Journal of *Marine Science and Engineering*

MDPI

Article

Screening of *Undaria pinnatifida* (Laminariales, Phaeophyceae) Lipidic Extract as a New Potential Source of Antibacterial and Antioxidant Compounds

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Abstract: The lipidic extract of *Undaria pinnatifida*, one of the worst invasive species, was investigated for its potential exploitation in biotechnological applications. The antimicrobial activity of the lipidic extract in three different portions (blade, sporophyll, and holdfast) was assessed by using the Kirby–Bauer method, while the antioxidant activity was evaluated by the TEAC, ORAC, and Folin–Ciocalteu assays. NMR spectroscopy and thin-layer chromatography were employed for the chemical characterization. The extracts showed antibacterial activity against several of the tested *Vibrio* species: *V. aestuarinus*, *V. fischeri*, *V. furnisii*, *V. inusitatus*, *V. litoralis*, and *V. mediterranei*, including some pathogens for farmed fish. Intriguing antioxidant activity was recorded, with the highest value in the blade (126.907 ± 28.993 mmol Trolox equivalent/g TEAC). Free, saturated, unsaturated, and polyunsaturated fatty acids were highlighted by 1D and 2D NMR spectroscopy. The presence of ω-3 and ω-6 PUFAs indicates the importance of this algal species in the food industry. We suggest the employment of *U. pinnatifida* as source of new and safer therapeutic agents to control fish and shellfish diseases due to vibriosis, as well as a source of natural antioxidants that are useful for human health, considering the growing interest in the development of strategies for invasive seaweed control.

Keywords: *Undaria pinnatifida*; bioactive compounds; vibriosis; antimicrobial activities; antioxidants; NMR

Citation: Stabili, L.; Acquaviva, M.I.; Cecere, E.; Gerardi, C.; Petrocelli, A.; Fanizzi, F.P.; Angilè, F.; Rizzo, L. Screening of *Undaria pinnatifida* (Laminariales, Phaeophyceae) Lipidic Extract as a New Potential Source of Antibacterial and Antioxidant Compounds. *J. Mar. Sci. Eng.* **2023**, *11*, 2072. https://doi.org/10.3390/jmse11112072

Academic Editor: Aurélie Blanfuné

Received: 30 August 2023
Revised: 19 October 2023
Accepted: 27 October 2023
Published: 30 October 2023

1. Introduction

Oceans cover 70% of our planet, hosting an astonishing wealth of biodiversity and offering crucial ecosystem services. This impressive species richness, linked to a high chemical diversity, represents a renewable resource of new bioactive compounds. Recently, due to modern technologies, several marine organisms, including algae, have been studied as sources of new therapeutic agents. In particular, seaweeds represent a remarkable source of bioactive compounds in the pharmaceutical, cosmetics, nutraceutical, biotechnological, and food fields. It is well known that crude extracts of seaweeds and their purified fractions exhibit antiviral [1], anticoagulant [2], anticancer [3], antioxidant [4], and anti-inflammatory activities [5]. Therefore, natural products from seaweeds could play an alternative role in drug discovery. Secondary metabolites are generally produced by chemical defense systems against the biotic stress of consumers, predators, and epibionts [6], as well as the abiotic stress of the neighboring environment (e.g., UV, desiccation, nutrient

availability), affecting their synthesis with direct or mediated effects [7]. The antimicrobials, a class of bioactive compounds, can prevent or contrast the development and growth of opportunistic microorganisms. Metabolites inhibiting viruses, bacteria, mycetes, and other epibionts can be extracted by green, brown, and red marine algae with antiviral, cytostatic, antihelmintic, antimycotic, and antibacterial potential. Thus, seaweeds represent an attractive source of potential biocompounds as alternatives to the conventional drugs that are useful to control multidrug-resistant strains of pathogenic microorganisms and new diseases. The protracted and indiscriminate employment of antimicrobial drugs has indeed led to therapeutic failures and resistant pathogens [8]. Today, there is a need to find new efficient biocompounds [9], both for new pathogens [7] and for classical microorganisms that become resistant to conventional drugs [10]. Since there are several multidrug-resistant bacterial strains among the microorganisms that have developed new strategies to elude the action of antibiotics, these potential substances are now receiving considerable attention from the pharmaceutical sector. A public health priority is to address the resistance to antibiotics among pathogens, exploring and developing effective and cheap natural antimicrobial compounds with minimal toxicity, higher bioavailability, and reduced side effects compared to existing antibiotics [11]. It is also useful to evaluate the marine antimicrobials for potential synergisms with traditional drugs [12]. In this context, although the beneficial effects of marine seaweeds have been well known for millennia in traditional medical treatment [9], antimicrobial substances secreted by algae were observed only in the second half of the 1910s [13]. Potent antimicrobial activities belonging to fatty acids, polysaccharides, pigments, phlorotannins, lectins, terpenoids, alkaloids, and halogenated compounds are exhibited by compounds from green, brown, and red algae [14].

Seaweeds are also a notable source of antioxidant compounds [4], since they are produced as a result of interactions with biological and environmental variables such as herbivory, light, depth, salinity, nutrient availability, and seasonality, as well as intrinsic factors such as algal tissue, age, and size [15]. The antioxidant potential of seaweeds, ascribed to natural products, belongs to an array of several structural classes of compounds, including phenolics, polysaccharides, and pigments [16–18]. Moreover, substances displaying antioxidant activity against free radicals and reactive oxygen species (ROS) have been found in brown, red, and green algae. In humans, the ROS, as products of oxygen metabolism and normal cellular functioning, are involved in several important biochemical processes, such as defense against infections, neurotransmission, gene regulation, vasodilation, and oxidative signaling [19,20]. However, changes in the balance between pro- and antioxidant reactions in cells can generate redox imbalance and oxidative stress, leading to great generation of ROS and free radicals, resulting in severe cellular damage [19,21–23]. Free radicals and ROS further interact with key organic molecules such as RNA, DNA, lipids, and proteins, disrupting their function or structure and subsequently leading to the onset of diseases, such as diabetes [24], atherosclerosis [25], rheumatoid arthritis [26], neurodegenerative diseases [27], inflammatory diseases [28], immune system disorders, aging, and cancer [29,30]. Both enzymatic and non-enzymatic antioxidants play an important role in the defense systems of organisms against free radicals [31]. However, small quantities of antioxidants are present in the body's cells, while natural or synthetic antioxidants may be introduced as a component of the diet, or as additives. Recently, health and safety concerns have been raised for synthetic antioxidants. Therefore, natural antioxidants have attracted attention and are broadly utilized today [32]. In particular, several compounds from seaweeds have been shown to be ecofriendly tools for the control of oxidative stress [16,33].

In this framework, in the present study, we investigated the antibacterial and antioxidant activity of the seaweed *Undaria pinnatifida* (Harvey) Suringar, a cold-temperate species belonging to the family Alariaceae that is native to Japan and also widely distributed in China and Korea [34]. As a consequence of its cultivation in the French Atlantic, starting from the late 1980s, invasive populations were detected in the Mediterranean Sea, New

Zealand, Tasmania, Argentina, and Australia [35]. In the Mediterranean Sea, this species was first found in the Thau Lagoon (France) in the early 1980s. Afterwards, it was observed near the fishing markets of Chioggia and Venice (northern Adriatic Sea, Italy) in the early 1990s, where it rapidly colonized the hard substrata, and from 1998 to 2010 in the Mar Piccolo of Taranto (Ionian Sea, Southern Italy). The species strongly settles on the substratum through thick rhizoids, and its eradication is very problematic and expensive [36]. The sporophytes, after their winter–spring growth, completely disappear in summer, when only microscopic gametophytes are present. In this context, we focused on the antioxidant and uninvestigated antibacterial activity of *U. pinnatifida* blades, sporophylls, and holdfasts against several *Vibrio* species, including some pathogens for farmed fish and some emerging pathogens (e.g., *Candida famata*, *Enterococcus* sp.) capable of developing resistance to conventional antibiotics. Moreover, for the first time, NMR spectroscopy was applied in order to provide a clear and unequivocal one-shot determination of the complete molecular contents in the specific *U. pinnatifida* extracts. The data were discussed in the light of new perspectives in order to encourage eradication programs and, at the same time, support recycling and biotechnological applications.

2. Materials and Methods

2.1. Study Site and Species Collection

Undaria pinnatifida was collected from the Venice Lagoon in March 2015, in aseptic containers at a depth of 0.5–2 m, and immediately transferred to the laboratory under controlled conditions (4 °C). Three replicates of about 500 g of fresh material were processed. The species was unequivocally identified based on morphological traits [34], such as the form of the sporophylls and the position of the sori (Voucher 47Fbis, TAR Herbarium, Consiglio Nazionale delle Ricerche, Istituto di Ricerca sulle Acque; Legit: Adriano Sfriso, Determinavit: Antonella Petrocelli).

2.2. Preparation of Lipidic Extracts from Macroalga

The seaweed samples were cleaned of epibiota and necrotic tissues with a mixture of ethanol and sodium hypochlorite [37]. The samples were further rinsed with sterile water to remove any associated debris. The seaweed was split into blades, sporophylls, and holdfasts. The freshly cleaned material was air-dried and finely granulated, and then 3 g of each sample was extracted in 150 mL of chloroform/methanol (2:1 at 55–60 °C for 24 h) using a Soxhlet apparatus. The extraction solvents were evaporated under vacuum at a controlled temperature. Then, 5 mg of extract was dissolved in 1 mL of ethanol (95% vol. J.T. Baker) and assayed for antimicrobial activity by the paper disc diffusion method [38].

2.3. Test Microorganisms

The antibacterial activity was tested against several microbial strains. In particular, we focused on human pathogenic microbial strains capable of developing resistance to conventional antibiotics. *Candida albicans*, *Candida famata*, *Enterococcus* sp., *Pseudomonas* sp., and *Staphylococcus* sp., kindly furnished by Vito Fazzi Hospital of Lecce, along with several *Vibrio* strains, including some pathogens for farmed fish (*Vibrio aestuarinus*, *V. alginolyticus*, *V. brasiliensis*, *V. chagasii*, *V. carchariae*, *V. corallilyticus*, *V. diazotrophicus*, *V. fischeri*, *V. fluvialis*, *V. furnisii*, *V. inusitatus*, *V. lentus*, *V. litoralis*, *V. mediterranei*, *V. metchnikovii*, *V. mimicus*, *V. natriegens*, *V. nereis*, *V. parahaemolyticus*, *V. splendidus*, and *V. vulnificus*) isolated and identified from seawater and algal samples [39–45] and conserved in the microbial collection (BioForIU https://www.unisalento.it/-/laboratorio-di-bio4iu, accessed on 1 January 2022) of the University of Salento, were used to test antibacterial activity.

2.4. Antimicrobial Activity

The Kirby–Bauer method [46] was used to assess the antimicrobial activity. Sterile 6 mm diameter paper discs (AA, Whatman International Ltd., Maidstone, Kent, UK) were wetted with several quantities of lipidic extract (10, 20, 30, 40, 60, 80, 100 µL) and then

left to air-dry at room temperature for 4 h [47]. Two discs were prepared as controls for each test: the former was wet with a carrier solvent, and the latter with an "extraction blank" represented by MeOH/$CHCl_3$ used as solvent in extraction and then dried and resuspended in ethanol. The positive controls were represented by the vibriostatic agent O/129 at a concentration of 10 µg, giving a growth inhibition diameter of 12 mm inhibition for vibrios, and by hen egg-white lysozyme at 0.76 mg/mL, giving a growth inhibition diameter of 12 mm for the other microbial strains. One hundred microliters of each microbial suspension (about 10^8 CFU mL^{-1}) was spread [48] on a specific agarized medium (Marine Agar 2216-Difco for environmental strains, Plate Count Agar-Difco for human pathogenic bacterial strains, and Sabouraud Dextrose Agar—Difco for yeasts) for each tested bacterial and fungal strain under sterile conditions; the Petri dishes inoculated with *Vibrio* species were incubated at 30 °C, while those with human pathogenic strains were incubated at 37 °C. A clear zone around the discs showed the antimicrobial activity, indicating the microbial growth inhibition. Then, the diameter of this clear zone was measured in millimeters.

2.5. Antioxidant Activity

2.5.1. Oxygen Radical Absorbance Capacity (ORAC) Assay

The ORAC assay was carried out as described by Stabili et al. [39]. The assay was performed in black-walled 96-well plates (Greiner-Bio One, Monroe, NC, USA) in a final volume of 200 µL. Using fluorescein (λEX = 485, λEX = 535) as a fluorescent probe and AAPH as a free-radical initiator, the protective effect of the extracts was evaluated by comparison of the area under the fluorescence decay curve (AUC) of the sample and the area of the blank (i.e., absence of antioxidant). The fluorescence intensity of fluorescein was assessed by using an Infinite200 Pro plate reader (Tecan, Männedorf, Switzerland) every minute, for a total of 80 min. A standard curve was constructed using 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox, Sigma-Aldrich, Oakville, ON, Canada, 1.5–10.5 µM). The estimates were reported as µmoles of Trolox equivalents (TE) per g of lipidic extract. All of the reaction mixtures were prepared in triplicate and, for each sample, at least three independent assays were carried out.

2.5.2. Trolox Equivalent Antioxidant Capacity (TEAC) Assay

The TEAC assay method described by Stabili et al. [39] was applied to a microplate reader. The absorbance reading of azinobis (3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS, Sigma-Aldrich) radical cations was assessed at 734 nm after 6 min from the initial mixing using an Infinite200 Pro plate reader (Tecan, Männedorf, Switzerland). A standard calibration curve of Trolox (0–16 µM) was constructed. The extracts were assessed in triplicate and in at least three separate dilutions. The inhibition of absorbance of ABTS•+ of the lipidic extract was measured at 734 nm using Magellan v 7.2 software. The percentage decrease in absorbance was plotted as a function of the concentration of Trolox, and the TEAC value was expressed as Trolox equivalents (in µmol) per g of lipidic extract.

2.5.3. Folin–Ciocalteu (F-C) Assay

The F-C assay was performed in algal lipidic extracts as described by Stabili et al. [39], in order to evaluate the total phenolic contents, expressed as gallic acid equivalents (mg-GAE)/g of lipidic extract. The assay was performed in triplicate using a microplate reader (Tecan, Infinite M200). A standard curve of gallic acid was performed in the range from 2.5 to 40.0 mg L^{-1} ($R \geq 0.9997$).

2.6. NMR Spectroscopy

The lipidic extracts from the blades, sporophylls, and holdfasts of *U. pinnatifida* were characterized by 1D and 2D NMR spectroscopy, as reported by Stabili et al. [49]. The 1D ^1H and 2D ^1H Jres, ^1H-^1H COSY, ^1H-^{13}C HSQC, and ^1H-^{13}C HMBC spectra were obtained at 298 K on a Bruker Avance III NMR spectrometer (Bruker Biospin, Milan, Italy) operating at

600.13 MHz for ^{1}H observation, equipped with a TCI cryoprobe (triple-resonance inverse cryoprobe) incorporating a *z*-axis gradient coil and automatic tuning/matching (ATM). A total volume of 600 µL ($CDCl_3$/CD_3OD) was obtained for each lipidic extract and transferred to a 5 mm NMR tube, using tetramethylsilane (TMS) as an internal standard (δ = 0.00). For each lipidic sample, a standard ^{1}H NMR experiment was run with the following parameters: 64 transients with a 2 s repetition delay, 64,000 data points, spectral width of 20.0276 Hz, 90° power pulse (p1) for 8 µsec, and a power level of 8.05 dB. The acquisition and processing of the spectra were performed using Topspin 3.6.1 software (Bruker Biospin, Milan, Italy). The resonances of the metabolites were assigned on the basis of data from the literature, and the eventual oxidized lipidic compounds were recorded in the range of 5.47–8.99 ppm [39,49–51]. Furthermore, a quantitative determination of the percentage of total fatty acids (FAs) was conducted by ^{1}H NMR spectroscopy. The FA concentration was obtained by integrating the NMR signals in the range of 0.86–0.90 ppm, relative to the CH_3 terminal methyl groups of the fatty acids, using the TMS resonance peak as an internal standard and the values already reported in the literature [52]. In the dry algal biomass, the percentages of the FAs related to the lipidic fraction were determined. Finally, for lipidic extracts of the blades, sporophylls, and holdfasts, the relative abundance was evaluated by analyzing selected distinctive unbiased NMR signals after spectral normalization [50].

2.7. Thin-Layer Chromatography

Thin-layer chromatography (TLC) analysis was performed on *U. pinnatifida* lipidic extracts (blade, sporophyll, and holdfast) to compare the data on pigments from the NMR analysis. Silica gel TLC plates were developed at room temperature with a mixture of hexane and acetone (3:2) as an eluent solution. Pigments directly detected by visible light from the TLC plates were identified by retention factors (Rf) [49,53].

3. Results

3.1. Antimicrobial Activity

The in vitro assays highlighted the presence of antibacterial activity in all of the tested *U. pinnatifida* lipidic extracts related to the three different portions (blade, sporophyll, and holdfast).

The obtained results of the antimicrobial action against the utilized bacterial strains are reported in Table 1, showing the diameters of microbial growth inhibition, ranging from 6.5 to 12 mm. In particular, the lipidic extract from *U. pinnatifida* blades revealed antimicrobial activity against several tested *Vibrio* species (*V. aestuarinus*, *V. chagasii*, *V. diazotrophicus*, *V. fischeri*, *V. furnisii*, *V. inusitatus*, *V. litoralis*, *V. mediterranei*, and *V. splendidus*). Also, the sporophyll and holdfast showed antibacterial activity against all of these vibrios except for three of them (i.e., *V. chagasii, V. diazotrophicus*, and *V. splendidus*). Interestingly, lipidic extracts from the sporophyll and holdfast showed antibacterial activity against the human pathogen *Staphylococcus* sp. All of the utilized yeasts (*Candida albicans* and *C. famata*) were unaffected in their growth by the seaweed lipidic extracts, as were the human pathogens *Enteroccus* sp., and *Pseudomonas* sp.

3.2. Antioxidant Activity

The antioxidant activity of the lipidic extracts from *U. pinnatifida*, as determined by the TEAC and ORAC assays, is reported in Table 2. The antioxidant capacity of the algal extracts evaluated by the ORAC assay was higher than the activity measured by the TEAC assay. The results revealed that the lipidic extracts from different parts of the algal thallus possessed different TEAC activities, while ORAC assays showed no significant differences between the blade, sporophyll, and holdfast extracts. In addition, to provide an exhaustive assessment of the algal extracts' antioxidant capacity, the Folin–Ciocalteu assay was performed (Table 2).

Table 1. Antimicrobial activity of the sporophyll, holdfast, and blade of *U. pinnatifida* lipidic extracts. Positive controls: vibriostatic agent O/129 (10 µ), growth inhibition diameter = 12 mm inhibition for vibrios; hen egg-white lysozyme (0.76 mg/mL), growth inhibition diameter = 12 mm for the other microbial strains.

	Algal Sporophyll—Diameter of Growth Inhibition (mm)							**Algal Holdfast—Diameter of Growth Inhibition (mm)**							**Algal Blade—Diameter of Growth Inhibition (mm)**						
Microbial Strain	**10 µL**	**20 µL**	**30 µL**	**40 µL**	**60 µL**	**80 µL**	**100 µL**	**10 µL**	**20 µL**	**30 µL**	**40 µL**	**60 µL**	**80 µL**	**100 µL**	**10 µL**	**20 µL**	**30 µL**	**40 µL**	**60 µL**	**80 µL**	**100 µL**
Candida albicans	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Candida famata	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Enterococcus sp.	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Pseudomonas sp.	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Staphylococcus sp.	0	7	7	7	7	7	7	0	0	7	7	7	7	7	0	0	0	0	0	0	0
Vibrio aestuarinus	7	7	8	8	8	9	9	8	8	8	8	8	9	9	0	0	6.5	6.5	7	7	7
Vibrio alginolyticus	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Vibrio brasiliensis	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Vibrio carchariae	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Vibrio chagasii	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	7	8
Vibrio corallilyticus	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Vibrio diazotrophicus	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	8
Vibrio fischeri	10	10	10	10	10	10	10	8	8	9	9	9	10	10	8	8	8.5	8.5	8.5	8.5	9
Vibrio fluvialis	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Vibrio furnisii	0	0	7	7	7	7	8	7	7	8	8	8	8	9	7	7	7	7	7	7	8
Vibrio inusitatus	7	7	7	8	8	8	10	6.5	7	7	7.5	8	9	10	0	0	7	7	8	9	9
Vibrio lentus	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Vibrio litoralis	8	8	9	8	8	8.5	11	7	7.5	9	9	10	10	12	7	7	8	8	8	9	10
Vibrio mediterranei	0	0	7	7	7	8	9	6.5	7.5	8	8	9	9	10	0	0	0	7	7	8	9
Vibrio metchnikovii	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Vibrio mimicus	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Vibrio natriegens	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Vibrio nereis	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Vibrio parahaemolyticus	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Vibrio splendidus	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	7	8	8
Vibrio vulnificus	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0

Table 2. Antioxidant activity and total phenolic contents of *U. pinnatifida* lipidic extracts, as determined by the TEAC, ORAC, and Folin–Ciocalteu assays. Data are the mean ± SD (n = 3). Different letters indicate significant differences at $p < 0.01$.

Sample	TEAC μmol Trolox Equivalent/g Extract	ORAC μmol Trolox Equivalent/g Extract	Folin–Ciocalteu (mgGAE/g Extract)
Blade	126.907 ± 28.993 [a]	165.53 ± 14.955 [a]	11.765 ± 0.505 [a]
Sporophyll	88.773 ± 15.599 [b]	189.597 ± 20.469 [a]	7.415 ± 0.145 [b]
Holdfast	73.187 ± 7.916 [c]	170.083 ± 12.062 [a]	5.949 ± 0.212 [c]

3.3. NMR Spectroscopy

Characteristic 1D ^{1}H NMR spectra of the *U. pinnatifida* blade, sporophyll, and holdfast lipidic extracts are shown in Figures 1 and 2, and the assignments are reported in Table 3. All of the ^{1}H NMR spectra of the lipidic fractions of the algae were characterized by the presence of phytosterols, triacylglycerols (TAGs), phospholipids, and unsaturated and saturated fatty acids (UFAs and SFAs, respectively). The phytosterols, such as fucosterol, the principal sterol present in *U. pinnatifida*, were identified at low frequencies, at 0.69–0.71 ppm (signal 1, Figure 1a–c). The presence of fucosterol was also confirmed by signals in the range of 5.74–5.81. The main lipid signals corresponding to the methyl groups (CH_3) of all fatty acid chains were observed at 0.88 ppm (signal 2, Figure 1a–c). Broad signals in the ranges of 1.26–1.34 (signal 4, Figure 1a–c), 1.59–1.65, and 2.26–2.37 ppm were revealed and identified as characteristic of n-CH_2 and the β- and α-methylene protons (–$\underline{CH_2}$-CH_2-COOH and $C\underline{H_2}$-COOH respectively) of all fatty acids (signal 6, Figure 1a–c). The UFAs were detected by resonance at 2.00–2.10 and 5.29–5.45 ppm (signals 5 and 11, respectively, Figure 1a–c), corresponding to allylic (–$C\underline{H_2}$CH=CH–) and olefinic (–C$\underline{H}$=C$\underline{H}$–) protons, respectively. In detail, ω-9 monounsaturated fatty acids (MUFAs) (such as oleic acid), ω-6 polyunsaturated fatty acids (PUFAs), and ω-3 PUFAs, with resonances at 2.01, 2.03, and 2.07 ppm, respectively, were identified. Furthermore, the presence of ω-6 PUFAs, such as linolenic acid, and ω-3 PUFAs, such as linoleic, arachidonic, and eicosapentanoic acids, was confirmed by signals of bis-allylic protons (–CH=CH–$C\underline{H_2}$CH=CH–) at 2.75–2.86 ppm (signals 3, 7, and 8, Figure 1a–c). The spectra also indicated the presence of phospholipids, such as phosphatidylcholines (PC), by the signal at 3.22 ppm due to the $N(CH_3)_3$. In addition, the signals at 4.30, 4.17, and 5.25 ppm were ascribed to TAGs—in particular to CH sn1–3 and CH2 sn-2 of the glycerol moiety—while the presence of diacylglycerols (DAGs) and monoacylglycerols (MAGs) was detected by the signals at 4.08–4.12 ppm and 3.66 (^{13}C 70.5 ppm), respectively (signals 9 and 10, Figure 1a–c). Moreover, a set of aromatic signals was observed consistent with the presence of dehydroabietic and abietic acids (7.16 ppm, 7.00 ppm, and 6.88 ppm). In addition, the signals of 7.58 ppm and 7.73 ppm were assigned to aromatic protons of alkaloid species (signal 12, Figure 2a–c). In the downfield regions, the signals due to pigments were recorded. In particular, signals at 9.55 ppm and 9.75–9.78 ppm were attributed to chlorophyll a and b, respectively (signals 13 and 14, Figure 2a–c), while other signals at 9.45 ppm and in the range of 9.62–9.66 ppm were assigned to chlorophyll derivatives, such as pheophytin a and b (signal 15, Figure 2a–c). Oxidized lipidic compounds were not detected in the range of 5.47–8.99 ppm. In the dry algal biomass, the percentages of fatty acid related to the lipidic fraction were 6.64% for the sporophyll, 5.02% for the blade, and 2.28% for the holdfast, and they were determined by the integration of the NMR signals in the range of 0.86–0.90 ppm, using the TMS resonance peak as an internal standard and considering the values already reported in the literature [52].

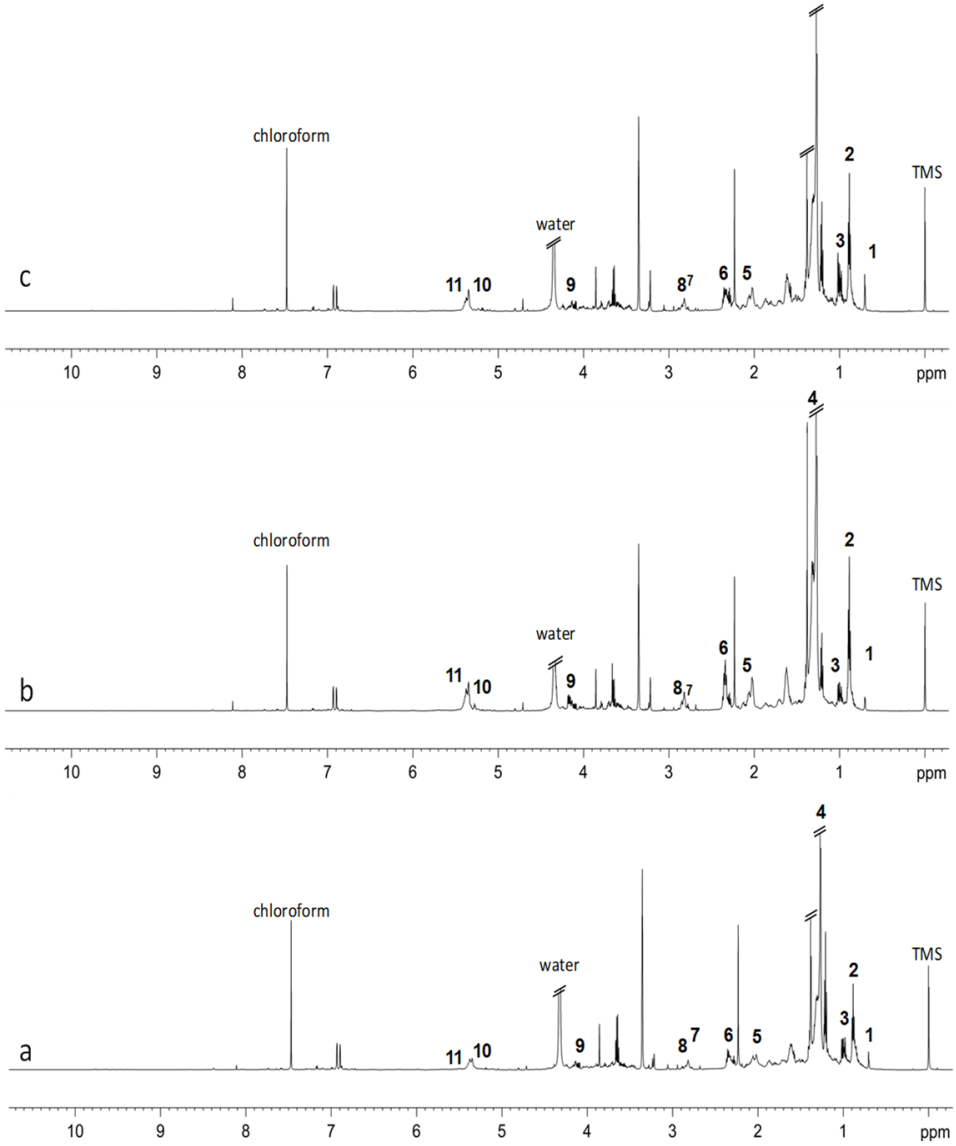

Figure 1. The ^{1}H NMR spectra of *U. pinnatifida* lipidic extracts in $CDCl_3$:CD_3CD: (**a**) ^{1}H NMR spectrum of the blade lipidic extract; (**b**) ^{1}H NMR spectrum of the sporophyll lipidic extract; (**c**) ^{1}H NMR spectrum of the holdfast lipidic extract (**1**. sterol; **2**. $-CH_3$ of all FAs; **3**. $-CH_3$ ω3 FAs; **4**. $-(CH_2)$n all FAs, **5.** CH_2–CH=CH for all UFAs; **6.** CH_2-COOH of FAs; **7.** =CH–CH_2=CH DUFAs; **8**. =CH–CH_2=CH PUFAs; **9.** sn 1,3 CH TGs, **10.** sn 2 CH_2 TGs, **11**. –CH=CH– vinyl groups of all UFAs).

Since the sporophyll, when compared to the blade and holdfast, was the compartment with the highest amounts of biocompounds, such as lipids [52], the relative contents of the different compounds in this compartment was calculated. In particular, by the integration of unbiased NMR signals with respect to pheophytin b, which was the least concentrated identified species, the relative abundances were reported as log2 fold change (FC) ratios (Table 4).

Furthermore, the relative abundances of the different biocompounds and pigments, through pairwise comparisons among the different compartments examined, were calculated as the log2 fold change (FC) ratio and are reported in Figure 3.

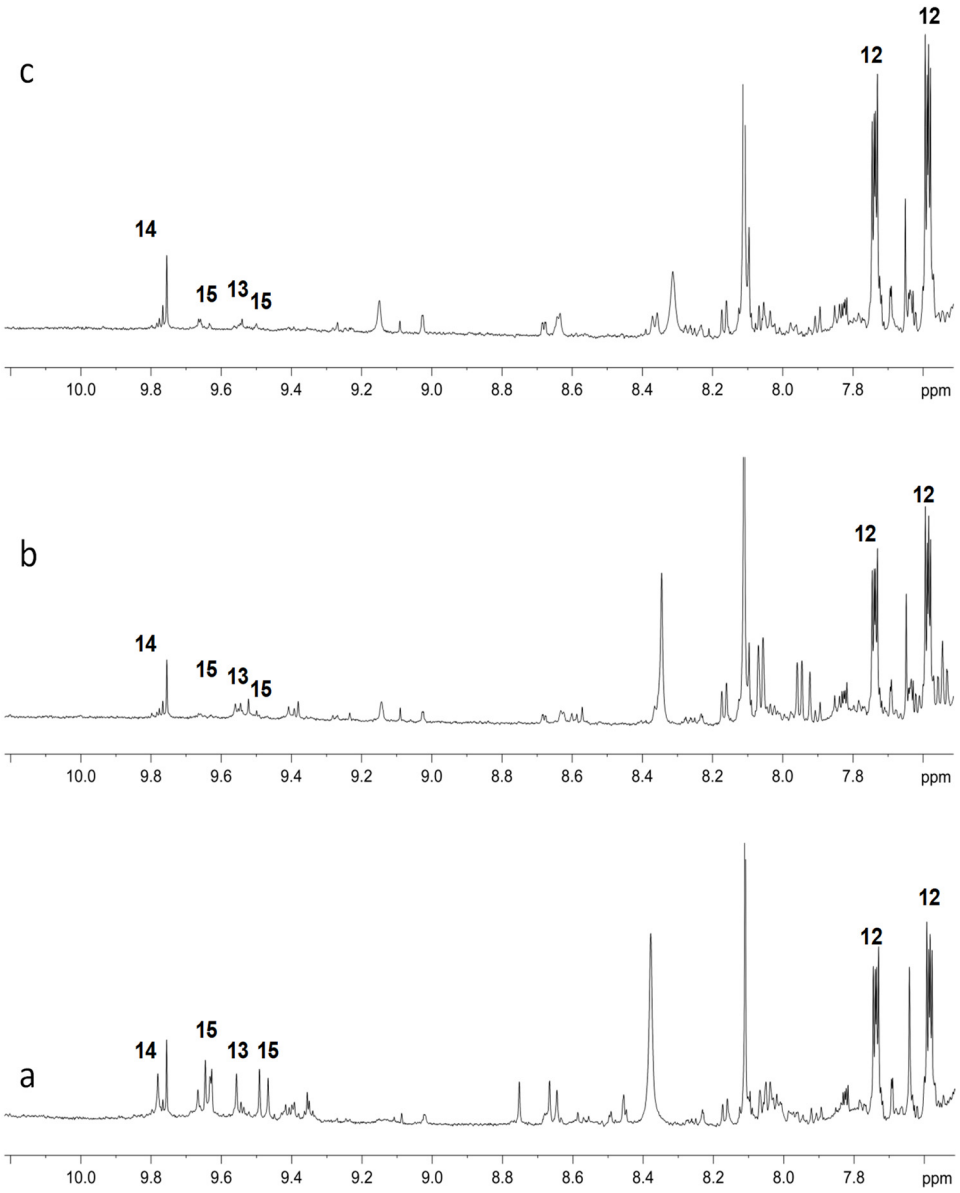

Figure 2. Expansion of the ^{1}H NMR spectra of *U. pinnatifida* lipidic extracts in $CDCl_3$:CD_3CD: (**a**) expansion of the ^{1}H NMR spectrum of the blade lipidic extract; (**b**) expansion of the ^{1}H NMR spectrum of the sporophyll lipidic extract; (**c**) expansion of the ^{1}H NMR spectrum of the holdfast lipidic extract (**12.** alkaloid species; **13.** chlorophyll a; **14.** chlorophyll b; **15** pheophytins).

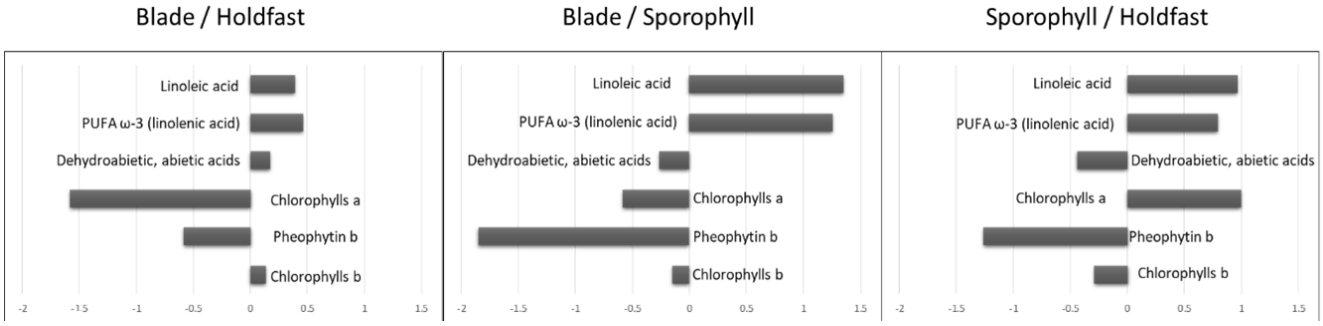

Figure 3. Relative abundance of biocompounds and pigments among blades, sporophylls, and holdfasts.

Table 3. Chemical shifts ^{1}H (ppm) and assignments of metabolite resonances in the ^{1}H NMR spectrum of *U. pinnatifida* lipidic extracts.

Compound	Assignment	δ^{1}H (ppm, Multiplicity)
Sterol	$-CH_3$	0.70
All FAs (SFAs, UFAs)	$-CH_3$	0.86–0.90
All FAs	$-(CH_2)-$	1.26–1.34 (m)
	$COOCH_2\underline{CH_2}$	1.59–1.65 (m)
UFAs	$-\underline{CH_2}CH=\underline{CH}-$	2.00–2.10
All FAs	$\underline{CH_2}-C=O$	2.26–2.37
ARA	CH_2-COOH	2.38
DUFA (linoleic acid)	CH_2	2.75–2.79 *
ω-3 PUFA (linolenic acid)	CH_2	2.79–2.86 *
MAGs		
DAGs	CHOCO	3.66
TGs		
	$OH-CH_2-CH$	4.08–4.12
	2′CHOCO	5.25 (m)
	CH_2 (sn1,3)	4.17, 4.30
DUFA	CH_2 (sn1,3)	4.28
ω-3 PUFA	CH (sn2)	5.28
All UFAs	CH=CH	5.30–5.45 (m)
Dehydroabietic and abietic acids	CH	6.88
	CH	7.00
	CH	7.16 *
Alkaloid species		7.58
		7.73
Chlorophyll a	CH-5	9.55 *
Chlorophyll b		9.75 *
		9.78
Pheophytin a		9.45
Pheophytin b		9.62
		9.66 *

FAs—fatty acids, SFAs—saturated fatty acids, UFAs—unsaturated fatty acids, ARA—arachidonic acid, DUFAs—diunsaturated fatty acids, PUFAs—polyunsaturated fatty acids, MAGs—monoacylglycerols, DAGs—diacylglycerols, TGs—triacylglycerols. Asterisks indicate the signals selected for the relative abundance comparison and evaluation among the different compartments of *U. pinnatifida* reported in Figure 3 and Table 4.

Table 4. Relative abundances of biocompounds in the sporophyll lipidic extract, reported as log (FC).

Biocompound	Log_2 (FC)
Chlorophyll b	1.85
Chlorophyll a	1.26
Dehydroabietic and abietic acids	6.11
ω-3 PUFA (linolenic acid)	9.32
Linoleic acid	7.00

3.4. Thin-Layer Chromatography

Thin-layer chromatography (TLC) analysis was performed on *U. pinnatifida* lipidic extracts (Figure 4), specifically on blade, sporophyll, and holdfast lipidic extracts. The TLC analysis revealed different pigment profiles among the three compartments of the seaweed analyzed. The blade was abundant in pigments compared to the sporophyll and holdfast. In particular, for the blade, two blurry bands corresponded to carotenes (extremely up), and two grey bands were recognized for pheophytins (a and b). Moreover, two other bands (blue–green and green) for chlorophylls (a and b) and three yellow bands for xanthophylls were also detected. The sporophyll and holdfast showed unclear bands, but they were comparable to those of the blade.

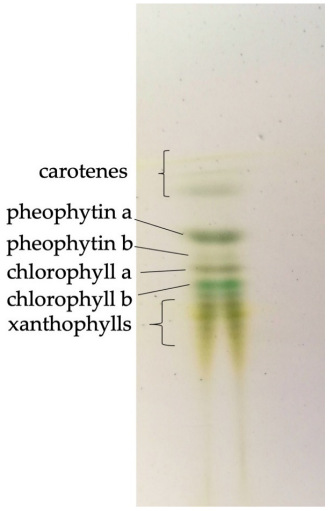

Figure 4. Thin-layer chromatography (TLC) plate of *U. pinnatifida* blade lipidic extract.

4. Discussion

Seaweeds represent a renewable source of bioactive compounds with several commercial applications in the pharmaceutical, medical, cosmetics, and food industries, as well as in agriculture [54]. In this scenario, studies on seaweeds from the Mediterranean Sea, and in particular from the southern Ionian Sea, have already been in progress for several years [39,49,55–57]. In the present study, we focused on the invasive brown macroalga *U. pinnatifida*, a successful invader of many temperate coasts around the world [58], commonly called "wakame" and cultivated in Japan since ca. 700 B.C., where it is one of the most used edible macroalgae [59]. In Italy, *U. pinnatifida* was first found near the fishing markets of Chioggia and Venice during the early 1990s, where it rapidly colonized the hard substrata [60], and then it was recorded within the Mar Piccolo of Taranto (Ionian Sea, Southern Italy) in 1998 [61]. Since the bioactivities and chemical features of seaweeds can depend on different variables, such as the specific part of the alga, local climate, depth, latitude, salinity, temperature, and collection time [56,62,63], in this study we considered *U. pinnatifida* collected in the Venice Lagoon, and we analyzed three different portions (blade, sporophyll, and holdfast) of this species. In particular, we ascertained whether the biomass related to these different portions could be used for several applicative purposes in order to encourage eradication programs and, at the same time, support recycling and biotechnological applications. In the lipidic extracts of the blade, sporophyll, and holdfast of the investigated alga, natural bioactive compounds that are potentially useful for the food and pharmaceutical industries were identified and characterized. In particular, the antibacterial and antioxidant activities of *U. pinnatifida* were assayed, and the chemical characterization was carried out by means of advanced analytical techniques such as multidimensional NMR spectroscopy and by thin-layer chromatography. The antibacterial activity of *U. pinnatifida* has already been investigated, focusing mainly on bacterial species such as *Escherichia coli*, *Bacillus subtilis*, *Candida albicans*, *Streptococcus mutans*, and *Staphylococcus aureus* [64]. Moreover, Zhang et al. [65] described virucidal activity, and Ferreira et al. [66] reported antimicrobial activity against *Pseudomonas aeruginosa* and *Staphylococcus aureus* in the phlorotannin-enriched extract from *U. pinnatifida*. Here, we increased the knowledge on this topic, focusing on several *Vibrio* species, including some pathogens for farmed fish and some of the human emerging pathogens that are capable of developing resistance to conventional antibiotics. The detection of uninvestigated antibacterial activity against different *Vibrio* species was a noteworthy result, since the diseases called vibriosis, which can affect both animals (mainly fish and mollusks) and humans, can be caused by different species of *Vibrio* [67]. This result is even more crucial when taking into account that the lipidic extracts from *U. pinnatifida* blades, sporophylls, and holdfasts exhibited great antibacterial activity against several *Vibrio* strains (i.e., *Vibrio aestuarinus*, *V. fischeri*, *V. furnisii*,

V. inusitatus, *V. litoralis*, and *V. mediterranei*). The blade was also capable of inhibiting the growth of *V. chagasii*, *V. diazotrophicus*, and *V. splendidus*. Vibrios exert great pressure on aquatic organisms in marine ecosystems, as well as in the seafood industry, posing growing problems related to bacterial pathogen contamination, the health of marine products, and risks to global food safety [68–70]. In the present research, the recorded antibacterial activity of the algal lipidic extracts against *V. aestuarianus*, *V. changasii*, *V diazotrophicus*, *V. furnissii*, *V. mediterranei*, and *V. splendidus* is a relevant finding, since these vibrios are considered to be relevant pathogens in aquatic systems. *Vibrio aestuarinus* has been isolated from estuarine waters and shellfish from the Oregon coast for the first time [71], and subsequently identified as a pathogen of the oyster *Crassostrea gigas* collected in the field and from a French hatchery [72]. Interestingly, *V. splendidus*, widespread in estuaries and seawater, is considered to be responsible for diseases of several aquatic organisms, including fishes, echinoderms, crustaceans, and bivalves. In the fish industry, *V. splendidus* is able to cause high mortality in mollusks and flatfish [73,74]. Recently, several environmental matrices and marine organisms are thought to be reservoirs or hosts of *Vibrio aestuarianus* and *V. splendidus* clade bacteria [75]. The bacterium *V. furnissii* is one of the non-cholera-*Vibrio* species that are pathogenic to humans [76], implicated in gastroenteritis due to the ingestion of undercooked contaminated seafood [77,78]. *Vibrio chagasii*, previously isolated from various environments, including marine sediments, seawater, *Artemia* rotifers, turbot larvae, and sea bass [78–80], has been reported as a bacterium involved in oyster, scallop, and mussel diseases [81–83]. More recently, it was held responsible for the massive mortality of *Argopecten purpuratus* scallop larvae during vibriosis outbreaks in commercial scallop larvae hatcheries in Northern Chile [84]. Among the diseases caused by *Vibrio* spp., larval vibriosis can seriously threaten both the fishing industry and the marine ecosystem, leading to high mortality of the organisms [85,86]. *Vibrio diazotrophicus* and *V. mediterranei* are known to affect the larvae of marine organisms and, interestingly, in the present study, we proved that their growth was inhibited by algal lipidic extracts. The bacterial strain *V. diazotrophicus* has been found in the gastrointestinal tracts of sea urchins collected in Nova Scotia, Canada, as well as on the surfaces of reeds growing in a drainage ditch in Kent, England [87]. It is an opportunistic pathogen capable of causing intestinal inflammation, immunocyte migration, and cytokine induction in the larvae of the sea urchin *Strongylocentrotus purpuratus,* leading to potential ecological consequences for the larvae's survival [85]. Furthermore, *V. mediterranei*, widely distributed in marine environments, is a potential emerging pathogen in marine organisms [88], recognized as a potential etiological agent of the yellow-spot disease of algae belonging to the genus *Pyropia* [89]. Recently, *V. mediterranei* has been observed to lead to mass mortality in bivalve larvae and juveniles in hatcheries, as reported in 2019 in the razor clam *Sinonovacula* in a shellfish hatchery in China [86]. In addition, within the Mediterranean Basin, *V. mediterranei* is also considered to be pathogenic to the noble pen shell *Pinna nobilis* [90]. *Vibrio fischeri* is known as a bioluminescent marine symbiont in the Hawaiian bobtail squid *Euprymna scolopes* and has been used as a model of symbiosis [91]. However, it was also isolated from shrimp farms located in peninsular India during a mass mortality event due to outbreaks of white-spot syndrome virus and shell disease, and it is considered to be a moderately opportunistic pathogen of shrimp, together with *Vibrio vulnificus* and *Photobacterium damsela* [92]. As for *V. inusitatus* and *V. litoralis*, little information is available on these strains; *Vibrio litoralis* was first isolated from seawater in a tidal flat of the Yellow Sea in Korea [93], while *V. inusitatus* was isolated from the guts of the abalones *Haliotis discus discus*, *H. gigantea*, *H. madaka*, and *H. rufescens* [94]. However, their role within the host has not yet been studied. In order to fight all of the above-mentioned infections and fish diseases, the research of innovative and sustainable additives is essential to reduce the consequences on human health and ecosystems. This is particularly important, since providing food for human beings and protecting the planet from degradation are two important goals to be reached according to the 2030 UN Agenda of Sustainable Development Goals (SDGs) [95]. In this scenario,

seaweeds appear to be a sustainable bioresource [14], and *U. pinnatifida* could represent a good candidate for exploitation in this field.

Interestingly, the lipidic extracts of the sporophyll and holdfast of *U. pinnatifida* showed antibacterial activity against the human pathogen *Staphylococcus* sp. Staphylococci are the most isolated bacteria in human nosocomial infections and foodborne illnesses worldwide, and they are implicated in serious systemic diseases. Recently, several studies have demonstrated their emerging antibiotic resistance. In this scenario, the discovery of antibacterial activity of *U. pinnatifida* against these microorganisms represents a challenge with implications due to the need to identify antimicrobial agents with new modes of action.

In the present study, neither of the yeasts (i.e., *C. albicans* and *C. famata*) tested with the three lipidic extracts of *U. pinnatifida* were sensitive to their action. However, it should be underlined that we used lipidic extracts to test the antibacterial activity, and this could explain the differences observed compared to other studies with different kinds of extracts of the examined algal species [64,96].

In order to deeply characterize the three lipidic extracts from *U. pinnatifida*, assess the presence of compounds potentially responsible for the detected activities, and detect the consequent possible biotechnological applications, multinuclear and multidimensional NMR spectroscopy and thin-layer chromatography were also performed. The peculiar aspect of the present work is indeed represented by the use, for the first time, of NMR spectroscopy for the analysis of *U. pinnatifida* lipidic extracts in order to obtain a clear and unequivocal one-shot determination of the complete molecular contents in the specific extracts without the need for prior component separations [97]. In this respect, the used methodology represents an untargeted analysis of the overall contents of the considered samples, eliminating possible bias due to the component separation stage [97]. In the present study, the sporophyll lipidic extracts of *U. pinnatifida* were characterized by higher lipid contents compared to the blades, in agreement with the findings of Boulom at al. [52]. Moreover, NMR analysis showed the presence of different classes of lipids, such as glycerol moieties of monoacylglycerols (MAGs), diacylglycerols (DAGs), triacylglycerols (TGs), and phospholipids, as already evidenced by other researchers [98]. This analysis mainly highlighted the presence of saturated (SAFAs) and unsaturated (UFAs) fatty acids. In particular, oleic acid prevailed among the monounsaturated fatty acids (MUFAs), and the signals of ω-6 and ω-3 polyunsaturated fatty acids (PUFAs) were also recorded. Interestingly, the presence of ω-6 PUFAs, such as linolenic acid, and ω-3 PUFAs, such as linoleic, arachidonic (AA), and eicosapentaenoic (EPA) acids, was highlighted in the lipidic extracts of *U. pinnatifida* grown in the Venice Lagoon. The presence of oleic acid in *U. pinnatifida*, in particular in the sporophylls, was already recorded by Boulom et al. [52], who studied the seasonal changes in lipidic contents in this seaweed species. Furthermore, relevant amounts of oleic acid were already found in other macroalgae, such as the red seaweeds *Gracilariopsis longissima*, *Gracilaria incurvata* [99], *G. tikvahiae*, *G. corticata* [100], *G. verrucosa* [101], and *Iridaea cordata* [102]. As reported in the literature, PUFAs, especially C18 (linolenic and linoleic acids) and C20 (AA and EPA), are contained in brown algae [103]. Some of the essential PUFAs, such as AA and docosahexaenoic acid (DHA), are components of the brain membrane's phospholipids. Vertebrates belonging to the class Mammalia need to obtain these acids from food supplements, as they cannot produce them by themselves. Interestingly, the main causes of human chronic diseases are driven by the inappropriate consumption of fatty acids [104]. Fish oil and food sources of animal origin contain ω-3 PUFAs, while vegetable oils mainly provide ω-6 PUFAs. In this context, it is crucial to point out the production of DHA and eicosapentaenoic acid (EPA) from marine seaweeds, since they show evidence of being an outstanding source of PUFAs, with a ω-6 FA:ω-3 FA ratio of less than 10, which is the value strongly recommended by the World Health Organization (WHO). This could be achieved through the consumption of some edible wellsprings of ω-3 and ω-6 FAs, useful for preventing inflammatory, cardiovascular, and neuro-chronic sickness [105]. Thus, based on our findings, we can also confirm that *U. pinnatifida* collected in the Venice Lagoon represents an excellent natural source of fatty

acids to be employed in enriched foods. The nutrient properties of this seaweed are well known, and for these reasons it is exploited as a healthy food or nutraceutical additive [98]. Moreover, the biomass of *U. pinnatifida* can be employed directly as a feed supplement in various breeding farms, such as fish farms or poultry farms, as already experienced for other algae [106,107]. Until now, *U. pinnatifida* has mainly been used as an additive for pigs and rats [108,109]. However, replacing approximately 5% of the typical feed with "wakame" in sea bream hatcheries leads to improvements in the growth rate and the nutrient absorption by breeding fish in aquaculture [110], since seaweeds added to conventional feed proved to be beneficial to fishes' growth, lipid metabolism, stress responses, and disease resistance, due to their lipid and protein contents [111]. Moreover, fatty acids from seaweeds could be employed as antibacterial agents in several fields [39,56,57,59]. Oleic, linoleic and linolenic acids have antibacterial activity and capability to inhibit pathogenic microbial growth, as also observed in other algae [56,112,113]. The antibacterial activity against *Vibrio* species in *U. pinnatifida* from the Venice Lagoon seems to be related to the occurrence of these fatty acids. In addition to fatty acids, it must also be highlighted that the NMR analysis of the *U. pinnatifida* extract showed the presence of terpene moieties, important defense compounds mainly found in herbal plants and diets [114]. For example, dehydroabetic acid and abietic acid are diterpene resin acids that show different biological activities [49,114], such as antimicrobial, antitumor, antiviral, and cytotoxic activities [114]. In particular, abietic acid has bacteriolytic actions related to the interaction and lysis of cell membranes [115]. By means of NMR analysis, the signals due to pigments were recorded. In particular, signals at 9.55 ppm and 9.75–9.78 ppm were attributed to chlorophylls *a* and *b*, respectively, while other signals at 9.45 ppm and in the range 9.62–9.66 ppm were assigned to chlorophyll derivatives, such as pheophytins *a* and *b*. Moreover, TLC analysis showed that *U. pinnatifida* blades were characterized by the presence of carotenes, pheophytins and chlorophylls (*a* and *b*), and xanthophylls. Natural pigments are a group of bioactive compounds that exhibit antioxidant activities [65,110,116,117]. Carotenoids, including five loliolide derivatives, have already been identified by Kimura and Maki [118] in *U. pinnatifida*. In the present study, the lipidic extracts of algal blades, sporophylls, and holdfasts showed good antioxidant properties in vitro. In particular, we employed the TEAC, ORAC, and Folin–Ciocalteu assays because the use of a single method hampers the description of the total antioxidant capacity of the extracts [119]. The TEAC and ORAC methods have different sensitivity to the different antioxidant molecules (polyphenols, UFAs, carotenoids, pheophytins, and chlorophylls) found in the algal extracts. In particular, the ORAC assay exerts greater sensitivity and specificity than the TEAC assay and is able to estimate a greater number of antioxidants present in the total extract [120]. This could explain our results, as the antioxidant activity of different portions of algae measured by the ORAC test was higher than that resulting from the TEAC test. Previous studies have reported that the antioxidant capacity of carotenoids is greater when tested by ORAC than by TEAC [120]; thus, carotenoids present in *U. pinnatifida* extracts are hypothesized to contribute significantly to the observed increased antioxidant activity of the lipidic extracts when measured by the ORAC assay. The blade extract showed the highest total polyphenol content. Similar total polyphenol contents have been reported for *U. pinnatifida* in previous studies [121,122]. Here, a good correlation between the results obtained using the Folin–Ciocalteu and TEAC assays was highlighted, and in particular, both tests indicated a higher antioxidant activity and phenol content of the blade extracts compared to the sporophyll and holdfast extracts. This result is consistent with the observations of a strong positive correlation between the TEAC assay and F-C assay [123]. Therefore, our results confirm *U. pinnatifida* as a natural source of functional molecules with good antioxidant properties, very interesting for the food industry in Western countries, which is focused on replacing the use of synthetics with natural antioxidants, as they are safer and, at the same time, provide bioactive properties, adding value to the final products [124–126].

Furthermore, the ^{1}H NMR spectra showed the presence of phytosterols—especially fucosterol, a sterol already reported by Boulom et al. [52]—in *U. pinnatifida* and alkaloids.

Phytosterols and alkaloids are bioactive compounds found in marine plants and algae with functional and nutraceutical properties that are useful to human health [127], including antifungal, antibacterial, anti-inflammatory, antitumor [128], antioxidant, anti-ulcerative, and antidiabetic properties [129,130].

In conclusion, the NMR-based method used herein enabled the one-shot recognition of several biocompounds in complex mixtures such as lipidic extracts of the invasive alga *U. pinnatifida*. The key aspect of the present work was the use of NMR spectroscopy, for the first time, to analyze the lipidic extracts of this specific alga and determine the distribution of its components. The high variety of valuable secondary metabolites discovered in the three lipidic extracts further encourages the exploitation of this algal species in different biotechnological fields, including nutraceuticals and pharmaceuticals. The obtained results call for further investigation, both in vitro and in silico, of the biological activity and possible pharmaceutical uses of the investigated extracts, which could be the pivotal theme of a further study. On the other hand, our results pave the way for the isolation of interesting molecules responsible for antimicrobial and antioxidant activities by performing integrated analytical approaches, such as HPLC, GC-MS, and LC-MS techniques, as well as in the light of eradication programs.

Author Contributions: Conceptualization, L.S. and L.R.; methodology, L.S., M.I.A., E.C., C.G., A.P., F.P.F., F.A. and L.R.; analysis, L.S., M.I.A., E.C., C.G., A.P., F.P.F., F.A. and L.R.; writing—original draft preparation, L.S. and L.R.; writing—review and editing, L.S., M.I.A., E.C., C.G., A.P., F.P.F., F.A. and L.R. All authors have read and agreed to the published version of the manuscript.

Funding: This study was partially supported by the "ON Foods—Research and innovation network on food and nutrition Sustainability, Safety and Security—Working ON Foods" project funded under the National Recovery and Resilience Plan (NRRP), Mission 4, Component 2, Investment 1.3—Call for proposals No. 341 of 15 March 2022 of the Italian Ministry of Universities and Research, funded by the European Union—NextGenerationEU, project code PE00000003, Concession Decree No. 1550 of 11 October 2022, adopted by the Italian Ministry of Universities and Research, CUP D93C22000890001 and by the "National Biodiversity Future Center (NBFC)", funded by European Union Next Generation EU (PNRR), Spoke 2 Activity 3 Task 3.2 CUP B83C22002930006.

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

Data Availability Statement: The data presented in this study are available in Tables 1–3.

Acknowledgments: The authors thank Adriano Sfriso, Alessandro Buosi, and Andrea Augusto Sfriso for their technical support in the Venice Lagoon.

Conflicts of Interest: The authors declare no conflict of interest.

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