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Research Article

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Assessment of *in vitro* antioxidant and *in vivo* anti-inflammatory activities of marine algae from Algerian coast

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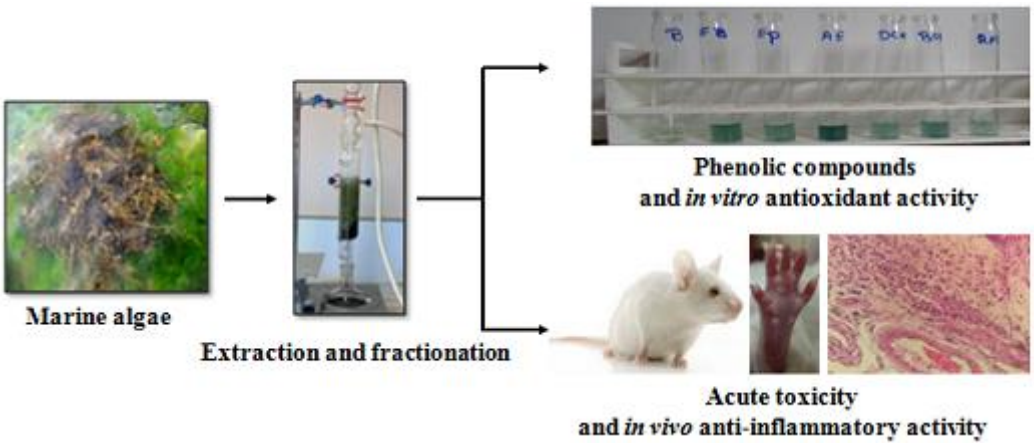
Highlights

- Crude extracts and fractions from three algal species *Sargassum vulgare*, *Cladostephus hirsutus* and *Rissoella verruculosa* were prepared.
- Total phenolic, flavonoid, flavonol and total condensed tannin contents were determined.
- The antioxidant activity of crude extracts and all fractions, using three assays, were evaluated.
- *In vivo* toxicological study and anti-inflammatory activity of crude extract of *Cladostephus hirsutus* were tested in Swiss albino mice.

Graphical Abstract



Abstract
The search for natural compounds with pharmaceutical activity indicated marine macroalgae as promising sources to supply novel compounds with potential bioactivities. In this study, three algae from the northwest coast of Algeria *Sargassum vulgare, Cladostephus hirsutus* and *Rissoella verruculosa* were investigated for their *in vitro* antioxidant activity and for their *in vivo* anti-inflammatory activity. Total phenolic, total flavonoid and flavonol, and condensed tannin contents were determined in crude extracts and petroleum ether, ethyl acetate (EA), dichloromethane and butanol fractions, and aqueous residues. Antioxidant activity was evaluated using several *in vitro* assays: Total Antioxidant Capacity, Ferric Reducing Antioxidant Power and diphenyl picryl hydrazyl radical scavenging activity. Acute toxicity of the crude extract of *C. hirsutus* was studied in Swiss albino mice and anti-inflammatory activity was evaluated using carrageenan-induced paw edema. Crude extracts revealed high total antioxidant activity ranging from 1.27 to 1.63 mg ascorbic acid equivalent/gram of dry matter for *R. verruculosa* and *C. hirsutus*, respectively. For the three algae, EA fractions showed the highest antioxidant activity by the three assays tested, with up to 67% of DPPH radical scavenging activity and an EC_{50} of 1.114 mg ml^{-1} by the ferric reducing power assay for the EA fraction of *R. verruculosa*. No signs of acute toxicity were observed in the crude extract of *C. hirsutus* at 150 and 300 mg/kg. Significant and dose-dependent activity was shown against acute inflammation with edema reduction of 58.86% and 71.01% at 150 and 300 mg/kg, respectively, after 6 hours of carrageenan administration.

Keywords: *Sargassum vulgare, Cladostephus hirsutus, Rissoella verruculosa,* Antioxidant activity, acute toxicity, anti-inflammatory activity.

Introduction

Marine organisms live in complex habitats exposed to extreme conditions, and in adapting to new environment surroundings, they produce a wide variety of specific and potent active substances that possess unique structural features that cannot be found elsewhere (Larsen et al., 2005; Eom et al., 2012). Marine algae, in particular, provide for an extraordinary reservoir of structurally diverse bioactive compounds (Zhang et al., 2012) such as carotenoid pigments (astaxanthin, zeaxanthin, β-carotene and fucoxanthin), polysaccharides (fucoidan, laminarins and alginic acid) and a series of polyphenolic compounds like catechins, phlorotannins, flavonoids, flavonols and flavonols glycosides (Rajauria et al., 2016). Many of these compounds have been demonstrated to possess a variety of biological activities, including antioxidant, antimicrobial, anticancer, anti diabetic, antiviral, cytotoxic, anti-thrombotic and anti-inflammatory effects (Lee et al., 2004; Demirel et al., 2009; Govindasamy et al., 2012). Among the most relevant compounds found in algae, antioxidants are the products that have attracted major interest (Zubia *et al*., 2009). Antioxidants play an important role in inhibiting and scavenging free radicals and thus providing protection to humans against inflammatory and neurodegenerative diseases (Butterfield et al., 2002; Khairy et al., 2015). Therefore, new interest has developed in searching for natural and safe antioxidative agents from marine algae. Reports on the biological activities of algal extracts from Algeria are very limited. Thus, the purpose of this work was to study the phytochemical composition and the *in vitro* antioxidant activity of crude extracts and several fractions of three selected algae, including two brown algae *Sargassum vulgare* and *Cladostephus hirsutus* and the red alga *Rissoella verruculosa*. The *in vivo* anti-inflammatory activity and acute toxicity of the crude extract of *Cladostephus hirsutus* were also investigated.

Materials and methods

Algal materials

Algae used in this study were *S. vulgare*, *C. hirsutus* and *R. verruculosa*. They were collected by handpicking from Ain Timouchent region situated in the Northwest Coast of Algeria (Latitude 35°18′ N, Longitude 1°28′ O) during June 2013. Samples collected were initially washed thoroughly with sea water to remove epiphytes and other dirt particles and transported to the laboratory immediately in plastic bags containing water, then rinsed with tap water followed by distilled water to remove salts, and shade dried at room temperature. Dried algae were ground in an electric mixer and stored at 4 °C for further experiments. Algal species were identified by Dr. Benguedda Wacila from the University of Tlemcen, Algeria and voucher specimens are deposited in the herbarium of our Laboratory of Natural Products.

Preparation of algal crude extracts and fractionation

Powdered samples (100 g) were extracted by using a Soxhlet apparatus with 500 mL methanol:chloroform (2:1 V/V) for 6 h, according to Duan et al. (2006). Extraction was repeated twice and the combined organic solution was evaporated under vacuum to obtain a crude extract, which was dissolved in 100 mL of 90% aqueous methanol. First fractionation was carried out with 3 × 100 mL petroleum ether. The aqueous methanol solution was evaporated under reduced pressure to give a semisolid, which was further dissolved in 200 mL distilled water and then extracted successively with 3 × 100 mL of ethyl acetate, 3 × 100 mL of dichloromethane and 3 × 100 mL of n-butanol, respectively. Resulting fractions were evaporated to dryness, to yield the petroleum ether (PE), ethyl acetate (EA), dichloromethane (DCM), butanol (BOH) fractions, and aqueous residue (AR).

1.1. Determination of phenolic compounds

Total phenolic content

Total phenol content (TPC) was determined by the spectrophotometric method based on the procedure described by Singleton and Rossi, (1965) using Folin-Ciocalteu reagent, with gallic acid as standard. 200 µL of sample were mixed with 1000 µL of Folin-Ciocalteu reagent (diluted with distilled water 1:9), and 800 µL of 7.5% sodium carbonate solution (Na_2CO_3). The mixtures were then incubated in the dark at room temperature for 30 min, absorbance was measured at 765 nm using a Specord 200 plus UV/VIS spectrophotometer. Estimation of the phenolic content was carried out in triplicate and results were expressed as milligrams of Gallic Acid Equivalents per Gram of Dry Matter (mg GAE/g DM).

Total flavonoids

Total flavonoid content (TFC) was determined using the colorimetric assay described by Zhishen et al. (1999). Briefly, 500 µL of samples were mixed with 1500 µL of distilled water and 150 µL of 5% sodium nitrate ($NaNO_2$) solution. After 5 min, 150 µL of 10% aluminum chloride ($AlCl_3$) was added. After 6 min of incubation, at room temperature, 500 µL of 1 M sodium hydroxide (NaOH) were added. The mixture was homogenized immediately and absorbance was measured at 510 nm against a blank. A calibration curve was prepared with catechin as standard and the results were expressed as milligram Catechin Equivalents per Gram of Dry Matter (mg CE/ g DM).

Total flavonols

Total flavonol content (TF) was determined according to Kumaran et al. (2007). Sample (250 µL) was mixed with 250 µL of $AlCl_3$ (2 mg/mL) and 1500 µL of sodium acetate (50 mg/ mL). The mixture was allowed to stand for 2.5 h at room temperature and absorbance was then read

at 440 nm. Quercetin was used as standard. Flavonol content was expressed as mg of Quercetin Equivalents per Gram of Dry Matter (mg QE/ g DM).

Condensed tannins

Condensed tannin content (CTC) was estimated using the vanillin assay described by Julkunen-Titto (1985). Briefly, 50 µL of sample were mixed with 1500 µL of 4% vanillin (prepared with methanol). After homogenization, 750 µL of concentrated HCl were added. The solution was shaken vigorously and left to stand at room temperature for 20 min in the dark. Absorbance was measured at 550 nm against blank. Catechin was used as standard. The amount of condensed tannins was expressed as milligram of Catechin Equivalents per Gram of Dry Matter (mg CE/ g DM).

In vitro antioxidant activity

Determination of total antioxidant capacity

Total antioxidant capacity (TAC) of crude extracts and fractions was determined according to Prieto et al. (1999). Briefly, 0.3 mL of sample was mixed with 3 mL of standard reagent (0.6 M sulfuric acid, 28 mM sodium phosphate and 4 mM ammonium molybdate) and incubated at 95 °C for 90 min. The sample was cooled down to room temperature and absorbance was measured at 695 nm against a blank. Ascorbic acid was used as standard. TAC of samples was expressed as milligrams of Ascorbic Acid Equivalents per Gram of Dry Matter (mg AAE/ g DM).

Ferric reducing antioxidant power (FRAP)

Reducing power of extracts was determined following the method of Oyaizu (1986). A 0.2 M phosphate buffer (pH 6.6) (2.5 mL) and 1% of potassium ferricyanide water solution (2.5 mL, $K_3[Fe(CN)_6]$) were mixed with 1 mL of extract at varying concentrations and incubated at 50 °C for 20 min. A 2.5 mL trichloracetic acid (10%) was added to the mixture, which was then centrifuged at 3000 rpm for 10 min. The upper layer of the solution (2.5 mL) was mixed with distilled water (2.5 mL) and a freshly prepared $FeCl_3$ solution (0.5 mL, 0.1%) and absorbance measured at 700 nm against a blank. An increased absorbance of the reaction mixture was taken to mean an increased reducing power. Ascorbic acid was used as positive control.

DPPH radical scavenging activity

Radical scavenging capacity of samples was evaluated as the ability to scavenge the free radical DPPH (diphenylpicrylhydrazyl). DPPH assay was realized according to Sanchez-Moreno et al. (1998). 50 µL of sample at different concentrations (0.5 to 6 mg/mL) was added to 1.950 mL of a 0.025 g/L DPPH methanolic solution. The mixture was vortexed and then left to stand at room temperature for 30 min in the dark. Absorbance was read at 515 nm against a blank, and the percentage of scavenging activity was calculated using the following formula:

$$\text{DPPH scavenging activity (\%)} = (A_{blank} - A_{sample} / A_{blank}) \times 100$$

Where: A_{blank} is the value of DPPH alone; A_{sample} is the value of DPPH mixed with the sample.

In vivo anti-inflammatory activity

Animals

Male Swiss albino mice (19-30g) were procured from the Pasteur Institute (Alger, Algeria).The animals were acclimated to laboratory condition for 10 days prior to the experiment and were allowed free access to a commercial pellet diet and water *ad libitum*. Before the day of assay, Swiss albino mice were deprived of food overnight and divided into six groups with six animals in each group.

Acute toxicity study

Acute oral toxicity test for the crude extract of *C. hirsutus* was conducted in accordance with OECD (Organization for Economic Cooperation and Development) guidelines. Doses of 150 mg/kg and 300 mg/kg were orally administered to the two groups of animals. Behavioral parameters (increase in activity, salivation, coma and convulsion) and mortality were observed closely for 24 h after extract administration (Paschapur et al., 2009).

Carrageenan-induced Paw Edema

The anti-inflammatory activity of crude extract of *C. hirsutus* against acute inflammation was examined by carrageenan-induced paw edema according to the method of Trovato et al. (2001). Each of the four animal groups was given orally either NaCl 0.9% (control), Diclofenac sodium, a nonsteroidal, anti-inflammatory drug, (50 mg/kg), or the crude extract of *C. hirsutus* (150 mg/kg and 300 mg/kg). One hour after oral administration of different drugs, acute inflammation is induced by injection of 0.1 ml of 0.5% carrageenan into the footpad of the hind paws of each mouse. The paw volume of mice was measured by plethysmometer, before and after injection of carrageenan at different time intervals (1, 2, 3, 4, 5 and 6h). The percentage inhibition (INH %) of the inflammation for each animal group was calculated by the following formula

$$INH\ \% = (D - Dt) / D0 \times 100$$

where D is the diameter of injected paw, D0 is the average inflammation (hind paw edema) of the control group of mice at a given time 0; and Dt is the average of diameters of hind paw edema of the drug treated (i.e. extract or reference diclofenac) mice at the same time (Marzocco et al., 2004).

Histopathological Examination

After 6 h, the animals were sacrificed and the carrageenan-induced edema feet were removed and fixed in a solution of 10% formalin. The material was embedded in paraffin, cut into sections, staind with hematoxylin eosin and viewed under the light microscope. Histological analysis was based on the edema and inflammatory cell infiltrate observed in epithelial and connective tissues.

Statistical analysis

Three replications of all assays were used to calculate means and standard deviations (SD). Data was evaluated using Pearson's correlation coefficient (*r*) to explore the relationship between phenolic compounds (total phenolic, flavonoid, flavonol and condensed tannin contents), and also between phenolic compounds and the antioxidant activities (total antioxidant capacity and ferric reducing antioxidant power).

Results and discussion

Crude extracts, fraction yields and phenolic compounds

Yields of the three algae extractions are given in Table 1. The yields of *S. vulgare*, *C. hirsutus* and *R. verruculosa* were 3.101 ± 0.556, 2.483 ± 0.164 and 1.953 ± 0.379%, respectively. The highest yields of the liquid-liquid extractions were obtained from PE fractions of *S. vulgare* (41.825 ± 0.004%) and *R. verruculosa* (27.803 ± 0.070%) and from AR of *C. hirsutus* (33.145 ± 0.156%), while the lowest yields were obtained from DCM fractions of all the three species. Considerable variations in extraction yield were found among different algae species. When compared to our study, the yield of the crude extract from *R. verruculosa* was comparable to the yield from the red alga *Euchema kappaphycus* (2.85%) (Ganesan et al., 2008). Whereas, higher yields are reported from the crude extracts of brown algae including *S. vulgare* (10.1%),

Padina pavonica (11.9%) (Khaled *et al*., 2012), *S. marginatum* (5.45%), *P. tetrastomatica* (12.31%) and *Turbinaria conoides* (5.76%) (Chandini *et al*., 2008). These considerable differences in the yields of the total extracts and fractions from various algae may be due to species-specific differences and varied extraction conditions, such as pH, varying solvent polarities, extraction time and temperature as well as the chemical compositions of the sample (Cho et al., 2011; López et al., 2011).

Table 1. Yield of the algae crude extracts and fractions.

	Yield (%)					
	CE	**PE**	**EA**	**DCM**	**BOH**	**AR**
S. vulgare	3.101±0.556	41.825±0.004	20.670±0.441	0.783±0.006	4.385±0.0146	17.865±0.157
C. hirsutus	2.483±0.164	23.278±0.093	18.082±0.003	1.044±0.013	7.652±0.037	33.145±0.156
R. verruculosa	1.953±0.379	27.803±0.070	10.599±0.077	1.075±0.011	10.266±0.031	27.619±0.240

CE: Crude extract; PE: Petroleum ether fraction; EA: Ethyl acetate fraction; DCM: Dichloromethane fraction; BOH: n-butanol fraction; AR: Aqueous residue.

Total phenolic, flavonoid, flavonol and condensed tannin contents of the three algae were given in Table 2. Various phenolic compounds including phenolic acids, flavonoids and tannins are commonly found in red, brown and green algae (Duan et al., 2006; Kuda et al., 2007). The highest TPC was observed in the crude extract of *R. verruculosa* (1.101 ± 0.007 mg GAE/g DM) followed by the crude extracts of *C. hirsutus* (1.042 ± 0.003 mg/g) and *S. vulgare* (0.662 ± 0.011 mg/g). EA fractions showed the highest amount in both brown algae while AR fraction was found to contain the highest TPC (0.686 ± 0.005 mg/g) in the red alga. Duan et al. (2006) observed higher phenolic contents (71.6 ± 0.00 mg GAE/g DM, 73.07 ± 0.00 mg GAE/g DM) in crude extract and EA fraction of Chinese red alga *Polysiphonia urceolata*. Chandini et al. (2008) reported the phenolic content of 0.29 GAE/g DM and 0.86 GAE/g DM in aqueous fraction of brown algae *S. marginatum* and *T. conoides*, respectively.

Crude extracts from all tested algae showed higher flavonoids content than fractions. TFC of *S. vulgare* is ranged from 0.021 ± 0.001 to 0.340 ± 0.005 mg CE/g DM. For *C. hirsutus*, it is ranged between 0.006 ± 0.000 and 0.427 ± 0.003 mg CE/g DM) and varied from 0.005 ± 0.001 to 0.399 ± 0.003 mg CE/g DM for *R. verruculosa*.

Crude extracts of *S. vulgare* and *C. hirsutus* exhibited higher TF (0.640 ± 0.041 mg QE/g DM and 0.559 ± 0.026 mg QE/g DM, respectively), compared to the crude extract of *R. verruculosa* and all the other fractions. TCT were higher in crude extracts of *S. vulgare* (2.636 ± 0.285 mg CE/g DM) and *C. hirsutus* (2.738 ± 0.008 mg CE/g DM), comparatively to *R. verruculosa* crude extract (1.520 ± 0.005 mg CE/g DM). In EA fractions of the two brown algae investigated, TCT were also significant with values of 2.164 ± 0.055 mg/g for *S. vulgare* and 2.783 ± 0.034 mg/g for *C. hirsutus*. The great variability observed in the phenolic contents in the algae could originate from external environmental factors such as herbivory, light, depth, salinity, nutrients, seasonality as well as intrinsic ones such as age, length, type of the tissues. All these factors could act on the spatio-temporal regulation of the phenolic metabolic expression inducing marked qualitative and quantitative variations among individuals at a very small scale, together with intra-individual variations (Connan et al., 2004).

Table 2. Phenolic compounds of crude extracts and fractions of *S. vulgare, C. hirsutus* and *R. verruculosa*.

Crude extracts and fractions	**TPC**	**TFC**	**TF**	**TCT**

S. vulgare	**CE**	0.662±0.011	0.340±0.005	0.640±0.041	2.636±0.285
	PE	0.182±0.004	0.087±0.002	0.287±0.005	0.800±0.025
	EA	0.280±0.009	0.140±0.001	0.248±0.012	2.164±0.055
	DCM	0.027±0.240	0.021±0.035	0.026±0.001	0.052±0.000
	BOH	0.029±0.310	0.022±0.003	0.074±0.001	0.060±0.001
	AR	0.104±0.003	0.021±0.001	0.002±0.000	0.009±0.000
C. hirsutus	**CE**	1.042±0.003	0.4277±0.003	0.5594±0.026	2.738±0.008
	PE	0.157±0.002	0.0943±0.001	0.205±0.004	0.814±0.003
	EA	0.525±0.001	0.278±0.004	0.286±0.009	2.783±0.034
	DCM	0.070±0.085	0.024±0.001	0.058±0.000	0.158±0.001
	BOH	0.096±0.012	0.006±0.004	0.063±0.004	0.068±0.003
	AR	0.144±0.006	0.017±0.006	0.006±0.000	0.049±0.001
R. verruculosa	**CE**	1.101±0.007	0.399±0.003	0.273±0.010	1.520±0.005
	PE	0.080±0.001	0.091±0.000	0.145±0.004	0.235±0.022
	EA	0.144±0.002	0.125±0.001	0.171±0.006	0.285±0.005
	DCM	0.008±0.002	0.005±0.001	0.005±0.000	0.027±0.007
	BOH	0.072±0.001	0.016±0.000	0.034±0.001	0.052±0.036
	AR	0.686±0.005	0.020±0.000	0.011±0.000	0.041±0.001

CE: Crude extract; PE: Petroleum ether fraction; EA: Ethyl acetate fraction; DCM: Dichloromethane fraction; BOH: n-butanol fraction; AR: Aqueous residue; TPC: Total Phenolic Content was expressed as mg of gallic acid equivalents per gram of dry matter (mg GAE/g DM); TFC: Total Flavonoid Content was expressed as milligram of catechin equivalents per gram of dry matter (mg CE/ g DM); TF: Total Flavonol content was expressed as mg of quercetin equivalents per gram of dry matter (mg QE/ g DM); TCT: Total Condensed Tannin content was expressed as milligrams of catechin equivalents per gram of dry matter (mg CE/g DM).

In vitro antioxidant activity

Total antioxidant capacity

TAC of the algae species is represented in Figure 1. Higher activities were observed in the crude extracts of *C. hirsutus* (1.63 ± 0.008 mg AAE/g DM) and *S. vulgare* (1.583 ± 0.042 mg/g), and to a less extent in the crude extract of *R. verruculosa* (1.272 ± 0.009 mg/g). EA fractions of three algae have the strongest TAC compared to all the other fractions. Chandini et al. (2008) noticed lower antioxidant activity (0.31, 0.08 and 0.17 mg AAE/g of seaweed on dry weight basis) in EA fraction of *S. marginatum*, DCM fraction of *P. tetrastomatica* and aqueous fraction of *T.* conoides, respectively.

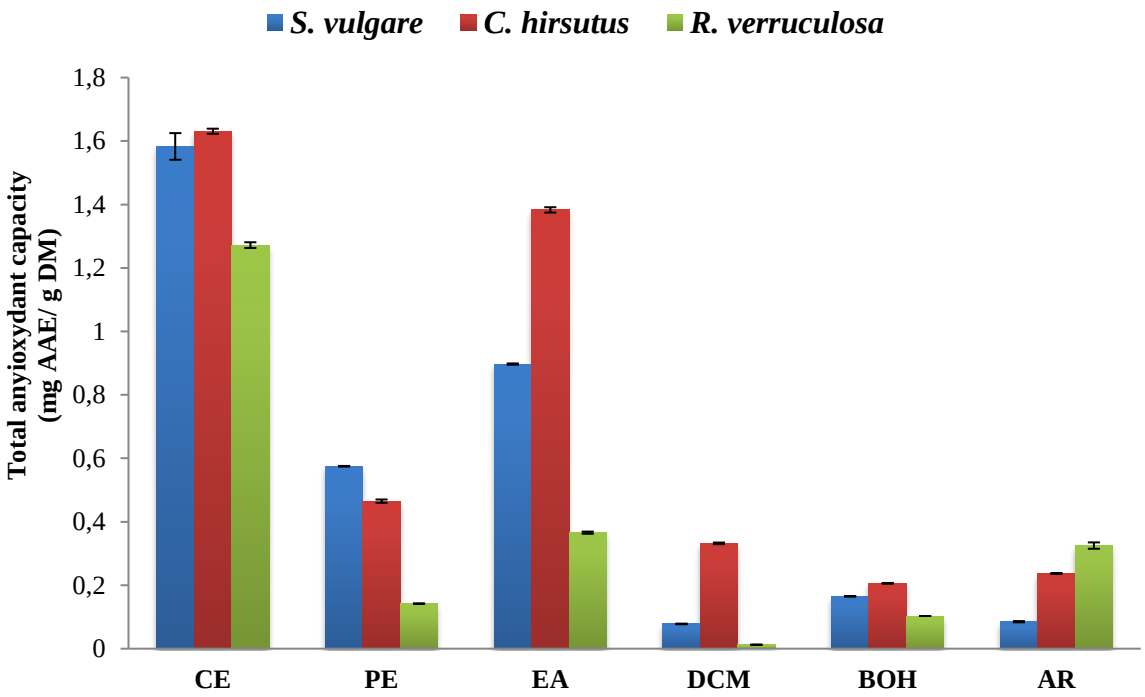


Figure 1. Total antioxidant capacity of crude extracts and fractions of *S. vulgare*, *C. hirsutus* and *R. verruculosa*.

CE: Crude extract; PE: Petroleum ether fraction; EA: Ethyl acetate fraction; DCM: Dichloromethane fraction; BOH: n-butanol fraction; AR: Aqueous residue; Total antioxidant activity was expressed as milligrams of ascorbic acid equivalents per gram of dry matter (mg AAE/ g DM).

Ferric reducing antioxidant power

Evaluation of the antioxidant activity by using FRAP assay is shown in Figure 2. Higher reducing power was observed in EA fractions of all tested algae as compared to crude extracts and other fractions. EA fraction of *R. verruculosa* showed the highest ability for reducing Fe^{+3} (EC_{50} = 1.114 ± 0.000 mg/mL) followed by EA fractions of *C. hirsutus* (EC_{50} = 1.276 ± 0.001 mg/mL) and *S. vulgare* (EC_{50} = 1.281 ± 0.006 mg/mL). The lowest activity was observed in PE fraction of *R. verruculosa* (10.195 ± 0.001 mg/mL) and AR of both brown algae. The reducing capacity of a compound may serve as a significant indicator of its potential antioxidant activity. Zhang et al. (2007) reported reducing power in the range of 0.07 to 11.60 mg AAE/g DM in 28 species of brown, red and green algae.

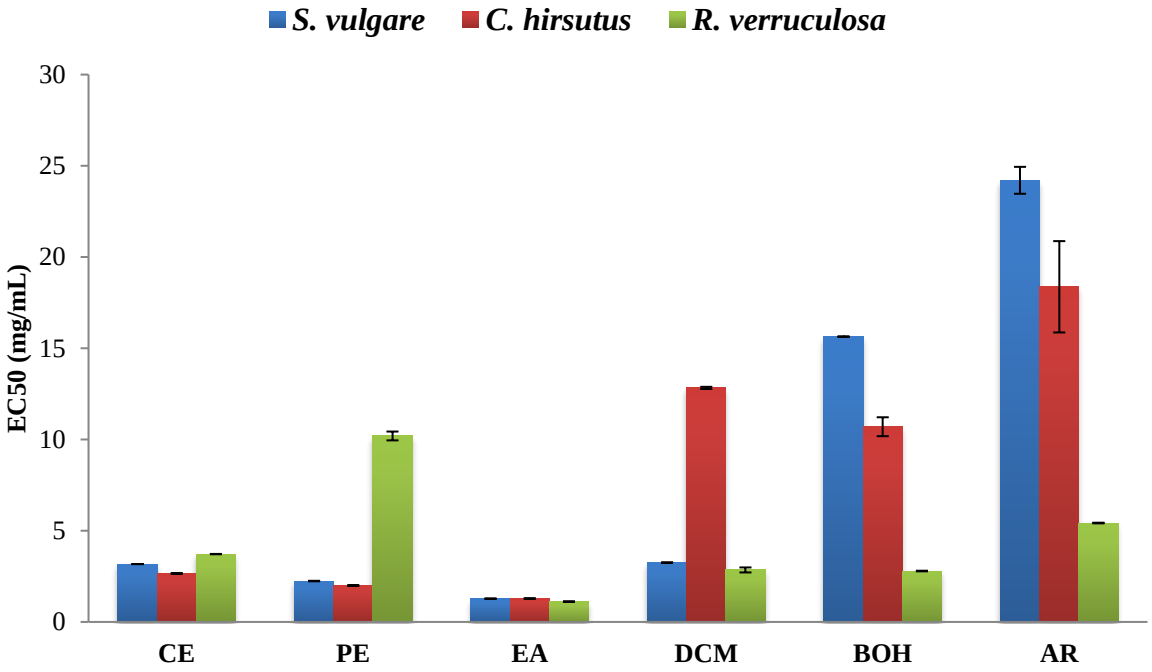


Figure 2. EC_{50} (mg/mL) concentrations of reducing power of crude extracts and fractions of *S. vulgare*, *C. hirsutus* and *R. verruculosa*.

CE: Crude extract; PE: Petroleum ether fraction; EA: Ethyl acetate fraction; DCM: Dichloromethane fraction; BOH: n-butanol fraction; AR: Aqueous residue.

DPPH radical scavenging activity

Free scavenging ability of the studied algae were expressed in percentage % and presented in Figure 3. DPPH radical scavenging activity was concentration dependent and increased with concentrations. Measured values of DPPH inhibition activity in the crude extracts and different fractions varied from 0.164 ± 0.179 to 67.877 ± 0.454%. EA fraction of *R. verruculosa* was found to have the maximum activity (67.8%) followed by EA fractions of *S. vulgare* and *C. hirsutus*, which had approximately the same value (43%). DPPH has been used extensively as a free radical to evaluate reducing substances and is a useful reagent for investigating the free radical scavenging activities of compounds (Duan et al., 2006). From the present findings, EA fraction of *R. verruculosa* has strong antioxidant activity 67.877 ± 0.454%, which were similar to those reported by Souza et al. (2011) who showed activity of 60% at the concentration of 5 mg/mL in the ethanolic extract of the red alga *Gracilaria birdiae*. On the other hand, Duan et al. (2006) noticed higher DPPH scavenging in crude extract, fractions and sub-fractions derived from *Polysiphonia urceolata*. Our findings are in agreement with those found by Duan et al. (2006) where the ethyl acetate-soluble fraction showed strongest activity. The activity of this fraction was observed to be significantly higher than that of crude extract itself. Indeed this is probably due to interactions between the compounds present in the extract that can exert an antagonistic effect between them (Khaled et al., 2012). In many studies, the antioxidant activity of whole algae, their parts, extracts and fractions has been attributed to the phenolic compounds. However, the observed activity could be a result of the complex composition of the macroalgal extracts (Balboa et al., 2013).

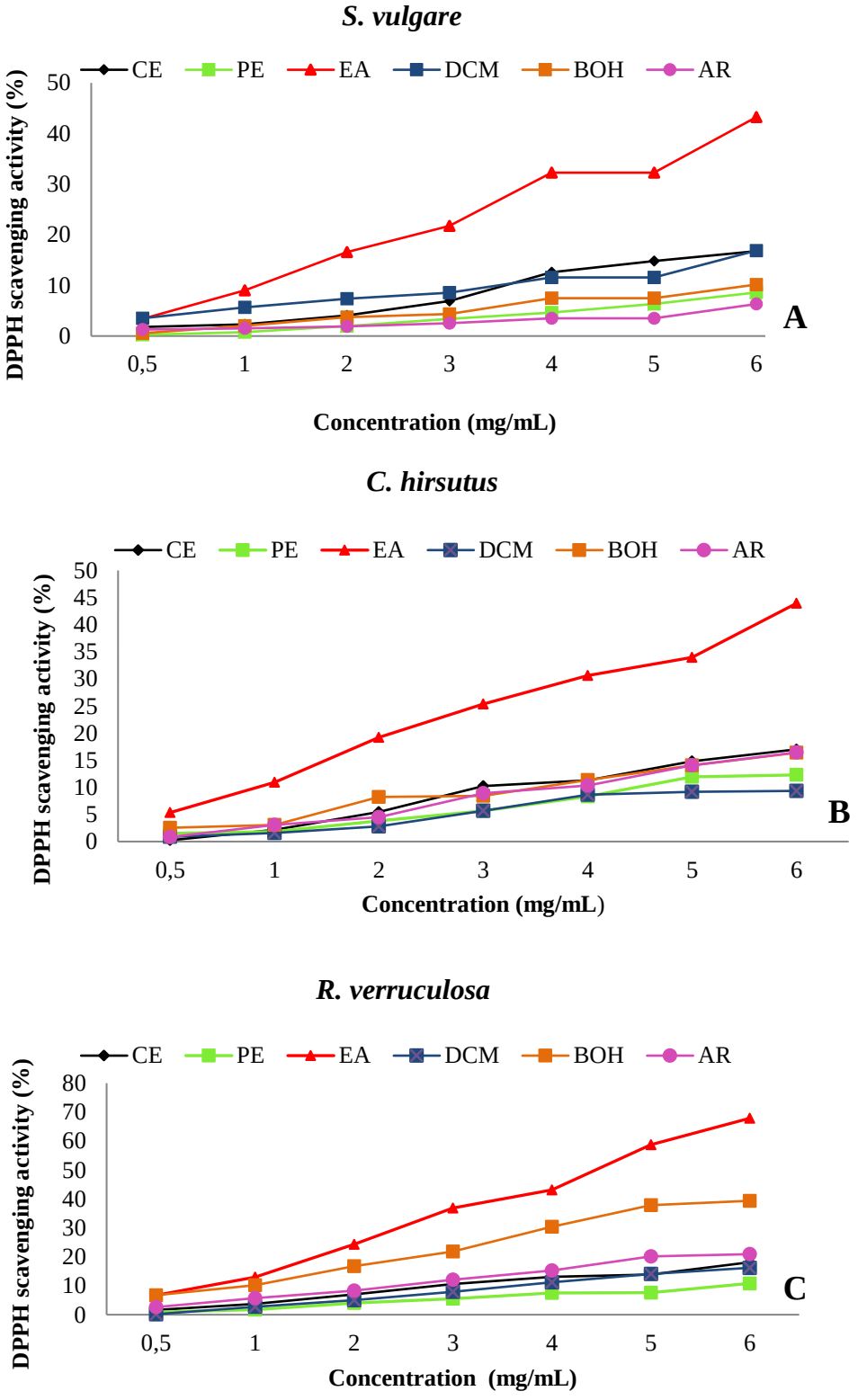


Figure 3. DPPH inhibition activity (%) of crude extracts and fractions at different concentrations; (A): *S. vulgare*; (B): *C. hirsutus*; (C): *R. verruculosa*.

Correlation between phenolic compounds and antioxidant activities

A Pearson's correlation between phenolic compounds and antioxidant assays are presented in Table 3. As shown in the table, there were strong positive correlations between total phenolic, flavonoid, flavonol and condensed tannin contents ($0.990 \geq r \geq 0.519$), and a high positive correlation between total antioxidant capacity and phenolic compounds ($r \geq 0.808$) in the three tested algae. Several studies have demonstrated a highly significant correlation between phenolic content and different antioxidant activity in algae extracts (Connan et al., 2006; Duan et al., 2006; Zhang et al., 2006; Zhang et al., 2007). A moderate negative correlation was obtained between phenolic content and ferric reducing antioxidant power ($-0.469 \geq r \geq -0.674$) in the brown algae, but no linear relationship was found between these compounds, total antioxidant capacity and ferric reducing antioxidant power ($0.042 \geq r \geq -0.062$) in the red alga. It was reported by Sabeena Farvin et al. (2013) that no clear correlation exists between TPC and Fe^{2+} chelating activity and thus phenolic compounds do not appear to be the major contributors to the metal chelating properties of the ethanolic extracts of some selected species of marine algae from Danish coast.

Table 3. Pearson correlation coefficient calculated among measured antioxidant activities and quantified chemical compounds.

	S. vulgare and C. hirsutus						***R. verruculosa***					
	TPC	**TFC**	**TF**	**TCT**	**TAC**	**FRAP**	**TPC**	**TFC**	**TF**	**TCT**	**TAC**	**FRAP**
TPC	1	0.977	0.883	0.874	0.933	-0.469	1	0.753	0.519	0.788	0.898	-0.011
TFC		1	0.929	0.936	0.976	-0.574		1	0.922	0.990	0.958	-0.055
TF			1	0.875	0.931	-0.638			1	0.866	0.808	0.042
TCT				1	0.975	-0.674				1	0.965	-0.062
TAC					1	-0.620					1	-0.133
FRAP						1						1

TPC: Total Phenolic Content; TFC: Total Flavonoid Content; TF: Total Flavonol content; TCT: Total Condensed Tannin Content; TAC: Total Antioxidant Capacity; FRAP: Ferric Reducing Antioxidant Power.

***In vivo* anti-inflammatory activity**

Acute toxicity

Within the 24-hour observation period, no mortality or changes in behavioral parameters such as increased activity, salivation, coma, or convulsions were observed after oral administration of CE of *C. hirsutus* at doses of 150 mg/kg and 300 mg/kg.

Carrageenan-induced Paw Edema

The effect of CE of *C. hirsutus* on carrageenan-induced paw edema in mice has been established. As illustrated in Figure 4, injection of 0.1 mL carrageenan (0.5%) produced a progressive increase in paw edema to a maximum volume at 6 h in the control group, the AUG % for the reference group declined at 2 and 4 h and gradually increased after the fourth hour. However, groups pretreated with CE (150 and 300 mg/kg) showed a significant reduction in AUG % as compared with the control and reference groups.

Percentage inhibition (INH %) of paw edema volume at different time intervals is shown in Figure 5. Edema was reduced by crude extract of *C. hirsutus* in a dose dependent manner till the end of sixth hour. Significant INH% of paw edema was observed with both doses tested (150 mg/kg and 300 mg/kg) with maximum INH% of 58.86% and 71.01%, respectively, at 6 hour while, Diclofenac sodium (50 mg/kg) prevented carrageenan-induced paw edema with a low INH% of 67.49% and 54.48% at 4 and 6 hour, respectively.

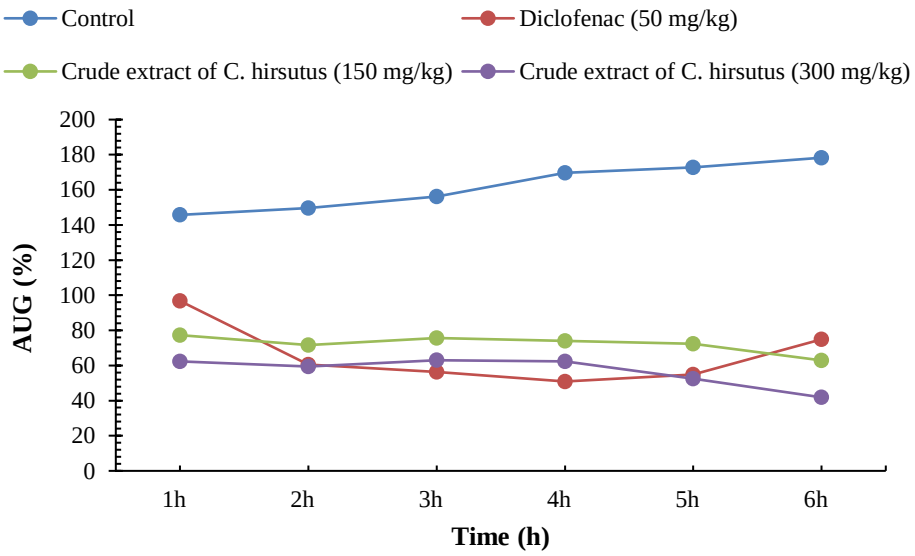


Figure 4. Percentage augmentation (AUG %) of edema after injection of carrageenan at different time intervals.

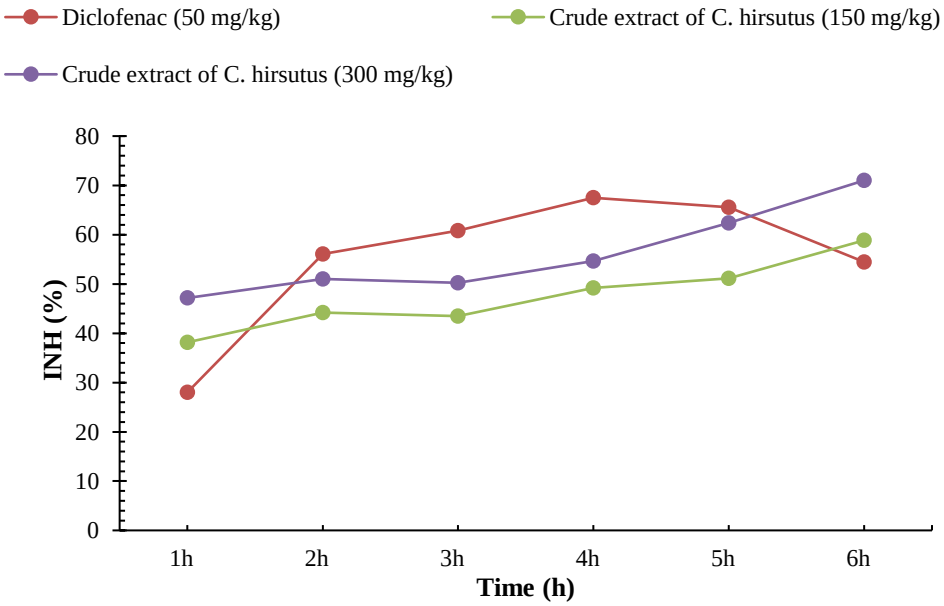


Figure 5. Percentage inhibition (INH %) of edema by crude extract of *C. hirsutus* (150 mg/kg and 300 mg/kg) at different time intervals determined with diclofenac sodium (50 mg/kg) as reference.

Histopathology Analysis

The histopathological results of edema paws 6 h after injection of carrageenan are illustrated in Figure 6. Control group showed massive accumulation of infiltrated inflammatory cells with predominance of polymorphonuclear neutrophils and edema formation (Figure 6A), reference group displayed moderate number of inflammatory cell infiltration (Figure 6B). While, less edema and only few inflammatory cell infiltration were observed in groups pretreated with CE (150 and 300 mg/kg) (Figure 6C, 6D).

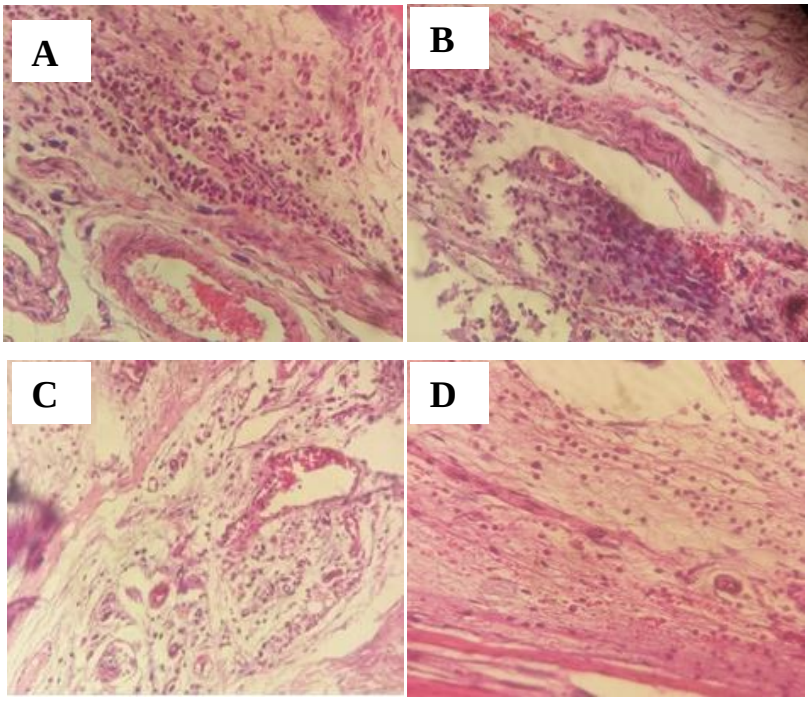


Figure 6. Histological evaluation of anti-inflammatory effect of crud extract of *C. hirsutus*. (A): control (NaCl 0.9%), (B): reference (diclofenac sodium 50 mg/kg), (C): CE of *C. hirsutus* at 150 mg/kg, (D): CE of *C. hirsutus* at 300 mg/kg. Each group was assessed at 200× magnification.

According to several works, different types of marine algae possess anti-inflammatory properties. (Ananthi et al., 2010; Cavalcante-Silva et al., 2012; Islam et al., 2013; Bitencourt *et al*., 2015). Unique compounds with anti-inflammatory activity have been identified in red, green and brown algae. These include polyphenols, sulfated polysaccharides, terpenes, fatty acids, proteins and several other bioactives (Fernando et al., 2016). To our knowledge, this is the first research into the toxicity and the anti-inflammatory effect of the extract of *C. hirsutus*. The data indicated that the crude extract of *C. hirsutus* produced a dose-dependent anti-inflammatory effect on carrageenan induced inflammatory paw edema in the mice and this effect was higher than that of the diclofenac sodium which is used as a reference drug. According to Dore et al. (2013), Fucan (SV1) sulfated polysaccharides from the brown algae *Sargassum vulgare* displays strong anti-inflammatory action at all concentrations tested in the carrageenan induced paw edema model, demonstrated by reduced edema and cellular infiltration; a tricyclic brominated diterpenoid isolated from the organic extract of the red algae *Laurencia glandulifera* shown a significant *in vivo* and *in vitro* anti-inflammatory activity (Chatter et al., 2011). Intraperitoneally injection of organic fractions chloroform (F-CHCl3) and ethyl acetate (F-AcOEt) of *C. sedoides* at different doses (25 and 50 mg/kg, (i.p)), exhibited a dose-dependent reduction of rat paw edema. The percentage of inhibition of edema, 3 h after carrageenan injection, ranged from 67.71% to 73.49% and from 67.74% to 74.58%, for F-$CHCl_3$ and F-AcOEt, respectively (Mhadhebi et al., 2011).

Conclusion

The results of the current study indicated that crude extracts and several fractions of the three algae *Sargassum vulgare, Cladostephus hirsutus* and *Rissoella verruculosa* contained phenolic compounds in various proportions and showed certain levels of *in vitro* antioxidant activity and *in vivo* anti-inflammatory activity without toxicity. Finally, we suggest that algae from the

Algerian coast could be a potential source of promising bioactive compounds and might be useful for developing natural antioxidants to combat oxidative stress and inflammatory diseases.

Conflict of Interest

The authors declare that they have no conflict of interest.

Author Contribution Statement

G. Aissaoui: Carried out the experiment and wrote the manuscript; N. Belyagoubi-Benhammou and L. Belyagoubi: analysed the data and corrected the manuscript; M. Benmahdjoub and K. Kerzabi-Kanoun: Contributed in experiment and discussion of antioxidant activity results; S. Mansour, N. Djebli and H. Bouakline: Contributed in experiment and discussion of anti-inflammatory activity results. W. Rahal-Benghedda: identified the algal species; F. Atik-Bekkara: Designed and supervised this research.

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