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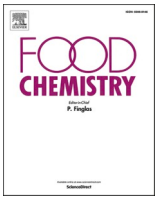

Phytochemical compounds with promising biological activities from *Ascophyllum nodosum* extracts using microwave-assisted extraction

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ABSTRACT

Phytochemical-rich antioxidant extracts were obtained from *Ascophyllum nodosum* (AN) using microwave-assisted extraction (MAE). Critical extraction factors such as time, pressure, and ethanol concentration were optimized by response surface methodology with a circumscribed central composite design. Under the optimal MAE conditions (3 min, 10.4 bar, 46.8 % ethanol), the maximum recovery of phytochemical compounds (polyphenols and fucoxanthin) with improved antioxidant activity from AN was obtained. In addition, the optimized AN extract showed significant biological activities as it was able to scavenge reactive oxygen and nitrogen species, inhibit central nervous system-related enzymes, and exhibit cytotoxic activity against different cancer cell lines. In addition, the optimized AN extract showed antimicrobial, and anti-quorum sensing activities, indicating that this extract could offer direct and indirect protection against infection by pathogenic microorganisms. This work demonstrated that the sustainably obtained AN extract could be an emerging, non-toxic, and natural ingredient with potential to be included in different applications.

1. Introduction

The increasing demand for functional foods, nutraceuticals, and cosmetics with natural ingredients has led academicians and industry towards the exploitation of natural and novel sources of bioactive compounds not only to replace the traditionally used synthetic ingredients but also to provide additional health benefits. In this scenario, brown algae have been gained attention in Western countries as they are a rich source of health-promoting compounds, such as dietary fiber, polyunsaturated fatty acids, polysaccharides, polyphenols, terpenoids, and minerals (Cassani et al., 2022). Although polysaccharides, especially alginate, lead the total commercial production of brown algae-derived compounds (Cikoš, Jerković, Molnar, Šubarić, & Jokić, 2021), the global market of the metabolites, such as phlorotannins, fucoxanthin, and fucoidan is also showing continuous growth (Thiyagarasaiyar, Goh, Jeon, & Yow, 2020).

Phlorotannins are a group of unique polyphenols synthesized in brown algae that have a complex polymerized structure of phloroglucinol units (monomer). According to the structural linkages between monomer units, phlorotannins can be classified into six classes (phlorethols, fuhalols, fucols, fucophlorethols, eckols, and carmalols) (Cassani et al., 2020; Tierney et al., 2014). The most studied sources are *Ecklonia* spp. and *Eisenia* spp. as they reported the highest content of phlorotannins (Cho et al., 2019). The European Food Safety Authority (EFSA) has approved the use of phlorotannins extracted from *Ecklonia cava* as food supplements (Commission Regulation (EU) 2017/2470) (Lähteenmäki-Uutela et al., 2021). Their physicochemical properties (water solubility) and associated biological activities (antioxidant, antimicrobial, anti-inflammatory, anti-cancer, etc.) support the use of phlorotannins as emerging ingredients with the multi-faceted potential to be included in the development of innovative foods, drugs, and

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Nomenclature

Abbreviations: Compounds

ABTS	2,2′-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid)
AChE	Acetylcholinesterase
BC	β-carotene
BuChE	Butyrylcholinesterase
DPPH	1,1-diphenyl-2-picrylhydrazyl
DMSO	Dimethyl Sulfoxide
Fx	Fucoxanthin
H_2O_2	Hydrogen peroxide
LB	Luria-Bertani Broth
L-DOPA	L-dihydroxyphenylalanine
LPS	Lipopolysaccharide
MAO-A	Monoaminoxidase Oxidase A
MAO-B	Monoaminoxidase Oxidase B
NADH	Nicotinamide Adenine Dinucleotide
NBT	Nitro Blue Tetrazolium
$O_2^{\bullet-}$	Superoxide anion
PE	Phloroglucinol Equivalents
PFF	Phlorofucofuroeckol
PMS	Phenazine Methosulfate
QE	Quercetin Equivalents
SRB	Sulforhodamin B
TFC	Total Flavonoid Content
TPC	Total Phenolic Content
$^{\bullet}OH$	Hydroxyl radical
$^{\bullet}NO$	Nitric Oxide

Abbreviations: Generic

AN	*Ascophyllum nodosum*
βCM	β-Carotene Bleaching Inhibition Method
CCCD	Circumscribed Central Composite Design
CNS	Central Nervous System
DP	Degree of Polymerization
DW	Durbin-Watson coefficient
E	Extract
EFSA	European Food Safety Authority
HAT	Hydrogen Atom Transfer
HPLC-DAD	High Performance Liquid Chromatography coupled to Diode Array Detector
IC_{50}	Half Maximal Effective Concentration
MAE	Microwave-Assisted Extraction
MAPE	Mean Absolute Percentage Error
MBC	Minimum Bactericidal Concentration
MIC	Minimum Inhibitory Concentration
MSE	Mean Squared Error
ns	Non-Significant
P/T	P/T - Pressure/Temperature
PLE	Pressurized Liquid Extraction
QS	Quorum Sensing
RMSE	Root Mean Square Error
ROS	Reactive Oxygen Species
RNS	Reactive Nitrogen Species
RSM	Response Surface Methodology
SD	Standard Deviation
SET	Single Electron Transfer
UAE	Ultrasound-Assisted Extraction

cosmetics.

Fucoxanthin is a carotenoid naturally occurring in brown algae responsible for their yellow to brown color. This pigment can account for more than 10 % of total lipids and due to its unique chemical structure, it has exhibited remarkable biological activities (Lourenço-Lopes et al., 2021; Shannon & Abu-Ghannam, 2017). For instance, fucoxanthin showed antioxidant activity as it was able to inhibit reactive oxygen species (ROS) production (Pangestuti, Ngo & Kim, 2013). In addition, this pigment showed cytotoxic effect against different cancer cell lines (colon, liver, leukemia, breast, cervical), without affecting healthy human cells (Wang, Xu, Bach & McAllister, 2019). Other activities, such as anti-obesity, anti-diabetic, anti-inflammatory, cardiovascular protection, antimicrobial, skin protection, and neuroprotective effects, have also been ascribed to this pigment (Lourenço-Lopes et al., 2021). In this context, fucoxanthin has potential to be used in different applications.

Despite some brown algae species have been widely studied as sources of phlorotannins and fucoxanthin, the potential of *Ascophyllum nodosum* (Linnaeus) Le Joli as a source of biologically active compounds compounds has not been greatly considered. In this regard, a small amount of research has focused on determining the most critical extraction conditions that enhance phytochemical component recovery and antioxidant activity from *A. nodosum* (AN) using powerful mathematical tools (Gisbert, Barcala, Rosell, Sineiro, & Moreira, 2021; Kadam et al., 2015; Liu, Luo, Wang, & Yuan, 2019). Most studies that do not use multivariate techniques result in underestimating extraction yields of phytochemicals (Boisvert, Beaulieu, Bonnet, & Pelletier, 2015; Yuan et al., 2018).

To fill this gap, the present work aimed to optimize the critical factors affecting the MAE of phytochemical compounds with antioxidant activity from AN using the RSM approach. Once the optimal conditions for MAE were identified, which enabled the maximization of extraction yield, phytochemical content, and antioxidant activity from AN, a comprehensive exploration of the extract's multifaceted potential under these optimized conditions was undertaken. This study included the evaluation of various biological activities, including antioxidant, antimicrobial, anti-quorum sensing, neuroprotective, and anti-inflammatory properties, as well as its cytotoxic effects against cancer cell lines.

2. Materials and methods

2.1. Sample collection

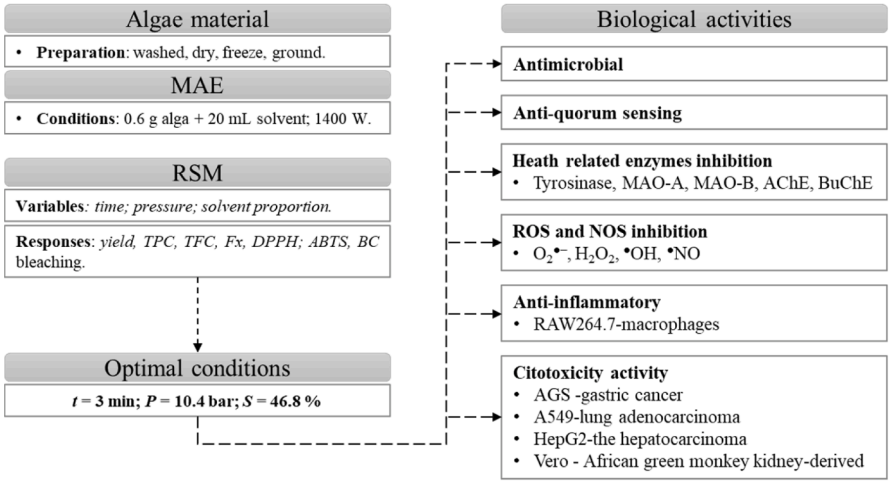
Fresh *Ascophyllum nodosum* (fifteen specimens) algae was generously supplied by Algas Atlánticas AlgaMar S.L. (Pontevedra, Spain, https://www.algamar.com) and collected from the northwest coast of Galicia, Spain, during the winter season of 2019. The algae samples were freeze-dried (−80 °C, 0.001 mbar, 3 days) and subsequently ground into a fine powder, which was then stored at −80 °C to facilitate subsequent analysis. A visual illustration outlining the procedural workflow employed in the research experiment is presented in part 1 of Fig. 1.

2.2. Optimization procedure of the MAE

2.2.1. MAE

To conduct the experiments, a Multiwave-3000 microwave reaction system (Anton Paar, Graz, Austria) was used. This system consists of an extraction chamber equipped with a MF 100-16 rotor with infrared temperature sensors, a pressure/temperature sensor (P/T) and a magnetic stirrer. In this case, 0.6 g of powder algae was added to the microwave vessels with 20 mL of extraction solvent, for a solid–liquid ratio of 30 g/L. A magnetic stirrer was added to each vessel to ensure a correct homogenization. The power was set at 1400 W. Once the extraction was finished, the vessels containing the samples were submerged in an ice bath for a duration of 5 min. Subsequently, the extraction solutions were

PART 1. WORKFLOW OF THE EXPERIMENTAL PROCEDURE



PART 2. GRAPHICAL REPRESENTATION OF THE EXTRACTION YIELD

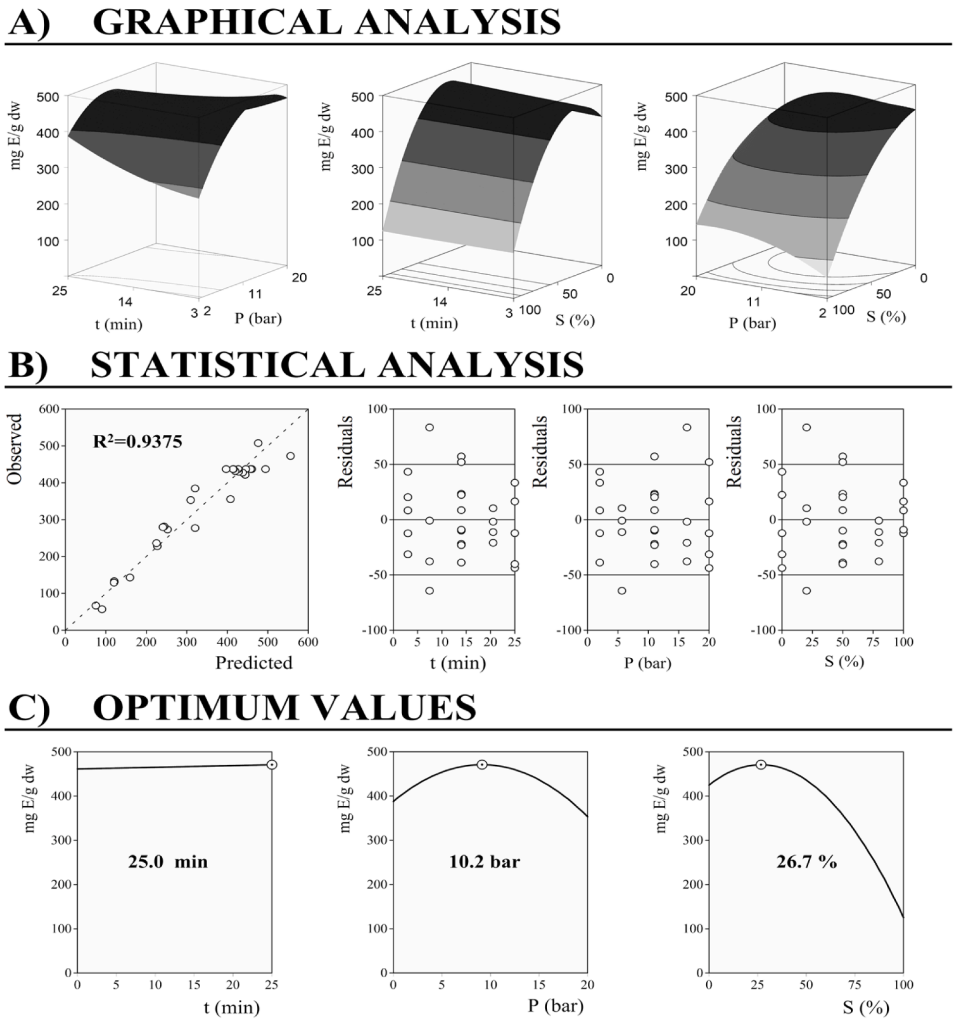


Fig. 1. **PART 1**: Schematic representation of the workflow of the experimental procedure of the research work. **PART 2**: Graphic representation of the MAE from AN as a function of the independent variables (X_1, X_2, and X_3) for the extraction yield (mg/g dw). (**A**) Graphical analysis representing 3D plots of the response surface predicted with the model of Eq. (2). (**B**): Statistical analysis (quadratic regression model, R^2) and residuals distribution as a function of each variable. (**C**): Single 2D responses of the variables (X_1, X_2, and X_3). The dots (○) represent the location of the optimum value. Lines and dots were generated by the Eq. (2).

transferred into Falcon® tubes and subjected to centrifugation at 8,800 × *g* for a period of 15 min. Finally, the resulting extracts were held at 80 °C for subsequent analysis.

2.2.2. Experimental design

The critical factors were time (*t* or X_1) ranging from 3 to 25 min, pressure (*P* or X_2) ranging from 2 to 20 bar and solvent proportion (*S* or X_2) from 0 to 100 % of ethanol in water. These operational factors were chosen based on previous screening studies (Pinela et al., 2016, Pinela et al., 2016).

To investigate the impact of these factors on the extraction yield, phytochemical content and antioxidant activity of AN alga exposed to MAE, RSM with a circumscribed central composite design (CCCD) was employed (Box, Hunter, & Hunter, 2005). The experimental design matrix comprised five levels of the critical factors, −α, −1, 0, 1, + α, where the value of α = 1.68 was selected to ensure a rotatable distribution of prediction variance (Teglia, Gonzalo, Culzoni, & Goicoechea, 2019). Consequently, a total of 28 experiments, including six replicates of central points were conducted. Experimental design matrix with the coded and actual levels of the three factors is depicted in Table 1.

2.2.3. Response variables

As previously mentioned, the effects of time (*t*), pressure (*P*), and ethanol concentration (*S*) on the phytochemicals' extraction from AN were simultaneously evaluated through extraction yield, phytochemical content and total antioxidant activity as explained below.

2.2.3.1. Extraction yield. The extraction yield was analyzed according to Silva et al. (2021). Results were expressed as grams of extract (E) per gram of algae dry matter (mg E/g dw).

2.2.3.2. Phytochemicals content. Total phenolic content (TPC) was determined following the spectrophotometric method described by Cassani et al. (2022). The results were expressed as mg of phloroglucinol equivalents (PE) per g of E (mg PE/g E).

Total flavonoid content (TFC) was assessed via a colorimetric method described by Barros, Carvalho, Morais, and Ferreira (2010), and the outcomes were reported as mg of quercetin equivalents (QE) per g of E (mg QE/g E).

For the analysis of fucoxanthin (Fx), high performance liquid chromatography coupled with a diode array detector (HPLC-DAD) was employed according to Garcia-Perez et al. (2022). Results were expressed as mg fucoxanthin/g E (mg Fx/g E).

2.2.3.3. Antioxidant activity assays. The evaluation of antioxidant activity was conducted by analyzing the concentration–time relationship using two single electron transfer (SET) assays, specifically the $ABTS^{\bullet+}$ and $DPPH^{\bullet}$ *in vitro* assays, as well as one hydrogen atom transfer (HAT) assay, namely the β-carotene bleaching method.

$DPPH^{\bullet}$ radical scavenging activity ($DPPH^{\bullet}$): The methodology applied was previously described by Cassani et al. (2022).

$ABTS^{\bullet+}$ radical scavenging activity ($ABTS^{\bullet+}$): The procedure for this activity was adapted from previous reports (Obón, Castellar, Cascales, & Fernández-López, 2005; Prieto, Curran, Gowen, & Vázquez, 2015; Re et al., 1999).

β-Carotene bleaching inhibition method (βCM): This method was performed according to Marco (1968) and Prieto, Rodríguez-Amado, Vázquez, and Murado (2012).

The experimental procedure involved the addition of 250 μL of the reagent and 50 μL of the sample into the wells of a plate to initiate the experimental process. By tracking the drop in absorbance as the reaction progressed, the reduction of $ABTS^{\bullet+}$ and $DPPH^{\bullet}$ (15 nM and 30 nM, respectively, in the final solution) and bleaching of β-Carotene (1 M in the final solution) were determined. The Weibull *(W)* cumulative distribution function (Weibull & Sweden, 1951), which measures response variation in relation to the concentration of the extracted antioxidant

Table 1
Experimental RSM results of the CCCD for the MAE optimization of the independent variables (X_1, X_2 and X_3) for the three response value formats assessed (*yield, phytochemical content, and antioxidant activity*). Variables are presented in natural values and codified ranges.

Run	Variable values						Responses						
	Code/natural values						*Extract*	*Phytochemical content*			*Antioxidant activity*		
	X_1: *t* (min)		X_2: *P(bar)*		X_3: *S (%)*		*Yield*	*TPC*	*TFC*	*Fx*	*DPPH•*	*ABTS•+*	*βCM*
							mg/g dw	*mg/g dw*	*mg/g dw*	*mg/g dw*	*nM R•/g dw*	*nM R•/g dw*	*μM βC/g dw*
1	−1	(7.5)	−1	(5.6)	−1	(20.3)	320.451	75.210	12.760	0.091	25.814	89.981	0.057
2	−1	(7.5)	−1	(5.6)	1	(79.7)	227.299	55.819	8.274	2.602	12.775	181.055	0.020
3	−1	(7.5)	1	(16.4)	−1	(20.3)	556.195	70.002	12.461	0.091	26.989	62.408	0.056
4	−1	(7.5)	1	(16.4)	1	(79.7)	243.886	52.667	11.261	1.877	15.942	78.041	0.021
5	1	(20.5)	−1	(5.6)	−1	(20.3)	437.934	73.136	13.062	0.091	28.156	112.638	0.053
6	1	(20.5)	−1	(5.6)	1	(79.7)	225.186	50.260	8.726	2.699	13.459	139.820	0.011
7	1	(20.5)	1	(16.4)	−1	(20.3)	428.137	49.288	12.000	0.091	20.437	60.704	0.044
8	1	(20.5)	1	(16.4)	1	(79.7)	252.464	48.620	12.741	1.167	16.638	88.926	0.018
9	−1.68	(3)	0	(11)	0	(50)	457.552	90.236	15.251	0.091	29.236	163.598	0.066
10	1.68	(25)	0	(11)	0	(50)	397.073	77.854	15.267	0.091	27.994	181.312	0.047
11	0	(14)	−1.68	(2)	0	(50)	240.996	63.097	9.198	1.104	20.701	190.121	0.030
12	0	(14)	1.68	(20)	0	(50)	407.849	52.653	12.134	0.091	25.553	76.468	0.038
13	0	(14)	0	(11)	−1.68	(0)	444.189	44.279	11.422	0.091	15.562	26.589	0.067
14	0	(14)	0	(11)	1.68	(100)	120.250	34.998	7.599	0.114	7.599	108.322	0.007
15	−1.68	(3)	−1.68	(2)	−1.68	(0)	320.531	42.605	10.181	0.145	15.156	38.436	0.077
16	−1.68	(3)	−1.68	(2)	1.68	(100)	75.437	18.4395	2.087	0.091	0.265	110.908	0.004
17	−1.68	(3)	1.68	(20)	−1.68	(0)	476.429	16.720	7.348	0.091	8.745	9.185	0.062
18	−1.68	(3)	1.68	(20)	1.68	(100)	120.769	13.196	7.777	0.619	2.602	1.461	0.026
19	1.68	(25)	−1.68	(2)	−1.68	(0)	419.532	32.041	11.828	0.097	15.092	55.721	0.074
20	1.68	(25)	−1.68	(2)	1.68	(100)	90.546	4.144	3.798	1.371	1.004	155.657	0.004
21	1.68	(25)	1.68	(20)	−1.68	(0)	309.547	6.830	10.469	0.091	7.282	24.993	0.033
22	1.68	(25)	1.68	(20)	1.68	(100)	159.542	2.232	11.149	0.132	3.000	15.838	0.018
23	0	(14)	0	(11)	0	(50)	427.289	77.848	13.975	0.097	28.288	143.749	0.039
24	0	(14)	0	(11)	0	(50)	494.435	77.848	13.975	0.098	28.288	143.749	0.040
25	0	(14)	0	(11)	0	(50)	414.218	77.848	13.975	0.091	28.288	143.749	0.035
26	0	(14)	0	(11)	0	(50)	460.569	74.820	13.847	0.091	29.433	145.990	0.042
27	0	(14)	0	(11)	0	(50)	415.435	78.134	15.651	0.100	24.630	138.575	0.040
28	0	(14)	0	(11)	0	(50)	445.792	75.953	15.130	0.102	24.598	133.307	0.049

mixture was used to analyze the dose–response effects (Prieto, Curran, Gowen & Vásquez, 2015). Thus, the effect of increasing concentrations of AN extract on the response can be generally expressed using Eq. (1):

$$W(A) = K\left\{1 - exp\left[- \ln(2)^{1-a}\left(\frac{2V_m}{Ka}A\right)^{a}\right]\right\} \quad (1)$$

where K is the asymptotic value related to averaged maximum value of antioxidant activity; V_m corresponds to a rate or average quantity of molecules (βC) or reduced radical molecules ($DPPH^{\bullet}$ and $ABTS^{\bullet+}$) per weight of extract obtained (μM βC/g dw for the βCM, nM R•/g dw for DPPH and $ABTS^{\bullet+}$); a is a shape parameter associated with the slope of potential profiles ($a < 1$), first order kinetic ($a = 1$) or various sigmoidal profiles ($a > 1$). For the purpose of this study, the V_m value was used as the response parameter, as it provides maximal predictability (Pinela et al., 2016, Pinela et al., 2016).

2.2.4. Mathematical modelling

The seven response variables were fitted to the following third-order mathematical model with interactive factors (Eq. (2)) for each response variable:

$$Y = b_0 + \sum_{i=1}^{n} b_i X_i + \sum_{\substack{i=1 \\ j>i}}^{n-1} \sum_{j=2}^{n} b_{ij} X_i X_j + \sum_{i=1}^{n} b_{iij} X_i^2 X_j^2 + \sum_{i=1}^{n} b_{iii} X_i^3 \quad (2)$$

where Y is the dependent variable (response variable) to be fitted, X_i and X_j are the independent variables, b_0 is the constant coefficient, b_i is the coefficient of linear effect, b_{ij} is the coefficient of interaction effect, b_{ii} the coefficients of quadratic effect, b_{iii} the coefficients of cubic effect, and n is the number of variables considered in the analysis.

The experimental responses for the RSM were yield (mg E/g dw), TPC (mg/g E), TFC (mg/g E) and Fx (mg/g E). Additionally, the parametric responses included the time-dose dependent parameter V_m of the antioxidant methods. This parameter denotes the average specific mass of extracted material necessary for capturing the radicals ABTS (nM R•/g dw), and $DPPH^{\bullet}$ (nM R•/g dw) or protecting βCM (μM βC/g dw). Higher *Vm* values indicate greater antioxidant activity. The relative standard deviation (RSD) values for the response variables were lower than 10 %.

2.2.5. Validation

A separate series of experiments was conducted using the predicted optimal factor levels to evaluate the simultaneous optimization's reliability. To this aim, AN extract was obtained under the identified optimal conditions, and all response variables were quantified to compare predicted with the actual experimental results.

2.3. Evaluation of biological activities of the optimized AN extract

The biological properties of AN extract obtained at optimized MAE conditions were evaluated, namely antimicrobial and anti-quorum sensing properties, anti-enzymatic and inhibitory activities against ROS and reactive nitrogen species (RNS), anti-inflammatory activities, and cytotoxic effects.

2.3.1. Antimicrobial activity

2.3.1.1. Strains and culture conditions. AN extract was individually assessed against a panel of target microorganisms in the food industry, specifically, two Gram-negative and two Gram-positive bacteria. the cultures of *Escherichia coli* ATCC 25158, *Pseudomonas aeruginosa* PA01 ATCC 15692, and *Staphylococcus aureus* ATCC 25923 were generously provided by the Center for Research and Development in Food Cryotechnology (CIDCA, CCT-CONICET La Plata, Argentina). Additionally, *Listeria innocua* (CIP 8011, CCMA 29) was provided by Facultad de Farmacia y Bioquímica (Buenos Aires, Argentina). The bacterial strains were cultured and properly diluted as described by (Pellegrini et al., 2014).

2.3.1.2. Disk diffusion method. The disk diffusion method was performed to qualitatively screen the inhibition activity of the AN extract (Pellegrini et al., 2014). The zones of inhibition were measured as an indicator of the AN extract's potential antimicrobial activity.

2.3.1.3. Broth microdilution method. Minimum inhibitory concentration (MIC) refers to the lowest concentration of the AN extract that effectively inhibits bacterial growth (Pérez, Falqué & Domíngues, 2016), while minimum bactericidal concentration (MBC) refers to the lowest concentration of the extract required to kill 99.9 % of the bacterial population. To determine the MIC and MBC values of the AN extract, concentrations ranging from 6.25 to 75 mg/mL were evaluated using the broth microdilution method, as described by Pellegrini et al. (2014). MIC was determined as the concentration of extract that effectively inhibited the bacterial growth, indicated by the presence of a clear well with no visible signs of bacterial growth. In addition, the MBC was determined by performing subcultures from the clear wells, which did not show growth.

2.3.2. Anti-quorum sensing activity

Chromobacterium violaceum ATCC 12472 (provided by Malbrán Institute, Argentina) was used as the biosensor strain in this study. *C. violaceum* releases a purple pigment called violacein in response to autoinducer synthesis (Pellegrini et al., 2014). The bacterial strain was cultured and properly diluted as described previously. Flask-incubation assays were performed following the methodology previously proposed (Pellegrini et al., 2014). The inhibition of violacein production could be attributed to two possibilities: (1) anti-quorum sensing activity, involving the quenching of quorum sensing (QS) signals, or (2) inhibition of cell growth. To differentiate between both possibilities, the antimicrobial activity against *C. violaceum* was analyzed. A seaweed extract with significant anti-QS activity should display a reduction of at least 50 % in the production of violacein without altering microbial growth (Pellegrini et al., 2014).

2.3.3. Inhibition of central nervous system related enzymes

The inhibition of tyrosinase, monoaminoxidase oxidase A (MAO-A) and B (MAO-B) acetylcholinesterase (AChE), and butyrylcholinesterase (BuChE) was studied since these enzymes are related to neurological disorders.

2.3.3.1. Tyrosinase inhibition. Tyrosinase inhibition was determined following the methodology described by Masuda, Yamashita, Takeda, and Yonemori (2005). The absorbance of the samples was assessed at 475 nm and the results were expressed as the % of inhibition.

2.3.3.2. Monoamino oxidase A and B inhibition. The inhibition activities of MAO-A and MAO-B were evaluated by monitoring the production of 4-hydroxyquinoline at 314 nm for 70 min. Kynuramine (3.75 mM) was served as the substrate of the enzymatic reaction, following the established methodology by Soares et al. (2021). Positive and negative controls were conducted using clorgyline, and blanks were prepared with buffer in place of extracts or enzymes, respectively. Results were expressed as % inhibition.

2.3.3.3. Cholinesterase inhibition. The AChE and BuChE inhibition activities by the AN extracts were determined following a modified version of the originally described by Ellman, Courtney, Andres, and Featherstone (1961) and adapted by Delerue et al. (2021). Results were expressed as % inhibition.

2.3.4. Antioxidant activity assessed by the reactive oxygen and nitrogen species inhibitory activity

The inhibitory activity of the optimized AN extract against ROS and reactive nitrogen species (RNS) was evaluated through four assays: the scavenging activity against superoxide anion ($O_2^{\bullet-}$), hydrogen peroxide (H_2O_2), hydroxyl radical ($^{\bullet}OH$), and nitric oxide ($^{\bullet}NO$).

2.3.4.1. Superoxide anion radical scavenging activity. This determination was carried out following a previously described methodology Oliveira et al. (2009). The results were expressed as the half maximal effective concentration (IC_{50}, mg/mL), indicating the AN extract concentration required to scavenge 50 % of the radical.

2.3.4.2. Hydrogen peroxide scavenging activity. This determination was performed following previous procedures (Gülçin, Mshvildadze, Gepdiremen, & Elias, 2006; Mancini et al., 2018). For each dilution, a blank sample was prepared by replacing H_2O_2 solution with the buffer. Additionally, a negative control was prepared using the buffer solution instead of the sample. Results were expressed as IC_{50} (mg/mL).

2.3.4.3. Hydroxyl radical scavenging activity. This determination was conducted based on the salicylic acid method as previously described (Geng, Ren, Lui, & Shang, 2012; Smirnoff & Cumbes, 1989). Blank samples were made by replacing H_2O_2 with demineralized water, while the negative control consisted of replacing AN extract with water. Results were expressed as IC_{50} (mg/mL).

2.3.4.4. Nitric oxide scavenging activity. This activity was analyzed following a diazotization reaction (Soares et al., 2021; Sun, Zhang, Broderick, & Fein, 2003). Blanks were prepared by adding 2 % phosphoric acid instead of the Griess reagent. The IC_{50} (mg/mL) value was used to express the results.

2.3.5. Anti-inflammatory activity

The anti-inflammatory activity of the optimized AN was evaluated as was explained in our previous work (Cassani et al., 2022). The results were quantified as IC_{50} (μg/mL), indicating the concentration of the extract needed to inhibit 50 % of the NO production induced by LPS stimulation.

2.3.6. Cytotoxicity

The cytotoxic impact of the AN extract was evaluated in four cell lines following the procedure explained in our previous study (Cassani et al., 2022). The results were expressed as IC_{50} (μg/mL), which denotes the concentration of AN extract required to inhibit 50 % of cell growth in the respective cell lines.

2.4. Numerical methods, statistical analysis, and graphical illustrations

The fitting procedures for the equations related to the experimental responses (yield, TPC, TFC and Fx) and parametric values (V_m) of the antioxidant methods ($ABTS^{\bullet+}$, $DPPH^{\bullet}$ and βCM) in RSM were conducted using a Microsoft Excel spreadsheet. The statistical tests and coefficient estimation followed a three-stage process: (1) the non-linear least-squares (quasi-Newton) method was used to estimate the coefficients by minimizing the sum of quadratic differences between the predicted and experimental values (Torrado et al., 2013); (2) '*SolverAid*' model was applied to determine the parametric confidence intervals and the significance of the coefficients, excluding those not statistically significant (p-value > 0.05) (Prieto et al., 2015); and (3) the model's uniformity was analyzed using different statistical criteria: R2 and R^2_{adj} assessed the variability of the dependent variable; the mean squared error (MSE), the root mean square error (RMSE) and the mean absolute percentage error (MAPE) were calculated to evaluate the agreement of the fitting and the Durbin-Watson coefficient (DW) was used to check the autocorrelation of the residuals of the model.

For the biological activity experiments involving the AN extract obtained under optimal conditions, triplicate experiments were performed, and the results were presented as means ± standard deviation (SD). To calculate the IC_{50} of the optimized AN extract in different biological activities, data were adjusted to non-linear regression models using Graph Pad prism version 8.0.1 (GraphPad Software Inc., San Diego, CA, USA). DeltaGraph v.7. was used to create the graphic illustrations from the obtained data.

3. Results and discussion

3.1. Optimization of MAE conditions by RSM

3.1.1. Model fitting and statistical analysis of the parametric values

Table 1 displays the mean values of response variables investigated, namely yield, phytochemical content, and antioxidant activity, for each of the 28 combinations of conditions (X_1, X_2 and X_3) employed in the MAE using CCCD. Experimental data were fitted to second or third-order polynomial models according to Eq. (2). Table 2 presents the estimated regression coefficients and outcomes obtained from the statistical fitting analysis. Predicted models for all responses showed statistical significance ($p < 0.05$). By considering solely the coefficients that were statistically significant ($p < 0.05$), the reduced forms of the polynomial equations for each of the investigated responses are as follows:

$$Y_{DW} = 437.21 + 22.60P - 86.94S - 42.23P^2 - 57.15S^2 - 12.78tP + 8.64tPS + 3.98t^2P^2S^2 \quad (3)$$

$$Y_{TPC} = 79.16 - 3.58t - 4.11P - 9.39S - 7.08P^2 - 13.53S^2 + 1.86S^3 + 2.06PS - 0.17t^2P^2S^2 \quad (4)$$

$$Y_{TFC} = 14.61 + 0.51t + 0.70P - 1.13S - 1.42P^2 + 0.13tP + 0.79PS + 0.12t^2P^2S^2 \quad (5)$$

$$Y_{Fx} = 0.35 - 0.14P + 1.48S + 0.30P^2 - 0.49S^3 - 0.08tP - 0.05tPS - 0.04t^2P^2S^2 \quad (6)$$

$$Y_{DPPH} = 27.56 - 6.68S - 1.65P^2 - 5.74S^2 + 1.37S^3 + 0.91PS \quad (7)$$

$$Y_{ABTS} = 144.35 + 4.79t - 26.08P + 15.51S - 5.20P^2 - 28.47S^2 - 8.43PS \quad (8)$$

$$Y_{\beta CM} = 0.0386 - 0.0035t - 0.0006P - 0.0156S - 0.0014tP + 0.0011tS + 0.0039PS \quad (9)$$

Overall, the linear, quadratic, and interactive effects resulted statistically significant ($p < 0.05$), whereas the cubic term associated with solvent concentration exhibited a significant influence on TPC, Fx and DPPH.

Regarding the evaluation of the models, different parameters were used to assess the adequacy of the fitting. The adjusted coefficient of determination (R^2_{adj}), as depicted in Table 2**B**, was within the range of 0.68 to 0.95, confirming that the experimental data obtained for all responses fit properly with the regression models. The DW statistic was calculated to assess autocorrelation in the residuals of the mathematical model. This statistic was in the range of 1.32–2.2, showing no substantial evidence of serial correlation (DW > 1) (Table 2**B**).

3.1.2. Influence of extraction conditions on response variables

Fig. 2 shows three-dimensional surface plots derived from the RSM analysis. These plots visualize the combined effects of two independent variables (t, P and S) on each response while keeping the remaining

Table 2

A) Parametric results of the third-order polynomial equation of Eq. [2] for MAE assessed and in terms of the extraction behavior for the three response value formats assessed (*Yield, Phytochemical content and Antioxidant Activity*). Variables are presented in codified ranges. The parametric subscript 1, 2 and 3 refers to the variables involved (X_1, X_2 and X_3), respectively. **B)** Statistical information of the fitting analysis according to the significance of the parameters ($\alpha = 0.05$). **C)** Optimum conditions in natural values that lead to optimal response values formats assessed.

Coefficients		Parametric responses to the central composite designs													
		Extract		*Phytochemical content*						*Antioxidant Activity*					
		Yield		*TPC*		*TFC*		*Fx*		*DPPH•*		*ABTS•+*		*βCM*	
A) Fitting coefficients obtained															
Intercept	b_0	437.213	±15.971	79.156	±1.419	14.612	±0.275	0.345	±0.161	27.560	±0.687	144.353	±5.884	0.038	±0.002
Linear effect	b_1	*ns*		−3.583	±0.687	0.506	±0.133	*ns*		*ns*		4.793	±3.319	−0.003	±0.002
	b_2	22.600	±7.737	−4.115	±0.687	0.702	±0.133	−0.145	±0.094	*ns*		−26.077	±3.319	−0.001	±0.002
	b_3	−86.944	±7.737	−9.393	±2.304	−1.129	±0.133	1.484	±0.316	−6.682	±1.298	15.51	±3.32	−0.016	±0.002
Quadratic effect	b_{11}	*ns*		*ns*		*ns*		*ns*		*ns*		*ns*		*ns*	
	b_{22}	−42.229	±12.452	−7.085	±1.106	−1.423	±0.214	0.300	±0.151	−1.655	±0.460	−5.196	±3.940	*ns*	
	b_{33}	−57.150	±12.452	−13.532	±1.106	*ns*		*ns*		−5.737	±0.460	−28.473	±3.940	*ns*	
Cubiceffect	b_{111}	*ns*		*ns*		*ns*		*ns*		*ns*		*ns*		*ns*	
	b_{22}	*ns*		*ns*		*ns*		*ns*		*ns*		*ns*		*ns*	
	b_{333}	*ns*		1.86	±0.907	*ns*		−0.487	±0.124	1.367	±0.511	*ns*		*ns*	
Interactive effect	b_{12}	−12.780	±5.492	*ns*		0.127	±0.094	−0.079	±0.067	*ns*		*ns*		−0.001	±0.001
	b_{13}	*ns*		*ns*		ns	ns	*ns*		*ns*		*ns*		0.001	±0.001
	b_{23}	*ns*		2.065	±0.488	0.793	±0.094	*ns*		0.909	±0.275	−8.433	±2.356	0.004	±0.001
	b_{123}	8.641	±3.390	*ns*		*ns*		−0.050	±0.041	*ns*		*ns*		*ns*	
	b_{112233}	3.985	±2.017	−0.167	±0.179	0.118	±0.030	−0.037	±0.018	*ns*		*ns*		*ns*	
B) Statistical information of the fitting analysis															
Obs		28		28		28		28		28		28		28	
df		20		19		20		20		22		21		21	
R^2		0.937		0.987		0.970		0.709		0.967		0.9320		0.896	
R^2adj		0.871		0.953		0.902		0.682		0.919		0.9048		0.821	
MEC		280.123		602.714		0.016		0.086		158.013		2397.5		0.003	
RMSE		16.731		24.553		0.126		0.526		12.572		48.96		0.050	
MAPE		12.150		12.782		6.551		8.921		12.413		15.23		5.072	
DW		2.228		1.321		2.047		1.896		2.179		1.659		2.093	
C) Optimal conditions and response values obtained															
Optimum conditions	X_1: *t* (min)	25.00	±2.00	3.00	±0.03	25.00	±2.25	21.25	±1.91	3.00	±0.24	25.00	±0.25	3.00	±0.18
	X_2: *P (bar)*	10.22	±0.51	9.19	±0.46	12.36	±0.25	2.00	±0.18	10.26	±0.92	2.00	±0.12	2.00	±0.10
	X_3: *S (%)*	26.74	±2.67	39.69	±1.98	42.38	±1.70	78.38	±7.84	35.05	±0.35	65.50	±5.90	0.00	±0.00
		mg/g dw		*mg/g dw*		*mg/g dw*		*mg/g dw*		*nM R•/g dw*		*nM R•/g dw*		*μM βC/g dw*	
Responses		471.00	±28.26	87.55	±1.75	15.72	±0.47	2.55	±0.18	29.33	±1.47	189.32	±15.15	0.08	±0.01

ns: non-significant coefficient; *Obs*: Number of observations; *df*: Number of degrees of freedom; R^2: Coefficient of determination; R^2adj: The adjusted determination coefficient for the model; *MSE*: The Mean Square of the Error; *RMSE*: The Root Mean Square of the Errors; *MAPE*: The Mean Absolute Percentage Error; and *DW*: The Durbin-Watson statistic.

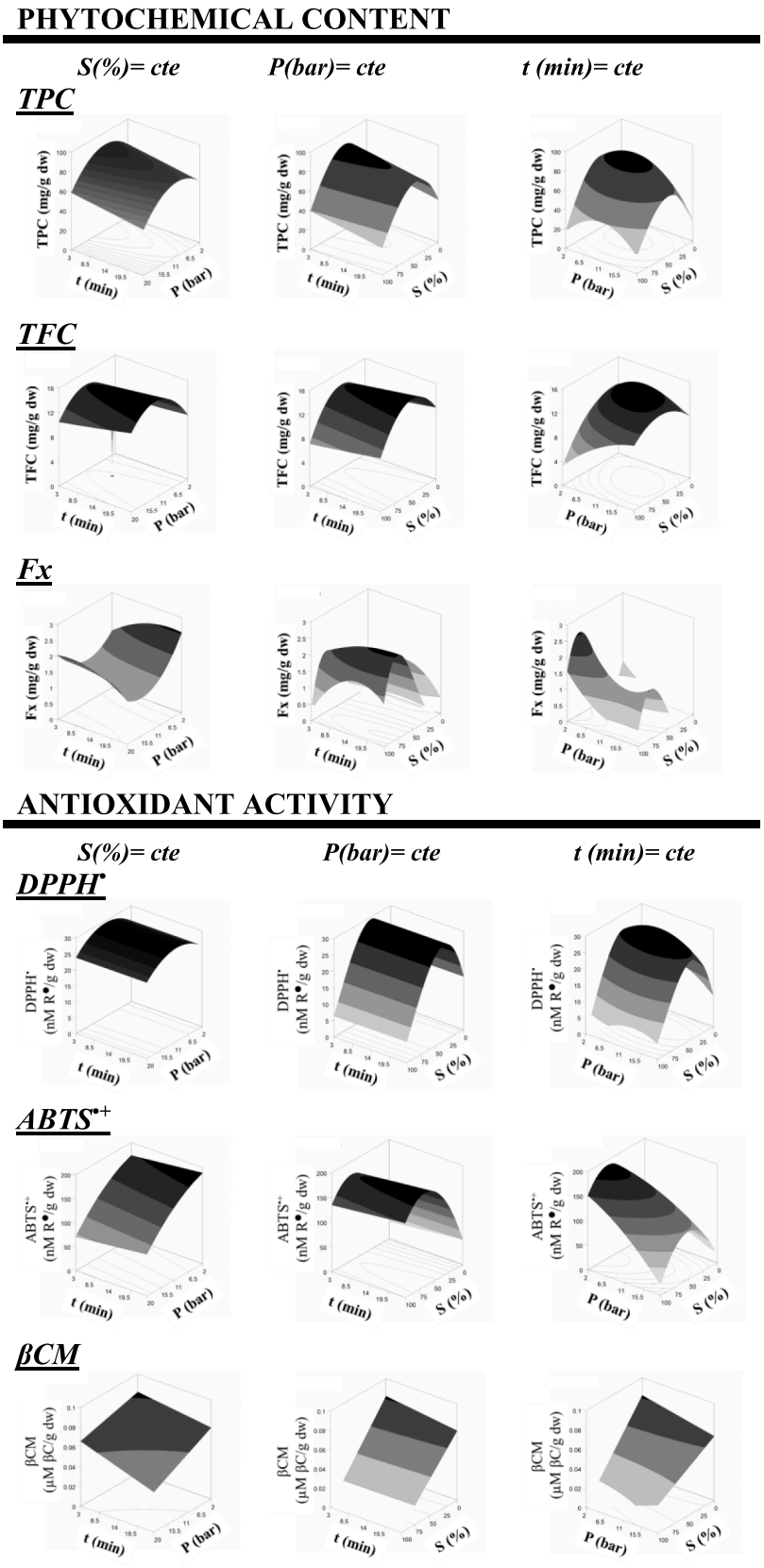


Fig. 2. Response surface plots depicting the combined effect of two independent variables while keeping the third variable at its central value for all the response variables examined.

variable at an intermediate value. These response patterns were obtained from the parametric values predicted by the third order polynomial Eq. [2]. Fig. 1**S**, on the other hand, depicts the distribution of residuals concerning each of the extraction variables. In every case, this distribution was randomly dispersed around zero with no presence of autocorrelation. Additionally, high correlation between the experimental and predicted values was observed suggesting the efficacy of the developed models (Eqs. (3) to (9)) to elucidate the phytochemical compounds and antioxidant activity of the AN extract through the fitted models.

Regarding extraction yield, the concentration of the solvent, both in its linear and quadratic terms, was the most influential factor impacting the yield (Table 2**A**). This observation indicated that using lower ethanol concentrations resulted in higher extraction yields (Fig. 1), suggesting that most of the extractable compounds were highly polar (e.g., polysaccharides, algae-derived polyphenols). Pressure additionally exerted an influence on the extraction yield, as higher pressure levels resulted in increased extraction yields (Fig. 1).

With regards to the phytochemical composition, it is important to highlight that while TPC displays a more complex model, incorporating a cubic term, in comparison to the model for TFC, the response surface plots effectively demonstrate a consistent pattern between the two (Fig. 2). Both TPC and TFC exhibited a steady increase with increasing pressure and solvent concentration, up to a specific value. However, beyond this value, there was a noticeable reduction in the levels of polyphenolic compounds (Fig. 2). Furthermore, increasing time exhibited a favorable impact on the extraction of TFC, while simultaneously exerting a detrimental influence on the recovery of TPC. The complex interrelation among the independent variables was unveiled by the statistical significance of the interactive terms within both the TPC and TFC models. It has been stated that AN seaweed is a rich source of phlorotannins and compounds belonging to the fuhalol, fucophlorethol, and phlorethols subclasses have been identified in this species (Sardari, Prothmann, Gregersen, Turner, & Nordberg Karlsson, 2020; Tierney et al., 2014). Despite other authors found that AN seaweed contains larger phlorotannins with degree of polymerization (DP) ranging from 6 to 13 phloroglucinol unit (Tierney et al., 2014), a more recent work reported the presence of shorter phlorotannins (DP from 3 to 6) (Sardari et al., 2020). These differences in the phlorotannins composition may explain differences in the polar properties of these compounds. Although phlorotannins are highly polar compounds, our findings indicate that an ethanol concentration of approximately 40 % is necessary for their effective extraction. Regarding the effect of pressure, increasing pressure levels result in a corresponding temperature rise, thereby inducing the absorption of energy by water molecules. The increased temperature within cells diminishes their structural integrity, rendering them susceptible to disintegration. This process subsequently accelerates the release of phenolic compounds and enhances their dissolution into the solvent. However, surpassing a specific pressure can result in the degradation of phenolic compounds.

On the other hand, solvent concentration was the predominant factor affecting the extraction of Fx, as evidenced by the statistical significance of its linear, quadratic, and cubic terms. Based on Fig. 2, Fx extraction was favored at low pressure level and higher solvent concentration, regardless of time assayed. Overall, these results indicated that the extraction efficiency of most compounds in AN was significantly determined by both the solvent polarity and the nature of the extracted compounds. While the extraction of most of the polyphenolic compounds was favored at intermediate ethanol concentrations (40 %), pigments such as Fx required a higher proportion of ethanol in the solvent for optimal extraction. In previous studies, ethanol (100 %) was the most efficient solvent in terms of extraction yield to recover pigments (including Fx) from AN (Garcia-Perez et al., 2022). This is attributed to the capacity of ethanol to dissolve compounds with heterogeneous chemical structures. In this way, ethanol can effectively extract components with polar properties (proteins and derivatives) and precipitate polysaccharides that could affect analytical determinations (Garcia-Perez et al., 2022). Similar to our results, other authors optimized Fx extraction from *Padina tetrastromatica* using UAE, finding that the highest Fx content was achieved at 50 °C for 30 min and using 80 % ethanol (Raguraman et al., 2018).

Regarding antioxidant activity, when contrasting the two SET assays, solvent concentration was the predominant factor affecting the antioxidant activity as assessed by the DPPH method. Interestingly, the response surface plots resulted from the DPPH assay are similar to those obtained for TPC (Fig. 2). In fact, the MAE conditions that led to the maximum antioxidant activity assessed by the DPPH method closely correspond to those associated with TPC. These results suggest that phlorotannins could be responsible for the antioxidant effect. The presence of numerous interconnected aromatic rings and several hydroxyl groups very close to each other available to act as hydrogen or electron donors may explain the high free radical scavenging activity of phlorotannins (Cassani et al., 2020). Consistently, ethanol concentration was the most significant factor affecting DPPH assay for phenolic extracts obtained by MAE from *Sargassum vestitum* (70 % ethanol led to the highest antioxidant activity values) (Dang, Bowyer, Van Altena, & Scarlett, 2018).

For the ABTS method, both pressure and solvent concentration demonstrated significant impacts on this property (Fig. 2). Notably, an increase in pressure was found to exert an adverse effect on the antioxidant activity as determined by the ABTS assay. This observation is consistent with the previously discussed findings concerning Fx, suggesting that this pigment could exhibit antioxidant activity.

With respect to βCM results, the influence of the independent variables resulted in a detrimental effect on the protection of β-carotene against oxidation. This deduction is derived from the statistical significance associated with their linear factors, indicating that to avoid degradation of this pigment, it is necessary to employ reduced values of time, pressure, and solvent concentration. Among the extraction conditions, solvent concentration was the main factor affecting the protection of β-carotene. Considering the hydrophobic nature of this pigment, it was expected that a higher concentration of ethanol within the solvent would be imperative to prevent its deterioration.

3.1.3. Individual and simultaneous optimization and validation of optimal conditions

Table 2**C** displays the optimal conditions of time, pressure and ethanol concentration that individually maximized every response variable (yield, TPC, TFC, Fx, DPPH, ABTS, and βCM). The predicted values for these responses were calculated using their optimal MAE conditions in their respective models. Based on these results, among the phytochemical compounds, the conditions that yield maximal TPC closely align with those resulting in TFC, differing primarily in terms of the duration of the extraction process. It is noteworthy that the longer time required to optimize TFC negatively impacted the extraction of TPC. In contrast to TPC and TFC, the ideal conditions to optimize the Fx extraction differed significantly, involving low-pressure levels and a high ethanol concentration. Likewise, the optimal conditions required to individually maximize each response associated with antioxidant activity were markedly different. This discrepancy suggests that these methodologies offer complementary insights, contributing to a complete comprehension of governing mechanisms in AN extract's antioxidant compounds. In conclusion, these findings collectively indicate that the optimal condition that maximizes a specific response could adversely influence the other responses. This emphasizes the need for the implementation of simultaneous optimization approaches.

In this context, simultaneous optimization was carried out on data modeled through RSM to obtain the maximum recovery of phytochemical compounds exhibiting antioxidant activity from the AN extract. Thus, the criterion applied was to maximize the responses predicted by the established models. The resultant optimal MAE conditions were determined as follows: a time of 3.0 min (t), a pressure of 10.4 bar

(*P*), and an ethanol concentration of 46.8 % (*S*). Consequently, the predicted values for each response under these optimal conditions were determined. A new set of assays was performed at the predicted optimal conditions to experimentally check the reliability of the developed models. Both predicted and experimental values are depicted below:

- Y_{DW}(mg/g dw) = 439.57 ± 17.58 (predicted); 580.57 ± 21.98 (experimental)
- Y_{TPC}(mg/g dw) = 86.45 ± 8.65 (predicted); 103.51 ± 24.74 (experimental).
- Y_{TFC}(mg/g dw) = 13.79 ± 0.28 (predicted); 15.44 ± 0.78 (experimental).
- Y_{Fx}(mg/g dw) = 0.20 ± 0.05 (predicted); 0.26 ± 0.01 (experimental).
- Y_{DPPH}(nM R•/g dw) = 28.19 ± 1.41 (predicted); 30.9 ± 2.51 (experimental).
- Y_{ABTS}(nM R•/g dw) = 137.34 ± 4.12 (predicted); 249.5 ± 111.75 (experimental).
- $Y_{\beta CM}$(μM βC/g dw) = 0.05 ± 0.01 (predicted); 0.11 ± 0.01 (experimental).

The experimental results exhibited higher values in contrast to the predicted values. This may be attributed to the use of an algae batch collected during a different season for validation. This discrepancy highlights the potential impact of environmental factors on the phytochemical composition of the algae.

The literature presents conflicting outcomes. Some authors reported higher TPC and extraction yield values (O'Sullivan et al., 2013; Yuan et al., 2018), whereas others have documented lower phlorotannin contents and extraction yield compared to our research (Liu et al., 2019; Toan et al., 2021). These differences can be attributed to variations in extraction conditions (solid-to-solvent ratio, solvent type and concentration, temperature/ power, time) as well as the collection season, geographical location, and environmental factors. The contrasting findings in the literature support optimizing extraction protocols for each species under study.

3.2. Biological activities of the optimized AN extract

3.2.1. Antimicrobial activity

Table 3 displays the findings of the antimicrobial activity of the optimized AN extract. AN extract was able to inhibit the growth of all tested bacteria with similar effectiveness between strains (MIC and MBC values of 11.90 mg/mL). Other authors who also worked with AN alga but using Soxhlet and acetone for extraction observed higher antibacterial effect against *S. aureus* ATCC 25925 and *E. coli* ATCC 25218 with MIC of 0.20 mg/mL and 0.40 mg/mL, respectively (Jiménez, O'Connell, Lyons, Bradley, & Hall, 2010). Similarly, higher antimicrobial effects were found against *E. coli* ATCC 25922 for extracts obtained by pressurized liquid extraction and ethanol, with 24.14 % growth inhibition at 500 μg/mL (Boisvert et al., 2015). In contrast, other researchers reported that AN extracts obtained using UAE and 0.1 M HCl had lower antibacterial activity (MIC for *E. coli* DSM 1103, *S. aureus* NCTC 8178 and *L. monocytogenes* NCTC 11994 of 596, 29.8 and 59.7 mg/mL, respectively) (Kadam et al., 2015). No reports regarding MBC determination in AN were found.

Different authors reported that phlorotannins are responsible for the antimicrobial activity of AN. For example, phlorotannins isolated from AN showed bacteriostatic and bactericidal effect against *E. coli* O157:H7 at 25 and 50 μg/mL, respectively (Wang, Xu, Bach, & McAllister, 2009). It was suggested that the antimicrobial effect of phlorotannins can be due to their interaction with bacterial proteins through hydrogen bonds and hydrophobic interactions, thus leading to cell membrane damage and cytoplasm release (Cassani et al., 2020). In addition, the antimicrobial activity of phlorotannins is also determined by the structure of the bacterial cell wall. Considering that gram-negative microorganisms have a high lipopolysaccharide content, they are generally more resistant to antimicrobial agents than gram-positive ones (Cassani et al., 2020). However, in this work, no differences in the antimicrobial activity between gram-positive and negative bacteria were found. Other compounds present in brown algae extracts such as sulfated polysaccharides, laminarian, and diterpenes have also revealed antimicrobial activity (Kandasamy, Khan, Evans, Critchley, & Prithiviraj, 2012). In this context, the mixture of bioactive compounds in the optimized AN

Table 3
Biological activities of the optimized AN extract: **A)** *In vitro* antimicrobial activity against Gram-negative and Gram-positive pathogenic bacteria (by agar diffusion and microdilution methods); **B)** Inhibitory activity against reactive oxygen (ROS) and nitrogen species (RNS); **C)** Inhibition of central nervous system related enzymes; **D)** Anti-inflammatory activity; **E)** Cytotoxic effects.

A: Antimicrobial activity			
	Inhibition (mm)	***MIC // MBC (mg/mL)***	
E. coli ATCC (25158)	7.25 ± 0.71	11.90 // 17.85	
P. aeruginosa (ATCC 15692)	7.00 ± 0.00	11.90 // 11.90	
S. aureus (ATCC 25923)	7.50 ± 0.71	11.90 // 11.90	
L. innocua (CIP 8011, CCMA 29)	7.75 ± 0.35	11.90 // 11.90	
B: Antioxidant activity (ROS, RNS)			
	IC_{50} (mg/mL)		***IC_{50} (μg/mL)***
Nitric oxide ($^{\bullet}NO$)	0.13		*Ascorbic acid (446 μg/mL)*
Superoxide anion ($O_2^{\bullet-}$)	0.08		*Ascorbic acid (160 μg/mL)*
Hydrogen peroxide (H_2O_2)	0.05		*Ascorbic acid (51 μg/mL)*
Hydroxyl radical ($^{\bullet}OH$)	>1 mg/mL		*Ascorbic acid (183 μg/mL)*
C: Enzymatic Inhibition			
	1 mg/mL	***2 mg/mL***	***IC_{50} (μg/mL)***
Tyrosinase	90.64 ± 2.85	87.96 ± 4.43	*Kojic acid (1.82 μg/mL)*
Monoaminoxidase oxidase A (MAO-A)	85.40 ± 0.40	87.33 ± 1.42	*Clorgyline (25 ng/mL)*
Monoaminoxidase oxidase B (MAO-B)	75.90 ± 1.33	78.24 ± 1.64	*Selegiline (2.10 μg/mL)*
Acetylcholinesterase (AChE)	19.84 ± 0.25	19.17 ± 11.20	*Galantamine (0.92 μg/mL)*
Butyrylcholinesterase (BUChE)	21.52 ± 0.55	15.07 ± 4.33	*Galantamine (4.92 μg/mL)*
D: Anti-inflammatory activity			
	IC_{50} (μg/mL)		***IC_{50} (μg/mL)***
RAW264.7	>400		*Dexamethasone (6.17 μg/mL)*
E: Cytotoxic effects			
	IC_{50} (μg/mL)		***IC_{50} (μg/mL)***
AGS	114.34 ± 5.81		*Ellipticine (<1 mg/mL)*
A549	282.07 ± 6.13		*Ellipticine (<1mg/mL)*
HepG2	60.58 ± 1.28		*Ellipticine (<1mg/mL)*
Vero	>400		*Ellipticine (<1mg/mL)*

Abbreviations: MIC, minimum inhibitory concentration; MBC, minimum bactericidal concentration; Vero, the African green monkey kidney-derived cell line; AGS, the human gastric cancer cell line; A549, the human lung adenocarcinoma cell line; HepG2, the human hepatocarcinoma cell line.

extract may have exerted a synergistic effect on the antimicrobial activity against the tested bacteria.

3.2.2. Anti-quorum sensing activity

Numerous bacterial strains that display resistance to multiple drugs are manifesting novel and diverse mechanisms to counteract antimicrobial effects. This phenomenon leads to a decline in the effectiveness of traditional antibiotics (Pérez, Falqué, & Domínguez, 2016). In this regard, strategies to inhibit the production of virulence factors associated to bacterial infections are chosen than killing microorganisms with antibiotics. For example, a less aggressive strategy to combat bacterial infections caused by antibiotic-resistant strains is the modulation of their pathogenesis mechanisms by inhibiting their cell-to-cell communication capacity, also known as 'quorum sensing' (Pellegrini et al., 2014; Tang, Wang, & Chu, 2020). The inhibition of QS mechanism can contribute to reduce de virulence of pathogens, thus controlling infections without promoting the appearance of resistant bacterial strains (Tomadoni et al., 2016).

QS-system regulates the violacein production by *C. violaceum*. Thus, decreased pigment production indicates disruption of the QS system (Tang et al., 2020). Fig. 3**A** depicts the inhibition of violacein production when exposing *C. violaceum* (biomonitor) to different concentrations of the optimized AN extract. Simultaneously, the *C. violaceum* viability (log CFU/ mL) was checked to verify that the inhibition of the purple pigment production was caused by the QS mechanism and not by microbial growth. A nonlinear dose–response relationship between violacein production and AN extract concentration was found ($R^2 = 0.947$). In addition, the optimized AN extract showed anti-QS activity at 2.5 mg/mL, meaning that it was able to interrupt intercellular communication, which was reflected by the inhibition of violacein production (about 60 %) without affecting cell viability of *C. violaceum*. Beyond this concentration, no further changes in violacein inhibition were noticed; however, significant reduction of bacterial counts (≈ 4 log CFU/mL) was found.

The anti-QS activity can be attributed to bioactive compounds present in the optimized AN extract. (Tang et al., 2020) found that phlorotannins from *H. fusiforme* not only was able to inhibit violacein production in *C. violaceum* 12,472 but also to reduce the production of virulence factors and interrupt the biofilm-forming ability associated with the *P. aeruginosa* pathogenicity. On the other hand, other authors found that a fucose-containing polymer rich fraction of *Ascophyllum nodosum* (L.) significantly reduced the gene expression of QS systems identified in *P. aeruginosa* (*i.e.*, lasI, lasR, rhlI, and rhlR) that are associated with the synthesis and regulation of virulence genes (Kandasamy et al., 2015). These authors proved that suppressing the bacterial QS leads to the inhibition of virulence factors secretion, toxic metabolites production and biofilm-forming capacity.

3.2.3. Inhibition of central nervous system related enzymes

Neurological disorders were considered the second leading cause of death worldwide and the leading cause of disability in 2016 (Feigin et al., 2019). Alzheimer's disease is the most prevalent neurological disorder characterized by a progressive and irreversible decline of brain functions that strongly impacts the life quality of patients (Barbosa, Valentão, & Andrade, 2020). Regulating the activity of key enzymes involved in neurological disorders is a therapeutic approach that can contribute to relieving symptomatology (Barbosa et al., 2020). In this context, the search of multi-target biological compounds from seaweed that may inhibit the central nervous system (CNS) related enzymes is a novel topic still initiating. Thus, the capacity of the optimized AN extract to interact with CNS-related enzymes that can trigger neurological disorders was studied (Fig. 3**B**).

The overexpression of tyrosine (involved in the synthesis of peripheral melanin) in substantia nigra could induce the formation of neuromelanin up to levels that affect normal cell function and thus, could initiate Parkinson degeneration (Carballo-Carbajal et al., 2019). The optimized AN extract showed a potent inhibitory activity against tyrosinase (90 % inhibition) under tested concentrations (Fig. 3**B** and Table 3). Up to our knowledge, this is the first study exploring the ability of AN seaweed to modulate CNS-related enzymes. Other studies using *Fucus* spp. found that *Fucus guiryi* and *F. serratus* extracts had strong inhibitory activity against tyrosinase with IC_{50} values of 47.99 and 47.66 μg/mL, respectively (Barbosa, Valentão, Ferreres, Gil-Izquierdo, & Andrade, 2020). Phlorotannins have been reported to bind proteins and form complexes with enzyme inhibition activity. The DP, position and number of hydroxyl groups, number of aromatic rings, and O-bridge linkages present in phlorotannins play key roles in exerting the inhibitory activity (Barbosa et al., 2020). Other authors found that, among phlorotannins isolated from *Ecklonia stolonifera* Okamura, compound 974-A (the one with the most complex structure) has the highest anti-tyrosinase activity. These authors concluded that the isolated phlorotannins could form bonds with tyrosinase residues through hydrogen bonds and hydrophobic interactions. In addition, phlorofucofuroeckol-A and eckol have been reported to inhibit tyrosinase activity and down-regulate the expression of enzymes related to melanogenesis (Manandhar, Seong, Kim, & Choi, 2019). Additionally, other researchers revealed that Fx from *Laminaria japonica* was able to inhibit tyrosinase activity, melanogenesis in melanoma and skin pigmentation in UVB-irradiated guinea-pigs (Shimoda, Tanaka, Shan, & Maoka, 2010).

On the other hand, the two isoforms of monoaminoxidases (MAO-A and MAO-B) catalyze the oxidation of certain neurotransmitters (serotonin, dopamine, noradrenaline) and endogenous amines (benzylamine and tyramine) to aldehydes. Decreases in these neurotransmitters have been related to cognitive dysfunction, the production of amyloid plaques and the damage of cholinergic neurons (Barbosa et al., 2020; Khatri & Juvekar, 2016). In the present study, AN extract showed high inhibitory activity against both isoforms (MAO-A and MAO-B), reaching 87 % and 78 % inhibition at the highest of extract concentration assayed, respectively (Fig. 3 and Table 3). Similarly, *F. guiryi* and *F. serratus* extracts were more effective to inhibit MAO-A than MAO-B isoform (Barbosa et al., 2020). A structure activity relationship between phloroglucinol, phlorofucofuroeckol (PFF-A), and dieckol and target proteins (including MAOs and G-couple protein receptors) was also observed (Seong et al., 2019). In this regard, those phlorotannins with more complex structure (like PFF-A and dieckol) showed more potent MAO inhibition activity than those with the simplest ones (eckol and phloroglucinol).

Acetylcholinesterase and butyrylcholinesterase are also critical enzymes in the catabolism of acetylcholine, decreasing its content in the brain and thus reducing cognitive function. Many studies reported that eckol-type phlorotannins, characteristic of *Ecklonia* spp., exert a significant AChe and BuChE inhibition activity (Kannan, Aderogba, Ndhlala, Stirk, & Van Staden, 2013; Lee & Jun, 2019). For example, 8,8′-bieckol exhibited the highest inhibitory activity against AChE, followed by dieckol and eckol. This was attributed to structural features of phlorotannins, 8,8′-bieckol being a hexamer containing 11 –OH groups (Lee & Jun, 2019). Additionally, a docking analysis revealed that 8,8′-bieckol form a hydrogen bond with the active site (ARG296) of AChE. In addition, most studies revealed that the inhibition activity of eckol-type phlorotannins was stronger against AChE than against BuChE (Choi et al., 2015; Lee & Jun, 2019; Yoon, Chung, Kim, & Choi, 2008). However, there are no reported data about the inhibitory capacity of AN against AChE and BuChE enzymes. In our work, a low inhibitory effect against AChE and BuChE was observed (around 20 % inhibition), even at the highest concentration assayed, regardless of the tested enzymes (Table 3). As it was previously mentioned, the AChE and BuChE inhibitory activity of phlorotannin was attributed to compounds belonging to eckol class. Polyphenolic profile of AN is mainly composed of fuhalol, fucophlorethol, and phlorethols (Sardari et al., 2020; Tierney et al., 2014), which have not been extensively investigated.

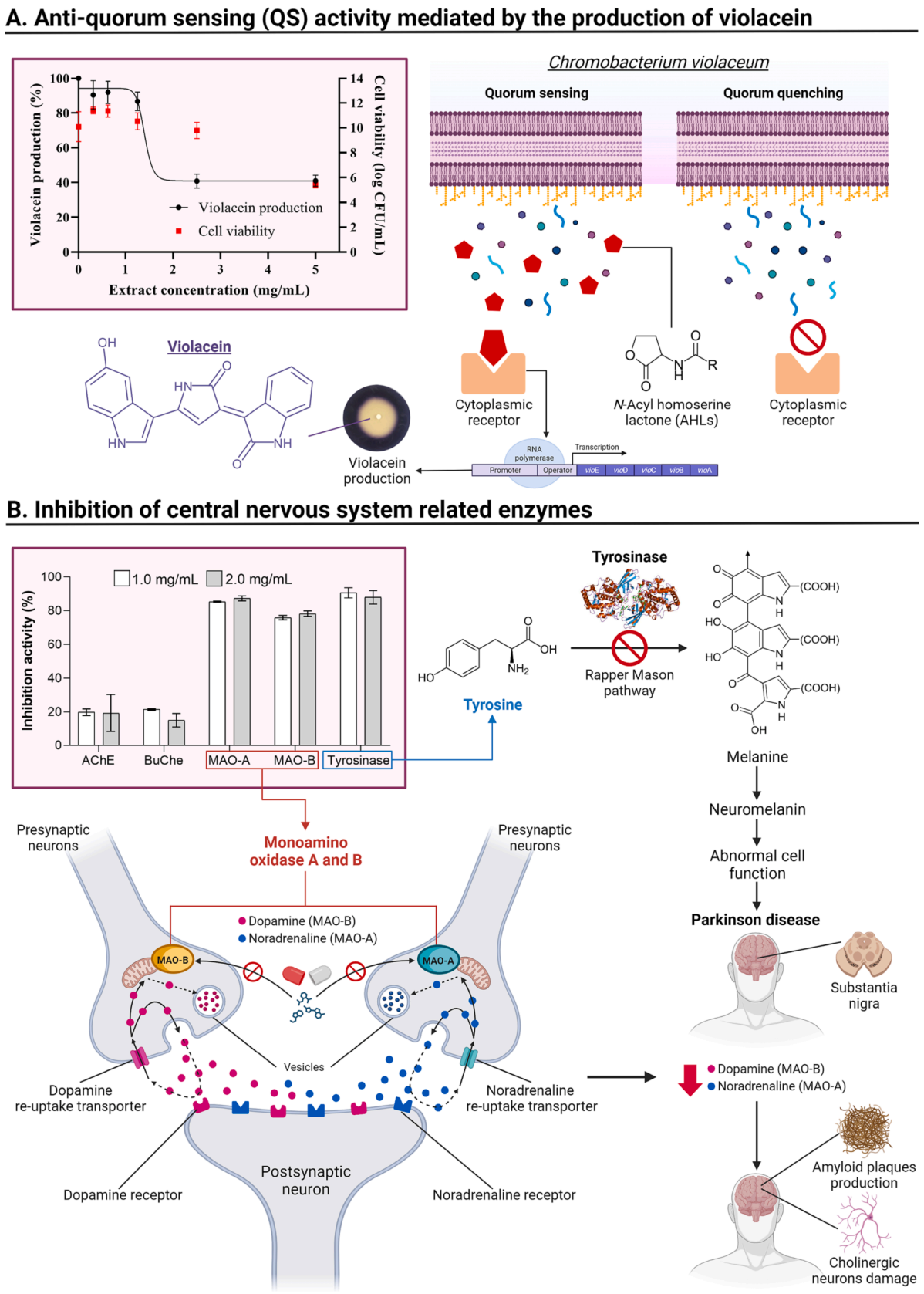


Fig. 3. Graphic representation of the results of the biological properties. **(A)** Anti-quorum sensing (QS) activity results and violacein production method. **(B)** Inhibition of CNS-related enzymes results and action mechanism of MAO-A, MAO-B and tyrosinase.

3.2.4. Reactive oxygen and nitrogen species inhibitory activity

ROS and RNS are unstable and can rapidly trigger chain reactions causing detrimental effects on health-relevant molecules, such as DNA, lipids and proteins (O'Sullivan et al., 2011). They include $O_2^{\bullet-}$, H_2O_2, $^{\bullet}OH$, and $^{\bullet}NO$, responsible for causing oxidative stress that leads to several diseases, such as cardiovascular and neurological disorders, and cancer. Table 3 shows the capacity of the optimized AN extract to scavenge ROS and RNS. The optimized AN extract showed potent scavenging activity against H_2O_2, $O_2^{\bullet-}$ and $^{\bullet}NO$ with IC_{50} values of 50, 79 and 130 μg/mL, respectively. To our knowledge, few studies have explored the ROS and RNS inhibitory activity of AN extracts. (Jiménez et al., 2010) reported that AN extract obtained using Soxhlet and acetone has a lower $O_2^{\bullet-}$ radical scavenging activity ($IC_{50} = 430$ μg/mL) than that observed in our work. Studies have been focused on analyzing ROS and RNS inhibitory activity of other algae species from the same family, such as *F. vesiculosus* and *F. spiralis*. Lower $O_2^{\bullet-}$ radical scavenging activity in *F. vesiculosus* extracts obtained through MAE (5 min, 75 °C, 57 % ethanol concentration) was reported with IC_{50} value of 527.30 μg/mL (Amarante et al., 2020). (Soares et al., 2021) found that the extracts obtained from *F. vesiculosus* using subcritical water extraction showed lower $O_2^{\bullet-}$ and $^{\bullet}NO$ scavenging activity than that in this work (IC_{50} values ranged from 100 to 20 and 125–250 μg/mL for $O_2^{\bullet-}$ and $^{\bullet}NO$, respectively). In contrast, insignificant scavenging effect against $^{\bullet}OH$ was found in our study with IC_{50} value > 1 mg/mL (Table 3). Similarly, the required effective concentration of AN extract to inhibit $^{\bullet}OH$ radicals scavenging activity in 50 % was 1.55 mg/mL (Jiménez et al., 2010).

The ROS and RNS scavenging activity reported in the optimized AN extract has been attributed to phlorotannins. Phlorotannins oligomers (3 to 8 monomeric units) have higher radical scavenging activity in comparison to polymeric fractions. In addition, increasing the DP leads to a decrease in the antioxidant activity of phlorotannins (Bogolitsyn et al., 2019). This was attributed to the formation of intra- and inter- molecular bonds between hydroxyl groups, which results in conformational changes of phlorotannins (Bogolitsyn et al., 2019). Other authors also reported that scavenging activity of phlorotannins from AN significantly depends on the molecular size and polarity characteristics of compounds, suggesting that phlorotannins fractions of low molecular size are more active than the larger ones (Blanc, Hauchard, Audibert, & Ar Gall, 2011). In addition, Fx has been reported to scavenge $O_2^{\bullet-}$, $ABTS^{\bullet+}$ and $DPPH^{\bullet}$ radicals in a dose-dependent manner (Zhang et al., 2014).

3.2.5. Anti-inflammatory activity and cytotoxicity

The anti-inflammatory and cytotoxic activities of the optimized AN extract are displayed in Table 3. Regarding the anti-inflammatory activity, AN extract showed an insignificant effect since IC_{50} value was higher than 400 μg/mL. On the other hand, the optimized AN extract had no cytotoxic effect against the healthy cell lines (Vero, $IC_{50} > 400$ μg/mL), ensuring its safety. This result suggests that AN extract could be used as a functional ingredient by the food, cosmeceutical, and pharmaceutical industries. In addition, cytotoxic activity against cancer cell lines showed promising growth inhibition of the human gastric (AGS), lung (A549) adenocarcinoma cell lines and highlighting a noteworthy impact on against hepatocarcinoma (HepG2) cells with IC_{50} of 60.58 μg/mL. Similarly, AN extracts had shown cytotoxic activity against Caco-2 colon cancer cell lines with IC_{50} of 114, 282 and 33 μg/μL, respectively (Nwosu et al., 2011). Interestingly, the highest effectiveness of the optimized AN extract against Caco-2 cells also affected the cell assembly of the culture, inducing impairment of the pyramidal islets classically associated with this cell line (Esclatine, Lemullois, Servin, Quero, & Geniteau-Legendre, 2000).

Some studies have revealed that phlorotannins exert cytotoxic activity and some key factors such as polarity and molecular size of compounds may significantly affect the expression of such activity (Gonçalves-Fernández, Sineiro, Moreira, & Gualillo, 2019). In this regard, previous studies reported that phlorotannins with more polar characteristics and low molecular size exhibit higher cytotoxic activity against a chondrogenic cell line (Gonçalves-Fernández et al., 2019). These authors found that tetrahydroxyfuhalol isolated from *B. bifurcata* has cytotoxic activity with IC_{50} of 100 μM. Fuhalol-type phlorotannins have been reported in AN and could be responsible for the cytotoxic effects against the different cancer cell lines observed in our study. On the other hand, there is evidence indicating that Fx inhibits the growth of other cancer cell lines (colon, breast, prostate, liver, leukemia, etc.) (Wang, Li, Dong, Zhu, & Cai, 2019). In addition, combining Fx with other drugs (*e.g.*, cisplatin, 5-fluorouracil, and tranexamic acid antagonists), enhances its cytotoxic effects (Wang et al., 2019).

4. Conclusions

The potential of AN as a source of biologically active compounds was studied, offering a rapid MAE procedure using ethanol to recover phytochemicals with promising biological activities.

Results indicated that RSM with CCCD proved to be an efficient strategy to find the optimal MAE conditions (3 min, 10.4 bar and 46.8 % ethanol) that maximized the recovery of phytochemical compounds (polyphenols and fucoxanthin) with enhanced antioxidant activity from AN. In addition, the AN extract obtained under optimal MAE conditions showed other biological activities. Results from antimicrobial and anti-QS activities suggested that the optimized AN extract could offer direct and indirect protection against infection by pathogenic microorganisms. Additionally, the optimized AN extract showed effective inhibitory activity against some critical central nervous system related enzymes (namely, tyrosinase, MAO-A and MAO-B). Besides, the obtained AN extract exhibited strong scavenging activity against ROS and RNS (namely, H_2O_2, $O_2^{\bullet-}$ and $^{\bullet}NO$). In addition, results regarding cytotoxic activity against cancer cell lines were promising since the optimized AN extract was active against three types of cancer cell lines without affecting healthy cell lines.

From these results, it can be concluded that the recovery of phytochemical compounds from AN using green extraction procedures presents a double opportunity: 1) adding AN extract to various products to improve their quality. Because of its antioxidant and anti-quorum sensing characteristics, AN extract can be used in functional foods by incorporating it into edible films and coatings. Because of its neuroprotective and antitumoral properties, AN extract can be used as a supplement in the nutraceutical industry. Furthermore, because of its antioxidant properties, the cosmetics industry can benefit by adding AN extract into sun creams and anti-aging products; and 2) to control the expansion of this algae through sustainable management.

CRediT authorship contribution statement

Lucía Cassani: Conceptualization, Methodology, Software, Validation, Formal analysis, Investigation, Data curation, Writing – original draft, Writing – review & editing, Visualization, Project administration, Supervision. **Aurora Silva:** Conceptualization, Methodology, Software, Validation, Formal analysis, Data curation, Writing – original draft, Visualization. **Maria Carpena:** Methodology, Software, Validation, Writing – original draft, Visualization. **María Celeste Pellegrini:** Methodology, Software, Validation, Investigation, Data curation. **Pascual García-Pérez:** Methodology, Software, Validation, Investigation, Data curation. **Clara Grosso:** Conceptualization, Methodology, Formal analysis, Investigation, Data curation, Funding acquisition, Project administration, Resources. **Maria Fátima Barroso:** Conceptualization, Methodology, Funding acquisition, Project administration, Resources, Supervision. **Jesus Simal-Gandara:** Conceptualization, Methodology, Funding acquisition, Project administration, Resources, Supervision. **Andrea Gómez-Zavaglia:** Investigation, Resources, Writing – review & editing, Supervision. **Miguel A. Prieto:** Conceptualization, Methodology, Software, Formal analysis, Investigation, Funding acquisition, Project administration, Resources, Writing – review & editing,

Visualization, Supervision.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.foodchem.2023.138037.

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