca*, highlighting the separation steps based on carbohydrate content analysis. After initial extraction, the crude ulvan underwent fractionation through anion exchange chromatography, where fractions were collected incrementally by increasing salt concentration. Each fraction was subsequently analyzed to determine carbohydrate content, allowing us to identify and categorize distinct ulvan fractions with varying molecular characteristics. This approach provided a systematic separation, resulting in fractions that could be further assessed for their specific biochemical properties and potential bioactivity.

Purifying ulvan via ion exchange chromatography efficiently removes contaminants and enables the separation of polysaccharides by charge groups. During this process, some pigmented compounds associated with the crude ulvan adhere to the column and are only eluted during a subsequent washing phase with NaOH, resulting in visibly altered colorations of the final ulvan fractions compared to the unpurified extract.

Following a similar approach to that used for *U. lactuca*, ulvan from *U. papenfussii* was likewise purified and fractionated using ion exchange chromatography, yielding three distinct fractions, labeled UP1-3, as depicted in (Fig. 2). Each fraction was differentiated based on its unique carbohydrate content and charge characteristics.

The purification and fractionation of ulvan via anion exchange chromatography involve using various chromatographic columns, with fractionation typically guided by a NaCl concentration gradient as the mobile phase. In a study by Li et al., ulvan was purified from *Ulva pertusa* using a Q Sepharose XL column; fractions were collected at increasing NaCl concentrations of 0, 0.5, and 1M, with the primary ulvan fraction isolated at 1 M NaCl [30]. Similarly, Glasson et al., [31] utilized a HiTrap Q FF column to purify ulvan from *Ulva ohnoi*, *Ulva tepida*, and *Ulva prolifera*, achieving yields of 1.45%, 1.29%, and 2.8% from the dried algae, respectively [31]. In our study, DEAE-macro prep resin was employed for ulvan fractionation. For both *U. lactuca* and *U. papenfussii*, three main fractions were obtained, with yields of 7.5%, 2.25%, and 0.47% for *U. lactuca*, and 7.14, 0.8, and 0.56% for *U. papenfussii*.

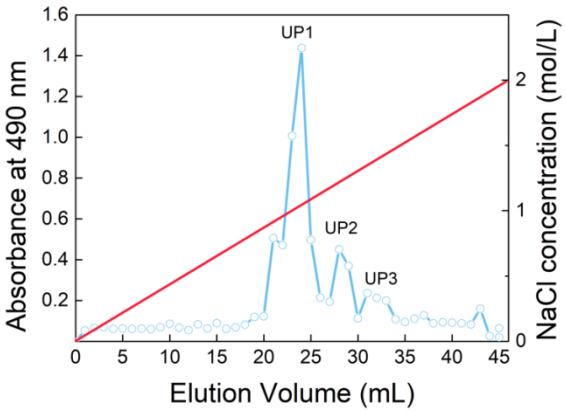

Figure 2. Anion exchange chromatography-based fractionation of ulvan extracted from *U. papenfussii*. Ulvan fractions (UP1, UP2, UP3) were separated using DEAE-macro prep column chromatography with a stepwise NaCl gradient. Fractionation was guided by total carbohydrate content determined via the phenol-sulfuric acid method. The resulting fractions exhibit distinct differences in monosaccharide and sulfate content

Composition of the ulvans

The monosaccharide composition and sulfate content of ulvan extracted from *U. lactuca* were analyzed in different fractions, including crude ulvan, UL1, UL2, and UL3. The results indicate significant variations in the distribution of neutral monosaccharides, uronic acids, and sulfate levels across these fractions (Table 1).

Table 1. Monosaccharide composition and sulfate content of ulvan from *U. lactuca*. The data are given in %w/w of total carbonhydrates analyzed in the ulvans

		Crude	UL1	UL2	UL3
Neutral Monosaccharide (%)	Rhamnose	32.25 ± 0.2	30.93 ± 5.6	32.44 ± 2.11	9.98 ± 5.03
	Galactose	0.88 ± 0.09	1.2 ± 0.23	1.96 ± 2.23	1.24 ± 1.31
	Glucose	2.41 ± 0.09	2.85 ± 2.32	1.39 ± 1.37	1.16 ± 0.46
	Xylose	2.43 ± 0.43	5.16 ± 3.63	5.06 ± 3.33	2.52 ± 1.38
Uronic acid (%)	Glucuronic acid	6.71 ± 1.08	7.97 ± 1.24	8.94 ± 1.59	7.16 ± 2.4
	Iduronic acid	8.55 ± 1.36	15.33 ± 2.07	11.87 ± 2.24	11.01 ± 5.56
Sulfate (%)		15.93 + 1.6	15.65 + 4.24	14.92 + 2.86	13.82 + 1.79

Rhamnose, a predominant monosaccharide in ulvan, varied significantly in all fractions. Its content peaked in UL2 (32.44 ± 2.11%) and was notably reduced in UL3 (9.98 ± 5.03%). This trend suggests that rhamnose concentration may decrease as ulvan is further fractionated. Galactose appeared in small quantities across all fractions. The highest percentage was detected in UL2 (1.96 ± 2.23%), while the lowest was observed in the crude extract (0.88 ± 0.09%). Glucose content exhibited a slight increase in UL1 (2.85 ± 2.32%) compared to the crude sample (2.41 ± 0.09%), followed by a progressive decline in UL2 (1.39 ± 1.37%) and UL3 (1.16 ± 0.46%). Xylose content peaked in UL1 (5.16 ± 3.63%) but was relatively lower in the other fractions, indicating a preferential association of xylose with specific ulvan components enriched in UL1. The analysis of uronic acids, which contribute to the bioactivity and structure of ulvan, revealed distinct patterns: Glucuronic acid was highest in UL2 (8.94 ± 1.59%) and lowest in the crude extract (6.71 ± 1.08%), suggesting that fractionation processes could concentrate glucuronic acid in particular fractions. Iduronic acid showed its highest level in UL1 (15.33 ± 2.07%) and was moderately reduced in UL2 (11.87 ± 2.24%) and UL3 (11.01 ± 5.56%). The high iduronic acid content in UL1 indicates that this fraction may retain structural regions rich in this monosaccharide. The sulfate levels, critical for ulvan's biological activities, decreased slightly across the fractions. The crude ulvan exhibited the highest sulfate content (15.93 ± 1.6%), followed by UL1 (15.65 ± 4.24%) and UL2 (14.92 ± 2.86%), with the lowest levels in UL3 (13.82 ± 1.79%). This reduction in sulfate content from crude to fractionated ulvans suggests that certain structural components rich in sulfate groups may be lost or concentrated in specific fractions during purification.

The fractionation of ulvan resulted in apparent differences in its monosaccharide composition and sulfate content, indicating a selective enrichment of specific components. Rhamnose and iduronic acid were most concentrated in UL2 and UL1, respectively, while UL3 showed a marked reduction in these sugars. The sulfate content declined progressively across the fractions, which may impact their bioactivity. These variations highlight the complexity of ulvan and its potential for diverse applications depending on the fraction and associated chemical composition.

The fractionation of ulvan from *U. papenfussii* revealed distinct compositional differences (Table 2). UP1 and UP2 were enriched in rhamnose (42.16% and 41.08%, respectively), glucuronic acid, and iduronic acid, with UP2 showing the highest sulfate content (28.46%), making it the most sulfated fraction. UP3, however, exhibited lower rhamnose (9.02%), glucose, and sulfate levels than the other fractions. Xylose was most abundant in UP2 (5.3%), while galactose remained consistently low across all fractions. These results highlight the unique structural characteristics of each fraction, potentially influencing their bioactivity and applications.

The polysaccharide composition of green seaweeds typically varies widely, with carbohydrate levels ranging from 38.6% to 61.4%, sulfates from 19.4% to 34.0%, and

uronic acids from 6.5% to 35.0% [32, 33]. In this analysis, fractions 1 and 2 of ulvan exhibited the highest rhamnose content, while fraction 1 of ulvan was notably enriched in uronic acids, aligning with trends observed in earlier studies [32, 33].

Table 2. Monosaccharide composition and sulfate content of ulvans from *U. papenfussii*. The data are given in % w/w of total carbonhydrates analyzed in the ulvans

		Crude	UP1	UP2	UP3
Neutral Monosaccharide (%)	Rhamnose	20.74 ± 1.54	42.16 ± 1.94	41.08 ± 4.55	9.02 ± 0.7
	Galactose	1.05 ± 0.18	0.97 ± 0.08	1.98 ± 1.21	0.81 ± 0.02
	Glucose	2.53 ± 0.44	0.58 ± 0.02	2.32 ± 1.76	0.77 ± 0.05
	Xylose	3.67 ± 0.41	3.89 ± 0.19	5.3 ± 2.49	2.64 ± 0.29
Uronic acid (%)	Glucuronic acid	9.23 ± 0.95	15.54 ± 1.02	15.29 ± 1.94	12.31 ± 0.73
	Iduronic acid	13.88 ± 0.78	16.39 ± 0.45	12.92 ± 0.77	9.95 ± 0.77
Sulfate (%)		13.39 + 0.63	16.25 + 2.43	28.46 + 3.63	20.15 + 2.47

Molecular properties of the ulvan-fractions

According to previous studies, the molecular weight (Mw) of ulvan extracted from various seaweed species exhibits considerable variability [34]. For instance, Mw values of 2000 kDa and 194 kDa have been reported for ulvan extracted from *Ulva armoricana* [35] and *Ulva intestinalis* [36]. The ulvan extracted from the species analyzed in this study demonstrates significantly higher molecular weights (Mw > 5×10^6 g/mol). This result is challenging to interpret, as the molecular weight of ulvan is influenced by factors such as seaweed species, harvest season, cultivation conditions, extraction methods, and analytical techniques [6, 31, 34, 37].

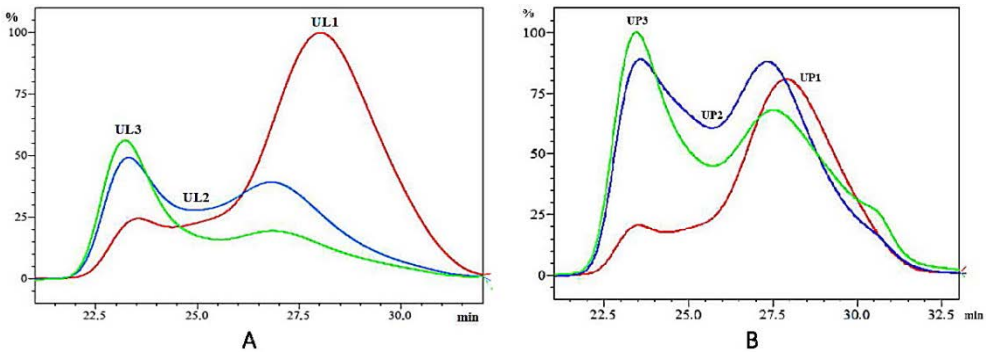

Figure 3. HPSEC chromatograms of *U. lactuca* (A) and *U. papenfussi* (B) polysaccharides. The molecular weight distribution of ulvan fractions was analyzed using high-performance size exclusion chromatography (HPSEC). Each curve corresponds to a different fraction: Fraction 1 (UL1/UP1 – red), Fraction 2 (UL2/UP2 – blue), and Fraction 3 (UL3/UP3 – green). Fraction 1 exhibited a predominance of low-molecular-weight polysaccharides, while Fraction 3 showed primarily high-molecular-weight components. The variation in peak distribution highlights the molecular heterogeneity among the ulvan fractions

The distribution of molecular weights across the ulvan fractions is presented in (Fig. 3). These findings indicate that low-molecular-weight ulvan molecules are predominantly separated in fraction 1, mainly consisting of neutral monosaccharides. Fraction 2 displays an almost equal distribution of high- and low-molecular-weight ulvan molecules. In contrast, fraction 3 primarily contains high-molecular-weight ulvan, predominantly composed of uronic acids. The result is similar to some previous publications [36]. This

observation provides valuable insights for selecting appropriate seaweed fractions and optimizing technologies for utilizing ulvan in food and pharmaceutical applications.

Bioactivity of the ulvan-fractions

The reducing power assay, total antioxidant capacity, and DPPH radical scavenging activities of ulvan fractions extracted from *U. papenfussii* and *U. lactuca* reveal notable differences across samples (Table 3). Among the fractions from *U. papenfussii*, UP2 exhibits the highest values in all three parameters, with a reducing power of 0.787 µg acid ascorbic/mg ulvan, a total antioxidant capacity of 5.343 mg acid ascorbic/mg ulvan, and a DPPH scavenging activity of 25.9%. UP1 and UP3 demonstrate lower antioxidant capacities, with UP1 showing the lowest reducing power and radical scavenging activity.

For ulvans from *U. lactuca*, UL2 displays superior antioxidant performance, with a reducing power of 0.819 µg acid ascorbic/mg ulvan, a total antioxidant capacity of 4.343 mg acid ascorbic/mg ulvan, and a DPPH scavenging activity of 29.5%. Conversely, UL1 has the lowest reducing power (0.286 µg acid ascorbic/mg ulvan) and total antioxidant capacity, though its DPPH scavenging activity (27.5%) remains relatively comparable to UL2. UL3 shows moderate performance in all parameters, indicating variability in antioxidant potential among the fractions.

Table 3. Reducing power assay, total antioxidant and DPPH radical scavenging activity of the ulvans

Samples		Reducing Power Assay (µg acid ascorbic/mg ulvan)	Total Antioxidant (mg acid ascorbic/mg ulvan)	DPPH Radical Scavenging Activity (Sc %)
U. papenfussii	UP1	0.433 ± 0.011	2.557 ± 0.037	20.3 ± 1.45
	UP2	0.787 ± 0.019	5.343 ± 0.052	25.9 ± 1.77
	UP3	0.391 ± 0.021	3.308 ± 0.102	23.6 ± 2.15
U. lactuca	UL1	0.286 ± 0.081	1.777 ± 0.029	27.5 ± 1.78
	UL2	0.819 ± 0.010	4.343 ± 0.041	29.5 ± 0.94
	UL3	0.568 ± 0.047	4.008 ± 0.066	21.8 ± 0.48

These results highlight the significant influence of extraction methods and ulvan fractions on antioxidant properties, which could guide the selection of fractions for specific functional applications in food or pharmaceutical products.

DISCUSSION

In ion exchange chromatography, the column resin forms a three-dimensional matrix that, beyond its ionic exchange properties, also influences separation by molecular weight. Consequently, the isolated ulvan fractions show molecular weight and chemical composition variation. The primary contributor to ulvan's negative charge is its sulfate groups, which means that the degree of sulfation and molecular weight significantly impact the elution behavior. As a result, the sulfation levels of fractions UL1, UL2, and UL3 for *U. lactuca* and UP1, UP2, and UP3 for *U. papenfussii* are expected to vary accordingly.

This study is the first to identify the main components of the uronic acid group of ulvan from two algae species growing in Vietnam. Glucuronic acid and iduronic acid are relatively difficult to separate using HPLC analysis. However, our results have shown specific separation and quantification. Interestingly, ulvan from Vietnamese algae is very rich in iduronic acid, a characteristic that has not been previously demonstrated. This feature may be attributed to the environmental conditions and the tropical climate in which the algae grow. These results need to be further investigated with expanded subjects to confirm the reproducibility of this characteristic.

The primary components [Rhamnose]:[Glucuronic acid + Iduronic acid + Xylose], is ~1.5:1:0.2, which aligns with the ideal

composition of ulvan, indicating that the analyzed ulvan sample comprises a combination of ulvanobiuronic acid and ulvanobioses. Interestingly, the iduronic acid content in this sample is significantly higher than that typically observed in ulvans extracted from the blade of Ulva species, while the sulfate content remains comparable [38]. Variations in yield and composition can be attributed to algal species, geographic origin, harvest season, and extraction methods [39].

Oxidative stress arises from an imbalance between oxidative activity and the body's antioxidant defenses, driven by reactive oxygen species. This imbalance can disrupt redox signaling pathways and regulatory processes, potentially causing molecular damage. It plays a key role in aging and the development of various diseases [40]. With growing interest in antioxidants, the activity of ulvan extracted using different methods has been increasingly explored. For example, Yuan et al. utilized microwave-assisted extraction to isolate ulvan, showing 27.6% scavenging activities against DPPH radicals and notable reducing power [41]. Li et al., [30] also reported that both purified ulvan and its highly sulfated variant, extracted with hot water, exhibited stronger radical scavenging capabilities than vitamin C [30]. In this study, when ulvan is in a highly purified state, charged groups such as carboxyl and sulfate form intramolecular bonds, which may hinder the interaction between DPPH radicals and the reducing agents, such as hydroxyl groups. However, the obtained results still show relatively high data, suggesting that the sulfate groups and large molecular weight have also enhanced the antioxidant activity.

The results from the data indicate that ulvan fractions from *U. lactuca* and *U. papenfussii* exhibit antioxidant activity. In vivo experiments in mice revealed that highly sulfated ulvan doses enhanced the activities of catalase, glutathione peroxidase, and superoxide dismutase while reducing malondialdehyde levels. These results suggest that ulvan effectively scavenges DPPH and ABTS radicals in vitro, likely due to its polysaccharide hydroxyl groups donating hydrogen ions to neutralize radicals [1]. The antioxidant activity of ulvan fractions correlate closely with their chemical composition, particularly the levels of uronic acids and sulfate groups. For instance, UL2 and UP2, exhibiting the highest DPPH and total antioxidant capacity, also contained relatively high sulfate and uronic acid content. Sulfate groups enhance antioxidant activity by increasing electron-donating ability and promoting radical scavenging, while uronic acids may contribute through metal chelation. These findings align with earlier reports indicating that sulfated and uronic-acid-rich polysaccharides tend to show stronger antioxidant properties. Therefore, the variation in antioxidant activity among ulvan fractions is likely due to differences in their structural composition. Within living systems, ulvan's antioxidant properties stem from its ability to regulate the activities of catalase, glutathione peroxidase, and superoxide dismutase. However, further studies are needed to clarify the molecular signaling pathways involved and to determine how ulvan influences the expression of these antioxidant enzymes in vivo [1].

Beyond its direct radical scavenging ability, ulvan may also exert antioxidant effects through the biological regulation of endogenous defense systems. Several studies have reported that ulvan stimulates the activity or gene expression of key antioxidant enzymes such as superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GSH-Px) in vivo and in vitro [12, 30]. These enzymes are crucial in neutralizing reactive oxygen species (ROS), thereby maintaining cell redox homeostasis. Moreover, ulvan's sulfate and uronic acid groups may interact with signaling pathways such as the Nrf2/ARE pathway, which governs cellular responses to oxidative stress. Although further studies are needed to confirm these mechanisms in detail, our findings support ulvan's multifunctional antioxidant potential.

CONCLUSION

This study has elucidated the chemical composition, molecular weight, and biological activity of ulvan extracted from the algae

species *U. papenfussii* and *U. lactuca*. The results demonstrate significant differences in the monosaccharide composition, sulfate content, and uronic acid levels of ulvan from both species, depending on the extraction fractions. Specifically, ulvan from *U. papenfussii* exhibited higher levels of iduronic acid and sulfate, whereas ulvan from *U. lactuca* showed a predominance of glucose and rhamnose. The molecular weight of the extracted ulvan was also significantly higher than in previous reports, suggesting the influence of the algal species, harvesting conditions, and extraction techniques.

The antioxidant activity analysis results indicate that ulvan from the UL2 and UP2 fractions exhibits superior capacity in DPPH free radicals, reducing power and overall antioxidant activity. These findings clarify the role of ulvan as a potential source of antioxidants suitable for development in food technology and pharmaceuticals. Furthermore, the differences in biological activity among the ulvan fractions suggest the potential to optimize the product by adjusting extraction techniques to achieve the desired performance.

However, further in-depth studies are needed to better understand the molecular mechanisms of ulvan in regulating the activity of antioxidant enzymes and identifying related biological signals. Additionally, exploring other biological properties of ulvan, such as anti-inflammatory or immunomodulatory effects, would open up greater application potential in the biomedical field.

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