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An extremely acidic environment microalga from Tinto River as a novel source of anti-inflammatory activity

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ABSTRACT

Coccomyxa onubensis (*C. onubensis*) is an acidotolerant microalga isolated from the extremely acidic Tinto River (Huelva), which contains high levels of metals in solution, mainly Fe and Cu. This makes *C. onubensis* an adequate candidate to easily cope with high levels of oxidative stress by increasing the levels of certain molecules and enzymes, which helps them elicit an adequate antioxidant biochemical response. Thus, *C. onubensis* is a promising source of bioactive compounds which exhibit *in vitro* anti-inflammatory activity, including fatty acids, (poly)phenolic compounds and carotenoids. In this study, the correlations between the antioxidant response and anti-inflammatory activity of cell extracts obtained from Fe (III)-stressed microalgal cultures were analyzed. The results suggested a direct relationship between the antioxidant capacity of the microalgal extracts and Fe (III) concentration in the culture medium. Consequently, the production of some of the target antioxidant molecules, including carotenes, xanthophylls and (poly)phenols, increased. The levels of these molecules increased the most in cell extracts obtained from microalgal cultures at 0.25 mM of Fe (III), which was correlated with a 50 % increase in the anti-inflammatory activity of the microalgal extracts in THP-1 differentiated human macrophages. Fe (III)-modulated oxidative stress allowed us to define culture conditions that can enhance the anti-inflammatory activity of *C. onubensis* extracts, which are enriched in valuable antioxidant molecules. Overall, this study highlighted the utility of a microalgal species from a highly acidic environment as a novel, natural source of anti-inflammatory agents, based on its ability to cope with the oxidative conditions of its habitat.

1. Introduction

Microalgae are a valuable source of natural antioxidants that can benefit human health. The antioxidants accumulated by microalgae, including unsaturated fatty acids, terpenoids, (poly)phenolic compounds, and phytosterols, can mitigate inflammatory symptoms (Araújo et al., 2020; Gallego et al., 2022). Therefore, culture conditions that facilitate the accumulation of antioxidant molecules can be used to develop biotechnological processes to produce cell extracts with high anti-inflammatory activity (Montero-Lobato et al., 2018). For this, extremophilic microorganisms can play a key role, i.e., the ability of these microorganisms to adapt to the harsh conditions of their habitats partly depends on the expression of secondary metabolic pathways, leading to the biosynthesis of unique molecules. Among these molecules, terpenoids, such as astaxanthin and lutein, (poly)phenolic compounds (e.g. caffeic and gallic acids), polyunsaturated fatty acids, and sterols (Ruiz-Domínguez et al., 2023) are important as they have anti-inflammatory effects. Therefore, microalgal extracts that contain them are valuable for applications in human health care (Navarro et al., 2016).

C. onubensis is an acidotolerant microalga (Garbayo et al., 2012), isolated from the Tinto River in the province of Huelva, Spain. This river

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has a highly acidic aquatic environment and contains dissolved metals, such as Fe (II and III) and Cu (II), at concentrations that are normally toxic to living forms. Despite these harsh conditions, the Tinto River has a wide variety of microorganisms (Fernández-Remolar et al., 2004). *C. onubensis* is adapted to the environment to tolerate pH values as low as 2.5 (Garbayo et al., 2012). This ability of microalgae to adapt to the acidic environment might be due to the impermeability of the plasma membrane to protons, among other reasons. This allows the cells maintaining a neutral cytoplasmic pH, also supported by a highly active ATP-synthase activity that pumps protons out of the cell. This leads to the maintenance of high proton concentrations in the extracellular medium, considerably increasing the osmotic pressure that also confers moderate halotolerance to acidotolerant microalgae (Gimmler et al., 1988; Gross, 2000). Maintaining a neutral intracellular pH in an acidic environment requires energy, which reduces the energy available for growth (Messerli et al., 2005). Low energy levels, along with the low availability of dissolved inorganic carbon in acidic water, may limit biomass productivity in large production processes. However, in an acidic environment, microalgae have developed strategies to compensate for this low inorganic carbon availability by evolving mechanisms to improve carbon uptake (Vaquero et al., 2013). This allows these extremophilic microorganisms, such as *C. onubensis*, to show moderate levels of productivity in acidic liquid cultures.

The membrane adaptability to the acidic environment might also partly explain the capability of *C. onubensis* to tolerate high concentrations of Fe (III) (as high as 30 g L^{-1}) in its natural habitat (Fernández-Remolar et al., 2004). Fe is necessary for microalgae as it plays a key role in cellular biochemistry due to its redox properties (Gao et al., 2022). However, Fe becomes highly toxic at high concentrations due to its oxidative action through the Fenton reaction, which leads to the production of reactive oxygen species (ROS). This reaction consists of an oxidation process catalyzed by transition metals, usually iron, in which highly ROS, such as the hydroxyl radical ($HO^{\bullet}$), are generated from hydrogen peroxide (H_2O_2), as shown in the following reaction (Gulcin and Alwasel, 2023).

$$\text{Fe (II)} + H_2O_2 \rightarrow \text{Fe (III)} + HO^{\bullet} + HO^{-}$$

The generation of hydroxyl radicals increases oxidative stress due to their reaction with lipids, proteins, and nucleic acids. The reaction between ROS and membrane lipids results in the loss of membrane permeability due to the partial saturation of unsaturated fatty acids of phospholipid molecules (Halliwell, 2006). Additionally, ROS can damage DNA, triggering genetic mutations that can lead to cell death (Ávila-Román et al., 2023). Microalgae respond to oxidative stress by inducing the activity of different antioxidant systems, including an increase in the activity of enzymes such as superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPX), and ascorbate peroxidase (APX), and the synthesis of low-molecular-weight antioxidant compounds, mainly glutathione, carotenoids, (poly)phenolic compounds, and flavonoids (Montero-Lobato et al., 2018).

Thus, microalgae biotechnology allows us to obtain cell extracts rich in antioxidants molecules which can be used to improve human and animal health (Navarro et al., 2016). For instance, (poly)phenolic compounds and carotenoids directly interfere with the transcription of TNFα (tumor necrosis factor-α), an inflammatory cytokine commonly used in assays to evaluate anti-inflammatory activity in suitable cell lines (*in vitro*), such as THP-1 (Araújo et al., 2020; Gallego et al., 2022) (Fig. 1). TNFα is used in anti-inflammatory activity assays of microalgal and algal extracts (Araújo et al., 2020; Ávila-Román et al., 2023). Nuclear factor kappa B (NF-κB) belongs to a family of inducible transcription factors and plays various evolutionarily conserved roles in the immune system (Gómez-Chávez et al., 2021). High levels of ROS induce the generation of oxidative stress inside cells. During exposure to oxidants, IκB (inhibitors of NF-κB) proteins bound to NF-κB are rapidly phosphorylated, leading to their ubiquitin-mediated proteasomal degradation and release from NF-κB (Linnewiel-Hermoni et al., 2014). Then, NF-κB is translocated to the nucleus where it binds to DNA sequences and activates the expression of pro-inflammatory cytokines (Gómez-Chávez et al., 2021).

To summarize, *C. onubensis* can adapt to its environmental (acidic pH and high concentrations of heavy metals) by activating metabolic pathways that promote the accumulation of antioxidant compounds, which have biotechnological applications (Montero-Lobato et al., 2018; Navarro et al., 2016). This ability of *C. onubensis* to adapt makes it a suitable model system for this study. In this study, we found a relationship between the antioxidant capacity of *C. onubensis* cells (that modulates oxidative stress levels), triggered by Fe (III), and the

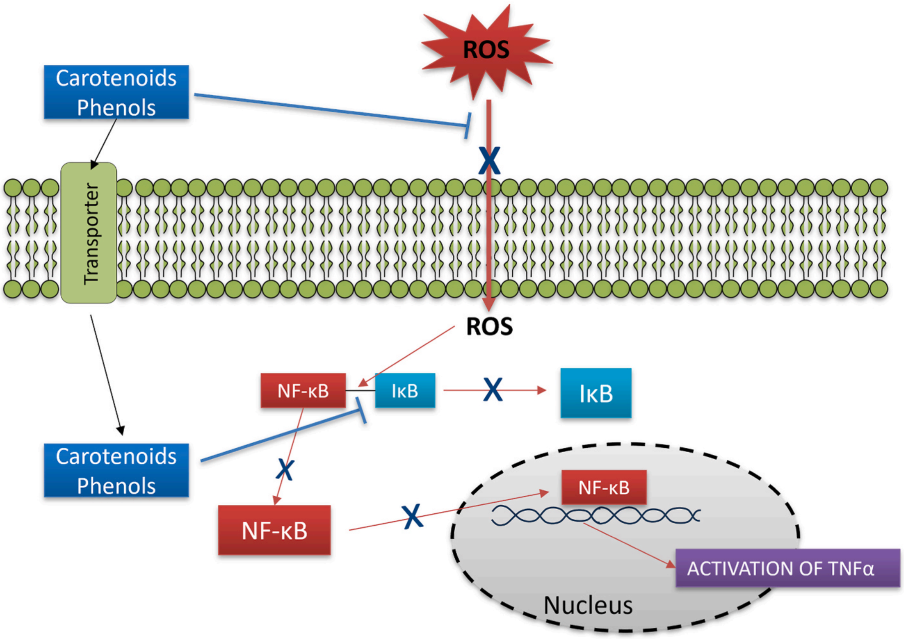


Fig. 1. A schematic representation of the mechanism underlying the anti-inflammatory effect of (poly)phenolic compounds and carotenoids through interaction with the transcription of TNFα (tumor necrosis factor-α); ROS: reactive oxygen species, NF-κB: transcription factor of TNFα, and IκB: inhibitor of NF-κB.

anti-inflammatory capacity of cell extracts rich in carotenoids and (poly)phenolic compounds.

2. Materials and methods

2.1. Microalgal strain and culture conditions

The organism used for this work was the microalga *Coccomyxa onubensis* ACCV1 (SAG 2510), an acidotolerant and halotolerant microalga isolated from the acidic waters of the Tinto River (Huelva) in the sampling area at latitude 37.5851153° and longitude −6.550754°, by the Algae Biotechnology research group (BITAL), of the Department of Chemistry "Professor Jose Carlos Vílchez Martín," from the Faculty of Experimental Sciences of the University of Huelva (Fuentes et al., 2016).

C. onubensis was grown in an optimized liquid culture medium, K9. The constituents of K9 (per liter) were as follows: 3.95 g K_2SO_4, 0.1 g KCl, 0.5 g K_2HPO_4, 0.41 g $MgCl_2$, 2.29 g KNO_3, 0.01 g $CaCl_2$, and 5 mL of Hutner traces, which were prepared as previously described (Garbayo et al., 2012), containing a Fe (II) concentration of 17.98 μM. Fe is more bioavailable at low pH than at neutral or basic pH, Fe (III) was used in this study because it is more soluble than Fe (II) (Johnson et al., 2012). *C. onubensis* cultures were prepared at an initial concentration of, approximately, 0.2 g L^{-1}, from a mother culture in the middle of the linear growth phase, the pH was adjusted to 2.5 and the cultures were subjected to different Fe (III) concentrations, added in the form of $FeCl_3 \cdot 6H_2O$ (VWR, Belgium) from 0 mM (control culture) to 2 mM. The cultures were established in a culture room at 25 °C ± 2. Light was supplied by white light fluorescent tubes, reaching the cultures at a constant light intensity of 150 μmol (photon)$\cdot m^{-2}$ s^{-1} for 24 h, and the cultures were bubbled with air enriched with 2.5 % (v/v) CO_2.

2.2. Growth and photosynthetic viability determination

Growth was followed by measuring optical density (OD) of the cultures at 750 nm in a Cary 60 UV–Vis spectrophotometer (Agilent Technologies, USA) equipped with temperature control system adjusted to 25 °C. The OD_{750} data expressed in absorption unit (UA) provided the information of culture turbidity, which is proportional to the amount of biomass present in the culture. In addition, dry weight of the biomass contained in 2 mL of culture medium was analyzed. This parameter was further used to express the antioxidant compounds content as mg$\cdot g^{-1}$ of biomass dry weight.

Photosynthetic viability was determined based on the measurement of chlorophyll fluorescence, the maximum quantum yield (F_V/F_M) of Photosystem II (PSII) according to published methodology (Maxwell and Johnson, 2000). Quantum yield (Qy) was measured by subjecting cultured samples of *C. onubensis* to darkness for 15 min and then placing them in the measurement chamber of the AquaPEN AP-C 100 portable amplitude-modulated pulse fluorimeter (Photon Systems Instruments, Drasov, Czech Republic). F_V represents the variable fluorescence and is calculated as $F_V = F_M - F_0$, being F_0 the minimum level of fluorescence observed after exposing cells to a non-actinic beam and acclimating them in the dark, while F_M represents the maximum fluorescence observed in cells after exposing them to a brief but saturating pulse of actinic light.

2.3. Electron microscopy

For transmission electron microscopical observations, the method described by Nishikawa et al. (2006) was performed. The microalgal cells were collected by centrifugation (1957×*g*, 1 min) from each culture (cultures treated with different Fe (III) concentrations, and control culture). The microalgal cells were fixed with 1 % glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.4) for 2 h at 4 °C. The cells were then washed three times for 5 min using the same buffer. The samples were postfixed with 1 % osmium tetroxide in 0.2 M cacodylate buffer at 4 °C for 1 h. Samples were washed with the same buffer, dehydrated in a graded ethanol series, and embedded in Epon 812 (Electron Microscopy Science, USA). Ultrathin sections of 80–90 nm obtained by an ultramicrotome (Leica, Germany) and placed on copper grids were stained with aqueous 1 % (w/v) uranyl acetate and lead citrate. Transmission electron micrographs were observed with a JEM 1011 (Jeol Ltd., Japan) electron microscope using an accelerating voltage of 80 kV. Several photographs of entire cells and of local detailed structures were taken at random, analyzed, and compared to investigate the effect of different concentrations of Fe (III) on different subcellular structures.

2.4. Chlorophyll and carotenoid determination

Chlorophyll and carotenoid content were determined as described by Robles et al. (2023). Culture samples, containing 1 or 2 mL, were centrifuged at 4400 rpm for 5 min, methanol was added to the pellet and the mixture was placed in an ultrasound bath (60 °C, 5 min) to weaken the microalgal cell wall. After another centrifugation step, the supernatant was collected and analyzed by UV/Visible spectrophotometry. Modified Arnon's equations were used to calculate the chlorophyll and carotenoid concentrations in the extracts. The extracts obtained were used to analyze the antioxidant capacity of the microalga and the determination of (poly)phenol and flavonoids compounds, see section 2.5 to 2.6. In addition, methanolic extracts were evaporated using a N_2 stream and resuspended in DMSO to 10 mg/mL of extract to analyze the anti-inflammatory activity exerted of *C. onubensis* extracts on THP-1 Mac cells, see section 2.7.

For specific carotenoid analysis and quantification, separation was performed by liquid chromatography (HPLC; Beckman System gold) using an RP-18 column with a flow rate of 1 mL/min and injection extract volume of 40 μL. The applied gradient was the following (solvent A; ethyl acetate and solvent B; acetonitrile/water, 9:1 v/v): 0–16 min, 0–60 % solvent A; 16–30 min, 60 % A; 30–35 min, 100 % A. For quantification, pigment standards supplied by DHI-Water and Environment (Denmark) were injected. Quantification of the selected pigments was based on comparison of peak areas obtained from methanolic extracts of *C. onubensis* with the areas obtained from the injected standards. Xanthophylls pools was determined as the summatory between lutein and neoxanthin concentration and, carotene pool corresponds to the sum between α- and β-carotene.

2.5. Antioxidant capacity

Antioxidant capacity of the methanolic extracts of microalga was determined by the modified version of the DPPH (2,2-diphenyl-1-picryl-hydrazyl-hydrate) free radical method described in Robles et al. (2023). The antioxidant capacity was determined by the decrease in absorbance at 515 nm of a methanolic solution of DPPH in the presence of the different methanolic samples of the microalga. A concentrated solution of DPPH (Sigma Aldrich, Darmstadt, Germany) in methanol of approximately 0.4 g L^{-1} was prepared and diluted with methanol to obtain an absorbance around 0.8. Next, 950 μL of the diluted DPPH solution was made to react with 50 μL of the methanolic sample. The absorbance at 515 nm at time 0 was then measured in a UV–vis spectrophotometer model Evolution 201 (Thermo Fisher Scientific, Walthman, MA, USA). Subsequently, the samples were allowed to stand for 30 min at room temperature and the absorbance of the sample at 515 nm was measured. Trolox (Fisher Scientific, Walthman, MA, USA) was used as an external standard. The antioxidant capacity was determined through the difference in absorbance at time 0 and time 30 min and was expressed as μmol eq-Trolox$\cdot g^{-1}$ biomass.

2.6. (Poly)phenols and flavonoids determination

Total (poly)phenols were determined using the procedure described by Georgé et al. (2005). Accordingly, (poly)phenolic compounds were oxidized by the Folin–Ciocalteu reagent (Panreac, Barcelona, Spain). The reaction was carried out in an alkaline medium; for this, sodium carbonate was added to the samples and the absorbance was measured at 725 nm by UV–vis spectrophotometry model Evolution 201 (Thermo Fisher Scientific, Walthman, MA, USA). Total (poly)phenolic content was determined as mg-eq gallic acid·L^{-1} using the equation obtained from a standard gallic acid 1-hydrate (Panreac, Barcelona, Spain) calibration curve.

Flavonoid compounds were determined by a spectrophotometric method described by Liu et al. (2002). The absorbance value was measured at 510 nm using UV–vis spectrophotometry model Evolution 201 (Thermo Fisher Scientific, Walthman, MA, USA). Total content of flavonoids was calculated as mg-eq catechin·L^{-1} using the equation obtained from a standard catechin, (+)-catechin hydrate (Sigma Aldrich, Darmstadt, Germany), calibration curve.

2.7. Anti-inflammatory activity in THP-1 differentiated macrophages (THP-1 Mac)

THP-1 cell line, kindly given by Dr. Nuno Santos (CBME, University of Algarve, Faro), was cultured according to ATCC instructions in RPMI Growth Medium (RPMI 1640 with L-Glutamine (Gibco, Waltham, MA, USA), 10 % heat-inactivated Fetal Bovine Serum (FBS, Biowest, Nuaillé, France), 1 % Pen-Strep (P/S, Gibco), at 37 °C in a humidified atmosphere containing 5 % CO_2. THP-1 macrophage differentiation (THP-1 Mac) was achieved by culturing cells in 25 ng/mL of phorbol 12-myristate 13-acetate (PMA) (Sigma-Aldrich, St. Louis, MO, USA) in complete RPMI for 48h.

THP-1 Mac cells viability exposed during 48h to 10 mg/mL of *C. onubensis* methanolic extracts from Fe (III)-added cultures, was determined using the CellTiter 96 cell proliferation assay (Promega, Madison, WI, USA), following manufacturer's instructions. The anti-inflammatory potential of *C. onubensis* methanolic extracts from Fe (III)-added cultures, was evaluated in 1×10^6 THP1-Mac cells, plated in 96 well plates with 200 μL media, by pre-treatment of the cells with 10 mg/mL of each extract during 24 h, followed by inflammation stimulation with LPS (100 ng/mL) (Sigma-Aldrich) for additional 24 h. THP-1 Mac cells without any treatment were used as non-inflamed control cells, and THP-1 Mac cells only exposed to LPS were used as positive inflammatory control. Cell culture media were collected for quantification of TNFα levels by ELISA (R&D Systems, Minneapolis, MN, USA) according to manufacturer's recommendations. The % of TNFα inhibition was calculated using the following formula: ([TNFα in LPS condition] − [TNFα in testing extracts + LPS condition]/[TNFα in LPS condition]) x 100.

2.8. Statistics

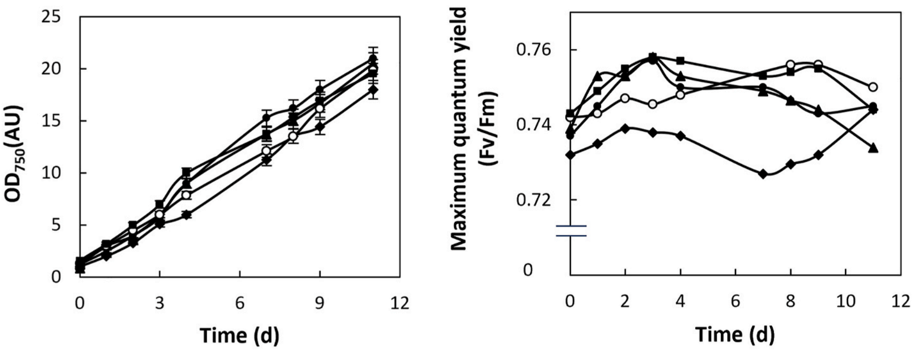
Unless otherwise stated, all data are presented as the mean of three replicates and the standard deviations are shown in the corresponding figures and tables. The data from the different treatments were evaluated by constructing univariate statistical models and performing the analysis of variance (ANOVA) (confidence of 95 % was considered for determining statistical significance). Tukey's test was also applied to identify significant differences between all the treatments studied, without reference to a specific control. Detailed results are presented in Tables B.1-B.9 attached to this article. All analyses were performed using the Minitab 22 software.

3. Results

3.1. Effect of Fe (III) concentration on the growth and viability of C. onubensis

C. onubensis is an acidotolerant microalga (Garbayo et al., 2012), isolated from the Tinto River in the province of Huelva, Spain. The Fe (III) concentration in the Tinto River can reach up to 30 g L^{-1} (Fernández-Remolar et al., 2004). In its natural habitat, *C. onubensis* can tolerate high Fe (III) concentrations; however, such levels are far from optimal for algal growth and can become toxic. Indeed, Fe (III) concentrations above 10 mM significantly reduced growth rates (data not shown, laboratory experiments). The experiments presented in this manuscript aimed to induce microalgal responses to oxidative stress produced by sublethal Fe (III) concentrations, without negatively affecting growth while thus allowing the cells to accumulate antioxidant molecules to counteract Fe (III)-induced ROS species. First, the growth and photosynthetic viability of the microalga *C. onubensis* were evaluated as a function of Fe (III) concentration and the duration for which the culture was exposed to Fe (III), within a range of sublethal concentrations of Fe (III) (Fig. 2). A significant time-dependent increase in the microalgal cell density (measured as OD at 750 nm) was found in Fe (III)-added cultures with respect to control cells (Fig. 2a). The maximum cell density was reached in the culture grown at the highest Fe (III) concentration tested (2 mM). The photosynthetic efficiency of photosystem II (quantum yield) in the microalgal cultures supplemented with Fe (III) is shown in Fig. 2b. This data provided information on the efficiency of the use of light by microalgal cells. A significant increase in quantum yield was recorded under increased Fe (III) concentrations; the highest quantum yield was recorded on day 3 of cultivation, which was approximately 3 % higher than the obtained in control culture cells.

Ultrastructural modifications in cells were analyzed through transmission electron microscopy (TEM) using cell samples obtained from cultures supplemented with Fe (III) (Fig. 3). The control culture (Fig. 3a)



(a) (b)

Fig. 2. Time-dependent evolution of **(a)** growth, measured as OD_{750}, and (**b**) the maximum photosynthetic efficiency of photosystem II (quantum yield, Fv/Fm) as a function of the concentration of Fe (III): 0 (-◆-), 0.25 (-●-), 0.5 (-▲-), 1 (-○-), and 2 (-■-) mM in cultures of the acidotolerant microalga *C. onubensis*. Data are the mean ± SD of three replicates.

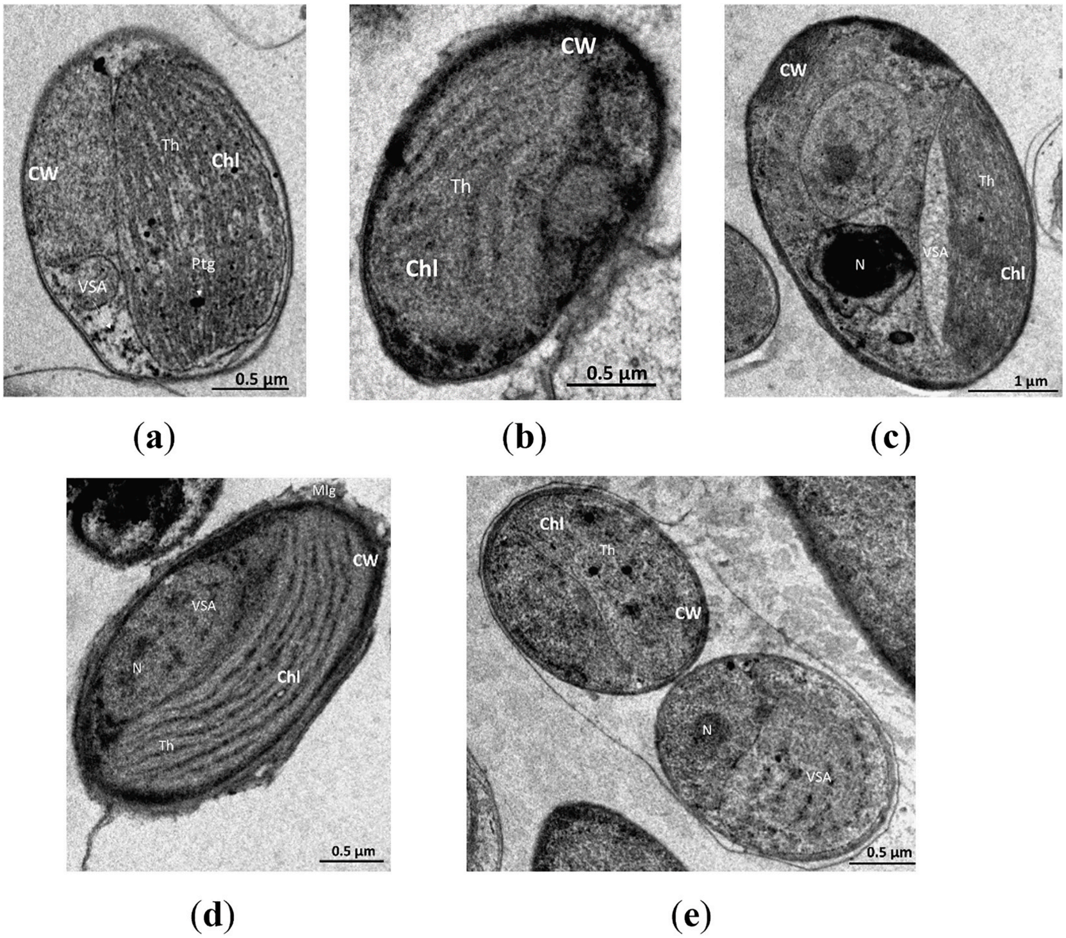


Fig. 3. Effect of Fe (III) concentration on the ultrastructure of the microalga *C. onubensis* through transmission electron microscopy images. (**a**) Control culture without Fe (III) supplement; cultures supplied with Fe (III) at concentrations of (**b**) 0.25 mM, (**c**) 0.5 mM, (**d**) 1 mM, and (**e**) 2 mM. Abbreviations: Chl: chloroplasts, Th: thylakoids, CW: cell wall, VSA: vesicular activity, N: nucleus, and Mlg: mucilage.

showed dark granules, which represent the accumulation or secretion of molecules that are typical of cellular activity in metabolically active cells, unlike the grayish and whitish granules that appear in stressed cells. The figure shows a unicellular microalga with a well-defined, regular, and spherical membrane, stable cytoplasm, and a typical large thylakoid-dense chloroplast that occupies approximately half of the total cell volume.

However, as Fe (III) concentration increased, the cell wall became thicker, which increased the rigidity of the cell membrane. Also, the increase in Fe (III) concentration led to the disorganization of cellular structures, as revealed by the widely separated (Fig. 3d) and unstructured thylakoid membranes (Fig. 3e). Additionally, black spots appeared in stressed cells and between thylakoid membranes, as shown in Fig. 3d. We also observed mucilage in Fig. 3e, which appeared as a viscous membrane-like structure around the algal membrane. Two cells undergoing cell division exhibiting the phenotype of cells under stress are illustrated in Fig. 3e.

3.2. Effect of Fe (III) concentration on non-enzymatic antioxidant response and anti-inflammatory activity of C. onubensis

The antioxidant capacity of the microalgal extracts was analyzed and correlated with its anti-inflammatory activity as a function of Fe (III) concentration. The anti-inflammatory activity was evaluated in THP-1 Mac cells and expressed as the percentage of TNFα inhibition, which reflected the capacity of each extract to decrease the accumulation of TNFα in LPS-treated THP-1 Mac cell culture media (Fig. 4). The cytotoxic effects of *C. onubensis* methanolic extracts obtained from Fe (III)-added cultures were tested; none of the tested extracts altered the viability of THP-1 Mac cells (Fig. A.1). In the early growth stage (day 4; Fig. 4), the antioxidant capacity decreased significantly and unexpectedly as Fe (III) concentration increased; the antioxidant capacity was the lowest for the 2 mM Fe (III) culture. Similarly, for the late cultivation stages (day 11), the antioxidant capacity of Fe (III)-added cultures was slightly lower than that of the control culture, irrespective of the concentration of Fe (III) present. Contrary to the pattern recorded in antioxidant capacity, a significant increase in anti-inflammatory activity was found, as determined by higher TNFα inhibition, in Fe (III)-added culture extracts compared to the control culture extracts and irrespective of the growth stage. The strongest inhibition of TNFα of, about, 50 % was found in the early growth cultivation period (day 4) and at the lower Fe (III) concentration added (0.25 and 0.5 mM), while control culture extracts on same cultivation period exerted 10 % inhibition of TNFα.

The variation in antioxidant capacity and anti-inflammatory activity as a function of Fe (III) concentration showed that the target antioxidant molecules of *C. onubensis* cultures were involved in ROS neutralization reactions, this is further analyzed in Fig. 5. The antioxidant capacity (Fig. 5a) decreased irrespective of the concentration of the target antioxidant molecules. The highest antioxidant capacity was found in cultures that were not exposed to Fe (III). A different pattern was found for anti-inflammatory activity (Fig. 5b), in which the highest values corresponded to the highest concentrations of the antioxidant molecules.

The overall contribution of the target antioxidant molecules to cope

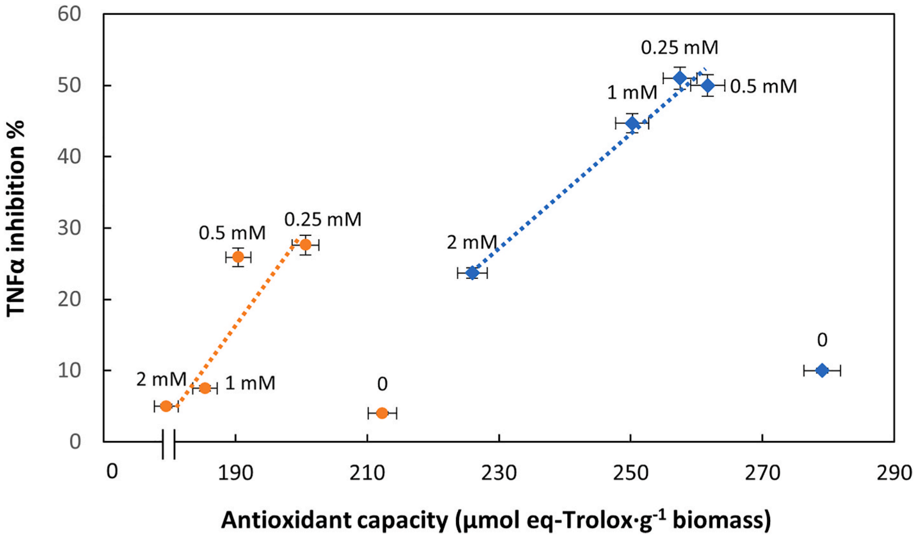
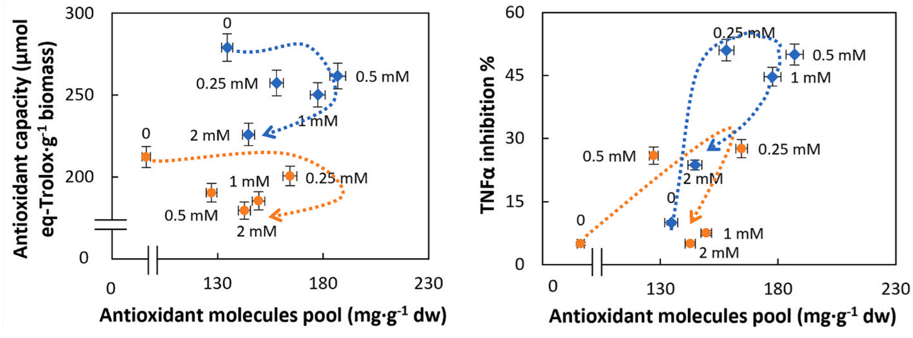


Fig. 4. Antioxidant capacity and anti-inflammatory activity exhibited by *C. onubensis* culture extracts as a function of Fe (III) concentration in the culture medium, after 4 days (blue diamond) and 11 days (orange circle) of exposure to Fe (III). Antioxidant capacity was measured as the capacity of the extracts to react with the DPPH radical. Anti-inflammatory activity was measured as the inhibition percentage of TNFα released by THP-1 MoM cells exposed to *C. onubensis* extracts. Further details are presented in the Materials and Methods section. Data are the mean ± SD of three replicates. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)



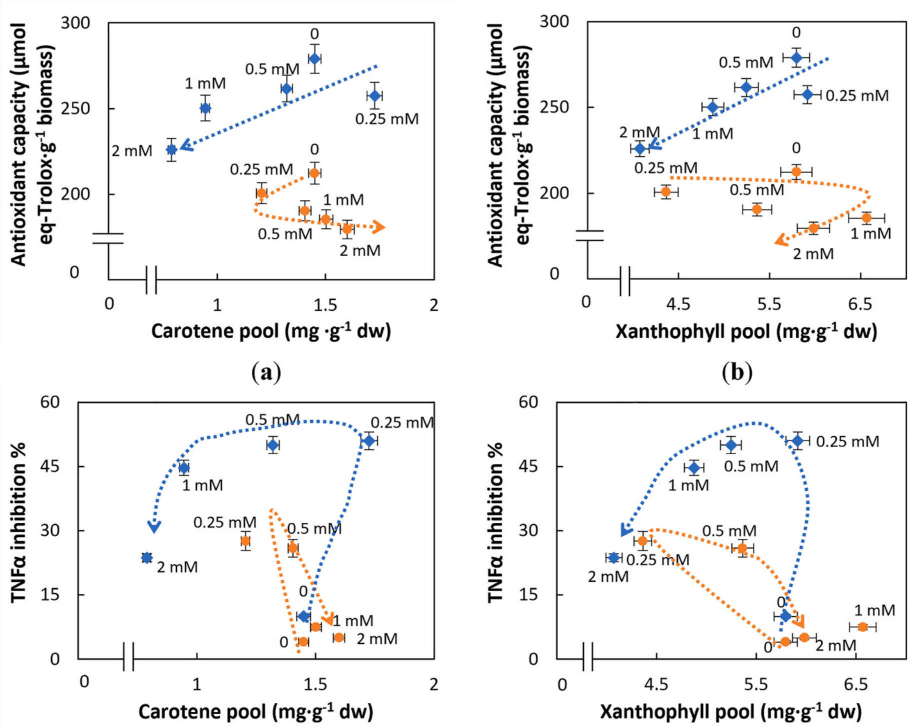
(**a**) (**b**)

Fig. 5. The analysis of the correlation between target antioxidant molecules (carotenes, xanthophylls, (poly)phenols, and flavonoids) and (**a**) the antioxidant capacity or (**b**) the anti-inflammatory activity in *C. onubensis* extracts, as a function of Fe (III) concentration in the culture medium of the microalga after 4 days (blue diamond) and 11 days (orange circle) of exposure to Fe (III). Data are the mean ± SD of three replicates. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

with Fe (III)-induced stress was evaluated by plotting the concentration data of each target molecule in the extract versus the total antioxidant capacity of the extracts (Fig. 6a, b, 7a, and 7b). Similar graphs were plotted to assess the contribution of each target molecule to the anti-inflammatory effects of *C. onubensis* cell extracts on the THP-1 Mac cells (Fig. 6c, d, 7c, and 7d). A similar pattern was obtained by plotting either (poly)phenols, carotenes, or xanthophylls against the total antioxidant capacity and anti-inflammatory activity of the extracts, i.e., only short-term exposure (day 4) at low Fe (III) levels and long-term exposure (day 11) at high Fe (III) levels led to similar or significantly higher intracellular levels of carotene, xanthophylls, and polyphenols than in control cultures. Additionally, a similar pattern was recorded for the antioxidant capacity of the microalga, i.e., an increase in Fe (III) concentration and exposure time significantly decreased the content of carotenes, xanthophylls, and polyphenols, and therefore, decreased the antioxidant capacity (Fig. 6a and b).

We found a different pattern for the anti-inflammatory effect on THP-1 Mac cells (Fig. 6c and d). In this case, the highest anti-inflammatory activities (blue line) were recorded in the microalgal cultures that were exposed to low Fe (III) levels (0.25 and 0.5 mM) for a short duration. However, the differences in the intracellular content of carotenes, xanthophylls, and (poly)phenols between the above-mentioned conditions and the control culture were minimal.

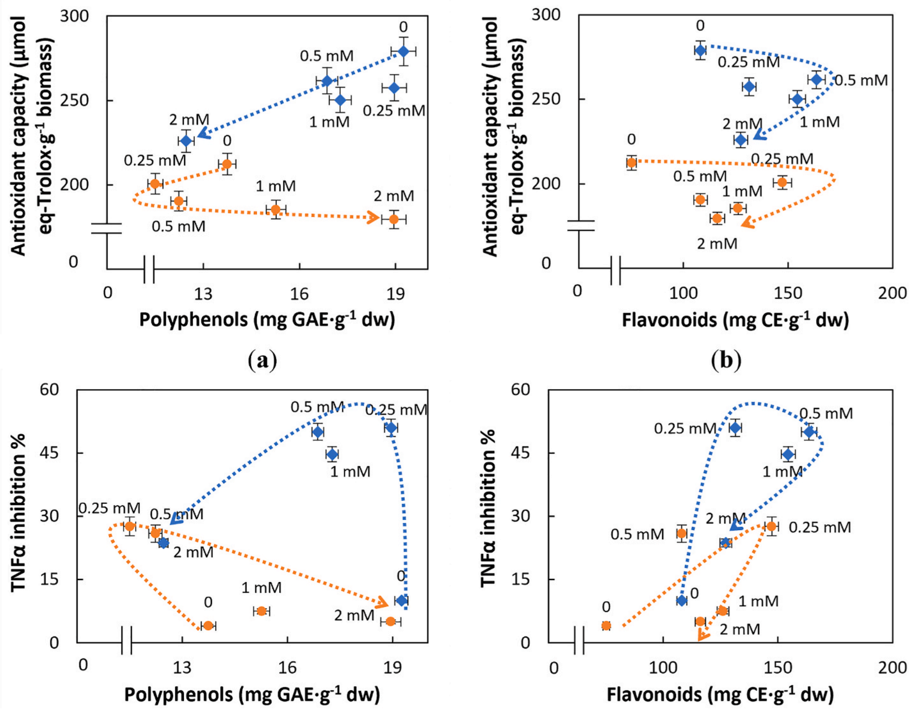
In contrast, the flavonoid content (Fig. 7b) increased significantly in *C. onubensis* cells as Fe (III) concentration increased (up to 0.5–1 mM); however, the antioxidant capacity in all sample extracts from Fe (III)-added cultures was lower than that in sample extracts from the control culture. The anti-inflammatory activity was also represented versus the flavonoid content (Fig. 7d). The highest concentration of flavonoids was found in cultures grown under low Fe (III) concentrations in the early growth stage. Under these conditions, *C. onubensis* extracts also showed the highest anti-inflammatory activity, which suggested that the flavonoids of *C. onubensis* play a more important role in inhibiting inflammation, compared to the contribution of carotenoids and phenolic compounds.



(a) (b)

(c) (d)

Fig. 6. Analysis of the correlations of carotene (a, c) and xanthophyll (b, d) with the antioxidant capacity (a, b) and anti-inflammatory activity (c, d) as a function of Fe (III) concentration in the culture medium of *C. onubensis* after 4 days (blue diamond) and 11 days (orange circle) of exposure to Fe (III). Data are the mean ± SD of three replicates. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)



(a) (b)

(c) (d)

Fig. 7. Analysis of the correlations of (poly)phenols (a, c) and flavonoids (b, d) with the antioxidant capacity (a, b) and anti-inflammatory activity (c, d) as a function of Fe (III) concentration in the culture medium of *C. onubensis* after 4 days (blue diamond) and 11 days (orange circle) of exposure to Fe (III). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

4. Discussion

As described in the Introduction section, an increase in the antioxidant capacity of the microalgal extracts can increase anti-inflammatory activity. This would enable planning strategies to develop ways to produce microalgal biomass with high anti-inflammatory activity by inducing microalgal cells to increase their antioxidant capacity. Thus, we studied the anti-inflammatory activity of *C. onubensis* extracts as a function of the concentration of Fe (III) added to the cultures. We also evaluated the correlations between the anti-inflammatory activity and the antioxidant capacity of the extracts. The results were expected to reveal the contribution of the antioxidant compounds of the acidotolerant microalga *C. onubensis* to the reduction of lipopolysaccharide (LPS)-induced inflammatory response in THP-1 differentiated macrophages (THP-1 Mac) human cells. The anti-inflammatory effects of *C. onubensis* extracts were analyzed and discussed as a function of key target antioxidant molecules that accumulated in the microalgal cells and were present in the extracts tested (carotenes, xanthophylls, and total (poly)phenolic compounds).

4.1. Effect of Fe (III) concentration on the growth and viability of C. onubensis

The results suggested that Fe (III) concentration of up to 2 mM can slightly increase the growth of *C. onubensis* (Fig. 2a). Photosynthetic efficiency is a relevant parameter for studying the toxic effects of Fe (III) on microalgae (Lysenko et al., 2020). Photosynthetic efficiency was determined by evaluating the quantum yield (Qy), which provides information on the efficiency of the photochemical events in photosystem II (PSII). The Qy values recorded were within 0.6–0.7 (Fig. 2b), which is typical for healthy cells (Maxwell and Johnson, 2000). This finding indicated that the Fe (III) concentration range selected for this study was below the lethal concentration range for the microalga. Additionally, the Fe (III) concentrations selected increased the photosynthetic viability of *C. onubensis*. This finding agreed with the notion that Fe plays a key role in the cellular biochemistry of microalgae due to its redox properties and its participation in fundamental processes, such as photosynthesis, respiration, nitrogen fixation, and DNA synthesis (Gao et al., 2022). Our results indicate that modulating Fe (III) levels in the culture medium can yield incubation conditions that are optimum for the growth and photosynthetic viability of *C. onubensis*.

Changes in the growth and photosynthetic viability of *C. onubensis* due to an increase in Fe (III) concentration can affect their ultrastructure at the cellular level, including modification of the number and appearance of lipid accumulating structures. These changes may also influence the anti-inflammatory activity exerted by the microalgal extracts on THP-1 Mac cells, as carotenoids and unsaturated fatty acids are antioxidant lipids that accumulate in microalgal cells. Such ultrastructural modifications in cells were analyzed through transmission electron microscopy (TEM) using cell samples obtained from cultures supplemented with Fe (III) (Fig. 3). Cell ultrastructure photographs showed that the membrane fluidity of Fe (III)-exposed cells decreased, as determined by an increase in the thickness of the cell wall (Fig. 3b–e). This might be a protective mechanism to prevent the entry of Fe (III) in the cell, thus minimizing oxidative stress (Ejaz et al., 2023). An increase in vesicular activity due to an increase in Fe (III) concentration indicated the accumulation of molecules inside the cells. Vesicular activity is not only related to the abundance of vesicles but also to the dilated cisternae of the smooth endoplasmic reticulum, which accumulate compounds synthesized *de novo* (Wan and Zhang, 2012). The smooth endoplasmic reticulum does not have associated ribosomes, and it is involved in key cell functions, such as lipid synthesis and detoxification (Miller and Zachary, 2017), which might include the toxic effects of Fe (III). Some studies have reported the presence of small black globules close to thylakoids in chloroplasts from plants grown in the presence of different stressors (Giacomelli et al., 2006). In this study, black spots occurred between the chloroplasts of Fe (III)-stressed cells, which might be related to the accumulation of lipidic compounds. Some researchers have found tocopherols and other lipid isoprenoid-related metabolites in the plastids of stressed cells (Laizet et al., 2004).

The ultrastructure of cells from cultures exposed to the highest Fe (III) concentration tested showed a more intense vesicular activity (Fig. 3d and e). Additionally, nucleolar activity was found in the nucleus, which implied that the cells synthesized molecules that protected these cells against the originated stress (Wan and Zhang, 2012; Miller and Zachary, 2017). The appearance of mucilage in stressed cells indicates that the cells are either about to divide or are under stress (Holzinger and Pichrtová, 2016). Related to the latter, Fig. 3e, shows two cells undergoing cell division that exhibit the phenotype of a stressed cell. Consequently, analysis of the microalgal cell ultrastructure in Fe (III)-exposed cultures showed that low-intensity oxidative stress might promote cell division under the tested conditions; this finding was also confirmed by an increase in the optical density of Fe (III)-stressed cultures (Fig. 2).

Therefore, the results suggested that Fe (III) was involved in the generation of oxidative stress in cells. This led to an increase in the antioxidant response of the microalga. Thus, an increase in the accumulation of antioxidant molecules, previously shown to modulate inflammatory reactions in cell lines (*in vitro*) (Araújo et al., 2020), was also expected.

4.2. Effect of Fe (III) concentration on non-enzymatic antioxidant response and anti-inflammatory activity displayed by C. onubensis

We assessed the non-enzymatic antioxidant responses of *C. onubensis* to cope with the oxidative stress generated in cultures to which Fe (III) was added. Although Fe plays a key role in living beings, it can be highly toxic at high concentrations as it can undergo redox reactions with various molecules in the cell and can also produce reactive oxygen species (ROS) through the Fenton reaction. Thus, adding Fe (III) can directly lead to oxidative stress, which can activate the response of the microalgal antioxidant system to balance ROS production. However, the results showed a decrease in the level of antioxidant capacity of Fe (III)-exposed microalgal cultures, which was inversely correlated with the anti-inflammatory effects of the microalgal extracts in THP-1 Mac cells.

The anti-inflammatory effects exerted by the control culture cell extracts of the highly acidic environment microalga *C. onubensis* on THP-1 Mac cells were not noticeable, with a maximum value of 10 % TNFα inhibition, which was similar to the values obtained for other microalgal species tested under standard conditions, despite the scarcity of studies on this topic (Gallego et al., 2022). However, cell extracts from *C. onubensis* cultures exposed to Fe (III) exerted anti-inflammatory effects that were quantitatively similar to the effects reported in a few microalgal species. Although the experimental approach followed was different from that described in our study, Gallego et al. (2022) investigated the an*ti-inflammatory effects of Haematococcus pluvialis, Isochrysis lutea, Nannochloropsis oceanica, and Porphyridium cruentum extracts on LPS-stimulated THP-1 cells, measured as* the level of secretion of *TNFα. The researchers found that only N. oceanica* extracts induced a significant reduction in TNFα secretion levels compared to that recorded in control cultures (LPS-induced TNFα secretion), with up to 48 % inhibition of secretion. Similarly, Tzachor et al. (2021) studied the anti-inflammatory effects of *Spirulina* extracts obtained from the biomass produced under different conditions of light quality and irradiance; they found a maximum reduction of 40 % in TNFα secretion levels in LPS-stimulated human monocytes. *Spirulina* is considered to be a “super-food”, particularly due to its beneficial effects on human health, including antioxidant, anti-inflammatory, and immunomodulatory effects. However, systematic studies on its anti-inflammatory effects on humans, indicated by a reduction in TNFα, IL-6, and CRP levels, are lacking (Calella et al., 2022).

The anti-inflammatory effects on THP-1 Mac cells by extracts of

C. onubensis obtained by exposing cultures to Fe (III) showed a 50 % reduction in TNFα secretion. This finding suggested that acidophilic microalgae might be excellent natural sources of anti-inflammatory compounds, partly because of their ability to adapt to oxidative stress due to the accumulation of a large number of anti-inflammatory molecules. Additionally, the anti-inflammatory capacity of the microalgal extracts could be improved by modulating oxidative stress during biomass production.

Assessing the content of the free radical DPPH, which is widely used to evaluate the ability of compounds to act as free-radical scavengers and hydrogen suppliers (Ávila-Román et al., 2023), is a rapid, simple, and inexpensive method for testing antioxidant capabilities. The antioxidant capacity of microalgal extracts obtained from Fe (III)-exposed cultures was lower than the antioxidant capacity of control cultures (Fig. 4). However, those extracts showed a higher capacity to mitigate pro-inflammatory reactions, measured as the TNFα content released in the THP-1 Mac cell culture medium. These results were similar to those obtained by Barone et al. (2021), who showed that the antioxidant response of the tested microalgal species decreased after exposure to 0.5 mM H_2O_2. This decrease in the antioxidant capacity in the referred study indicated the role of the activity of the enzymatic antioxidant system in the neutralization of ROS. Similarly, results obtained by our group under similar conditions showed an increase in SOD activity in *C. onubensis* extracts obtained from cultures exposed to different Fe (III) concentrations, with activity values approximately 60 % higher than those recorded in control cultures (Robles et al., 2023). However, considering that Fe (III) induces an enzyme-based antioxidant response in *C. onubensis,* in this study, we aimed to more comprehensively understand the non-enzymatic antioxidant system that contributes to the anti-inflammatory activity of the microalgal extracts. For this, we analyzed the variation in the content of the target antioxidant molecules in *C. onubensis* cultures as a function of the added Fe (III) and correlated it with the antioxidant capacity and anti-inflammatory activity exerted by the microalgal extracts (Figs. 5–7).

As shown in Fig. 5, the findings suggested that the microalgal extracts obtained from Fe (III)-exposed cultures contain molecules that can mitigate inflammatory responses, but these molecules may not exert antioxidant effects, as previously described, i.e., molecules involved in the antioxidant response of *C. onubensis* to cope with Fe (III) stress-derived ROS might not act against DPPH, and thus, they may not reflect the antioxidant capacity of the microalga. This might be the case for (poly)phenols and carotenoids (terpenoids), which are strong antioxidant scavengers of ROS (Montero-Lobato et al., 2018), whose content in *C. onubensis* extracts from Fe (III)-stressed culture cells generally decreased, compared to cell extracts from control cultures (Figs. 6 and 7). Thus, we hypothesized that the actual content and antioxidant capacity of (poly)phenols and carotenoids might be underestimated.

In relation with the later, the OH groups in the phenol structure, which must react with ROS to exhibit ROS-scavenging activity, are also required to react with DPPH. Gulcin and Alwasel (2023) studied the interaction between the DPPH radical and the (poly)phenolic group in usnic acid, which has two -OH units. They inferred that the withdrawal of H atoms from the (poly)phenolic -OH groups by a reactive radical species is a thermodynamically favorable chemical transformation, which can occur under high oxidative pressure. This is also coherent with the lower antioxidant capacity obtained in Fe (III)-added cultures of microalgal extracts and the proposed structural changes in (poly) phenols. Therefore, part of the (poly)phenolic content in the microalgal extracts, especially in extracts from Fe (III)-stressed cultures, would not be acting in reducing chemically the DPPH molecule; thus, resulting in a lower antioxidant capacity, as obtained for extracts with high Fe (III) levels.

However, (poly)phenols and carotenoids (terpenoids) can decrease the inflammatory response measured in terms of LPS-induced secretion of TNFα by THP-1 macrophages (Araújo et al., 2020; Gallego et al., 2022). Linnewiel-Hermoni et al. (2014) reported that carotenoid-oxidized derivatives, but not the intact carotenoid structures, can mediate the inhibition of NFkB. Moreover, derivatives that contain α,β-unsaturated carbonyl groups interact with higher affinity with the active site of NFkB following the Michael addition reaction (Linnewiel-Hermoni et al., 2014). This concept provides insights into new approaches to studying the anti-inflammatory potential capacity of natural compounds and also explains our findings of considerably lower antioxidant capacity and higher anti-inflammatory activity in extracts from microalgal cell cultures exposed to high levels of Fe (III)-induced oxidative stress.

Consequently, we hypothesized that structural changes in target antioxidant molecules after reacting with ROS might explain the observed reduction in antioxidant capacity and increase in anti-inflammatory activity. This hypothesis was supported by the results presented in Figs. 6 and 7 and schematically represented in Fig. 8, ie., the data obtained allowed us to suggest the existence of a direct relationship between these structurally oxidized compounds and the increase in anti-inflammatory activity. This hypothesis matched the results reported by Havaux (2014); ROS, especially singlet oxygen, produced in chloroplasts under stress, can oxidize β–carotene, leading to the production of various oxidized derivatives, such as β–cyclocitral or β–ionone, containing α,β-unsaturated carbonyl groups. Similarly, after

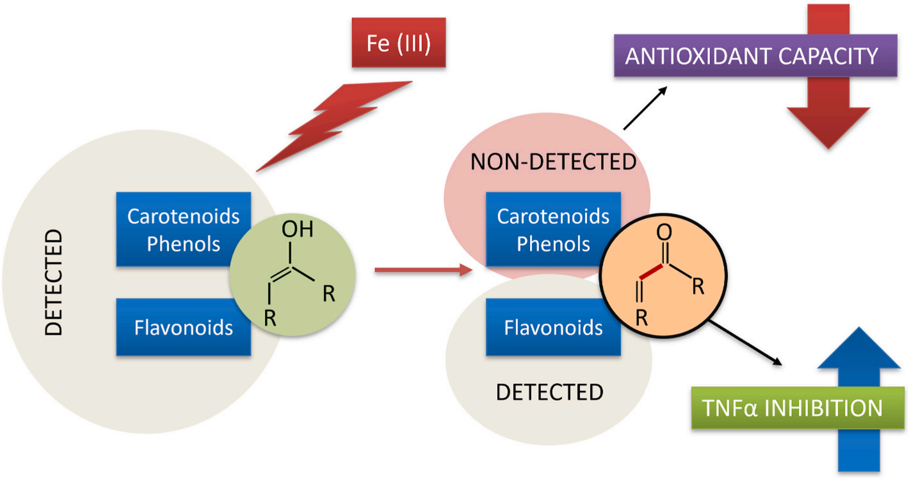


Fig. 8. A schematic representation of the mechanism underlying the oxidative Fe (III) stress-derived structural changes in antioxidant molecules produced by *C. onubensis*, and illustration of the subsequent effect on the antioxidant capacity and anti-inflammatory activity exerted by its extracts.

reacting with ROS, (poly)phenolic compounds and xanthophylls may lose their hydroxyl groups to form α,β-unsaturated carbonyl groups (Hunyadi, 2019; Rajashekar, 2023). These structural changes can cause the actual contents of the carotenoids identified by HPLC to be underestimated (Fig. 6), based on the changes in polarity. As (poly)phenolic compounds are determined spectrophotometrically based on the reaction of their hydroxyl groups with the Folin-Ciocolteu reagent (Pérez et al., 2023), fewer hydroxyl groups in (poly)phenolic compounds might be available for that reaction, which might result in lower levels of detection.

Additionally, these proposed structural changes might favor the (bio)synthesis of derived molecules containing α,β-unsaturated carbonyl groups; the active domain of NFkB may have a higher affinity for these groups, as discussed above. Thus, the expression of TNFα might be inhibited, resulting in a higher anti-inflammatory activity of the extracts.

In contrast to the pattern obtained with carotenes, xanthophylls, and (poly)phenols, the measurement procedure of flavonoids might not be affected by reaction with ROS, as a direct correlation was found between the flavonoid content and anti-inflammatory activity (Fig. 7d). This occurred probably because the determination of flavonoids is mediated by the oxidation of (poly)phenolic ring-hydroxyl groups to the carbonyl groups, which are also involved in the formation of Al (III)-flavonoid chelates, detected by spectrophotometry (Shraim et al., 2021). The transformation of hydroxyl groups into carbonyl groups in flavonoids by reacting with ROS does not hinder their detection. This fact explained why a significant increase in flavonoid content was recorded under moderate levels of Fe (III)-induced oxidative stress in *C. onubensis* cultures.

To summarize, the information obtained in this study can improve our understanding of the role of the target antioxidant molecules analyzed (carotenes, xanthophylls, (poly)phenols, and flavonoids) in neutralizing ROS to alleviate the oxidative stress generated by Fe (III) in cultures of the acidotolerant microalga *C. onubensis*. We also found a relationship between an increase in the oxidative status of the microalga and an increase in the capacity of the microalgal extracts to mitigate the inflammatory response of THP-1 Mac cells. Structural changes have been proposed in xanthophylls, (poly)phenols, and flavonoids when reacting with ROS, consisting of oxidation of their hydroxyl groups to α,β-unsaturated carbonyl groups forming their respective derivative molecules. In the case of β–carotenes, these structural changes could be caused by bond breakage, leading to the formation of some oxidized products, such as β–cyclocitral or β–ionone, containing α,β-unsaturated carbonyl groups. These molecular changes could accordingly explain the lower detection of these molecules based on their chemical interference with the analytical determination procedure. Additionally, the suggested loss of hydroxyl groups could explain the decrease in their reactivity with the DPPH radical, which decreased the antioxidant capacity of the extracts. Flavonoids are an exception to this behavior; the possible oxidation of their hydroxyl groups seems to not interfere with their quantification procedure. However, the proposed increase in α,β-unsaturated carbonyl groups of target antioxidant molecules due to an increase in the Fe (III)-mediated oxidative status of the microalgal cells is suggested to promote a stronger interaction with the active domain of NFkB; thus, inhibiting the expression of the inflammatory cytokine TNFα. In line with the results and discussion of this study, the algal meroditerpenoid amentadione, an α,β-unsaturated carbonyl terpenoid (carotenoid derivative), was found by some researchers to inhibit NFκB signaling pathways. Their findings strongly suggest that amentadione is a novel anti-inflammatory factor with high therapeutic potential (Araújo et al., 2020). The presence of the α,β-unsaturated carbonyl structure in amentadione strongly suggests that this group is a key chemically active moiety, which confers anti-inflammatory activity to carotenoids and phenolic compound-oxidized derivatives.

Therefore, Fe (III)-mediated induction of oxidative stress can be used to produce cell extracts with high capacity to reduce inflammatory responses by increasing the synthesis of antioxidants, such as terpenoids, (poly)phenolic compounds, and flavonoids. Although our findings were limited to the action of microalgae in a highly acidic environment, Fe (III)-based stress might have a broader application in other microalgal species.

Author contributions

Conceptualization, M.R., C.S.B.V., D.C.S and C.V.; methodology, M.R., C.S.B.V., R.T. and I.G.; investigation, M.R., C.S.B.V. and R.T.; writing—original draft preparation, M.R., C.S.B.V., D.C.S, J.M.V. and C.V.; writing—review and editing, all authors; supervision, D.C.S. and C.V.; project administration, C.S.B.V., D.C.S., I.G. and C.V.; funding acquisition, C.S.B.V., D.C.S. ad C.V. All authors have read and agreed to the published version of the manuscript.

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Declaration of competing interest

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

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.plaphy.2025.109815.

Data availability

Data will be made available on request.

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