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Comparative analysis of *in vitro* antioxidant capacities of mycosporine-like amino acids (MAAs)

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ABSTRACT

The most common Mycosporine-like Amino Acids (MAAs) in red algae (asterina-330, shinorine, palythine, palythinol, and porphyra-334) were evaluated regarding their *in vitro* antioxidant capacities, comparing them with synthetic (BHT and Trolox) and natural (ascorbic acid, gallic acid, *p*-cumaric acid, quercetin, and rutin) antioxidants. Folin-Ciocalteu, $ABTS^{\cdot+}$, and FRAP assays have the same reaction mechanism of electron transfer and usually show a positive correlation. However, the antioxidant capacities for the MAAs were discrepant between these assays. While porphyra-334, shinorine, and palythine were as active as, or more active than synthetic phenolic antioxidants (Trolox and BHT) in Folin-Ciocalteu assay, the antioxidant potentials of MAAs were lower than those of the standard compounds in $ABTS^{\cdot+}$ and FRAP assays. The pH-dependent antioxidant power was hypothesized as an explanation for this distinct behavior and was evaluated through $ABTS^{\cdot+}$ assay. Alkaline pH allows antioxidant capacities similar to Trolox in $ABTS^{\cdot+}$ assay, supporting our hypothesis. Comparing to standard compounds, MAAs showed low activity using ORAC assay and were pro-oxidant in β-carotene/linoleic acid assay. Therefore, imino-MAAs present relevant *in vitro* antioxidant capacity under specific pH conditions in assays based on electron transfer.

1. Introduction

Mycosporine-like amino acids (MAAs) are nitrogenous secondary metabolites of low molecular weight (< 400 Da) highly water-soluble [1]. The maximum absorption of these compounds occurs in the ultraviolet (UV) region in the range of 310 to 360 nm [2]. MAAs are common in marine organisms, including single-cell organisms (*e.g.*, dinoflagellates and cyanobacteria), animals (*e.g.*, corals, fish, and crustaceans), and marine macroalgae (mainly Rhodophyta), as well as in some land organisms (fungi and lichens) [3].

Structures of MAAs have a cyclohexenone or cyclohexenimine ring linked to an amino acid, an amino alcohol or an amino group [4]. The cyclohexenone ring is present in the oxo-MAAs, while the cyclohexenimine ring is characteristic of the imino-MAAs. The biosynthesis of these compounds is still unclear. There are evidences of an origin from the shikimate pathway [5], as well as from the pentose phosphate pathway [6]. Regardless of the pathway, 4-deoxygadusol is the common precursor of all MAAs [6,7]. The addition of glycine amino acid to 4-deoxygadusol originates the mycosporine-glycine, an oxo-MAA. Further additions of amino acids to mycosporine-glycine give rise to primary imino-MAAs (*e.g.*, shinorine and porphyra-334 with addition of serine and threonine amino acids, respectively) [4], while additional modifications at amino acid side-chains give rise to secondary imino-MAAs (*e.g.*, asterina-330 and palythinol) [4].

Associated with their high light absorbing property, there are strong evidences suggesting photoprotective function of MAAs against UV radiation [8–11]. Field studies have shown high positive correlation between the concentration of MAAs and the levels of UV radiation in the environment [11–13]. In laboratory conditions, induction in the synthesis of these compounds has also been reached with the increase of UV radiation [13–15]. Furthermore, photochemical and photophysical studies have demonstrated that MAAs absorb UV radiation and eliminate it almost completely as heat, without generating free radicals [16,17]. Although there is little evidence, other functions have been attributed to MAAs, such as osmolytes, animal reproduction and fungal sporulation regulators, chemical signals, and accessory pigments in photosynthesis [4,18]. Nevertheless, among these less-studied functions, the antioxidant capacity of these compounds has been the most accepted by the scientific community.

Antioxidants are compounds that can prevent or attenuate oxidative

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stress on organisms or materials susceptible to oxidation, such as food and cosmetics. Oxidative stress can be induced by, among others, high UV radiation, desiccation, and toxicity by heavy metals [19]. Some studies have shown an increase of MAAs in situations of oxidative stress without the participation of UV radiation, supporting the hypothesis of an antioxidant function of these compounds [12,20–25]. For example, Yakovleva et al. [22] observed the induction of mycosporine-glycine synthesis in the coral *Platygyra ryukyuensis* under thermal stress conditions, and Torres et al. [23] observed a significant increase in the concentration of palythinol in red seaweed *Gracilariopsis tenuifrons* cultivated under high intensities of photosynthetically active radiation (PAR).

In vitro studies have also demonstrated the antioxidant capacity of some MAAs, suggesting results from low to high antioxidant activity [26,27]. However, a comparative evaluation between the antioxidant potential of MAAs with other compounds of recognized antioxidant capacity, such as phenolic compounds and synthetic antioxidants, has not been reported. In addition, as postulated by Wada et al. [7], many *in vitro* antioxidant assays commonly employed in several studies have never been performed with MAAs. Since each *in vitro* antioxidant assay presents distinct reaction mechanisms [28–30], the evaluation of a compound through several assays can help understanding how this particular compound acts as an antioxidant.

In this context, the main goals of the present study were (i) to evaluate, through *in vitro* assays (DPPH, ferrous ion-chelating, Folin-Ciocalteu, $ABTS^{\cdot+}$, FRAP, β-carotene/linoleic acid, and ORAC), the antioxidant capacity of the imino-MAAs (asterina-330, shinorine, palythine, palythinol, and porphyra-334) isolated from the red seaweed *Gracilaria domingensis*, and (ii) to compare these antioxidant activities with those of standard compounds (L-ascorbic acid, butylated hydroxytoluene, *p*-coumaric acid, gallic acid, quercetin, rutin, and Trolox).

2. Materials and methods

2.1. Chemicals and reagents

2,2′-Azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) diammonium salt (ABTS; Cat. No. a1888), 2,2′-azobis(2-methylpropionamidine) dihydrochloride (AAPH; Cat. No. 440914), 2,2-diphenyl-1-picrylhydrazyl (DPPH; Cat. No. d9132), 2,4,6-tris(2-pyridyl)-s-triazine (TPTZ; Cat. No. 93285), 3-(2-pyridyl)-5,6-diphenyl-1,2,4-triazine-4′,4″-disulfonic acid sodium salt (Ferrozine; Cat. No. 82950), β-carotene (Cat. No. 22040), Folin-Ciocalteu reagent (Cat. No. 47641), linoleic acid (Cat. No. l1012), polyoxyethylenesorbitan monopalmitate (Tween® 40; Cat. No. 9005-66-7), and sodium fluorescein (Cat. No. 46960) were purchased from Sigma-Aldrich®. HPLC grade solvents were purchased from JT Baker®, and other chemicals were of analytical grade. The following standard compounds, purchased from Sigma-Aldrich®, were used in this study: L-ascorbic acid (≥99% of purity, Cat. No. a5960), gallic acid (purity ≥ 97.5%, Cat. No. g7384), *p*-coumaric acid (purity ≥ 98%, Cat. No. c9008), butylated hydroxytoluene (BHT; purity ≥ 99%, Cat. No. w218405), quercetin (purity ≥ 95%, Cat. No. q4951), rutin (purity ≥ 94%, Cat. No. r5143), and Trolox (purity of 97%, Cat. No. 238813).

2.2. Algal material

The red macroalga *Gracilaria domingensis* (Kützing) Sonder ex Dickie (Gracilariaceae, Rhodophyta) was collected on Northeast Brazilian coast at Morro de Pernambuco beach (Ilheus, Bahia State; 14°48′21.6″S, 39°01′25.6″W). The thalli were washed in tap water to remove sand and epibionts. Soon after, they were completely oven-dried at 40 °C for seven days. Dried algal samples were powdered in a knife mill (sieve of 30 mesh) (Fortinox® STAR FT 80).

2.3. Isolation and characterization of MAAs

The powdered algal-material was defatted and depigmented in hexane and subsequently dichloromethane. The resulting dry powder was macerated in methanol (10%; w/v) at 50 ± 5 °C for 8 h. Then, the methanolic extract was dried in rotatory evaporator under reduced pressure (< 45 °C) and desalted adding methanol as an anti-solvent. The desalted sample was fractionated by semi-preparative High-Performance Liquid Chromatography with Diode-Array Detector (semi-HPLC-DAD - Agilent 1200 series). The equipment was equipped with a semi-preparative column (Zorbax RX-SIL HILIC, 9.4 mm × 250 mm, 5 μm). The flow rate was 4 mL·min^{-1}, temperature of column at 30 °C, and the imino-MAAs were monitored at 330 nm. Aliquots of 100 μL of desalted samples (400 mg·mL^{-1} in methanol) were injected in the method A, which consisted of acetonitrile (solvent A) and 0.2% acetic acid (solvent B) in isocratic elution with 68% A for 20 min. Three fractions were collected and re-fractioned using the same semi-HPLC-DAD conditions with adjusts at mobile phase. The injection volume of the fractions at 20 mg·mL^{-1} in methanol was 50 μL. Fraction 1, enriched of palythine and palythinol, and fraction 2, enriched of palythinol and asterina-330, were analyzed using the method B, as follows: acetonitrile (solvent A) and 0.2% acetic acid (solvent B), beginning with 80% A until 55% A in 15 min, returning to 80% A in 1 min. The isolation of shinorine and porphyra-334 from fraction 3 were conducted using the method C, which consisted of acetonitrile (solvent A) and acetonitrile: methanol: water (10:10:80) (pH 2.2 adjusted with trifluoroacetic acid - TFA) (solvent B) in isocratic elution with 80% A for 10 min. Fig. 1 summarizes all the steps for the isolation and purification of asterina-330, shinorine, palythine, palythinol, and porphyra-334.

The MAAs were identified by HPLC-DAD coupled to Mass Spectrometry (HPLC-DAD-MS/MS). The HPLC-DAD-MS/MS was performed in a Shimadzu's integrated LC system (controller: CBM-20A, pumps: LC-20AD, detector: SPD-M20A, oven: CTO-20A and auto-injector: SIL-20AC) coupled to Amazon speed ETD ion trap mass spectrometer (Bruker Daltonics) equipped with an Electrospray Ionization (ESI) source. The ESI parameters were capillary voltage = 4.5 kV; pressure of nebulizer gas = 27 psi; the flow rate and temperature of drying gas were 7 L·min^{-1} and 300 °C, respectively. The scanned mass ranged between *m/z* 100 and 1000. MS-spectra were recorded in positive ESI mode. Column used was the HILIC (Zorbax RX-SIL, 4.6 mm × 250 mm, 5 μm). The mobile phase consisted of acetonitrile (solvent A) and 0.2% acetic acid (solvent B) in isocratic elution with 68% A for 25 min at constant flow rate of 1 mL·min^{-1}. Detection was performed at 330 nm.

Shinorine, palythine, and porphyra-334 were also analyzed and their structures confirmed through ^{1}H and ^{13}C NMR (Nuclear Magnetic Resonance) in Bruker Avance III (500.13 MHz for ^{1}H NMR and 125.77 MHz for ^{13}C NMR) at room temperature. For NMR analyzes, the compounds were dissolved in D_2O (Sigma-Aldrich®) (10 mg·mL^{-1}), and DSS (4,4-dimethyl-4-silapentane-1-sulfonic acid) was the internal standard.

2.4. Determination of antioxidant capacity

Antioxidant assays were performed on a microplate reader (BioTek™, Synergy™ H1) with 96-well microplates. Ultrapure water and methanol were used as negative controls. Standard compounds were diluted in methanol and the MAAs in ultrapure water. According to the availability of the mass obtained from the isolation procedures, the highest concentrations tested for MAAs were 0.46 mM for asterina-330, 0.20 mM for shinorine, 0.27 mM for palythine, 0.22 mM for palythinol (+palythine), and 0.95 mM for porphyra-334. From these higher concentrations, dilutions were made for the construction of dose–response curves. The antioxidant capacities were expressed in mmol of Trolox equivalents (TE) per mol of compound, IC50 (concentration of