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Chemical Characterization, Antibacterial Activity, and Safety Profile of the Lipid Extract from *Sargassum filipendula* against Multidrug-Resistant Bacteria

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Abstract

Antimicrobial resistance in multidrug-resistant (MDR) bacteria necessitates new therapeutic alternatives, and marine macroalgae represent an underexplored source of bioactive lipids. This study characterized the lipid extract (LE) of *Sargassum filipendula* and evaluated its antibacterial and cytotoxic profiles against MDR hospital isolates. LE composition was determined by GC-MS, and its thermal behavior by thermogravimetric analysis (TGA) and differential scanning calorimetry (DSC). Antibacterial activity was evaluated using the broth microdilution method (CLSI M07) against *Acinetobacter baumannii*, *Escherichia coli*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, *Staphylococcus aureus*, and coagulase-negative staphylococci, followed by determination of the minimum bactericidal concentration. Cytotoxicity was assessed in L929 and MDBK cell lines using MTT, Neutral Red, and Lactate Dehydrogenase assays; Hemolytic activity was evaluated using erythrocytes; and selectivity indices (SI) were calculated for each bacterial species. Palmitic acid (61.07%) and oleic acid (15.90%) were the predominant fatty acids. TGA revealed a total mass loss of 82% across two decomposition stages (296°C, 445°C), and DSC detected no phase transitions, indicating the absence of amorphous or crystalline domains. The LE exhibited bacteriostatic activity against all isolates, with MIC values ranging from 1.18 mg/mL (*S. aureus*) to 2.5 mg/mL (*P. aeruginosa*). Cytotoxicity assays yielded IC_{50} values ranging from 0.16 to 1.25 mg/mL, and the hemolytic IC_{50} was 12.81 mg/mL. However, the SI values remained below the safety threshold for all tested species (1.45–3.90). The *S. filipendula* LE is a candidate source of antibacterial compounds against MDR bacteria, although purification processes and *in vivo* validation are required to establish its therapeutic potential.

Keywords: Antibiotics; Bacterial resistance; Brown macroalgae; Phaeophyceae; Seaweed

Introduction

Antimicrobial resistance represents one of the greatest threats to global health, accounting for more than 4.71 million deaths annually, with projections reaching 39 million by 2050 (Barrantes Jiménez et al. 2022; Naghavi et al. 2024; Laitin et al. 2024). This phenomenon occurs as bacteria, fungi, viruses, and parasites develop over time with new ways to resist antimicrobial treatments (Reynolds et al. 2022; Gauba and Rahman 2023).

Among the 12 families of clinically relevant bacterial pathogens most frequently associated with healthcare-associated infections (HAIs), according to the WHO List of Bacterial Priority Pathogens (BPPL) (World Health Organization 2024), multidrug-resistant (MDR) Gram-positive bacteria (GPB), such as methicillin-resistant *Staphylococcus aureus* (MRSA) and vancomycin-resistant *Enterococcus* spp. (VRE), stand out. In addition, MDR Gram-negative bacilli (GNB), such as *Klebsiella pneumoniae*, *Escherichia coli*, *Pseudomonas* spp. and *Acinetobacter baumannii*, producers of extended-spectrum β-lactamases (ESBL) (Rello et al. 2019; Mulani et al. 2019; Butler et al. 2022; World Health Organization 2024).

Given the growing need for new antibiotics (ATB), natural compounds, especially those derived from seaweed, have attracted interest for their therapeutic properties in addition to their nutritional value (Fialho 2015; Manoj Kumar and Arunkumar 2023; Adarshan et al. 2023). The genus *Sargassum* spp. (class Phaeophyceae), brown macroalgae rich in pigments such as fucoxanthin (Luna-Pérez et al. 2023; Widyaswari et al. 2024), is notable for the presence of fucoidans, sulfated polysaccharides with multiple pharmacological activities, including antibacterial, antiviral, antioxidant, anticoagulant, and anticancer (Manoj Kumar and Arunkumar 2023; Widyaswari et al. 2024; Gupta et al. 2024), antiproliferative(Costa et al. 2011), and antiparasitic and antibacterial (Telles et al. 2018; Hamed et al. 2018)

In addition, *Sargassum* spp. contains several bioactive compounds such as proteins, amino acids, phenolic compounds, tannins, carotenoid pigments, peptides, enzymes, vitamins, and lipids, including polyunsaturated fatty acids (PUFAs) such as eicosapentaenoic, arachidonic, linoleic, and octadecatetraenoic acids (Kendel et al. 2015; Manzelat et al. 2018; Biris-Dorhoi et al. 2020; Vicente et al. 2022; Silva et al. 2024). Studies indicate that PUFAs from seaweed have promising antimicrobial effects (Manivannan et al. 2011; Shannon and Abu-Ghannam 2016; Alves et al. 2020). Thus, this study aims to evaluate the antibacterial activity of the lipid extract (LE) of the brown macroalga *S. filipendula* against

MDR hospital bacteria and to investigate its chemical composition, thermal stability, and cytotoxicity in mammalian cell models.

Materials and methods

Lipid extraction and characterization of S. filipendula

Collection of Sargassum filipendula biomass

Samples of *S. filipendula* were obtained from the Phycology Laboratory (LAFIC) of the Department of Botany at the Federal University of Santa Catarina (UFSC). For this purpose, fronds from adult specimens of *S. filipendula* were collected on rocky shores at Sambaqui Beach, Florianópolis-SC (coordinates 27º29'26" South and 48º32'18" West) at a depth of 1 m, transported in coolers to the LAFIC facilities, washed in distilled water, cleaned (epibiont organisms were removed manually), weighed (fresh drained weight), and kept frozen (-40 °C) until use.

Lipid extraction

The extraction of lipids from *S. filipendula* was performed at the Laboratory of Innovation and Solutions in Chemistry (INNOVASCHEM) at Federal University of Pelotas (UFPel), conducted according to the Bligh & Dyer method (Bligh and Dyer 1959), in which initially, 10 g of the sample was dried and ground with a mortar and pestle, weighed, and then 60 mL of a chloroform:methanol (1:2) mixture was added. The closed bottles were homogenized for 1 h and centrifuged for 20 min at 3000 rpm. Subsequently, the aqueous phase was removed and the solvent in the oil phase was evaporated using a rotary evaporator, leaving only the EL in the bottle, which was eluted in hexane to remove impurities and again evaporated in the rotary evaporator. This LE was separated and kept away from light at -20°C for further analysis.

Gas Chromatography Coupled with Mass Spectrometry (GC-MS) Assay

The LE of *S. filipendula* was derivatized using boron trifluoride (BF3). During this procedure, the LE was eluted in hexane and 250 mg were weighed in a glass flask, and 6 mL of a 2% m/v methanolic sodium hydroxide solution were added. The flask was then left in a reflux system for 5 min under agitation. Next, 5 mL of the BF3/methanol (MeOH) derivatizing agent was added, and reflux was maintained for 5 min Subsequently, the solution was transferred to a separation funnel, where 20 mL of hexane was added until complete separation of the phases. The lower (aqueous) phase was then discarded, and the upper phase was filtered with sodium sulfate into a glass flask. The solution was placed in a rotary evaporator for complete evaporation of the solvent. Finally, the solution was eluted with 1 mL of High-Performance Liquid Chromatography (HPLC) grade hexane (≥99.9) and transferred to a vial, where it was analyzed by Gas Chromatography Coupled with Mass Spectrometry (GC-MS).

To obtain the composition of the fatty acids (FA) present in *S. filipendula* oil, a gas chromatograph (Shimadzu, model GC-2010) coupled to a mass spectrometer detector (GCMS-QP2020) was used, equipped with an SP 2560 fused silica capillary column (100 m x 0.25 mm x 0.10 μm). The injected sample volume was 8 μL in a 1:50 ratio and in split mode. The interface and ion source temperatures were 280 °C and 200 °C, respectively. For the injector, the temperature was set at 200 °C using helium carrier gas with a flow rate of 3 mL min^{-1}. For the column, the initial temperature was 100 °C, remaining at this condition for 5 min. Subsequently, the temperature was increased at a constant heating rate of 5 °C/min to 250 °C, totaling 55 min of analysis time. The mass spectrometer operated in scan mode in the range of 35-700 m/z for the identification of substances. The analyses were performed at INNOVASCHEM at UFPel.

Thermogravimetric analyses (TGA)

Thermogravimetric analyses (TGA) were performed using a TGA Q5000 (TA Instruments, USA). The instrument was calibrated using Calcium oxalate monohydrate ($CaC_2O_4 \cdot H_2O$, 99.9%). Thermal characterization analyses were carried out at a heating rate of 10 °C min^{-1} up to 650 °C. The sample mass used in each experiment was approximately 3 mg. An inert atmosphere of N_2 was maintained at a flow rate of 25 mL min^{-1}. No prior drying of the samples or isothermal step was performed before the analyses. The

analyses were conducted at the Laboratory of Ionic Liquids and Nanomaterials (LABLINm), Department of Chemistry, Federal University of Santa Maria, RS, Brazil.

Differential Scanning Calorimetry (DSC)

Thermal behavior was investigated by Differential Scanning Calorimetry (DSC) in order to evaluate the thermal properties of the samples, which are directly related to phase transitions and the corresponding enthalpy changes. The phase transitions were determined using Modulated Temperature Differential Scanning Calorimetry (MTDSC) on a DSC Q2000 (TA Instruments, USA). The oil sample mass used in each analysis ranged from 1 to 5 mg and was weighed using a Sartorius M500P analytical balance with a precision of ±0.001 mg. Both samples were subjected to three heating and cooling cycles. The analysis was performed over a temperature range from −80 °C up to 300°C. The heating rate was 10 °C min^{-1}, and nitrogen (N_2) was used as the inert purge gas at a flow rate of 50 mL min^{-1}. The analyses were conducted at the LABLINm, Department of Chemistry, Federal University of Santa Maria, RS, Brazil.

Antibacterial activity

Multidrug-resistant (MDR) bacterial isolates

Three bacterial isolates were selected from each species that showed resistance to at least three classes of antibiotics, including four GNB: *A. baumannii*, *E. coli*, *K. pneumoniae*, *P. aeruginosa*, and two GPC: *S. aureus* and coagulase-negative staphylococci (CoNS). The MDR isolates were provided by two hospitals in the city of Pelotas-RS, Brazil, and stored in the Bacteriotheque of the Microbiology and Bioprospecting Laboratory of the Department of Microbiology and Parasitology (DEMP) of the Institute of Biology (IB) of the UFPel. The resistance profile was analyzed using the VITEK 2 – Biomerrieux and BD PHOENIX automation systems. The epidemiological and susceptibility profile of these isolates has been previously characterized by Jara et al. (2021).

Broth microdilution: Minimum inhibitory concentration (MIC)

The antimicrobial activity of *S. filipendula* LE was analyzed by identifying the MIC (minimum inhibitory concentration) following the guidelines in manual M07-A12 (Clinical and Laboratory Standards Institute (CLSI) 2024). The MIC assay was employed as an endpoint susceptibility method, adequate for the initial screening stage of *S. filipendula* LE antibacterial potential; kinetic time-kill assays, which would characterize the rate and pattern of bacterial death over time, were not performed in the present study and are proposed as a direction for future investigation of this extract. A solution with a concentration of 10mg/mL was prepared by dissolving 50 mg of the algae LE in ethyl alcohol p.a. and then adding 3.5 mL of Müller-Hinton broth (MHB). In sterile 96-well microdilution plates, previously filled with 100 µL of CMH, 100 µL of the dilution of *S. filipendula* algae LE (10 mg/mL) was added to the second column. Ten successive dilutions (from 5 to 0.01 mg/mL) were then performed, from column two to column 11. resulting in a final volume of 100 µL in each well. An initial bacterial suspension was prepared from a colony by diluting it in 3 mL of saline solution and adjusting the turbidity to 0.5 on the McFarland scale (1.5×10^7 CFU/mL). To obtain the working concentration of 1.5×10^8 CFU/mL, 0.5 mL of this initial suspension was added to 4.5 mL of MHB. Next, 5 µL of the inoculum was added to the wells of the second to 12th columns. The plates were then incubated at 37°C in an oven for 24 h. The first column was used as a negative control with only CMH, while the last column (12th) was used as a positive control, with CMH and the inoculum containing the microorganism of interest. A commercial ATB was not included as an additional positive control in the susceptibility assay, since the bacterial isolates used had already undergone prior automated identification and antimicrobial susceptibility testing (VITEK 2 – Biomérieux/BD Phoenix), confirming their MDR profile (item 2.1). Given this pre-established resistance, the inclusion of a clinically active reference ATB would not add discriminatory value to the assay; instead, assay validity across all plates was guaranteed by the internal negative (broth only) and positive (broth plus inoculum) growth controls. Subsequently, the MIC was evaluated colorimetrically, with the addition of 40 µL/well of 0.015% 2.3,5-triphenyltetrazolium chloride (TTC) dye and incubated in an oven at 37°C for 30 min. Visual assessment involved observing the presence or absence of pink coloration, where the absence of color indicated antimicrobial activity by the alga, whereas wells containing active microorganisms exhibited pink coloration. The tests were performed using two biological replicates, with three technical replicates

conducted on different days for each bacterial isolate. The visual analyses were performed by a single experienced analyst.

Minimum Bactericidal Concentration (MBC)

After reading the MIC test, the Minimum Bactericidal Concentration (MBC) was verified using the Brain Heart Agar (BHA) plating method with a 5µL sample from the well corresponding to the MIC and the following. The plate was incubated at 37°C in an oven for 24 h to determine the bactericidal or bacteriostatic concentrations. As the MBC determination reflects a single endpoint reading rather than a continuous kinetic profile, results should be interpreted as bactericidal/bacteriostatic classification at 24 h, with dynamic time-kill behavior left for subsequent studies. Observational analyses of colony growth (bacteriostatic) or the absence thereof (bactericidal) were performed by a single analyst on all isolates in duplicate and in three replicates on different dates.

Cytotoxic Activity

Evaluation of Hemolytic Activity (HA)

Hemolytic activity (HA) was evaluated according to the protocol described by Vaucher et al. (2010) using defibrinated sheep red blood cells. The red blood cells were washed and resuspended three times in phosphate-buffered saline (PBS) until a 4% (v/v) suspension was achieved. The LE of *S. filipendula* was prepared in PBS at an initial concentration of 22.3 mg/mL. From this solution, 1 mL was added to the 4% red blood cell suspension, and serial dilutions were performed until a final concentration of 0.179 mg/mL was reached. The samples were incubated at 37 °C for 2 h under continuous agitation in a shaker incubator. The samples were then centrifuged at 2000 rpm for 10 min, and the supernatant was carefully transferred to 96-well plates. As a negative control, a 2.3% (v/v) solution of pure ethanol (EtOH p.a.) in distilled water was used, while the positive control consisted of Triton X-100. Hemoglobin release was quantified using a spectrophotometer (Hitachi U-1100), with absorbance readings in the range of 425 to 450 nm. The assays were performed in triplicate.

To assess potential interference from the coloration of the *S. filipendula* LE, a color control assay was performed. This was conducted using serial dilutions, replicating the concentrations and setups used in the duplicate HA assay (Supplementary Fig. 1).

Cell viability

Murine fibroblast (L929) and Madin-Darby-Bovine-Kidney (MDBK) cells, acquired from the American Type Culture Collection (ATCC, Rockville, USA), were cultured in Modified Eagle Medium (MEM, pH 7.4) supplemented with 10% (v/v) fetal bovine serum (FBS), penicillin/streptomycin (100 U/mL), and amphotericin B (0.1%). Cell suspensions were dispensed at 100 µL per well into 96-well culture plates and maintained under standard conditions (37°C, humidified atmosphere with 5% CO_2) until they reached 80–90% confluence.

Cell treatment

S. filipendula LE was initially solubilized in EtOH p.a. and subsequently diluted in MEM medium, obtaining final concentrations of 2.5; 1.25; 0.625; 0.312; 0.156; 0.078; 0.039; 0.020; 0.010; 0.005; 0.002 mg/mL. In 96-well plates, 100 µL of MEM medium was dispensed into all wells except the first column, where 200 µL of the initial LE solution was placed (2.5 mg/mL); two-fold serial dilutions were then carried out by transferring 100 µL sequentially from well to well down each row, generating the concentration gradient described above. For a maximum concentration of 2.5 mg/mL of LE, a solution containing 2.3% EtOH p.a. was prepared, and dilution to the lowest concentration (0.002 mg/mL) resulted in 0.002% EtOH in the medium. For cell viability tests, cells were seeded in 96-well plates, using 5×10^3 cells/well (30,000 cells/well), and exposed to treatments for 24 h. In addition to quantitative analyses, cells were monitored via light microscopy to observe morphological changes and assess cellular integrity. The negative controls consisted of cultures treated only with vehicle (EtOH in MEM) at the same concentrations used to dissolve the LE. All assays were performed in quadruplicate to ensure reproducibility.

MTT cytotoxicity assay

Cell viability was determined by the MTT (3-(4,5-dimethylthiazol-2-yl)-2.5-diphenyltetrazolium bromide) method, based on the enzymatic reduction of tetrazolium salt to formazan crystals by metabolically active mitochondria according to the methodology described by Mosmann (1983). After treatment, the cells were washed with balanced saline solution without calcium and magnesium (CMF, pH 7.4) and incubated with MTT (0.5 mg/mL) for 4 h under controlled conditions. The crystals formed were solubilized in absolute EtOH (50 µL/well), and the absorbance was measured at 570 nm using a microplate reader (Biotex ELX800). The tests were performed in quadruplicate. Viability was expressed as a percentage relative to the control (untreated cells), according to the equation:

$$Viability\ (\%) = \frac{(OD570\ treated)}{(OD570\ control)} x100$$

Where:

OD_{570} of treated: Average absorbance of cells exposed to LE from *S. filipendula*.

OD_{570} of control: Average absorbance of cells treated only with vehicle (EtOH in MEM).

Evaluation of ethanol cytotoxicity in MTT

To evaluate the cell viability of the MDBK and L929 cell lines in the presence of EtOH, the cells were exposed to different dilutions of EtOH p.a. in MEM medium, corresponding to the same concentrations used in the treatments with *S. filipendula* LE. The concentrations tested were: 2.3; 1.15; 0.575; 0.287; 0.143; 0.071; 0.035; 0.017; 0.008; 0.004, and 0.002% (v/v). The negative control consisted of cells maintained only in MEM, without the addition of EtOH. The cell viability assay was conducted using the MTT method, following the same protocol and experimental conditions adopted for the tests with *S. filipendula* LE. The experiments were performed in quadruplicate and conducted simultaneously with the samples treated with the extract in order to minimize inter-experimental variations. Cell integrity and morphology were monitored using optical microscopy.

Cytotoxicity assessment in Neutral Red (NR)

Cell viability was determined using the Neutral Red (NR) assay, based on the ability of viable cells to take up and accumulate the dye in their lysosomes, as described by Borenfreund and Puerner (1985). After the incubation period with the treatments, the culture medium was removed and 200 µL of 0.033% (v/v) NR solution was added to each of the wells of the 96-well plate. The plates were incubated at 37 °C in a humid atmosphere with 5% CO_2 for 2 h. After this time, the dye was removed, and the cells were washed with 100 µL of MEM medium without SFB. Next, 100 µL of the fixation and extraction solution composed of 1% acetic acid and 50% EtOH (v/v) was added. The plates were shaken for 10 min to promote uniform dye release. The optical density was then measured at 540 nm in a microplate reader, using wells without cells as a reference. Cell viability (%) was calculated using the equation:

$$Viability\ (\%) = \frac{(OD540\ treated)}{(OD540\ control)} x100$$

Samples that showed values close to 100% viability, compared to the negative control (untreated cells), were considered non-cytotoxic. All tests were performed in quadruplicate.

Cell viability Lactate dehydrogenase (LDH)

The method for determining lactic dehydrogenase (LDH) activity was performed using the supernatant from dilutions of 2.5 to 0.002 mg/mL, collected 24 h after cell treatment during the MTT assay, to assess the plasma membrane integrity of L929 and MDBK cells (Decker and Lohmann-Matthes 1988). LDH activity in the supernatant was measured using the commercial LDH® reagent (Labtest, Diagnostic) in Cobas Miras-Roche® biochemical equipment. This assay is based on the reaction catalyzed by LDH, which converts pyruvate to lactate concomitantly with the oxidation of NADH to NAD+, and the kinetics of this reaction are accompanied by a decrease in absorbance at 340 nm, reflecting cell integrity. The values were determined as a percentage of LDH released Concentrations of 2.5 to 0.313 mg/mL did not conform to the sigmoidal model used for nonlinear regression and were therefore excluded from IC_{50} curve fitting. Values were determined as the percentage of LDH released. The assays were performed in triplicate.

Calculation of the Selectivity Index (SI)

The selectivity index (SI) was calculated to evaluate the pharmacological safety of *S. filipendula* LE, considering the relationship between cytotoxicity and antimicrobial activity. The SI was determined by the ratio between the concentration causing 50% effect (IC_{50}) in eukaryotic cells and the MIC against bacteria, according to the formula:

$$SI = \frac{IC50}{MIC}$$

A value of IS ≥ 10 was adopted as a criterion indicative of a safe therapeutic window, i.e., effective antimicrobial concentrations associated with low cellular toxicity. The IC_{50} values were obtained based on MTT, NR, and LDH assays performed on MDBK and L929 cell lines, while the MICs were determined by antimicrobial activity tests as described previously.

Statistical analysis

The data from the TGA and DSC were processed using TA Universal Analysis 2000 software (version 4.5, TA Instruments, USA). MIC and MBC determinations were carried out in duplicate, each with three technical repetitions performed on separate days, yielding six independent readings per isolate (n = 6); these values are reported as mean ± standard deviation (SD). Because the CLSI M07-A12 (2024) microdilution protocol relies on a twofold, stepwise dilution series, the resulting SD is inherently discrete rather than continuous: replicates that converged on the same dilution step produced an SD of zero, whereas replicates spanning adjacent steps produced non-zero values that were not rounded, accurately capturing the reproducibility of the assay at the resolution allowed by the method. Experiments using the LE were performed in triplicate for the HA and LDH assays, and in quadruplicate for the MTT and NR cytotoxicity assays. Data were analyzed using two-way analysis of variance (ANOVA) with the GraphPad Prism software, version 8.0 (GraphPad Software, San Diego, USA). Results are presented as mean ± standard error of the mean (SEM), and statistical comparisons among means were performed using Sidak's multiple comparison test. Differences were considered statistically significant at $p < 0.05$.

Declaration Regarding Human Experimentation

This research did not involve direct experimentation on human subjects. The use of clinical bacterial isolates was conducted in accordance with the ethical approvals specified in the Ethical Approval section.

Results

Lipid extract profile of S. filipendula

The characterization of the LE of *S. filipendula* biomass by GC-MS revealed a profile dominated by saturated fatty acids (SFA). Hexadecanoic acid was the major component, accounting for 61.07%. Among the other SFA, myristic acid (C14:0; 5.18%) and stearic acid (C18:0; 3.59%) also stood out, confirming the predominance of saturated linear chains in the extract. Monounsaturated fatty acids (MUFA) represented 23.27% of the total, the main constituent being oleic acid (C18:1; 15.90%), followed by palmitoleic acid (C16:1; 4.99%) and vaccenic acid (C18:1; 2.38%). PUFA accounted for 6.90% of the lipid fraction, including linoleic acid (C18:2; 2.05%), arachidonic acid (C20:4; 2.19%), and docosapentaenoic acid (C22:5; 2.66%) (Table 1).

Table 1 about here

Thermal analyses

Thermogravimetric analyses (TGA)

The TGA curves of *S. filipendula* LE are presented in (Fig. 1) and the corresponding thermal parameters are summarized in Table 2. For *Sargassum* LE, an initial mass loss of approximately 20% is observed, which is attributed to the evaporation of volatile compounds such as residual solvents and/or

water. The thermal decomposition occurs in two main stages, with decomposition temperatures (Td_1) at 296 °C and (Td_2) at 445 °C, resulting in a total mass loss of approximately 82%.

Fig. 1 and Table 2 about here

Differential Scanning Calorimetry (DSC)

The DSC analysis of *Sargassum* LE did not show any significant thermal events within the evaluated temperature range (−80 to 300 °C). This behavior indicates the absence of detectable phase transitions, such as glass transition, crystallization, or melting processes, suggesting that these LE do not exhibit measurable amorphous or crystalline domains under the experimental conditions (Fig. 2).

Fig. 2 about here

Resistance Profile of Selected Bacteria

In this study, the bacteria were tested for sensitivity to 39 ATBs, spanning 16 distinct classes. The isolates were screened for MDR when they demonstrated resistance to at least three ATB classes. Three bacterial isolates from each species were chosen, totaling nine MDR bacteria, which were subsequently characterized regarding their resistance profiles as shown in Table 3.

This approach allowed for a representative evaluation of the resistance diversity among the analyzed bacteria.

Table 3 about here

Antibacterial activity

The bacteriostatic activity of *S. filipendula* was observed in all bacteria tested. The mean MIC across the three biological isolates (a, b, c) of each species ranged from 1.18 to 2.5 mg/mL. The MICs observed for each bacterial species are described in Table 4.

Table 4 about here

Each isolate-level MIC value (a, b, c) represents the mean of six independent readings (n = 6): three bacterial isolates were assessed per species, and each isolate was evaluated in two independent assay replicates performed on the same day, each carried out with three technical repetitions on separate dates. The species-level mean ± standard deviation (SD) was calculated from the three isolate-level means (n = 3 biological replicates, isolates a, b, c). MBC values reported as ">2.50" indicate that no bactericidal endpoint was reached at any concentration up to the highest tested (2.50 mg/mL); under these conditions, the MBC/MIC ratio exceeded the threshold of 4 conventionally used to distinguish bactericidal from bacteriostatic action (Pankey and Sabath 2004; Wald-Dickler et al. 2018), consistent with the bacteriostatic profile observed for all isolates.

Cytotoxicity analyses

The hemolytic potential of LE *S. filipendula* was evaluated using red blood cells to estimate selective toxicity in blood cells. In Fig. 3, the IC_{50} value obtained was 12.81 mg/mL, significantly higher than the effective antimicrobial concentrations (1.04–2.5 mg/mL). This result indicates that LE does not cause significant hemolysis at bacteriostatic concentrations, suggesting low toxicity to erythrocytes and a favorable safety profile for systemic applications. It was observed that at a concentration of 22.3 mg/mL there was a significant increase in the percentage of HA, while at concentrations between 11.5 and 0.179 mg/mL there was a significant reduction in HA.

Fig. 3 about here

The MTT cell viability assay demonstrated that exposure of MDBK and L929 cells to different concentrations of EtOH, ranging from 2.3% down to 0.002%, did not result in a significant reduction in cell viability at any of the tested concentrations (Fig. 4a and 4b). Most cells-maintained viability close to 100%, comparable to the untreated control group, indicating that EtOH, at the concentrations used to dilute the *S. filipendula* LE, did not exhibit relevant toxicity to the MDBK and L929 cell lines under the experimental conditions.

Fig. 4 about here

For the MTT, NR, and LDH assays, the highest concentrations of LE (2.5 and 1.25 mg/mL for MTT and NR; 2.5 to 0.313 mg/mL for LDH) were excluded from the dose–response graphs and from IC_{50} estimation. This exclusion reflects the curve-fitting behavior of the data rather than an optical artifact: a dedicated photographic color-interference control, performed across the dilution range used in the hemolytic assay (23.5 to 0.179 mg/mL; Supplementary Fig. 1), confirmed that the extract's intrinsic pigmentation was not visually detectable below 11.5 mg/mL — well above the highest concentration used in any of the cytotoxicity assays (2.5 mg/mL). At these top concentrations, MTT viability had already reached a plateau near the assay floor, consistent with near-maximal cytotoxicity, while the NR data for MDBK cells and the LDH data at these same concentrations did not conform to the Gaussian distribution assumed by the four-parameter nonlinear regression model used for IC_{50} estimation. As an additional quality control, product-only blank wells (LE at each tested concentration, without cells or detection reagent) were included in every colorimetric assay to quantify and subtract any background absorbance intrinsic to the extract, and serial dilutions were used throughout to characterize the extract's absorbance behavior across the full concentration range.

In the MTT assay, which was used to evaluate the cell viability of MDBK and L929 cell lines after exposure to *S. filipendula* LE, a significant reduction in viability was observed at concentrations between 0.313 and 0.002 mg/mL (Fig. 5a and 5b). The IC_{50} values were 0.6229 mg/mL for MDBK and 1.252 mg/mL for L929, indicating greater resistance of L929 cells to LE compared to MDBK cells. The difference in cell

sensitivity suggests that the cytotoxic effect of LE varies according to cell type, possibly due to metabolic or membrane permeability differences.

Fig. 5 about here

The cytotoxicity evaluation using the NR method analyzed the ability of viable cells to incorporate the dye into their lysosomes. No significant reduction was observed at any of the tested concentrations; however, significant cell proliferation was observed at concentrations ranging from 0.039 to 0.002 mg/mL in MDBK cells (Fig. 6a). In L929 cells, a significant reduction in cell viability was observed at concentrations of 0.625 and 0.156 mg/mL, with no significant increase at concentrations ranging from 0.078 to 0.002 mg/mL (Fig. 6b). Consequently, an IC_{50} value of 0.1605 mg/mL was determined for L929 cells (Fig. 6b); however, for MDBK cells (Fig. 6a), it was not possible to calculate an IC_{50} value because the data did not follow a Gaussian distribution. The viability of LDH release into the culture medium was used as an indicator of plasma membrane damage and cell death. The results observed in MDBK and L929 cells treated with LE from *S. filipendula* demonstrated a significant reduction in cell viability for all concentrations tested from 0.156 to 0.002 mg/mL. The IC_{50} values were 0.3848 mg/mL for MDBK and 0.2664 mg/mL for L929 (Fig. 7a and 7b). The low concentration required to induce LDH release in L929 suggests that these cells are more susceptible to membrane damage by the extract. In contrast, MDBK cells exhibited greater resistance, reinforcing the existence of a wider safety window for this cell type.

Figures 6 and 7 about here

Cell Viability IC_{50}

S. filipendula LE showed low toxicity to MDBK and L929 cells, according to MTT, NR, and LDH tests. IC_{50} values varied between different tests and cell types, ranging from 0.1605 to 1.252 mg/mL (Table 5); an IC_{50} could not be determined for MDBK cells in the NR assay (see Limitations). In addition, the HA test was performed, which indicated that LE did not cause significant hemolysis at the concentrations tested. The IC_{50} for HA was 12.81 mg/mL, which is higher than the antimicrobial concentrations tested in Table

5. This suggests that even at relatively high concentrations, LE has low toxicity to blood cells. Overall, MDBK cells tended to be more resistant to LE than L929 cells, as reflected in the NR and LDH assays; the MTT assay, however, showed the opposite pattern, underscoring that relative cell-line sensitivity was not fully consistent across endpoints.

Table 5 about here

The results of the SI evaluation presented in Table 6 demonstrated that, although the mean antimicrobial concentrations of the *S. filipendula* LE (1.18 to 2.5 mg/mL) are close to the doses affecting cell viability, the observed toxicity differs among the cell types evaluated, indicating a potentially usable therapeutic window. Based on these values, renal cells (MDBK) exhibited greater resistance to LE than fibroblasts (L929)—a pattern consistent with that observed in the NR and LDH assays, and contrary only to the findings of the MTT assay (see Discussion and Limitations); this suggests that the toxic effect of LE may vary depending on the cell line, possibly reflecting metabolic differences or intrinsic defense mechanisms. For this calculation, the IC_{50} used for each cell line corresponded to the mean of all cytotoxicity endpoints assessed for that cell line: MTT, NR, LDH, and HA for L929 (n = 4); and MTT, LDH, and HA for MDBK (n = 3), since an IC_{50} could not be determined in the NR assay for this cell line (see Limitations). Furthermore, the low hemolysis detected at bacteriostatic concentrations strengthens the safety profile of LE for blood components, giving it an advantage for systemic applications.

Table 6 about here

Discussion

Our results indicate that the biomass of *S. filipendula* exhibits a diversity of FA, whose lipid profile is dominated by SFA such as hexadecanoic acid (palmitic acid, C16:0), which was the major component. There was also a substantial contribution of monounsaturates such as oleic acid (C18:1), and the presence of polyunsaturates such as linoleic (C18:2), arachidonic (C20:4), and docosapentaenoic (C22:5) acids. This

pattern is in line with the literature reporting a similar profile in congeneric species, which may reflect both taxonomic and ecological factors influencing lipid biosynthesis in marine macroalgae.

FA present in the LE of *S. filipendula* are recognized for playing relevant biological functions in complex systems. The literature demonstrates that many FA possess bactericidal properties. Long-chain unsaturated FA, such as linoleic acid, are well-known for inhibiting GNB, including *E. coli* and *K. pneumoniae* (Padmini et al. 2020). Notably, studies have shown that palmitic acid, in particular, possesses remarkable biological properties, which include cytotoxic activity mediated by the induction of oxidative stress and the regulation of apoptotic pathways. (Harada et al. 2002; Martins de Lima et al. 2006; Un et al. 2022) Studies suggest that high concentrations of C16:0 can influence programmed cell death processes via the generation of reactive oxygen species and the activation of pro-apoptotic pathways in in vitro tumor cells, suggesting therapeutic potential (Rocha et al. 2021). On the other hand, in the antimicrobial field, the potential of FA and their derivatives as antimicrobial agents have been widely demonstrated (Padmini et al. 2020). FA from marine algae, for example, act by destroying the bacterial cell membrane, inhibiting electron transport and oxidative phosphorylation, and compromising nutrient uptake and the enzymatic activity of microorganisms (Zheng et al. 2005; Smith et al. 2010)

Considering that the antibacterial activity observed in this study was evaluated for the crude LE, it is unlikely to result from the action of a single FA. Beyond the major saturated and monounsaturated components, palmitic and oleic acids, the PUFA identified in *S. filipendula*, linoleic, arachidonic, and docosapentaenoic acids, have also been reported to exert antibacterial effects through membrane-disruptive mechanisms, including increased permeability, lipid peroxidation, and interference with membrane-associated enzymes, and may therefore contribute additively or synergistically to the overall bioactivity of the extract (Yoon et al. 2018; Das 2018). Arachidonic acid in particular has been shown to permeabilize and disorganize bacterial membranes in both Gram-positive and Gram-negative species through an oxidative, lipid-peroxidation-dependent mechanism, reinforcing its plausible contribution to the effect observed here. Given this complexity, bioassay-guided fractionation represents a logical next step to identify the compounds or fractions responsible for the antibacterial activity of *S. filipendula* LE and to evaluate whether selective enrichment of specific bioactive FA could enhance antibacterial potency while preserving, or improving, the SI.

The MIC values obtained for *S. filipendula* LE (1.04–2.50 mg/mL) should be interpreted with the understanding that this is a crude, multi-component natural extract rather than an isolated compound, and its potency cannot be benchmarked directly against purified ATBs on a mass-for-mass basis. Clinical susceptibility breakpoints established by CLSI/EUCAST (Clinical and Laboratory Standards Institute (CLSI) 2026; European Committee on Antimicrobial Susceptibility Testing 2026) were designed for single, well-defined antimicrobial agents and are not intended to serve as a direct comparator for hydrophobic, multi-component extracts such as LE. Indeed, MIC values in the mg/mL range are commonly reported for crude seaweed extracts, reflecting the fact that antimicrobial activity in macroalgae typically arises from the combined contribution of several compound classes, FA, phlorotannins, pigments, among others, rather than a single dominant molecule (Pérez et al. 2016). Consistent with this, the natural product literature recommends that crude extracts showing activity in this concentration range be advanced to bioactivity-guided fractionation, so that the active constituent(s) can be isolated and their potency reassessed on a purified basis (Gonzalez-Pastor et al. 2023; Calefi et al. 2025). The MIC range reported here should therefore be regarded as consistent with an early-stage, unfractionated screening result that supports further fractionation, rather than being read as an indicator of weak antibacterial potential in the sense applied to purified antimicrobial agents.

The TGA of the LE of *S. filipendula* revealed a thermal decomposition pattern with well-defined characteristics. Initially, a mass loss of approximately 20% was observed, which is attributed to the evaporation of volatile compounds, including residual solvents and/or water. This initial stage is crucial for understanding the composition and purity of the extract.

The main thermal degradation of the *Sargassum* LE occurred in two distinct stages, identified by their decomposition temperatures (Td_1) at 296 °C and (Td_2) at 445 °C. These stages indicate the presence of different components in the extract that degrade in distinct temperature ranges, contributing to a total mass loss of approximately 82% at the end of the heating process. Similar compositional dependence has been reported for algal materials, whose thermochemical profiles vary among species and matrices (Cassani et al. 2022). TGA studies of brown and red seaweeds have also reported species-dependent mass-loss regions around 250 °C (Mohammed et al. 2021) and between 160 and 300 °C (Khan et al. 2022). These studies support the interpretation that algal materials exhibit matrix-dependent decomposition profiles, although they do not directly reproduce the Td values observed for the *S. filipendula* LE.

Additionally, DSC was used to investigate the thermal behavior of the *S. filipendula* lipid extract, focusing on phase transitions. DSC analysis did not detect significant thermal events in the evaluated temperature range, which varied from −80 °C to 300 °C. The absence of phase transitions, such as glass transition, crystallization, or melting, suggests that the LE does not possess discernible or measurable amorphous or crystalline domains under the experimental conditions employed. This result is indicative of a material that does not exhibit detectable phase organization or crystallization within the studied thermal range. The data presented, including decomposition temperatures, mass loss, and the absence of phase transitions, are unprecedented for LE of *S. filipendula*. This information is fundamental for future biotechnological applications, in defining temperature limits for processing and storage, and contributes significantly to the knowledge of the physicochemical properties of this extract.

From an applied standpoint, thermal characterization of natural LE is not merely descriptive: the TGA decomposition profile defines the temperature window within which the material remains chemically stable, informing decisions on drying, sterilization, and storage conditions, while the absence of DSC transitions supports classifying the extract as an amorphous system, a feature directly relevant when selecting excipients and delivery strategies (e.g., emulsions, liposomes) for downstream pharmaceutical or cosmetic formulation. Combined, these thermoanalytical outputs are widely regarded as an essential preliminary step in the quality assessment and pre-formulation characterization of plant- and algae-derived bioactive extracts (Pirsaheb et al. 2023).

The cell walls of GPCs and GNB differ mainly in the thickness of the peptidoglycan layer. According to Nazzaro et al. (2013), in GPC, this layer is thick and associated with proteins and teichoic acid-hydrophobic components that facilitate the penetration of equally hydrophobic molecules, such as lipids, into the cell cytoplasm. In contrast, GNB bacteria have a more complex cell wall, with a thin layer of peptidoglycan and an outer membrane composed of lipoproteins, lipopolysaccharides, and lipid A. This additional barrier hinders the entry of hydrophobic molecules, making these bacteria more resistant to lipophilic antibiotics and other toxic substances (Marino et al. 2001; Burt 2004; Gutierrez et al. 2008; Chorianopoulos et al. 2008)

Currently, scientific literature presents a scarcity of information on the antibacterial activity of LE from *S. filipendula* and also on the methodology used in our study. A study by Mofeed et al. (2022) investigated the organic extract of *S. filipendula* at a concentration of 100 µg/mL in relation to strains of *E.*

coli (ATCC 8739), *Salmonella typhimurium* (ATCC 14028), and *S. aureus* (ATCC 6538) strains, using scanning electron microscopy to observe micrographs of the cytomorphology of the bacteria under study in comparison with controls without treatment with the organic extract to obtain data on bacterial inhibition. The results were promising for all strains studied, with inhibitions of 91.4% for *E. coli* (ATCC 8739), 82.5% for *S. aureus* (ATCC 6538), and 65% for *S. typhimurium* (ATCC 14028).

Telles et al. (2018) conducted research on antimicrobial and antibiofilm activity using the methodology proposed by Melo-Silveira et al. (Melo-Silveira et al. 2011) by measuring the difference in absorbance at 600 nm at the end and beginning of the incubation period in 96-well microtiter plates. The research covered five extracts of *S. filipendula* tested against *Trichomonas vaginalis* (ATCC 30236), *Staphylococcus epidermidis* (ATCC 35984), and *K. pneumoniae* (clinical isolate). The results indicated that three of the extracts showed antiparasitic potential, acting against the protozoan *T. vaginalis* and reducing the number of live protozoa by approximately 50%, suggesting their potential use as antiparasitic agents, while none of the extracts were able to inhibit the growth of *S. epidermidis* and *K. pneumoniae*, having no antimicrobial activity. However, one of the extracts (SF0.5V) from *S. filipendula* stood out for its ability to inhibit biofilm formation, reducing this formation by about 50% for *S. epidermidis*, and two extracts (SF0.7V and SF1.0V) showed antibiofilm action in *K. pneumoniae*. The variation in biofilm inhibition effects can be attributed to the distinct characteristics between *S. epidermidis*, a GPC and GNB and *K. pneumoniae*, which has an outer membrane in addition to the cell wall, resulting in different interactions compared to *S. epidermidis*.

The two articles cited above(Telles et al. 2018; Mofeed et al. 2022) are the only studies conducted with *S. filipendula* related to antibacterial activity, one for antibiofilm activity with absorbance reading and the other for visualization under electron microscopy. Both were conducted using a different methodological approach from the present study, which characterizes the current work as the first to seek the minimum inhibitory concentrations of these algae against MDR of human origin. Research on antibacterial activity has been recurrent, but in studies with other species of the algae *Sargassum* spp. or other genera of algae.

Another study was found with extract from the algae genus *Sargassum* sp. conducted by Alreshidi et al. (2023) with promising results for antibacterial and antifungal activities in all isolates tested. The authors tested nine bacteria (*S. aureus* sensitive and resistant to methicillin, *E. coli*, *S. epidermidis* ATCC

12228, *K. pneumoniae*) and one yeast (*Candida albicans*) in two different techniques by well diffusion determination of the mean zone of growth inhibition and MIC and the minimum bactericidal/fungicidal concentration (MBC/MFC) test; in addition to antibiofilm activities with the crystal violet technique against seven isolates of *S. aureus*. It is worth noting that, in the biofilm test with *Staphylococcus* spp. strains, concentration dependence was observed, with the highest percentage recorded for *S. epidermidis.*

Although LE from other species of macroalgae and from *Sargassum* spp. itself, extracted with polar solvents or with a focus on antitumor activity, have been extensively investigated, no articles were found that report the cytotoxicity of LE from *S. filipendula* in MDBK and L929 cells using the combined methods of MTT, NR, and LDH, in addition to HA tests with red blood cells, as performed in the present study. This lack of data prevents direct comparison of the results found, making the cytotoxicity profile obtained a new and essential piece of data for the risk assessment of *S. filipendula* LE in the literature.

It should be noted that the MTT test performed with the EtOH vehicle (15% v/v), used to dissolve the LE, confirmed that the solvent did not interfere with the viability of L929 cells at the final concentrations tested, which ranged from 2.3% to 0.002% EtOH. Thus, any changes observed in cell viability during treatment can be attributed exclusively to the compounds present in LE, which strengthens the reliability of the experimental model and the conclusions about the biological activity of *S. filipendula* LE.

The results indicate that the toxicity profile of LE differed between the two cell lines and was not fully consistent across assays: MDBK cells tended to show greater resistance than L929 cells in the NR and LDH assays, whereas the MTT assay showed the opposite pattern (IC_{50} = 0.6229 mg/mL for MDBK vs. 1.252 mg/mL for L929). In the LDH assay, IC_{50} values were 0.3848 mg/mL for MDBK and 0.2664 mg/mL for L929; an IC_{50} could not be determined in the NR assay for MDBK cells due to non-Gaussian data distribution (see Limitations). This difference in toxicity profile between cell lines and endpoints is particularly relevant, as it suggests that LE from *S. filipendula* may exhibit tissue- and mechanism-dependent selectivity. The lowest IC_{50} value found in the NR assay for the L929 cell line (0.1605 mg/mL) indicates that changes in lysosomal integrity and metabolic activity of these cells occur at lower concentrations of *S. filipendula* LE. This divergence between the neutral red and MTT assays likely reflects the fact that each method probes a distinct cellular parameter: MTT reduction depends on mitochondrial enzymatic activity (Ghasemi et al. 2021), whereas the neutral red assay reflects the functional integrity of the lysosomal membrane (Repetto et al. 2008). In comparison, the IC_{50} value obtained in the LDH assay

for L929 was 0.2664 mg/mL, signaling that damage to plasma membrane integrity, characterized by cell lysis, occurs at intermediate concentrations of LE. These data suggest that LE toxicity initially acts on cytoplasmic organelles, such as lysosomes, interfering with cellular metabolism, and that damage to the plasma membrane, detected by the LDH assay, may occur later. This sequence indicates that the metabolic effects detected in the NR and MTT assays may precede cell lysis.

In addition, the hemolysis test with red blood cells showed a significant IC_{50} value of 12.81 mg/mL, higher than the antimicrobial concentrations tested (1.04 to 2.5 mg/mL), indicating low HA of LE and suggesting safety for systemic use.

This favorable hemolysis profile should nonetheless be reconciled with the comparatively lower SI values obtained in the adherent cell assays. This discrepancy may indicate that the components of *S. filipendula* LE interact differently with suspended erythrocytes than with adherent mammalian cells, or that adherent cell types are inherently more sensitive to the cytotoxic constituents of the extract than red blood cells. Such dissociation between cytotoxicity endpoints is well documented in the toxicology literature; comparative studies using the same panel of assays have similarly reported differential sensitivity among endpoints, with NR and MTT typically detecting cytotoxic events at concentration thresholds distinct from those revealed by LDH leakage or hemolysis (Fotakis and Timbrell 2006). This reinforces that a single assay is insufficient to characterize the safety profile of a crude, multi-component LE, and that the tiered, multi-assay, multi-cell-line approach adopted here provides a more complete safety picture than any individual test in isolation.

Therefore, the combined analysis of the MTT, NR, and LDH assays provides a more detailed understanding of the cytotoxic profile of LE from *S. filipendula*. According to the ISO 10993-5 standard (Fotakis and Timbrell 2006), a substance is classified as cytotoxic when it causes 30% or more reduction in cell viability. Since NR-based viability remained above this threshold across all tested concentrations, *S. filipendula* LE cannot be considered cytotoxic by this criterion. This finding corroborates the MTT assay results and reinforces the comparatively greater resistance of MDBK cells observed across endpoints. This differentiation in sensitivity between cell models highlights the importance of developing formulations that can mitigate toxicity in susceptible cells while preserving the antimicrobial efficacy of LE.

The lack of previous data on the SI of *S. filipendula* LE highlights the relevance of this contribution, as SI is a crucial measure for estimating the safety of antimicrobial agents under development.

SI values ranged from 1.449 (*P. aeruginosa* in L929 cells) to 3.903 (*S. aureus* in MDBK cells) (Table 6), remaining below the conventional threshold of 10 adopted for a safe therapeutic margin across all isolate–cell line combinations. This indicates that the concentrations of *S. filipendula* LE required to exhibit antimicrobial activity are close to, or in most cases higher than, the concentrations that affect the viability of mammalian cells. For this calculation, the IC_{50} used for each cell line corresponded to the mean of all cytotoxicity endpoints assessed for that cell line, MTT, NR, LDH, and HA for L929, and MTT, LDH, and HA for MDBK, since an IC could not be determined in the NR assay for MDBK cells (see Limitations), rather than a single endpoint, in order to avoid over-reliance on any one assay; as discussed below, the NR and LDH-derived IC_{50} values in particular carry specific methodological caveats that should be considered when interpreting these composite estimates. The values obtained suggest that, despite effective antimicrobial activity against MDR isolates, the cytotoxic and antimicrobial concentration ranges of LE overlap considerably, indicating a narrow safety margin that will require further optimization before therapeutic application. Although the *in vitro* results provide an important basis, there is a need for complementary *in vivo* and pharmacokinetic studies to better understand the safety and clinical efficacy of LE. These innovative data are fundamental to support future formulations and strategies that seek to maximize the therapeutic benefit of *S. filipendula* with lower toxicity.

Conclusion

S. filipendula LE demonstrated bacteriostatic activity *in vitro* against all hospital-acquired MDR isolates tested, with the most pronounced activity observed against *S. aureus*, followed closely by *A. baumannii* and *E. coli*.

Chemical characterization revealed that the LE is predominantly composed of the SFA palmitic acid, together with oleic acid and other unsaturated FA, linoleic, arachidonic, and docosapentaenoic acids, previously reported to exhibit antibacterial activity, providing a basis for future structure–activity investigations. Given that the extract is a complex mixture, the antibacterial effect observed here is more likely attributable to the combined, additive or synergistic action of these FA than to a single major constituent; bioassay-guided fractionation and testing of isolated FA will be essential in future studies to

establish definitive structure–activity relationships and identify the principal contributor(s) to the antimicrobial effect.

Thermal analyses, in turn, complement the understanding of the LE of S. *filipendula*. TGA revealed the presence of volatile compounds and indicated a multi-stage thermal decomposition profile, with substantial mass loss at elevated temperatures, providing insights into its thermal stability. In contrast, DSC did not detect significant thermal events, suggesting the absence of distinct phase transitions, such as glass transition, crystallization, or melting, indicating that the LE does not possess measurable amorphous or crystalline domains under the experimental conditions.

Regarding safety, cytotoxicity assays (MTT, NR, and LDH) performed in MDBK and L929 cell lines, together with the HA assay, indicated low hemolytic potential at the concentrations required for antimicrobial activity, with the ethanol vehicle control confirming that the solvent itself did not interfere with cell viability and thus supporting the reliability of the results obtained.

However, the comprehensive pharmacological safety evaluation, which included the SI, revealed values ranging from 1.449 to 3.903, well below the threshold conventionally adopted for a safe therapeutic margin ($SI \geq 10$), indicating that the antimicrobial and cytotoxic concentration ranges of the LE overlap considerably. These findings reflect a preliminary, screening-level characterization, and the SI values obtained here should be interpreted as a starting point rather than a definitive indicator of therapeutic potential; further studies, including bioassay-guided fractionation, structure–activity assessment, and formulation strategies aimed at widening the therapeutic window, are needed before conclusions about clinical applicability can be drawn.

Future investigations should include time-kill kinetic assays, biofilm inhibition assays, membrane permeability and depolarization assays, ultrastructural analyses, and resistance induction studies, in addition to the bioassay-guided fractionation noted above. These approaches would substantially strengthen the biological relevance of the findings and provide a more comprehensive understanding of the antibacterial mechanism and therapeutic potential of this extract.

Taken together, these findings position *S. filipendula* LE as an early-stage candidate among marine-derived antimicrobial agents in the context of the global challenge of bacterial resistance; realizing this potential will depend on the fractionation, optimization, and safety studies outlined above.

Limitations

For the MTT, NR, and LDH assays, the highest LE concentrations tested were excluded from curve fitting and IC_{50} estimation (2.5 and 1.25 mg/mL for MTT and NR; 2.5 to 0.313 mg/mL for LDH). This choice was based on the behavior of the dose–response data — a toxicity plateau for MTT, and a non-Gaussian distribution that prevented reliable sigmoidal curve fitting for the NR assay in MDBK cells and for the LDH assay at these concentrations — rather than on optical interference: a dedicated color-interference control (Supplementary Fig. 1) confirmed that the extract's pigmentation was not visually detectable within this concentration range. For each assay, curve fitting was based on a minimum of two concentrations above the estimated IC_{50} and several concentrations below it, which supported the reliability of the IC_{50} estimates despite the exclusion of the highest concentrations.

The exclusion of the highest concentrations had different implications depending on the cytotoxicity endpoint. For the MTT assay, the highest concentration retained already produced near-maximal cytotoxicity in both cell lines, consistent with a toxicity plateau; exclusion of further concentrations would therefore be unlikely to shift the IC_{50} estimate. For the NR assay, cell viability remained at or above 100% throughout the tested range in both MDBK and L929 cells, and the MDBK data did not conform to the Gaussian distribution assumed by the four-parameter nonlinear regression model; the reported IC_{50} value for L929 (0.1605 mg/mL) therefore represents an extrapolation beyond the concentration range actually tested and should be interpreted with substantial caution. According to the ISO 10993-5 standard (ISO 10993-5:2009(en) 2026), a substance is classified as cytotoxic when it causes a reduction in cell viability of 30% or more; since NR-based viability remained above this threshold across the entire tested range, *S. filipendula* LE cannot be considered cytotoxic by this criterion. This divergence from the MTT result is not an artifact, but reflects the fact that the two assays probe distinct cellular compartments: mitochondrial metabolic activity in the case of MTT (Ghasemi et al. 2021), and lysosomal membrane integrity in the case of NR (Repetto et al. 2008). For the LDH assay, the data at the highest concentrations likewise did not fit the sigmoidal model, and viability remained low across the retained range without recovering toward baseline, indicating that the upper (low-toxicity) portion of the dose–response curve was not captured; the reported IC_{50} values for this assay may therefore underestimate the true threshold. Taken together, these patterns indicate that MTT provided the most robust individual IC_{50}

estimate for *S. filipendula* LE, whereas the NR- and LDH-derived values should be regarded as preliminary approximations pending confirmation with orthogonal, non-colorimetric methods such as flow cytometry. Nonetheless, to avoid over-reliance on a single endpoint and to provide a conservative, comprehensive estimate of cytotoxic potential, the SI calculation reported in Table 6 was based on the mean IC_{50} across all cytotoxicity endpoints assessed for each cell line (MTT, NR, LDH, and HA for L929; MTT, LDH, and HA for MDBK, since an IC_{50} could not be determined in the NR assay for MDBK cells), rather than on the MTT assay alone; the composite estimate should nonetheless be interpreted in light of the NR- and LDH-related caveats discussed above.

Purification of the extract, or the adoption of complementary techniques capable of resolving the plateau and non-Gaussian behavior observed at the highest tested concentrations, such as flow cytometry-based assays, may represent a useful approach for the LE of *S. filipendula* to obtain a more complete dose–response characterization at the upper end of the tested range, in addition to potentially enhancing the biological activity of the extract. Future studies incorporating these strategies would allow a more precise evaluation of its intrinsic cytotoxicity. Nonetheless, in-depth studies remain essential to ensure the efficacy and safety of any such formulation.

Data availability

Data will be made available on request.

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Ethical approval

This study was approved by the Research Ethics Committees (CEP) under numbers 2.961.379 and 2.985,372 and by the National Research Ethics Commission (CONEP) under number 2.880.831 (Brazil Platform Approval).

Declaration of competing interest

The authors have no competing interests to declare that are relevant to the content of this article.

Declaration on generative AI and AI-assisted technologies in the manuscript preparation process

During the preparation of this work, the author(s) used Gemini and ChatGPT for English language editing and grammatical refinement. Following the use of this tool/service, the author(s) reviewed and edited the content as needed and take(s) full responsibility for the content of the published article.

Table 1 – Fatty acid profile of lipid extract from *Sargassum filipendula* determined by GC-MS, including identified fatty acids, chemical formulas, and relative abundances (%)

IUPAC Nomenclature	Fatty Acid Name	Chemical Formula (C:n)	Saturation Type	Retention Time (min)	Relative Area (%)
Methyl tetradecanoate	Myristic acid	C14:0	SFA	20.302	5.18
Methyl (9Z)-hexadec-9-enoate	Palmitoleic acid	C16:1	MUFA	26.179	4.99
Methyl hexadecanoate	Palmitic acid	C16:0	SFA	26.876	61.07
Methyl (9Z,12Z)-octadeca-9,12-dienoate	Linoleic acid	C18:2	PUFA	32.022	2.05
Methyl (9E)-octadec-9-enoate	Oleic acid	C18:1	MUFA	32.206	15.90
Methyl (11Z)-octadec-11-enoate	Vaccenic acid	C18:1	MUFA	32.370	2.38
Methyl octadecanoate	Stearic acid	C18:0	SFA	33.030	3.59
Ethyl (5Z,8Z,11Z,14Z)-eicosa-5,8,11.14-tetraenoate	Arachidonic acid	C20:4	PUFA	36.740	2.19
Methyl docosapentaenoate (all-Z isomer)	Docosapentaenoic acid	C22:5	PUFA	36.939	2.66
Σ SFA			69.84		
Σ MUFA			23.27		
Σ PUFA			6.90		

SFA= saturated fatty acid; MUFA= monounsaturated fatty acid; PUFA= polyunsaturated fatty acid. Relative area values (%) were calculated from peak areas in the total ion chromatogram and represent a single GC-MS run per extract.

Table 2 Thermogravimetric characteristics of the *Sargassum filipendula* lipid extract: mass loss by volatilization and decomposition temperatures across four thermal phases

Oil	**Volatile (mass loss) (%)**	**T_{d1} (°C)**	**T_{d2} (°C)**	**T_{d3} (°C)**	**T_{d4} (°C)**	**Mass loss (%)**
Sargassum filipendula	20	296	445	-	-	82 %

Td1, Td2: temperatures (°C) at which each thermal degradation event occurs, identified from the derivative thermogravimetric (DTG) curve. Volatile: initial mass loss (%) attributed to evaporation of residual solvents and/or water prior to thermal decomposition. Mass loss (%): cumulative mass loss at 650 °C. A single representative run is shown.

Table 3 – Susceptibility profile of the three bacterial isolates of each species for each class of antimicrobials tested in two hospitals in the city of Pelotas, Brazil, 2018–2019

BACTERIA (nº Isolates)	CLASSES OF ANTIBIOTICS															
	AMINOGLYCOSIDES	β-LACTAMASE INHIBITORS	CARBAPENEMS	CEPHALOSPORINS	GLUCOPEPTIDES	GLYCYLCYCLINES	LINCOSAMIDES	LIPOPEPTÍDEOS	MACROLIDES	NITROFURANS	OXAZOLIDINONES	PENICILLINS	POLYPEPTIDES	QUINOLONES	RIFAMPICINES	TETRACYCLINES
Acinetobacter baumannii (a)	S	R	R	R	NA	NA	NA	NA	NA	R	NA	R	S	R	NA	NA
Acinetobacter baumannii (b)	R	R	R	R	NA	S	NA	NA	NA	NA	NA	R	S	R	NA	NA
Acinetobacter baumannii (c)	R	R	S	R	NA	NA	NA	NA	NA	R	NA	R	NA	R	NA	NA
Escherichia coli (a)	S	R	R	R	NA	NA	NA	NA	NA	S	NA	R	NA	S	NA	NA
Escherichia coli (b)	R	R	S	R	NA	S	NA	NA	NA	NA	NA	R	S	R	NA	NA
Escherichia coli (c)	S	R	S	R	NA	NA	NA	NA	NA	S	NA	R	NA	R	NA	NA
Klebsiella pneumoniae (a)	S	R	R	R	NA	S	NA	NA	NA	NA	NA	R	NA	R	NA	NA

Klebsiella pneumoniae (b)	R	R	R	R	NA	NA	NA	NA	NA	R	NA	R	NA	R	NA	NA
Klebsiella pneumoniae (c)	R	R	R	R	NA	NA	NA	NA	NA	R	NA	R	NA	R	NA	NA
Pseudomonas aeruginosa (a)	S	R	R	R	NA	NA	NA	NA	NA	NA	NA	NA	R	R	NA	NA
Pseudomonas aeruginosa (b)	S	S	S	R	NA	NA	NA	NA	NA	NA	NA	NA	R	S	NA	NA
Pseudomonas aeruginosa (c)	R	R	R	R	NA	NA	NA	NA	NA	NA	NA	NA	S	R	NA	NA
Staphylococcus aureus (a)	S	NA	S	S	S	S	R	S	R	NA	S	R	NA	NT	S	S
Staphylococcus aureus (b)	S	NA	NA	S	S	S	R	NT	R	NA	S	R	NA	R	R	NT
Staphylococcus aureus (c)	S	NA	NA	S	S	S	R	NT	R	NA	S	R	NA	R	R	NT
Coagulase-negative staphylococci *(a)*	R	NA	NA	NT	R	S	R	S	NT	NA	S	R	NA	I	R	NT
Coagulase-negative staphylococci *(b)*	NT	NA	NA	R	S	NT	R	NT	R	NA	NT	R	NA	R	R	NT
Coagulase-negative staphylococci *(c)*	NT	NA	NA	R	S	NT	R	NT	R	NA	NT	R	NA	R	I	NT

Susceptibility patterns to 16 antimicrobial classes among the 18 MDR clinical isolates used in this study (3 independent isolates per bacterial species), obtained through automated testing platforms VITEK 2 (BioMérieux) and BD Phoenix (Becton Dickinson) at two reference hospitals in Pelotas, Brazil, between 2018 and 2019. MDR classification followed the criterion of resistance to three or more antibiotic classes. Abbreviations: R, resistant; S, susceptible; I, susceptible with increased exposure (intermediate category); NT, not tested; NA, not applicable (antibiotic class absent from the automated panel for the corresponding species); GNB, Gram-negative bacilli; GPC, Gram-positive cocci. This table details, isolate by isolate, the resistance pattern underlying the MDR classification applied to each strain, providing the rationale for their selection for the antibacterial (MIC/MBC) assays conducted with the *Sargassum filipendula* lipid extract. Only strains fulfilling this MDR threshold were carried forward to the antimicrobial evaluation.

Table 4 – Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC) of *Sargassum filipendula* LE against multidrug-resistant bacterial isolates

Species	Isolate	MIC (mg/mL) isolate mean (n=6)	Species mean MIC ± SD (mg/mL) (n = 3 isolates)	MBC (mg/mL)	Predominant action
Acinetobacter baumannii	(a)	2.50			
Acinetobacter baumannii	(b)	1.25	1.67 ± 0.72	>2.50	Bacteriostatic
Acinetobacter baumannii	(c)	1.25			
Escherichia coli	(a)	1.25			
Escherichia coli	(b)	2.08	1.67 ± 0.42	>2.50	Bacteriostatic
Escherichia coli	(c)	1.67			
Klebsiella pneumoniae	(a)	2.50			
Klebsiella pneumoniae	(b)	2.50	2.22 ± 0.48	>2.50	Bacteriostatic
Klebsiella pneumoniae	(c)	1.67			
Pseudomonas aeruginosa	(a)	2.50			
Pseudomonas aeruginosa	(b)	2.50	2.50 ± 0.00	>2.50	Bacteriostatic

Pseudomonas aeruginosa	(c)	2.50			
Staphylococcus aureus	(a)	1.04			
Staphylococcus aureus	(b)	1.25	1.18 ± 0.12	>2.50	Bacteriostatic
Staphylococcus aureus	(c)	1.25			
Coagulase-negative staphylococci	(a)	2.08			
Coagulase-negative staphylococci	(b)	2.08	2.08 ± 0.00	>2.50	Bacteriostatic
Coagulase-negative staphylococci	(c)	2.08			

Each isolate-level MIC value (a, b, c) represents the mean of six independent readings (n = 6): three bacterial isolates were assessed per species, and each isolate was evaluated in two independent assay replicates performed on the same day, each carried out with three technical repetitions on separate dates. The species-level mean ± standard deviation (SD) was calculated from the three isolate-level means (n = 3 biological replicates, isolates a, b, c). MBC values reported as ">2.50" indicate that no bactericidal endpoint was reached at any concentration up to the highest tested (2.50 mg/mL); under these conditions, the MBC/MIC ratio exceeded the threshold of 4 conventionally used to distinguish bactericidal from bacteriostatic action [34, 35], consistent with the bacteriostatic profile observed for all isolates.

Table 5 – Determination of IC_{50} values (Mean and Median) of the lipid extract from *Sargassum filipendula* in cytotoxicity assays (MTT, NR, LDH) using MDBK and L929 Cells, and Hemolytic Activity (HA) assay in red blood cells

Assays	Cell IC_{50} (mg/mL)		Mean IC_{50} (mg/mL)	Median IC_{50} (mg/mL)	Cells IC_{50} (mg/mL)		Mean IC_{50} (mg/mL)	Median IC_{50} (mg/mL)
	MDBK	**Red blood cells**			**L929**	**Red blood cells**		
HA		12.81	4.6059	0.6200		12.81	3.622	0.7590
MTT	0.6229				1.252			
NR	-				0.1605			
LDH	0.3848				0.2664			

MTT: [3-(4,5-dimethylthiazol-2-yl)-2.5-diphenyltetrazolium bromide], NR: Neutral Red, LDH: Lactate Dehydrogenase, HA: Hemolytic Activity; L929: mouse fibroblast cell line; MDBK: bovine kidney cell line. The Mean and Median IC_{50} (mg/mL) columns represent the overall summary statistics across the cytotoxicity assays performed for each cell line: MTT, LDH, and HA for MDBK cells (n = 3; NR excluded, as an IC_{50} could not be determined for this assay), and MTT, NR, LDH, and HA for L929 cells (n = 4).

Table 6 – Evaluation of the Selectivity Index of *Sargassum filipendula* lipid extract, based on the mean IC_{50} across all cytotoxicity endpoints: Correlation between cytotoxicity in MDBK and L929 cells and antimicrobial activity

Bacterial species (isolates)	Mean CIM bacterial species (mg/mL)	Mean Cell L929 IC_{50} (mg/mL)	Mean Cell MDBK IC_{50} (mg/mL)	SI Cell L929	SI Cell MDBK	Therapeutic margin
Acinetobacter baumannii	1.67	3.622	4.605	2.169	2.757	SI < 10
Escherichia coli	1.67			2.169	2.757	SI < 10
Klebsiella pneumoniae	2.22			1.632	2.074	SI < 10
Pseudomonas aeruginosa	2.50			1.449	1.842	SI < 10
Staphylococcus aureus	1.18			3.069	3.903	SI < 10
Coagulase-negative staphylococci	2.08			1.741	2.214	SI < 10

SI: Selectivity Index; MTT: [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide]; MDBK: bovine kidney cell line; L929: mouse fibroblast cell line. Mean Cell IC_{50} values correspond to the mean of all cytotoxicity endpoints assessed for each cell line (MTT, NR, LDH, and HA for L929; MTT, LDH, and HA for MDBK, since an IC_{50} could not be determined in the NR assay for MDBK cells) rather than a single assay, in order to provide a comprehensive, conservative estimate of cytotoxic potential (see Limitations for methodological caveats regarding the NR- and LDH-derived values).

Fig. 1 Thermogravimetric analysis (TGA) curve of the lipid extract from *Sargassum filipendula*. A single representative run is shown; the two thermal decomposition events identified (Td1, Td2) are indicated by dashed lines

Fig. 2 Differential Scanning Calorimetry (DSC) curve of the lipid extract from *Sargassum filipendula* (Cycles 1–3). A single representative run is shown. The absence of endothermic or exothermic peaks indicates no detectable phase transitions (glass transition, crystallization, or melting) within the evaluated range, consistent with an amorphous lipid system

Fig. 3 Hemolytic activity of the *Sargassum filipendula* lipid extract on sheep erythrocytes. Dotted line indicates 50% hemolytic activity (IC_{50}). Data are mean ± SEM (n = 3 biological replicates); two-way ANOVA with Sidak's post-hoc test. **** $p < 0.0001$ compared to the positive control (C+, Triton X-100). C− = negative control (2.3% ethanol in PBS).

Fig. 4 (a) MDBK. Effect of ethanol (vehicle control) on MDBK cell viability, assessed by the MTT assay. Data are mean ± SEM (n = 4 biological replicates); two-way ANOVA with Sidak's post-hoc test. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; ns = not significant compared to the cell control (CC).

(b) L929. Effect of ethanol (vehicle control) on L929 cell viability, assessed by the MTT assay. Data are mean ± SEM (n = 4 biological replicates); two-way ANOVA with Sidak's post-hoc test. ns = not significant compared to the cell control (CC).

Fig. 5 (a) MDBK. Cytotoxic activity of the *Sargassum filipendula* lipid extract on MDBK (bovine renal) cells assessed by the MTT. Dotted line indicates 50% viability (IC_{50}). Data are mean ± SEM (n = 4 biological replicates); two-way ANOVA with Sidak's post-hoc test. ** $p < 0.01$; **** $p < 0.0001$; ns = not significant compared to the cell control (CC).

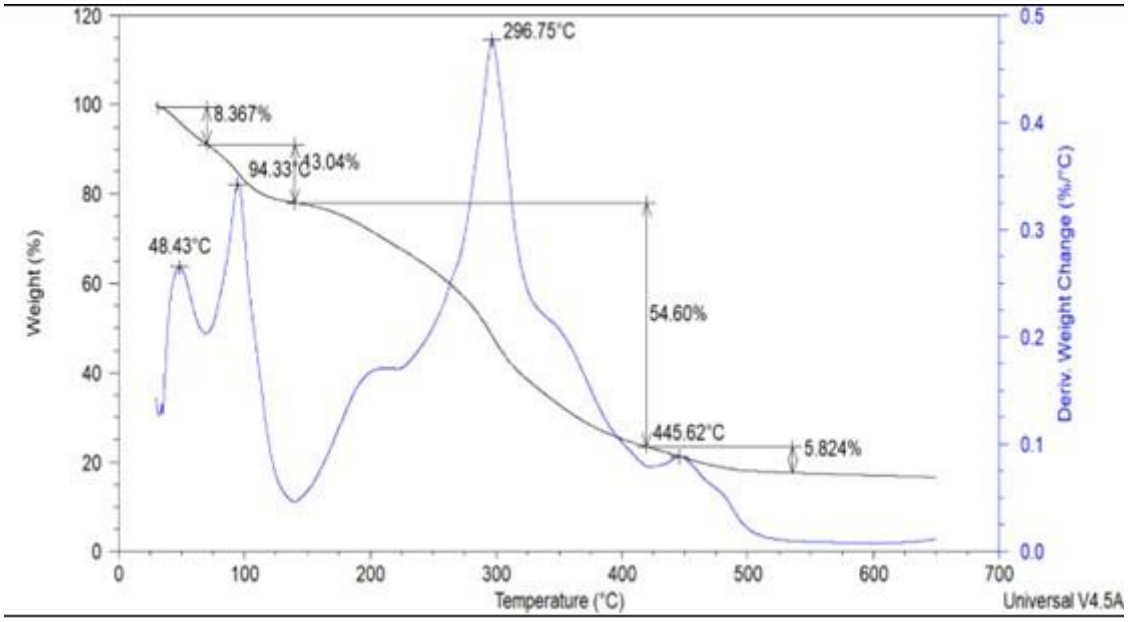
(b) L929. Cytotoxic activity of the *Sargassum filipendula* lipid extract on L929 (murine fibroblast) cells assessed by the MTT. Dotted line indicates 50% viability (IC_{50}). Data are mean ± SEM (n = 4 biological replicates); two-way ANOVA with Sidak's post-hoc test. **** $p < 0.0001$; ns = not significant compared to the cell control (CC).

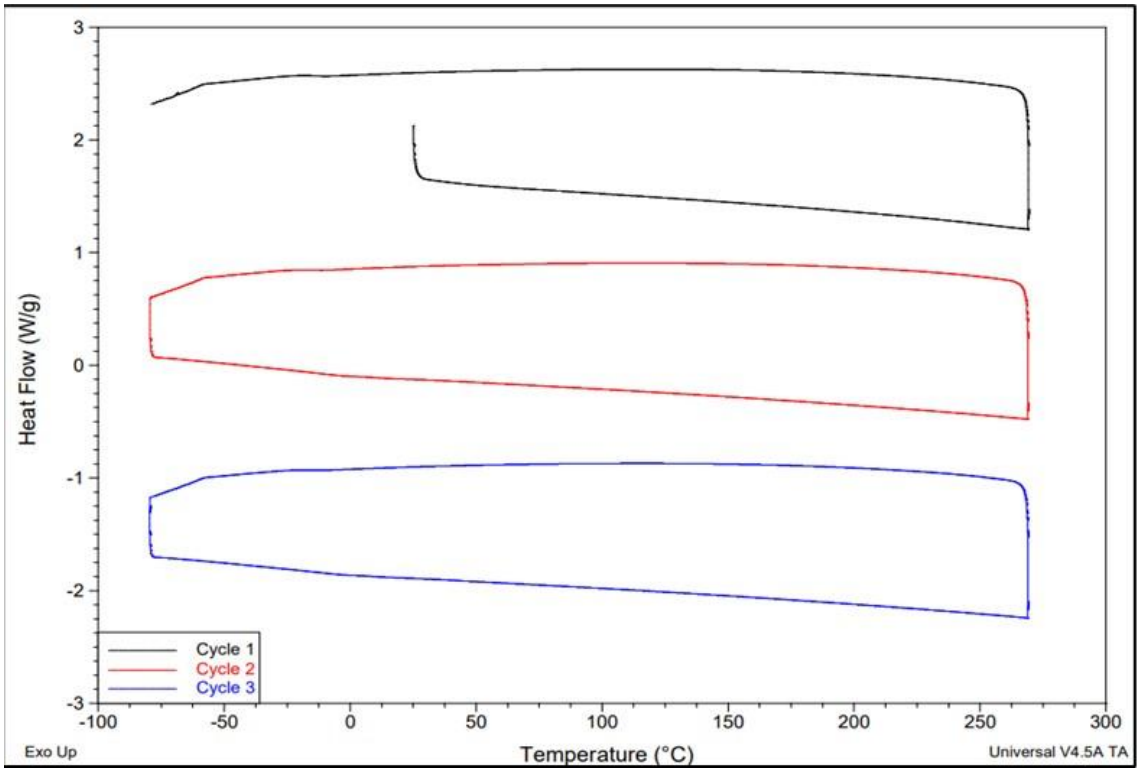
Fig. 6 (a) MDBK. Cytotoxic activity of the *Sargassum filipendula* lipid extract on MDBK (bovine renal) cells assessed by the Neutral Red assay. Dotted line indicates 50% viability (IC_{50}). Data are mean ± SEM (n = 4 biological replicates); two-way ANOVA with Sidak's post-hoc test. *p <0.05; *** p < 0.001; ns = not significant compared to the cell control (CC).

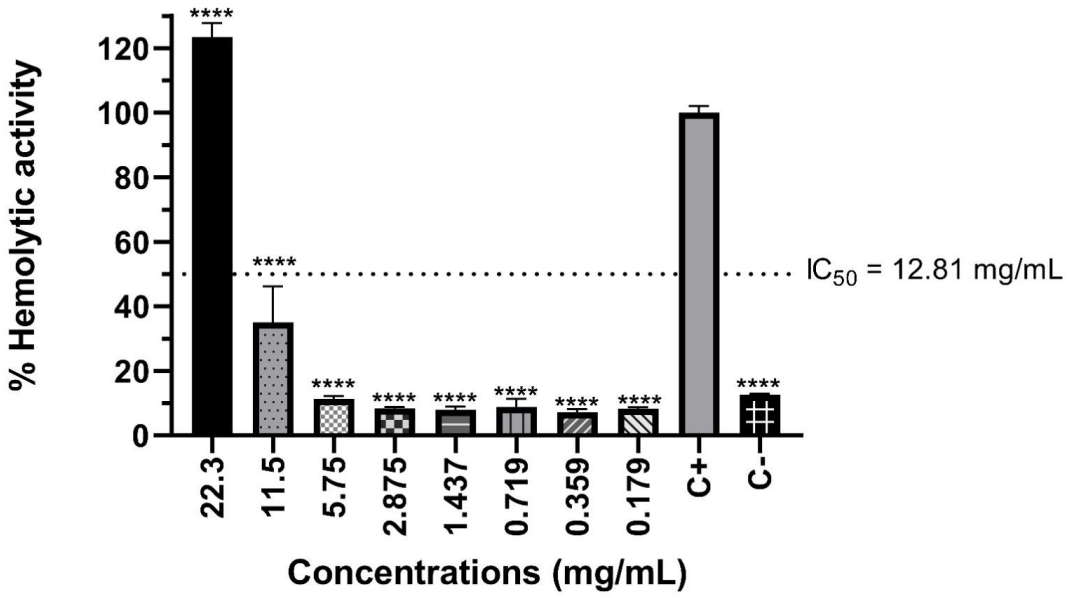
(b) L929. Cytotoxic activity of the *Sargassum filipendula* lipid extract from on L929 (murine fibroblast) cells assessed by the Neutral Red assay. Dotted line indicates 50% viability (IC_{50}). Data are mean ± SEM (n = 4 biological replicates); two-way ANOVA with Sidak's post-hoc test. *p<0.05; **p < 0.01; *** p < 0.001; ns = not significant compared to the cell control (CC).

Fig. 7 (a) MDBK. Cytotoxic activity of the *Sargassum filipendula* lipid extract on MDBK (bovine renal) cells assessed by the Lactate Dehydrogenase release assay. Dotted line indicates 50% viability (IC_{50}). Data are mean ± SEM (n = 3 biological replicates); two-way ANOVA with Sidak's post-hoc test. **** p < 0.0001 compared to the cell control (CC).

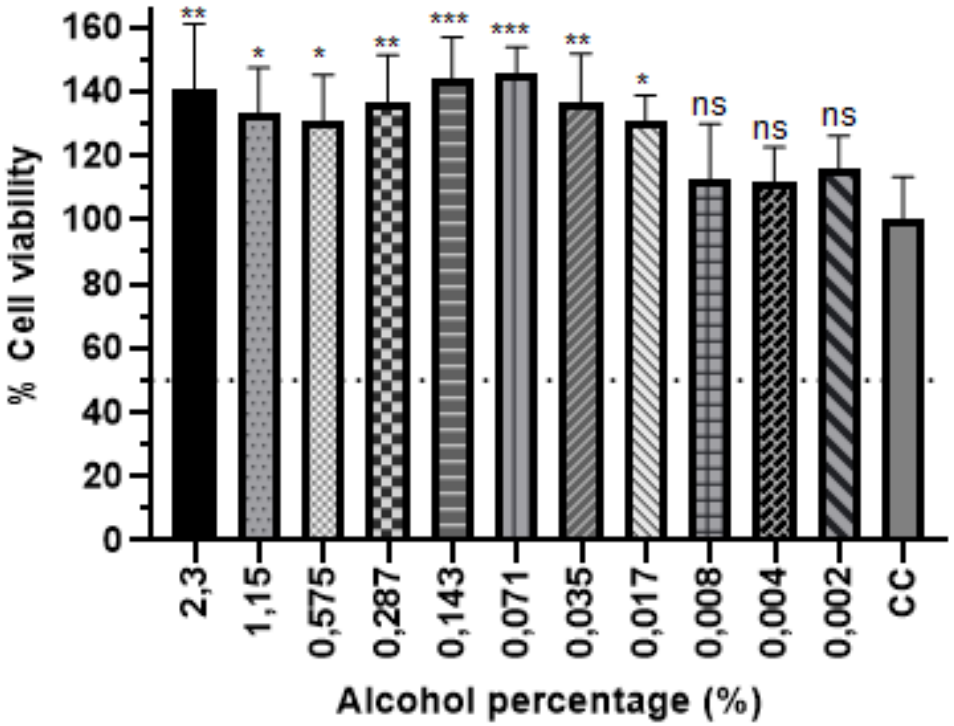
(b) L929. Cytotoxic activity of the *Sargassum filipendula* lipid extract on L929 (murine fibroblast) cells assessed by the Lactate Dehydrogenase release assay. Dotted line indicates 50% viability (IC_{50}). Data are mean ± SEM **** p < 0.0001 compared to the cell control (CC).


296.75°C
8.367%
94.33°C
13.04%
48.43°C
54.60%
445.62°C
5.824%
Weight (%)
Deriv. Weight Change (%/°C)
Temperature (°C)
Universal V4.5A

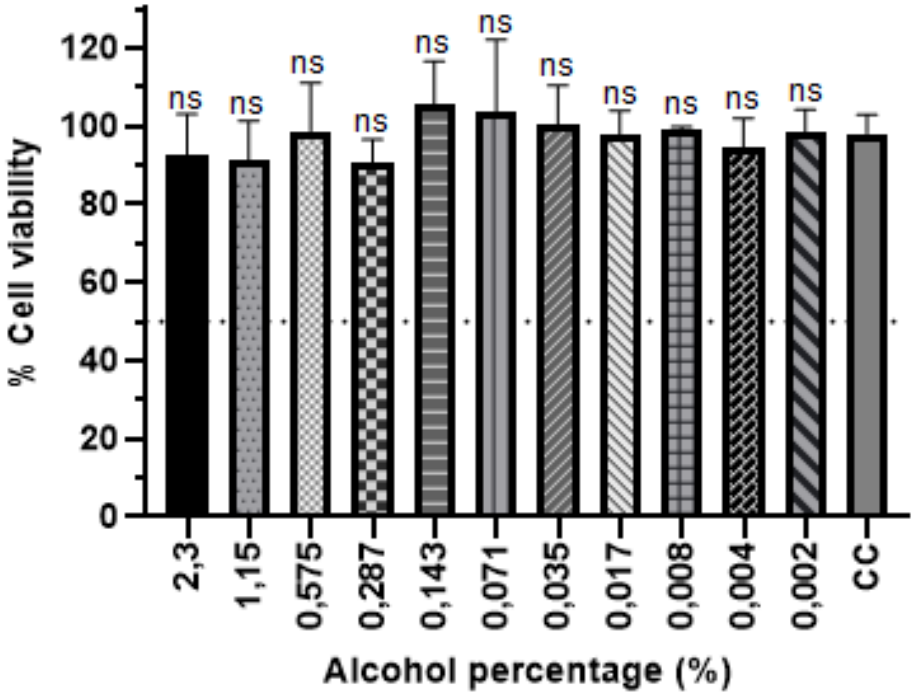

Heat Flow (W/g)
Temperature (°C)
Exo Up
Universal V4.5A TA
Cycle 1
Cycle 2
Cycle 3
3
2
1
0
-1
-2
-3
-100
-50
0
50
100
150
200
250
300


% Hemolytic activity
120
100
80
60
40
20
0
****
****
****
****
****
****
****
****
****
IC50 = 12.81 mg/mL
22.3
11.5
5.75
2.875
1.437
0.719
0.359
0.179
C+
C-
Concentrations (mg/mL)

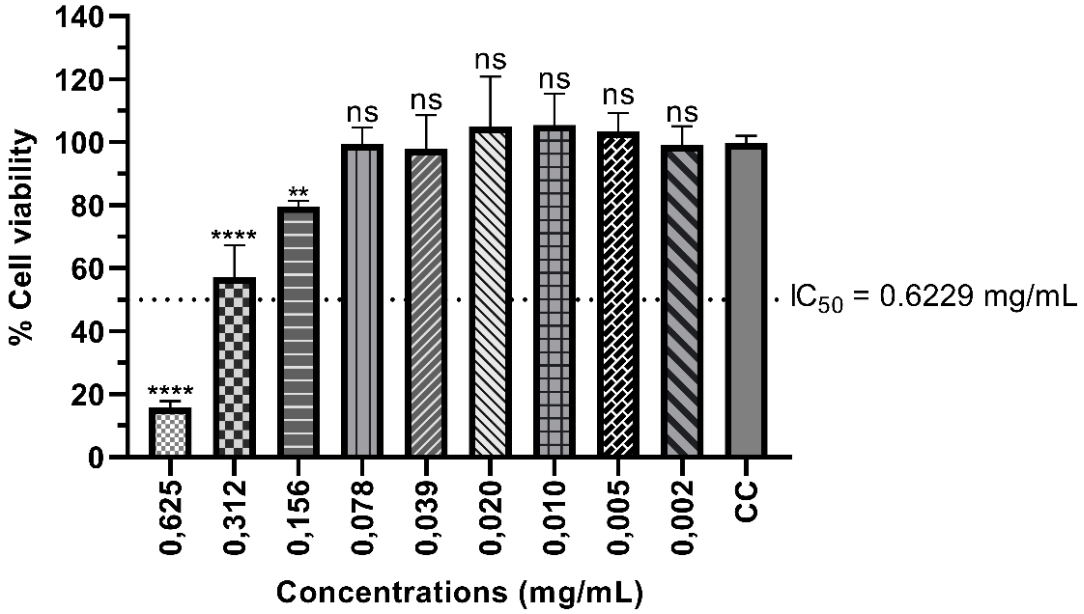
(a)


% Cell viability
160
140
120
100
80
60
40
20
0
**
*
*
**
***
***
**
*
ns
ns
ns
2,3
1,15
0,575
0,287
0,143
0,071
0,035
0,017
0,008
0,004
0,002
CC
Alcohol percentage (%)

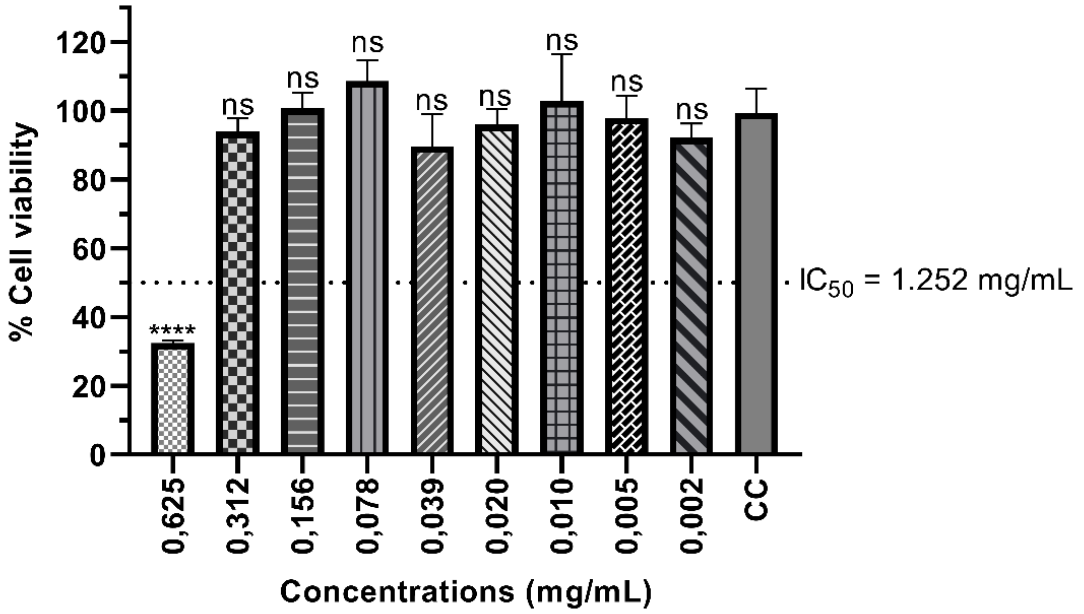
(b)


% Cell viability
120
100
80
60
40
20
0
ns
ns
ns
ns
ns
ns
ns
ns
ns
ns
ns
2,3
1,15
0,575
0,287
0,143
0,071
0,035
0,017
0,008
0,004
0,002
CC
Alcohol percentage (%)

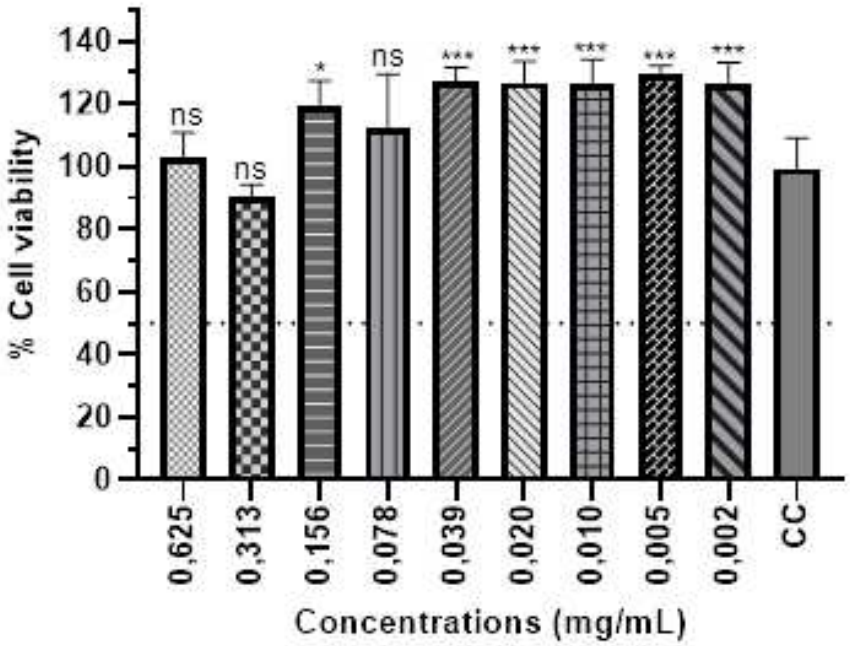
(a)


% Cell viability
140
120
100
80
60
40
20
0
****
****
**
ns
ns
ns
ns
ns
ns
0,625
0,312
0,156
0,078
0,039
0,020
0,010
0,005
0,002
CC
Concentrations (mg/mL)
IC50 = 0.6229 mg/mL

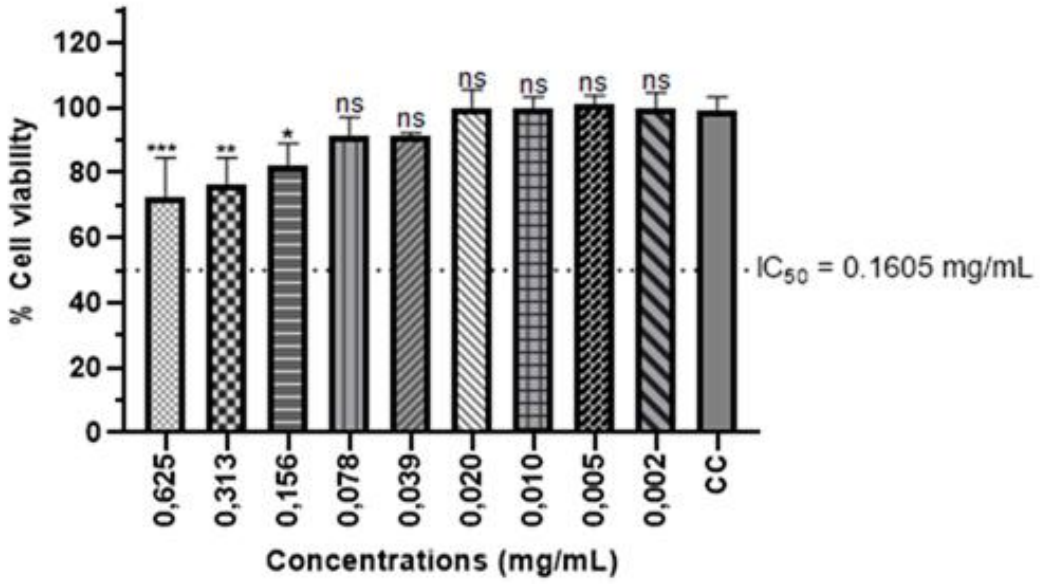
(b)


% Cell viability
120
100
80
60
40
20
0
****
ns
ns
ns
ns
ns
ns
ns
ns
0,625
0,312
0,156
0,078
0,039
0,020
0,010
0,005
0,002
CC
Concentrations (mg/mL)
IC50 = 1.252 mg/mL

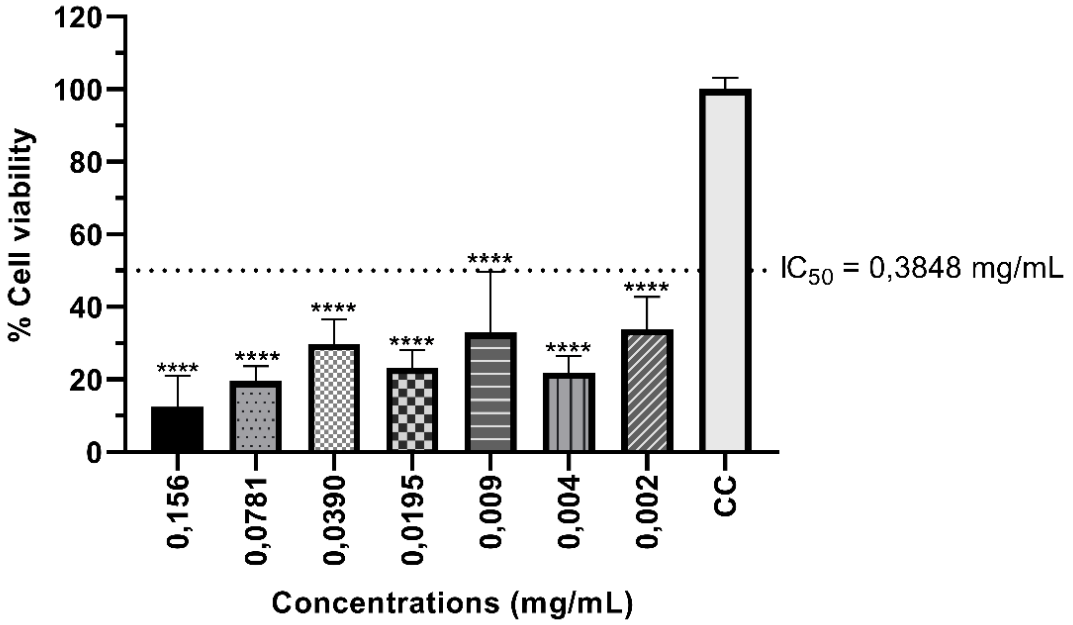
(a)


% Cell viability
140
120
100
80
60
40
20
0
ns
ns
*
ns
***
***
***
***
***
0,625
0,313
0,156
0,078
0,039
0,020
0,010
0,005
0,002
CC
Concentrations (mg/mL)

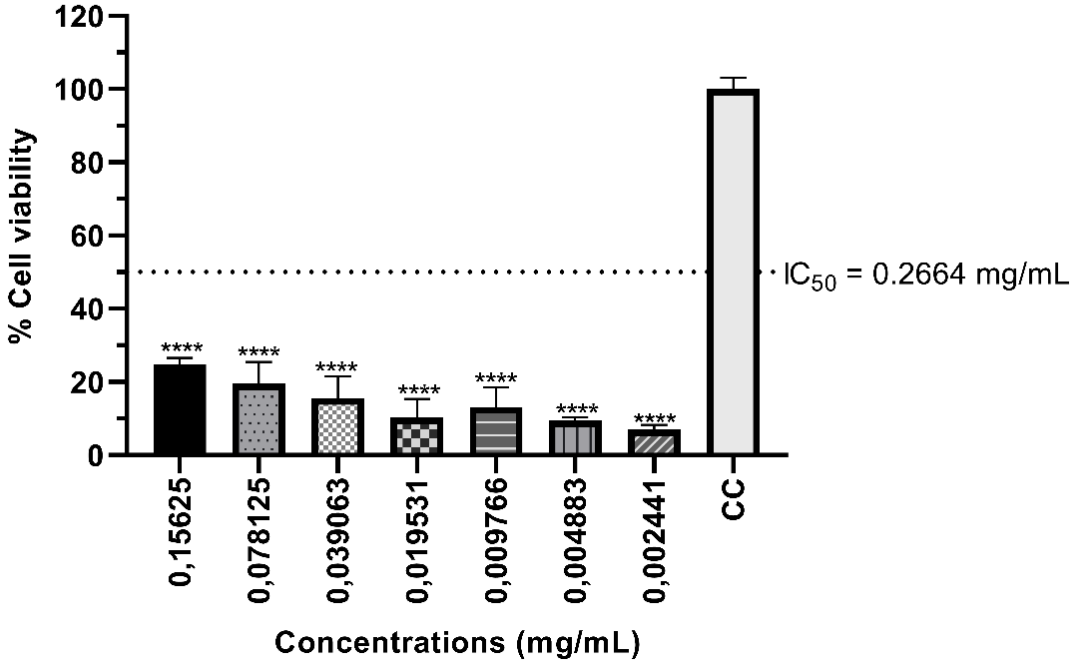
(b)


% Cell viability
120
100
80
60
40
20
0
**
**
*
ns
ns
ns
ns
ns
ns
0,625
0,313
0,156
0,078
0,039
0,020
0,010
0,005
0,002
CC
Concentrations (mg/mL)
IC50 = 0.1605 mg/mL

(a)


120
100
80
60
40
20
0
% Cell viability
****
IC50 = 0,3848 mg/mL
0,156
0,0781
0,0390
0,0195
0,009
0,004
0,002
CC
Concentrations (mg/mL)

(b)


120
100
80
60
40
20
0
% Cell viability
****
IC50 = 0.2664 mg/mL
0,15625
0,078125
0,039063
0,019531
0,009766
0,004883
0,002441
CC
Concentrations (mg/mL)

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- GraphicResumeSargassum240826.jpeg
- Suppl.Fig.1.docx