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A comparative study of metabolites profiles, anti-inflammatory and antioxidant activity of methanolic extracts from three *Arthrospira* strains in RAW 264.7 macrophages☆

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ABSTRACT

Arthrospira (spirulina), is a cyanobacterium endowed with antioxidant and anti-inflammatory activity, but strain-dependent production of bioactive compounds may impact these properties. In LPS-stimulated RAW 264.7 macrophages, we compared the antioxidant and anti-inflammatory activity of methanolic extracts from two *A. platensis* (F&M-C256 and F&M-C260), and an *A. maxima* (F&M-C258) strains. F&M-C260 (5–25 μg/mL) concentration dependently inhibited LPS-induced NO release ($p < 0.01$) while F&M-C256 was effective only at the highest concentration ($p < 0.01$) and F&M-C258 had no activity. Both *A. platensis* strains significantly suppressed iNOS and COX-2 expression and PGE_2 production, while *A. maxima* lacked effects. Only F&M-C260 up-regulated miR-223 expression ($p < 0.001$). *A. maxima* showed the highest inhibition of NLRP3 and IL1-β expression ($p < 0.001$) and IL1-β secretion ($p < 0.01$). All extracts displayed direct antioxidant activity and counteracted LPS-induced SOD2 overexpression without affecting HO-1 expression. Metabolites profiles, explored by HPLC-UV and HPLC-HRMS, revealed that F&M-C260 and F&M-C256 were the richest in β-cryptoxanthin and monogalactosyl monoglycerides respectively, with minor differences in zeaxanthin and β-carotene content.

These results suggest that the antioxidant activity of the extracts was due to a direct reducing effect rather than to the transcriptional activation of SOD2 and HO-1 and that their differential and multitarget anti-inflammatory effects were correlated to differences in metabolites profiles.

1. Introduction

Temporally restricted inflammatory response is an essential protective measure against invading pathogens and damaged cells, however, the perpetuation of a state of low-grade, chronic inflammation, can increase the risk for various non-communicable diseases such as cancer, metabolic, cardiovascular, autoimmune, and neurodegenerative diseases [1]. Oxidative stress, caused by an imbalance between the production and accumulation of reactive oxygen and nitrogen species and antioxidant defense mechanisms, can contribute to the development and progression of inflammation by activating proinflammatory pathways and the release of multiple pro-inflammatory mediators [2]. Furthermore, there is an extensive crosstalk between transcriptional pathways during oxidative stress and inflammation as exemplified by

☆ This research article is in memory of Prof. M. R. Tredici who passed away while this paper was being prepared. Prof. Tredici was an enlighten scientist and a benchmark for all of us with his dedication, knowledge, and research in the field of microalgae biotechnologies and their applications.

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[1] Mario R. Tredici passed away on June 17, 2022.

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the negative regulation exerted by the redox- sensitive transcription factor nuclear factor erythroid 2-related factor 2 (Nrf2), on nuclear factor-kappa B (NF-κB) and on the nucleotide-binding domain-like receptor protein 3 (NLRP3) inflammasome signaling [3,4]. By regulating the expression of several antioxidant and cytoprotective enzymes such as heme oxygenase 1 (HO-1) and super-oxide dismutase 2 (SOD2), Nrf2 is also a master regulator of endogenous antioxidant response and cellular defense [5]. Moreover, nonsteroidal anti-inflammatory drugs such as acetyl salicylic acid and celecoxib, activate Nrf2 and HO-1 expression further supporting the interplay between antioxidant and anti-inflammatory effects [6,7].

The cyanobacterial genus *Arthrospira*, commercially known as spirulina, has a long history of being used as a food source since it contains high amounts of macro and micronutrients such as carbohydrates, lipids, proteins, vitamins, and minerals [8]. Animal studies demonstrated that in addition to their nutritional value, *Arthrospira platensis* (*A. platensis*) and *Arthrospira maxima* (*A. maxima*), the best-known representatives of the genus, exert numerous health benefits including hypoglycemic, hypocholesterolemic and blood pressure reducing effects [9–13]. Some meta-analysis of clinical trials also showed that *Arthrospira* supplementation has a favorable impact on cardiovascular and metabolic markers by reducing lipid and glucose levels and blood pressure in patients with metabolic syndrome and diabetes [14–16]. As critical components of metabolic and cardiovascular diseases, the effects of *Arthrospira* supplementation on oxidative stress and inflammation were also investigated. A recent meta-analysis of controlled clinical studies indicated that the supplementation with *Arthrospira* of not specified species, significantly reduced proinflammatory interleukin (IL)-6 levels and lipoperoxidation in non-overweight individuals [17]. The treatment with *A. platensis* and *A. maxima* extracts or whole biomass also exerted anti-inflammatory and antioxidant effects in experimental models of arthritis, periodontitis, and inflammatory bowel diseases [18–26].

Several studies have attributed the anti-inflammatory effects of *Arthrospira* genus to its water-soluble pigments (phycobiliproteins) content and above all, phycocyanin, whereas the role of bioactive, non-water-soluble compounds including unsaturated fatty acids, carotenoids, phenolic compounds, and chlorophylls, is largely unexplored [9,27–29]. Strain-dependent production of vitamins, fatty acids, and polyphenols by *Arthrospira* genus, has been described [30]. Different content of phycobiliproteins, chlorophylls, polyphenols, and carotenoids were also reported in *A. platensis* strains from Algeria, Chad, and the USA [31].

Therefore, we hypothesized that species or strain dependent differences in the composition and abundance of non-water-soluble compounds can impact the anti-inflammatory and antioxidant activity of different *Arthrospira* strains and their potential application to treat inflammatory-based diseases.

To test this hypothesis, we compared the antioxidant and anti-inflammatory effects of methanolic extracts from three *Arthrospira* strains, *A. platensis* F&M-C256, *A. platensis* F&M-C260, and *A. maxima* F&M-C258, on LPS-induced inflammation in RAW 264.7 macrophages, the most applied cellular model for the screening of anti-inflammatory compounds when challenged with inflammatory stimuli such as lipopolysaccharide (LPS) [32,33]. Furthermore, the molecular pathways regulating the observed effects were investigated and correlated to metabolites profiles of each ex-tract.

2. Materials and methods

2.1. Microalgae cultivation and production

A. platensis F&M-C256, *A. platensis* F&M-C260 and *A. maxima* F&M-C258 belong to the Fotosintetica & Microbiologica (F&M) S.r.l. Culture Collection (Florence, Italy). The strains were cultivated in 1-L vertical glass tubes bubbled with an air: CO_2 (98:2, v:v) mixture under continuous light (100 μmol photons m^{-2} s^{-1}) in Zarrouk medium [34] (macroelements in g L^{-1}: NaCl 1, K_2HPO_4 0.5, $NaNO_3$ 2.5, $K2SO_4$ 1, $NaHCO_3$ 16.8; $MgSO_4$ $7H_2O$ 0.2, $CaCl_2$ $2H_2O$ 0.042; micronutrients in mg L^{-1}: H_3BO_3 2.86, $MnCl_2$ $4H_2O$ 1.81, $ZnSO_4$ $7H_2O$ 0.222, $CoCl_2$ $6H_2O$ 0.035, $CuSO_4$ $5H_2O$ 0.08, Na_2MoO_4 $2H_2O$ 0.23; iron solution in mg L^{-1}: Na_2EDTA 29.75, $FeSO_4$ $7H_2O$ 24.9). At the end of the batch, the cultures were harvested by centrifugation (Neya 8, Neya, Modena, Italy) at 2100 *g* for 15 min at room temperature (RT) and washed with 1 g L^{-1} NaCl solution to remove residual salts from the culture medium. The biomasses were then frozen, lyophilized, and powdered in a mortar.

2.2. Microalgal extract preparation

An aliquot of 250 mg of each lyophilized *Arthrospira* biomass was extracted in 30 mL of methanol, overnight, at RT. The mixture was then sonicated for 2 cycles of 3 min in ice, followed by a 3-min resting interval by using an ultrasonic homogenizer (Sonopuls HD 2070, Bandelin Electronic, Germany), set at 100 % power. The solvent was separated from the biomass by filtration on filter membrane. The residual biomass was extracted again with 15 mL of methanol at 37 °C for 4 h; then, the exhausted biomass was removed by filtration on filter membrane and the extract (30 + 15 = 45 mL) was evaporated under vacuum using the Concentrator 5301 (Eppendorf, Hamburg, Germany), at RT. The dry residue was solubilized in DMSO (Sigma Aldrich, Milan, Italy) [35]. For cells treatment, each methanolic extracts was diluted in complete cell culture medium to obtain the appropriate concentrations to be tested with a final concentration of DMSO <0.1 %.

2.3. HPLC-UV and HPLC-HRMS characterization of the microalgal extracts

One mL of methanolic extract was added with 1 mL of water and then extracted three times with 2 mL of hexane. After centrifugation in a Jouan BR4i centrifuge for 6 min at 4680*g*, hexane was transferred in a new vial and concentrated under a gentle stream of nitrogen, at RT and protected by light, to 1 mL final volume for the HPLC-UV analyses. These analyses were performed using a modular HPLC 1050 (Hewlett Packard Italy, Cernusco sul Naviglio, Milan, Italy), composed by a quaternary pump, an autosampler and a UV detector. The column was a YMC C30 (Waters Italy, Milan, Italy), using methanol:water:methyltbutyl ether (81:10:9, *v/v/v*) as eluent A and methyltbutylether:methanol (90:10, v/v) as eluent B. Linear gradient elution was done starting from 100 % A to 10 % A in 16 min and maintained for 5 min, then returning at initial condition and equilibrating for 8 min. The flow rate was 1 mL/min; volume injection was 20 μL. Data were recorded at 450 nm.

For HPLC-HRMS/MS analysis 1 mL of the methanolic extract was diluted with 1 mL of LC-MS grade water containing 0.1 % formic acid. The analyses were carried out using a HPLC Ultimate 3000 Dionex coupled to a LTQ Orbitrap mass spectrometer equipped with an electrospray (ESI) interface (Thermo Scientific, Bremen, Germany). The column was a Kinetex EVO C18, 5 μm, 100 × 2.1 mm (Phenomenex Italy, Anzola dell'Emilia, Bologna, Italy), operating at 0.27 mL/min and thermostated at 35 °C. LC-MS grade water and acetonitrile, both containing 0.1 % formic acid, were used as phase A and phase B, respectively. A linear gradient elution from 5 % B to 97 % B in 18.4 min was used, then maintained for 5.1 min and re-equilibrated at initial conditions for 7.5 min. Injection volume was 10 uL. Data were acquired in data dependent acquisition in positive ion mode. A high-resolution scan (60,000 resolving power at 400 *m/z*) from 200 to 1500 m/z in the orbitrap analyzer was combined with five MS/MS experiments in the ion trap analyzer selecting the five most intense precursor ions detected in the previous HRMS scan. After two consecutive MS/MS experiments, the MS pre-cursor ions were included in a dynamic exclusion list for 20 s. The LTQ Orbitrap was calibrated immediately before the analyses and two lock masses (*m/z* 214.08963 e 391.284287) were used for HRMS acquisition.

2.4. Total antioxidant capacity

Total antioxidant capacity in the methanolic extracts was determined by using the Folin-Ciocalteu reagent, as described by Everette et al. with few modifications [36]. Briefly, sample extracts (60 μL) and Folin-Ciocalteu's reagent (60 μL) were mixed and incubated at room temperature for 5 min. After incubation, 60 μL of Na_2CO_3 (10 % *w/v*) was added and the reaction mixture was further incubated for 60 min at room temperature in the dark. After incubation, the absorbance was measured at 760 nm. Gallic acid was used as standard and total antioxidant capacity was expressed as mg gallic acid equivalents (GAE)/g of methanolic extracts.

2.5. In vitro model of inflammation

RAW 264.7 macrophages were purchased from the American Tissue Type Culture Collection (Manassas, VA, USA) and cultured in Dulbecco's modified Eagle's medium (Thermo Fisher Scientific, Milan, Italy) with 10 % fetal bovine serum (FBS) (Thermo Fisher Scientific) and 100 U/mL penicillin-streptomycin (Thermo Fisher Scientific), in 5 % CO_2 at 37 °C. To evaluate the effect of the extracts on cell viability, MTS assay was used as previously described [37]. Macrophages were treated with lipopolysaccharide (1 μg/mL, Sigma-Aldrich, Milan, Italy) and with *A. platensis* F&M-C256, *A. platensis* F&M-C260 and *A. maxima* F&M-C258 methanolic extracts (25 μg/mL) for 18 h at 37 °C, and then were harvested for RNA extraction. The cell media were collected and stored at −20 °C for interleukin-1β (IL-1β) and prostaglandin E2 (PGE2) determination.

2.6. Total RNA extraction and qRT-PCR for gene expression

Total RNA was extracted from cell lysates using the Nucleo Spin® RNA kit (Ma-cherey-Nagel, Bethlehem, PA, USA) according to the manufacturer's instructions. First-strand cDNA synthesis was performed using the Revert Aid RT Kit (Thermo Scientific, Whaltam, USA). qRT-PCR assays were carried out in Rotor-Gene®qPCR System (Qiagen) using SsoAdvanced Universal SYBR Green Supermix (Biorad). Briefly, each reaction was performed in a final volume of 10 μL containing 1 μL of the cDNA, 1 μL of forward and 1 μL of reverse primers, 5 μL of SsoAdvanced universal SYBR Green supermix and 1 μL of Nuclease-free water. Primers were designed based on the mouse GenBank sequences for Il-1β, COX-2, iNOS, NLRP3, HO-1 and SOD2 (Table 1). The amplification protocol comprised an initial heat activation at 95 °C for 30 s, followed by 35 cycles of denaturation at 95 °C for 15 s and combined annealing/extension 60 °C for 30 s. The relative expression of mRNA was normalized by Ribosomal protein large P1 (Rplp-1) and calculated by the $2^{-\Delta\Delta Ct}$ method [38]. Five independent experiments were performed.

Table 1
Primer sequences.

Gene	Primer forward	Primer reverse
Rplp-1	5′-ATCTACTCCGCCCTCATCCT-3′	5′-CAGATGAGGCTCCCAATGTT-3′
COX-2	5′-TCCTCCTGGAACATGGACTC-3′	5′-CCCCAAAGATAGCATCTGGA-3′
IL-1β	5′-CAGGCAGGCAGTATCACTCA-3′	5′-AGGCCACAGGTATTTTGTCG-3′
iNOS	5′-AGACCTCAACAGAGCCCTCA-3′	5′-GCAGCCTCTTGTCTTTGACC-3′
NLRP3	5′-TGGGTTCTGGTCAGACACGAG-3′	5′-GGGGCTTAGGTCCACACAGAA-3′
SOD2	5′-ACCCAAAGGAGAGTTGCTGGA-3′	5′-ATGTGGCCGTGAGTGAGGTT-3′
HO-1	5′-GGCTGC CCTGGAGCAGGAC-3′	5′-AGGTCACCCAGGTAGCGGGT-3′

Rplp-1: Ribosomal protein large P1; COX-2: cyclooxygenase-2; interleukin-1 beta (IL-1β); iNOS: inducible Nitric Oxide Synthase; NLRP3: NLR family pyrin domain containing 3; SOD2: superoxide dismutase 2;HO-1:heme oxygenase-1.

2.7. qRT-PCR for miR-223 expression

miR-223 expression was measured as described previously [39], with few modifications. Briefly, cDNA was synthesized using the miRCURY LNA RT kit (Qiagen, Hilden, Germany). RT-quantitative PCR (qPCR) was performed using miRCURY LNA SYBR Green PCR kit (Qiagen) and primers from miRCURY LNA miRNA PCR Assays (mir-223-3p: cat. n. YP00205986, Qiagen; RNU6b: cat. n. YP00203907, Qiagen). cDNA was diluted at the ratio of 1:10. The analyses were performed using the Rotor-Gene Q thermal cycler (Qiagen). Each sample was tested in triplicate. RNU6b was used as reference miRNA. The calculation of relative expression was performed using $2-\Delta\Delta Ct$ [35]. Five independent experiments were performed.

2.8. Determination of PGE2 and IL-1β in the culture media

After treatments, culture media were collected and IL-1β and PGE2 were assayed using commercially available ELISA kit (Cayman Chemicals, Ann Arbor, MI, USA), according to the manufacturer's instructions, and expressed as pg/mL.

2.9. Statistical analysis

All data represent the results of five independent experiments. Data were analyzed using GraphPad Prism Software, version 6.0 (GraphPad Software, Inc., San Die-go, CA, United States). Results are expressed as mean ± SEM. Statistical analyses were performed by one-way analysis of variance (ANOVA) followed by Dunnett's multiple comparisons post hoc test for multiple comparisons. A p value <0.05 was considered statistically significant.

3. Results

3.1. Characterization of the microalgal extracts

The three methanolic extracts were analyzed by HPLC-UV and HPLC-HRMS and MS/MS. Zeaxanthin and β-cryptoxanthin were the most abundant carotenoids present in the three extracts together with β-carotene; the relative intensities of the carotenoids signals showed that zeaxanthin abundance was similar in the three extracts (Fig. 1, bottom trace, panels A-C) while β-cryptoxanthin was approximately 1.8 times more abundant in *A. platensis* F&M-C260, compared to *A. platensis* F&M-C256 and *A. maxima* F&M-C258 (Fig. 1, top trace, panels A-C). Other carotenoids like diatoxanthin and diadinoxanthin, were detected and their accurate masses, as $[M+H]+$, also revealed in the HRMS analyses: in general, their content was slightly less abundant in *A. platensis* F&M-C256 extract (data not shown). The HPLC-HRMS analyses showed a clear difference in the glycolipid content of the three extracts: in *A. platensis* F&M-C256 the accurate masses of several monogalactosyl monoglycerides, as sodium adducts, were clearly detected, and confirmed by MS/MS fragmentation [40] (Fig. 2, panel A). In *A. platensis* F&M-C260 some of them were also detected but at significant lower intensities, comparing the areas of the chromatographic peak of the specific ion (Fig. 2, panel B). In *A. maxima* F&M-C258 these ions were not detectable. Traces of digalactosyl lipids were detected only in *A. platensis* F&M-C256. In the three extracts multicharged ion species were present, but we were not able to assign an identity to these molecules, potentially attributable to small peptides (between 1 and 5 kDa molecular weight).

3.2. Effects of microalgal extracts on RAW 264.7 macrophages viability

Preliminary experiments to evaluate the effects of methanolic extracts of *A. platensis* F&M-C256, *A. platensis* F&M-C260, and *A. maxima* F&M-C258 on cell viability were carried out using MTS test. RAW 264.7 macrophage cells were treated with each extract at different

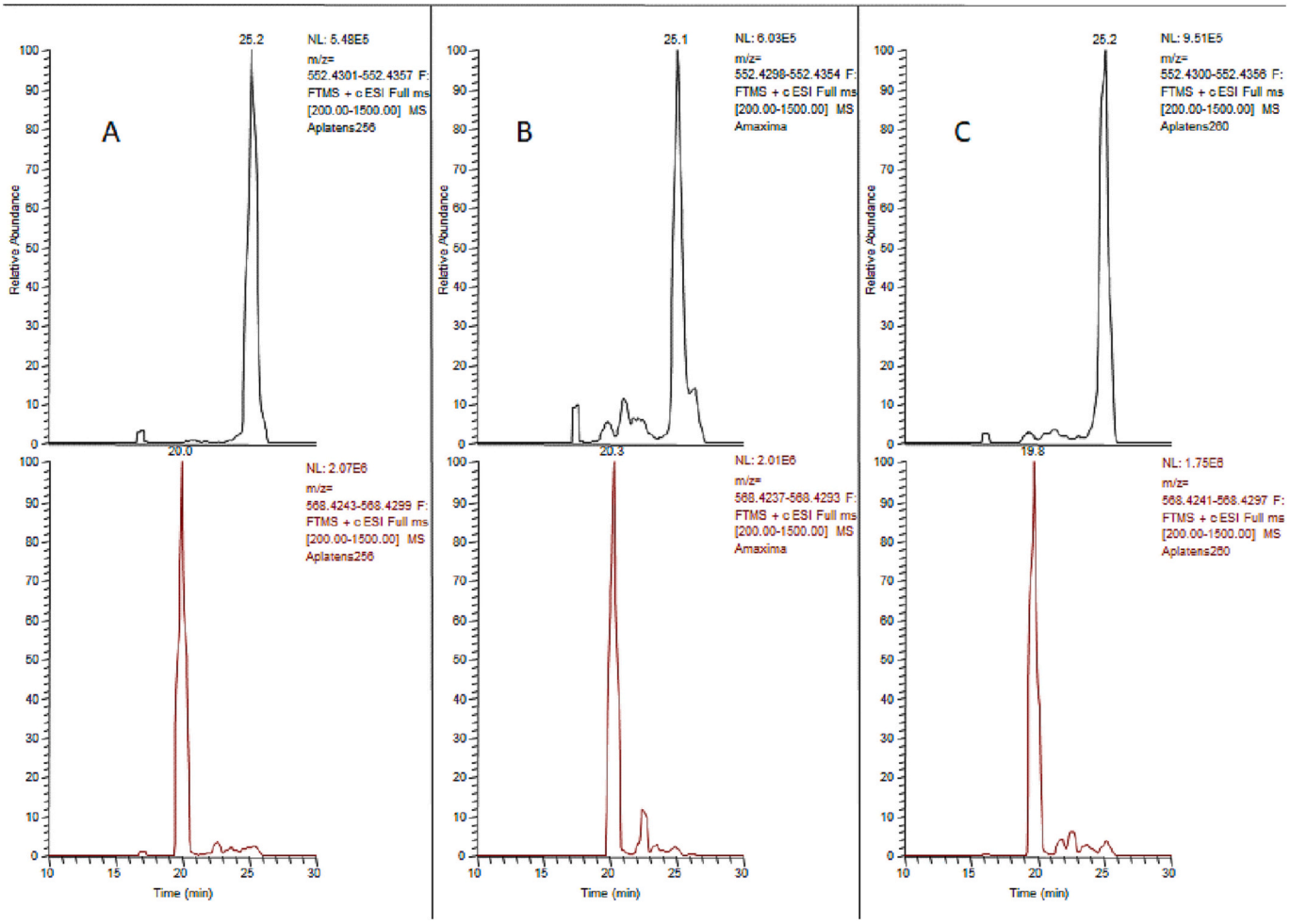

Fig. 1. Extracted ion chromatograms of M+ ions of β-cryptoxanthin (top trace) and zeaxanthin (bottom trace) in *A. platensis* F&M-C256 (A), *A. maxima* F&M-C258 (B) and *A. platensis* F&M-C260 (C) obtained by HPLC-ESI HRMS analysis of methanolic extracts. The accurate masses of the ions were extracted in a 5 ppm window.

concentrations (5–10-25 μg/mL) for 18 h. None of the extracts reduced cell viability even when tested at 25 μg/mL (Fig. 3), that was therefore selected as the highest non-toxic concentration to be used for further analyses on anti-inflammatory activities.

3.3. Comparative effects of microalgal extracts on nitric oxide (NO) production

LPS treatment significantly increased the production of NO compared to control cells ($p < 0.001$) (Fig. 4). The anti-inflammatory drug celecoxib (3 μM) used as a positive control, significantly reduced LPS induced NO production ($p < 0.001$); despite being much less effective than celecoxib, the methanolic extract of *A. platensis* F&M-C260 significantly attenuated NO production in a concentration dependent manner ($p < 0.01$), whereas *A. platensis* F&M-C256 was effective only at 25 μg/mL ($p < 0.01$). Of note, *A. platensis* F&M-C260 extract at a concentration of 5 μg/mL, was as much effective as *A. platensis* F&M-C256 extract at a concentration of 25 μg/mL in reducing NO production. *A. maxima* F&M-C258 extract did not show any significant effect on NO production (Fig. 4). Because of these results, further mechanistic evaluations were carried out testing each extract at a final concentration of 25 μg/mL.

3.4. Comparative effects of microalgal extracts on iNOS/COX-2 mRNA expression and PGE2 production

In consistence with the results on NO production, *A. platensis* F&M-C260 extract showed the highest activity in reducing LPS-induced inducible nitric oxide synthase (iNOS) expression ($p < 0.001$) followed by *A. platensis* F&M-C256 ($p < 0.001$), while *A. maxima* F&M-C258, did not show any significant effect (Fig. 5, panel A). Interestingly, *A. platensis* F&M-C260 halved LPS-induced iNOS expression reaching the levels obtained with the treatment with 3 μM of celecoxib, used as a reference anti-inflammatory drug (Fig. 5, panel A). Compared to LPS, cyclooxygenase-2 (COX-2) expression was not affected by *A. maxima* F&M-C258 extract but significantly reduced by *A. platensis* F&M-C260 ($p < 0.001$) and to a lesser extent, by *A. platensis* F&M-C256 ($p < 0.05$) (Fig. 5, panel B). Accordingly, as shown in Fig. 5 panel C, the extract of *A. platensis* F&M-C260 and that of *A. platensis* F&M-C256 (both $p < 0.001$), decreased LPS-induced production of prostaglandin E2 (PGE2) whereas *A. maxima* F&M-C258 was ineffective.

The expression of microRNA (miR)-223 was significantly reduced by LPS compared to controls ($p < 0.01$) and this effect was completely reversed by celecoxib ($p < 0.01$) and by *A. platensis* F&M-C260 extract ($p < 0.001$) but not by *A. maxima* F&M-C258 or by *A. platensis* F&M-C256 (Fig. 5, panel D).

3.5. Comparative effects of microalgal extracts on NLRP3/IL-1β axis

LPS significantly induced the mRNA expression of NLRP3 ($p < 0.001$, Fig. 6, panel A) and IL-1β ($p < 0.001$, Fig. 6, panel B) and the release of IL-1β ($p < 0.001$, Fig. 6, panel C) compared to control cells. Celecoxib 3 μM significantly reduced the expression of NLRP3 ($p < 0.001$) and IL-1β ($p < 0.001$) as well as the secretion of IL-1β ($p < 0.001$) compared to LPS

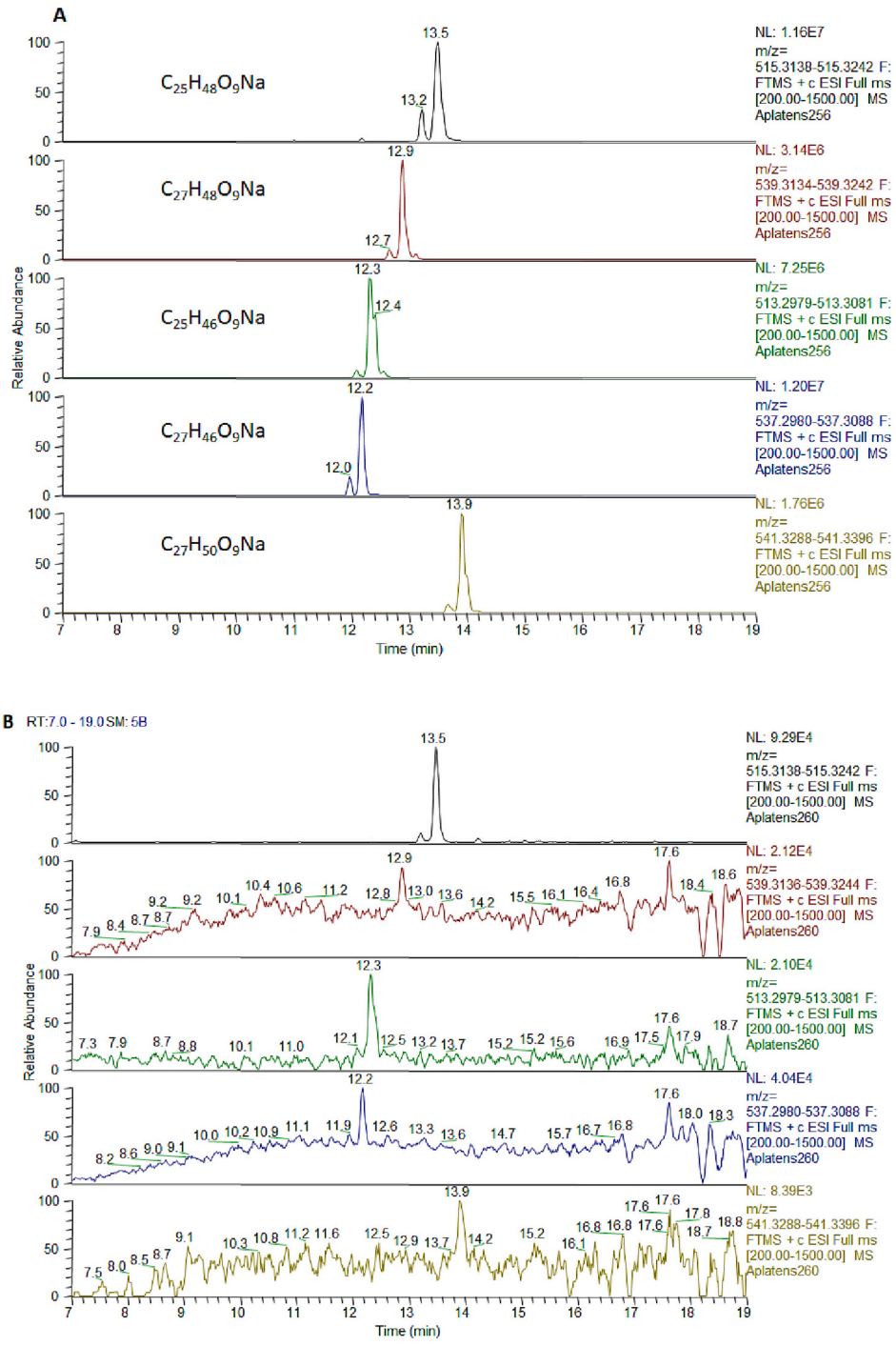

Fig. 2. Extracted ion chromatograms of the *m/z* values attributed to the sodium adducts of MGMG species, obtained by HPLC-ESI HRMS analysis of the methanolic extracts. The accurate mass of each species was extracted in a 5 ppm window. The corresponding molecular formulae are shown. The MGMGs ions were very intense in *A. platensis* F&M-C256 extract (A), while in *A. platensis* F&M-C260 extract (B) were poorly detectable. In *A. maxima* F&M-C258 extract, these MGMGs were not detectable.

treated cells (Fig. 6, panel A, B and C). *A. platensis* F&M-C260 and *A. platensis* F&M-C256 extracts significantly reduced NLRP3 (both, $p < 0.001$) and IL-1β expression ($p < 0.01$ and $p < 0.05$, respectively) but the one from *A. maxima* F&M-C258, was the most effective in counteracting LPS induced NLRP3 ($p < 0.001$) and IL-1β expression ($p < 0.001$) (Fig. 6, panel A and B). Indeed, *A. maxima* F&M-C258 extract significantly reduced IL-1β secretion ($p < 0.01$) at levels quite similar to those of celecoxib, while *A. platensis* F&M-C260 and *A. platensis* F&M-C256 did not exert any significant effect (Fig. 6, panel C).

3.6. Total antioxidant capacity and comparative effects of microalgal extracts on HO-1 and SOD2 expression

The most active methanolic extract was the one from *A. platensis* F&M-C256 that showed a total antioxidant activity significantly higher compared to that from *A. maxima* F&M-C258 (5.24 ± 0.11 μg GAE mg $-$ 1 vs 3.62 ± 0.04 μg GAE mg $-$ 1, $p < 0.05$) but similar to that from *A. platensis* F&M-C260 (4.47 ± 0.07 μg GAE mg $-$ 1). The relative abundance of β-cryptoxanthin and monogalactosyl mono-glycerides seem associated to the higher antioxidant activity of *A. platensis* F&M-C256 and F&M-C260 and to their higher inhibitory activity against the inflammatory mediators PGE2 and NO (Supplementary Fig. 1). On the contrary, antioxidant properties seem inversely associated with anti

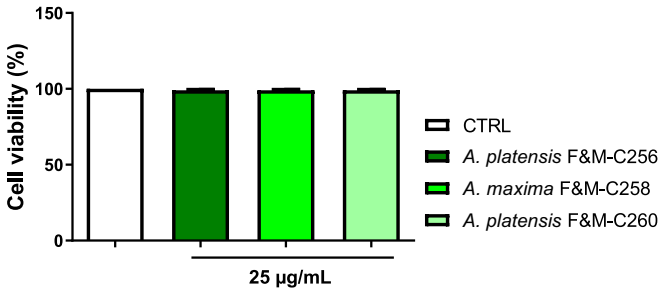

Fig. 3. Effect of *A. platensis* F&M-256, *A. maxima* F&M-C258 and *A. platensis* F&M-C260 methanolic extracts at 25 µg/mL on RAW 264.7 macrophages viability. Results are expressed as percentage of cell viability relative to untreated control cells (CTRL). Data are shown as means ± Standard Error of the Mean (SEM) ($n = 5$).

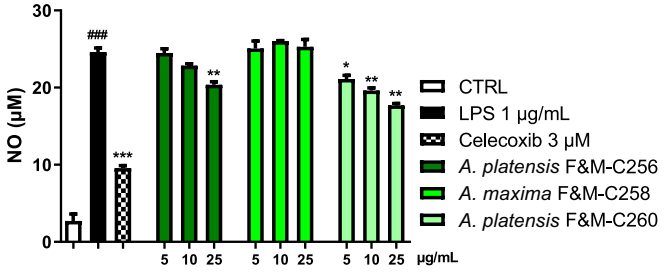

Fig. 4. Effect of *A. platensis* F&M-256, *A. maxima* F&M-C258 and *A. platensis* F&M-C260 meth-anolic extracts on nitric oxide (NO) production in RAW 264.7 macrophages stimulated with lipo-polysaccharide (LPS) for 18 h. $^{###}$ $p < 0.001$ vs control cells (CTRL); *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$ vs LPS by one-way ANOVA and Dunnett's multiple comparisons test. Data are shown as means ± SEM ($n = 5$).

NLRP3/IL-1β activity since *A. maxima* F&M-C258 extract showed the lower antioxidant activity but the higher suppression of IL-1β secretion (Supplementary Fig. 1).

Following stimulation by LPS, compared to control cells, a significant decrease in HO-1 expression was observed (Fig. 7, panel A, $p < 0.01$), whereas that of SOD-2 was increased (Fig. 7, panel B, $p < 0.05$). Celecoxib significantly increased the expression of HO-1 (Fig. 7, panel A, $p < 0.01$) but did not modify that of SOD2 compared to LPS (Fig. 7, panel B). Despite not significantly, treatment with *A. platensis* F&M-C260, *A. platensis* F&M-C256 and *A. maxima* F&M-C258 counteracted LPS-induced SOD2 overexpression without affecting HO-1 compared to LPS alone (Fig. 7, panel A and B).

4. Discussion

Inhibition of chronic, low-grade inflammation has been regarded as a beneficial strategy in preventing and treating non-communicable diseases, one of the major causes of morbidity and mortality in the world [1]. Macrophages are key components of innate immunity and play a critical role in minimizing injury or infection by aiding in the elimination of invading pathogens and damaged cells, thereby restoring tissue homeostasis, and resolving inflammation [41]. The innate immune response is typically triggered through the activation of pattern recognition receptors that recognize pathogen-associated molecular patterns, such as LPS, or damage-associated molecular patterns produced by injured cells [42]. By binding to toll-like receptors (TLRs) and particularly TLR4, LPS activates the NF-kB signaling cascade that culminates in the transcription of iNOS and COX-2 and in the downstream production of several proinflammatory cytokines and mediators such as NO, PGE2, tumor necrosis factor-α (TNF-α), IL-6, and IL-1β [43]. As such, LPS-stimulated RAW 264.7 macrophages are a widely used model for the screening of anti-inflammatory compounds and their mechanism of action [35,44,45].

Research conducted so far documented the anti-inflammatory effects of whole biomasses from *A. platensis* or *A. maxima* with no tentative

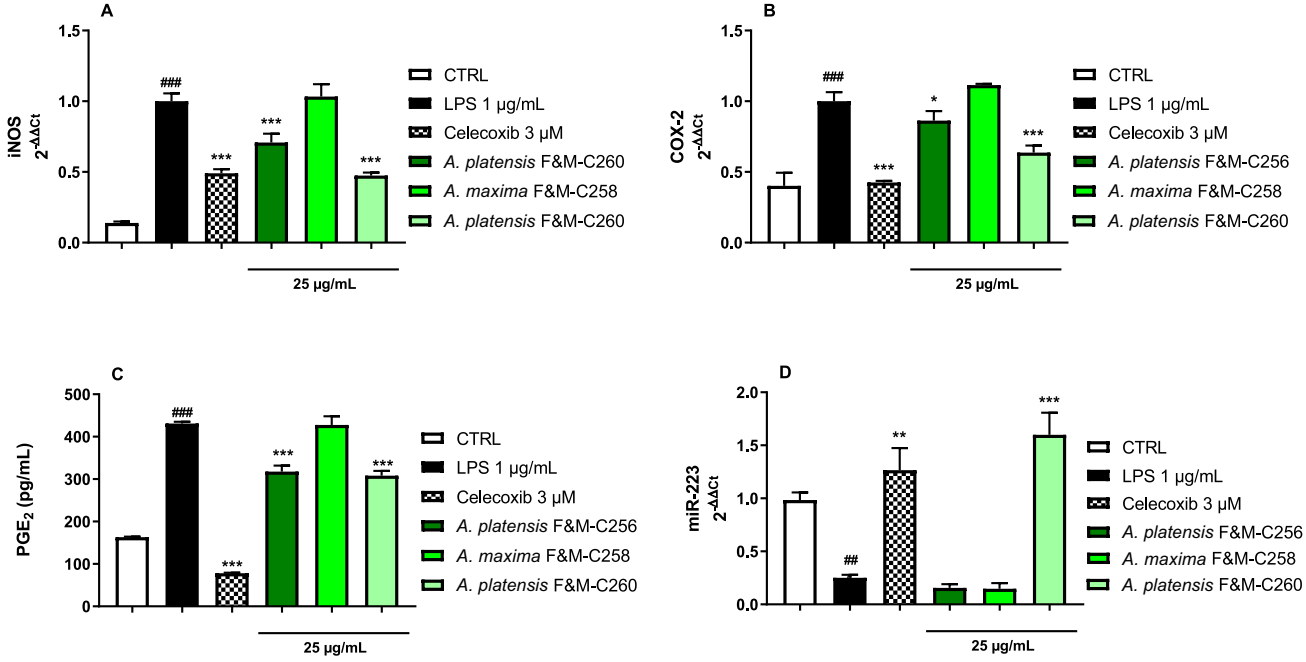

Fig. 5. Effect of *A. platensis* F&M-256, *A. maxima* F&M-C258 and *A. platensis* F&M-C260 methanolic extracts on iNOS (A) and COX-2 (B) mRNA expression, quantified by real-time PCR, in RAW 264.7 stimulated with lipopolysaccharide (LPS) for 18 h. Effect of the three methanolic extracts on PGE2 production (C). MicroRNA 223 (miR-223) expression in RAW 264.7 macrophages stimulated with LPS for 18 h and treated with the three methanolic extracts (D). $^{###}$ $p < 0.001$; $^{##}$ $p < 0.01$ vs control cells (CTRL); *$p < 0.05$; ** $p < 0.01$; ***$p < 0.001$ vs LPS by one-way ANOVA and Dunnett's multiple comparisons test. Data are shown as means ± SEM ($n = 5$).

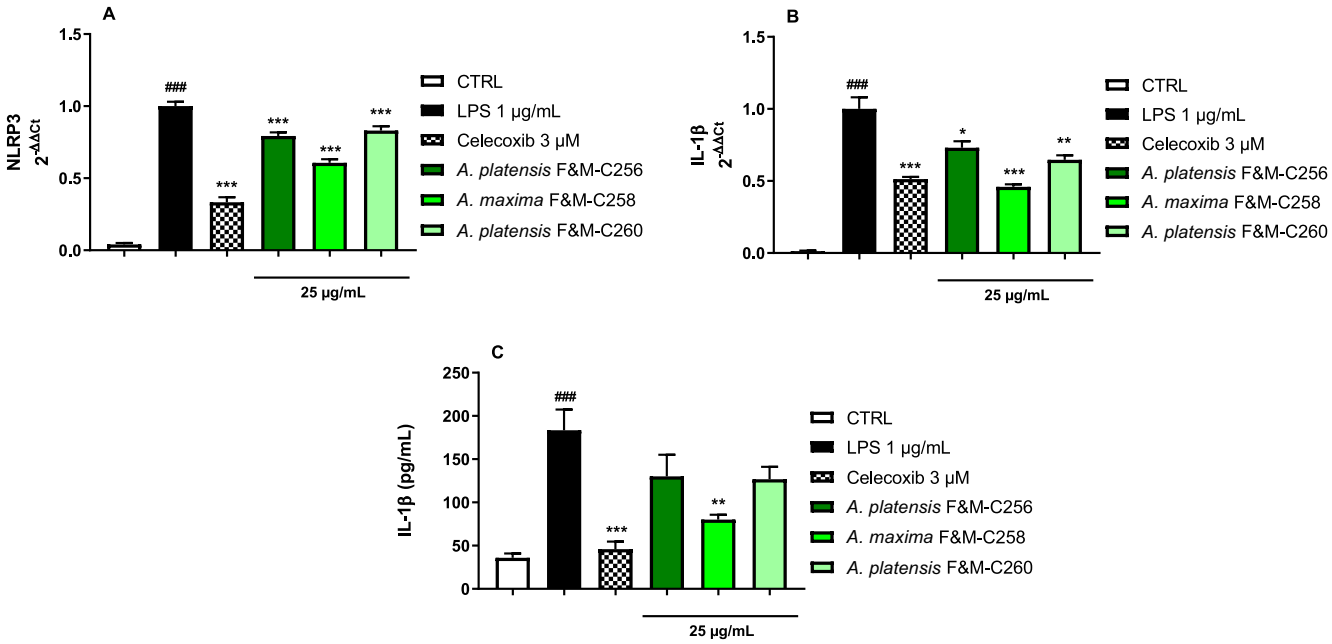

Fig. 6. Effect of *A. platensis* F&M-256, *A. maxima* F&M-C258 and *A. platensis* F&M-C260 meth-anolic extracts on NLRP3/IL-1β axis in RAW 264.7 stimulated with lipopolysaccharide (LPS) for 18 h. NLRP3 (A) and IL-1β (B) mRNA expression quantified by real-time PCR. Effects of *A. platensis* F&M-C256, *A. maxima* F&M-C258 and *A. platensis* F&M-C260 methanolic extracts on IL-1β release in the medium (C). $^{###}$ $p < 0.001$ vs control cells (CTRL); * $p < 0.05$; **$p < 0.01$; ***$p < 0.001$ vs LPS by one-way ANOVA and Dunnett's multiple comparisons test. Data are shown as means ± SEM ($n = 5$).

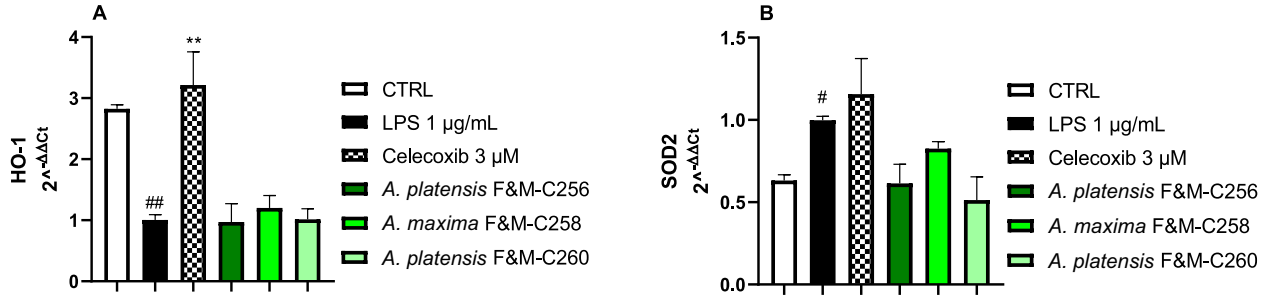

Fig. 7. Effect of *A. platensis* F&M-256, *A. maxima* F&M-C258 and *A. platensis* F&M-C260 meth-anolic extracts on HO-1 and SOD2 expression in RAW 264.7 stimulated with lipopolysaccharide (LPS) for 18 h. HO-1 (A) and SOD2 (B) mRNA expression quantified by real-time PCR. # $p < 0.05$ vs control cells (CTRL); $^{##}$ $p < 0.01$ vs control cells (CTRL); **$p < 0.01$ vs LPS by one-way ANOVA and Dunnett's multiple comparisons test. Data are shown as means ± SEM ($n = 5$).

characterization of the compounds responsible [18–23]. Other authors investigated the anti-inflammatory activity of water-soluble compounds: an aqueous extract from *A. platensis* attenuated intestinal barrier dysfunction and inflammation by exerting antioxidant effects and by suppressing the expression of COX-2, iNOS, and various inflammatory mediators [24]; phycocyanin and carbohydrates were deemed responsible of the observed effects. Similarly, *A. platensis* aqueous extract ameliorated colonic mucosal damage by suppressing free radicals' levels and the expression of several inflammatory cytokines. Bioactive compounds identified in this extract were proteins, phycocyanin, phycoerythrin and allophycocyanin [25]. Cho et al. also demonstrated that phycocyanin was the major active component of *A. maxima* against retinal degeneration [23].

Unlike previous investigations, in this study, we explored the potential anti-inflammatory activity of non-water-soluble compounds, providing the first evidence that methanolic extracts from three different *Arthrospira* strains exert anti-inflammatory effects through multi-target mechanisms that are partly overlapping and partly more strain-specific, probably depending on their different chemical composition.

A. platensis F&M-C256 and to a lesser extent *A. platensis* F&M-C260, exerted significant protective effects mainly via inhibiting iNOS/COX-2 inflammatory pathway. Similar results were obtained in rats treated with an ethyl acetate extract from *A. platensis* to reduce paw oedema [46]. Accordingly, a meta-analysis of randomized controlled trials demonstrated that carotenoids such as astaxanthin, lutein, zeaxanthin and β-cryptoxanthin reduced inflammatory biomarkers [47]. Zeaxanthin and β-cryptoxanthin are both endowed with anti-inflammatory and antioxidative effects: in a colitis rat model, zeaxanthin suppressed NF-κB activation, inhibited iNOS and COX-2 expression and the production of several proinflammatory mediators [48]; β-cryptoxanthin, also called provitamin A, reduced oxidative stress and inflammation and polarized macrophages toward an anti- inflammatory phenotype [49]. Moreover, β-cryptoxanthin inhibited PGE2 production via the downregulation of COX-2 and directly suppressed the activity of the inhibitor of NF-κB kinase [50]. Through bioassay-guided fractionation, Tan et al. identified carotenoids, apocarotenoids, flavonoid glycosides and galactolipids (monogalactosyl-diacylglycerols, digalactosyl-diacylglycerol, and sulfoquinovosyl-diacylglycerol) as the anti-inflammatory components of six commercial spirulina supplements [51]. The bioactive effects of galacto-lipids in inflammatory processes appear to involve NO suppression through the downregulation of iNOS [52,53]. According to our findings and to literature data [49–53], the inhibition of iNOS-COX-

2 inflammatory pathway by *A. platensis* F&M-C256 and *A. platensis* F&M-C260, may likely be ascribed to their relative abundance in monogalactosyl monoglycerides and β-cryptoxanthin, respectively; it could be also speculated that monogalactosyl monoglycerides and β-cryptoxanthin might synergize each other, with other carotenoids or with other hitherto uncharacterized compounds, enhancing the anti-inflammatory effects of the single compound. This is consistent with the evidence that a phytocomplex such as an extract, could be endowed with higher activity than single components due to additive and/or synergistic effects [35,54,55]. Hence, the lack of ability of *A. maxima* F&M-C258 to inhibit iNOS and COX-2 expression and NO production appears to be associated with both the absence of monogalactosyl mono-glycerides and its minor content of β-cryptoxanthin, against a similar abundance of other carotenoids and β-carotene compared to the other two strains.

Piovan et al. also recently demonstrated that an acetone extract of *A. platensis* containing chlorophylls, pheophytins, and carotenoids, mainly β-carotene and zeaxanthin, reduced neuroinflammation through reduced NF-κB activation and enhancement of Nrf2 [56], a pivotal transcription factor orchestrating the antioxidant response through transcriptional control of HO-1 and other antioxidant enzymes such as SOD2 [5–7]. In this study, we investigated both direct and indirect antioxidant effects demonstrating that compared to *A. maxima, A. platensis* F&M-C256 and F&M-C260 showed higher direct antioxidant capacity measured by the Folin-Ciocalteu assay; despite being widely used for measuring total phenolics content, the Folin-Ciocalteu reagent can react toward other compounds and have been used to measure total antioxidant capacity, with good correlations with other assays [36,57]. Conversely, none of the microalgal extracts had indirect antioxidant effects via HO-1 and SOD2 genes, suggesting that the possible contribution of antioxidant properties to the observed anti-inflammatory effects may be based on their direct reducing activity rather than on the transcriptional activation of Nrf2-driven genes. The relative abundance of β-cryptoxanthin and monogalactosyl mono-glycerides, together with other carotenoids and β-carotene, appear associated to the higher antioxidant activity of *A. platensis* F&M-C256 and F&M-C260 and to their higher inhibitory activity against PGE2 and NO production, proinflammatory mediators downstream activation of iNOS-COX-2 pathway. On the contrary, the higher the antioxidant properties and the relative abundance of β-cryptoxanthin and monogalactosyl mono-glycerides, the lower the anti-NLRP3/IL-1β activity. By mediating the secretion of IL-1β/IL-18 proinflammatory cytokines, NLRP3 inflammasome is a critical component of the innate immune system and a promising therapeutic target for a wide range of inflammatory diseases [58,59]. Our results indicate that the anti-inflammatory activity of *A. maxima* F&M-C258 extract seems mostly mediated by the inhibition of NLRP3/IL-1β pathway, as previously demonstrated for an ethanolic extract from *A. maxima* rich in chlorophyll-a [26]. Moreover, recent research found that β-carotene directly binds to NLRP3, thereby suppressing inflammasome activation [60]. However, our chemical characterization did not provide a clear association between *A.maxima* F&M-C258 composition and its higher activity against NLRP3/IL-1β signaling since comparable amounts of β-carotene were detected in the three extracts while signals potentially attributable to chlorophyll-a were detected, but not unequivocally identified.

In recent years, epigenetic factors such as microRNAs (miRNAs), have been found to be involved in regulating gene expression and in coordinating various biological processes that drive inflammation [61–63]. MiR-223 is implicated in many inflammatory disorders, infections, and cancers [64]. Mechanistically, miR-223 inhibits IL-1 receptor-associated kinase 1 (IRAK-1) and sequentially, the activation of NF-κB and production of pro-inflammatory mediators [65,66]. MiR-223 is as a regulator of the inflammatory response, it induces macrophage polarization toward an anti-inflammatory phenotype, and miR-223 deficient macrophages, are hypersensitive to LPS stimulation [67]. According to our results, *A. platensis* F&M-C260 is the sole, out of the three *Arthrospira* strains, to be able to induce the expression of miR-223 upon LPS challenge, likely in virtue of its higher β-cryptoxanthin content.

5. Conclusions

In this study, we demonstrated that three methanolic extracts from different strains of the genus *Arthrospira* show distinct metabolites profiles and exert anti-inflammatory effects through a multi-target activity, suggesting that *Arthrospira* strains are potential source of treatments for inflammatory diseases. The underlying mechanisms involve several pro-inflammatory targets and a direct reducing effect rather than the transcriptional activation of antioxidant enzymes; furthermore, the degree of bioactivity against each target seems to be species and strain specific and associated to their different metabolites' composition and abundance. In particular, the relative abundance of β-cryptoxanthin and monogalactosyl mono-glycerides, together with other carotenoids and β-carotene, seems responsible of the higher inhibitory activity of *A. platensis* F&M-C256 and F&M-C260 toward iNOS-COX-2 inflammatory pathway while neither the chemical characterization of *A. maxima* nor its antioxidant activity, provided clear associations with its higher activity against NLRP3/IL-1β signaling.

Further studies comparing the antioxidant and anti-inflammatory activity of each extract against that of identified active compounds tested alone or in standardized combinations are required to specifically determine which metabolite(s) present in *Arthrospira* extracts may actually contribute to the observed effects and whether simultaneous synergistic, additive, or even antagonistic interactions take place.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.algal.2023.103171.

Statement of informed consent

No conflicts, informed consent, or human or animal rights are applicable to this study.

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CRediT authorship contribution statement

Elisabetta Bigagli: Conceptualization; Methodology; Validation; Writing - original draft; Writing - review & editing; Supervision. Mario D'Ambrosio: Validation; Investigations; Visualization. Lorenzo Cinci: Methodology; Investigations. Giuseppe Pieraccini: Methodology; Investigations; Validation; Writing - review & editing; Riccardo Romoli: Methodology; Investigations; Validation. Natascia Biondi: Methodology; Resources; Writing - review & editing. Alberto Niccolai: Resources; Writing - review & editing; Liliana Rodolfi: Methodology; Resources; Writing - review & editing. Mario R. Tredici: Methodology; Resources; Funding acquisition; Cristina Luceri: Conceptualization; Methodology; Validation; Formal Analysis; Resources; Writing - review & editing; Supervision; Funding acquisition.

Declaration of competing interest

A. platensis F&M-C256, *A. platensis* F&M-C260 and *A. maxima* F&M-C258 belongs to the Culture Collection F&M S.r.l. culture collection, where M.R.T. had and L.R. has a financial interest. The other authors have no conflicts of interest.

Data availability

The data that support the findings of this study are available on request from the corresponding author C.L. (cristina.luceri@unifi.it).

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