

Proceeding Paper

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

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- [†] Presented at the 2nd International Electronic Conference on Antibiotics—Drugs for Superbugs: Antibiotic Discovery, Modes of Action and Mechanisms of Resistance, 15–30 June 2022; Available online: <https://eca2022.sciforum.net/>.



Citation: Silva, A.; Cassani, L.; Lourenço-Lopes, C.; Morais, S.L.; Cao, H.; Garcia-Oliveira, P.; Garcia-Perez, P.; Carpena, M.; Domingues, V.; Barroso, M.F.; et al. Antimicrobial Activity of Crude Extracts from *Ascophyllum nodosum* Obtained by Microwave-Assisted Extraction. *Med. Sci. Forum* **2022**, *12*, 19. <https://doi.org/10.3390/eca2022-12724>

Academic Editor: Marc Maresca

Published: 15 June 2022

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Abstract: *Ascophyllum nodosum* (Linnaeus) Le Jolis is a brown alga from the Fucaceae family and a 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 were found in the macroalgae composition, which makes this alga particularly interesting for exploring potential biological activities. Among sustainable extraction technologies, microwave-assisted extraction (MAE) has many advantages such as a short extraction time and fewer solvent requirements. 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. Therefore, in this work, analytical conditions of MAE: t = 5 min; pressure = 10.5 bar; ethanol concentration (37%) as solvent were applied to obtain a polyphenol-rich extract from *A. nodosum*. The antimicrobial effect of the resulting extract against five food-borne 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 with a concentration of 400 µg/mL and an inhibition halo of 11.79 ± 1.92 mm.

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

1. Introduction

Nowadays, consumer demand is focused on natural, safe, and diverse food sources. Moreover, the growing interest in functional foods is leading the scientific community to 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 oriental countries, but they have emerged as an important nutrition alternative because, apart from their nutritional properties, they have shown biologically active properties such as antioxidant, antimicrobial, antitumor, and anti-inflammatory properties [1–3]. *A. nodosum* is a 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 is 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) [4]. Regarding solvents, MAE requires the use of polar solvents. In that sense, ethanol emerges as a non-toxic solvent, adequate to achieve high extraction yields [5] and has already proven to be capable of extracting bioactive molecules from alga matrix with proven antioxidant and antimicrobial capacity [6].

Food safety is a crucial topic for human health. Food-borne 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 food-borne pathogens [7]. This problem led to the research of technologies and alternative antimicrobial 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 cleaned and washed with demineralized water, before freeze-drying. Then, algae were triturated and stored at $-20\text{ }^{\circ}\text{C}$ 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 the literature and previous works [8].

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 $37\text{ }^{\circ}\text{C}$. 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 [9].

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 $0.20\text{ }\mu\text{m}$ 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 [9,10]. Determinations were conducted in triplicate.

2.3.4. Microdilution Assay

Minimal inhibitory concentration (MIC) determination was performed by the microdilution method [9], using multiplate reader equipment (Biotek Synergy HT). The test was performed 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 10⁶ 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. nodosum* extracts was tested against five common food-borne contaminants [11] *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). The results obtained showed that *A. nodosum* extract has an antimicrobial capacity, with greater inhibition against the Gram-positive strains, *B. cereus* and *S. aureus*, but was still active against one of the Gram-negative strains *S. enteritidis*. Previous studies have stated this differential interaction of algae polyphenols with Gram-positive and Gram-negative bacteria [12] and in general, Gram-positive bacteria are more sensitive to phlorotannins than Gram-negative bacteria [13].

Table 1. Inhibition halos obtained by plate diffusion method.

Gram	Microorganism	Inhibition Zone (mm)
positive	<i>S. epidermidis</i>	NI
	<i>B. cereus</i>	9.31 ± 1.50
	<i>S. aureus</i>	11.79 ± 1.92
negative	<i>E. coli</i>	NI
	<i>S. enteritidis</i>	5.7 ± 1.8
	<i>P. aeruginosa</i>	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 species, except for *E. coli*.

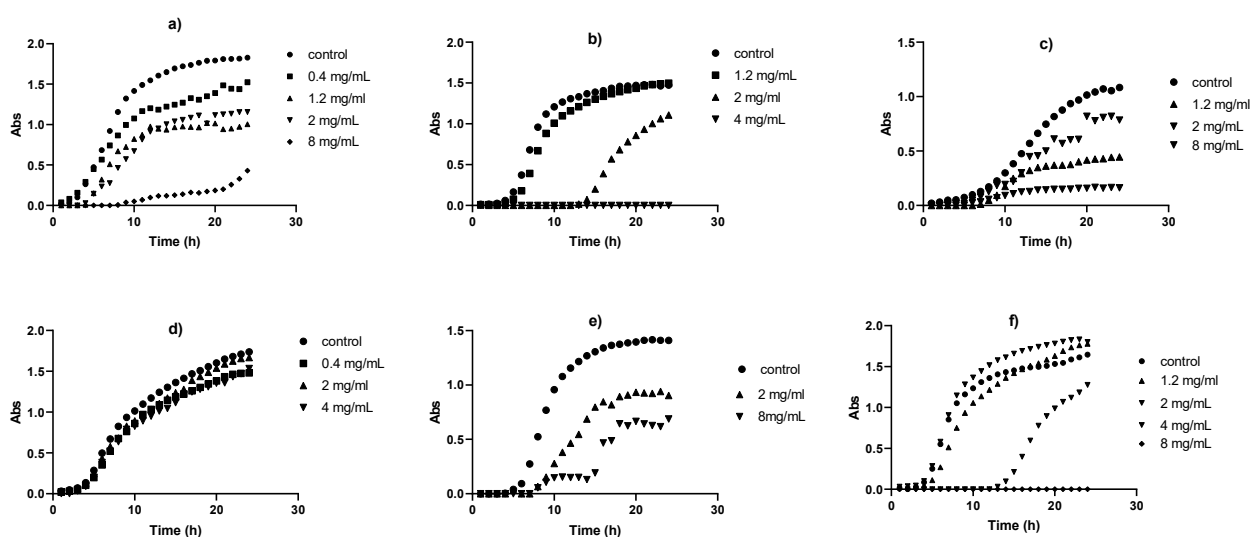


Figure 1. Growth curves for (a) *S. aureus*; (b) *B. cereus*; (c) *S. epidermidis*; (d) *E. coli*; (e) *P. aeruginosa*; and (f) *S. enteritidis*.

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 [13]. 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, therefore, it was concluded that the inhibitory effect achieved was due to the bacteriostatic effect, given that no cellular death was achieved.

It is also important to highlight that despite no inhibition activity being 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 for following the microorganism growth over time, whereas in the plate diffusion method, only the endpoint is observed. Furthermore, it was previously stated that the microdilution method is the most sensitive one [14].

By observing the growth curves, it is possible to deduce that bacterial inhibition is mostly achieved by lag time extension. Through the 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 [15,16]. The bioactive molecules present in the extract are postponing this phase.

4. Conclusions

The 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 for inferring that the antimicrobial action is attained due to the 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.

Author Contributions: Conceptualization, A.S. and M.F.B.; methodology, A.S., C.L.-L., L.C., P.G.-O., P.G.-P. and M.C.; formal analysis, A.S., M.F.B. and L.C.; investigation, A.S., L.C. and M.F.B.; resources, M.A.P., J.S.-G. and M.F.B.; data curation, A.S., S.L.M. and M.A.P.; writing—original draft preparation, A.S., S.L.M. and V.D.; writing—review and editing, L.C., M.A.P. and M.F.B.; visualization, H.C. and

V.D.; supervision, M.F.B. and M.A.P.; funding acquisition, J.S.-G. and M.A.P. All authors have read and agreed to the published version of the manuscript.

Funding: The authors are grateful to the Ibero-American Program of Science and Technology (CYTED—AQUA-CIBUS, P317RT0003), to the Bio Based Industries Joint Undertaking (JU) under grant agreement No 888003 UP4HEALTH Project (H2020-BBI-JTI-2019) that supports the work of C. Lourenço-Lopes. The authors would like to thank the EU and FCT for funding through the programs UIDB/50006/2020; UIDP/50006/2020. This work has received funding from the Argentinean Agency for Scientific and Technological Promotion [PICT(2020)/1602].

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

Data Availability Statement: Not applicable.

Acknowledgments: The research leading to these results was supported by MICINN supporting the Ramón y Cajal grant for M.A. Prieto (RYC-2017-22891) and the Juan de la Cierva Incorporación for Hui Cao (IJC2020-046055-I), Xunta de Galicia for supporting the program EXCELENCIA-ED431F 2020/12, the post-doctoral grant of L. Cassani (ED481B-2021/152), and the pre-doctoral grants of P. Garcia-Oliveira (ED481A-2019/295), and M. Carpena (ED481A 2021/313). The research leading to these results was supported by the European Union through the “NextGenerationEU” program supporting the “Margarita Salas” grant awarded to P. Garcia-Perez. The authors are grateful to the AlgaMar enterprise (www.algamar.com accessed on 20 January 2022) for the collaboration and algae material provision.

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

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