

# Inhibitory Effect of *Spirulina maxima* on the Azoxymethane-induced Aberrant Colon Crypts and Oxidative Damage in Mice

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## ABSTRACT

**Background:** *Spirulina maxima* (Sm) is a cyanobacterium well known because of its high nutritive value, as well as its anti-inflammatory, anti-hyperlipidemic, antioxidant, and anti-genotoxic activities.

**Objective:** To determine the capacity of Sm to inhibit the induction of aberrant colon crypts (AC), as well as the level of lipid peroxidation and DNA oxidative damage in mice treated with azoxymethane (AOM).

**Materials and Methods:** Sm (100, 400, and 800 mg/kg) was daily administered to animals by the oral route during 4 weeks, while AOM (10 mg/kg) was intraperitoneally injected to mice twice in weeks 2 and 3 of the assay. We also included a control group of mice orally administered with distilled water along the assay, as well as other group orally administered with the high dose of Sm. **Results:** A significant decrease in the number of AC with the three tested doses of Sm, with a mean protection of 51.6% respect to the damage induced by AOM. Also, with the three doses of the alga, we found a reduction in the level of lipoperoxidation, as well as in regard to the percentage of the DNA adduct 8-hydroxy-2'-deoxyguanosine.

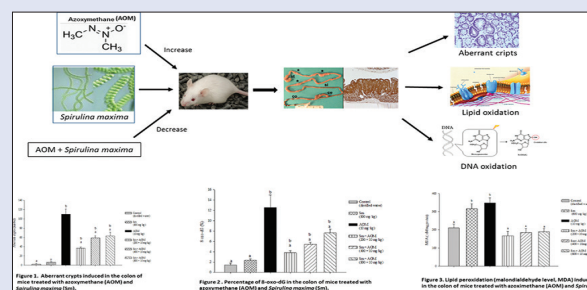
**Conclusion:** Sm possesses anti-precarcinogenic potential *in vivo*, as well as capacity to reduce the oxidative damage induced by AOM.

**Key words:** Azoxymethane, chemoprevention, colon damage, mice, *Spirulina*

## SUMMARY

- Azoxymethane (AOM) induced a high number of colon aberrant crypts in mouse. It also increased the level of peroxidation and of DNA oxidation in the same organ.

- Spirulina maxima* significantly reduced the number of AOM-induced colon aberrant crypts in mouse. It also reduced the AOM-induced lipid and DNA oxidation in mouse.
- The results suggest a chemopreventive potential for the tested algae.



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## INTRODUCTION

Studies have thoroughly found that colorectal cancer is one of the main causes of morbidity and mortality in most of the countries. This malignancy has been considered as the third most frequent and the fourth leading cause of death from cancer worldwide.<sup>[1]</sup> This information is in line with the fact that lifestyle, dietary factors, and genetic influences are major contributors for its development, but also that modifications of carcinogenic factors coupled with the implementation of chemoprevention are important targets for the future approach to manage such disease.

The strategy of chemoprevention in colon cancer is in close relationship with the development of biomarkers of risk, as well as of those for early detection. In this context, the validation of a number of genetic, epigenetic, and protein markers is in progress.<sup>[2,3]</sup> Likewise, it is known that molecular damage may result in increased proliferation of normal crypts that can give rise to the well described aberrant crypts (AC) or AC foci; structures that have been identified as preneoplastic lesions formed during the histopathological development of cancer and are clearly recognized as microscopically elevated crypts above the normal colonic

mucosa, with thickened epithelia, altered luminal openings, and they are circumscribed from adjacent normal crypts.<sup>[4]</sup>

Azoxymethane (AOM) is an alkylating agent specifically used to induce mouse and rat colon carcinogenesis through the initial expression of AC. This chemical is initially transformed by cytochrome P450 2E1 to methylazoxymethanol, and then the glucuronic conjugates of such compound are hydroxylated in the mucosa of the colon and

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metabolized by bacterial flora to the biomolecules damaging agents like methyl diazonium and methyl carbonium ion.<sup>[5,6]</sup>

In the pathogenesis of colon cancer, reactive oxygen species (ROS) such as oxygen, hydroxyl radicals, and hydrogen peroxide may play a significant role, and AOM is known to be able to induce such an effect, for example, by increasing the lipoperoxidation and inducible nitric oxide synthase levels as well as by reducing the level of glutation.<sup>[7]</sup> However, as colon carcinogenesis is a multifactorial process, other events occur, such as genetic and epigenetic damages, as well as alterations in proliferative, inflammatory or apoptotic events.<sup>[8]</sup>

*Spirulina maxima* (Sm) is a microscopic filamentous cyanobacterium, well known because of its high nutritive value, its relative absence of toxicity in a number of assays, and because of its beneficial biomedical effects observed in different *in vitro* and *in vivo* studies. For the present report, it is relevant to mention that *Spirulina* has shown anti-inflammatory and anti-hyperlipidemic properties, as well as antioxidant, anti-genotoxic, and anti-neoplastic activities.<sup>[9-11]</sup>

Based on the aforementioned information, we made the present study with two main purposes: Initially, to determine the capacity of Sm for inhibiting the development of AC in the colon of AOM treated mice, and secondly, to evaluate whether such an effect could be related, at least partially, with the antioxidant potential of the cyanobacterium; for this last purpose, we evaluated its effect on the level of lipid peroxidation, as well as on the formation of the DNA adduct, 8-hydroxy-2'-deoxyguanosine (8-oxo-dG) in the colon of AOM treated mice.

## MATERIAL AND METHODS

### Chemicals and animals

The following chemicals were purchased from Sigma Chemicals (St Louis MO, USA): AOM, phosphate buffer saline (PBS), proteinase K, ribonuclease (RNase), trichloroacetic acid (TCA), trizma base, albumin standard, thiobarbituric acid (TBA), and methylene blue. Anti-8-oxo-dG (4354-MC-050) was purchased from Trevigen, Inc. (Gaithersburg, Md., USA). Formaldehyde, HCl, NaCl, sodium citrate, xylene, and ethanol were acquired from Fermont (Mexico City, México).

Sm was supplied by Alimentos Esenciales para la Humanidad, S. A. de C. V. (Mexico City, México). The algae corresponded to the bulk production batch SDW-9714, of standardized quality. In the present report, we show a summary of the chemical composition provided by the manufacturer [Table 1]. However, other phytochemicals with interesting properties such as different polyphenols have also been found in the alga.<sup>[12]</sup>

The study was made in 90 Swiss Webster male mice with a mean weight of 28.5 g. The animals were placed in metallic cages at 22°C ± 2°C, 50–60% relative humidity and in a 12 h dark-light cycle. Mice were permitted to freely consume food (Rodent Lab Chow 6800, Purina) and water.

### Experimental protocol

The experiment was approved by the Committee of Ethics of the National School of Biological Sciences. Six groups with 15 mice each were organized for the assay and treated as follows: One group was daily administered by the oral route with 0.3 ml of distilled water during the 4 weeks of the assay; another group was intraperitoneally (ip) injected AOM (10 mg/kg) twice a week; the 2<sup>nd</sup> and 3<sup>rd</sup> week of the assay, three other groups were daily administered by the oral route with 100, 400, and 800 mg/kg of Sm each during the 4 weeks of the assay, and they were also ip injected 10 mg/kg of AOM, twice a week, in the 2<sup>nd</sup> and 3<sup>rd</sup> week of the assay; finally, a last group was daily administered by the oral route with the high tested dose of Sm (800 mg/kg). Sm was suspended, and AOM diluted in distilled water. The used model has been reported to be efficient

**Table 1:** Chemical constituents in *Spirulina maxima*

Compound	Amount per 100 g
Protein	65 g
Total lipids	6 g
Saturated fat	0 g
Monounsaturated fat	0 g
Polyunsaturated fat	6 g
Cholesterol	0 g
Carbohydrates	16.4 g
Sodium	900 mg
Calcium	1 g
Phosphorous	800 mg
Magnesium	400 mg
Iron	150 mg
Potassium	1.4 g
Zinc	3 mg
Beta carotene	201 mg
Vitamin E	10 mg
Vitamin B1	3.5 mg
Vitamin B2	4 mg
Vitamin B3	14 mg
Vitamin B5	0.1 mg
Vitamin B6	0.8 mg
Vitamin B12	0.25 mg
Inositol	64 mg
Phycocyanin	15 mg
Chlorophyll	1.1 mg
Carotenes	0.37 mg
Gamma-linolenic acid	1000 mg
Alpha-linolenic acid	800 mg

for the induction of AC with AOM, as well as for its chemoprevention,<sup>[13]</sup> moreover, the short time of the assay may be advantageous when no further progress of carcinogenesis is required in the study.

### Determination of aberrant crypts

At the end of the indicated treatment, mice were cervically dissected, their colon dissected and washed in distilled water. The AC determination was made in eight animals per group. For this purpose, we extended each colon in a Petri dish with solidified paraffin at the bottom and fixed it in 10% formaldehyde for 24 h. Then, the organs were stained with methylene blue (diluted in PBS) for 15 min, and the number (single or multiple) AC, as well as their distribution, was registered at a ×100.

### Immunohistochemical determination of 8-hydroxy-2'-deoxyguanosine

We follow the method described by Moreira *et al.*<sup>[14]</sup> with some modifications. Colon tissue sections, 3 µm thick, were deparaffinized and hydrated gradually. Each sample was incubated with proteinase K (50 µg/ml, 1-h at 37°C). Then, the sections were incubated with RNase A 100 µg/ml containing 150 mM NaCl and 15 mM sodium citrate at 37°C for 1-h. The tissue was washed twice with PBS, and after the DNA

denaturation with HCl 2 N for 5 min, the process was neutralized with trizma base 1 M for 5 min, and the tissue was washed twice in PBS. Nonspecific binding was blocked by incubating the slides for 60 min in 10% goat serum in PBS. Primary antibodies anti-8-oxo-dG diluted 1:250 were incubated overnight at 4°C followed by secondary antibody immunoglobulin, fluorescein-linked whole antibody at 37°C for 2 h. Tissue images were captured by an epifluorescent microscope (Axioskop 50 C. Zeiss) equipped with a filter ( $\gamma = 365\text{--}395$  nm) for detecting 4',6-diamidino-2-phenylindole blue staining nuclei, and another ( $\gamma = 450\text{--}490$  nm) for detecting the 8-oxo-dG adducts by the fluorescein isothiocyanate green color. Fluorescent samples were analyzed in a Zeiss-510 laser scanning confocal microscope (Carl Zeiss, Inc. Jena, Germany) to confirm the coexistence of 8-oxo-dG in the nucleus. After confirming the co-localization patterns, we captured tissue images through an optical microscope (Olympus 1 × 70, Olympus Europe GmbH, Hamburg, Germany). Then, tissue images were quantified in ten randomly selected fields (×1000) per individual sample. Adduct positive nuclei were quantified with respect to the total number of nuclei using image analysis software (Analysis Soft Imaging System GmbH) (Soft Imaging System GmbH Analysis<sup>®</sup>, Münster, Germany).

### Determination of lipid peroxidation

Lipid peroxidation was assessed by measuring malondialdehyde (MDA) content as described by Buege and Aust,<sup>[15]</sup> with minor modifications, in the colons of the seven mice which were not used in the previous studies. Briefly, 100 mg of each colon was homogenized in 1 ml of cold PBS (pH 7.0), and 0.5 ml of each sample was added to 1 ml of a reagent containing TCA, TBA and HCl (15% w/v, 0.375% w/v and 0.25 N, respectively). The solution was then heated for 30 min in a boiling water bath. After cooling, the flocculent precipitate was removed by centrifugation at 1000 g for 10 min. A Bio-Mini Shimadzu spectrophotometer was used to determine absorbance at 532 nm. A sample containing reagent only was used as blank. The MDA content was calculated using an extinction coefficient of  $1.56 \times 10^{-5} \text{ m}^{-1}/\text{cm}$ . The protein content was determined using the Coomassie blue method,<sup>[16]</sup> with Bovine serum albumin as standard.

### Statistical analysis

Statistical significance regarding AC and lipoperoxidation data was obtained by applying an analysis of variance (ANOVA) followed by the Holm–Sidak test; in the case of 8-oxo-dG results, an ANOVA and the Student–Newman–Keuls tests were applied. For this purpose, we used the software SigmaStat version 3.4 (Systat Software, Inc. California, USA).

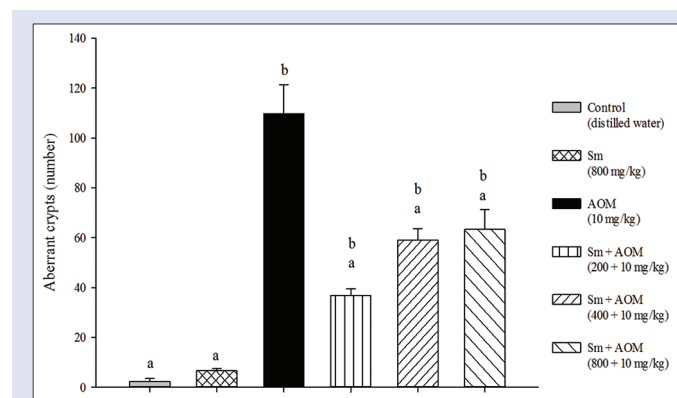
## RESULTS

In regard to weight, control mice, as well as those treated only with Sm, had a moderate but constant increase along the assay, showing an elevation of about 5 g at the end of the experiment (from 27.4 g to 32.4 g in the control group, and from 27.4 g to 33.2 g in the Sm treated group). The other three groups, however, suffered a detainment in their weight gain at the 3<sup>rd</sup> week of the experiment, an effect that seemed to be related to the AOM toxicity; therefore, the mean weight in these groups at the end of the study (29.3 g) was similar to that registered at the beginning (28.2 g).

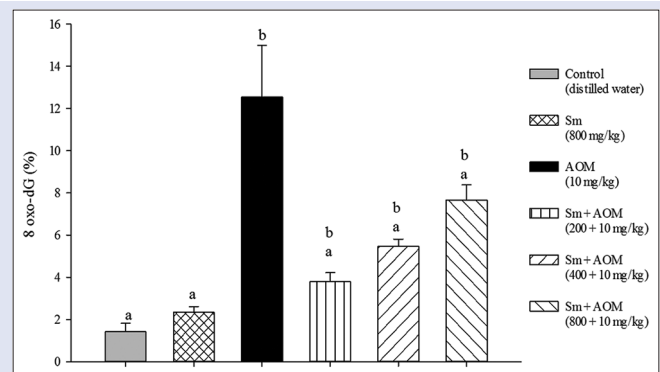
The quantification of AC is shown in Figure 1. A very low number of AC were observed in control mice, as well as in those administered 800 mg/kg of Sm; on the contrary, mice administered AOM showed a significant increase in the number of AC. Animals treated with both the carcinogen and the alga revealed a statistically significant decrease in the number of AC. The protection with respect to the AOM treated animals reached 66.4%, 46.2%, and 42.3% for the doses of 200, 400, and 800 mg/kg, respectively. The presence of AC foci was low, as expected because of the short time of our assay. The distribution of AC along the colon showed a higher number in the median part of the organ, a concordant result with other studies, for example,<sup>[17]</sup> followed by the distal and the proximal parts.

Data obtained regarding the induction of 8-oxo-dG are indicated in Figure 2. The percentage of adducts found in control mice was about 1% and that observed in animals administered 800 mg/kg of Sm was a little more than 2%, without statistical significance with respect to the untreated group. In contrast, the colon of mice treated with AOM showed a strong increase in the level of adducts. On the contrary, in animals administered the three tested doses of Sm besides the carcinogen, we determined a significant protection, which corresponded to 69.7%, 56.4%, and 38.9% for the doses of 200, 400, and 800 mg/kg of Sm, respectively.

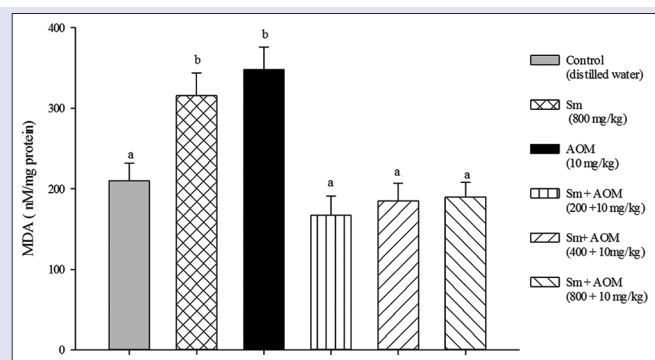
Results concerning lipoperoxidation are indicated in Figure 3. We determined that the AOM treated group increased about 80% the level observed in control animals. As to the effect of Sm on the lipoperoxidation induced in AOM treated mice, our results showed a significant reduction with the three tested doses, which induced a level slightly lower than the observed in the control animals. However, it was interesting to note an unexpected oxidative effect with the high dose of Sm (800 mg/kg), which induced about 50% more lipoperoxidation than the control value.



**Figure 1:** Aberrant crypts induced in the colon of mice treated with azoxymethane and *Spirulina maxima*. Each bar represents the mean ± sampling distribution means of eight mice per group. <sup>a</sup>Statistically significant difference with respect to the value of the azoxymethane group, and <sup>b</sup>with respect to the control group. Analysis of variance and Holm–Sidak test,  $P < 0.05$



**Figure 2:** Percentage of 8-hydroxy-2'-deoxyguanosine in the colon of mice treated with azoxymethane and *Spirulina maxima*. Each bar represents the mean ± sampling distribution means of 8 mice per group. <sup>a</sup>Statistically significant difference with respect to the value of the azoxymethane group, and <sup>b</sup>with respect to the control group. Analysis of variance and Student–Newman–Keuls tests,  $P < 0.05$



**Figure 3:** Lipid peroxidation (malondialdehyde level) induced in the colon of mice treated with azoximethane and *Spirulina maxima*. Each bar represents the mean  $\pm$  sampling distribution means of seven mice per group. <sup>a</sup>Statistically significant difference with respect to the azoxymethane group, and <sup>b</sup>with respect to the control group. Analysis of variance and Holm–Sidak tests,  $P < 0.05$

## DISCUSSION

Anticancer studies with *Spirulina* or its main protein constituent have been made especially in *in vitro* approaches although a few reports in experimental animals and in human subjects have also been published. In the first case, studies in stomach and blood cellular lines have shown such an effect by the alga and have also suggested that the reported activity was mainly related to water-soluble polysaccharides rather than with proteins,<sup>[18,19]</sup> however, similar anticancer potential has also been detected for the water-soluble alga biliprotein, C-phycoyanin, in the human hepatoma cell line, where it was determined anti-proliferative and apoptotic effect by the agent.<sup>[20]</sup> Therefore, it is highly possible that in the described effect, various components of *Spirulina* may act alone or in combination; this suggestion is supported by the observed anti-carcinogenic increase when phycocyanin is combined with external compounds, such as topotecan or piroxicam.<sup>[21,22]</sup>

Experimental animals have also suggested the potential of the cyanobacterium or its constituents for inhibiting the development of cancer. Reports on the effect of whole *Spirulina* against the presence of AC are quite limited. Chen and Zhang<sup>[23]</sup> however studied its effect in 1,2-dimethylhydrazine treated mice and rats and found a heterogeneous response. The positive effect was observed from the ninth to the 16<sup>th</sup> week of the assay but not from the 21<sup>st</sup> to the 24<sup>th</sup> week. Therefore, this information strongly suggested the pertinence of new studies on the matter. In regard to the constituent of *Spirulina*, C-phycoyanin, which is a selective cyclooxygenase-2 inhibitor, it was shown to reduce the presence of AC and dysplasia in the colon of dimethylhydrazine treated rats, an effect that was related to an increase in mitochondrial-dependent apoptosis.<sup>[24,25]</sup> The chemopreventive capacity of the alga has been studied in other organs; *Spirulina platensis* was shown to reduce liver tumorigenesis in rats treated with dibutyl nitrosamine, probably because of apoptotic induction, decrease in cell proliferation, and anti-mutagenic effects.<sup>[26]</sup> Besides, the cyanobacterium combined with *Dunaliella* was shown to prevent the development of oral cancer in hamsters treated with 7,12-dimethylbenz[a] anthracene.<sup>[27]</sup> Based on the mentioned hamster results, researchers have performed at least one study on human oral carcinogenesis.<sup>[28]</sup> In their report, the authors determined that 45% of their study cohort showed complete regression of leukoplakia after 1-year of taking *Spirulina* supplement.

In the present report, we established a chemopreventive effect of Sm on the induction of AC. This organism together with *S. platensis* and *Spirulina fusiformis* are the most investigated species of the alga due to their nutritional value and therapeutic potential. Our results established

that Sm is a useful agent to prevent the initial colon precarcinogenesis damage in mice, an effect that could be observed after 2 weeks of mucosal damaging by AOM.

Explanations for the protective capacity of *Spirulina* or phycocyanin have been related with effects on molecules connected with apoptosis, cell proliferation, cell cycle signaling, immune stimulation or anti-oxidation. In the present study, we explored the participation of the alga in the last mentioned area, which can be an important chemopreventive activity in light of the fact that oxidative stress can contribute to different pathogenic events including colorectal cancer. In this case, its development has been related to a number of inflammatory diseases where reactive oxygen and nitrogen species produced by inflammatory cells can interact with key genes involved in carcinogenic pathways, such as p53, or various DNA repair genes.<sup>[29]</sup> The increase in oxidative stress during colon carcinogenesis has been related to two general events: One refers to granulocyte activation with release of ROS, and stimulation of cytokines, some of which may also produce ROS; the other event refers to the production of hydrogen peroxide by malignant cells.<sup>[30]</sup> In our research, we found a significant increase in the level of lipoperoxidation by AOM, a process where MDA and other reactive molecules are produced and may interact with DNA altering its structure and functioning. Other authors have also found elevated levels of lipid peroxidation in AOM treated mice.<sup>[31]</sup> Besides, we determined an increase of DNA oxidation due to AOM, as shown by the elevated amount of 8-oxo-dG adducts, which correspond to alterations correlated with the development of carcinogenesis *in vivo*.<sup>[32]</sup> Therefore, it is possible that the observed cellular oxidative damage could be partially involved in the precarcinogenic damage induced by AOM. In this context, it is also interesting to note that elevation in the levels of 8-oxo-dG has been reported not only in patients with colon cancer but also in those with benign adenoma,<sup>[30]</sup> suggesting that such event is produced early during the carcinogenesis process, a finding which agrees with our data.

In regard to the effect of Sm, we determined a significant decrease in the number of AC in concordance with the observed reduction of cellular oxidation. This suggests that in our present experimental conditions, the antioxidant potential of Sm could be involved in the prevention of carcinogenic damage by AOM. Such conclusion is in line with results of studies showing that the administration of different antioxidants reduces colon cancer induced with AOM,<sup>[33-35]</sup> as well as with the antioxidant capacity of various species of *Spirulina*, and several of its components, including carotenoids, phycobiliproteins, and vitamins.<sup>[36,37]</sup> However, we observed a lipid peroxidation increase with the highest tested dose of Sm, an unexpected result that suggests that other mechanisms are also participating in the observed protection against the effect of AOM. Such result deserves specific research for examining its real toxicological significance and its meaning with respect to the safety of ingesting the alga; moreover, because *Spirulina* consumption has extended in cancer patients or survivors of the disease.<sup>[38]</sup> Furthermore, the dose was observed to be the least effective in the oxidative and precarcinogenic protection against AOM, suggesting then a specific range for the beneficial capacity of Sm. For the present, a possibility could be that such dose of the alga caused an alteration in the balance of the oxido-reduction state in intestinal mice cells. Besides, a possible involvement in this toxic observation may have been related with the well-documented property of a number of antioxidants (some of them present in *Spirulina*), which, in specific doses and particular chemical interactions can give rise to the opposite, a prooxidant activity and cell deleterious effects. This process has been reported in chemicals such as flavonoids, proteins, terpenes, ascorbic acid, or tannins, among other compounds, where variable amount of oxidative stress can be produced; moreover, *Spirulina* has also been reported to contain microcistin and

anatoxin-a, potent toxic agents known to affect the liver and the brain, respectively that could also be another source of mouse molecular disturbance processes.<sup>[39-41]</sup>

## CONCLUSION

Sm is an alga with nutritive value that has also shown a number of biomedical beneficial activities, including anti-oxidative and anti-mutagenic effects; therefore, it is relevant to determine its anticancer capacity, particularly *in vivo*. The present study collaborates with this purpose demonstrating that Sm was able to inhibit the development of colon AC in mice treated with AOM. These histopatologic anomalies correspond to preneoplastic lesions preceding the development of adenomas and carcinomas in the colon of both rodents and humans. Our results, therefore, clearly suggest a potential of the alga as a candidate for chemopreventive purposes. In this type of agents, it is indispensable to determine their mechanism of action, as they may act in various forms to counteract the colon carcinogenesis; in our assay, we also established a role for the antioxidant potential of Sm. Finally, our present report suggests the importance of confirming its protective capacity against tumor development, as well as of determining its various mechanisms of action, as steps prior to continuing with its chemopreventive characterization.

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## Conflicts of interest

There are no conflicts of interest.

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