

Evaluation of Antioxidant Activity of *Spirulina (Arthrospira) maxima* During Acute and Chronic Inflammation *In Vivo*

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ABSTRACT

Introduction: In recent years, the use of food supplements like co-adjuvants in the treatment of various diseases has increased; being one of the most studied and consumed the algae *Spirulina maxima*, which possesses important demonstrated biological effects including antioxidant and anti-inflammatory activities.

Objective: This study sought to determine if there is a direct link between these two pharmacological mechanisms in models of acute and chronic inflammation *in vivo* using a trademark of *Spirulina* powder.

Methods: The acute anti-inflammatory effect of *Spirulina* was evaluated in the model of sub-plantar edema induced by carrageenan and its antioxidant activity was determined in a model of experimental arthritis induced with complete Freund's adjuvant.

Results: For acute inflammation, *Spirulina* at 400 mg/kg generated an anti-inflammatory effect (51%) similar to indomethacin (58%) in fifth hour, whereas in the chronic model, also dose of 400 mg/kg demonstrated an anti-inflammatory effect on days 14 and 21 of the study (51% and 38%), close to phenylbutazone effect in the same days (53% and 52%), as well this dose showed an increase in body weight gain during the experiment compared to CFA control group. Finally, 400 mg/kg dose of *Spirulina* decreased protein carbonyl content (69%) and lipid peroxidation (81%) in edema tissue compared to arthritic mice without treatment. And for antioxidant enzymes activities this dose decrease SOD activity (42.98%) and increase CAT (3216%) and GSH-Px (130%) activities when compared to CFA control group.

Conclusion: Thus, it was demonstrated that *Spirulina* at 400 mg/kg dose generated an anti-edematous effect associated to antioxidant activity *in vivo* since it decreases oxidative damage over biomolecules of sub-plantar edema tissue during chronic inflammation, as well increase and regulate the antioxidant enzymatic endogenous response.

Keywords: *Spirulina maxima*, Acute inflammation, Chronic inflammation, Oxidative stress

Abbreviations: 2,4-DNPH: 2,4-Dinitrophenylhydrazine; ANOVA: Analysis of Variance; BIRMEX: Biological Laboratories Reagents México; BW: Body Weight; CAT: Catalase Enzyme; CFA: Complete Freund's; COX-2: Cyclooxygenase 2; EtOAc: Ethyl Acetate; EtOH: Ethanol; GSH: Reduced Glutathione; GSH-Px: Glutathione Peroxidase; HCl: Hydrochloric Acid; IBD: Inflammatory Bowel Disease; i.g.: intragastric; IMC: Indomethacin; iNOS: induced Nitric Oxide Synthase; MDA: Malondialdehyde; MEC: Molar Extinction Coefficient; OS: Oxidative Stress; PBS: Phosphate Buffer Solution; PBZ: Phenilbutazone; PGE₂: Prostaglandin E2; RM: Repeated Measures; ROS: Reactive Oxygen Species; s.c.: subcutaneous; SEM: Standard Error of the Mean; Sm: *Spirulina maxima*; SNK: Student Newman Keuls; SOD: Superoxide Dismutase Enzyme; TBA: Thiobarbituric Acid; TBARS: Thiobarbituric Acid Reactive Substances; TCA: Trichloroacetic Acid; TP: Total Proteins; UV/Vis: Ultraviolet/Visible

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INTRODUCTION

Inflammation is a beneficial body reaction caused by a stimuli [1-3], this could be external (induced by a biological or physical damage) or internal (auto-immune pathologies), with the objective of identify and isolate the damaged area, eliminate the causal agent and finally reset the homeostasis in the affected zone. This immune process has two well-known phases: acute and chronic, both characterized by vasodilatation, macrophages migration, reactive oxygen species (ROS) production and edema, also it exhibits two pharmacological pathways: the cellular and the oxidative one, in this last one is where the respiratory burst takes place for the generation of ROS and other free radicals with high reactivity [2-6]. Nowadays, the use of food supplements as co-adjuvants in the treatment of diseases has increased, due to allopathic medicine failure [7-11]. Algae from *Spirulina* genus have been one of the most studied as a source of active compounds [12-15], which did not show toxicity [16-18]. Also its properties have been proven through scientific studies: anti-viral [19], hypoglycemic [20], hypolipidemic [21], anti-cancer [22], anti-anemic [23], and anti-inflammatory [24,25]. Previous work demonstrated that laboratory cultivated *S. maxima* protects against oxidative damage in joint tissue as well as serum during arthritis in rats [26].

The aim of this work was to demonstrate the ability of *Spirulina maxima* to generate an anti-inflammatory effect right on the paw edema tissue, through its antioxidant activity *in vivo* over oxidative stress associated to Complete Freund's Adjuvant (CFA)-induce arthritis in mice. Also its beneficial effect during acute inflammation was evaluated.

METHODS

Reagents and chemicals

All chemicals used in this study were analytical grade and were purchased from Sigma-Aldrich (St. Louis, MO, E.U.A.). λ -Carrageenan (No. CAT 9064-57-7), Complete Freund's Adjuvant (No. CAT F5881, 1 mg/mL), Bradford reagent (No. CAT B6916), trichloroacetic acid (No. CAT T6399), thiobarbituric acid (No. CAT S564508), hydrochloric acid (No. CAT H1758), 2,4-dinitrophenylhydrazine (No. CAT D199303), ethyl acetate (No. CAT 270989), ethylic alcohol (No. CAT E7023), guanidine (No. CAT PH016683), epinephrine (No. CAT 1236970), superoxide dismutase (No. CAT S5395), acetic acid (No. CAT 1005706), hydrogen peroxide (No. CAT 216763), glutathione reductase (No. CAT G9297), HEPES (No. CAT H3375), sodium azide (No. CAT 769320), and NADPH (No. CAT N5130).

Biological material

Lyophilized powder of *Spirulina maxima* (Sm) was obtained from Solarium Biotechnology Company, S.A., Santiago, Chile. According to the company procedures complete *S.*

maxima biomass was cultivated and put in distilled water and then subjected to defrost and freeze cycles, then stirred to obtain an aqueous extract with all the cellular components in suspension and finally put in a freeze dryer (Quanta-S Model) coupled to a vacuum system (Leybold Rotary Vane Model) to lyophilize the water at a temperature of -50°C and obtain the final dried sterile powder.

Laboratory animals

Male CD1 mice (weighing 20 ± 5 g) were acquired from BIRMEX (Biological Laboratories Reagents México, D.F.), with a 7 day conditioning period, 12 h light-dark cycles, at a temperature of $25 \pm 2^{\circ}\text{C}$, with 55-80% humidity, food (Lab Rodent Chow) and water *ad libitum*. The experimental protocol was carried out according to the National Commission of Scientific Investigation and Bioethics and the Postgraduate and Research Secretariat of the National Polytechnic Institute (Project no. 20121433) and following all the guidelines mentioned in the Mexican Official Norm (NOM-062-ZOO-1999) in relation to the handling and use of laboratory animals.

Inflammation murine models *in vivo*:

Carrageenan-induced edema in mice: This model was performed as described by Dominguez-Ortiz et al. [27] and Garcia-Rodriguez et al. [28]. All experimental groups were formed randomly with CD1 male mice ($n=8$). Treated groups received by intragastric (i.g.) route indomethacin (IMC) (10 mg/kg) and Sm (400 and 800 mg/kg), 1 h prior to the subcutaneous (s.c.) injection of carrageenan (20 μl , 2% dissolved in sterile isotonic saline solution), prepared at the moment. The reference drug and *S. maxima* were solubilized in Tween80:water (1:9) and carrageenan control received only vehicle. The percentage of inhibition was calculated by comparing the measurement of the paw edema at different times (1, 2, 3, 5 and 7 h) (E_t) using a digital micrometer (Mitutoyo model 293-831) and value of time zero (baseline) (E_0). Results were analyzed with the formula described by Olajide et al. [29]:

$$\% \text{ Inhibition} = \frac{(E_t - E_0) \text{ carrageenan control} - (E_t - E_0) \text{ treated}}{(E_t - E_0) \text{ carrageenan control}} \times 100$$

CFA-induced experimental arthritis in mice: This model was carried out according to Rasool et al. [25]. All experimental groups control and treated ($n=8$) were injected s.c. with 25 μl of CFA in the hind paw in day zero (E_0). Treatment groups were administered by i.g. route with PBZ (100 mg/kg) and Sm (400 and 800 mg/kg) daily from day 7 to 21, because physical clinical signs of experimental arthritis begin to be observed on day 7. All samples were solubilized in Tween 80:water (1:9) and group of arthritic animals without treatment only received vehicle. Development of paw edema were measured at different times (1, 4, 7, 14 and 21 days) (E_t) using a digital

micrometer (Mitutoyo model 293-831) and the value of day zero (baseline) (E_0). Body weight (BW) gain was also measured the same days. Percent inhibition of edema in each group was calculated from 7 to 21 day comparing with CFA group without treatment as follows:

$$\% \text{ inhibition} = \frac{((E_t - E_0) \text{ CFA group} - (E_t - E_0) \text{ Treated group})}{(E_t - E_0) \text{ CFA group}} \times 100$$

Oxidative stress (OS) measurement: For OS evaluation, animals with CFA-induced experimental arthritis, were euthanized on the day 21 of the experiment, later sub-plantar edema tissue was obtained and placed in an ice bath, after this 500 mg were homogenized in 2 ml of cold phosphate buffer solution (PBS, pH 7.3), from which appropriated aliquots were taken for the determination of the concentration of carbonyl proteins and the lipid peroxidation rate. 1 ml of the remaining homogenate was centrifuged (12500 rpm for 15 min at 4°C) and 25 μ l of the supernatant was taken for the quantification of total proteins (TP) by the Bradford's technique [30] and the rest of the supernatant was used for the antioxidant enzymes assays. All the OS measures were realized in an ultraviolet-visible (UV-VIS) spectrophotometer (Shimadzu Model UV-1700 Double Beam Scanning).

Lipid peroxidation: Evaluation of lipid peroxidation process was performed by the modified method of Buège and Aust [31]. To an aliquot of 500 μ l of homogenate were added 1 ml of thiobarbituric acid reactive substances (TBARS) reagent (trichloroacetic acid (TCA 16%), thiobarbituric acid (TBA 0.5%), hydrochloric acid (HCl 0.3 N), mixed by vortexing and then incubated under boiling conditions (at 92°C for 15 min). Later the samples were placed in ice bath for 10 min and then centrifuged (4000 rpm for 10 min at 4°C). Finally, the absorbance of the supernatant was measured at 535 nm against a TBARS reagent blank (without homogenate). The results were expressed as moles of malondialdehyde (MDA)/mg of protein/g of tissue, using the molecular extinction coefficient (MEC) of $1.56 \times 10^5 \text{ M}^{-1}\text{cm}^{-1}$.

Carbonyl proteins: This assay was performed according to the method described by Parvez and Raisuddin [32], in which to a 300 μ l of homogenate, 300 μ l of 20% TCA were added, and centrifuged (11500 rpm for 5 min at 4°C). The supernatant was discarded and 150 μ l of 2,4-dinitrophenylhydrazine (2,4-DNPH) (10 mM in HCl 2 M) were added to the precipitated and reconstituted by vortexing for 5 min and subsequently incubated in a water bath for 1 h at 37°C. After that the samples were centrifuged (11000 rpm for 10 min at 4°C), the supernatant was discarded and the precipitated was washed with 1 ml of a solution of ethyl acetate:ethanol (EtOH-AcOEt, 1:1) and vortexed for 5 min, repeating this step for three times. The final precipitated was dissolved in 1 ml of guanidine (6 M in 0.2 mM PBS, pH 2.3) and vortexed for 5 min, then the samples were incubated at 37°C for 40 min and after

centrifuged (5500 rpm for 5 min at 4°C). Finally, the supernatant was measured at 360 nm. As reagent blank 1 ml of HCl 2 M was used. The results were expressed as moles of reactive carbonyls CO^{\bullet} /mg of protein/g of tissue, according the MEC $21000 \text{ M}^{-1}\text{cm}^{-1}$.

Antioxidant enzymes: Superoxide dismutase (SOD) was determined as described by Misra and Fridovich [33], measuring absorbance at 30 s and at 5 min and the difference between these two values was used to calculate adrenochrome concentration with MEC of $4020 \text{ M}^{-1}\text{cm}^{-1}$ at 480 nm. Data was extrapolated in a calibration curve ($y = -0.004x + 0.0518$; $R^2 = 0.9536$) and results were expressed as SOD International Units (IU)/g tissue. Catalase (CAT) activity was evaluated as described by Radi et al. [34], measuring absorbance at time zero and at 1 min and difference was employed to calculate hydrogen peroxide (H_2O_2) denaturalization with MEC of $0.043 \text{ mM}^{-1}\text{cm}^{-1}$ at 240 nm; results were expressed as mmol H_2O_2 consumed/min/g tissue. According to this method, one CAT IU/g tissue represents 1 μ mol of H_2O_2 consumed/min/g tissue. Glutathione peroxidase (GSH-Px) activity was determined according to Plagia and Valentine [35], measuring absorbance at time zero and at 1 min with MEC of $6.2 \text{ mM}^{-1}\text{cm}^{-1}$ for 360 nm; results were expressed as mmol NADPH consumed/min/g tissue.

STATISTICAL ANALYSIS

SigmaPlot ver. 12.0 statistical software (2011-2012) was utilized for analysis of results. Data is presented as standard error (\pm) of the mean (SEM). Data from sub-plantar edema diameter in both models of inflammation (acute and chronic) and body weight gain during experimental arthritis were analyzed with a repeated measures (RM) two-way analysis of variance (ANOVA), while results of oxidative stress parameters were analyzed with one-way ANOVA. The difference between means was determined by the Student Newman Keuls (SNK) post hoc test. Results considered statistically significant were those that showed a value $p < 0.05$, according to the confidence interval destined for biological tests (95%).

RESULTS AND DISCUSSION

Acute inflammation

For acute inflammation model, we found that IMC generated a statistical decrease ($p < 0.05$), over sub-plantar edema formation in the first 2 hours after the inoculation of the carrageenan (37 and 41%) compared to carrageenan control at the same hours (0.42 ± 0.02 and 0.44 ± 0.04 mm, respectively), while Sm at 400 and 800 mg/kg caused an inhibition of 23 and 37%, respectively in the second hour compared to those mice injected with carrageenan and administered only with the vehicle (0.44 ± 0.04 mm) (Figure 1). Furthermore, it was observed that Sm generated significant edema inhibition at both doses (400 and 800 mg/kg) of 51 and 24%, respectively, at the fifth hour of

measure, being the first dose similar in inhibition to that shown by IMC (58%) ($p < 0.05$), when compared the three groups to carrageenan control group values at the same hour

(0.67 ± 0.03 mm). In this model of acute inflammation this stage (5 h), is characterized by the release of free radicals by the immune cells and leukocyte infiltration (**Figure 1**).

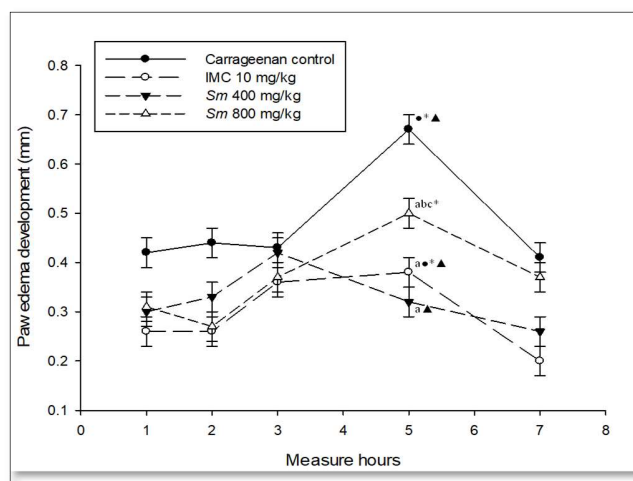


Figure 1. Anti-inflammatory activity of *Spirulina maxima* on carrageenan-induced acute paw edema (mm).

Data is presented as mean \pm standard error (SE); data is shown as millimeter (mm) of the edema size compared to baseline measure (time zero), also in parentheses is indicated the percent of inhibition of edema compared to carrageenan control. Treatments were administrated orally. Statistical analysis RM two-way ANOVA, SNK post hoc test ($P < 0.05$). ^a vs. carrageenan control; ^b vs. IMC; ^c vs. Sm 400 mg/kg; ^{*} vs. 1 h; ^{*} vs. 2 h; [▲] vs. 3 h; [♦] vs. 5 h. IMC: Indomethacin; Sm: *Spirulina maxima*; NE: No Effect ($n=8$)

This effect described above could respond to the suppression of pro-inflammatory cytokines or vasodilators like histamine, which are known to be generated and released at this time due to the pharmacokinetic of this acute inflammation model. *S. maxima* effect on interleukins was demonstrated by Romay et al. [36] and Gemma et al. [37], in which *Spirulina* decreased the concentration of tumor necrosis factor-alpha (TNF- α) and tumor necrosis factor-beta (TNF- β), key mediators to initiate the inflammatory process.

The previously described effect may be due to the antioxidant activity of the components of *Spirulina maxima*, because it is known that their inactive ROS, being these chemo-attractants during the immune process and reduce in this way the leukocyte migration and metabolic activity of neutrophils [38,39]. Another possible mechanism through which this alga inhibited the edema growth, would be by the suppressive effects of its constituents like the phycocyanin over the expression of induced nitric oxide synthase (iNOS) and cyclooxygenase 2 (COX-2), both important enzymes in the development of the edema generation [40-43] and thus the synthesis of prostaglandin E2 (PGE2)[44]. To elucidate these important pharmacological steps further experiments need to be done, such as *in vivo* evaluation of

myeloperoxidase in this acute inflammation model or the *in vitro* determination of COX-2 and iNOS activities, as well nitric oxide concentration.

Chronic inflammation

Although there was no group of healthy mice as a negative control in various publications where work has been done with experimental arthritis induced with CFA, it has been shown that this adjuvant undoubtedly generates a measurable and visible severe inflammatory condition compared to healthy animals, both in rats and mice, and more than our vehicle group it was our CFA control without treatment to compare the anti-edematous and antioxidant effects [26]. During the development of this experiment, PBZ-treated group showed a statistical increase in BW at day 14 (263.23%) and 21 (170.31%), compared to arthritic mice without treatment in the same days (0.94 ± 0.39 and 1.28 ± 0.56 g, respectively). Furthermore, animals administered with Sm at 400 mg/kg exhibited a significant increase of BW on days 14 (138.30%) and 21 (329.69%) when compared to CFA-induced control group that only was administered with vehicle, while the higher tested dose of Sm (800 mg/kg), did not show a positive effect in the gain of BW during the chronic inflammation model (**Table 1**).

Table 1. Effect of *Spirulina maxima* on body weight gain during chronic inflammation (g).

Treatments	Time of measure (days)				
	1	4	7	14	21
CFA control	0.22 ± 0.15	0.14 ± 0.11	0.08 ± 0.06	0.94 ± 0.39	1.28 ± 0.56 ^{•*▲}
PBZ 100 mg/kg	0	0.18 ± 0.07	1.02 ± 0.14 ^{•*}	3.42 ± 0.13 ^{ab•*▲}	3.46 ± 0.19 ^{ab•*▲}
Sm 400 mg/kg	0	0.06 ± 0.03	1.12 ± 0.06 ^{•*}	2.24 ± 0.25 ^{ab•*▲}	5.50 ± 0.56 ^{ab•*▲♦}
Sm 800 mg/kg	0	0	0.54 ± 0.24	0.62 ± 0.21 ^{bc}	0.24 ± 0.11 ^{abc}

Data is presented as mean ± Standard error (SE); data is shown as grams (g) of body weight gain. Treatments were administrated orally since day 7. Statistical analysis RM two-way ANOVA, SNK post hoc test ($P < 0.05$). ^a vs CFA control; ^b vs PBZ; ^c vs. Sm 400 mg/kg; [•] vs. 1 day; ^{*} vs. 4 days; [▲] vs. 7 days; [♦] vs. 14 days

CFA: Complete Freund's Adjuvant; PBZ: Phenylbutazone; Sm: *Spirulina maxima* (n=8)

In the model of chronic inflammation induced by CFA, one of the general indicators that there is in fact an immune process is the loss of BW due to the hyperalgesia generated by pro-inflammatory cytokines [26,39]. The weight loss can be explained in two ways, due to pain produced during chronic inflammation, provoking the immobility of the animals and leading to the alteration of normal feeding habits [45] or by bad absorption syndromes of nutrients in the intestine [46]. In this study, the treatment with *Spirulina maxima* at lower dose was able to maintain BW and although this alga has not been used on inflammatory bowel disease (IBD) models, the same protective effect was found in isolated natural products such as curcumin, which generated the inhibition of TNF-β, IL-1β and IL-6,

decreased the immune response and favored both absorption and transport of nutrients [47] and it is known that *Spirulina maxima* has molecules that inhibit this cytokines.

For paw edema formation induced by CFA, PBZ-treated group showed a significant decrease in size on day 14 and 21 (53.14 and 52.18%, respectively) ($p < 0.05$), compared to CFA un-treated group (1.59 ± 0.10 and 1.27 ± 0.09 mm, respectively). Animals that were administered with the 400 mg/kg dose of Sm, showed a significant greater inhibition at day 14 (51.22%) than in day 21 (37.97%) when compared to CFA control group; however, higher evaluated dose of Sm (800 mg/kg) only generated a statically moderate anti-inflammatory effect in both days (37.92 and 26.21%, respectively), than the dose of 400 mg/kg (**Figure 2**).

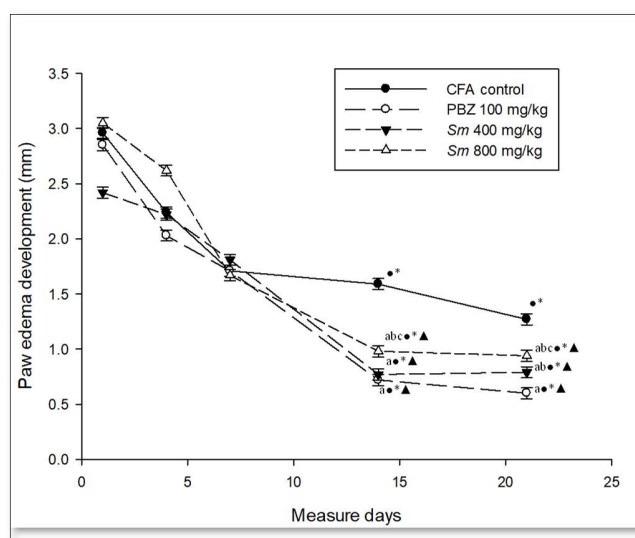


Figure 2. Effect of *Spirulina maxima* on CFA-induced chronic paw edema (mm).

Data is presented as mean ± standard error (SE); data is shown as a millimeter (mm) of the edema size compared to baseline measure (time zero), also in parentheses is indicated the percent of inhibition of edema compared to CFA control. Treatments were administrated orally since day 7. Statistical analysis RM two-way ANOVA, SNK post hoc test ($P < 0.05$). ^a vs CFA control; ^b vs. PBZ; ^c vs. Sm 400; [•] vs. 1 day; ^{*} vs. 4 days; [▲] vs. 7 days; [♦] vs. 14 days
CFA: Complete Freund's Adjuvant; PBZ: Phenylbutazone; Sm: *Spirulina maxima* (n=8)

The inhibitory effect generated by Sm on the edema development, is probably, among other possible pharmacological mechanisms, due to the suppression of ROS during the oxidative phase of chronic inflammation [39]. During an immune process there exist two pathways in which it begins and is exacerbated, one that depends on enzymes like COX-2 and another that is oxidative, the last one is related to the generation of free radicals. Today is known that OS is involved in the development of various human immune pathologies related to both acute and chronic inflammation [6].

OS microenvironment in edema tissue

The tests of OS in edema tissue of arthritic animals showed that CFA control group exhibited high levels of oxidized proteins ($21.84 \pm 1.87 \mu\text{m CO}^*/\text{g tissue}$), and an increased

lipid peroxidation ($2.24 \pm 0.43 \mu\text{m MDA/g tissue}$), while PBZ-treated group exhibited a statistical decrease in the concentration of carbonyl proteins (41.70%) and lipid peroxidation (71.87%) ($p < 0.05$). Furthermore, Sm administered groups at both doses 400 and 800 mg/kg had a statistical decrease in the concentration of oxidized proteins (69.49 and 59.29%, respectively), while for lipid peroxidation Sm treated arthritic animals exhibited a significant decrease in a non-dose dependent effect as well as in the oxidation of proteins, in which 400 mg/kg dose caused a greater decrease (80.87%) while higher tested dose 800 mg/kg generated a lesser antioxidant effect protecting lipid from oxidative damage (49.13%), when both groups were compared to CFA un-treated group of arthritic mice (**Table 2**).

Table 2. Effect of *Spirulina maxima* over the oxidative damage induced by CFA on sub-plantar edema tissue.

Treatments	Oxidized Proteins ($\mu\text{mol of CO}^*/\text{g tissue}$)	Lipid Peroxidation ($\mu\text{mol of MDA/g tissue}$)
CFA control	21.84 ± 1.87	2.24 ± 0.43
PBZ 100 mg/kg	12.73 ± 1.26^a (41.70%) ↓	0.63 ± 0.06^a (71.87%) ↓
Sm 400 mg/kg	6.66 ± 1.30^{ab} (69.49%) ↓	0.43 ± 0.04^{ab} (80.87%) ↓
Sm 800 mg/kg	6.51 ± 0.87^{ab} (59.29%) ↓	1.1 ± 0.11^{abc} (49.13%) ↓

Data is presented as mean \pm Standard error (SE); data in parentheses indicate the percent inhibition of oxidative process compared to CFA control at day 21. Statistical analysis one way ANOVA, SNK post hoc test ($P < 0.05$). ^a vs. CFA control; ^b vs. PBZ; ^c vs. Sm 400 mg/kg

CFA: Complete Freund's Adjuvant; PBZ: Phenylbutazone; Sm: *Spirulina maxima* (n=8)

In this context food supplements like *Spirulina maxima* has a large number of components with antioxidant activity, such as phycobiliproteins and unsaturated fatty acids like the gamma linolenic acid and these ones also have a suppressing effect over pro-inflammatory cytokines, such as TNF- α , IL-1 β , IL-6 and IL-12 [12,40,48]. By inhibiting these chemical mediators of the inflammation, the effect could be at different levels of the immune process, such as the generation of free radicals or the differentiation and maturation of T cells, that are characteristic of a chronic inflammatory process [46,49].

An increase of LPO and PCC indicates that there is an important and directly relation between the experimental monoarthritis onset and the OS, in which this last one helps in the maintenance and exacerbation of the chronic immune process [50]. During the development of this research work, arthritic animals treated with *Spirulina maxima*, had decreased their oxidized proteins levels, as also the rate of lipid peroxidation. This can be explained by the fact that this

alga contains several bioactive compounds, including C-phycocyanin, which possess great antioxidant activity reported by Romay et al. [51] and confirmed by Riss et al. [52]. Other substances with high antioxidant activity are beta carotene and gamma linolenic acid [41,42].

For the antioxidant response, those mice that were from the CFA control group exhibited an antioxidant enzymatic activity for SOD of $2257.05 \pm 1.96 \text{ IU/g tissue}$, CAT $60.01 \pm 0.03 \text{ UI/g tissue}$ and GSH-Px of $3.09 \pm 0.20 \mu\text{mol NADPH/g tissue}$, while PBZ-treated arthritic mice showed for SOD activity an increase of 80.53% ($p < 0.01$), for CAT enzyme a statistical decrease of 66.62% ($p < 0.05$) and for GSH-Px enzyme a significant decrease of 68.28% compared to CFA control group (**Table 3**). On the other hand, those arthritic mice treated with *Spirulina maxima* generated a regulation on the antioxidant enzymatic response in a no dose-dependent manner, where dose of 400 mg/kg showed a statistical decrease in SOD activity of 42.98% ($p < 0.05$), however for CAT and GSH-Px activities it generated a

significant increase of 3216% and 130%, respectively compared to CFA control group. Arthritic mice group treated with *S. maxima* at dose of 800 mg/kg showed a lower beneficial effect and different behavior compared to the previous described dose over the antioxidant response in

sub-plantar edema tissue with an increase for SOD enzyme activity (89.27%), and a decrease for CAT and GSH-Px activities of 49.94% and 35.28%, respectively when compared to CFA control group (Table 3).

Table 3. Effect of *Spirulina maxima* over the antioxidant response during chronic inflammation induced by CFA in sub-plantar edema tissue.

Treatments	Superoxide dismutase (UI/g tissue)	Catalase (UI/g tissue)	Glutathione peroxidase (μmol NADPH/g tissue)
CFA control	2257.05 ± 1.96	60.01 ± 0.03	3.09 ± 0.20
PBZ 100 mg/kg	4074.55 ± 6.32 ^a ↑ (80.53%)	20.03 ± 0.01 ^a ↓ (66.62%)	0.98 ± 0.25 ^a ↓ (68.28%)
Sm 400 mg/kg	1287.05 ± 4.70 ^{ab} ↓ (42.98%)	1990.01 ± 0.38 ^{ab} ↑ (3216%)	7.08 ± 0.10 ^{ab} ↑ (130%)
Sm 800 mg/kg	4272.05 ± 0.92 ^{ac} ↑ (89.27%)	30.04 ± 0.02 ^{abc} ↓ (49.94%)	2.00 ± 0.20 ^{abc} ↓ (35.28%)

Data is presented as mean ± Standard error (SE); data in parentheses indicate the percent inhibition of oxidative process compared to CFA control at day 21. Statistical analysis one way ANOVA, SNK post hoc test (P<0.05). ^a vs. CFA control; ^b vs. PBZ; ^c vs. Sm 400 mg/kg

CFA: Complete Freund's Adjuvant; PBZ: Phenylbutazone; Sm: *Spirulina maxima* (n=8)

This behavior is commonly known due to the existence of link between oxidative stress and inflammatory processes, where these last ones have an oxidative pathway for the pathogen elimination as well for the signaling during immune response, this occurs when the host organism could not regulate the inflammatory response so this triggers a chain of cellular and immune reactions which establish an oxidative stress and makes the pass from acute to chronic inflammation [53]. It has been proven that an increase in ROS amounts at articular level stimulates a greater expression of TNF-α in blood, favoring in this way the propagation of the inflammatory chronic response due to this interleukin acts as a chemo-activator of T lymphocytes and activated macrophages [54]. Also it has been proven that the antioxidant enzymatic defenses are compromised during a chronic inflammatory process such as arthritis, where levels of reduced glutathione (GSH) are below the normal values generated in articular tissue and also in systemic level [55].

In the articular tissue of arthritic mice treated with *Spirulina maxima* we found a significant decrease so much of oxidized proteins as well lipid peroxidation, and generated also an increase of both enzymes CAT and GSH-Px at dose of 400 mg/kg, while for SOD activity a decreased was observed, these results match to those published by Kuriakose and Kurup in 2011 [56], where a treatment with *S. laxissima* stimulates a greater antioxidant enzymatic response in which the only activity of SOD could not be consider as beneficial to the host organism since it only reduces the reactivity of the anion superoxide for a molecule of hydrogen peroxide

(which is still cytotoxic), so the coupled system of SOD-CAT or SOD-GSH-Px is needed.

This mechanism could be explained due to this observed antioxidant effect is generated by the cellular components of these cyanobacteria such as gamma linoleic acid which has demonstrated biological activities like antioxidant, anti-inflammatory and anti-proliferative [57,58]. These cyanobacteria also possesses an important content of amino acids like cysteine which is a precursor molecule of the GSH, which increase the levels of this compound in the host body, as well as glutathione reductase enzyme [59].

In the other hand, it's well-known that *S. maxima* has also a large number of essential minerals needed as enzymatic co-factors such as selenium [60]. Another important component of these cyanobacteria is the ferredoxin which are molecules in charge of reduce NADP to NADPH, important co-factor in the regulation of the organism's oxide-reduction environment [61].

The antioxidant activity of *S. maxima* has been widely studied and supported in diverse pre-clinical experimental animal models of oxidative stress where the beneficial effect of these cyanobacteria was reported previously, for example Canchihuaman et al. [62] in a lead-induced liver oxidative stress model, *S. maxima* treatment decrease lipid peroxidation and favored the antioxidant enzymatic activity in liver tissue.

For everything described previously, *S. maxima* capacity to generate an anti-inflammatory effect and antioxidant activity is due to the ability of its components to inhibit the pro-inflammatory cytokine TNF- α , which favored the immune response and also increases transcription of oxidative stress during inflammation via the NF- κ B pathway, as well the antioxidant effect of this cyanobacteria is due to its molecular and cellular components such as polyunsaturated fatty acids and antioxidant enzymes like glutathione reductase [54,63].

Finally through all the experiments 400 mg/kg was the best dose, which generated both anti-edematous effect in both acute and chronic models, increase BW gain in arthritic mice, and reducing oxidative damage. In previous works same dose of *Spirulina fusiformis*, showed beneficial effects in various chronic inflammation models induced by zymosan [64], collagen [39] and CFA-induced experimental arthritis in rats [26,65].

This beneficial effect at dose of 400 mg/kg is due may be to saturation on gastric (20%) and intestinal (80%) absorption of *Spirulina* constituents such as proteins and polyunsaturated acids which increase their bio-availability when this alga is orally consumed, nonetheless in previous published works this kind of biological effect is well-known as hormesis, where a bi-phasic dose-response is observed due to the phenomena of saturation in the absorption site, for this reason lower doses allow the perfect balance between absorption and blood concentration of the evaluated compounds, allowing their distribution in the body and increasing their bioavailability [66], however pharmacokinetics of *Spirulina* remain unknown and need further studies [63].

CONCLUSION

In conclusion due to what we have previously described and found in this research work, we demonstrated that, in fact exists a direct relationship between the antioxidant activity of *Spirulina maxima* and its anti-inflammatory properties *in vivo* on a located zone in which develops a chronic immune process, exhibiting a higher anti-inflammatory effect at dose of 400 mg/kg as well as a greater ability to regulate the oxidative stress develop within these immune processes. Thus *Spirulina maxima* could work as a co-adjuvant in the treatment of chronic degenerative diseases related to acute and chronic inflammation by inhibiting the oxidative damage over biomolecules and regulating the antioxidant enzymatic endogenous response in compromised tissue.

CONFLICT OF INTERESTS

The authors declare that there is no conflict of interest regarding the publication of this paper.

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