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ORIGINAL ARTICLE

Evaluation of the Genotoxicity and Cytotoxicity of Semipurified Fractions from the Mediterranean Brown Algae, *Dictyopteris membranacea*

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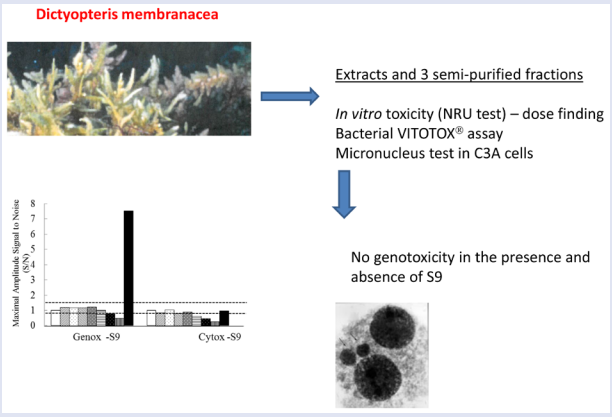
ABSTRACT

Dictyopteris membranacea, a species of Mediterranean brown algae,is believed to have potential pharmacological and nutritional applications. However, such potentials only make sense when devoid of any adverse health consequences. The present study should be seen in this context. It aimed at evaluating the genotoxicity and cytoxicity of its organic extract (F_0) and semi purified fractions (F_4,F_5,and F_6).Extracts were tested using the bacterial Vitotox® test and micronucleus assay in different concentrations (from 1.25 µg/mL up to 100 µg/mL, depending on the test and the extract). Applied concentrations were based on a preliminary dose-finding test with the neutral red uptake assay. The results show that all extracts were not genotoxic in the presence or absence of a rat metabolic enzyme fraction (S9). This is encouraging and justifies further investigations on the therapeutic and other values of this algae.

Key words: Cytotoxicity, *Dictyopteris membranacea*, genotoxicity, micronucleus, test, Vitotox test



SUMMARY

- *Dictyopteris membranacea* extracts and some of their semi purified fractions have important antibacterial properties.
- The organic extract (F_0) and semi purified fractions (F_4, F_5, and F_6) were not genotoxic according to the bacterial Vitotox test.
- They were also not genotoxic according to the micronucleus test in human C3A cells.
- Applied concentrations were based on the *in-vitro* neutral red uptake (NRU) test.

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INTRODUCTION

The oceans of the world occupy two-thirds of the planet's surface and provide a rich source of natural products. Marine macroalgae or seaweeds make up a large fraction of these natural products. Farmed or foraged from the wild, seaweeds are used for different purposes, including their application in traditional medicine. Several studies have shown interesting pharmacological and nutritional applications of seaweeds[1-5] and therapeutic properties of bioactive metabolites isolated from algae.[6,7] For this reason, new compounds are continuously isolated from marine resources and are investigated to unravel their biological properties.[8] Brown algae (Phaeophyceae) are potential sources of ingredients with nutritional and/or therapeutic properties. They are already known to contain relatively more antioxidants than green and red algae,[9-12] and their extracts also show higher antibacterial activities.[13] *Dictyopteris membranacea*, a species of Mediterranean brown algae collected from the Tunisian coast, is furthermore well-known for its exceptional odoriferous capacity and is currently being investigated for its potential therapeutic use. Its usage in the development of a new therapeutic means can, however, only be envisaged provided it is devoid of harmful effects. The aim of this study is, therefore, to investigate the genotoxicity of the organic extract and its semipurified fractions of *Dictyopteris membranacea*. This is important as genotoxic agents may, among others, induce hereditary diseases or cancer. Natural compounds that may be envisaged to

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become ingredients of food additives or health care products should, therefore, be devoid of any genotoxic properties.

MATERIALS AND METHODS

Sample collection and preparation of the organic extract

Dictyopteris membranacea plants were collected in June (summer) from the Mediterranean Sea in various areas of the coastal region of Monastir (Tunisia) at a depth between 2 m and 5 m. The collected samples were rinsed with seawater and distilled water and then transported in cool boxes to the laboratory. The cleaned material was air dried to dryness in the shade at 30°C. Finally, the dried samples were powdered and stored at −20°C until use. Identification of specimens was carried out in the National Institute of Marine Sciences and Technologies (Salamboo,Tunisia).The organic extract of *Dictyopteris membranacea* was prepared by maceration of finely powdered material packed in small bags (5cm×10cm) of Whatman filter paper no. 1 with methanol and dichloro methane. This was done three times with intervals of 48 hours. The organic extract was concentrated to solvent free by evaporation in a rotating evaporator (Buchi, B-480) at 40°C and then stored at −20°C until use.

Purification of the organic extract

Extracts of *Dictyopteris membranacea* were purified using C18 cartridges (Sep-pack, Supelco), by gradient elution with acetonitrile, acetone, and dichloromethane–methanol to give three semi purified fractions (respectively, F_4, F_5, and F_6). The solvents were removed from the fractions using rotating evaporator at 35°C. The fractions were stored at −20°C until use. The organic extract (F_0) and the fractions F_4, F_5, and F_6 were diluted to the desired final concentration immediately prior to manipulation.

The neutral red uptake (nru) assay

The NRU test measures cell viability based on the property of living cells to be able to take up neutral red dye into their lysosomes.[14] Dying cells have altered membrane properties preventing them to take up neutral red (NR). The dye is applied to the cells and the NI_{50} concentration (50% reduction of uptake) is determined by measuring OD_{540}. This test was performed according to well-known standard methods. Human C3A cells were plated in 96 well plates (40.000 cells per well) and incubated in Dulbecco's Modified Eagle Medium (DMEM) + 10% Fetal Bovine Serum (FBS) for 24 hours at 37°C and 5% CO_2. Next, extracts were added in different concentrations for another 24 hours. Cells were then washed with phosphate buffer saline (PBS) after which 200 µl 0.05 mg/mL neutral red solution was added. After 3 hours, cells were again washed in PBS to remove the remaining dye. Addition of 200 µL ethanol/acetic acid (50/1) resulted in the release of the dye from the cells that were placed on a stirring plate until a homogenous color was formed (approximately 1 hour). The optical density (OD) was measured with a spectrophotometer. The OD_{620} measured as a reference value was subtracted from the OD_{540} which is the optical density at the wavelength at which maximal absorption of NR occurs. Absorption of non treated cells was given a 100% value to which data from exposed cells were compared. Sodium Dodecyl Sulfate (SDS) in concentrations of 0.42, 0.35, 0.28, 0.21, 0.14, and 0.07 mM was used as a positive control.

Genotoxicity and cytotoxicity assays

The organic extract and fractions were tested using the Vitotox® test which is a genotoxicity test based on SOS-induction.[15] It gives information on both genotoxicity and toxicity of a sample. A detailed description of the Vitotox® test is given elsewhere.[15-17] In summary, this test employs two different constructs of *Salmonella typhymurium* TA 104. One has a luciferase gene under the control of a modified recN promoter, which leads to light production when DNA is damaged (TA 104-recN2-4 strain or Genox strain), whereas the second one contains *lux*-genes under the control of a constitutive promoter so that the light production is not influenced by genotoxic compounds (pr1 or Cytox strain). It serves as an internal control wherein if the light production goes up, the test compounds affect the *lux* gene in a different way than by damaging the DNA. Furthermore, a decrease in light production would indicate a toxic response.

Light measurements were performed in a luminometer (Modulus Microplate Multimode Reader from Turner Biosystems) at 30°C every 5 minutes in each well during a 4-hour period after addition of the extract or fractions to the bacteria. Concentrations used were based on preliminary dose-finding and toxicity tests using the NRU assay. The extracts were dissolved in dimethyl sulfoxide to give a stock solution of 1 mg/mL, and serial two fold dilutions were made resulting in a tested concentration range from 100 µg/mL to 1.25 µg/mL. The samples were tested with and without the presence of a rat metabolic enzyme fraction (S9). 4-Nitroquinoline oxide (4-NQO, 4ppb) was used as a positive control without S9 metabolic activation, whereas benzopyrene (Bap, 8ppm) was used as the positive control requiring S9 metabolic activation.

The signal to noise ratio (S/N) or, specifically, the light production of exposed bacteria divided by the light production of nonexposed bacteria is automatically calculated for each measurement. S/N is calculated for both strains separately and the ratio between the maximum S/N values of the exposed over the control strain.

A substance is considered genotoxic when:

- max S/N (genox)/max S/N (cytox) >1.5
- max S/N in genox shows a good dose–effect relationship
- max S/N (genox/cytox) shows a good dose–effect relationship

A substance is considered cytotoxic when S/N in the cytox strain decreases and becomes considerably lower than 0.8.

The micronucleus assay

The micronucleus test[18] was conducted in human C3A cells that were cultivated as described above, exposed to the test material after 24 hours, and then treated 24 hours later with 4.5 µg/mL cytocholasin B to block the cells in telophase, so as to be sure that cells were divided and, hence, may show induced micronuclei. Fixation occurred after a total cultivation time of 72 hours. Slides were then stained with DAPI (4',6-diamidino-2-phenylindole) and analyzed with a Zeiss Axio Plan fluorescence microscope. Approximately 2000 binucleated cells were scored for the presence of micronuclei. MMS (methyl methane sulfonate; 15 µg/mL) was used as a positive control. Samples were tested in concentrations that were based on the results of the neutral red uptake test, but cytotoxicity was also determined using the Cytokinesis-Block Proliferation Index (CBPI) which is calculated as the number of mononucleated cells + 2*x* number of binucleated cells + 3*x* number of multinucleated cells divided by the total number of cells. The CBPI was determined after examination of 500 acridine orange stained slides. Cytotoxicity is then calculated as:

Cytotoxicity (=cytostasis) = 100 – 100{($CBPI_T$– 1)/($CBPI_C$ – 1), where $CBPI_T$is the CBPI of the test compound (extract or fraction) and $CBPI_C$ the CBPI of the unexposed control cells. This cytotoxicity evaluation usually goes along with the micronucleus test using cytochalasin B. It helps in determining appropriate test concentrations for the *in vitro* micronucleus (MN) test.[19]

The highest concentration used should aim to achieve 55 ± 5% cytotoxicity and, i.e. show a reduction in CBPI up to 45± 5% of the concurrent negative control.[20]

Statistical analysis

No statistics are needed for the NRU test where we only determined NI_{50} values. The same holds true for the Vitotox® test where a dose–effect relationship and S/N ratio (genox over cytox strain) reaching levels over 1.5 and requirements as outlined before are sufficient to decide about the presence or absence of genotoxicity. The Kastenbaum and Bowman[21] tables were used for determining statistically significant deviations from (unexposed) controls. This binomial test was found adequate for the purpose of the present investigation. Here, the frequency of cells with micronuclei was compared between exposed cells and their unexposed controls. This means that we compared each test concentration with its negative control only. At this point, we did not take multiple comparisons into account.

RESULTS AND DISCUSSION

In this paper, we report on the *in vitro* cytotoxicity and genotoxicity of organic extracts of *Dictyopteris membranacea* which is at present extensively investigated with regard to their potential beneficial properties. The laboratory at the Faculty of Pharmacy (University of Monastir) is for example involved in studies on their antimicrobial and antitubercular activity. From such studies we already know that fractions F_4 (acetonitrile) and F_5 (acetone), especially, have important antibacterial properties (unpublished results).

The neutral red uptake and micronucleus tests were conducted in C3A cells because these cells largely conserved both phase I and phase II metabolic capacities.[22] Tests can, therefore, be conducted in the absence of S9.

The results of the neutral red uptake test are summarized in Table 1 where NI_{50} concentrations are given. F_4 presents the lowest concentration of NI_{50} and the highest concentration is for the organic extract (F_0). Concentrations tested were based on the results of the NRU test. Cytotoxicity determination based on CBPI showed that all concentrations were accurate as the maximum percent cytostasis fluctuated between 32.71% (F_0) and 52.04% (F_6) (see Table 2).

Table 1: Concentrations of 50% reduction of neutral red uptake (NI_{50}) for the organic extract and the fractions F_4, F_5, and F_6

Fraction	Solvent	NI_{50} (µg/ml)
Methanolic extract F_0	Methanol	142.5
F_4	Acetonitrile	21.16
F_5	Acetone	51.2
F_6	Dichloromethane-methanol (1:1)	83.1

The Vitotox® test is a test for bacterial genotoxicity based on SOS induction. Its results correlate well with the Ames assay.[15,23,24] The Vitotox® test was found very suitable and highly efficient for (high throughput) screening of chemicals to determine their genotoxic potential.[16,17] It was also shown to be particularly useful in the research or prescreening phase in the pharmaceutical industry.[23,25] The test is usually more sensitive than the Ames assay as it detects lower concentrations of a genotoxic compound.[15-17] It also requires only limited amounts of a test compound. This is one of the major advantages of the test for prescreening purposes where usually only small amounts of the test compound are available (e.g., in the discovery phase of a new pharmaceutical agent).

As expected, the positive control 4-NQO was found to be genotoxic as light production was induced over an S/N of 1.5, and no cytotoxicity (S/N ~1) was found. Benzo(α)pyrene also showed genotoxicity without being cytotoxic in the presence of metabolic activation. This can be seen in Figures 1 and 2. These figures also show that the organic extract F_0 and the fractions F_4, F_5, and F_6 were not genotoxic in the presence and absence of S9 (S/N <1.5). Cytotoxicity was, however, found at the higher doses.Without addition of S9, cytotoxicity was obvious (S/N in the cytox strain well below 0.8) for the organic extract at >50 µg/mL, and respectively at >50, >5, and >25 µg/mL for F_4, F_5, and F_6. Cytotoxicity was also obvious in the presence of S9 and found at >50, >25, >100, and > 25 mg/mL for the organic extract F_4, F_5, and F_6, respectively.

Table 2: Micronucleus test applied to the organic extract F_0 and its fractions F_4, F_5, and F_6.

	Number of MN	Number of BN cells	MN/2000 cells	CBPI	CBPI (% of control)	Cytostasis
Unexposed control	32	2663	24.0	1.538	-	-
MMS (15µg/ml)	68	1940	70.1 (P<0.05)	1.582	102.83	-8.18
F_0 (12.5 µg/ml)	19	17,6	22.3	1.586	103.09	-8.92
F_0 (25 µg/ml)	19	1933	19.7	1.482	96.33	10.41
F_0 (50 µg/ml)	9	2012	8.9	1.524	99.09	2.60
F_0 (100 µg/ml)	15	1314	22.8	1.362	88.56	32.71
F_4 (2.5 µg/ml)	26	2041	25.5	1.52	98.8	3.35
F_4 (5 µg/ml)	25	2036	24.6	1.526	99.32	2.23
F_4 (10 µg/ml)	25	1929	25.9	1.514	98.41	4.46
F_4 (15 µ/ml)	6	987	12.2	1.322	85.93	40.15
F_5 (3.125 µg/ml)	29	2271	25.5	1.546	100.49	-1.49
F_5 (6.25 µg/ml)	21	1466	28.6	1.512	98.28	4.83
F_5 (12.5 µg/ml)	13	2159	12	1.554	101.01	-2.97
F_5 (25 µg/ml)	13	1083	24	1.292	83.98	45.72
F_6 (6.125 µg/ml)	28	3313	25.3	1.556	110.36	-3.35
F_6 (12.5 µg/ml)	17	2132	15.9	1.544	101.14	-1.11
F_6 (25 µg/ml)	22	2138	20.6	1.458	94.77	14.87
F_6 (50 µg/ml)	11	1456	15.1	1.258	83.59	52.04

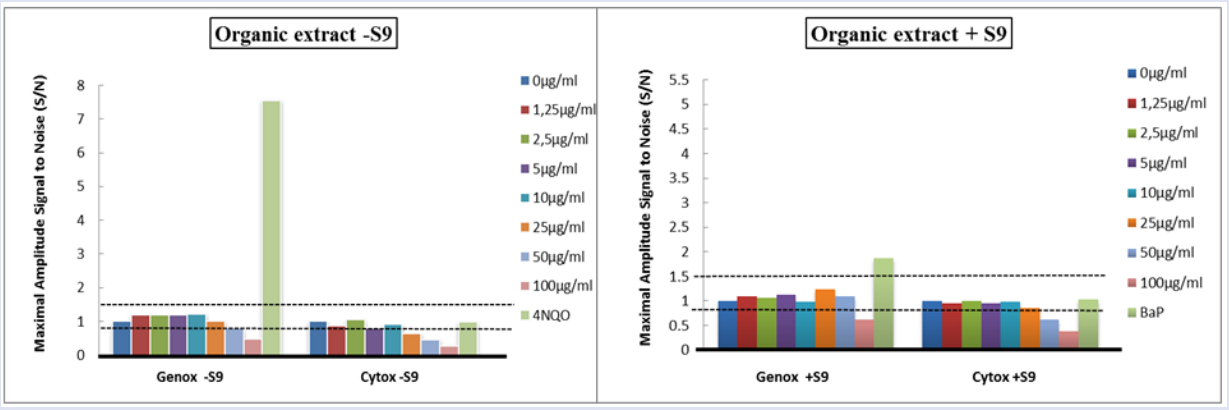

Figure 1: VITOTOX® test results for the organic extract F_0 in the absence and presence of S9. The horizontal line at S/N = 1.5 indicates the threshold level for genotoxicity in the Genox strain, whereas the line at S/N=0.8 gives the level below which S/N is indicative of a toxic response in the Cytox strain. 4-NQO and Bap = positive control.

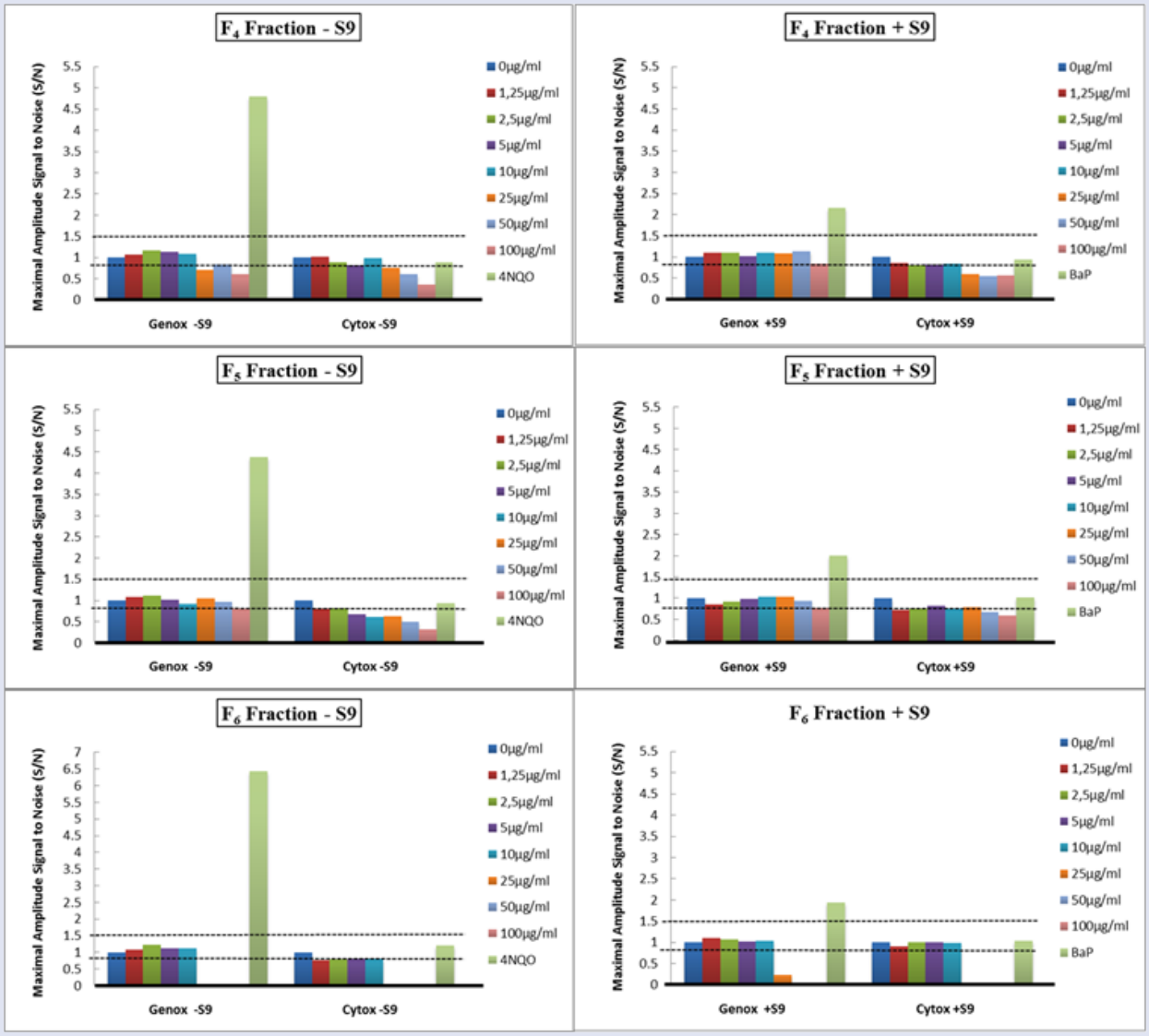

Figure 2: VITOTOX® test results for the fractions F_4, F_5, and F_6 in the absence and presence of S9 (see also legend of Figure 1 for details).

Bacterial cytotoxicity in the Vitotox® test strain was, thus, not totally in agreement with the results from the NRU test and CBPI/cytostasis determinations. This is not surprising as the different assays measure cytotoxicity based on totally different endpoints. It should also be noted that we often found a cytotoxic (or cytotoxic-like) response with the Vitotox® test when complex mixtures were studied. This already occurred at concentrations that were non-toxic or subtoxic according to the NRU test or at concentrations that did not affect the growth of Umu-C Salmonella typhimurium (TA 1535) bacteria.[16,17,26] This is probably due to an interaction of the sample or some of its constituents with the metabolic pathways in which the luciferase operon is involved.[17] This can at first sight be seen as a disadvantage of the Vitotox® test. We, however, previously demonstrated that this test can perfectly be used for such samples (extracts) and be valuable for the (pre)screening of large numbers of samples, including, for example, extracts from plants.[27] It should be noted that the Vitotox® test often enables genotoxicity testing at much lower concentrations than other test systems and that Vitotox® results are, therefore, often valid even when only low(er) concentrations could be tested.[28] The Vitotox® test, hence, remains valuable as a first screening test.For this reason, the test was applied to our extract and semi purified fractions. However, this test is only a rapid indicator test for DNA damage which means that further mutation tests in mammalian/human cells may be necessary yet. We, therefore, also conducted the micronucleus assay which is now recognized as one of the most successful and reliable assays for genotoxic carcinogens. A micronucleus is formed during the metaphase/anaphase transition of mitosis (cell division). It may arise from a whole lagging chromosome (aneugenic event leading to chromosome loss) or an acentric chromosome fragment detaching from a chromosome after breakage (clastogenic event) which does not integrate in the daughter nuclei. Scoring of micronuclei in telophase cells is, therefore, a convenient way to investigate the potential of an agent to induce structural and numerical chromosome aberrations. Together with the Vitotox® test, we cover gene mutations (which the Vitotox® test detects due to its high concordance with the Ames assay) and structural and numerical chromosome mutations.

The results of the micronucleus test are given in Table 2. It can be seen that only the positive control (15 µg/mL MMS) induced micronuclei in a statistically significant way. The organic extract (F_0) and its semi purified fractions (F_4, F_5, and F_6) from the Mediterranean brown algae, *Dictyopteris membranacea,* were shown not to significantly induce micronuclei in the C3A cells.

In conclusion, according to our results, the organic extract and the F_4, F_5, and F_6fractions are not genotoxic.To the best of our knowledge, this is the first report on the evaluation of the genotoxic and cytotoxic effects of the tested algae extract or fractions. It shows that they can (so far) be considered safe in terms of their potential *in vitro* genotoxic properties. This is encouraging with respect to further investigations on their therapeutic value.

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Conflicts of interest

There are no conflicts of interest

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