



Ulvan as A New Trend in Agriculture, Food Processing and Medicine

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Author's contribution

The sole author designed, analyzed and interpreted and prepared the manuscript.

Article Information

DOI: 10.9734/AJFAR/2020/v6i430105

Editor(s):

(1) Dr. Luis Enrique Ibarra Morales, University of Sonora, Mexico.

Reviewers:

(1) Renato Teixeira Moreira, Education and Technology of Ceará, Brazil.

(2) Levent Bat, University of Sinop, Turkey.

(3) V. Ramasubramanian, Ayya Nadar Janaki Ammal College, India.

Complete Peer review History: <http://www.sdiarticle4.com/review-history/57551>

Original Research Article

Received 17 March 2020

Accepted 24 May 2020

Published 12 June 2020

ABSTRACT

In this work the researcher is looking for natural, new, safe, cheap and available compounds that can be used as antimicrobial and antioxidant in the medical and agricultural fields. A sulfated polysaccharide, Ulvan was extracted from *Ulva lactuca* and purified by cold or hot water-extraction and ethanol-precipitation with yield = 5:10% (w-w). Its appearance and touch like gel. Characterization of active groups of Ulvan was achieved by FT-IR spectroscopy, its content of sulfate = 3.998% (w-w) was determined by Ion Chromatography (IC), its molecular weight = 532.221 Kilo Daltons was determined by Gel permeation chromatography, its thermal stability was evaluated by DSC-TGA, its viscosity = 18 was determined by viscometer apparatus and its antimicrobial activity was studied against human, plant, animal, fish and poultry pathogens. Ulvan showed antioxidant activity with $IC_{50} = 3.04$ mg/ml. Its biological activity as an antioxidant and antimicrobial may be a good indication for the use of Ulvan against various pathogens as a preservative in food processing and cosmetics and as antioxidant and antimicrobial in medicine against human pathogen.

Keywords: *Ulvan; extraction; purification; antimicrobial; antioxidant; FT-IR.*

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1. INTRODUCTION

Every day, scientists are searching for new sources that help in resistance of huge amount of parasites that cause diseases in plants, animals, fish, poultry and human. Oceans and seas are good sources of new creatures that should be used for extraction new active compounds. Algae are good marine organisms that contain many useful phytochemicals. The largest families of algae are green, brown and red algae and are distinguished with their polysaccharides. Their polysaccharides contain unique chemical structure that can't be found in higher plants. *Ulva lactuca* (a green macro algal called also sea lettuce or green laver) is characterized by sulfated polysaccharides called Ulvan [1,2] Ulvan percentage varies as a result of origin, season, species, maturity and growth conditions [3] Ulvan is extracted from the cell walls structure of thallus of green marine macro algal *Ulva* with yield 8 to 29% of dry weight of *Ulva* [4] Ulvan is used in medicine for enhancing gastrointestinal immunity, dropping blood glucose and lipids, reducing colorectal cancer and cardiovascular risks and as antiviral, antioxidant and as anticancer and has also hepatoprotective effects [2] and as prebiotic in food processing [5] and in the Bio control Activity of *Debaryomyces hansenii* and *Stenotrophomonas rhizophila* against Fruit Rot of *Cucumis melo* L. [6].

Free radicals and reactive oxygen species (ROS) includes O^{\cdot} , $O^{\cdot}H$, LOO^{\cdot} cause damage to biomolecules, oxidative stress and responsible for diseases such as cancer, diabetes, cardiovascular, atherosclerosis, cataracts, neurological disorders, ageing diseases and other diseases. Antioxidants facilitate as a defense against free radicals that generated as byproducts of biological reactions, pollutants and radiation. Antioxidants protect the body from oxidative damage by scavenging the free radicals. Natural antioxidants such as sulphated polysaccharide Ulvan extracted from *Ulva lactuca* is a good antioxidant against free radicals that cause many diseases [7,8].

As a result of the biological activity of Ulvan, proved in this work, it should be used as antioxidant and as antimicrobial agent in the medical field for its effectiveness against human pathogens, as preservatives in cosmetics and packaged foods and as prebiotics in the agricultural field, especially the food processing.

2. MATERIALS AND METHODS

2.1 Collection of Algae (*Ulva lactuca*) as Described in [9]

Algae collected from the Mediterranean (Abu Qir) in the spring of 2018 and cleaned with sea water to remove sand pebbles, epiphytes and shells, then the algae were transported to the laboratory in plastic bags, washed with a dilute solution of sodium chloride and then distilled water. Algae were shade dried, put and grounded in an electric mixer and stored in the refrigerator for further use. *Ulva lactuca* was recognized by a Professor of Microbiology and phycology, Faculty of Science, Zagazig University. As follows:

Division: Chlorophyta

Subdivision: Chlorophytina

Class: Cladosiphorophyceae

Family: Ulvaceae

e.g: *Ulvalactuca*

2.2 Extraction and Purification of Ulvan Polysaccharides

Ulvan polysaccharide was extracted by using methods of cold or hot water-extraction and ethanol-precipitation [10], with some modifications. 10 g of *Ulva lactuca* were extracted with petroleum ether for two hours, and further extracted with 80% ethanol at 90°C for 2 h. After filtering, the residue was further extracted three times with bi-distilled water at 100°C for 2 h. Then all extracts were combined, concentrated using a rotary evaporator at 55°C and filtered. The extract was deproteinized from 8 to 10 times using the Sevag reagent. The polysaccharide mixture was deproteinized following the Sevag method as described by [11]. Briefly, the polysaccharides was mixed with Sevag reagent, 5:1 (v: v) $CHCl_3$: n-But OH and stirred for 30 min. Then, the mixture was centrifuged and the upper polysaccharide solution was collected. The polysaccharide solution was further deproteinized with Sevag reagent for 8 to 10 times until there was no white layer between polysaccharide solutions and the polysaccharide was free of proteins as scan by UV Spectra in 260 nm and 280 nm. After removal of the Sevag reagent, the extract was precipitated by adding ethanol (four times the volume of aqueous extract), and the mixture was kept overnight at 4°C to yield the polysaccharide. The precipitate was collected by centrifugation at 6,000 rpm for 15 min, washed successively with petroleum

ether, acetone and ethanol, petroleum ether to remove fat-soluble molecules. Extraction with ethanol removed the monosaccharides and phenolic compounds and other polar parts in order to remove these impurities completely. The procedure of precipitation was repeated, and then dissolved in water and dialyzed against deionized water for 72 h, freeze-drying to yield the crude polysaccharide.

The polysaccharide yield (%) was calculated using the following equation:

$$\text{Yield of polysaccharide (\%)} = \left[\frac{\text{Weight of polysaccharides (g)}}{\text{Weight of raw material (g)}} \right] \times 100$$

2.3 Chemical Analysis (Table 1)

Total carbohydrate was estimated spectrophotometrically at 490nm by phenol-sulphuric acid method of [12] using glucose as standard.

Sulfate content was measured using Ion Chromatography (ICS-1100-Thermo Dionex, USA) in the central lab of faculty of Science Ain Shams uni.in Egypt.

The viscosity of Ulvan sample (1%) was elucidated by Ostwald's viscometer according to the method of [4] and the obtained values were expressed as millipascal in second (mPa.s).

2.3.1 UV-visible spectroscopy analysis

UV-VIS was used to prove the success of the purification step of Ulvan polysaccharides.

UV-VIS was determined by Shimadzu UV-1800 in the central lab of faculty of Science, Ain Shams University in Egypt (Fig. 1).

2.3.2 FT-IR spectroscopy (Fourier transform infrared spectrometer) of Ulvan

The chemical composition of active groups of the sulphated polysaccharides were determined by NICOLET- 6700- FT/IR- Thermo Scientific and the spectra were recorded in the wavelength interval of 4000 to 400 cm⁻¹ in the central lab of faculty of Science Ain Shams University in Egypt (Fig. 2) and (Table 2).

Gel Permeation Chromatography were applied for determining molecular weight of Ulvan by Waters-2410 Reactive Index Detector – Temperature Control Module – 515 HPLC Pump

in Egyptian Petroleum Research Institute(EPRI) in Egypt.

2.4 Thermal Analysis of Ulvan

DSC (Differential Scanning Calorimeter) analysis and TGA (Thermo Gravimetric Analysis) of the same sample (DSC-TGA).

Thermal decomposition analysis of Ulvan was studied using SDTQ600 TG-DSC instrument (TA Company, USA) in National Research Center (NRC), Egypt (Fig. 3).

2.5 Biological Activity of Ulvan

2.5.1 Antioxidant activity of Ulvan polysaccharides (1 mg/ml)

Adding 100,200,300,400,500,600,700,800,900, 1000 µl of Ulvan (1 mg/ml) and raised to 1 ml by ethanol and were added to 4 ml of DPPH (0.1 mM of 2,2'-biphenyl picrylhydrazyl (DPPH) . After 30 minutes of incubation period at room temperature in the dark, the absorbance was read against the blank at 517 nm. Inhibition of free radical DPPH was calculated according to the following equation as described in [9].

$$\% \text{ Scavenging activity} = \left(\frac{A_{\text{control}} - (A_{\text{sample}}/A_{\text{control}})}{A_{\text{control}}} \right) \times 100$$

2.5.2 Antimicrobial activity of Ulvan polysaccharides by Kirby-Bauer method

Antimicrobial activity of the tested samples was determined using a modified Kirby-Bauer disc diffusion method [13] Briefly, 100 µl of the test bacteria/fungi were grown in 10 ml of fresh media until they reached a count of approximately 10⁷ cells/ml for bacteria or 10⁵ cells/ml for fungi [14] 100 µl of microbial suspension was spread onto agar plates corresponding to the broth in which they were maintained.

Isolated colonies of each organism that might be playing a pathogenic role should be selected from primary agar plates and tested for susceptibility by disc diffusion method [15,16]. Of the many media available, NCCLS recommends Mueller-Hinton agar due to: it results in good batch-to-batch reproducibility. Disc diffusion method for filamentous fungi tested by using approved standard method (M38-A) developed by the [17] for evaluating the susceptibilities of filamentous fungi to antifungal agents. Disc

diffusion method for yeasts developed by using approved standard method (M44-P) by the (NCCLS, 2003). Plates inoculated with filamentous fungi as *Aspergillus flavus* at 25°C for 48 hours; Gram (+) bacteria as *Staphylococcus aureus*, *Bacillus subtilis*; Gram (-) bacteria as *Escherichia coli*, *Pseudomonas aeruginosa* they were incubated at 35-37°C for 24-48 hours and yeast as *Candida albicans* incubated at 30°C for 24-48 hours and, then the diameters of the inhibition zones were measured in millimeters [13] Standard discs of Ampicillin (Antibacterial agent), Amphotericin B (Antifungal agent) served as positive controls for antimicrobial activity but filter discs impregnated with 10 µl of solvent (distilled water, chloroform, DMSO) were used as a negative control. The agar used is Mueller-Hinton agar that is rigorously tested for composition and Ph. Further the depth of the agar in the plate is a factor to be considered in the disc diffusion method. This method is well documented and standard zones of inhibition have been determined for susceptible and resistant values. Blank paper disks (Schleicher & Schuell, Spain) with a diameter of 8.0 mm were impregnated 10µ of tested concentration of the stock solutions. When a filter paper disc impregnated with a tested chemical is placed on agar the chemical will diffuse from the disc into the agar. This diffusion will place the chemical in the agar only around the disc. The solubility of the chemical and its molecular size will determine the size of the area of chemical infiltration around the disc. If an organism is placed on the agar it will not grow in the area around the disc if it is susceptible to the chemical. This area of no growth around the disc is known as a "Zone of inhibition" or "Clear zone". For the disc diffusion, the zone diameters were measured with slipping calipers of the National Committee for Clinical Laboratory

Standards [15] Agar-based methods such as Etest and disk diffusion can be good alternatives because they are simpler and faster than broth-based methods [18,19] (Table 3).

2.6 Statistical Analysis

The statistical package SPSS (version 20) was used for statistical analysis. The medium inhibition concentration (IC₅₀) for determining antioxidant activity were calculated by linear regression analysis [20].

3. RESULTS AND DISCUSSION

Ulva lactuca is a green macro algal characterized by chlorophyll a,b pigments and polysaccharide Ulvan. Ulvan is a heteropolysaccharide composed of sulfate, rhamnose, xylose, glucuronic acid and iduronic acid [21]. Ulvan was extracted and purified by water-extraction and ethanol-precipitation method with yield 5:10g/100g of dry weight of *Ulva lactuca*. Carbohydrates determined calorimetrically by the phenol-sulfuric acid method using D- glucose as a standard was 26.41% and Sulfate determined by Ion Chromatography (IC) was 3.998% (Table 1).

Table 1. Chemical composition of Ulvan

Total carbohydrates	26.41%
Yield of sulfated polysaccharides	5-10%
Sulfate	3.998%

UV-VIS spectra with no peaks between 260:280 nm proved the success of purification step of extracted Ulvan and the peaks 418.5 and 440.5 are according to the sulfate content in Ulvan (Fig. 1).

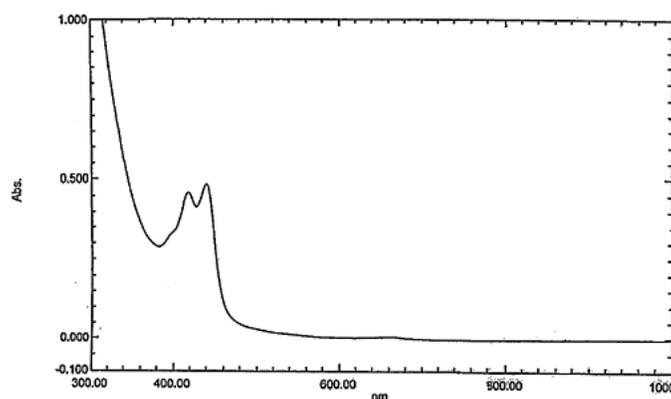


Fig. 1. UV-VIS of purified (deproteinated) Ulvan

3.1 Viscosity of Ulvan

Ulvan exhibited a gel – like behavior with viscosity = 18.

FT-TR spectrum of Ulvan indicated the chemical composition of the polysaccharides Ulvan by peaks summarized in (Table 2 and Fig. 2).

3.2 Gel Permeation Chromatography

Molecular weight of Ulvan was determined by Gel Permeation Chromatography = 532.221 Kilo Daltons as proved by [22] and mentioned that sulfated polysaccharides have high

molecular weight values higher than 100 Kda.

3.3 Thermal Analysis of Ulvan in (Fig. 3)

DSC-TGA of Ulvan was carried out to observe weight loss with temperature. The extracted polysaccharide Ulvan was subjected to heating from room temperature to 1000°C. The first thermal degradation of Ulvan at around 153.62°C and 224.73°C respectively. This disintegration accompanied by about 67.52% of mass loss. The second thermal decomposition of Ulvan took place at about 399.19°C and 423.32°C with a loss of mass about 26.02% respectively. The complete breakdown with a further loss of mass at about 562.5°C.

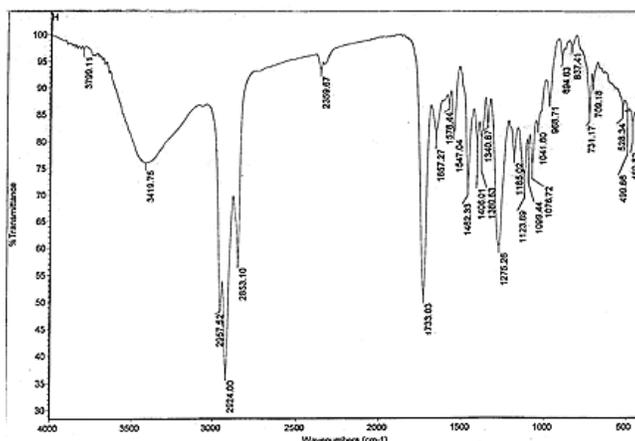


Fig. 2. FT-TR spectrum of Ulvan

Table 2. FT-IR of Ulvan

Frequency (cm ⁻¹)	Bond/ stretching	Functional groups
3419.75&3799.11	O-H stretch	Polysaccharides
2853.1 & 2924 & 2957.52	Many asymmetrical & symmetrical C-H stretch of aliphatic CH ₂	Polysaccharides
1657.27 & 1733.03	-COO- stretch of uronic acid	Polysaccharides
1406.01 & 1462.33	Symmetric stretch of –COO- & stretch of C-O within –COOH	Polysaccharides
1380.63	C-H bending of aliphatic CH ₂	Polysaccharides
1275.26	Sulfate group	Polysaccharides
1099.44 & 1123.89 & 1185.02 1041.8 & 1076.72	Region of ring vibrations overlapped with stretching vibrations of (C-OH) side groups and the (C-O-C) glycosidic bond vibrations	Polysaccharides
837.41 & 894.03	α-dominating configuration of the sugar units sulfate ester	Polysaccharides
499.66 & 528.34 & 709.18 & 731.17	Indicated sulfate ester groups (C-S-O) in the axial of ether and in equatorial primary sulfate group and S-O of sulfate group	Polysaccharides

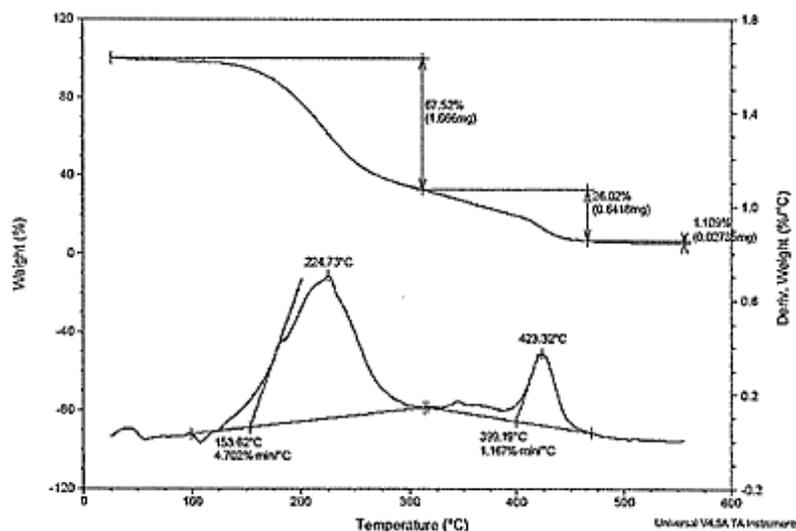


Fig. 3. DSC-TGA of Ulvan

Table 3. Antimicrobial activity of Ulvan

Microorganism	Standard	Inhibition zone diameter (mm/mg)
<i>Bacillus subtilus</i>	G+ 32	16
<i>Escherichia coli</i>	G- 30	17
<i>Klebsiella sp.</i>	G- 18	ve
<i>Pseudomonas aeuroginasa</i>	G- 28	19
<i>Staphylococcus aureus</i>	G+ 26	16
<i>Streptococcus faecalis</i>	G+ 30	17
<i>Neisseria gonorrhoeae</i>	G- 25	17
<i>Alternaria alternate</i>	F 15	ve
<i>Aspergillus fugigatus</i>	F 14	ve
<i>Candida ablicans</i>	Y 16	10
<i>Fusarium oxysporum,</i>	F 14	ve
<i>Penicellium sp.</i>	F 15	ve
<i>Saccharomyces cerevisiae</i>	Y 14	16

F = Fungus, G+ = Gram positive bacterium, Y = Yeast, G- = Gram negative bacterium, ve = negative

3.4 Antioxidant Activity

DPPH was examined using concentration = 1mg/1ml as described in [9].

DPPH free radical compound used to determine free radical scavenging ability of compounds by measuring the decrease in absorbance of the DPPH radical caused by scavenging of the hydroxyl radical through hydrogen donation from Ulvan with IC₅₀ = 3.04 mg/ml. The scavenging activities of sulfated polysaccharides Ulvan increased with increasing concentrations of Ulvan. Sulfate groups of Ulvan may be responsible for the antioxidant activity of Ulvan polysaccharides [23].

3.5 Antimicrobial Activity of Ulvan was Summarized in (Table 3)

Ulvan (100 mg/1 ml) showed higher antimicrobial activity against bacteria and yeasts than fungi. It showed higher antimicrobial activity against human pathogens such as *Candida ablicans* and all tested bacteria and against fish pathogens such as *Staphylococcus aureus*, *Escherichia coli*, *Pseudomonas aeuroginasa*. Ulvan also showed higher antimicrobial activity against animal pathogens such as *Candida ablicans* and *Bacillus subtilus*. It also showed antimicrobial activity against plant pathogens such as *Pseudomonas aeuroginasa* and *Bacillus subtilus*.

The results of antimicrobial activity of Ulvan were summarized in Table 3 where the control was DMSO, but the standard of antibacterial activity was Ampicillin and the standard of antifungal activity was Amphotericin B.

Ulvan showed higher antimicrobial activity against human, plant, poultry, fish and animal pathogens and that suggested Ulvan can be used in food processing as prebiotic [5] and as preservative in packaged food and cosmetics and in the pharmaceutical industry and medical applications against human pathogens [2].

4. CONCLUSION

In this work, I hope to highlight on heteropolysaccharide, Ulvan, extracted and purified from marine macro algal *Ulva lactuca* obtained from Egyptian shores by hot water-extraction and ethanol-precipitation. Its chemical composition was proved by spectroscopic methods. The biological activity of Ulvan as antioxidant activity and as antimicrobial activities were examined against human, plant, fish, poultry and animal pathogens and gave a good idea for using Ulvan as preservative in cosmetics and packaged food or prebiotic in food processing and also as antioxidant and as antimicrobial against human pathogens in the medical field. This work to find new sources that have unique chemical compounds that cannot be found in higher plants and explore their biological activity as antimicrobial and as antioxidant, but Ulvan need more researches to explore its role in medicine, cosmetics and food processing and other industrial fields, because Ulvan is a unique structure present in *Ulva lactuca* only and not in other algae or higher plants.

COMPETING INTERESTS

Author has declared that no competing interests exist.

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