EFFECT ON THE SKIN OF EXTRACTS ISOLATED FROM THE PORTUGUESE COAST'S *SARGAÇO*

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Introduction and Aims

Sargaço is a designation from the northern Portuguese coast assigned to a mixture of seaweeds that grows on rocky platforms and is loosen and run aground on the beach due to tidal movement. It mainly includes brown algae, but also red and green algae. Traditionally, it was collected by local farmers and fishermen, dried and applied in agriculture, or sold fresh for industry. Today, the traditional collection of *sargaço* is practically extinct.

However, these macroalgae continue to run aground on the beach. This accumulation on shore brings several constraints not only for vacationers, due to the algal accumulation in the sand and sea and bad odour from algal decomposition, but also ecologically, making the habitat of many species practically anoxic. On the other hand, this natural biomass, rich in organic matter, minerals and various bioactive compounds, can be a valuable resource for Man. Nevertheless, it is currently being wasted and underexploited.

The project VALSAR – Valorisation of Sargaço on the Portuguese north coast aims to investigate and explore the potential of these stranded macroalgae and enhance this natural biomass, contributing to sustainable development and diversifying the local economy of coastal communities.

Methods & References

Specimens' collection and preparation of extracts | Specimens of *sargaço* were collected at Vila do Conde, at Praia de Mindelo and Praia A-Ver-O-Mar in October 2021 and February 2022. The harvest took place one hour before the low tide and all algae washed ashore on the beach or loosened in the water were collected. The algae were collected into plastic baskets and then stored in refrigerated coolers. A sample of each seaweed that constitutes *sargaço* was stored in a plastic bag for posterior identification. The seaweeds were washed with seawater to remove sand and epiphytes and then with distilled water to remove the excess of salt. The different specimens of algae were identified by naked eye observation and microscope observation following the identification guides (1,2). The washed *sargaço* was dried at 65°C in the oven. It was then milled and stored for the future preparation of extracts. Aqueous extracts were prepared as described by Sousa et al. (3) through the weighting of 12 g of milled dried seaweed mass and then wetted with 100 mL of distilled water and added into a blender for 3 min (automatic programs: auto clean and smoothie). Afterwards, the solution was filtered in a Buchner funnel, with a nylon net set (mesh dimension: 1 mm), connected to a kitasato flask, under vacuum; followed by another vacuum filtration with a Gooch funnel (porosity: G3). The solution was freezed and lyophilized before the further analysis.

Cell culture and treatments | Mouse leukaemic monocyte macrophage cell line (RAW 264.7 - ATCC number TIB-71) were cultured in DMEM medium (pH 7.2), supplemented with fetal bovine serum (FBS, 10%), glucose (up to 25 mM), sodium bicarbonate (17.95 mM), penicillin (100 U/mL) and streptomycin (100 µg/mL) (4). Mouse skin fibroblasts (3T3, obtained from ATCC CRL-1658) and Human keratinocytes cell line (HaCaT, CLS 300493, acquired from DKFZ) were grown in DMEM medium (pH 7.2) supplemented with heat inactivated FBS (10%), glucose (up to 25 mM), sodium bicarbonate (35.9 mM), penicillin (100 U/mL) and streptomycin (100 µg/mL) (4). The cells were maintained at 37 °C in a humidified atmosphere of 95% air and 5% CO2. Before reaching confluence (every two days), the cells were detached with a cell scrapper (RAW 264.7) or with trypsin (3T3 and HaCaT) and further subcultured in fresh culture media (4). The RAW 264.7 (50000/well), 3T3 (10000/well) and HaCaT (20000/well) cells were plated in duplicates in a 96-well plate in a final volume of 0.2 mL/well, for 24 h. The next day, the medium was replaced by fresh culture medium with extracts. Both February and October extracts (*A Ver o Mar Fev* (AVM Fev) & *A Ver o Mar Out* (AVM Out), respectively) were diluted in culture medium to a final concentration of 10 mg/mL or 5 mg/mL and filtered with a 0.2 µm cellulose acetate syringe filter. Different concentrations of the extracts were tested ([mg/mL], as depicted in the figures), for 24 h.

Metabolic capacity (Alamar blue assay) | Cell metabolism was assessed using Alamar Blue (resazurin) reduction colorimetric assay (4,5). After 24 h of incubation with the extracts, the medium was replaced with culture medium with 50 µM of resazurin solution (in sterile PBS). After 1h30, the absorbance was read at 570 and 620 nm with a Synergy HT multi-mode microplate reader. Metabolically active cells reduce resazurin (a non-fluorescent blue dye) into resorufin (pink colored and fluorescent form) and, hence, their number correlates with the magnitude of dye reduction. Results were expressed as a percentage of control.

Determination of nitric oxide (NO) production (Griess assay) | The macrophages RAW 264.7 were simultaneously exposed to the extracts and to the pro-inflammatory stimuli lipopolysaccharide (LPS, 100 ng/mL, added 30 min after the extracts), for 24h. NO production was further determined through the colorimetric Griess assay (4,5), that measures nitrite accumulation in the culture supernatants. Briefly, equal volumes of cell culture supernatants and Griess reagent [1% (w/v) sulphanilamide in 5% (w/v) phosphoric acid and 0.1% (w/v) N-(1-naphthyl)-ethylenediamine dihydrochloride] were mixed and incubated at room temperature (RT) for 30 min. The absorbance was measured at 550 nm in a multi-mode microplate reader. Nitrite concentration was calculated through a regression analysis of a sodium nitrite standard curve.

References | *1. Pereira, L. 2022. MACOI: Portuguese Seaweeds Website. http://macoi.uc.pt/; 2. Pereira, L. 2009. Guia ilustrado das macroalgas: conhecer e reconhecer algumas espécies da flora portuguesa. Imprensa da Universidade de Coimbra, Coimbra, Portugal, p. 90. DOI: 10.14195/978-989-26-0397-1; 3. Sousa, T., Cotas, J., Bahcevandziev, K., Pereira, . L. (2020). Effects of "sargaço" extraction residues on seed germination. Millenium - Journal of Education, Technologies, and Health, 2(13), 29–37. https://doi.org/10.29352/mill0213.03.00279; 4. Moreira, P., Sousa, F.J., Matos, P., Brite,s G.S., Gonçalves, M.J., Cavaleiro, C., Figueirinha, A., Salgueiro, L., Batista, M.T., Branco, P.C., Cruz, M.T., Pereira, C.F. (2022) Chemical composition and effect against skin alterations of bioactive extracts obtained by the hydrodistillation of Eucalyptus globulus leaves. Pharmaceutics, 14(3), 561. https://doi:10.3390/pharmaceutics14030561; 5. Silva, A., Oliveira, A.S., Vaz, C.V. et al.(2020). Anti-inflammatory potential of Portuguese thermal waters. Sci Rep. 10, 22313. https://doi.org/10.1038/s41598-020-79394-9*

Results

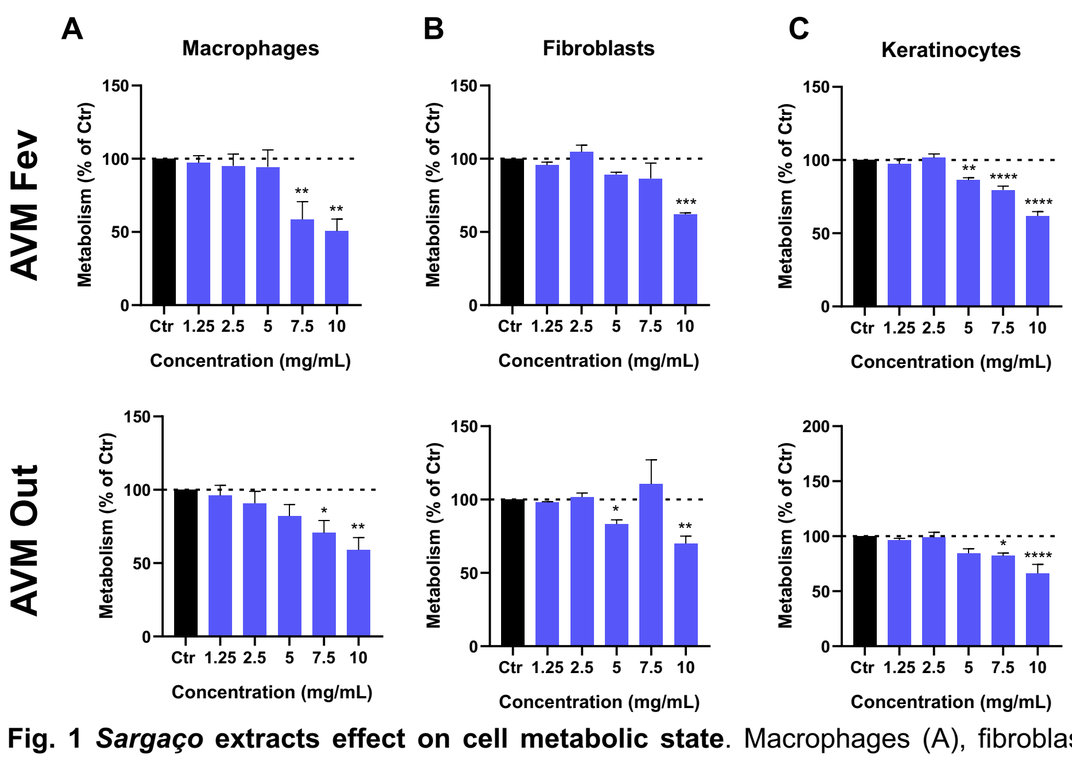


Fig. 1 *Sargaço* extracts effect on cell metabolic state. Macrophages (A), fibroblasts (B) and keratinocytes (C), were plated and exposed to different concentrations of the extracts for 24 h and Alamar blue assay was further performed to assess cell metabolism. Data correspond to the means ± SEM of at least three independent experiments and are represented as % of control cells (Ctr, black bars). Statistical analysis: one ANOVA with Dunnett's multiple comparisons test; $p < 0.05$ was considered significant: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$, compared to Ctr.

Both *sargaço* extracts preserved the cell metabolic capacity of macrophages, fibroblasts or keratinocytes, and induced a significant increase in nitric oxide (NO) levels in macrophages

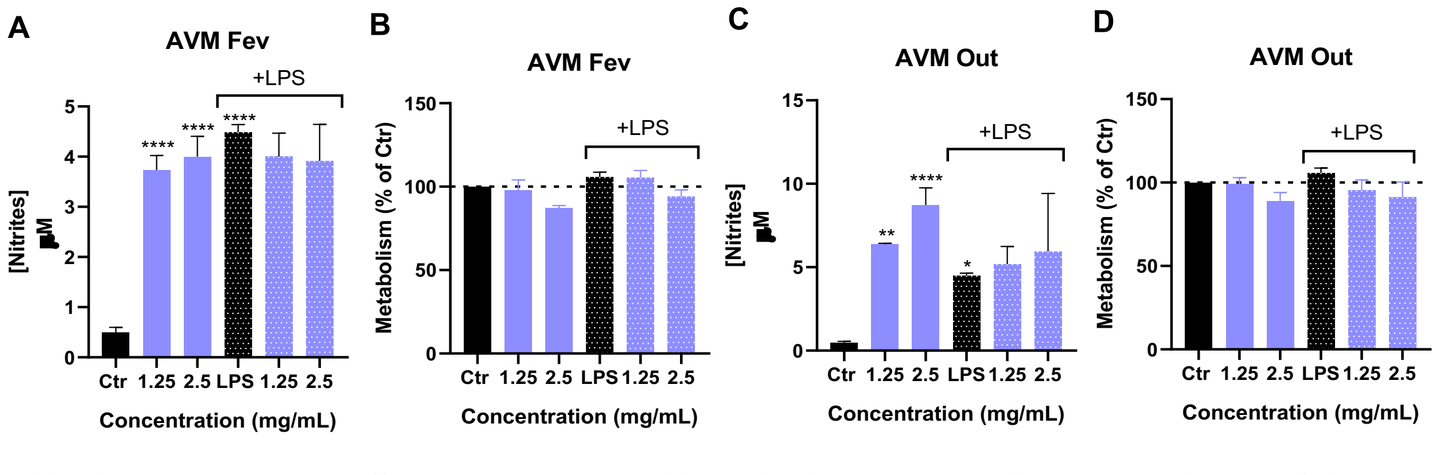
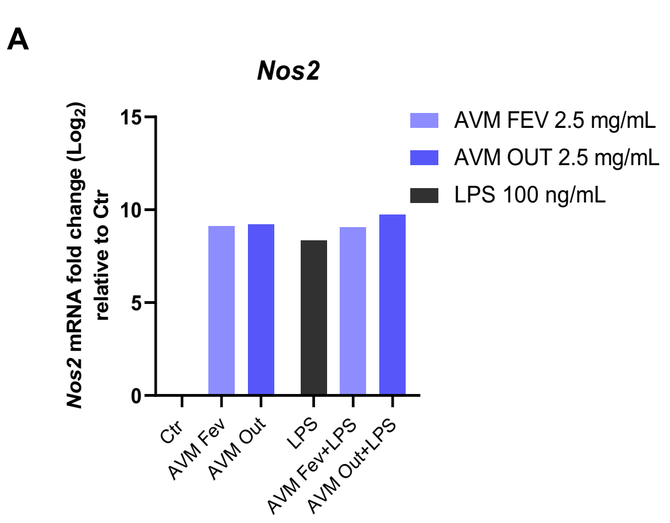


Fig. 2 *Sargaço* extracts effect on macrophage NO production upon an inflammatory stimulus. Cells were plated and exposed to different concentrations of the extracts for 24 h, in the presence or absence of LPS (100 ng/mL) and nitrites levels in the supernatant were measured by Griess assay (A and C). Cell metabolism was also evaluated (B and D). Data correspond to the means ± SEM of two to seven independent experiments and are represented as nitrite concentration (in µM). Statistical analysis: one ANOVA with Tukey's multiple comparison test; $p < 0.05$ was considered significant: * $p < 0.05$, ** $p < 0.01$ and **** $p < 0.0001$, compared to Ctr.



Both *sargaço*-derived extracts upregulated the pro-inflammatory gene *iNOS* in macrophages and the antioxidant gene *HMOX1* in keratinocytes

Fig. 3 *Sargaço* extracts effect on *Nos2* and *HMOX1* gene expression. The expression of the anti-inflammatory gene *Nos2* (A) and the antioxidant gene *HMOX1* (B) was evaluated in macrophages and keratinocytes, respectively, by real-time RT-PCR. The cells were exposed to 2.5 mg/mL of *sargaço* extracts (with or without 100 ng/mL of LPS – macrophages only), for 6 h. Data correspond to one independent experiment. mRNA levels are expressed relative to control cells (log2=0).

Conclusions

Findings suggest that *sargaço* exhibits a pro-inflammatory profile, and that its use as an antimicrobial or coadjuvant agent should be explored.

Although preliminary, the results also suggest that *sargaço* extracts could trigger antioxidant responses.

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