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Antimicrobial Activity of Crude Extracts from *Ascophyllum nodosum* Obtained by Microwave Assisted Extraction †

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Abstract: *Ascophyllum nodosum* (Linnaeus) Le Jolis is a brown alga from the Fucaceae family and the unique species from the *Ascophyllum* genus. This brown alga is an edible macroalga from the North Atlantic Ocean, commonly found on the European north-western coast. High-value bioactive molecules such as pigments, polyphenols, and phlorotannin [1,2] have been found in the macroalgae composition, which turns this alga particularly interesting for exploring potential biological activities. Among sustainable extraction technologies, microwave-assisted extraction (MAE) has many advantages such as short extraction time and less solvent requirement. On the other hand, ethanol and water are eco-friendly solvents that have already been proven to be effective for obtaining bioactive compounds with antimicrobial capacity [3]. Therefore, in this work, analytical conditions of MAE: t = 5 min; pressure = 10.5 bar; ethanol concentration (37%) as solvent was applied to obtain a polyphenol-rich extract from *A. nodosum*. The antimicrobial effect of the resulting extract against five foodborne microorganisms (*Bacillus cereus*, *Escherichia coli*, *Salmonella enteritidis*, *Pseudomonas aeruginosa*, *Staphylococcus aureus*), and the opportunistic bacteria *Staphylococcus epidermidis* was assessed. The antimicrobial activity was performed through the Kirby-Bauer disk diffusion susceptibility test protocol and the microdilution method. The analytical results indicated that the *A. nodosum* extract was effective against all tested bacteria except for *Escherichia coli*. The highest antimicrobial activity was found against *Staphylococcus aureus* presenting inhibitory capacity since a concentration of 400 µg/mL and an inhibition halo of 11.79 ± 1.92 mm.

Keywords: *Ascophyllum nodosum*; crude extract; antimicrobial activity

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1. Introduction

Nowadays, consumers demand is focused on natural, safe, and diverse food sources. Moreover, the grown interest in functional foods is leading the scientific community to the search for nutraceutical characteristics in natural products as a novel approach to a healthier lifestyle. Macroalgae are traditionally part of the human diet especially in the

oriental countries, but they have been emerged as an important nutrition alternative, because apart of nutritional properties, they have shown biological active properties such as antioxidant, antimicrobial, antitumor, and anti-inflammatory [4–6]. *A. nodosum* is perennial edible alga belonging to the *Ochrophyta* phylum, that can be found in cold waters, with special abundance on sheltered rocky shores of the north Atlantic ocean. This alga is a rich source of underexploited bioactive compounds. Microwave-assisted extraction (MAE) is a non-thermal emerging technique that has been applied to extract bioactive compounds from macroalgae. Some works reported higher antioxidant capacity in *A. nodosum* extracts obtained with MAE when compared to extracts obtained by traditional extraction techniques (maceration) [7]. Regarding solvent, MAE requires the use of polar solvents. In that sense, ethanol emerges as a non-toxic solvent, adequate to achieve high extraction yields [8], and has already shown to be capable of extracting bioactive molecules from alga matrix with proven antioxidant and antimicrobial capacity [3].

Food safety is a crucial topic for human health. Foodborne pathogens are responsible, worldwide, for an enormous number of diseases. For instance, it is estimated that pathogens caused 37.2 million ailments annually in the United States of America (USA), of which, 9.4 million were caused by foodborne pathogens [9]. This problem led to the research of technologies, and alternative antimicrobials compounds that minimize the triggering of microorganisms, increase food safety and prolong shelf life. In this work, the antimicrobial activity of a polyphenolic-rich extract from *A. nodosum* obtained through microwave-assisted extraction was analyzed.

2. Materials and Methods

2.1. Sample Preparation

Brown algae were hand-picked from the coasts of Galicia, by Algas Atlánticas Algamar SL company located in Pontevedra, Spain. Algae were carefully clean and washed with demineralized water, before freeze-drying. Then, algae were triturated and stored at −20 °C until use.

2.2. Microwave-Assisted Extraction

MAE was carried out using a multiwave-3000 microwave extraction system (Anton-Paar, Germany). The biomass to solvent ratio was 30 g L^{-1} and the extraction was performed at 10.5 bar for 5 min using aqueous ethanol 37% *v*/*v*) as solvent. These conditions were selected based on literature and previous works [10].

2.3. Bioassays

2.3.1. Microorganisms and Cultures

Cultures of *S. aureus* (ATCC 25923) *B. cereus* (ATCC 14579), *P. aeruginosa* (ATCC 10145); *S. enteritidis* (ATCC 13676) were provided by Selectrol, Buckingham, UK; *E. coli* (NCTC 9001), and *S. epidermidis* (NCTC 11047) were supplied by Microbiologics, Minnesota USA. The stock cultures were inoculated into 10 mL of Mueller-Hinton broth (MHB) (Biolife Milan, Italy) and grown overnight at 37 °C. After the inoculum, concentration was normalized to the 0.5 MacFarland standard (0.09 to 0.110 optical density at 600 nm) by dilution in fresh MHB [11].

2.3.2. Extract Preparation

A. nodosum extract obtained by MAE was dissolved in dimethyl sulfoxide (DMSO) (Sigma-Aldrich Steinheim, Steinheim am Albuch, Germany) and brought up to 20 mg/mL concentration. Then, the solubilized extract was sterilized by passing through a 0.20 μm syringe filter.

2.3.3. Kirby-Bauer Plate Diffusion Test

Petri dishes containing Mueller-Hinton agar were divided into four quadrants and seeded with 50 μL of the microorganism culture and spread with sterile swabs. The test was made by putting 15 μL of DMSO (negative control) in the center of the plate and placing 15 μL of each test extract in three quadrants, and 15 μL of lactic acid 40% (*v*/*v*) (Sigma-Aldrich Steinheim, Germany) (positive control). Petri dishes were incubated at 37 °C for 24 h and the inhibition zone diameter was measured with a digital caliper rule [11,12]. Determinations were conducted in triplicate.

2.3.4. Microdilution Assay

Minimal inhibitory concentration (MIC) determination was performed by the microdilution method [11], using multiplate reader equipment (Biotek Synergy HT). The test was done using an extract concentration ranging from 1.2 to 8.0 mg/mL. For that, a 96-well round-bottom sterile plate was filled with a total volume of 250 μL, containing 10^6 Colony Forming Units (CFU), algae extract (100 μL), and fresh MHB media. Negative controls contained medium with extract samples and no bacteria and positive control wells were prepared with inoculated medium without extract samples. Microplates were incubated for 24 h at 37 °C at 630 nm. Determinations were conducted in triplicate.

3. Results and Discussion

The potential antimicrobial activity of *A. nodosun* extracts was tested against five common foodborne contaminants [13] *E. coli*, *B. cereus*, *S. aureus*, *S. enteritidis*, *P. aeruginosa*, and an opportunistic bacteria *S. epidermidis*. The antimicrobial capacity was firstly evaluated by the plate diffusion assay. These preliminary studies were carried out with an extract concentration of 20 mg/mL (Table 1). Results obtained showed that *A. nodosun* extract has antimicrobial capacity, with greater inhibition against the Gram-positive strains, *B. cereus* and *S. aureus,* but still active against one of the Gram-negative strains *S. enteretidis.* Previews studies have stated this differential interaction of algae polyphenols with Gram-positive and Gram-negative bacteria [14] and in general, Gram-positive bacteria are more sensitive to phlorotannins than Gram-negative [15].

Table 1. Inhibition halos obtained by plate diffusion method.

Gram	Microorganism	Inhibition Zone (mm)
positive	*S. epidermidis*	NI
	B. cereus	9.31 ± 1.50
	S. aureus	11.79 ± 1.92
negative	*E. coli*	NI
	S. enteritidis	5.7 ± 1.8
	P. aeruginosa	NI
NI-No inhibition detected		

The evolution of six microorganisms' growth curves in the presence of *A. nodosum* extract is presented in Figure 1. As can be seen from Figure 1, it is possible to confirm that there is an effective inhibition due to a decrease in the bacterial growth rates of all the tested microorganism's species except for *E. coli*.

For the Gram-positive bacteria, the MIC values were higher than 8.0, 4.0, and 8.0 mg/mL, respectively. It is worth mentioning that 0.4 mg/mL of alga extract produced a 20% inhibition effect in *S. aureus*. Concerning the Gram-negative strains, the MIC values obtained were higher than 8.0 mg/L for *P. aeruginosa* and 8.0 mg/mL for *S. enteritidis.* No inhibition effect was observed against *E. coli*. The MICs obtained from the Gram-negative bacteria were higher than the ones determined for Gram-positive strains. This can be attributed to the external membrane of Gram-negative microorganisms as an effective

barrier to many active molecules, including antibiotics [15]. A few inoculation loops were taken from the wells without measurable bacterial growth (4 mg/mL *B. cereus*; 8 mg/mL *S. enteritidis*) and spread on a Muller-Hinton agar medium. After incubation at 37 °C overnight, the existence of viable colonies was observed, so it was concluded that the inhibitory effect achieved was due to the bacteriostatic effect, given to no cellular dead was achieved.

It is also important to highlight that despite no inhibition activity was detected against *S. epidermidis* and *P. aeruginosa* in the plate diffusion method, different growth rates were observed in the microdilution tests (Figure 1). These results can be explained by the fact that the microdilution method allows following the microorganism growth over time, whereas in the plate diffusion method only the endpoint is observed. Furthermore, it has been previously stated that the microdilution method is the most sensitive one [16].

By observing the growth curves, it is possible to deduce that the bacterial inhibition is mostly achieved by lag time extension. Through lag phase, the microorganisms adjusted to the new environment, and cells start to adapt to the metabolic functions and synthesize the components necessary for growth [17,18]. The bioactive molecules present in the extract are postponing this phase.

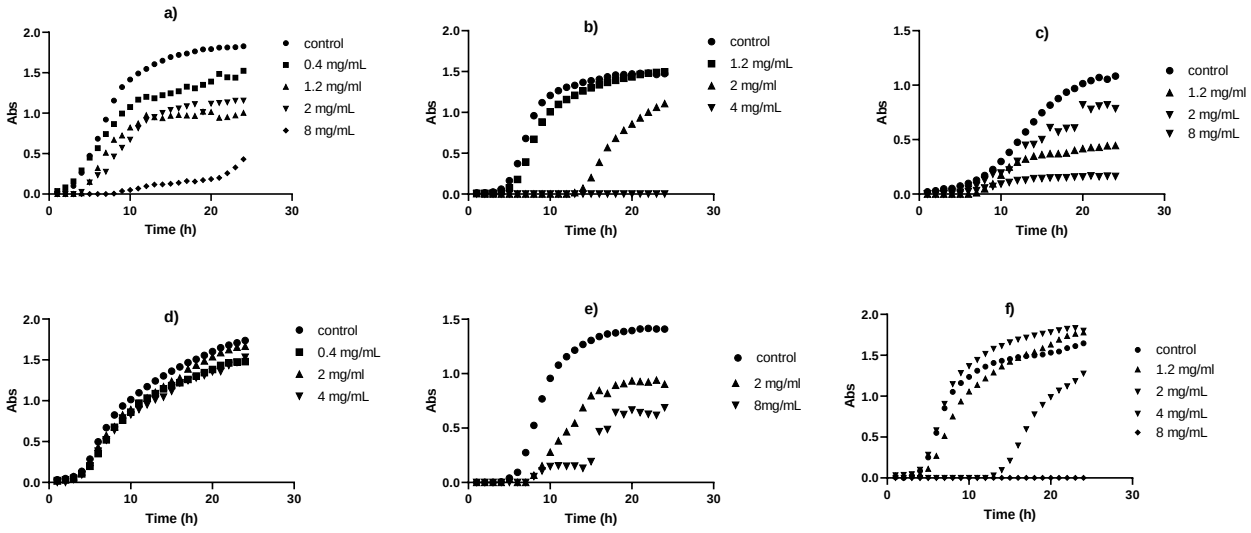

Figure 1. Growth curves for (**a**) *S. aureus*, (**b**) *B. cereus*; (**c**) *S. epidermidis*; (**d**) *E. coli*; (**e**) *P. aeruginosa*; (**f**) *S. enterititis*.

4. Conclusions

Results from the present work indicated that *A. nodusum* was effective against all tested bacteria except for *E. coli* disclosing the potential of *A. nodusum* as an antimicrobial agent. Furthermore, the growth curves obtained allow inferring that the antimicrobial action is attained by lag time extension. Thus, it is possible to expect that the incorporation of this extract into food products could be useful in the control of food-borne infections.

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

References

1. Sardari, R.R.R.; Prothmann, J.; Gregersen, O.; Turner, C.; Karlsson, E.N. Identification of phlorotannins in the brown algae, saccharina latissima and ascophyllum nodosum by ultra-high-performance liquid chromatography coupled to high-resolution tandem mass spectrometry. *Molecules* **2021**, *26*, 43. https://doi.org/10.3390/MOLECULES26010043.
2. Garcia-Perez, P.; Lourenço-Lopes, C.; Silva, A.; Pereira, A.G.; Fraga-Corral, M.; Zhao, C.; Xiao, J.; Simal-Gandara, J.; Prieto, M.A. Pigment Composition of Nine Brown Algae from the Iberian Northwestern Coastline: Influence of the Extraction Solvent. *Mar. Drugs* **2022**, *20*, 113. https://doi.org/10.3390/md20020113.
3. Silva, A.; Rodrigues, C.; Lourenç, C.; Silva, S.A. Screening for bioactive properties on brown algae from the northwest Iberian Peninsula. *Foods* **2021**, *10*, 1915.
4. Jiménez, J.; O'Connell, S.; Lyons, H.; Bradley, B.; Hall, M. Antioxidant, antimicrobial, and tyrosinase inhibition activities of acetone extract of Ascophyllum nodosum. *Chem. Pap.* **2010**, *64*, 434–442. https://doi.org/10.2478/s11696-010-0024-8.
5. Riou, D.; Colliec-Jouault, S.; Pinczon du Sel, D.; Bosch, S.; Siavoshian, S.; Le Bert, V.; Tomasoni, C.; Sinquin, C.; Durand, P.; Roussakis, C. Antitumor and antiproliferative effects of a fucan extracted from ascophyllum nodosum against a non-small-cell bronchopulmonary carcinoma line. *Anticancer Res.* **1996**, *16*, 1213–1218.
6. Ahmad, T.; Eapen, M.S.; Ishaq, M.; Park, A.Y.; Karpiniec, S.S.; Stringer, D.N.; Sohal, S.S.; Fitton, J.H.; Guven, N.; Caruso, V.; et al. Anti-Inflammatory Activity of Fucoidan Extracts In Vitro. *Mar. Drugs* **2021**, *19*, 702. https://doi.org/10.3390/md19120702.
7. Yuan, Y.; Zhang, J.; Fan, J.; Clark, J.; Shen, P.; Li, Y.; Zhang, C. Microwave assisted extraction of phenolic compounds from four economic brown macroalgae species and evaluation of their antioxidant activities and inhibitory effects on α-amylase, α-glucosidase, pancreatic lipase and tyrosinase. *Food Res. Int.* **2018**, *113*, 288–297. https://doi.org/10.1016/j.foodres.2018.07.021.
8. Carpentieri, S.; Soltanipour, F.; Ferrari, G.; Pataro, G.; Donsì, F. Emerging Green Techniques for the Extraction of Antioxidants from Agri-Food By-Products as Promising Ingredients for the Food Industry. *Antioxidants* **2021**, *10*, 1417. https://doi.org/10.3390/antiox10091417.
9. Scallan, E.; Hoekstra, R.M.; Angulo, F.J.; Tauxe, R.V.; Widdowson, M.-A.; Roy, S.L.; Jones, J.L.; Griffin, P.M. Foodborne Illness Acquired in the United States—Major Pathogens. *Emerg. Infect. Dis.* **2011**, *17*, 7–15. https://doi.org/10.3201/eid1701.P11101.
10. Chamorro, F.; Cassani, L.; Lourenço-Lopes, C.; Carreira-Casais, A.; Carpena, M.; Echave, J.; Baamonde, S.; Fernández-Saa, F.; Otero, P.; Garcia-Perez, P.; et al. Optimization of Bioactive Compounds with Antioxidant Activity of Himanthalia elongata by Microwave Assisted Extraction Using Response Surface Methodology. *Chem. Proc.* **2021**, *5*, 70.
11. Paz, M.; Gúllon, P.; Barroso, M.F.; Carvalho, A.P.; Domingues, V.F.; Gomes, A.M.; Becker, H.; Longhinotti, E.; Delerue-Matos, C. Brazilian fruit pulps as functional foods and additives: Evaluation of bioactive compounds. *Food Chem.* **2015**, *172*, 462–468. https://doi.org/10.1016/j.foodchem.2014.09.102.
12. Clinical and Laboratory Standards Institute. *Performance Standards for Antimicrobial Disk Susceptibility Tests*; Approved Standard—Eleventh Edition; Clinical and Laboratory Standards Institute: Malvern, PA, USA, 2012; Volume 32, ISBN 1562387812.
13. Silva, A.; Silva, S.A.; Lourenço-Lopes, C.; Jimenez-Lopez, C.; Carpena, M.; Gullón, P.; Fraga-Corral, M.; Domingues, V.F.; Barroso, M.F.; Simal-Gandara, J.; et al. Antibacterial Use of Macroalgae Compounds against Foodborne Pathogens. *Antibiotics* **2020**, *9*, 712. https://doi.org/10.3390/antibiotics9100712.
14. Stern, J.L.; Hagerman, A.E.; Steinberg, P.D.; Mason, P.K. Phlorotannin-protein interactions. *J. Chem. Ecol.* **1996**, *22*, 1877–1899. https://doi.org/10.1007/BF02028510.

15. Besednova, N.N.; Andryukov, B.G.; Zaporozhets, T.S.; Kryzhanovsky, S.P.; Kuznetsova, T.A.; Fedyanina, L.N.; Makarenkova, I.D.; Zvyagintseva, T.N. Algae Polyphenolic Compounds and Modern Antibacterial Strategies: Current Achievements and Immediate Prospects. *Biomedicines* **2020**, *8*, 342. https://doi.org/10.3390/biomedicines8090342.
16. Scorzoni, L.; Benaducci, T.; Almeida, A.M.F.; Silva, D.H.S.; Bolzani, V.S. ; Mendes-Giannini, M.J.S. Comparative study of disk diffusion and microdilution methods for evaluation of antifungal activity of natural compounds against medical yeasts Candida spp and Cryptococcus sp. *J. Basic Appl. Pharm. Sci.* **2007**, *28*, 25–34.
17. Liu, S. How Cells Grow. In *Bioprocess Engineering*; Elsevier: Amsterdam, The Netherlands, 2017; pp. 629–697.
18. Rolfe, M.D.; Rice, C.J.; Lucchini, S.; Pin, C.; Thompson, A.; Cameron, A.D.S.; Alston, M.; Stringer, M.F.; Betts, R.P.; Baranyi, J.; et al. Lag Phase Is a Distinct Growth Phase That Prepares Bacteria for Exponential Growth and Involves Transient Metal Accumulation. *J. Bacteriol.* **2012**, *194*, 686–701. https://doi.org/10.1128/JB.06112-11.