https://doi.org/10.48047/AFJBS.6.15.2024.9582-9599

African Journal of Biological Sciences

Journal homepage: http://www.afjbs.com

Research Paper Open Access

The properties of astaxanthin extracted from Algerian green microalga *Haematococcus pluvialis*

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Volume 6, Issue 15, Sep 2024

Received: 15 July 2024

Accepted: 25 Aug 2024

Published: 05 Sep 2024

doi: 10.48047/AFJBS.6.15.2024.9582-9599

Abstract

The present study aimed to investigate the antioxidant, analgesic and anti-inflammatory properties of astaxanthin extracted from a new Algerian strain of *Haematococcus pluvialis* (*H. pluvialis*). Antioxidant activity of astaxanthin was evaluated with FRAP, β-carotene bleaching and FTC methods. Analgesic activity was determined by acetic acid-induced abdominal constrictions model. Anti-inflammatory activity was determined using I*n vitro*, *In vivo* and *In silico* methods. Results showed that iron reducing capacity astaxanthin evaluated with FRAP test was about 3.7 times higher than that recorded with the standard, ascorbic acid. The β-carotene bleaching test showed that this carotenoid was more effective on inhibition of the lipid peroxidation than BHA one. The lipid peroxidation inhibition rate of astaxanthin was twice higher than that of the ascorbic acid. Oral administration of astaxanthin in mice showed its ability to reduce abdominal constrictions induced by acetic acid. The efficacy of astaxanthin in preventing protein denaturation and erythrocytes membrane injury was important. It inactivated the cyclooxygenase 2 and exhibited a significant inhibition against carrageenan induced inflammation. Moreover, there was no evidence of toxicity in mice given the algal extract. This suggests that the astaxanthin from *H. pluvialis* has interesting biological properties and could be used safely in food and pharmaceutical industries.

Keywords: *Haematococcus pluvialis*, astaxanthin, inflammation, analgesia, antioxidant activity.

Introduction

Oxidative molecules with very high reactivity, such as free radicals and reactive oxygen species (ROS), are produced by normal aerobic metabolism in organisms for sustaining life processes

(Higuera-Ciapara et al., 2006). However, physiological stress, air pollution, tobacco smoke, exposure to chemicals or ultraviolet (UV) light can enhance the production of such agents. Phagocytes can also generate an excess of free radicals to aid in their defensive degradation of the invader (Guérin et al., 2003). However, the overproduction of ROS could disturb cellular redox balance, resulting in cell injury or apoptosis (Zhang et al., 2017). These species can also react with macromolecules such as proteins, lipids and nucleotides through chain reactions (Yang et al., 2013), resulting in development of various diseases such as cancer, atherosclerosis, neurodegenerative, cardiovascular and inflammatory diseases (Babar et al., 2011).

It is well known that inflammation is the response of the body to harmful physical, chemical or biological stimuli (Gul et al., 2022). It involves a complex process and is characterized in vascular tissues by redness, warmth, swelling, protein denaturation, membrane alteration, an increase of vascular permeability and several tissue damage (Ferrero-Millani et al., 2007). Anti-inflammatory drugs especially non-steroidal anti-inflammatory drugs (NSAID) are commonly used to attenuate symptoms and suppress enzyme activities. Unfortunately, studies have demonstrated that the use of these drugs leads to several adverse side effects, such as gastric ulcers (Oguntibeju, 2018). For this reason, it is necessary to seek search for new safe anti-inflammatory molecules.

Several scientific works showed that a diet rich in antioxidants can protect the organism against the damages caused by free radicals and consequently, prevents several diseases (Zubia et al., 2007). Carotenoids in general are very beneficial, and in particular, while those derived from microalgae, are considered as excellent antioxidants, they can be exogenously supplied to the cells, re-establishing the levels of oxidative stress by neutralizing the excess of free radicals and reactive species of oxygen and nitrogen (Raposo et al., 2015). The red ketocarotenoid astaxanthin (3,3′-dihydroxy-β-β′-carotene-4,4′-dione) attracts considerable interest (Kobayachi et al., 1997), and is recognized as a good candidate for the prevention of intracellular oxidative stress (Regnier et al., 2015). It has been reported that its antioxidant activity is 10 times more important than other carotenoids such as zeaxanthin, lutein, canthaxanthin and β-carotene and 100 times higher than α-tocopherol (Miki, 1991). The antioxidant properties of astaxanthin are believed to have a key role in several other properties such as prevention of inflammatory diseases, diabetes, cardiovascular disease, cancer, ulcer gastric, aging and neurodegenerative disorders (Guérin et al., 2003; Sadoud et al., 2023). The fresh-water microalga *Haematococcus pluvialis* (*H. Pluvialis*) is one of the richest sources of astaxanthin accumulating up to 2–3% of dry weight and constitutes from 85 to 88% of total carotenoid (Tripathi et al., 1999 ; Ranga Rao et al., 2009).

Algerian Microalgae remains unvaluable natural bioressources, hence the interest of conducting this

study, aiming to evaluate the antioxidant, analgesic and anti-inflammatory activities of astaxanthin, produced under nitrogen starvation and strong light by an Algerian *H. pluvialis* strain isolated from freshwater.

1. Materials and methods

Animals

In vivo experiment involved male *Mus musculus* mice aged 07 weeks and weighting 27 ±3 g provided by Pasteur Institute of Algiers in Algeria. The animals were housed in polypropylene cages equipped with a free access feeding and watering system, under controlled temperature (23±10°C), relative humidity (50±10%) and 12 hours light/dark cycle. They received standard pellet diet. The experiments were conducted early in the morning on fasting mice. The animals were reared, handled, and used according to the recommendations of the Algerian Ethics Committee for Research on Animals at University Abdelhamid Ibn Badis, of Mostaganem (ECRA/AIBUM).

Microalga culture and astaxanthin determination

The strain of *H. pluvialis* was obtained from a strain previously isolated in our laboratory (Sadoud et al., 2019). It was grown in Bold's Basal Medium (BBM) under continuous illumination of 40 µmol photons $m^{-2}S^{-1}$ at 25±2°C for 8 days. The atmospheric carbon dioxide inside the flask was maintained at 1.5% (V/V) generated from a bicarbonate/carbonate mixture. The culture was centrifuged at 3000 g for 10 min, after that cells were resuspended in a fresh BBM medium without nitrogen source (0 g/mL $NaNO_3$) at a concentration of 105 cells/mL and exposed to a continuous light intensity of 200 µmol photons $m^{-2}S^{-1}$ during two weeks, to enhance the accumulation of astaxanthin in this microalga (Wan et al., 2014).

Astaxanthin contained in the enkysted cells of *H. pluvialis* was extracted and determined according to slightly modified procedure (Sadoud et al., 2019).

Antioxidant activity of astaxanthin produced by *H. pluvialis*

Ferric Reducing Antioxidant Power (FRAP)

The Ferric Reducing Antioxidant Power (FRAP) assay was carried out according to the procedure described by Benzie and Strain (1996). The FRAP assay is based on the reduction of ferric (Fe^{3+}) to ferrous (Fe^{2+}) ions at low pH which causes the formation of a colored ferrous-tripyridyltriazine complex, and it is quantified spectrophotometrically at 593 nm. Briefly, 200 µL of astaxanthin was added to 3000 µL freshly prepared FRAP reagent (300 mM sodium acetate buffer, pH 3.6, 10.0 mM TPTZ solution and 20.0 mM $FeCl_3$.6H2O solution in a ratio of 10:1:1(V/V/V). The reaction mixture was incubated at 37 °C for 30 min. The absorbance of mixture was measured at 593 nm. Solutions containing ferrous ions ($FeSO_4$) at known concentrations were used for calibration curve.

β-Carotene-linoleic acid assay

The β-carotene bleaching assay was carried out as described by Kelin and Tepe (2008). In brief, 0.5 mg of β-carotene was dissolved in 1 mL of chloroform. 25 µL of linoleic acid and 200 mg of Tween 40 were added to the mixture. Chloroform was completely removed using a rotary evaporator. The resulting mixture was diluted with 100 mL distilled water saturated with oxygen with a vigorous shaking to make an emulsion. 2.5 mL of this emulsion were dispensed into different test tubes, 350µL of various concentrations of astaxanthin (6.25, 12.5, 25, 50, 100 and 200 µg/mL) were added to the mixture, and incubated for 48 h at room temperature. The same procedure was repeated with synthetic antioxidant, BHT, as positive control and a blank (containing DMSO instead of extract solution). After incubation period, absorbance of the mixtures was measured at 490 nm. The bleaching rate of β-carotene was calculated according to equation below.

$$AAR = ((A_{Control} - A_{Sample})/A_{Control}) \times 100$$

Where:

$A_{Control}$: is the absorbance of the control containing all reagents except the test compound

A_{Sample} : is the absorbance of the test compound.

Ferric thiocyanate (FTC) method in linoleic acid emulsion

The antioxidant activity of astaxanthin was evaluated according to the FTC method as described by Glüçin et al. (2004). The linoleic acid oxidation generates peroxides which oxidize the Fe2+ of the ferric thiocyanate into Fe3+ giving rise to a colored complex which has a maximal absorbance at 500nm. Briefly, 1 mL of a 50 µg/mL astaxanthin solution was added to a mixture of 1 mL of 2.5% (V/V) ethanolic solution of linoleic acid, 2 mL of saline phosphate buffer (0.05 M, pH7.0) and 1 mL of distilled water, then the mixture was stirred and incubated at 40°C. 0.1 mL of this mixture was added to 9.7 mL of ethanol (75%: V/V), 0.1 mL of potassium ferricyanide (30%: W/V) and 0.1 mL of 0.02 M ferric chloride in hydrochloric acid (3.5%: W/V), and left 10 min at room temperature; then the absorbance was measured at 500 nm. This step was repeated every 24h until the control reached its maximum absorbance. Solutions of DMSO and ascorbic acid (50µg/mL) were used as negative (blank) and positive (standard) controls, respectively.

Analgesic activity

The analgesic activity of astaxanthin was investigated using the acetic acid-induced abdominal writhing according to Koster et al. (1959) method. Animals were divided into five groups of five mice each one. Animals of negative control group were given intraperitoneally 10mL/kg of body weight (B.W) a normal saline. Animals of positive control group received 50 mg/kg of body weight a standard drug Ibuprofen. The two remaining groups were treated orally with 50 and 100 mg of

astaxanthin/kg of body weight, respectively. 30 minutes after administration of drug and *H. pluvialis* DMSO-AE, 0.6% (V/V) acetic acid (0.2 mL/20 g of body weight) was given intraperitoneally to all the mice to induce abdominal constrictions. The number of spasms observed in each mousse was counted over a period of 15 minutes. The percentage protection against abdominal spasms was calculated using formula above.

$$\text{Parentage of protection} = ((N_{nc} - N_t)) / N_{nc}) \text{ X } 100$$

Where

N_{nc} : Mean of writhes number observed in negative control group

N_t : Mean of writhes number observed in animals treated with astaxanthin or Ibuprofene.

Anti-inflammatory activity

In vitro anti-inflammatory activity

Inhibition of protein denaturation

The effect of astaxanthin on inhibition of protein denaturation was carried according to the protocol described by Hai Trieu et al. (2021). Briefly, 2 mL of different concentrations of astaxanthin were mixed with 2.9 mL of phosphate buffered saline (pH 6.4) and 100 µL of fresh egg albumin. The mixture was incubated at 37°C for 15 min. the reaction mixture was then heated at 70°C for 10 min in a water bath. After cooling, the absorbance of mixture was monitored at 660 nm. The same procedure was used for the diclofenac sodium which was used as a positive control.

Human red blood cell (HRBC) membrane stabilization assay

The effect of astaxanthin on Inhibition of HRBC membrane stabilization was carried out according to the method described by Shinde et al. (1999). To prepare the erythrocyte suspension, blood was collected from healthy human volunteer donator in heparinized tubes (volunteer mustn't consumed any NSAIDs 2 weeks prior experiment). Blood samples were centrifuged at 3000 rpm for 15 min and the supernatant was removed, cells were then washed three times with sterile saline solution. After centrifugation, blood cells volume was reconstituted as a concentration of 10% (V/V) with 10 mM sodium phosphate buffer pH 7.4 solution.

Heat-induced hemolysis test was carried out following Gunathilake et al. (2018) method. Briefly, 0.05 mL of blood cell suspension, 0.05 mL of *H. pluvialis* extract and 2.95 mL phosphate buffer (pH 7.4) solution were mixed. Mixtures were then incubated at 54 °C for 20 min in water bath then centrifuged at 2500 rpm for 5 min. The same procedure was used for the diclofenac sodium which was used as a positive control. The absorbance of the supernatant was measured at 560 nm and the percentage inhibition of hemolysis was calculated.

$$\text{Percentage inhibition of hemolysis} = (1 - A_c/A_t) \times 100$$

Where:

A_c: is the absorption of the control,

A_t: is the absorption of test sample.

In vivo anti-inflammatory activity

In Vivo anti-inflammatory activity of astaxanthin extracted from *H. pluvialis* was investigated on carrageenan induced paw edema as described by Winter et al. (1962) with slight modifications. The mice were randomly divided into five groups of five animals each one.

Astaxanthin dissolved in physiological saline (0.9%) at doses of 50 and 100 mg/kg of body weight were given orally to groups 3 and 4, respectively. Group 1 served to the negative control group was given with physiological saline (0.9%) at an equivalent volume. The second group (2) was treated with Diclofenac Sodium (10 mg/kg) as the reference drug. 30 minutes after treatment, animals were injected with 0.1mL of 1% (W/V) carrageenan in physiological saline into the plantar side of the right hind paw of each mouse. The paw thickness was measured using a vernier caliper at hourly interval after carrageenan administration for 6h and the percentage of inhibition of paw oedema was calculated using the fomula.

$$\text{Percentage inhibition (\%)} = (V_c - V_t) \text{ X } 100 / V_c$$

Where :

V_t : increase in paw thickness in animals treated with astaxanthin or Diclofenac sodium.

V_c : increase in paw thickness in the negative control group at the same time

In silico anti-inflammatory activity

In silico study of the anti-inflammatory activity of astaxanthin and Diclofenac Sodium was appraised against cyclooxygenase-2 (COX 2) through molecular docking approach. The 3D structure of cyclooxygenase-2 was downloaded from Protein Data Bank (www.rcsb.org). A structure of human COX2 in good resolution (2.41 Å) was chosen (PDB ID: 5IKQ). The macromolecule was then prepared using Autodock tools. The Chemical structure of astaxanthin was drawn via chemsketch softwar, then minimized and optimized using Chem 3D software. The grid box was defined according to the biding site of the downloaded COX-2 enzyme. The docking calculation was carried out using autodock vina. The visualization of the interactions and pose view between ligands and the COX-2 enzyme, Discovery Biovia software was used. Besides ataxanthin, Diclofenac Sodium was used as control in molecular docking. The RMSD (Root Mean Square Deviation) value was calculated.

Acute toxicity

Acute toxicity test was carried out as described by OECD guidelines (Organization for Economic Co-operation and Development no. 425) (2008). Single high dose of astaxanthin at 500 mg/kg B.W. was administered to mice by the oral route. The mice were observed during the first 3 h and after 24 h for any toxic manifestations such as changes in physical appearance, salivation, increased locomotors activity, convulsion coma and death.

Statistical analysis

The data collected were subject to descriptive statistical analysis. Analysis of variance (ANOVA) was performed using Past software version 3.19, followed by t-test. Differences at $P < 0.05$ are considered to be statistically significant. The results are presented as arithmetic mean ± standard deviation (x ± SD).

Results and discussion

Antioxidant activity of astaxanthin

Ferric Reducing Antioxidant Power (FRAP).

Antioxidant activity of astaxanthin, BHT and ascorbic acid evaluated by FRAP essay based on the reducing power of an antioxidant and measuring its ability to reduce the ferric ions (Fe^{3+}) to ferrous ions (Fe^{2+}), confirmed our results showing that all tested samples demonstrated an important capacity to reduce the complex ferric tripyridyltriazine (Fe^{3+}-TPTZ) to ferrous tripyridyltriazine (Fe^{2+}-TPTZ)). The highest ability to reduce Fe^{3+} to Fe^{2+} ($P<0.001$) was revealed by astaxanthin (221.50±8.18 mM Fe^{+2}/g) followed by BHT (135.22±6.07 mM Fe^{+2}/g) and ascorbic acid (59.09±4.22 mM Fe^{+2}/g) (figure 1).

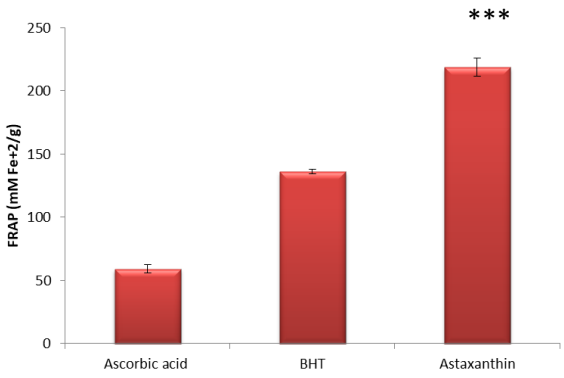

Figure 1. FRAP antioxidant potential test of ascorbic acid, BHT and astaxanthin; ***: significant difference at $P<0.01$ between astaxanthin and standard antioxidants (BHT and Ascorbiv acid) (x ± SD, n= 3).

β-Carotene-linoleic acid assay

The antioxidative activity of astaxanthin determined by the β-carotene bleaching method measured the ability of an antioxidant to prevent lipid peroxidation. Generally, the β-carotene bleaching can be decreased in presence of an antioxidant. However, our results exhibited an increased behavior in the percentage of inhibition of β-carotene bleaching and the extract or BHT concentration (figure 2). With all tested concentrations, astaxanthin was more efficient ($p<0.05$) on inhibition of the lipid peroxidation compared to the BHT. The Percentage of inhibition of β-carotene bleaching values recorded were 29.86±1.91, 36.16±1.36, 48.78±2.11, 68.38±4.26 and 88.15±2.78%, in presence of 6.25, 12.5, 25, 50 and 100 µg/mL of astaxanthin, respectively, compared to 7.22±1.16, 12.90±1.95, 22.54±2.90, 39.10±2.03% and 58.51±3.78% recorded with the same concentrations of BHT. A concentration of 200 µg/mL of astaxanthin inhibited almost totally the β-carotene bleaching (98.12±1.15%).

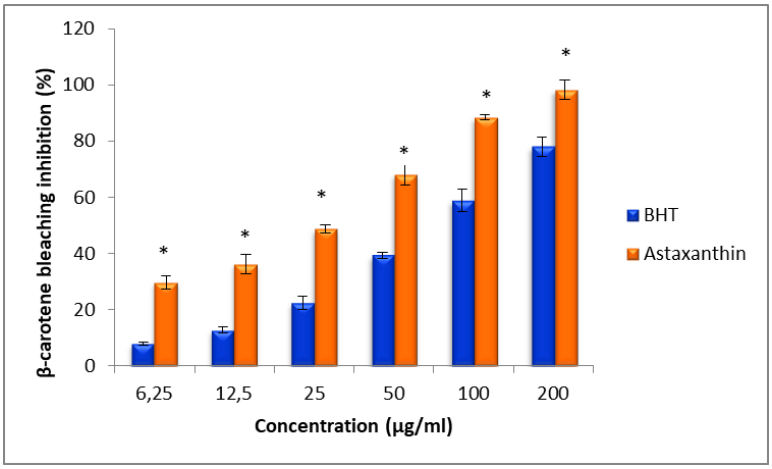

Figure 2. β-carotene bleaching inhibition (%), *: significant difference between BHT and astaxanthin at same concentrations at $P<0.05$ (x ± SD, n= 3)

Ferric thiocyanate method in linoleic acid emulsion (FTC)

The ferric thiocyanate (FTC) method quantifies peroxides produced during the initial phase of lipid peroxydation. In this FTC test, linoleic acid oxidation led to an increase in the amount of peroxides in the negative control (without antioxidant) which remained higher than those obtained in the positive control (ascorbic acid) and astaxanthin trial.

Inhibition rates of linoleic acid peroxidation recorded with algal extract were 81.5±2.7, 78.81±3.06, 67.33±2.92 and 48.38±4.48% obtained after 24, 48, 72 and 96h of incubation, respectively, compared to 53.24±4.04, 46.98±3.9, 31.07±3.76 and 23.65±2.38% obtained with ascorbic acid, at the same period of incubation, respectively (Figure 3).

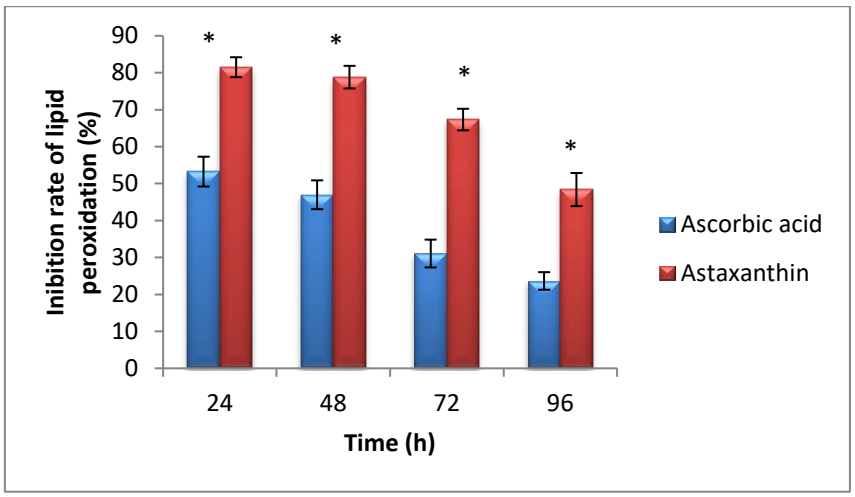

Figure 3. Inhibition rate of linoleic acid peroxidation, *: significant difference between astaxanthin and ascorbic acid at same times at P<0.05 (x ± SD, n= 3).

In agreement with our findings, Zhao et al. (2016) incriminate algal astaxanthin content in neutralization of free radicals, thereby stopping the radical chain reaction and delaying the formation of hydroperoxides.

The role of astaxanthin derived from *H. pluvialis* microalga which represents 80 to 99% of total carotenoids produced by this species on the prevention of lipid peroxidation, as previously reported by Goto et al. (2001) and Shah et al. (2016). Yang et al. (2013) and Focsan et al. (2017) indicate that astaxanthin is also known as an antioxidant with powers exceeding those of α-tocopherol and other carotenoids such as: lutein, β-carotene or lycopene.

The reducing power of astaxanthin was obtained by Zhao et al. (2009) who reported that this extract act as a good electron donor, and its ability to reduce the ferric ions can be explained by the presence of hydroxyl and carbonyl groupements in its structure. Besides, Ranga Rao et al. (2009) reported that 9 ppm of *H. pluvialis* extract exhibited an inhibition percentage of 89% in β-carotene bleaching test. The authors explained these results by the high level of astxanthin in the extract.

In vivo analgesic activity

The table 1 illustrating the effect of astaxanthin on acetic acid-induced abdominal spasms in mice showed that astaxanthin and Indomethacin inhibited significantly (P<0.05) abdominal spasms comparatively to the negative control group. No significant difference (P>0.05) was observed between group treated with Indomethacin and group treated with 100 mg/kg of body weight of atsaxanthin. However, treatment of animals with 150 mg/kg of body weight of astaxanthin prevented spasms at 100%.

Table 1. Percentage of inhibition (%) of acetic acid-induced abdominal spasms in mice (x ± SD, n= 5); **: significant difference at P<0.05.

Animal group	Control positive group (treated with Indomethacin)	Group treated with 50 mg/kg B.W of astaxanthin	Group treated with 100 mg/kg B.W of astaxanthin	Group treated with 150 mg/kg B.W of astaxanthin
Percentage of inhibition (%)	76.67±3.33^{b}	64.03±4.67^{a}	75.40±3.84^{b}	100±00^{c}

Our results corroborated those of Kuedo et al. (2016) who observed that mice treated with 150 mg of astaxanthin/kg of body weight showed significant reduction in paw edema induced in hind paw and an increase in thermal paw withdrawal latency, furthermore, the same authors showed a significant reduction in the amount of myeloperoxidase enzyme and lipid peroxidation products in the paw. Additionally, Sharma et al. (2018) demonstrated that astaxanthin has an effective role in the treatment of chronic neuropathic pain, it significantly ameliorates behavioral and biochemical alterations with a decrease of oxidative stress.

Anti-inflammatory activity

In vitro anti-inflammatory activity

In vitro anti-inflammatory activity of astaxanthin and Diclofenac Sodium (standard drug) was assessed upon inhibition of protein denaturation and HRBC membrane stabilization. Both astaxanthin and Diclofenac Sodium showed a potent ability to protect the protein denaturation and the HRBC membrane stabilization against heat treatment in a dose -dependent way (table 2). However, astaxanthin exhibited high capacity ($p<0.05$) on protection of protein denaturation than Diclofenac Sodium. Indeed, low ($P<0.05$) IC50 value 598.97±10.70 µg/mL was recorded with astaxanthin compared to 775.44±21.37 µg/mL recorded with Diclofenac Sodium. Nevertheless, Diclofenac Sodium was more efficient ($P<0.05$) in red blood cell membrane stabilization (IC50= 427.62±16.19 µg/mL) than astaxanthin (IC50=810.49±33.64 µg/mL).

Table 2. Effect of astaxanthin and Diclofenac Sodium on inhibition of protein denaturation and hemolysis; letters indicate significant difference at P<0.05 between values of the same test (x ± SD, n= 3).

Concentration	Inhibition percentage of protein	Inhibition percentage of

(µg/mL)	denaturation, %		hemolysis, %	
	Astaxanthin	Diclofenac Sodium	Astaxanthin	Diclofenac Sodium
1000	69.47±1.11^{f}	56.55±1.18^{e}	55.32±0.86^{d}	71.79±0.99^{e}
500	51.43±2.56^{d}	48.53±2.77^{d}	41.35±1.98^{c}	57.64±1.24^{d}
250	30.87±2.04^{c}	23.30±1.26^{b}	36.94±0.85^{b}	42.15±1.41^{c}
125	20.80±2.07^{b}	14.53±2.15^{a}	23.42±1.79^{a}	35.22±0.88^{b}
IC50 value, µg/mL	598.97±10.70*	775.44±21.37	810.49±33.64$^{\#}$	427.62±16.19

*: significant difference between IC50 values of astaxanthin and Diclofenac Sodium (protein denaturation test), $^{\#}$: significant difference between IC50 values of astaxanthin and Diclofenac Sodium (HRBC membrane stabilization test).

Denaturation of proteins is defined as a modification in their structural integrity, it occurs when they are exposed to some external stimuli such as heat treatment. Protein alteration generates auto-antigens causing various inflammatory arthritic diseases as reported by Sangeetha et al. (2016). Furthermore, HRBC membrane stabilization was studied in *In vitro* anti-inflammatory activity of compounds because of the analogies between the erythrocyte and lysosomal membranes. Samina et al. (2020) indicated that the stabilization of the lysosomal membrane would prevent the release of the lysosomal contents which causes tissue inflammation and damage. Therefore, compounds which can prevent protein denaturation or erythrocyte membrane lysis would be considered as anti-inflammatory agents.

The anti-inflammatory activity of Diclofenac Sodium; is very obvious, as long as it inhibits endogenous prostaglandins synthesis by blocking cyclooxygenase enzymes, but also prevents erythrocyte membrane lysis and denaturation of proteins, reported in previous studies by Na'imah, (2019).

Boukhari et al. (2013) showed that the erythrocyte is more exposed to secondary damage through lipid peroxidation induced by free radicals when its membrane is injured. Therefore, astaxanthin possess a very potent antioxidant activity due to its molecular structure and would be used in the treatment of inflammatory disorders (Ranga Rao et al., 2013). Moreover, Suganya et al. (2017) studying *In vitro* anti-inflammatory activity of the encapsulated and non-encapsulated astaxanthin, using different methods such as inhibition of albumin denaturation, membrane stabilization method against heat induced hemolysis, protein inhibitory action and HRBC membrane stabilization method observed that both encapsulated and non-encapsulated astaxanthin exhibited high anti-

inflammatory activities. Astaxanthin as an antioxidant pigment possess specific anti-inflammatory properties and seems to be more able way in controlling inflammatory conditions, through its role in neutralizing the free radicals and minimizing the oxidative damages.

In vivo anti-inflammatory activity

As shown in table 3, both, Diclofenac Sodium and astaxanthin suppress significantly ($P<0.05$) the carrageenan-induced paw edema.

The administration of 50 mg/kg of body weight astaxanthin showed very important percentages of inhibition through time in comparison with negative control group, the percentages of inhibition recorded in this group from the first to the 6th hour ranged between 30.6±1.2 and 49.8±1.2%. However, the dose of 100 mg/kg of body weight astaxanthin seemed to prevent carrageenan-induced paw edema in the same way as Diclofenac sodium. Indeed, after the 4th hour of carrageenan-inducing paw edema, no significant differences ($P<0.05$) were observed between percentages of inhibition in the control positive group and group treated with 100 mg/kg of body weight of astaxanthin.

Table 3. Inhibition percentage of carrageenan-induced paw edema through time; (x ± SD, n= 5); letters indicate significant difference at $P<0.05$.

Time (h)	Positive control group 10 mg/kg of Diclofenac Sodium	Group treated with 50 mg/Kg of astaxanthin/Kg BW	Group treated with 100 mg/Kg of astaxanthin /Kg BW
1	32.73±0.39^{a}	30.72±1.69^{a}	36.90±0.48^{a}
2	37.01±0.81^{a}	29.41±0.99^{a}	44.30±0.84^{b}
3	51.32±0.85^{c}	42.71±0.89^{b}	48.64±0.80^{c}
4	56.72±1.45^{e}	46.40±1.42^{b}	57.69±0.50^{e}
5	62.71±1.19^{e}	48.16±0.86^{b}	62.40±0.33^{e}
6	63.59±1.84^{e}	47.91±0.24^{b}	60.89±1.15^{e}

Carrageenan inducing paw edema in mice was used to determine the *In vivo* anti-iflammatory activity of Astaxanthin. The edema is explained by release of inflammatory mediators such as: serotonin, bradykinin, histamine, and prostaglandins, which induce vasodilatation and increase vascular permeability and produce edema as explained by Kuropakornpong et al. (2020).

Carrageenan-induced edema is believed to be biphasic; the first one is characterized by the production of inflammatory mediators such as: serotonin, histamine, bradykinins and

prostaglandins, while the late phase is sustained by cytokines synthesis; TNF-α, interleukins (IL-1β, IL-6), ROS and other inflammatory mediators, including nitric oxide (NO) and prostaglandin E2 (PGE2) which are synthesized by nitric oxide synthetase and cyclooxygenase (COX) respectively, and involved in several diseases such as: rheumatoid arthritis, asthma and atherosclerosis as reported by Boughton-Smith et al. (1993); Ritchlin et al. (2003).

Kuedo et al (2016) previously showed that astaxanthin administered orally to mice inhibits both phases of inflammation. The work of Lee et al (2003) revealed that astaxanthin inhibited the formation of pro-inflammatory mediators and cytokines in LPS-stimulated RAW 264.7 cells, decreasing the serum concentration of NO, PGE2, TNF-α and IL-1β in LPS-treated mice, NF-κB and iNOS. In the same context, Miyachi et al. (2014) in their study on the role of astaxanthin in chronic inflammation caused by *Escherichia coli* O55 LPS in human gingival keratinocytes, demonstrated that the localization of nuclear factor κB and the level of IL-6 and TNF-α tended to decrease. Chan et al (2012) reported that the production of ROS, IL-6 and TNF-α was significantly reduced in blood after astaxanthin intake in diabetic rats.

In silico anti-inflammatory activity

Cyclooxygenase-2 (COX-2) is one of the main enzymes involved in inflammation; hence, its use as the target protein for the simulation of the anti-inflammatory activity of ligands. The ΔG corresponds to the amount of energy needed to bind the receptor and the ligand (Patil et al., 2010).

Table 4. Molecular docking of COX-2 enzyme (5IKQ) with astaxanthin and Diclofenac Sodium.

Ligand	**ΔG (kcal/mol)**	**RMSD (Å)**
Astaxanthin	-8.4	1.3
Diclofenac Sodium	-6.2	0.6

Our results revealed that astaxanthin was the top-scored ligand with a −8.4 kcal mol^{-1} than Diclofenac Sodium (ΔG= -6.2 kcal mol^{-1}). The RMSD (Root Mean Square Deviation) obtained are 1.3 and 0.2 Å with astaxanthin and Diclofenac Sodium, respectively (table 4). If the RMSD is ≤ 2, the copy ligand position is similar to native ligand, so a valid docking method is used to predict COX-2 interactions with ligands (Na'imah, 2019).

The 2D diagrams of the ligands (Astaxanthin and Diclofenac Sodium) docked on the active site for cyclooxygenase-2 protein (5IKQ) are shown in figure 4. Astaxanthin and Diclofenac Sodium inhibit the COX-2, hence, the interactions that occur between COX-2 and astaxanthin are alkyl and pi-alkyl

interactions. They are established through HIS 91, TYR 91, HIS95 and PRO514 amino acids of the active site of the receptor. Diclofenac acid interacts with three amino acids of the active site connecting astaxanthin, PRO 154 through Pi-Alkyl interaction, ASP 157 through Pi-Anion interaction and ASP 133 through conventional hydrogen bond (figure 4).

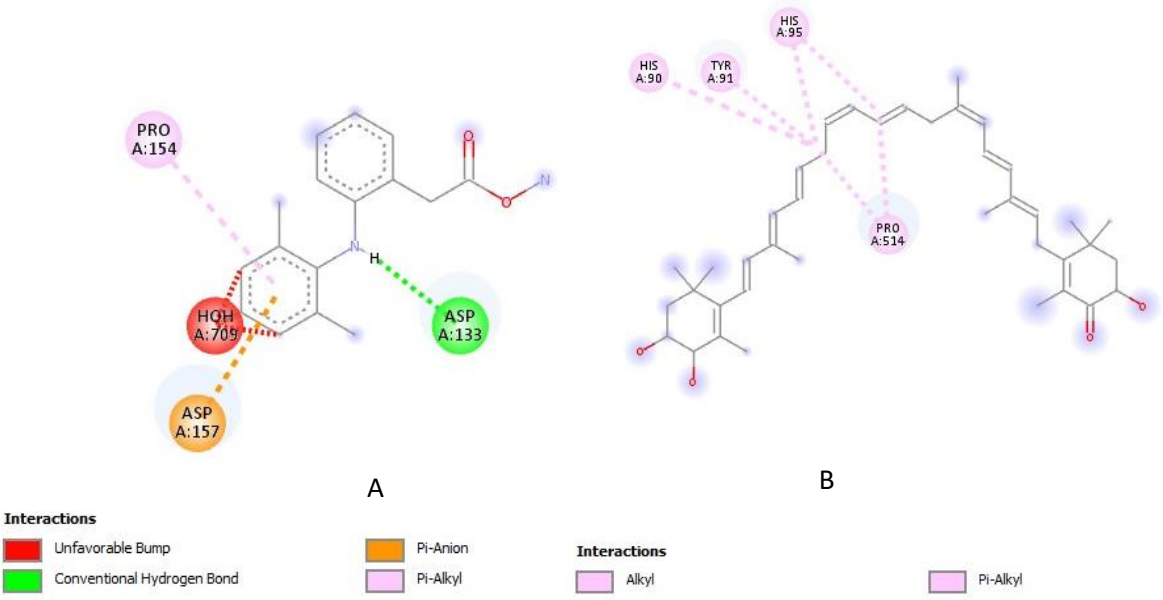

Figure 4. Interaction between COX2 (5IKQ), Diclofenac Sodium (A) and astaxanthin (B).

Concerning, *In silico* activity, Yatam et al. (2018) reported that the interactions protein–ligand are essential for the stabilization of the complex, as they enhance the binding affinity and biological activities of the complexes molecules, which helps to stabilize their biochemical environment as reported by Patil et al. (2010). Cyclooxygenases-2 is a stimulus enzyme in inflammation, so the activity inhibition of this enzyme will be a target for the treatment of inflammation.

Acute toxicity

During 24h of observation, no clinical signs of toxicity, neither mortality were observed for all animals given 500mg astaxanthin /Kg of body weight.

In similar findings, John et al. (2008) demonstrated that DL50 of *H. pluvialis* extract is superior to 12000 mg/kg. Thereby, Regnier et al. (2015) confirmed the safety of *H. pluvialis*.

Conclusion

This study investigated the antioxidant, analgesic and anti-inflammatory activities of astaxanthin extracted from an isolated *H. pluvialis* microalga. Results revealed that astaxanthin showed a strong antioxidant activity. Furthermore, it exerted an important activity on inhibition of acetic acid-induced abdominal spasms in mice. In addition, astaxanthin exhibited an important anti-inflammatory activity demonstrated by *In vitro*, *In vivo* and *In silico* methods. These results suggest that more research on astaxanthin is needed due to the fact that this microalga is a promising source of potent antioxidant, and could have other medicinal, therapeutical and nutraceutical uses.

Conflict of interest

The authors declare that they have no conflict of interest.

References

Babar, N. H. S, Oberoi D. S, Uppal, P. and Patil, R. T (2011). Total phenolic content and antioxidant capacity of extracts obtained from six important fruit residues. Food Research International, 44(1), 391–396. https://doi.org/10.1016/j.foodres.2010.10.001

Benzie, I. F. F. and Strain J. J. (1996). The Ferric Reducing Ability of Plasma (FRAP) as a Measure of Antioxidant Power "The FRAP Assay". Analytical Biochemistry, 239, 70–76. https://doi.org/10.1006/abio.1996.0292

Boughton-Smith, N. K., Deakin, A. M. Follenfant, R. L., Whittle, B. J. and Garland, L. G. (1993). Role of oxygen radicals and arachidonic acid metabolites in the reverse passive Arthus reaction and carrageenin paw oedema in the rat. British Journal of Pharmacology, 110, 896–902.

Bukhari, I. A., Alhumayyd, M. S., Mahesar ,A. L. and Gilani, A.H. (2013). The analgesic and anticonvulsant effects of piperine in mice. Journal of Physiology and Pharmacology, 64, 789

Chan, K., Pei-Jain, P. and Meichin ,Y. (2012). Anticoagulatory and Anti-inflammatory Effects of Astaxanthin in Diabetic Rats. Food Sciences, 77 (2), 123-146. https://doi.org/10.1111/j.1476-5381.1993.tb13897.x

Ferrero-Millani, L., Nelsen, O. H., Anderson, P. S. and Girardin, S. E. (2007). Chronic inflammation: Importance of NOD2 and NALP3 in interleukin-1 beta generation. Clininical Experiment Immunology. 147, 227- 235. http://doi.org/10.1111/j.1365-2249.2006.03261.x

Focsan, A. L. Polyakov, N. E. and Kispert, L. D. (2017). Photo protection of *Haematococcus pluvialis* algae by astaxanthin: unique properties of astaxanthin deduced by EPR, optical and electrochemical studies. Antioxydants, 6(4), 1-17. https://doi.org/10.3390/antiox6040080

Goto, S., Kogure, K., Abe, K., Kimata, Y., Kitahama, K., Yamashita, E. and Terada, H. (2001). Efficient radical trapping at the surface and inside the phospholipid membrane is responsible for highly potent antiperoxidative activity of the carotenoid astaxanthin. Biochimestry Biophysics Acta, 1512, 251-258. https://doi.org/10.1016/S0005-2736(01)00326-1

Guerin, M., Huntley, M E., and Olaizola, M. (2003). *Haematococcus* astaxanthin: applications for human health and nutrition. Trends in Biotechnology, 21, 210–216. https://doi.org/10.1016/S0167-7799(03)00078-7

Gul, Z. Akbar, A. and Leghari, S. K. (2022). Elucidating therapeutic and biological potential of Berberis baluchistanica Ahrendt bark, leaf, and root extracts. Frontiers in Microbiology, 13, 823673–823673. https://doi.org/10.3389/fmicb.2022.823673

Gülçin, İ., Şat, İ. G., Beydemir, Ş., Elmastaş, M. and Küfrevioğlu, Ö. İ. (2004). Comparison of antioxidant activity of clove (Eugenia caryophylataThunb) buds and lavender (Lavandula stoechas L.). Food Chemestry, 87, 393- 400.

Higuera-Ciapara, I., Valenzuela, L. and Goycoolea, F.M . (2006). Astaxanthin: a review of its chemistry and applications. Food Science and Nutrition, 46 (2), 185–196. https://doi.org/10.1080/10408690590957188

Kelin, K. and Tepe, B. (2008). Chemical composition, antioxidant and antimicrobial properties of essential oils of three Salvia species from Turkish flora. Journal of Bioresource Technology, 99, 4096-4104. https://doi.org/10.1016/j.biortech.2007.09.002

Kobayashi, M., Kurimura, Y., Sakamoto, Y. and Tsuji T (1997). Selective extraction of astaxanthin and chlorophyll from the green alga *Haematococcus pluvialis*. Biotechnology Techniques, 11, 657–660. https://doi.org/10.1023/A:1018455209445

Koster, R., Anderson, M. and De Beer, E (1956). Acetic acid for analgesic screening. Federation Proceedings, 18, 412-417.

Kuedo, Z., Sangsuriyawong, A., Wanwimol Klaypradit, W., Varomyalin Tipmanee, V. and Onpathompikunlert P (2006). Effects of Astaxanthin from Litopenaeus Vannamei on Carrageenan-Induced Edema and Pain Behavior in Mice. Molecules 21 (382), 1-14. https://doi.org/10.3390/molecules21030382

Kuropakornpong, P., Itharat, A., Panthong, S., Seewaboon, Sireeratawong, S. and Ooraikul B. (2020). In Vitro and In Vivo Anti-Inflammatory Activities of Benjakul: A Potential Medicinal Product from Thai Traditional Medicine. Evidence-Based Complementary and Alternative Medicine. 14, 9760948. https://doi.org/10.1155/2020/9760948

Lee, S.J., Bai, S.K., Lee, KS, Namkoong, S., Na, H.J. and Ha, K.S. (2003). Astaxanthin inhibits nitric oxide production and inflammatory gene expression by suppressing I (kappa) B kinase-dependent NF-kappaB activation. Molecules and Cells, 16, 97-105.

Miki, W. (1991). Biological functions and activities of animal carotenoids. Pure and Applied Chemistry, 63, 141-146. https://doi.org/10.1351/pac199163010141

Miyachi, M., Tomonori, M., Kazunari, A. and Izumi, M. (2015). Anti-inflammatory effects of astaxanthin in the human gingival keratinocyte line NDUSD. Clinical Biochemistry, 56 (3), 171–178. https://doi.org/10.3164/jcbn.14-109

Na'imah, J. (2019). In silico study of COX-2 on indomethacin and diclofenac as nonsteroidal antiinflammatory drugs (NSAIDs). Farmasains : Jurnal Farmasi dan Ilmu Kesehatan, 4(1), 31-36. https://doi.org/10.22219/farmasains.v4i1.7767

OECD (2008). Guidelines for testing of chemicals. Guidance. Acute oral toxicity – up and down procedure (UDP). Available at: http://iccvam.niehs.nih.gov/SuppDocs/FedDocs/OECD/OECDtg425.pdf.

Oguntibeju O. (2018). Medicinal plants with antiinflammatory activities from selected countries and regions of Africa. Journal of Inflammatory Research, 11, 307- 317. https://doi.org/10.2147/JIR.S167789

Patil, R., Das, S., Stanley, A., Yadav, L., Sudhakar, A. and Varma, A.K. (2010). Optimized hydrophobic interactions and hydrogen bonding at the target-ligand interface leads the pathways of drug-designing. PLoS ONE, 5:e12029. https://doi.org/10.1371/journal.pone.0012029

Ranga Rao, A., Sarada, R., Baskaran, V. and Ravishankar G. A. (2009). Identification of carotenoids from green alga *Haematococcus pluvialis* by HPLC and LC-MS (APCI) and their Antioxidant properties. Journal of Microbiology and Biotechnology, 19(11), 1333–1341.

Raposo, M. F., Bernardo de Morais, A. M. M. and Costa de Morais, R.M.S. (2015). Carotenoids from Marine Microalgae: A Valuable Natural Source for the Prevention of Chronic Diseases, Marine Drugs, 13, 5128-5155. https://doi.org/10.3390/md13085128

Regnier, P., Bastias, J., Rodriguez-Ruiz, V., Caballero-Casero, N., Caballo, C. and Sicilia D. (2015). Astaxanthin from *Haematococcus pluvialis* Prevents Oxidative Stress on Human Endothelial Cells without Toxicity. Marine Drugs, 13, 2857-2874.

https://doi.org/10.3390/md13052857

Ritchlin, C. T., Haas-Smith, S. A., Li, P., Hicks, D. G. and Schwarz , E. M. (2003). Mechanisms of TNF-α- and RANKLmediated osteoclastogenesis and bone resorption in psoriatic arthritis. Journal of Clinical Investigation, 111, 821−831

https://doi.org/10.1172/JCI16069

Sadoud, M., Mokhtar, M., Bouamar, S., Bouziane, N., Belabbas, M. and Riazi, A. (2019). Nitrate starvation associated with strong light induces astaxanthin acéacumulation in a new green microalga *Haematococcus pluvialis* isolated from Algerian freshwater. South Asian Journal of Experimental Biology, 9(2), 40-49. https://doi.org/10.38150/sajeb.9(2).p40-49

Samina, Y., Arkajyoti, P., Tarannum, N., Atiqur, R., Sarkar, F., Akhter, Ibne Wahed, M .I., Talha, B. I. and Shafayet A. S. (2020). Membrane stabilization as a mechanism of the anti-inflammatory activity of ethanolic root extract of Choi (Piper chaba). Clinical Phytoscience, 6, 59. https://doi.org/10.1186/s40816-020-00207-7

Sangeetha, G. and Vidhya, R. (2016). In vitro anti-inflammatory activity of different parts of Pedalium murex (L.). International Journal of Herb Medicine, 4, 31–6

Shah, M. M. R., Liang, Y., Cheng, J. J. and Daroch, M. (2016). Astaxanthin-producing green microalga Haematococcus pluvialis: from single cell to high value commercial products. Front Plant Science, 7, 531-545.

http://doi.org/10.3389/fpls.2016.00531

Sharma, K., Sharma, D., Sharma, M., Sharma ,N., Bidve, P., Prajapati, N., Kalia, K. and Tiwari, V. (2018). Astaxanthin ameliorates behavioral and biochemical alterations in in-vitro and in-vivo model of neuropathic pain. Neuroscience Letters, 674, 162–170. https://doi.org/10.1016/j.neulet.2018.03.030

Stewart, J. and Lingnell, A. (2008). Safety assessment of astaxanthin-rich microalgae biomass: acute and subchronic toxic¬ity studies in rats. Food Chemestry and Toxicology, 46, 3030–3036. https://doi.org/10.1016/j.fct.2008.05.038

Suganya, V., Anuradha, V., Syed Ali, M., Sangeetha, P. and Bhuvana, P. (2017). In vitro anti-inflammatory activity of microencapsulated and non-encapsulated astaxanthin. International Journal of ChemTech Research. 10(10), 186-195.

Tripathi, U., Sarada, R. and Ravishankar, G. A (1999). Production of astaxanthin in Haematococcus pluvialis in various media. Bioressource Technology, 68, 197–199. https://doi.org/10.1016/S0960-8524(98)00143-6

Wan, M., Zhang J., Hou, D., Fan, J., Li Y. and Huang J (2014). The effect of temperature on cell growth and astaxanthin accumulation of Haematococcus pluvialis during a light–dark cyclic cultivation. Bioresource Technology, 167, 276 283. https://doi.org/10.1016/j.biortech.2014.06.030

Winter, C. A, Risley, G. A. and Nuss, G. W. (1962). Carrageenan- induced oedema in hind paw of the rat as an assay for anti-inflammatory drugs. Proceedings of the Society for Experimental Biology and medicine, 3, 544–547. https://doi.org/10.3181/00379727-111-27849

Yang, Y., Kim, B. and Lee, J. Y. (2013). Astaxanthin Structure, Metabolism, and Health Benefits. Journal of Human Nutrition and Food Science, 1, 1003.

Yatam, S., Gundla, R., Jadav, S.S., reddy Pedavenkatagari , N., Chimakurthy, J. and Kedam, T. (2018). Focused library design and synthesis of 2-mercapto benzothiazole linked 1, 2, 4-oxadiazoles as COX2/5-LOX inhibitors. Journal of Molecular Structure, 1159 193-204. https://doi.org/10.1016/j.molstruc.2018.01.060

Zhang, L., Xu Y., Li, Y, Bao, T., Gowd, V. and Chen, W. (2017). Protective property of mulberry digest against oxidative stress, a potential approach to ameliorate dietary acrylamide-induced cytotoxicity. Food Chemestry, 230, 306–315. https://doi.org/10.1016/j.foodchem.2017.03.045

Zhao, L., Chen, G., Guanghua, Zhao, G. and Hu, X (2009). Optimization of Microwave-Assisted Extraction of Astaxanthin from *Haematococcus Pluvialis* by Response Surface Methodology and Antioxidant Activities of the Extracts. Separation Science and Technology, 44:1, 243-262. https://doi.org/10.1080/01496390802282321

Zubia, M., Robledo, D. and Freile-Pelegrin, Y. (2007). Antioxidant activities in tropical marine macroalgae from the Yucatan Peninsula, Mexico. Journal of Applied Phycology, 19, 449–458.