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Phytochemical and in vitro antimicrobial and genotoxic activity in the brown algae *Dictyopteris membranacea*

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ABSTRACT

The emergence and increase of microbial resistance towards many conventional therapeutics necessitate the investigation and discovery of new natural sources of antimicrobial agents. In this study the Mediterranean brown alga, *Dictyopteris membranacea*, was investigated. The total phenolic, flavonoid and tannin contents were quantified and antibacterial, antifungal and antitubercular activities of the crude extract and semi-purified ethanol, acetone and methanol/CH_2Cl_2 fractions were determined. The antimicrobial activity was determined by the agar diffusion method against six strains of Gram-positive, two strains of Gram-negative bacteria and one yeast strain. The MIC and MBC or MFC values served to identify the bactericidal and bacteriostatic effects of all prepared fractions against the different strains. The acetone fraction presented a source of phenolic (112 mg GAE/g of sample) and tannin (130 mg CAE/g sample) compounds whereas the ethanol fraction showed the highest concentration of flavonoids (120 mg QE/g). Ethanol and acetone fractions exhibited considerable antibacterial, antifungal and antitubercular activities with a good correlation between the phytochemical composition of all prepared fractions and the different biological activities.

This study on the phytochemical and in vitro antimicrobial activity of this brown alga was supplemented by an in vitro investigation of the potential genotoxicity of the extract and fractions using the bacterial Vitotox test and micronucleus test on human C3A cells. The results show that all prepared fractions were not genotoxic in the presence or absence of S9.

Overall, the results are therefore encouraging and justify further investigations on the therapeutic and other values of this alga.

1. Introduction

Plants produce a vast number of natural compounds, called secondary metabolites. These are crucial to plant development and the interaction of a plant with its biotic and abiotic environment (Kutchan, 2001). Medicines derived from natural products are widely used and account for more than 30% of therapeutic agents presently prescribed in clinics (Yang et al., 2008). New antimicrobial agents and strategies for their use in the treatment of serious Gram-negative and Gram-positive infections are necessary because of the emergence of multidrug resistance in common pathogens, the rapid emergence of new infections, and the potential for use of multidrug-resistant agents in bioweapons (Spellberg et al., 2003; Shah, 2005; Gulçin et al., 2008; Gülçin et al., 2010). Seaweeds are a rich source of natural products and may be incorporated into the human diet, be used in cosmetic products and as traditional medicine (Fisch et al., 2003). There are many examples of their therapeutic properties such as the Australian seaweed *Delisea pulchra* may interfere with bacterial colonization (Cappitelli and Sorlini, 2008); sulfated saccharides from both red (Rhodophyceae) and green (Chlorophyceae) macroalgae inhibit some DNA and RNA enveloped viruses (Cappitelli and Sorlini, 2008; Kazłowski et al., 2012). Brown macroalgae (Phaeophyceae) exhibit relatively more antioxidant compounds than green and red algae (Al-Amoudi et al., 2009; Batista Gonzalez et al., 2009; Costa et al., 2010; Cox et al., 2010), and their extracts also show higher antibacterial activity (Valchos et al., 1997). Moreover, polyphenols and phlorotannins from brown algae have antioxidant, antibacterial and anti-algae properties (Shibata et al., 2006; Kuda et al., 2007).

The Mediterranean brown alga *Dictyopteris membranacea* (Stackhouse) Batters (Phaeophyta, Dictyotaceae), is well known for its exceptional odoriferous capacity due to diterpenes which act as pheromones in sexual reproduction (Stratmann et al., 1992). To our

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knowledge, there are no reports on the possible pharmacological applications or the antimicrobial properties of this species. Thus, the aim of the present study was to investigate the ability of crude extract and its semi-purified fractions of *D. membranacea* to reduce the growth of various potential pathogenic microorganisms. As their use in therapeutic products can only be envisaged provided they are devoid of harmful effects, the genotoxicity of these prepared fractions was also investigated.

2. Methods

2.1. Sample collection and preparation of the crude extract

D. membranacea was collected in June 2010 from the Mediterranean Sea at various locations along the coastal region of Monastir (Tunisia) at a depth between 2 and 5 m. Samples were rinsed with sea-water and distilled water and transported in cool boxes to the laboratory. The cleaned material was then dried in the shade at 30 °C for 1 week until completely dry. The dried samples were powdered with a grinder (Type FORPLEX) and stored at −20 °C until analysis. Identification of specimens was carried out in the National Institute of Marine Sciences and Technologies (Salamboo, Tunisia). The crude extract of *D. membranacea* was prepared by packing the fine powdered material in small bags (5 cm × 10 cm) made of Whatman No. 1 filter paper and placed in methanol and dichloromethane (1:1, v/v) for 48 h at room temperature. This was repeated three times. The crude extract was concentrated to dryness by evaporation in a rotating evaporator (Buchi, B-480) at 40 °C and then stored at −20 °C until analysis.

2.2. Purification of the crude extract

The crude extract of *D. membranacea* was semi-purified, using C_{18} cartridges (Sep-pack, Supelco), by gradient elution with different organic solvents in the order of decreased polarity: ethanol, acetone and methanol/CH_2Cl_2 (1:1) to give three semi-purified fractions: ethanol, acetone and methanol/CH_2Cl_2 fractions. Organic solvents were removed from the fractions using rotating evaporator at 40 °C. The fractions were stored at −20 °C until analysis. All the prepared fractions were diluted in 10% DMSO to the desired final concentration (1 mg/ml).

3. Phytochemical analysis

3.1. Total phenolic content (TPC)

The total phenolic content of the crude extract and its semi-purified fractions were estimated using the method of McDonald et al. (2001). Briefly, 100 μl aliquot of sample was mixed with 2.0 ml 2% Na_2CO_3 and allowed to stand for 2 min at room temperature. After incubation, 100 μl 50% Folin–Ciocalteu's phenol reagent was added, and the reaction mixture mixed thoroughly and allowed to stand for 30 min at room temperature in the dark. A blank contained 100 μl methanol instead of the extract. Absorbance was measured at 720 nm on a UV spectrometer (Jenway 6505) against the reagent blank, and TPC was standardized against gallic acid (GA) and expressed as mg (GA) equivalent per g of sample (mg GAE/g) (Baiano et al., 2009). All measurements were performed in triplicate.

3.2. Total flavonoid content (TFC)

The total flavonoid content was determined by the $AlCl_3$ colorimetric method (Chang et al., 2002). A 500 μl aliquot of sample (1 mg/ml) was mixed with 1.5 ml methanol, 0.1 ml 10% $AlCl_3$ solution, 0.1 ml potassium acetate (1 M), and 2.8 ml distilled water. After 30 min incubation at room temperature, the absorbance was measured at 415 nm versus a prepared water blank using a Jenway 6505 UV spectrophotometer. Quercetin was used as a reference standard and the total flavonoid content was expressed as milligram of quercetin equivalents (mg QE/g). There were three replicates.

3.3. Total tannins content (TTC)

Analysis of the total tannin content was carried out according to the method of Broadhurst and Jones (1978) and as modified by Xu and Chang (2007). Three ml 4% methanol vanillin solution and 1.5 ml concentrated hydrochloric acid were added to 50 μl sample. The mixture was allowed to stand for 15 min and the absorption was measured at 500 nm against methanol, which was used as a blank. The amount of condensed tannin was calculated and expressed as milligram catechin equivalents (mg of CAE/g sample). All samples were analyzed in triplicate.

4. In vitro evaluation of antimicrobial activity

4.1. Microbial strains and growth conditions

The yeast *Candida albicans* American Type Culture Collection (ATCC) 10231 and 8 bacterial strains including the Gram-positive *Staphylococcus aureus* (ATCC 6538), *Enterococcus faecium* (ATCC 19434), *Streptococcus agalactiae*, *Bacillus subtilis*, *Enterococcus faecalis* (ATCC 292129) and the Gram-negative *Escherichia coli* (ATCC 8739) and *Salmonella typhimurium* (ATCC 14028) were used to assess the antimicrobial properties of the extracts of *D. membranacea*. *S. agalactiae* and *B. subtilis* were isolated from clinical samples in the Institut National des Sciences Appliquees (Tunis, Tunisia) and all other strains were obtained from the Institut Pasteur (Paris, France). Bacterial strains were cultured overnight at 37 °C in Mueller–Hinton agar (MHA). *Mycobacterium tuberculosis* H37Rv ATTC 27294 is sensitive to the five first line antituberculosis drugs (streptomycin, isoniazid, rifampin, ethambutol, and pyrazinamide) and was transformed earlier with a pSMT1 plasmid harboring the lux genes of *Vibrio harveyi* (Snewin et al., 1999; Cappoen et al., 2012).

4.2. Disk diffusion method

For the determination of the antimicrobial activity, the disk diffusion method was used according to the National Committee for Clinical Laboratory Standards (NCCLS, 1997). Briefly, a suspension of the tested organism (108 CFU/ml) was spread on the MHA solid media plate. Filter paper disks (6 mm in diameter) were soaked with 15 μl extract and placed on the inoculated plates. After being kept at 4 °C for 2 h, they were incubated at 37 °C for 24 h for bacteria and at 30 °C for 48 h for the yeast. Disk containing standard concentration of ampicillin (10 μg/ml) was used as positive growth control. The definite zone of inhibition of any dimension surrounding the paper disk was measured accurately using a metric scale or a sliding caliper. This test was conducted in triplicate. The zone of inhibition indicates the degree of sensitivity of bacteria to an extract according to the following criteria (Barros et al., 2007):

- Diameter ≤ 7 mm: no antimicrobial activity
- 7 mm ≤ diameter ≤ 9.9 mm: low antimicrobial activity
- 10 mm ≤ diameter ≤ 11.9 mm: modest antimicrobial activity
- 12 mm ≤ diameter ≤ 15 mm: high antimicrobial activity
- 15 mm < diameter: strong antimicrobial activity.

4.3. Microdilution method

A broth microdilution method was used to determine the minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) (Berche et al., 1991; NCCLS, 1999; Bassole et al., 2003).

All tests were performed in Mueller Hinton broth (MHB). Bacterial strains were cultured overnight at 37 °C in MHA and the yeasts were cultured overnight at 30 °C in Sabouraud Dextrose Broth (SDB). Test strains were suspended in MHB to give a final density of 5.105 CFU/ml. All the prepared fractions were dissolved in 10% DMSO and geometric dilutions ranging from 15.62 to 500 μg/ml of each extract, were prepared in a 96-well micro titer plate, including one growth control (MHB + 10% DMSO). Plates were incubated under normal atmospheric conditions at 37 °C for 24 h for bacteria and at 30 °C for 48 h for yeasts.

The MIC is defined as the lowest concentration of the extract at which the microorganism does not demonstrate visible growth. The microorganism growth was evaluated by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. This test assesses the enzymatic reduction of the tetrazolium dye MTT, to its insoluble formazan, which has a purple color. To determine MBC or MFC, 10 μl broth was taken from each well and inoculated in MHA for 24 h at 37 °C for bacteria or in SDA for 48 h at 30 °C for yeasts. The MBC and MFC are defined as the lowest concentration of the extract at which the inoculated microorganism was completely killed (99.99%). Each test was performed in three replicates and repeated twice. The antibacterial effect is bactericidal (fungicidal) or bacteriostatic (fungistatic) was calculated according to the ratio: $(\frac{MBC}{MIC})$ for the bacterial strains and $(\frac{MFC}{MIC})$ for the yeast (Berche et al., 1991). Effects are according to the ratios given below.

$1 \le (\frac{MBC}{MIC}) \le 2$: Bactericidal effect $1 \le (\frac{MFC}{MIC}) \le 2$: Fungicidal effect

$4 \le (\frac{MBC}{MIC}) \le 16$: Bacteriostatic effect $4 \le (\frac{MFC}{MIC}) \le 16$: Fungistatic effect

4.4. Monitoring mycobacterial growth by luminometry

The minimal inhibitory concentration (MIC) against mycobacteria of all the prepared fractions was evaluated by testing serial dilutions (12.5, 25, 50, 100, 200 and 400 μg/ml). The in vitro assay was based on a method in which luminescent mycobacteria transformed with pSMT1 luciferase reporter plasmid were used (Snewin et al., 1999).

All the prepared fractions were solubilized in DMSO at stock concentrations of 10 mM. Serial dilutions of each compound were made in liquid 7H9 medium [Middlebrook 7H9 broth based (Difco)] + 10% FCS (Gibco). Volumes of 20 μl of the serial dilutions were added in triplicate to 96-well, flat-bottomed microwell plates. The bacterial suspension was made by thawing and dissolving a frozen Mycobacteria pellet in 7H9–10% FCS. The dissolved pellet was passed through a 5.0 μm filter (Millipore) to eliminate clumps and left for 1 h to recover at 37 °C, 5% CO_2. Next, the bacterial suspension was diluted in 7H9–10% FCS to obtain 50,000 Relative Light Units (RLU)/μl and a volume of 180 μl of bacteria was added to each well. Bacterial replication was analyzed by luminometry after 6 days of culture. The bacterial suspension from each well was collected, and brought in a 2.5 ml Eppendorf (Difco). To measure the luminescence, 100 μl 1% n-decanal in ethanol was added to the Eppendorf tube and light emission was measured over 10 s using a Turner Modulus Single Tube Luminometer from Biosystems.

5. In vitro toxicity test

5.1. Vitotox test®

This bacterial genotoxicity test is performed in genetically modified *S. typhimurium* strains. One of them is called the Genox strain and is used to reveal genotoxicity; the other (designated as the Cytox strain) is used as a control strain to avoid false positive results and give an indication of toxicity. The Vitotox test is based on SOS induction. The Genox strain contains a lux gene from *Vibrio fischeri* which is linked to a modified recN gene that will be induced following DNA damage. Consequently, lux will also be induced and light emission therefore reflects DNA damage. In the Cytox strain light production is constitutive and not related to DNA damage. An influence on light emission here is therefore not the result of DNA damage. A detailed description of the test is given elsewhere (Verschaeve et al., 1999; Verschaeve, 2005, 2013). In short, it can be assumed that there is genotoxicity when the signal to noise ratio (S/N = ratio of light emission in exposed bacteria vs. unexposed controls) increases in the Genox strain in a dose-dependent way and reaches S/N values of ≥1.5. However, if S/N in the Cytox strain also increases light emission is most probably due to an indirect effect of the test agent on the Lux operon and not due to genotoxicity. The effect seen in the Genox strain may therefore not reflect genotoxicity. A considerable decrease of S/N in the Cytox (and possibly Genox) strain (S/N ˂ 0.9) may indicate toxicity.

5.2. The micronucleus test

This well-known test was performed according to the standard protocols. Briefly, human C3A cells are cultivated in the presence of the test agent and blocked in telophase (binucleated cell stage) with Cytochalasin B. The presence of one or more micronuclei in the telophase cells indicates the presence of structural chromosome aberrations (chromosome fragments), or numerical chromosome aberrations (loss of a whole chromosome due to an aneugenic event). This test therefore is an easy chromosome and genome mutation test. A detailed description of this test can be found elsewhere (e.g., Fenech, 1993).

6. Statistical analysis

With respect to the investigation of the phytochemical and in vitro antimicrobial activities the data are presented as the mean ± standard error (s.e.m). Statistical analysis was performed using Student's t-test. The determinations of antimicrobial activity were conducted in triplicate and the statistical analyses were done by one-way ANOVA followed by Dunnet's test. All samples were analyzed in triplicate.

For the Vitotox test no replication is needed as a S/N ≥ 1.5 indicates genotoxicity (see Section 5.2). Statistical tables from Kastenbaum and Bowman (1970) were used to estimate statistical significant deviations from untreated control cells.

7. Results

The highest phenolic content in *D. membranacea* was measured in the acetone fraction and the lowest in the methanol/CH_2Cl_2 fraction (Table 1). The ethanol fraction contained the highest concentration of flavonoids and the acetone fraction contained the lowest amount of flavanoids. The acetone fraction showed the highest concentration of tannins and the ethanol fraction the lowest (Table 1).

The crude extract, ethanol and acetone fractions are moderately active against all strains tested in the disk diffusion assay (Table 2). A relatively high antimicrobial activity of crude extract was determined against *S. agalactiae*, *B. subtilis* and *E. faecium* with diameters of the zone of inhibition of respectively 17.5, 17 and 18.33 mm. The lower activity of crude extract was observed against Gram-negative bacteria (10–11.5 mm) and the fungal strain (11.66 mm) with MIC = 62.5–125 μg/ml and MBC = 125–500 μg/ml and MFC = 500 μg/ml (Table 3).

Table 1
Extraction yield and total phenolic (TPC), flavonoid (TFC) and tannin (TTC) contents of *Dictyopteris membranacea* crude extract and its semi-purified fractions.

Extract	YIELD (%)	TPC[a]	TFC[b]	TTC[c]
Crude extract	15.03 ± 2.15	24 ± 0.07	65 ± 0.03	21.43 ± 0.05
Ethanol fraction	7.35 ± 1.33	61 ± 0.05	120 ± 0.04	55.24 ± 0.03
Acetone fraction	11.73 ± 1.50	112 ± 0.05	55 ± 0.03	130.47 ± 0.02
Methanol/CH_2Cl_2 fraction	8.82 ± 0.86	24 ± 0.02	60 ± 0.02	30.52 ± 0.05

Results are presented as mean ± SD (n = 3). Significant difference obtained with: $p < 0.01$.

Table 2
Antimicrobial activity measured using the disk diffusion assay of the crude extract and its semi-purified fractions from *D. membranacea*: diameter of inhibition zone.

Microbial strains		Inhibition zone (mm)*				
		Crude extract	Ethanol fraction	Acetone fraction	Methanol/CH_2Cl_2 fraction	Ampicillin (10 μg/ml)
Gram (+)	*S. aureus*	14 ± 0.4	23.5 ± 0.2	22.5 ± 0.8	12 ± 0.6	40 ± 0.9
	S. agalactiae	17.5 ± 0.6	18 ± 0.7	19.66 ± 1.1	NA	38 ± 0.1
	B. subtilis	17 ± 0.1	19.66 ± 1.2	26 ± 0.4	NA	43 ± 0.2
	E. faecium	18.33 ± 1.1	21.66 ± 0.8	24 ± 0.4	10 ± 0.8	40 ± 0.4
	E. faecalis	13 ± 0.6	12 ± 0.2	13 ± 0.4	NA	37 ± 1.1
Gram (−)	*S. typhimurium*	10 ± 0.6	15.5 ± 0.2	14 ± 0.4	NA	33 ± 0.0
	E. coli	11.5 ± 0.6	13.66 ± 0.2	16.5 ± 0.4	NA	21 ± 1.1
Yeast	*C. albicans*	11.66 ± 0.4	14 ± 0.5	15.33 ± 0.2	NA	29 ± 0.3

(*) diameter of inhibition zone (mm) including disk diameter of 6 mm, NA: not active, values are means (mm. ± SD) of triplicate determination, values with different letters differ significantly: $p < 0.01$.

Ethanol and acetone fractions exhibited the highest antimicrobial activity against all the strains tested, particularly against Gram-positive bacteria tested in the disk diffusion assay (Table 2). Antimicrobial activity of methanol/CH_2Cl_2 fraction was only against two Gram-positive bacteria *S. aureus* and *E. faecium* (Table 2) with MIC values 250 and 500 μg/ml respectively (Table 3). Neither the Gram-negative bacteria (*E. coli*, and *S. typhimurium*) nor the yeast *C. albicans* were inhibited by this fraction. The acetone and ethanol fractions were the most potent with the lowest MIC and MBC values compared to the other prepared fractions (Table 3). The MIC values obtained against Gram-positive bacteria were greater than those against Gram-negative bacteria. All the prepared fractions showed the highest activity against *S. aureus* and moderate activity against the other strains. The MFC/MIC ratios also show that it is possible to define the antimicrobial effect of each extract (Table 3). Crude extract had a fungistatic effect against *C. albicans*, a bactericidal effect against *S. aureus*, *E. faecium*, *E. faecalis* and *S. typhimurium* and a bacteriostatic effect against *S. agalactiae*, *B. subtilis* and *E. coli*. Acetone fraction had a fungicidal effect against *C. albicans*, a bactericidal effect against *S. aureus*, *S. agalactiae*, *E. faecium*, *E. faecalis* and *S. typhimurium* and a bacteriostatic effect against *B. subtilis* and *E. coli*. Ethanol fraction had a fungistatic effect against *C. albicans*, a bactericidal effect against *S. agalactiae*, *E. faecalis*, *S. typhimurium* and *E. coli* and a bacteriostatic effect against *S. aureus*, *B. subtilis* and *E. faecium*.

Antitubercular activity could only be observed at high extract concentrations. The acetone fraction reduced in vitro growth of *M. tuberculosis* by 50% at 191.10 μg/ml while the critical concentration at which 90% of *M. tuberculosis* growth was inhibited was 387.38 μg/ml (Table 4). For the other fractions tested, the antitubercular activity was not significant. Furthermore, the results revealed that the acetone fraction showed a high bioactivity against the *M. tuberculosis* H37Rv. The critical concentration at which the *M. tuberculosis* growth is at 90% and 99%, could not be defined for the crude extract and methanol/CH_2Cl_2 fraction, although ethanol fraction reduced the bacillary growth by 90% and 99% at 365.10 μg/ml and 397.62 μg/ml, respectively (Table 4).

The Vitotox test was performed with sample concentrations between 0 and 120 μg/ml where the highest concentration as well as some lower concentrations showed toxicity. The positive controls 4-nitroquinoline oxide (4NQO; in absence of S9) and benzo(α)pyrene (BaP; in the presence of S9) were genotoxic (S/N > 1.5 in the Genox strain). Concentrations of 50 and 100 μg/ml crude extract showed toxicity in both the Genox and Cytox strain (S/N well below 0.8). Other concentrations were not toxic (except 25 μg/ml without S9 where S/N was also lower than 0.8). There was also no DNA damage independent effect on the lux operon as there was no increased light production in the Cytox strain (S/N remain approximately 1). There was no indication of genotoxicity as S/N never increased above the 1.5 level (Fig. 1).

A similar result was obtained with the other fractions. The higher concentrations showed some degree of toxicity (S/N < 0.8 in the Cytox strain) and there was no dose-dependent increase of S/N in the Genox strain. All S/N values remained approximately 1 (data not shown).

The micronucleus test also showed no genotoxic effect in human C3A cells for any of the samples. The positive control (15 μg/ml methyl methanesulfonate (MMS)) was clearly genotoxic (more than 70 micronuclei per 2000 binucleated cells) compared to 24 in the unexposed control cells ($p < 0.05$). The micronucleus frequencies of the crude extract and its ethanol, acetone and methanol/CH_2Cl_2 fractions fluctuated, showing an apparent protective effect (lower micronucleus frequencies compared to the unexposed control cells) for some of the

Table 3
Antimicrobial activity measured using the microwell assay of the crude extract and its semi-purified fractions from *D. membranacea*: MIC and MBC (or MFC).

		Gram (+)					Gram (−)		Yeasts
		S. aureus	*S. agalactiae*	*B. subtilis*	*E. faecium*	*E. faecalis*	*S. typhimurium*	*E. coli*	*C. albicans*
Crude extract	MIC	62.5	125	62.5	62.5	500	250	125	125
	MBC or MFC	125	500	250	125	500	500	500	500
	R*	2	4	4	2	1	2	4	4
Ethanol fraction	MIC	15.62	125	62.5	31.25	250	62.5	125	62.5
	MBC or MFC	62.5	250	250	125	500	62.5	250	250
	R*	4	2	4	4	2	1	2	4
Acetone fraction	**MIC**	15.62	62.5	31.25	31.25	125	62.5	62.5	62.5
	MBC or MFC	15.62	125	125	62.5	250	125	250	125
	R*	1	2	4	2	2	2	4	2
Methanol/CH_2CL_2 fraction	MIC	250	NA	NA	500	NA	NA	NA	NA
	MBC or MFC	>500	NA	NA	>500	NA	NA	NA	NA
	R*	–	–	–	–	–	–	–	–

MIC: the minimum inhibitory concentration (μg/ml); *MBC*: the minimum bactericidal concentration (μg/ml); *MFC*: the minimum fungicidal concentration (μg/ml); *NA*: not active, R*: the ratio (MBC/MIC) for the bacterial strains and (MFC/MIC) for the yeast.

Table 4
Antitubercular activity of the crude extract and its semi-purified fractions from *D. membranacea*.

Extract	Critical concentrations (μg/ml)		
	GI_{50}	GI_{90}	GI_{99}
Crude extract	236.69	NI	NI
Ethanol fraction	220.55	365.10	397.62
Acetone fraction	191.10	387.38	NI
Methanol/CH_2Cl_2 fraction	292.82	NI	NI

NI: not identified.

prepared fractions and concentrations (Fig. 2). Differences were however not statistically significant and there was never a significant increase in micronucleus frequency in the exposed vs. the control cells. Thus, the bacterial Vitotox test as well as the micronucleus test in human C3A cells showed that the crude extract and fractions were not genotoxic.

8. Discussion

From a quantitative point of view, it is difficult to compare our results with those reported previously due to variations in the extraction solvent and conditions used, as well as different assays used for the phytochemical analysis.

Phenolic compounds are integral structural components of cell walls in brown algae, as well as playing other roles such as protection from UV radiation and defense against grazing (Pavia et al., 1997). The yield in polyphenols in *D. membranacea* may indicate that they could have a protective role and antimicrobial activity against different pathologic strains (Rodrigues et al., 2011). Catechin is a type of natural phenol and antioxidant compound. It belongs to the group of flavan-3-ols (flavanols) which is part of the flavonoids. Several studies have found that catechins exhibit stronger antibacterial effects on Gram-positive bacteria than on Gram-negative bacteria (Toda et al., 1990; Ikigai et al., 1993). Tannins are natural polyphenols that have the ability to precipitate proteins and alkaloids (Hagerman et al., 1997; Amarowicz, 2007). Phlorotannins are a type of tannin found in brown algae. Contrary to hydrolysable or condensed tannins, these compounds are oligomers of phloroglucinol (polyphloroglucinols) (Shibata et al., 2004). Some phlorotannins have the ability to oxidize and form covalent bonds with some proteins and are anti-plasmin inhibitors and antioxidants (Nakayama et al., 1989; Fukuyama et al., 1990; Nakamura et al., 1996). In addition, these compounds have a pronounced bactericidal activity against pathogenic bacteria (Nagayama et al., 2002).

The quantity of these compounds in *D. membranacea* may explain the antioxidative and antimicrobial properties of different prepared fractions. The contents of each fraction with polyphenol, flavonoids and tannins depend on the extraction solvent and its polarity. The total phenolic (TPC), flavonoid (TFC) and tannin (TTC) contents of the crude extract were determined relative to the dried seaweed material. Acetone fractions with the highest phenolic and tannin contents had the highest antimicrobial activity. These levels are low in the methanol/CH_2Cl_2 fraction, which may explain its lack of antimicrobial effect. The antibacterial and antifungal activities of the ethanol fraction may be due to its high level of flavonoid content.

A major problem in antimicrobial chemotherapy is the increasing appearance of antibiotic resistance, which leads to the inadequacy of antimicrobial treatments (Valero and Salmeroj, 2003). Our results support previous findings that Gram-positive bacteria are more sensitive to seaweed extracts than gram-negative bacteria (Sahin et al., 2004; Jang et al., 2010). The resistance of Gram-negative bacteria to antibacterial agents can be explained by the different structures of the cell and the presence of a lipopolysaccharide-containing outer membrane in Gram-negative bacteria (Sawer et al., 1997; Gao et al., 1999; Jang et al., 2010). Extracts of marine brown algae exhibited antibacterial activity and antimicrobial activity (Funahashik et al., 2001, Harada et al., 2002, Kamenarska et al., 2009).

Etahiri et al. (2001) and Kubo et al. (1992) reported that extracts or substances derived from seaweeds, especially from Phaeophyceae, have antibacterial activity against strains of *S. aureus*. In addition, acetone extraction has been reported to result in higher antimicrobial activity than ethanol and methanol/CH_2Cl_2 extraction. The present study shows that organic solvents always have higher efficiency in extracting antibacterial compounds and that acetone as a solvent proved best suited for the extraction of the antibacterial constituent(s) from the algae. Most of the soluble components in seaweeds had high polarity. Connan et al. (2006) showed high levels of total phenolic content in fucoid seaweed species. The highest amount was found in the 70% acetone extract of *Fucus vesiculosus* (24.2 mg gallic acid equivalent/g extract). The present results indicate that the acetone semi-purified

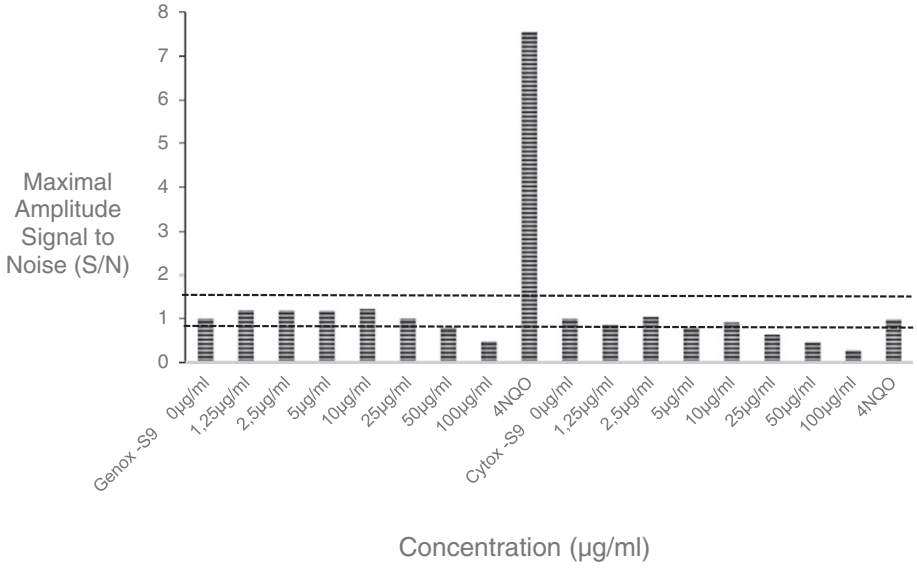

Fig. 1. Example of VITOTOX® test results for the crude extract of *Dictyopteris membranacea* in the absence of S9. The horizontal line at S/N = 1.5 indicates the threshold level for genotoxicity in the Genox strain, whereas the line at S/N = 0.8 gives the level below which S/N is indicative of a toxic response in the Cytox strain. 4-NQO = positive control.

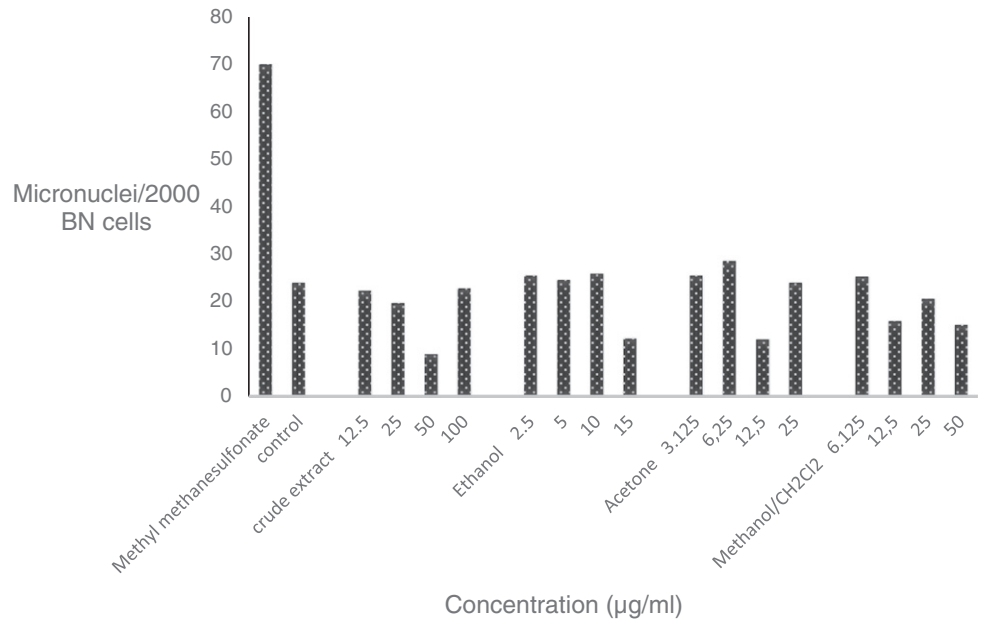

Fig. 2. Micronucleus test applied to the crude extract of *Dictyopteris membranacea* and its semi-purified fractions (ethanol, acetone and methanol/CH_2Cl_2 fraction); Methyl methanesulfonate (MMS): positive control.

fraction of *D. membranacea* has good antibacterial activity against some bacterial strains, which could have an importance for further research.

There is a positive correlation between phenolic content and antimicrobial activities (Oki et al., 2002). The high phenolic content of the semi-purified acetone fraction (112 mg GAE/g sample) shows the relationship between phenolic content and antimicrobial activity.

With respect to the investigation of the phytochemical and antimicrobial activities of the *D. membranacea* extract and its fractions, we can conclude that this activity varied significantly within concentrations and within strains. In general, the strong antimicrobial activity was related not only to a high content of antimicrobial component such as polyphenols and flavonoids, but also to the presence of the synergy between all compounds of each extract. Although the antimicrobial activity was only mild to poor for the crude extract, it was shown that purification to semi-purified ethanol and acetone fractions increased the potency.

For the genotoxicity study, we used the bacterial Vitotox test, which correlates very well with the Ames assay (Muto et al., 2006; Westerink et al., 2009) and hence can be considered a gene mutation test even if it is actually based on SOS induction. The micronucleus tests was conducted with human C3A hepatic cells because they largely conserved both phase I and phase II metabolic capacities (Kelly, 1994). Tests can therefore be conducted in the absence of S9. The combination of Vitotox and micronucleus tests thus allows a screening of the different types of genetic effects. According to our results, the crude extract and the ethanol, acetone and methanol/CH_2Cl_2 fractions are not genotoxic. To the best of our knowledge, this is the first report on the evaluation of the genotoxic 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.

In the next step of this research, we therefore envisage to purify and isolate the active metabolite(s) from the *D. membranacea*. Identification or characterization of the active ingredient may result in the discovery of a new class of therapeutics that might be used as antimicrobials.

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