

Gastric ulcer prevention, harmlessness and antioxidant activity of astaxanthin extracted from a new Algerian strain of *Haematococcus pluvialis*

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Abstract

Astaxanthin is a high-value carotenoid (3, 3' dihydroxy- β carotene-4, 4'-dione) with multiple biological properties of interest. It is produced by a microalga, *Haematococcus pluvialis*, in substantial amounts especially under stressful conditions such as nitrate starvation and high-light intensity. The present study investigated the gastric ulcer prevention, harmlessness, and antioxidant activity of dimethyl sulfoxide-extracted (DMSO) astaxanthin (DMSO-AE) of a newly isolated *Haematococcus pluvialis* Algerian strain. The experiment was carried out using the ethanol-induced gastric ulcer model in mice. Changes in behavior, physical appearance, convulsion, and death rate were regularly monitored during the first 3h and after the next 24h. Antioxidant activity of *H. pluvialis* DMSO-AE was evaluated with DPPH (2, 2'-diphenylpicrylhydrazyl) method. Ethanol-induced gastric ulcer was significantly ($P < 0.05$) reduced in mice treated with 250 and 500 μ g of *H. pluvialis* DMSO-AE/Kg BW, when compared to the negative and the positive control groups. Histopathological examination of stomach sagittal sections of *H. pluvialis* DMSO-AE pretreated mice did not show any modification of tissue architecture. There was no evidence of toxicity or changes in the behavior or the mortality rate of the mice at the administrated dose of 500 mg *H. pluvialis* DMSO-AE/Kg BW. The DPPH scavenging activity of *H. pluvialis* DMSO-AE used at a concentration of 200 μ g/mL, was about 89.97% with an IC₅₀ value of 25.82 μ g/mL. These results highlighted the astaxanthin protective effects on ethanol-induced gastric ulcers and lipid peroxidation which open up the prospects for the use of this carotenoid in the food, pharmaceutical, and cosmetic industry.

Keywords: *Haematococcus pluvialis*, Astaxanthin, Gastric ulcer, Antioxidant activity, Mice

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Introduction

Gastric ulcer is a chronic disease affecting up to 10% of the world's population (Kuna et al., 2019). It is damage in the gastric or duodenal mucosa down to the submucosa (Karaoglan et al., 2018) and is characterized by different a stages of necrosis, neutrophil infiltration, blood flow reduction and inflammation (Da Silva et al., 2013). Its occurrence depends on exposure to several aggressive factors such as stress, non-steroidal anti-inflammatory drugs (NSAIDs) uptake, smoking, alcohol consumption, *Helicobacter pylori* infection, and high secretion of hydrochloric acid (Hudson and Christopher, 1993; De Souza et al., 2018; Kuna et al., 2019). This disease may be treated using several synthetic anti-ulcer drugs such as proton-pump inhibitors, H₂-receptor antagonist, antacid, and prostaglandin (Khushtar et al., 2016). However, the wide use of these drugs is associated with a range of side effects namely bone fracture (Yang et al., 2018), stroke (Sherwood et al., 2015) and renal disorder, among others (Khushtar et al., 2016). Furthermore, over intake of these drugs may be related to the risk of developing ulcer cancer (Cheung et al., 2017). The above-mentioned limitations of use and side effects of conventional ulcer drugs have led to look for new alternative therapeutic substances from more effective and safer natural sources like plants and microorganisms (bacteria, fungi, microalgae) that can be used in ulcer gastric treatment (Lavinya et al., 2012).

Astaxanthin, a xanthophyll carotenoid, is a very powerful antioxidant with a unique molecular structure with hydroxyl and keto groups on both ends (Guerin et al., 2003). Moreover, this carotenoid has several biological properties such as protection against cancer, inflammation, cardiovascular diseases, and diabetes (Yang et al., 2013; Ranga Rao et al., 2014). Likewise, epidemiological studies have shown that high consumption of astaxanthin has reduced gastric ulcer disease (Kamath et al., 2008).

Synthetic astaxanthin is a petrochemical product that is much more widespread on the world market than natural astaxanthin which is extracted from salmon, trout, krill, yeast or even algae (Capelli et al., 2013; Ranga Rao et al., 2014). The green microalga *H. pluvialis* is believed to be the best source of natural astaxanthin, since it accumulates up to 1–5% of cell dry weight under unfavorable (stressful) growth conditions (He et al., 2007) such as salinity (Torres-Carvajal et al., 2017), high temperature (Giannelli et

al., 2015), strong light and nutrient starvation like nitrogen or phosphorus (Nagaraj et al., 2012). Under these stressful conditions, *H. pluvialis* cells undergo a dormant stage and accumulate astaxanthin as a protective mechanism for their survival beyond 40 years without nitrogen or phosphorus (Capelli et al., 2013).

Stronger antioxidant activity of natural astaxanthin compared to that of the petrochemical form has been previously reported by Capelli et al. (2013) who estimated it 50 and 20 times higher in singlet oxygen quenching and free radical elimination, respectively; while human benefits were not determined yet. The goal of this study is to investigate the *in vitro* antioxidant activity and *in vivo* gastroprotective effect of astaxanthin, produced under absolute nitrogen starvation and strong light by an Algerian *H. pluvialis* strain isolated from freshwater, on ethanol-induced gastric ulcer in mice.

Material and Methods

All reagents used in this study were obtained from Sigma-Aldrich (St Louis, Mo USA).

Microalga culture and astaxanthin determination

H. pluvialis was obtained from our laboratory collection of the previously isolated strain (Sadoud et al., 2019), and grown, first on Bold's Basal medium (BBM) containing 250 mg/L NaNO₃ for 10 days under continuous illumination of 40 μmol photons m⁻²S⁻¹ at 25 ± 2°C and bicarbonate/carbonate mixture generated carbon dioxide atmosphere (1.5%: V/V) inside the flask. Cells were then transferred to fresh modified absolutely nitrogen-free BBM medium at final cell concentration of 10⁵ cells/mL and exposed to continuous illumination of 200 μmol photons m⁻²S⁻¹ at 25 ± 2°C to induce astaxanthin accumulation as previously described by Sadoud et al. (2019).

Extraction and determination of Astaxanthin were carried out using the slightly modified (Sadoud et al., 2019) method of Wan et al. (2014). Astaxanthin accumulated in the encysted cells was extracted using dimethyl sulfoxide (DMSO). To do so, 5 mL of culture of encysted cells were centrifuged (3000 g / 15 min). The issued pellet was treated with a 5% (w/v) methanolic hydroxide potassium solution at 65°C for 15 min, and then washed three times with 2 mL of distilled water. Carotenoids were extracted with 5 mL of preheated (55 °C) DMSO vigorously mixed for 10 min to break the cells and recover the maximum of cell



pigments content. This procedure was repeated at least three times until the cell debris were almost colorless. Absorbance of the extract was measured at 490 nm, and astaxanthin concentration (AC) was calculated using the following formula (1) (Davies, 1976):

$$AC \text{ (mg/mL)} = 4.5 (A_{490}) \cdot V_a / V_b \quad (1)$$

Where: AC: Astaxanthin concentration, A₄₉₀: Absorbance of the extract at 490 nm, V_a: Volume of extract, V_b: Volume of the algal culture.

Experimental animals

Seven-week-old male *Mus musculus* mice weighing 25g to 30g were purchased from Pasteur Institute (Algiers, Algeria) and acclimatized for two weeks in plexiglass cages in an air-conditioned room at $23 \pm 1^\circ\text{C}$ and $55 \pm 10\%$ relative humidity and 12h light-dark cycle. Female mice were excluded from the experiment because of the possible interference of hormones and estrous cycle in data variability (Becker et al., 2005). They were fed ad libitum with a standard pellet diet (Animal Food, Bouzereah, Algiers, Algeria) and tap water. The experiment was conducted following the recommendations of the Algerian Ethics Committee for Research on Animals of Abdelhamid Ibn Badis University of Mostaganem, Algeria (AECRA/AIBUM).

Gastroprotective determination of *H. pluvialis* DMSO astaxanthin extract

Gastroprotective effect of the *H. pluvialis* DMSO astaxanthin extract (*H. pluvialis* DMSO-AE) was carried out as described by Kamath et al. (2008) with a slight modification. Animals were divided into 5 groups of 6 mice each and were fasted overnight (16h) before the experiment.

The different groups of mice were all given the standard diet and tap water ad libitum. Group 1 (G1) received nothing else (sham control); whereas groups G2 and G3 orally received in addition either 1mL of 0.9% (P/V) saline solution (G2: negative control) or 30mg omeprazole/Kg BW (G3: positive control). The assay groups (G4 and G5) were orally given 250 and 500µg *H. pluvialis* DMSO-AE/Kg BW, respectively. Then 1 hour after, 1 mL of absolute ethanol (90%: V/V) was orally administered to all mice groups, except the sham control (G1). One hour after induction of gastric ulceration, all mice were anesthetized with chloroform and the abdomen was opened; the stomachs were rapidly removed, excised along the greater curvature,

washed with saline solution (0.9% P/V), and stored at 4°C until analysis.

Macroscopic evaluation of stomach tissues

Cleaned stomachs were fixed on a glass slide and the mucosa was macroscopically examined for red coloration, spot ulcer, hemorrhagic lesions, deep ulcers, or perforation, and then stomach samples were photographed using a digital camera. The images were registered and the total ulcerative lesions in each stomach were measured using Image-J software (1.51 d). The ulcerated area rate in each stomach and the protection percentage against ulcer (I %) were calculated using the following formulas (2) and (3) (Salga et al., 2011) :

$$\text{Ulcerated Area Rate (\%)} = \text{UAR \%} = (TLa / TSa) \cdot 100 \quad (2)$$

Where: TLa : total lesions area, TSa : total stomach area

$$(I \%) = \text{protection percentage against gastric ulcer} = [(UAR_{\text{control}} - UAR_{\text{assay}}) / UAR_{\text{control}}] \cdot 100 \quad (3)$$

Where: UAR_{control} : Ulcerated Area Rate in a negative control group, UAR_{assay} : Ulcerated Area Rate in assay group

Microscopic evaluation of stomach tissues

Stomach tissues were fixed in formalin 10% (V/V) for 24h, embedded in paraffin, and sectioned into 5 µm thick slices that were stained with hematoxylin and eosin (H&E); then the gastric lesions were analyzed using an optical microscope (Chung et al., 2008).

Acute toxicity test

Acute toxicity test was carried out as described by OECD guidelines (Organization for Economic cooperation and Development no. 425) (2008) using 2 groups of five mice each: the assay group and the control group received a limit dose of 500 mg DMSO-AE of *H. pluvialis*/Kg BW and a saline solution, respectively. Changes in behavior (salivation and locomotor activity), physical appearance, convulsion, and death rate were regularly monitored during the first 3h and after the next 24h.

In vitro determination of antioxidant capacity of the DMSO extract of *H. pluvialis*

DPPH radical scavenging activity



The DPPH radical scavenging activity of *H. pluvialis* DMSO astaxanthin extract was determined as originally described by Blois (1958). 1mL of different concentrations of the *H. pluvialis* DMSO-AE (6.25, 12.5, 25, 50, 100, and 200 µg/mL) was mixed with 1mL of a 0.004% (p/v) DPPH methanolic solution, and left 30 min in the dark at 27°C. The absorbance was measured at 517nm against a DMSO solution (negative control) in comparison to ascorbic acid or butylhydroxytoluene (BHT) as standards at the same concentrations (positive control). The percentage inhibition of free radical DPPH (I%) was calculated using the following formula (4) :

$$I\% = [(A_{\text{control}} - A_{\text{assay}}) / A_{\text{control}}] \cdot 100 \quad (4)$$

Where: A_{control} is the absorbance of the negative control; A_{assay} is the absorbance of *H. pluvialis* DMSO-AE, ascorbic acid or BHT.

H. pluvialis DMSO-AE, ascorbic acid or BHT concentration inhibiting 50% of DPPH scavenging activity (IC_{50}) was obtained graphically.

Results and Discussion

The results of the present study confirm the previously reported (Sadoud et al., 2019) accumulation of astaxanthin (about 67.25 ± 2.28 mg/L) in nitrogen-free medium and under strong light exposure.

Gastroprotective effect of *H. pluvialis* DMSO astaxanthin extract

Macroscopic aspect of gastric mucosa

Figure 1 illustrates the gastric mucosa appearance of all the mice groups used in the present experiment. Gastric mucosa of ethanol given mice (G2: negative control), but not those treated with *H. pluvialis* DMSO-AE (G4 and G5) or omeprazole (G3: positive control), was markedly damaged, and characterized by perforations, deep ulcerations, visible bleeding strips, and edema (Fig.1B); while that of sham control (G1 mice group) did not show any damage (Fig.1A). Omeprazole (30mg/Kg BW) (G3: positive control: Fig.1C) or *H. pluvialis* DMSO-AE (250 and 500µg/Kg BW) (G4, Fig.1D and G5, Fig.1E, respectively) treatment of ethanol-induced gastric ulcer remarkably reduced damage of the gastric mucosa comparatively

to the negative control group (G2). Sequelae of mild damage with slight interstitial hemorrhagic lesions of the gastric mucosa of these G4 and G5 mice groups were still observed after treatment (Fig.1D and 1E).

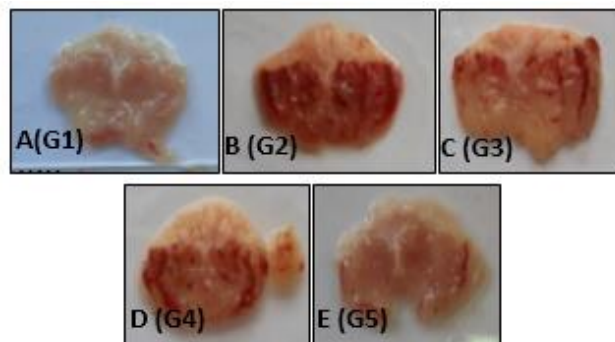


Figure-1. Macroscopic aspects of mice gastric mucosa:

A: sham control (G1); B: untreated ethanol-induced gastric ulcer (G2: negative control); C: omeprazole (30mg/Kg BW) treated ethanol-induced gastric ulcer (G3: positive control); D and E: ethanol-induced gastric ulcer treatment with doses of 250 (assay group G4) and 500µg *H. pluvialis* DMSO-AE/Kg BW (assay group G5).

This is in line with the results of Kamath et al. (2008) who reported this same dose-dependent effect of *H. pluvialis* extract on ethanol-induced gastric lesions in rat. These authors attributed this effect to an inhibition of proton-potassium (H^+ , K^+ -ATPase) pump, a regulation of mucin content, and an increase of antioxidant status.

Determination of Ulceration Area Rate

As shown in figure 2, the ulceration area rate (UAR) in *H. pluvialis* DMSO-AE-untreated gastric ulcer mice (G2: negative control) reached 62.25 ± 2.56 % and was significantly ($P < 0.001$) higher than those treated with 30 mg omeprazole (24.49 ± 0.89), 250 µg (45.08 ± 1.54) or 500 µg *H. pluvialis* DMSO-AE/Kg BW ($17.68 \pm 0.75\%$) in G3, G4 and G5 mice groups, respectively.

H. pluvialis DMSO-AE effect is likely dose-dependent since its increase provides better protection against an ethanol-induced gastric ulcer in mice compared to the reference drug (omeprazole).

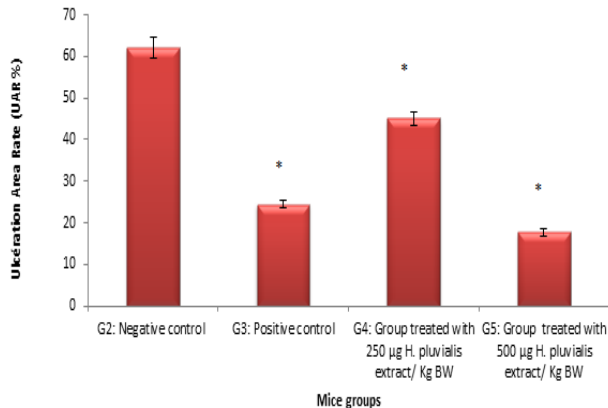


Figure-2. Ulceration Area Rate (UAR %) of ethanol-induced gastric ulcer mice. Values represent the mean \pm SD (n = 6).

H. pluvialis DMSO-AE protection rate against gastric ulcer

The protection rate of *H. pluvialis* DMSO-AE against ethanol-induced gastric ulcer is about 27.58 ± 2.5 and $71.59 \pm 2.19\%$ when used at a concentration of 250 and 500µg/Kg BW, respectively; whereas that of omeprazole was 60.65 ± 4.15 (Fig.2).

Histopathological examination

Gastric mucosa histopathological examination of sham control mice group (G1) showed intact well-organized cell structure (figure 3 A and B); whereas that of negative control one (G2: untreated ethanol-induced gastric ulcer) was markedly damaged showing deep tissue changes with necrosis, hemorrhage, neutrophil infiltration in submucosa, inflammation, edema, and loss of the epithelial cells continuity (figure 3 C, D, and E). Several studies have shown involvement of ethanol in gastric mucosal damage characterized by gastric injuries via dehydration which disrupts mucosal cell barriers and cytotoxicity (Kim et al., 2005a). Therefore, ethanol causes depletion of the gastric wall mucosal barrier via Reactive Oxygen Species (ROS) and cytokine upward regulation, which promotes oxidative stress-induced damage in gastric tissue, inflammatory reactions, and epithelial cell apoptosis. Thus, ethanol is actually one of the most frequent causes of gastric ulceration (Chen et al., 2019; Liu et al., 2020).

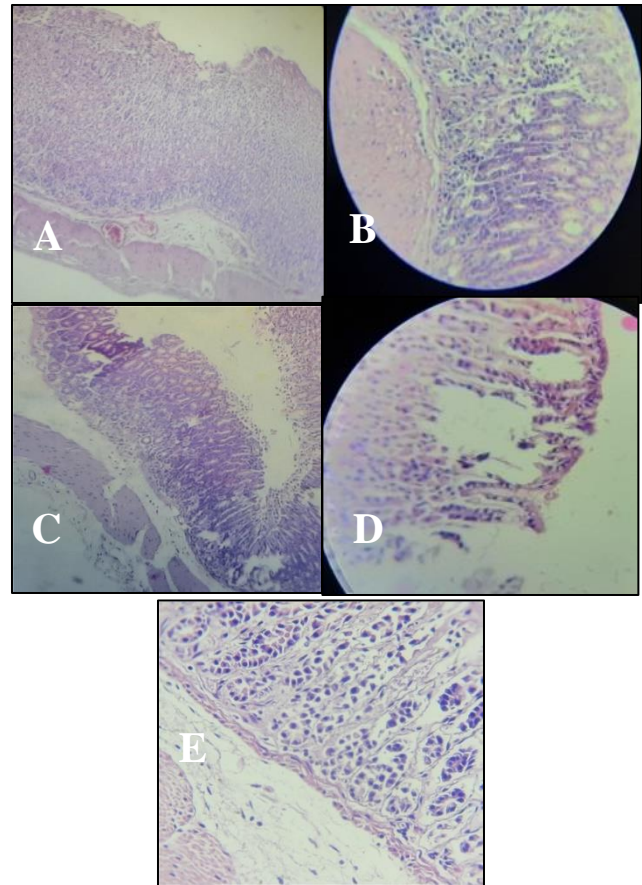


Figure-3. Histopathological examination of sectioned stomachs of sham control (G1) (A, B) and untreated ethanol- induced gastric ulcer mice (G2: negative control) (C, D, E) (G10x100).

Ethanol induces lesions and injuries to the gastric mucosa and causes a reduction in bicarbonate secretion and inflow of gastric blood with depletion of gastric wall mucus and an increase in the release of histamine, the influx of calcium and sodium ions, generation of reactive oxygen species (ROS) and leukotrienes, resulting in severe gastric damage (Balan et al., 2015).

Histopathological examination of stomach sections of mice given absolute ethanol and pretreated with 30 mg omeprazole/Kg BW (G3: positive control mice group) has shown an important reduction in gastric injury and erosion as compared with the negative control mice group (G2) (fig. 4 A and B). These findings are in agreement with those of Raish et al. (2021) who observed that omeprazole pretreatment of ethanol-induced gastric ulcer in rat led to a reduction in lesions in the gastric wall mucosa, a decrease in the gastric volume and free acidity, and an increase in the gastric

pH and mucosa levels. Thus, Omeprazole, a proton pump inhibitor, ensures good protection of gastric mucosa and is an effective drug widely used in the treatment of gastric disorders related to gastric acid secretion (Liu et al., 2020).

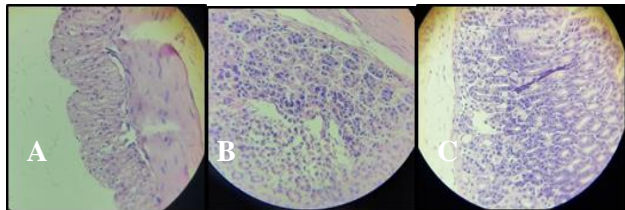


Figure-4. Histopathological examination of sectioned stomachs of ethanol-induced gastric ulcer mice (G3: positive control) pretreated with 30 mg omeprazole/kg BW (A, B,C) (G10x100).

A significant ($P<0.05$) reduction in ethanol-induced gastric lesions was observed in *H. pluvialis* DMSO-AE pretreated mice groups (G4 and G5 groups). Microscopic examination of the gastric mucosa of mice pretreated with 250 μ g of *H. pluvialis* DMSO-AE/kg BW (G4) has shown minor damage. Gastric epithelium surface is protected from ethanol erosion and necrotic lesions; the leukocytes infiltration is reduced compared to that of the negative control group (G2) and a moderate inflammation of the submucosa was observed in the G4 group (Fig.5 A, B, and C).

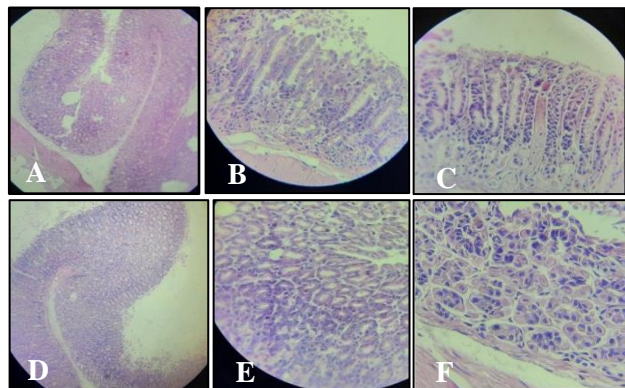


Figure-5. Histopathological examination of sectioned stomachs of ethanol-induced gastric ulcer mice pretreated with 250 (A, B, C) (G4 group) or 500 μ g *H. pluvialis* DMSO-AE/kg BW (D, E, F) (G5 group) (G10x100).

However, the histopathological aspect of the gastric mucosa of 500 μ g *H. pluvialis* DMSO-AE /kg BW pretreated mice group (G5) has shown intact gastric mucosa and absence of gastric ulcer indicator signs such as leucocytes infiltration, inflammation, edema

formation, erosion or epithelium destruction. The preservation of the continued formation of the gastric epithelium after pretreatment of mice with 500 μ g of *H. pluvialis* DMSO-AE/kg BW shows the efficiency of this dose to ensure excellent protection of the integrity of gastric mucosa against ethanol harmfulness (Fig.5 D, E, and F). Similar observations have been reported by Murata et al. (2012) and Kim et al. (2005a, b) who have shown that treatment of mice and rats with astaxanthin significantly ($P<0.05$) reduced gastric lesions induced by ethanol, hydrochloric acid secretion or drug consumption.

The ethanol-induced gastric ulcer can be promoted by free radicals production and lipid peroxidation which unbalances the ratio between cellular antioxidant and oxidant capacity, thereby increasing oxidative stress (Kim et al., 2005b). The preventive effect of astaxanthin on ulcer gastric damage is related to its high antioxidant potential in removing free radicals species involved in gastric lesions (Kamath et al., 2008). Kim et al. (2005a) have shown that the gastric mucosa of *Xanthophylomyces dendrorhousis* astaxanthin orally given rats was significantly protected from ethanol-induced damage and in turn from lipid peroxidation. The histopathological examination has shown that in the present study, gastric lesions disappeared after *H. pluvialis* DMSO-AE treatment. Kamath et al. (2008) study on astaxanthin has shown that this carotenoid exhibits a dose-dependent protective effect on ethanol-induced gastric ulcer which might be due to an increase in the levels of the antioxidant enzymes such as superoxide dismutase, catalase, and glutathione peroxidase in stomach homogenate. Furthermore, astaxanthin ensures a gastroprotective effect by regulating gastric acid secretion (Kim et al., 2005a, b; Kamath et al., 2008). Astaxanthin provides a protective effect against gastric lesions induced by non-steroidal anti-inflammatory drugs such as indomethacin and naproxen, suggesting that this carotenoid may represent an alternative strategy for gastric ulcer treatment (Kim et al., 2005a).

Acute toxicity

The harmlessness of 500mg/Kg *H. pluvialis* DMSO-AE was evidenced by the absence of clinical toxicity signs during the whole experiment period for all mice. This is in agreement with observations made by Regnier et al. (2015) on *H. pluvialis* astaxanthin harmlessness. These authors have reported no changes either in the morphology or in the viability of human

vascular cells when cultured in presence of up to 10 μM astaxanthin concentrations.

Antioxidant activity of the *H. pluvialis* DMSO astaxanthin extract DPPH assay

Radical scavenging activity of *H. pluvialis* DMSO-AE is dose-dependent in that it reaches respective values of 49.35 ± 1.45 , 64.35 ± 4.98 , $78.61 \pm 2.23\%$, and $89.07 \pm 2.23\%$ for extract concentrations of 25, 50, 100 and $200 \mu\text{g/mL}$, respectively.

The concentration of *H. pluvialis* DMSO-AE extract required to scavenge 50% of the initial DPPH radicals (IC_{50}) was calculated in order to be compared to that of the controls (BHT and ascorbic acid). Obtained results have shown that *H. pluvialis* DMSO-AE IC_{50} ($24.56 \mu\text{g/mL}$) is significantly ($P < 0.01$) lower than that of BHT ($141.61 \mu\text{g/mL}$) and ascorbic acid ($68.82 \mu\text{g/mL}$), reflecting the high anti-free radical capacity of the algal extract (Fig. 6).

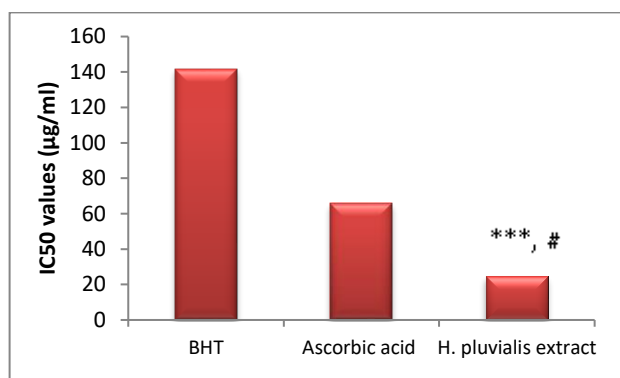


Figure-6. IC_{50} values ($\mu\text{g/mL}$) of BHT, ascorbic acid and *H. pluvialis* DMSO astaxanthin extract Values are the mean \pm SD (n = 3).

*** ($P < 0.001$) difference *H. pluvialis* extract Vs BHT, # ($P < 0.05$) difference *H. pluvialis* extract Vs ascorbic acid

This is in line with the results of Zhao et al. (2016) who reported the related high *H. pluvialis* extract DPPH scavenging capacity to the richness of this microalga in hydrogen donor molecules which can convert free radicals to stable products and terminate the radical chain reaction. Astaxanthin is the carotenoid that represents such molecules (Shimada et al., 1992) and its production by *H. pluvialis* is increased by the applied unfavorable environmental conditions herein such as nitrate starvation and strong light exposure. Ranga Rao (2011) and Zhao et al. (2016) reported similar results that they attributed the same interpretation; while Karppi et al. (2007) also

recorded the high scavenging capacity of astaxanthin that Dose et al. (2016) attribute to the presence of carbonyl and hydroxyl functional groups. Astaxanthin is also known as an antioxidant whose potency exceeds that of α -tocopherol and other carotenoids such as lutein, β -carotene or lycopene (Yang et al., 2013; Focsan et al., 2017). This suggests that the antioxidant potential of astaxanthin may be one of the mechanisms responsible of its gastro-protective activity, since ulcer gastric process is related to oxidative stress (Kim et al., 2005a).

Conclusion

This investigation evidenced that a new Algerian strain of *Haematococcus pluvialis* DMSO-astaxanthin extract is a powerful gastroprotective extract whose harmlessness at a dose of 500mg/Kg BW has been herein demonstrated in mice. By the way, it has been also shown that this algal extract has a dose-dependent antioxidant activity. These biological properties are likely related to the astaxanthin content of the algal extract which depends on the unfavorable growth (absolute nitrate starvation and strong light exposure) conditions of *H. pluvialis*. These findings suggest that *H. pluvialis* is an Algerian endemic alga that is easy to grow, and whose exploitation for the astaxanthin production could be of interest.

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Conflict of Interest: None.

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Contribution of Authors

Sadoud M: Conceived idea, participated in microalgae culturing, histopathological experiment and manuscript write up

Bouamar S: Reviewed literature, helped in determination of the antioxidant activity of the astaxanthin extract

Bouziane N: Participated in data collection, data treatment and statistical analysis

Medjkane M: Helped in housing and maintenance of mice and astaxanthin extraction

Riazi A: Conceived idea, designed research methodology and final editing of the manuscript

