

# Chemopreventive and Antioxidant Effect of Polyphenol Free *Spirulina maxima* and Its Hydrolyzed Protein Content: Investigation on Azoxymethane Treated Mice

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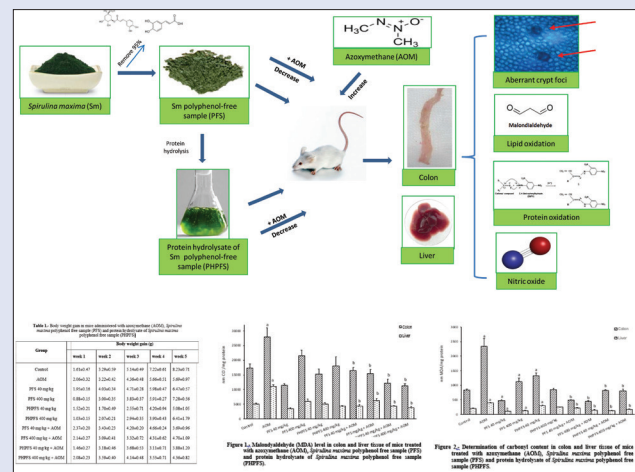
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## ABSTRACT

**Background:** *Spirulina maxima* (Sm) is known to have nutritive value as well as a number of potentially useful biomedical properties. **Objectives:** The initial purpose of this report was to evaluate the inhibitory effect of the alga (without its polyphenol content), on the induction of azoxymethane (AOM)-induced colon aberrant crypts (AC) in mouse. Besides, we hydrolyzed the protein content of such mixture. Our second aim was to determine the inhibitory potential of this last plant mixture on the AOM-induced colon AC in mouse. Moreover, we also determined the effect of the two indicated Sm samples on the oxidative damage caused by AOM in the colon and liver of treated mice. **Materials and Methods:** The experiment lasted 5 weeks. At the end, we registered the level of AC, nitric oxide, and the lipid and protein oxidation. **Results:** Our results showed the following: (1) the carcinogen increased more than 18 times the amount of the AC found in the control group. (2) On the contrary, the two tested mixtures of Sm produced a significant reduction over this damage (about 45%). (3) The two tested Sm mixtures were generally able to reduce the oxidative stress markers although with variable effects which go from 59% to 100% with respect to the control mice. **Conclusion:** Therefore, the present report established that the tested Sm fractions have mouse colon anticarcinogenic potential, partially related with their antioxidant capacity. Our report also suggested the need to further evaluate specific Sm chemicals as chemopreventive agents.

**Key words:** Antioxidation, Chemoprevention, Mouse, *Spirulina maxima* fractions



## INTRODUCTION

Cancer has been reported to be the cause of 12% of deaths worldwide, and colon cancer as the third most common type of cancer.<sup>[1]</sup> The incidence of colon cancer in Mexico has tripled in the last 10 years, and about seven patients are reported to die every day as a result of the disease.<sup>[2]</sup> In the last two decades, evidence has been provided regarding the importance of lifestyle, diet, and genetic factors in the development of the disease;<sup>[3]</sup> moreover, it has been observed that alterations in the mentioned aspects can be related with molecular and cellular disturbances which can induce genetic or epigenetic changes during the multistep process of carcinogenesis. Among these alterations, oxidative stress has been reported as one of the key factors in activating the disease, in light of the fact that high concentrations of reactive oxygen and nitrogen species can severely attack the structure and function of lipids, proteins, and DNA.<sup>[4,5]</sup> Moreover, the prolonged transit time of food chemicals in the colon also allow high cell exposure to oxidized molecules from different sources, thereby increasing the risk of oxidative stress and the induction of mutations in

the colon epithelial cells, which in further steps may contribute to the development of carcinogenesis.<sup>[6]</sup>

One of the first morphological manifestations during the process of colon carcinogenesis is the induction of aberrant crypts (AC). Such lesions, which can be observed as single or multiple crypts, have been validated as useful tissue biomarkers for studying both, carcinogenesis and its prevention.<sup>[7]</sup> These lesions are distinguished by their increased size, thicker epithelial lining, and increased pericryptal zone, and they can be observed in humans as well as in rodents.<sup>[8]</sup>

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Due to the high social and health impact of colon cancer, efforts to prevent, de-accelerate, or revert the disease have been developed using natural or synthetic agents. In these efforts, a number of agents with antioxidant properties have been used because of their capacity to trap free radicals with the concomitant inhibition of molecular or cellular damage. In these studies, a number of molecules have been identified with the capacity to inhibit genotoxic alterations and reduce carcinogenesis.<sup>[9-11]</sup> In this context, *Spirulina maxima* (Sm), a cyanobacteria with high nutritive value, has been reported to also possess a number of beneficial activities such as antiviral, antigenotoxic, antihypertensive, and antioxidant effects, among others.<sup>[12,13]</sup> By using this cyanobacteria, our laboratory demonstrated a significant inhibitory effect on the number of AC induced by AOM in mouse, as well as a reduction in the oxidative level of various biomolecules;<sup>[14]</sup> moreover, in another report we found an *in vitro* antioxidant effect of polyphenol-free Sm by applying three different techniques, and interestingly, in this study an increase of such activity was observed after a protein sequential hydrolysis with pepsin/pancreatin,<sup>[15]</sup> a finding which suggested the release of peptides with stronger antioxidant activity as previously reported by other authors.<sup>[16]</sup>

The present report is a continuation of studies regarding the biological actions of Sm. In this study we initially obtained an Sm polyphenol-free sample (PFS) in an attempt to eliminate, as much as possible, the participation of these chemicals in our results because of the well-documented effect of polyphenols on antioxidant and chemopreventive activities.<sup>[17,18]</sup> Then, the obtained mix was applied to AOM-treated mice in order to determine its preventive capacity on the induction of colon AC, as well as in the oxidative damage of lipids, proteins, and nitric oxide (in both colon and liver tissue). Our second aim was to enzymatically hydrolyze the proteins of the previously tested mix, a procedure that may increase the biological function of the obtained peptides;<sup>[19]</sup> then, we measured its potential for inhibiting the number of AC in AOM-treated mice, besides its capacity for inhibiting the oxidation of lipids, proteins, and nitric oxide in the colon and liver of the same animals.

## MATERIAL AND METHODS

### Chemicals, *Spirulina maxima*, and laboratory mice

AOM, pepsin, pancreatin, phosphate buffered saline (PBS), dimethyl sulfoxide (DMSO), Bradford reagent, AOM, thiobarbituric acid (TBA), trichloroacetic acid (TCA), 2,4-dinitrophenylhydrazine (DNPH), guanidine, sulfanilamide, and N-1-(naftil) etilendiamine dichloride were acquired from Sigma Chemicals (St Louis MO, USA). Ethyl acetate, formic acid, methanol, hydrochloric acid (HCl), and ethylene blue were purchased from Baker (Phillipsburg NJ, USA). Sm was obtained from Los Andes Co. (Quito, Ecuador). Its chemical composition was provided by the manufacturer and described in our previous report.<sup>[14]</sup>

For the assay we used 110 male mice (ICR) with a mean weight of 23 g (Harlan Laboratory, Mexico City). Mice were placed in polycarbonate cages at 24°C, 12 h dark-light cycles, 50% relative humidity, and with free access to water and food (Rodent Chow 5001, Purina). The experiment was approved by the Committee of Ethics and Biosecurity of the National School of Biological Sciences, and was started after a week of animal adaptation according to the previously indicated conditions.

### Preparation of Sm polyphenol-free sample

The elimination of polyphenols was carried out by means of six consecutive acetone extractions at 4°C under constant agitation. Each extraction, which lasted 30 min, was made with a sample-solvent relationship of 0.5--5 w/v. At the end of the process we eliminated the solvent. By using this procedure, we removed more than 95% of phenolic compounds.

### Protein hydrolysate of Sm polyphenol-free sample (PHPFS)

The obtained alga without phenols was treated with a sequential hydrolysis using pepsin and pancreatin.<sup>[20]</sup> The protein suspension in water (5% w/v) was adjusted to pH 2.5 with HCl. Pepsin was then added in a 1:20 w/w enzyme--substrate relationship. After 90 min of reaction, the pH was modified to 7.5 with NaOH, and pancreatin was added in the same proportion as that previously used for pepsin. Temperature along the process was maintained at 37°C. Finally, the obtained suspension was lyophilized.

### Experimental design in mice

For a 5-week assay, 10 groups with 11 animals each were organized. In the control group, besides the ingestion of the standard food and water, mice received no chemical during the assay. The next groups that were similarly fed and allowed to drink water received specific agents, in this case, a second group was intraperitoneally (ip) injected AOM (5 mg/kg) twice a week, in weeks 3 and 4 of the assay; two other groups were intragastrically (ig) administered PFS everyday with the doses of 40 and 400 mg/kg, respectively; two other groups were ig administered PHPFS everyday 40 and 400 mg/kg, respectively; two more groups were also ig administered PFS everyday with the doses of 40 and 400 mg/kg, however, in these groups we also ip administered AOM (5 mg/kg) twice a week in weeks 3 and 4 of the assay; finally the last two groups were ig administered PHPFS daily with the doses of 40 and 400 mg/kg, respectively, plus ip injected AOM twice a week in weeks 3 and 4 of the study.

The aforementioned experimental groups were designed to provide information concerning the effect of the carcinogen (AOM) on the number of AC and on the level of molecular oxidation, and consequently, to detect a deleterious effect with which the protective effect of PFS and PHPFS could be compared. In our assay, the tested low dose of the alga was based on recommendations as to its daily consumption by humans,<sup>[21]</sup> whereas the high dose corresponded to the therapeutic range determined by various authors.<sup>[22]</sup> In our experiment, the weight of the animals was registered every other day.

At the end of the study, mice were cervically dislocated, and the liver and colon dissected for their analysis. Five mice of each group were used to determine total protein content, lipid and protein oxidation, and nitric oxide. For the first two analyses, tissue homogenate from liver and colon was prepared with PBS 0.01 M at pH 7.4 in a 1:10 ratio, and for the determination of nitric oxide a small portion of colon and liver was placed in fetal bovine serum with 15% DMSO and frozen at -70°C until use. The other six animals per group were used to examine the induction of colon crypts along the whole organ.

### Quantification of AC

For the quantification of AC we generally followed the procedure described by Bird.<sup>[23]</sup> The colon of each mouse was longitudinally opened, extended, and fixed with pins in the bottom of Petri dishes containing solidified paraffin, then, each colon was immersed in 10% formaldehyde made in PBS 0.01 M at pH 7.4, placed in the Petri dish with mucosal side up, and left for 24 h at room temperature. After 24 h, the tissue was stained for 20 min with methylene blue 0.25% made in PBS 0.01 M at pH of 7.4, and placed in a Petri dish with the mucosal side up. Then, the number and type of AC (single or multiple) were identified with a Nikon Eclipse 80 (Nikon Co., Japan) at a 100× magnification.

### Total protein determination

For this determination, we followed the method described by Bradford.<sup>[24]</sup> Briefly, 100 µL of homogenate (colon or liver tissue) were centrifuged at

9000 rpm for 10 min, 10  $\mu\text{L}$  of the obtained supernatant were mixed with 90  $\mu\text{L}$  of deionized water and 2.5 mL of Bradford's reagent after which the mix was agitated for 5 min. Samples were spectrophotometrically read at 595 nm against a blank made with 100  $\mu\text{L}$  of deionized water plus 2.5 mL of Bradford's reagent. The results were interpolated in a bovine serum albumin standard curve (0.1–1.0 mg/mL) and expressed as mg protein/g tissue.

### Determination of lipid peroxidation

This determination was registered in terms of TBA reactive substances (TBARS) according to the method of Buege and Aust.<sup>[25]</sup> To 500  $\mu\text{L}$  of homogenate from each tissue we added 2 mL of the reaction mixture (TCA–TBA–HCl) at 15% w/v, 0.375 w/v, and 0.25 N, respectively. The mix was boiled for 15 min, cooled in an ice bath for 10 min and centrifuged at 4000 rpm for 10 min. Then, the supernatant was spectrophotometrically read at 532 nm against a reference blank. The concentration of malondialdehyde (MDA) was calculated by using an extinction coefficient of  $1.56 \times 10^5 \text{ M}^{-1} \text{ cm}^{-1}$ . The results were expressed as nmol MDA/mg protein.

### Determination of oxidized proteins

This measurement was made through the quantification of the reactive carbonyl content according to the method of Levine *et al.*<sup>[26]</sup> We added 500  $\mu\text{L}$  of DNPH (10 mM in HCl 2 M) to 200  $\mu\text{L}$  of the tissue homogenate. The mixture was placed at room temperature in the dark for 1 h, and the generated hydrazones were precipitated with 500  $\mu\text{L}$  of 20% TCA. Each sample was centrifuged three times at 9000 rpm for 10 min; each time, the suspension was washed with 1 mL of ethyl acetate–ethanol 1:1. The pellet was resuspended in 1 mL of hydrochlorate guanidine 6 M, incubated at 37°C for 15 min, and centrifuged at 9000 rpm for 10 min. For each sample, we concurrently followed the same procedure with a blank incubated with 500  $\mu\text{L}$  of HCl 2M without DNPH. The carbonyl content was spectrophotometrically registered in a range from 350 nm to 375 nm, and its concentration was calculated by using  $22000 \text{ M}^{-1} \text{ cm}^{-1}$  as the coefficient of molar absorbance. Results were expressed as nmol of CO/mg protein.

### Nitric oxide determination

The tissue that was previously kept at -70°C was unfrozen and washed with cold PBS 0.01 M at pH 7.4. From each sample we prepared a homogenate (1:4) by adding cold PBS. The mix was centrifuged for 20 min at 4000 rpm and the supernatant was treated with the Griess reaction to determine the concentration of nitrites.<sup>[27]</sup> For this purpose, 300  $\mu\text{L}$  of the Griess reactive plus 600  $\mu\text{L}$  of distilled water were added to 100  $\mu\text{L}$  of the obtained supernatant. The mixture was then measured at 540 nm. As standard we used  $\text{NaNO}_2$  0.1 Mm in a range from 0.9  $\mu\text{mol}$  to 10  $\mu\text{mol}$ . The results were expressed as  $\mu\text{mol}$  of nitrite/g of tissue.

### Statistical analysis

Statistical analysis for the weight data was carried out with a two-way ANOVA followed by the Holm–Sidak test. In the case of the three different oxidative/antioxidative assays and the evaluation of AC number, we used a one-way ANOVA followed by the Student–Newman–Keuls test. The determinations were made in a SPSS 11.0.1 computer program (SPSS Statistical Software Inc, Chicago, IL, USA).

## RESULTS

### Weight gain

At the beginning of the assay the weight of the animals was  $24.21 \pm 0.67$ . During the experiment all groups showed a continuous weight gain, although the control and those administered with the two samples alone

showed the better interweek increase. Noteworthy was the fact that the weight of the AOM-treated group did not diminish during the period of its administration. [Table 1] presents the body weight gain registered throughout the 5-week treatment, and shows that the lower increase was determined in the groups treated with the two tested mixtures together with AOM.

### Colon crypts

The number of AC produced with AOM, as well as its reduction by the tested PFS and PHPFS are shown in Table 2. In control animals a low level of all registered lesions was observed, a level, however, which was significantly elevated in mice receiving AOM. Table 2 also clearly shows a lack of AC inducing potential by both tested Sm fractions. In fact, the results determined for the two different mixtures (with the two doses), were in the range detected for the control mice, with a mean of  $3.7 \pm 0.4$  AC. With respect to the low dose of PFS plus AOM, we determined a protection of 20% in comparison with the damaging effect of the carcinogen, and with the high dose of PFS the protective effect on the carcinogen activity was of 73%. In regards to PHPFS, the observed protection was significant with the two tested doses, showing a more marked effect (56%) with the low dose.

In regard to multiple crypts, notably AOM was the more deleterious followed by PFS plus AOM which, interestingly, was also the less effective in preventing the damage. As to the distribution of AC along the colon we observed that they were mainly distributed in the median and distal parts of the organ (82%), a finding that has also been reported by other authors.<sup>[28,29]</sup>

### Oxidative stress

Results of the lipid peroxidation obtained in colon and liver tissue are indicated in Figure 1. Regarding this parameter, a significant increase was found due to the action of AOM in comparison with the control level. In mice treated with the carcinogen, at least one duplication of the basal effect was reached, although in colon the increase was more marked. Concerning the effect of the two Sm samples, we found that the high dose of PFS and the low dose of PFPH induced a certain increase of lipoperoxidation in the colon over the control level. Most of the other groups of mice, including those treated with the two Sm mixtures plus the carcinogen were found in a level similar or even lower to the one determined in control data, indicating a strong antioxidant capacity of the tested mixtures, which could reach 100% effectiveness over the AOM treated animals.

With respect to protein oxidation we found at least one duplication of the amount of reactive carbonyl content induced by AOM in comparison with the result obtained in control animals, an effect that was observed in both, colon and liver tissues [Figure 2]. In regard to the Sm fractions tested alone, data obtained in both organs showed values that were around the range registered for the control mice except for 400 mg/kg of PFS in the colon. Our results showed a significant reduction of the carbonyl content in regards the effect of the two tested Sm mixtures against the AOM treated mice. In general, it could reach around 100% effectiveness over the AOM treated animals, resulting in a better effect with the PHPFS fraction.

Figure 3 shows the results obtained with respect to the quantification of nitrites in colon and liver. This indirect measurement of nitric oxide showed a significant induction of the biomarker by AOM in the two organs. Regarding the two Sm samples, when tested alone, PFS showed no increase of nitrites; however, the administration of PHPFS produced a significant increase in the colon and a lower but clear increase in the liver. Nevertheless, when the effect of the two mixtures was tested over AOM-treated mice, except for PFS at 40 mg/kg, in all other groups we found a significant inhibition in the level of nitrites which goes from 58.9% to 100%.



**Table 1:** Body weight gain in mice administered azoxymethane (AOM), *Spirulina maxima* polyphenol free-sample (PFS), and protein hydrolyzate of *Spirulina maxima* polyphenol free-sample (PHPFS)

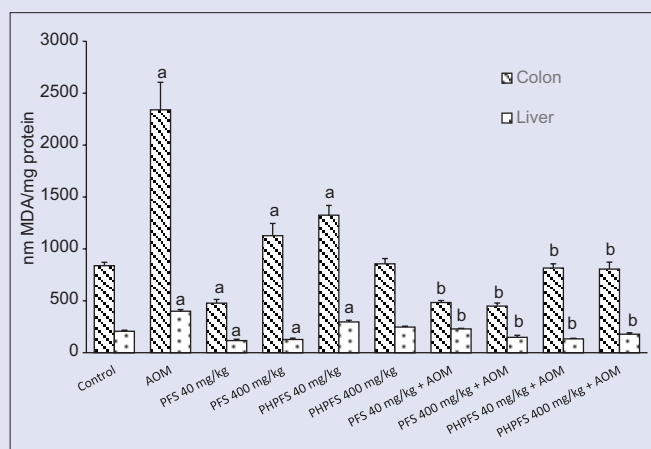
Group	Body weight gain (g)				
	Week 1	Week 2	Week 3	Week 4	Week 5
Control	1.61±0.47	3.29±0.59	5.14±0.49	7.22±0.61	8.23±0.71
AOM	2.06±0.32	3.22±0.42	4.36±0.48	5.66±0.51	5.69±0.97
PFS 40 mg/kg	1.95±0.16	4.03±0.34	4.71±0.28	5.98±0.47	6.47±0.57
PFS 400 mg/kg	0.88±0.15	3.00±0.35	3.83±0.37	5.91±0.27	7.28±0.56
PHPFS 40 mg/kg	1.52±0.21	1.70±0.49	2.55±0.71	4.20±0.94	5.08±1.05
PHPFS 400 mg/kg	1.03±0.15	2.07±0.21	2.94±0.35	3.90±0.43	6.41±1.79
PFS 40 mg/kg + AOM	2.37±0.20	3.43±0.23	4.20±0.20	4.66±0.24	3.69±0.96
PFS 400 mg/kg + AOM	2.14±0.27	3.09±0.41	3.32±0.72	4.31±0.62	4.70±1.09
PHPFS 40 mg/kg + AOM	1.46±0.27	3.18±0.46	3.68±0.53	3.11±0.71	3.88±1.20
PHPFS 400 mg/kg + AOM	2.08±0.23	3.59±0.40	4.14±0.48	3.53±0.71	4.36±0.82

Weight gain was calculated as the difference between the initial body weight and the weight achieved in each treatment week. Each value represent the mean±SD registered in five mice per group. Statistical analyses were carried out to compare the treated groups vs. controls at the same week of treatment. No significant differences were found with a two-way ANOVA followed by the Holm-Sidak test

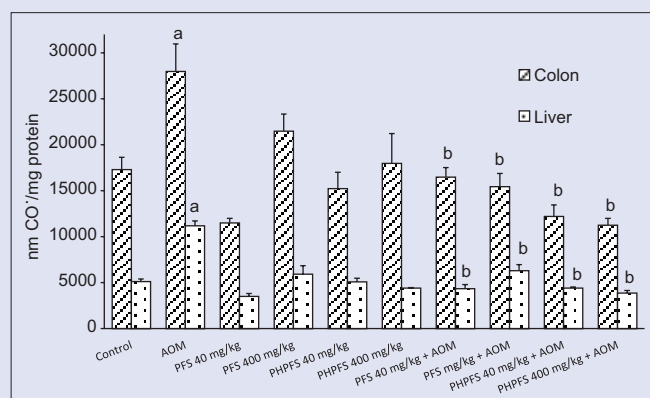
**Table 2:** Colon aberrant crypts (AC) induced in mice with azoxymethane (AOM), *Spirulina maxima* polyphenol-free sample (PFS), and protein hydrolyzate of *Spirulina maxima* polyphenol-free sample (PHPFS)

Group	AC/colon	1	2	3	4
Control	3.67±0.42	3.67±0.42			
AOM	68.50±4.40 <sup>a</sup>	44.83±2.68 <sup>a</sup>	9.67±1.45 <sup>a</sup>	1.17±0.17 <sup>a</sup>	0.17±0.1 <sup>a</sup>
PFS 40 mg/kg	4.5±0.43 <sup>b</sup>	4.5±0.43 <sup>b</sup>			
PFS 400 mg/kg	3.0±0.37 <sup>b</sup>	3.0±0.37 <sup>b</sup>			
PHPFS 40 mg/kg	3.17±0.31 <sup>b</sup>	3.17±0.31 <sup>b</sup>			
PHPFS 400 mg/kg	4.83±0.31 <sup>b</sup>	4.83±0.31 <sup>b</sup>			
PFS 40 mg/kg + AOM	55.50±2.32 <sup>ab</sup>	49.67±2.64 <sup>ab</sup>	2.67±0.80 <sup>b</sup>	0.41±0.17	
PFS 400 mg/kg + AOM	18.17±1.64 <sup>ab</sup>	17.83±1.51 <sup>ab</sup>	0.17±0.1 <sup>b</sup>		
PHPFS 40 mg/kg + AOM	30.17±2.63 <sup>ab</sup>	29.83±2.55 <sup>ab</sup>	0.17±0.1 <sup>b</sup>		
PHPFS 400 mg/kg + AOM	45.17±1.85 <sup>ab</sup>	42.83±2.39 <sup>ab</sup>	1.17±0.54 <sup>b</sup>		

Each value represent the mean±SD from five mice per group, <sup>a</sup>statistically significant in comparison with the control group, and <sup>b</sup>with respect to the AOM treated group. One way ANOVA and Student-Newman-Keuls test was used for statistical analysis, P<0.05



**Figure 1:** Malondialdehyde (MDA) level in colon and liver tissue of mice treated with azoxymethane (AOM), *Spirulina maxima* polyphenol-free sample (PFS) and protein hydrolyzate of *Spirulina maxima* polyphenol-free sample (PHPFS). Each bar corresponds to the mean ±SDM of five mice per group, <sup>a</sup>statistically significant difference with respect to the control value, and <sup>b</sup>with respect to the AOM treated group. One way ANOVA and Student-NewmanKeuls tests, P < 0.05

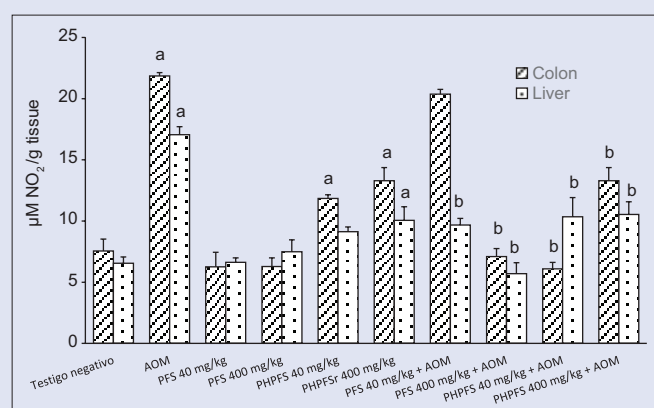


**Figure 2:** Determination of carbonyl content in colon and liver tissue of mice treated with azoxymethane (AOM), *Spirulina maxima* polyphenol-free sample (PFS) and protein hydrolyzate of *Spirulina maxima* polyphenol-free sample (PHPFS). Each bar corresponds to the mean ±SDM of five mice per group, <sup>a</sup>statistically significant difference with respect to the control value, and <sup>b</sup>with respect to the AOM treated group. One way ANOVA and Student-Newman-Keuls tests, P < 0.05

## DISCUSSION

Plants belonging to lower or higher taxonomical categories have demonstrated a variety of biomedical properties. In the case of *Spirulina*,

this highly nutritive alga has also shown important biological qualities with potential medical application, including a chemopreventive effect. In this last field, a significant inhibitory effect of Sm has been reported on the colon AC induced by AOM in mouse.<sup>[14]</sup> Such result was obtained in a short, 4-week assay, where authors found a mean reduction of 51.6%



**Figure 3:** Nitrite content in the colon and liver tissue of mice treated with azoxymethane (AOM), *Spirulina maxima* polyphenol-free sample (PFS), and protein hydrolysate of *Spirulina maxima* polyphenol-free sample (PHPFS). Each bar corresponds to the mean  $\pm$  SDM of five mice per group, <sup>a</sup>statistically significant difference with respect to the control value, and <sup>b</sup>with respect to the AOM treated group. One way ANOVA and Student–Newman–Keuls tests,  $P < 0.05$

on the crypts produced with AOM; moreover, the authors also found a significant decrease exerted by the alga on the oxidative damage induced by AOM in lipids, and in DNA. Therefore, the mentioned report is a clear antecedent to the present study. Chen *et al.*<sup>[30]</sup> also reported an inhibitory effect of *Spirulina* on rat colon AC, although with variable response along the evaluated time, and Mathew *et al.*<sup>[31]</sup> as well as Shwartz *et al.*<sup>[32]</sup> studied the preventive potential of *Spirulina* in animal and human oral cancer. More recently there has been manifested interest in determining the effect of *Spirulina* constituents. For example, polysaccharides from *Spirulina platensis* have been studied and shown to inhibit the proliferation of ascitic hepatoma cells as well as to reduce lung metastasis of B16-BL6 melanoma cells.<sup>[33,34]</sup> Studies made on the effect of one of the main proteins of the alga, phycocyanin, have demonstrated an inhibitory effect on the growth of human leukemia K562 cells, as well as on the number of AC induced by 1, 2-dimethylhydrazine dihydrochloride (DMH) in rats.<sup>[35,36]</sup> These reports confirm the relevance of continuing research regarding the chemopreventive effect of different parts or specific constituents of the alga.

The carcinogen AOM is usually administered at higher doses than the one used in the present report (5 mg/kg) to initiate colon carcinogenesis; however, in our study we confirmed its capacity to induce mouse colon crypts with such low dose, as has also been demonstrated by other authors.<sup>[37,38]</sup> The number of crypts registered in our assay was in the range of the mentioned reports. However, interestingly in our 5-week assay, single crypts were mostly induced, in contrast with more multiple crypts found by the mentioned authors in assays with at least 2 more weeks of duration. In fact, both results are congruent with the histological evolution of colon cancer. Besides, in spite of the fact that AOM is known as a colon-specific carcinogen, it is metabolized in the liver; therefore, we deemed it relevant to evaluate its oxidative potential in both, colon and liver tissue.

In regard to *Spirulina*, the first aim of the present assay was to provide information on the chemopreventive capacity of the selected fractions. The removal of polyphenols in the first fraction is sustained because of the well-documented knowledge concerning their antioxidant and chemopreventive capacity.<sup>[39]</sup> These authors describe polyphenols as a large group of substances that include phenolic acids, flavonoids, stilbenes, and lignans, and they show that even in a single step, in the first (initiation) phase of carcinogenesis, they may act through the inhibition of

procarcinogens, by intercepting procarcinogens, inhibiting procarcinogen activation, inducing the cytochrome CYP1A2, or by modulating phase-II biotransformation enzymes; moreover, these agents may also act on apoptosis or cell proliferation processes, among other actions.<sup>[18,40]</sup> Without these chemicals, our results showed significant protection against the AC induced by AOM, mainly with the high-tested dose (400 mg/kg). This effect, however, may be attributed to a number of chemicals, including polysaccharides, vitamins, chlorophyll, or proteins, or to a synergism among them. In fact, some of these chemicals when tested alone have previously manifested antioxidant or chemopreventive effects.<sup>[33–36]</sup>

Our other approach was to concentrate the polyphenol-free protein from the algae and generate fragmentation through a sequential hydrolytic process. The rationale for such procedure lies in the observation that by experimentally mimicking the digestive process, peptides and amino acids of different size and composition with strong beneficial properties have been obtained.<sup>[41]</sup> With this strategy, we observed a significant reduction in the damage induced by AOM with the two tested doses.

However, even though the two tested SM fractions showed a significant chemopreventive potential, no clear difference in their effect could be observed, suggesting that more doses should be tested, in a wider range, so as to provide a clearer conclusion on this point; this is advisable, moreover, because other authors have demonstrated strong anticancer and antioxidant effects of whey, soy, cheese, or egg protein hydrolyzates in comparison with the nonhydrolyzated protein.<sup>[42–45]</sup>

The protective effect of *Spirulina* has been related to various lines of action including genetic and epigenetic changes that affect the signaling for proliferation, apoptosis, cell cycle, or those that increase the blocking or capture of oxidative molecules which may produce DNA and cell damage, thereby contributing to the reduction of the carcinogenic risk.<sup>[46–48]</sup> Our assay is in line with this last effect because of the observed decrease exerted by the two Sm samples on the oxidative potency of lipids, proteins, and nitric oxide, which suggested that severe molecular and cellular damage could be inhibited reducing the risk of carcinogenesis. This could be done, for example, by inhibiting the action of MDA and other reactive metabolites that are known to interact with DNA and alter its structure and functioning<sup>[49]</sup> by decreasing protein oxidation, and in turn, avoiding its abnormal folding, loss of function, aggregation, and cell death,<sup>[50]</sup> or by inhibiting nitric oxide, which in sustainable amounts generated by overexpression of inducible nitric oxide synthase, can cause DNA damage or impairment of DNA repair.<sup>[51]</sup> Nevertheless, we found it interesting to note some oxidative potential by the tested Sm, a finding that could be related with the pro-oxidant effect exerted by one or more components of the alga or to the liberation of peptides/amino acids with this property. Although the alga is usually reported as lacking any oxidant potential, the present data was similar to that found in our previous report,<sup>[14]</sup> suggesting then, that this aspect deserves a particular approach.

## CONCLUSION

Our study demonstrated the antiprecarcinogenic activity of the two tested Sm mixtures, and suggested that a number of its constituents may have synergistically participated in the preventive action. Besides, we also determined the antioxidant potential of the two fractions which indicated that this effect may be involved in its chemoprevention. However, as no clear dose response was observed, and an oxidative effect was registered for Sm, widening the range of tested doses seems pertinent in order to reach a more trustworthy conclusion on this point, before, or parallel to, evaluation of the effect by specific chemicals in the studied Sm mixtures.

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## Conflicts of Interest

There are no conflicts of interest.

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