

## Evaluation of tyrosinase enzyme inhibition, antioxidant activity and total phenols of seaweeds from the state of Rio de Janeiro

## Avaliação da inibição da enzima tirosinase, atividade antioxidante e fenóis totais de algas marinhas do estado do Rio de Janeiro

## Evaluación de la inhibición de la enzima tirosinasa, la actividad antioxidante y los fenoles totales de las algas marinas del estado de Río de Janeiro

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### ABSTRACT

Total phenolic content, antioxidant activity and inhibition of tyrosinase enzyme by seaweed extracts were evaluated. The tyrosinase enzyme is responsible for the oxidation of tyrosine during melanin biosynthesis and its dysfunction is associated with conditions such as albinism, vitiligo and melanosis, motivating interest in the discovery of molecules capable of inhibiting this enzyme. The total phenolic content showed a relevant correlation with the antioxidant activity results for the extracts and fractions tested. *Ulva lactuca* (UL), *Kappaphycus alvarezii* (KA) and cultivated *Sarcocornia ambigua* (SC) showed the best enzyme inhibition results, with the UL being fractionated by classical column chromatography. The UL fractions tested showed a reduction in tyrosinase enzyme inhibition, suggesting that the results for the crude extract were due to synergism. The hydrophilic fraction of UL was analysed by  $^{13}\text{C}$  NMR spectroscopy, and the spectrum showed signals characteristic features of sucrose, confirming data already described for this species.

**Keywords:** seaweeds, total phenols, antioxidant activity, tyrosinase inhibition

### RESUMO

O teor fenólico total, a atividade antioxidante e a inibição da enzima tirosinase por extratos de algas marinhas foram avaliados. A enzima tirosinase é responsável pela oxidação da tirosina durante a biossíntese da melanina e sua disfunção está associada a condições como albinismo, vitiligo e melanose, motivando o interesse na descoberta de moléculas capazes de inibir essa enzima. O teor fenólico total mostrou uma correlação relevante com os resultados da atividade antioxidante para os extratos e frações testados. *Ulva lactuca* (UL), *Kappaphycus alvarezii* (KA) e *Sarcocornia ambigua* (SC) cultivada apresentaram os melhores resultados de inibição enzimática, com a UL sendo fracionada por cromatografia em coluna clássica. As frações de UL testadas mostraram uma redução

na inibição da enzima tirosinase, sugerindo que os resultados para o extrato bruto foram devidos ao sinergismo. A fração hidrofílica de UL foi analisada por espectroscopia de RMN  $^{13}\text{C}$ , e o espectro mostrou sinais característicos da sacarose, confirmando dados já descritos para esta espécie.

**Palavras-chave:** algas marinhas, fenóis totais, atividade antioxidante, inibição da tirosinase

## RESUMEN

Se evaluó el contenido fenólico total, la actividad antioxidante y la inhibición de la enzima tirosinasa por extractos de algas marinas. La enzima tirosinasa es responsable de la oxidación de la tirosina durante la biosíntesis de la melanina y su disfunción está asociada a afecciones como el albinismo, el vitiligo y la melanosis, lo que motiva el interés por el descubrimiento de moléculas capaces de inhibir esta enzima. El contenido fenólico total mostró una correlación relevante con los resultados de la actividad antioxidante de los extractos y fracciones analizados. *Ulva lactuca* (UL), *Kappaphycus alvarezii* (KA) y *Sarcocornia ambigua* (SC) cultivada mostraron los mejores resultados de inhibición enzimática, fraccionándose la UL mediante cromatografía en columna clásica. Las fracciones de UL analizadas mostraron una reducción en la inhibición de la enzima tirosinasa, lo que sugiere que los resultados del extracto crudo se debieron a un efecto sinérgico. La fracción hidrofílica de UL se analizó mediante espectroscopia de RMN de  $^{13}\text{C}$ , y el espectro mostró señales características de la sacarosa, lo que confirma los datos ya descritos para esta especie.

**Palabras clave:** algas marinas, fenoles totales, actividad antioxidante, inhibición de la tirosinasa

## 1 INTRODUCTION

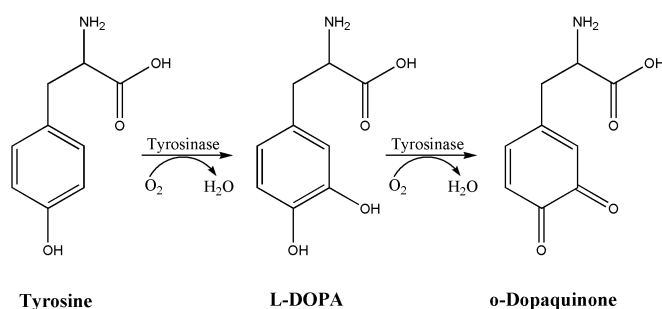
Tyrosinase is an enzyme found in several organisms, including mammals, where it plays a crucial role in the first step of the synthesis of melanin, the pigment responsible for the colour of skin, hair and eyes. Its function is to catalyse the oxidation of the amino acid tyrosine to dopaquinone, an essential intermediate in the synthesis of melanin. Melanin plays an important role in protecting the skin against ultraviolet (UV) radiation, helping to prevent DNA damage, and reducing the risk of skin cancer<sup>[1]</sup>.

However, in other organisms, tyrosinase may have functions other than those found in mammals, such as in plants, where it is involved in the synthesis of phenolic compounds, and in fungi, where it catalyses the synthesis of fungal melanin, which helps protect them against UV radiation and pathogens<sup>[2]</sup>.

In humans, tyrosinase dysfunction is associated with several diseases, including albinism, vitiligo and melanosis. Albinism is a rare genetic disorder in which there is insufficient or no production of melanin due to mutations in the gene encoding tyrosinase or other genes involved in melanin synthesis. Vitiligo is an autoimmune disease that causes the destruction of melanocytes, the cells responsible for producing melanin, resulting in white patches on the skin. In addition, tyrosinase dysfunction can increase susceptibility to infectious diseases, as melanin plays an important role in defence against pathogenic micro-organisms<sup>[3]</sup>.

The mechanism of action of tyrosinase in the conversion of tyrosine to dopaquinone involves the oxidation of tyrosine in two steps. First, tyrosine is hydroxylated to form L-DOPA (3,4-dihydroxyphenylalanine), an important intermediate in neurotransmitter synthesis. L-DOPA is then oxidised by tyrosinase to form dopaquinone, an essential intermediate in the synthesis of melanin (Figure 1). The redox reaction in the presence of oxygen is mediated by copper ions present in the active site of the enzyme, which act as electron acceptors, allowing the oxidation of tyrosine<sup>[4]</sup>.

Figure 1: Mechanism of action of the tyrosinase enzyme.



Therapies currently used to treat hyperpigmentation, such as the use of hydroquinone and kojic acid, have been considered unsatisfactory due to several side effects, such as high cytotoxicity and mutagenicity, low absorption capacity by the skin, instability of formulations, among others<sup>[5]</sup>.

Therefore, the development and discovery of tyrosinase enzyme inhibitor compounds that minimise the undesirable effects of currently used therapies is relevant in several fields and can be used as skin lightening agents, in the treatment of hyperpigmentation conditions, as well as in the food industry to prevent enzymatic browning of fruits and vegetables<sup>[6]</sup>.

Therefore, understanding the structure, function and regulation of the tyrosinase enzyme is essential to explore its role in different organisms, as well as its implications in areas such as biology, medicine and industry.

Literature reports indicate that seaweeds are sources of antioxidant compounds, including phenolic compounds<sup>[7]</sup>, which may represent potential sources of compounds that inhibit the tyrosinase enzyme.

Therefore, the aim of this work was to evaluate the antioxidant capacity, total phenolic content and activity on the tyrosinase enzyme *in vitro*, of extracts and fractions of marine macroalgae available along the coast of the State of Rio de Janeiro.

## 2 RESULTS AND DISCUSSION

The results of the extract yield, the evaluation of the total phenolic content and the total antioxidant activity are described in Table 1 (Supplementary Material).

Both *Sarcocornia ambigua* samples presented the highest levels of total phenols, with values for the cultivated and wild samples equal to  $32.66 \pm 2.39$  and  $31.23 \pm 1.92$  mg of EAG/g of ethanolic extract, respectively. , as well as the lowest EC<sub>50</sub> values, with values of  $0.25 \pm 0.00045$  and  $0.22 \pm 0.00008$  mg/mL, respectively.

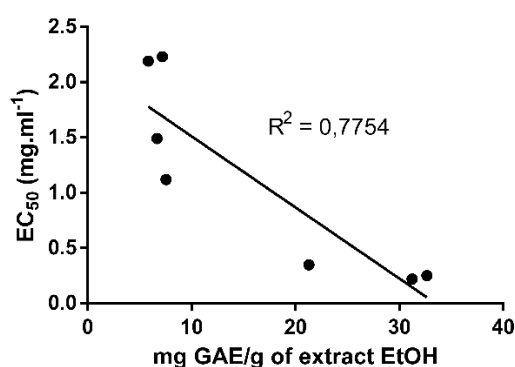
*Ulva flexuosa* showed a total phenolic content equal to  $21.29 \pm 0.40$  mg of EAG/g of ethanolic extract and EC<sub>50</sub> equal to  $0.35 \pm 0.00028$  mg/mL.

*Cladophora sp.* showed a total phenolic content equal to  $10.78 \pm 0.64$  mg of EAG/g of ethanolic extract. However, it was not possible to determine its EC<sub>50</sub> against DPPH•, since to the high absorption of its extract in the wavelength range required by the experiment, even at low concentrations, exceeded the reading capacity of the equipment used.

Finally, *Sargassum vulgare*, *Ulva lactuca*, *Kappaphycus alvarezii* and *Dictyota menstrualis* showed the lowest levels of total phenolics, with values between  $5.84 \pm 0.05$  and  $7.54 \pm 0.13$  mg of EAG/g of ethanolic extract, and the highest EC<sub>50</sub> values, between  $1.12 \pm 0.00436$  and  $2.23 \pm 0.00429$  mg/mL.

These results show that the antioxidant capacity of the analysed extracts varies according to the total phenolic content, which led us to draw a graph in order to evaluate the correlation trend between these factors. (Figure 2).

Figure 2: Correlation graph between total phenol content and total antioxidant activity.



As expected, a correlation was observed between antioxidant activity and the total phenolic content ( $R^2 = 0.7754$ ) of the extracts studied confirming data from the literature linking these two factors.

Tyrosinase enzyme assays at a concentration of 666  $\mu\text{g/ml}$  of hydroalcoholic extracts of UF, UL, KA, DM, SS and SC algae showed inhibition percentages of 41.7, 75.0, 64.0, 43.0, 46.0 and 73.6%, respectively. The  $\text{IC}_{50}$  values of UL, KA and SC were determined and showed values equal to 252, 530 and 504  $\mu\text{g/ml}$  respectively. (Figures 3a-c).

Figure 3a: Inhibition curve of the UL extract as a function of concentration.

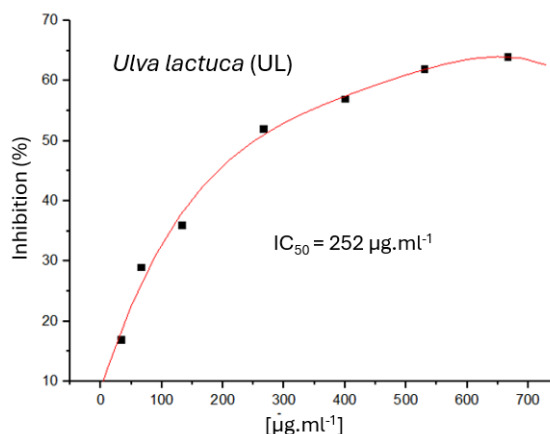


Figure 3b: Inhibition curve of the SC extract as a function of concentration.

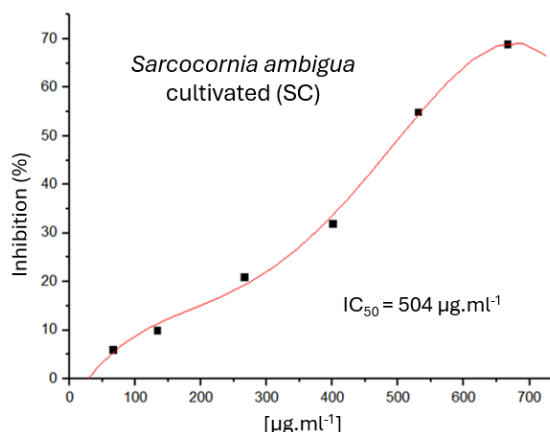
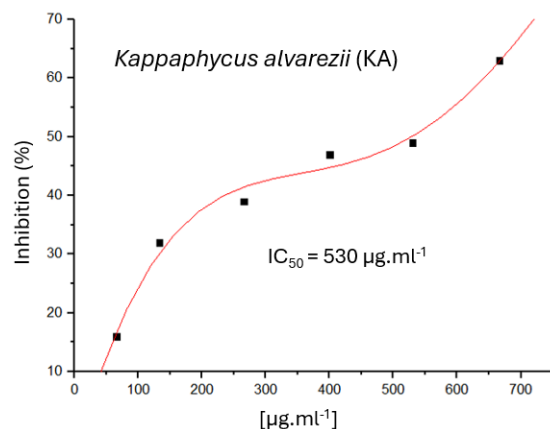


Figure 3c: KA extract inhibition curve as a function of concentration.



Since it showed the lowest IC<sub>50</sub> value, the UL extract was fractionated by liquid-liquid partition by resuspending the crude extract in a solvent system containing methanol and water (4:1), followed by extraction with hexane, producing a lipophilic and another hydrophilic fraction. The hydrophilic fraction obtained (FPUL) was evaluated for antioxidant activity, showing an EC<sub>50</sub> of 3.3 mg/mL and 38% inhibition of tyrosinase at a concentration of 666 µg/mL. The composition of the FPUL fraction was analysed by NMR spectroscopy. The <sup>13</sup>C-DEPTQ NMR spectrum obtained from the FPUL fraction shows signals with chemical shifts at 104, 92 and 82 ppm (Figure 1 - Supplementary Material), which are characteristic of sucrose, as already previously described for this species<sup>[8]</sup>.

### 3 CONCLUSIONS

The results of this research showed that the tested extracts present significant antioxidant activity that may be related to the total phenolic content.

The study also showed that the tyrosinase inhibitory effect of the hydroethanolic extract of *Ulva lactuca* was better than that of the other algae tested. Sucrose is probably not the main contributor to the tyrosinase inhibitory effect of these extracts, as tyrosinase activity decreased when the aqueous fraction was tested.

Finally, crude algae extracts, or the main components of their extracts, may be potential candidates for the development of effective and safe treatments for skin diseases related to the tyrosinase enzyme. However, more research is needed before these inhibitors can be used for practical applications.

### EXPERIMENTAL SECTION

#### Collection and preparation of extracts

In this work, seven species of seaweed were studied, among which *Ulva lactuca* and *Ulva flexuosa* were collected in the Quebra-Mar region, located in the Barra da Tijuca neighborhood, and *Cladophora* spp. was collected on Barra de Guaratiba beach, both in the Municipality of Rio de Janeiro. *Dictyota menstrualis* and *Sargassum vulgare* were collected on the Azeda and Azedinha beaches, located north of the municipality of Armação dos Búzios. *Kappaphycus alvarezii* was collected in the Ilha Grande region, in the municipality of Angra dos Reis, and *Sarcocornia ambigua*, which was evaluated in wild samples, collected on Barra de Guaratiba beach, and samples cultivated at D'alga Aquicultura Urbana, both in the Municipality of Rio de Janeiro.

The plant material was dried in a circulating air oven at 55°C for 48 hours and then ground in a rotor mill. Extraction was carried out by decoction of 100 g of dry plant material in 200 ml of

80% ethanol for two hours at 80°C, followed by vacuum filtration and concentration of the extracts obtained by distillation at reduced pressure.

Due to their high concentration of mineral compounds, the extracts obtained were solubilised in absolute methanol and filtered to remove insoluble compounds, followed by concentration by distillation under reduced pressure.

### **Determination of total phenol content**

The Folin-Ciocalteu method is based on the interaction of reducing substances with the Folin-Ciocalteu reagent, which contains phosphotungstic acid ( $\text{H}_3\text{PW}_{12}\text{O}_{40}$ ) and phosphomolybdic acid ( $\text{H}_3\text{PMo}_{12}\text{O}_{40}$ ), which are yellow in color. In an alkaline environment, reducing substances, such as phenolic substances, are deprotonated, giving rise to the phenolate anion. This anion is capable of reducing the Folin-Ciocalteu reagent, forming tungsten oxide ( $\text{W}_8\text{O}_{23}$ ) and molybdenum oxide ( $\text{Mo}_8\text{O}_{23}$ ), which are blue in color, with absorption at a maximum wavelength of 760 nm, which makes it possible to quantification of the formation of these compounds by spectrophotometry<sup>[9]</sup>.

A standard curve of gallic acid at concentrations between 10 and 100 mg/L was prepared by reacting with appropriate volumes of Folin-Ciocalteu reagent and 10% sodium carbonate for 60 minutes. After the reaction time, the samples were read in a spectrophotometer at 760 nm. Blank samples, containing only the reagents and the solvent used in the extractions, and the extracts were equally treated, and the absorbances obtained for the samples were substituted into the straight line equation of the standard curve, to obtain the concentration values of acid equivalents gallic acid (EAG) in each extract<sup>[10]</sup>.

### **Assessment of total antioxidant activity (%TAA) using the DPPH method**

The DPPH• method consists of evaluating the antioxidant capacity of a substance, according to its ability to reduce radical species. DPPH• (2,2-diphenyl-picrylhydrazyl) is a radical that has a purple color, with absorption at a maximum wavelength of 518 nm, commonly used to identify hydrogen donors and phenolic compounds present in natural products<sup>[11]</sup>. After reacting with an antioxidant, DPPH• is reduced, forming 2,2-diphenyl-picrylhydrazine (DPPH), yellow in color. Therefore, DPPH• consumption can be monitored by the decrease in absorbance at 518 nm, in a spectrophotometer<sup>[12]</sup>.

In this way, the antioxidant activities of the studied algae extracts were determined, in different concentrations, against an ethanolic solution of 0.3 mM DPPH, with the percentages of antioxidant activity being determined in relation to a control containing only the radical solution. In

parallel, blanks were prepared for each concentration in order to determine the absorbance of the samples. After 30 minutes of reaction, the absorbance readings of the samples, blanks and control were taken using a spectrophotometer at 518 nm. Subsequently, concentration x %AAT curves were constructed, and using the straight line equation obtained, the EC<sub>50</sub> values were determined. The %AAT values were calculated using the following formula<sup>[13]</sup> (OLIVEIRA, 2020):

$$\%TAA = 100 - ((Abs_s - Abs_b) \times 100) / Abs_c$$

Where, Abs<sub>s</sub>, Abs<sub>b</sub> e Abs<sub>c</sub> are, respectively, the absorbances of the sample, the blank and the control.

### Assessment of tyrosinase enzyme inhibition

The inhibition of the tyrosinase enzyme was evaluated using the method described by Amorim and collaborators<sup>[14]</sup>, in order to evaluate the activity of crude extracts on the tyrosinase enzyme at a fixed concentration of 666 µg/mL. Stock solutions (10.0 mg/mL) were prepared in DMSO, and aliquots were added to the solution containing L-DOPA (0.17 mM), EDTA (0.022 mM) and tyrosinase (50 – 100 units) in PBS (pH = 7.4), with a final volume equal to 1.5 mL. Absorbance was determined using a spectrophotometer at 475 nm, at room temperature below 27 °C, immediately after preparing the solutions, and the formation of L-DOPA was monitored for 30 minutes.

The percentage of inhibition was calculated according to the equation:

$$\% \text{ inhibition} = \{[(B_{30} - B_0) - (Am_{30} - Am_0)] / (B_{30} - B_0)\} \times 100$$

Where, B<sub>0</sub> e B<sub>30</sub> are the absorbance of L-DOPA + tyrosinase at times t = 0 e 30 min, respectively, while Am<sub>0</sub> e Am<sub>30</sub> are the absorbance of L-DOPA + tyrosinase + extracts, at times t = 0 and 30 min, respectively.

The extracts that showed an inhibition percentage greater than 60% at a concentration equal to 666 µg/mL had their IC<sub>50</sub> values determined from the curve equation generated for the inhibition results at different concentrations, with ascorbic acid being used as a control. positive.

The results presented in this study correspond to the average of three replications (n = 3) ± standard deviation of the mean. The correlation coefficient between the total phenol content and the efficient concentration of each extract, EC<sub>50</sub>, was determined using the GraphPad Prism 6.0 program, and the tyrosinase enzyme inhibition curves were constructed using the Microcal Origin 7.5 program.

## Supplementary Material

**Table 1:** Results of extract yield per 100 g of wet algae, evaluation of total phenolic content and total antioxidant activity.

**Figure 1** –  $^{13}\text{C}$  NMR spectrum of the FPUL fraction.

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## Author Contribution Statement

**Rodrigo Cesar Fernandes Barbosa:** Collection of seaweed, preparation of extracts, fractionation and evaluation of total phenolic content and antioxidant activity, writing and editing.

**Marcia Cristina Campos de Oliveira:** Evaluation of the inhibition of the tyrosinase enzyme and project supervision.

## Conflict of Interests

The authors have no conflict of interest to disclose.

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## SUPPLEMENTARY MATERIAL

Table 1: Results of extract yield per 100 g of wet algae, evaluation of total phenolic content and total antioxidant activity.

| Seaweed                      | Code | Extract yield (g) | Total phenols<br>(mg GAE.g <sup>-1</sup> extract EtOH ± SD) | EC <sub>50</sub> ± SD (mg.ml <sup>-1</sup> ) | μM Trolox.g <sup>-1</sup> extract ± SD |
|------------------------------|------|-------------------|-------------------------------------------------------------|----------------------------------------------|----------------------------------------|
| <i>S. ambigua</i> cultivated | SC   | 1.25              | 32.66 ± 2.39221                                             | 0.25 ± 0.00045                               | 55.59 ± 0.24                           |
| <i>S. ambigua</i> wild       | SS   | 1.37              | 31.23 ± 1.92118                                             | 0.22 ± 0.00008                               | 60.79 ± 3.07                           |
| <i>Cladophora</i> sp.        | CL   | 1.53              | 10.78 ± 0.63506                                             | ND                                           | 84.02 ± 2.45                           |
| <i>S. vulgare</i>            | SV   | 1.17              | 6.67 ± 0.20900                                              | 1.49 ± 0.00280                               | 88.64 ± 3.12                           |
| <i>U. flexuosa</i>           | UF   | 2.46              | 21.29 ± 0.40105                                             | 0.35 ± 0.00028                               | 55.5 ± 1.5                             |
| <i>U. lactuca</i>            | UL   | 2.22              | 7.54 ± 0.12649                                              | 1.12 ± 0.00436                               | 47.41 ± 2.78                           |
| <i>K. alvarezii</i>          | KA   | 0.98              | 7.2 ± 0.16278                                               | 2.23 ± 0.00429                               | 54.24 ± 1.29                           |
| <i>D. menstrualis</i>        | DM   | 2.01              | 5.84 ± 0.04869                                              | 2.19 ± 0.00311                               | 35.20 ± 1.46                           |
| Gallic acid                  | AG   | -                 | -                                                           | 0.002 ± 0.00001                              | -                                      |

Figure 1 – <sup>13</sup>C NMR spectrum of the FPUL fraction.

