Modulatory Effect of *Chaetomorpha gracilis* on Erythrocytes Functions and Metabolic Disorders in Mice Fed a High-cholesterol Diet

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Abstract: The current study was carried out to estimate the protective effect of methanolic extract of *Chaetomorpha gracilis* (MECG) against High Cholesterol Diet (HCD) induced erythrocyte damage in mice. The results of the in vitro assay showed that MECG have higher antioxidant capacities in the DPPH, TAC, ABTS, NBT, NO⁻ inhibition assays. The HPLC analysis confirmed that this potential antioxidant seems to be due to the active compounds, in particular polyphenols, flavonoids. HCD promoted oxidative stress with a rise the level of malonaldehyde (MDA), advanced oxidation protein product (AOPP) levels and a significant decrease of the Vitamin C content, as well the antioxidant enzyme activities such as superoxide dismutase and glutathione peroxidase. In addition, HCD treatment caused significant lipid profile disorders via increase the cholesterol, triglycerides and LDL levels and reduction HDL-Ch level. A statistically significant decrease of Mg²⁺ and Ca²⁺ ATPase activities accompanied with a severe damage in the erythrocytes structure and hematological parameters alterations were also noted in hypercholesterolemic mice. Pre-treatment with MECG significantly restored biochemical markers and pathological lesions. It can be suggest that supplementation of MECG displays high potential to quench free radicals and attenuates high cholesterol diet induced erythrocytes oxidative stress and related damages.

Key words: *Chaetomorpha gracilis*, high cholesterol diet, oxidative stress, erythrocytes, mice

1 Introduction

Raised cholesterol is estimated to cause 4.4 million deaths (7.9% of total) and 40.4 million DALYs (2.8% of total). Nowadays, consumption of high-cholesterol diets (HCDs) is a crucial risk factor leading to metabolic disorders such as hyperlipidemia, hypertension and cardiovascular disease⁷. In fact, the excessive accumulation and storage of fat in the body increase the occurrence of hypercholesterolemia⁷. Obesity is absolutely related with high cholesterol fed, thus increase in growing mortality, damaged life quality and massive expenditure on healthcare system⁷. Besides, many studies have reported that the high cholesterol diet are strictly associated with elevated plasma levels of cholesterol, triglycerides and huge changes in the profiles of serum lipoproteins such as LDL and/or HDL cholesterol⁴. As a result, lipids structure is supposed to be the substrate for lipid peroxidation upon exposure to oxygen species compound by insufficient scavenging and/or depressed antioxidant enzymes⁹. Consequently, Hypercholesterolemia causes oxidative stress resulting in increased lipid peroxidation in multiple tissues.

Abbreviations: AI, Atherogenic index; AOPP, Advanced oxidation protein products; BW, Body weight; CAT, Catalase; GPx, Glutathione peroxidase; HCD, High Cholesterol Diet; HDL-Ch, High density lipoproteins of cholesterol; Hb, Hemoglobin; Ht, Hematocrit; LDH, Lactate dehydrogenase; LDL-Ch, Low density lipoproteins of cholesterol; LPO, Lipid peroxidation; MCHC, Mean corpuscular hemoglobin concentrations; MECG, methanolic extract of *Chaetomorpha gracilis*; Plt, Platelet; RBC, Red blood cells; ROS, Reactive oxygen species; SOD, Superoxide dismutase; TAC, Total antioxidant capacities; TBARS, Thiobarbituric acid reactive substances; TBS, Tris-buffer saline; T-Ch, Total cholesterol; TG, Triacylglycerols; Vit C, Vitamin C; WBCs, White blood cells.

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Accepted February 9, 2021 (received for review April 17, 2020)
Journal of Oleo Science ISSN 1345-8957 print / ISSN 1347-3352 online
http://www.jstage.jst.go.jp/browse/jos/ http://mc.manuscriptcentral.com/jjocs

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organs\textsuperscript{3}. Free radicals are generated over cellular respiration in the body and many factors are documented to perturb the balance between reactive oxygen species (ROS) generation and cellular defense mechanisms, lead to alteration of cell function and tissue injury\textsuperscript{7}.

Recently, it has been approved that erythrocyte can generate endogenous ROS\textsuperscript{8}. In addition, the erythrocytes is considered as dynamic promoter for the oxidative stress due to the high cellular levels of oxygen and hemoglobin (Hb) and a significant level of polyunsaturated fatty acids in their membranes\textsuperscript{9,10}. Exposure of erythrocytes to Oxidative stress leads to the enzymatic and nonenzymatic depletion, lipid peroxidation and inhibition of ATPase activities\textsuperscript{11}.

Owing to hypercholesterolemia-induced oxidative stress, the RBCs are susceptible to alteration of membrane integrity and fragility, increases of osmotic fragility\textsuperscript{11}.

A great deal of research work has explored many drugs to manage severe cases of hypercholesterolemia in humans. Herbal medicines derived from plant extracts constitute an indispensable part of the traditional medicine and are being increasingly utilized to treat a wide variety of clinical diseases. Marine algae have been extensively explored to possess a large range of biological activities\textsuperscript{11}.

Recently, there is a rising interest to use natural vegetal products identified from marine algae for the improvement their major therapeutic role in the treatment of obesity and other metabolic diseases\textsuperscript{12}. The ability of natural products derived marine origins to protect against obesity was few documented and could be a precious source of therapeutic agents for the development of value-added health food products and for human consumption due to higher nutritional value. Phenolic compounds found in marine algae are known principally as a mechanism of adaptation for oxidative stress. A novel approach supported a novel way for the active molecules possess hypolipidemic effects\textsuperscript{12}.

The green algae Chaetomorpha gracilis is filamentous algae has been reported to possess high levels of antioxidant molecules\textsuperscript{13}. From previous experimental studies performed on Chaetomorpha gracilis aqueous extract, it seems that most of its pharmacological action is due to its antioxidant activity to trap free radicals and/or inhibit lipid peroxidation\textsuperscript{13}. For this reason, in the current study, we report the phytochemical composition and antioxidant effects of methanolic extract of Chaetomorpha gracilis in several in vitro systems. In addition, we determined on evaluating protective of side effects by of erythrocytes damage in mice fed high cholesterol diet.

2 Materials and Methods
2.1 Algal material

Fresh Chaetomorpha gracilis alga was collected in April 2016, at Al Mahres, Sfax, Tunisia. The algal was identified and authenticated by senior botanist, Pr. Mohamed Chayeb, Department of biology at the Faculty of Science of Sfax, Tunisia.

2.2 Extraction procedure

After collection, they were thoroughly washed and then shade dried. The dried samples were crushed in an electric mill till a powder was obtained. Then, 200 g from the obtained powder was extracted in 1000 mL of methanol (absolute). After 48 hours, all extracts were filtered. Then the dried extracts were kept in the dark at +4°C in order to evaluate the composition of Chaetomorpha gracilis extract. After filtration with Whatman filter paper, the resultant ethanolic extract was evaporated to drying using Rotary evaporator. The dry extract was stored at 4°C.

2.3 Chemical reagents

All reagents used in the current study were of analytical grade. Cholesterol and all chemicals were purchased from Sigma Chemical (St. Louis, France).

2.4 Animals

Forty male Wistar mice weighing 28–30 g aged 60 days were obtained from the "Central Pharmacy of Tunis" (SIPHAT). The animals were kept in an air-conditioned room temperature 21 ± 1°C and relative humidity of 40% with a 12 h light/dark cycle. All mice had free access to drinking water and diet with standard pelleted diet food. The general guidelines for the use and care of living animals in scientific investigations were followed (Council of European Communities, 1986). The handling of the animals was approved by the Tunisian Ethical Committee of the Faculty of Science of Sfax.

2.5 In vitro study

2.5.1 Quantification of phenolic classes

2.5.1.1 Determination of total phenolic

Total phenolic content in MECG was determined by Singleton et al.\textsuperscript{14} using Folin-Ciocalteu colorimetric method. The absorbance was measured at 760 nm. The phenolic content in the extract were expressed in mg of gallic acid equivalents per g of dry weight of extract.

2.5.1.2 Determination of total flavonoid

The flavonoid content in the methanolic extract was determined using a colorimetric method described by Ordonez et al.\textsuperscript{15}. After 30 min, the absorbance was measured at 430 nm. The total flavonoid content in the MECG was expressed in mg quercetin equivalent per g of dry weight of extract.

2.5.2 In vitro antioxidant assays

2.5.2.1 Total antioxidant capacities (TAC)

The total antioxidant capacities (TAC) were determined method of Prieto et al.\textsuperscript{16}. The ascorbic acid was used as a
reference standard and the antioxidant activity of the samples was expressed as milligrams of ascorbic acid equivalents per mL.

2.5.2.2 Determination of DPPH radical scavenging activity

The antioxidant activity of the extract was determined in terms of hydrogen-donating or radical-scavenging ability, using the stable radical 1,1-diphenyl-2-picrylhydrazyl (DPPH), according to the method of Son and Lewis. The inhibition free radical DPPH was calculated as:

\[ \text{IC}_{50} = \frac{(A_{control} - A_{sample})}{A_{control}} \]

The results were expressed as IC50 (inhibition level was 50% DPPH radical).

2.5.2.3 Antioxidant activity by ABTS+ assay

The ability of sample to scavenge the ABTS+ free radical was determined according to Re et al. The ABTS (7 mM in 20 mM sodium acetate buffer, pH 4.5) was added to potassium persulfate (2.45 mM) and incubated 12–16 h in the dark (4°C). The solution was then diluted to an absorbance of 0.7 ± 0.01 at 734 nm to form the test reagent. The methanolic extract were added to 3 mL of the reagent and the mixture incubated at 30°C for 30 min. The capacity of scavenging of ABTS radical was calculated as:

\[ \% \text{Inhibition} = \left( \frac{A_{control} - A_{test}}{A_{control}} \right) \times 100 \]

Where Absorbance of the control reaction and Atest is the absorbance of the extract reaction.

2.5.2.4 Superoxide anion scavenging activity (NBT test)

The effect of MECG on the production of superoxide anion was determined according to the method described by Yagi et al. The absorbance was read at 560 nm. All solutions were prepared in a phosphate buffer (1 M, pH 7.4). Scavenging of the superoxide anion was monitored by the absorbance decrease at 560 nm by spectrophotometry. The capacity of scavenging of superoxide anion radicals was calculated as:

\[ \% \text{superoxide anion scavenging} = \left( 1 - \text{absorbance sample} - \text{absorbance control} \right) \times 100 \]

2.6 HPLC analysis conditions

For determination Chaetomorpha gracilis methanolic composition by high performance liquid chromatography (HPLC) analysis, C-18 reversed-phase column (Zobrax, 250 x 4.6mm) was used. The mobile phase consist of acetonitrile water (98:2 v/v) as solvent A and glacial acetic and water (58:2 v/v) as solvent B. For the preparation of the ethanol extract by dissolving in 1 mL of methanol making a final concentration of 25 mg/mL. Before starting HPLC analysis, all the solutions prepared were filtered with Whatman paper (Ø 0.45 μm). The diluted extract was injected directly and chromatograms were monitored at 280 nm. The flow rate was set at 0.9 mL/min and 20 μL of extract was injected. The phenolic acids and flavonoids were found at 280 nm and 360 nm respectively to the differences in their absorption spectra were utilized for their quantification, by comparing their chromatograms to reference substances.

2.7 In vivo study

2.7.1 Experimental procedure

The mice were divided into four groups of 10 mice each as follows:

- Control: received distilled water and standard diet.
- HCD: mice received HCD (2% cholesterol powder + 0.2% cholic acid/kg chow diet) to persuade hypercholesterolemia for 6 weeks, with cholic acid which promotes processing of dietary fat according to Akdogan et al.
- HCD + MECG: mice received HCD and treated with green algae extract MECG (250 mg/kg of body weight/daily) by gavage for 6 weeks.
- MECG: mice received Chaetomorpha gracilis extract (250 mg/kg body weight/daily) dissolved in distilled water by gastric gavage route.

After the 6 weeks of treatment, the animals of different groups were weighed and sacrificed by cervical decapitation to avoid stress.

2.7.2 Blood samples preparation

Blood samples were collected into EDTA tubes, some of which were immediately used for the determination of hematological parameters and blood smear and the others were centrifuged at 2,200 × g for 15 min. Plasma samples were then removed, and the sediments containing erythrocytes were suspended in phosphate buffer saline solution (0.9% NaCl in 0.01 M phosphate buffer, pH 7.4) and centrifuged as reported by Sinha et al. This process was repeated twice. After removing cells' debris by centrifugation at 1,000 × g for 15 min, the hemolysates were collected and stored at −80°C until biochemical analysis.

2.7.3 Hematological study

Hematological parameters (WBC, RBC, Ht, Hb, MCHC and platelet number) were determined by an automated cell Coulter MAXM (Beckman Coulter, Inc, Fullerton, CA). A blood smear is a blood analysis used to appear the abnormalities in blood cells. The blood smear was performed with a drop of fresh blood. The smears were stained according to May-Grunwald-Giemsa. The morphological criteria of individual cells were performed using a microscope at 10x, 40x and 100x magnification.

2.7.4 Analysis of plasma lipids

Plasma lipid parameters such as total cholesterol (T-Ch), triacylglycerol (TG), LDLcholesterol (LDL-Ch) and high-density lipoprotein-cholesterol (HDL-C) levels were determined by were measured by autoanalyzer (Erba xl 200, Mannheim, Germany) using Erba diagnostic kit.

2.7.5 Evaluation of biochemical markers

The albumin, α2, β1, β2 and γ globulins contents were
determined with capillary® electrophoresis using commercial kit CapillarysSebia in the pathological laboratory of Sfax Hospital. In addition, the activities of Ca\(^{2+}\) and Mg\(^{2+}\) ATPases in the erythrocyte and platelets cells were determined according to Hjerken and Pan\(^{22}\) and Ohinishi et al.\(^{23}\) methods respectively.

2.7.6 Osmotic fragility test
With the method of Godal and Heisto\(^{24}\), the range for osmotic fragility of erythrocytes been estimated in terms of lysis in hypotonic saline solution. Hb content of the supernatant was measured spectrophotometrically at 540 nm. The level of lysis in each tube was compared with a 0.1 % NaCl tube (100 % lysis).

2.7.7 Determination of protein content
Protein content was determined according to Lowry et al.\(^{25}\) using bovine serum albumin as standard.

2.7.8 Effect of MECG on oxidative stress markers
2.7.8.1 Evaluation of erythrocytes lipid peroxidation
The lipid peroxidation was estimated by measuring the thiobarbituric acid-reactive substances (TBARS) level according to Fraga et al.\(^{26}\).

2.7.8.2 Evaluation of erythrocytes protein peroxidation
Advanced oxidation protein products (AOPP) content in erythrocytes was measured using the method of Witko et al.\(^{27}\). The absorbance was determined at 340 nm. The concentration of AOPP for each sample was calculated using the extinction coefficient of 261 cm\(^{-1}\) mmol\(^{-1}\), and the results were expressed as micromoles per milligram of protein.

2.7.8.3 Estimation of antioxidant enzyme activities
Superoxide dismutase (SOD) was measured according to Rotruck et al.\(^{28}\). The developed blue color reaction was determined at 560 nm. Units of SOD activity were expressed in the amount of required to inhibit the reduction of NBT (4-nitro blue tetrazolium chloride) by 50%. Glutathione peroxidase (GPx) activity was measured according to Kakkar et al.\(^{29}\), using hydrogen peroxide as substrate and the reduced glutathione. The GPx activity was expressed as nmole of GSH per mg of protein.

2.7.8.4 Erythrocytes vitamin C level
Ascorbic acid content was determined using the method described by Jacques-Silva et al.\(^{30}\).

2.8 Statistical methods
The data are obtained from ten animals per group and differences were calculated by a one-way analysis of variance (ANOVA) followed by the Fisher’s protected least significant difference test as a post hoc test for comparison between groups ([treated groups (HCD) vs Control] and [(HCD + MECG) vs (HCD)]). Data are expressed as means ± standard deviation (SD) and the p value is considered statistically significant when <0.05.

![HPLC profile of phenolic acids (λ = 280 nm) and flavonoids (λ = 280) from C. gracilis methanolic extract (MECG). The HPLC separation of the active compounds was carried out on C-18 reverse phase HPLC column (Zorbax, 250mm _4.6 mm, particle size 5 µm) on an elution gradient at 30°C. The mobile phase was composed of solvent A: water and acetonitrile (98:2 v/v), and solvent B: water and 2% glacial acetic acid (pH = 2.6) (58:2 v/v). The gradient elution used was 0-5 min 5% A, 25-30 min 35% A, 35-45 min 70% A. The flow rate amounted to 0.9 mL/min with an injection volume of 20 µL.](image-url)
3 Results

3.1 In vitro study

3.1.1 Amounts of polyphenols and flavonoids

The data show that methanolic extract of *C. gracilis* had a content of total phenols $= 59.18 \pm 3.88$ mg GAE/g dry extract.

The total flavonoids were expressed as mg of quercetin equivalents per gram of dry matter using the equation of the calibration curve. Our results show that the MECG had a flavonoid content $= 33.81 \pm 1.68$ mg QE/g MECG.

Qualitative estimation of phytoconstituents in the methanolic extract of *Chaetomorpha gracilis* indicated the presence of phenolic acids and flavonoids. Figure 1 shows the presence of six compounds identified in MECG which contain gallic acid (9.65%), rutin (5.88%), quercetin (0.32%), apigenin (0.53%), kampferol (0.42%), naringenin (0.65%).

3.1.2 Antioxidant properties of MECG

Our data shows that the MECG Exhibited a higher total antioxidant capacity (11.62 mg EAG/g dry extract). We have to contain as much quantity of antioxidants compounds as equivalents of ascorbic acid to effectively reduce the oxidant in the reaction matrix. The 50% scavenging concentration on DPPH radical (IC50) values of MECG and BHT were 0.87 and 0.69 mg/mL, respectively. Superoxide anion was also scavenged (Fig. 2). In addition, the antioxidant activity of MECG was compared to that of trolox, a well-known antioxidant, using two different assays, namely superoxide oxygen radicals inhibition and ABTS scavenging ability. Figure 1 shows that AERO is a good scavenger of superoxide anion and ABTS radicals compared to Vit C as standard.

3.2 Effects of MECG on hematological study

HCD induced an increase in the levels of RBCs, WBCs, Hb, Ht, platelets and MCHC in erythrocytes ($p \leq 0.01$) compared to control group. MECG attenuated these alterations by about $-68\%$ of RBC, $-37\%$ of WBC respectively compared to positive control. Treatment by MECG alone didn’t change the hematological parameters of normal mice (Table 1).

From the histopathological point of view, blood smears from mice in the HCD group showed an abnormal appearance of blood cell. The pathological lesions induced by the administration of high cholesterol diet were remarkably alleviated by MECG (Fig. 3).

3.3 The effect of MECG on body weight

Table 2 illustrated the impact of *Chaetomorpha gracilis* extract on body weight of hypercholesterolemic mice. MECG 250 mg/kg did not affect the body weight gain. Nevertheless, there was a significant increase ($p<0.01$) in the body weight gain of hypercholesterolemic animals when compared with the control. HCD groups pre-treated with MECG reduced body weight reaching the control value.

![Fig. 2](image-url)  
**Fig. 2** Antioxidative properties of MECG: (a) DPPH radical scavenging activity; (b) ABTS $^+$ radical scavenging activity; (c) NBT radical scavenging activity; (d) TAC: total antioxidant capacity.
3.4 The effect of MECG on lipid profile

The results observed after treatment with MECG on various lipid markers in the groups under study are given in Table 3.

Hypercholesterolemia caused a significant increase in total cholesterol, triglycerides, LDL levels, while HDL level was decreased compared to controls untreated mice. In addition, HCD caused a significant increase in AI index. Treatment with MECG (250 mg/kg) after to hypercholesterolemia induction protect the rise total cholesterol, triglycerides, LDL levels and reduce the drop of HDL compared to the HCD damaged mice. When a dose of MECG (250 mg/kg) given for 30 days this did have a significant effect on any of the parameters studied in these experiments.

3.5 Osmotic fragility

Results presented in Fig. 4 revealed an increase in osmotic fragility of erythrocytes in HCD treated rats com-
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J. Oleo Sci. 70, (6) 837-848 (2021)

pared to untreated animals. In fact, the increase of osmotic fragility as a result of hypercholesterolemia administration were largely protected in the MECG group.

Table 2 Effect of Chaetomorpha gracilis methanolic extract on body weight of control and treated mice groups.

| Parameters & treatments | Controls | HCD | HCD+MECG | MECG |
|-------------------------|----------|-----|----------|------|
| Initial body weight (g) | 33.2 ± 1.24 | 32.12 ± 1 | 32.12 ± 3.33 | 32.5 ± 2.27 |
| Final body weight (g)   | 35.77 ± 2.4 | 41.15 ± 3.9** | 35.74 ± 0.65** | 34.5 ± 1.59 |
| Body weight gain (%)    | 2.57 ± 0.43 | 9.03 ± 0.3** | 3.62 ± 0.2** | 1.5 ± 0.29 |

Values are means for eight mice in each group.
HCD and treated groups vs control group: *p < 0.05; **p < 0.01
HCD group vs (HCD+MECG) group: +++p < 0.01

Table 3 Effects of HCD and/or MECG treatment for 30 days on lipid profiles in mice erythrocytes.

| Parameters & treatments | n=10 | C | HCD | HCD+MECG | MECG |
|-------------------------|------|---|-----|----------|------|
| 1T-Ch                   | 3.7 ± 0.20 | 4.84 ± 0.27** | 4.04 ± 0.24** | 3.38 ± 0.21 |
| 2TG                    | 1.19 ± 0.13 | 2.14 ± 0.16** | 1.5 ± 0.02** | 1.1 ± 0.05 |
| 3LDL-Ch                | 0.83 ± 0.03 | 1.82 ± 0.02** | 1.03 ± 0.02** | 0.83 ± 0.01 |
| 4HDL-Ch                | 218 ± 0.15 | 1.02 ± 0.08** | 1.87 ± 0.03** | 197 ± 0.07 |
| 5AI                    | 0.69 ± 0.03 | 3.74 ± 0.32** | 1.11 ± 0.21** | 0.71 ± 0.02 |

C: Control mice; HCD: Treated mice with high cholesterol diet; (HCD+MECG): mice treated with methanolic extract of Chaetomorpha gracilis and treated with high cholesterol diet; (MECG): mice treated with methanolic extract of Chaetomorpha gracilis.

1T-Ch: Total cholesterol (mmol/L); 2TG: Triglycerides (mmol/L); 3LDL-Ch: Low density lipoproteins of cholesterol (mmol/L); 4HDL-Ch: High density lipoproteins of cholesterol (mmol/L); 5AI: Atherogenic index = (T-Ch − LDL-Ch)/HDL-Ch.

3.6 Effects of MECG on biochemical markers

High cholesterol diet induced hyperlipidemia resulted in significant (p<0.05) increase Ca^{2+} and Mg^{2+} ATPases activities in erythrocyte cells (38% and 41%, respectively) compared to the normal control group as shown in Fig. 5.

The effect hypercholesterolemia on serum immunoglobulin level is shown in Table 4. There was significant increase in α2, β1, β2, and γ globulins levels following hypercholesterolemia compared to the normal control group. These alterations were significantly alleviated by MECG administration.

3.7 Oxidative damage markers in erythrocytes

Results presented in Table 5 revealed an increase in TBARS and AOPP level in HCD-treated mice compared to untreated animals. In fact, the decrease in erythrocytes TBARS and AOPP concentrations as a result of HCD administration were largely protected in the MECG groups.

It is worth noting that there were no significant differences in the TBARS and AOPP levels in the treated group with only MECG (250 mg/kg BW) compared to healthy mice.

Fig. 4 The activity of Mg^{2+} ATPase (a) and Ca^{2+} ATPase in the erythrocytes of mice treated by HCD, MECG or their combination. Values were expressed as mean ± SEM of 10 mice in group. *p<0.05, **p<0.01, ***p<0.001 compared with control, ‘p<0.05, ‘’p<0.01 compared with HCD treated group (CCl4).
3.8 Effects of MECG on antioxidant enzymes activities and acid ascorbic level

Comparison of oxidative stress markers (antioxidant enzymes activities) in erythrocytes of treated mice with the control animals indicated a significant decrease in SOD and GPx activities (Table 5) levels after the induction of oxidative stress.

Treatment with the MECG at doses of 250 mg/kg BW prevented a full protection from oxidative damage when compared to that in the HCD group. Pretreatment with MECG (250 mg/kg) resulted in almost. MECG itself in a dose of 250 mg/kg BW did not result in any significant change in the antioxidant enzymes activities in the animals compared to control mice.

4 Discussion

A high cholesterol diet is related to combination of environmental and genetic factors resulting in the increase of diverse metabolic diseases\(^{31}\). Indeed, hypercholesterolemia is necessarily combined with aggravation of inflammation and oxidative stress\(^{32}\). In this current study, we evaluated the potential effect of *Chaetomorpha gracilis* methanolic extract on hypercholesterolemia caused oxidative stress and also to explore it is a beneficial effect against biochemical, hematological and immunology disorders in mice.

In the present work, the haematological results of the HCD-treated mice showed large disorders as evidence by increase in Pt, Hb, Ht and WBC, indicating an activation of the defense mechanism compared to the control group. Since red blood cell counts are much greater than leucocyte counts or platelet counts, a significant increase in white blood cells or platelet is required to cause an increase in RBCs\(^{33}\). Indeed, the increase platelet count is considered as a marker of vascular disease similar to microangiopathy and macro angiopathy\(^{34}\). High WBC count

### Table 4

Effects of HCD and/or MECG treatment for 30 days on plasma proteins (albumin + globulin).

| Parameters & treatments | n=10 | C | HCD | HCD+MECG | MEGC |
|-------------------------|-----|---|-----|----------|------|
| albumin                 | 19.21 ± 2.16 | 26.86 ± 2.76** | 20.06 ± 2.5** | 19.85 ± 2.31 |
| α₁ (g/L)                | 13.17 ± 1.63 | 9.48 ± 0.45** | 12.24 ± 0.64** | 13.03 ± 1.28 |
| β₁ (g/L)                | 6.23 ± 1.13 | 3.45 ± 0.65* | 6.03 ± 0.73** | 6.32 ± 0.91 |
| β₂ (g/L)                | 6.23 ± 1.76 | 8.92 ± 1.4** | 7.02 ± 1.36 | 6.18 ± 0.68 |
| γ (g/L)                 | 4.16 ± 0.18 | 6.03 ± 1.12** | 4.74 ± 0.85** | 4.19 ± 0.21 |

C: Control mice; HCD: Treated mice with high cholesterol diet; (HCD+MECG): mice treated with methanolic extract of *Chaetomorpha gracilis* and treated with high cholesterol diet; (MECG): mice treated with methanolic extract of *Chaetomorpha gracilis*.

Values are expressed as means ± SE: n=8 in each group.

(HCD) compared to control (C) group: *p < 0.05; **p < 0.01; ***p < 0.001

(HCD+MECG) group compared to HCD group: ‗p < 0.05; ‗p < 0.01; ‗‘p < 0.001
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Table 5  Effects of HCD and/or MECG treatment for 30 days on oxidative stress markers in mice erythrocytes.

| Parameters & treatments | n=10 |
|-------------------------|------|
|                         | C    | HCD | HCD+MECG | MECG |
| ^1TBARS                 | 3.7±0.20 | 4.84±0.27** | 4.04±0.24* | 3.38±0.21 |
| ^2AOPP                  | 1.19±0.13 | 2.14±0.16** | 1.5±0.02** | 1.1±0.05 |
| ^3SOD                   | 6.04±0.55 | 3.86±0.88** | 5.91±0.56** | 6.17±0.97 |
| ^4GPX                   | 3.07±0.26 | 1.43±0.16** | 2.46±0.16** | 3.29±0.28 |
| ^5Vit C                 | 34.23±3.19 | 15.27±2.43** | 29.86±1.21*** | 35.03±2.99 |

C: Control mice; HCD: Treated mice with high cholesterol diet; (HCD+MECG): mice treated with methanolic extract of *Chaetomorpha gracilis* and treated with high cholesterol diet; (MECG): mice treated with methanolic extract of *Chaetomorpha gracilis*.

^1TBARS: Thiobarbituric acid reactive substances (nmol/mg protein); ^2AAOP: advanced oxidation protein products (µmol/mg of protein); ^3SOD, Superoxide dismutase (U SOD/mg of protein); ^4GPx: Glutathione peroxidase (nmol/GSH/min/mg of protein); Vit C: Vitamin C (µg ascorbic acid/g tissue).

Values are expressed as means ± SE: n=8 in each group.

(HCD) compared to control (C) group: *p<0.05; **p<0.01; ***p<0.001
(HCD+MECG) group compared to HCD group: ′p<0.05; ″p<0.01; ″″p<0.001

signal some features metabolic syndrome such as cardiovascular disease and it can be correlated to the activation of inflammation from excessive consumption of lipids.

The present study showed that the dietary cholesterol induced an increase in body weight compared to the control group. This examination is in agreement with the study of Ahen et al. who showed that the consumption of a HCD facilitates the development of a positive energy balance and leads to an increase in visceral fat deposition. But, our data found efficacy of MECG in decreasing the body weight gains. The anti-obesity effect of dietary green algae was reported by several studies. Li et al. reported that dietary supplementation with *Codiumium cylindricum* inhibited fat absorption, moderated body weight gain and perirenal white adipose tissue caused by a 78 days high fat dietary course. In fact, the reduction of weight was attributed to the inhibitory effect on fact absorption of dietary fiber contained in the green algae and anti-obesity active compounds such as carotenoids. However, the molecular mechanism underlying the inhibitory effect of this extract was uncertain.

In addition, high cholesterol fed caused a significant increase in the triglyceride, total Cholesterol, and LDL levels and decrease in HDL level. Rise in the cholesterol concentrations might be due to the accumulation of lipids in the arterial wall, leading to atherosclerotic plaque progress. Furthermore, the rise of LDL level is according to a down regulation in LDL-c receptors by fat incorporated in the diet.

Erythrocytes and platelets have minimal capacity for storage of cholesterol but they have a high capacity for exchange of cholesterol with plasma. Pretreatment with MECG (250 mg/kg) significantly reduced the elevated levels of total cholesterol, triglycerides, LDL and increased of HDL level as compared to HCD group. The methanolic extract of *Chaetomorpha gracilis* also revealed significant hypercholesterolemia effect was due to the presence of the phenolic compounds such as acid gallic, rutin, queercetion. Reinforcing this idea, Padma et al. reported the attenuation of lipid metabolism and the increase of HDL in hyperlipidemia in rats by quercetin. Previous studies have been proved that cholesterol content of the erythrocyte plasma membrane is mainly sensitive to serum cholesterol. In fact, several research studies have observed in gene-targeted rodent models of dyslipidemia, the presence of erythrocyte abnormalities. Our Findings also observed that the erythrocyte membrane of hypercholesterolemia mice was relatively more fragile than that of the normal mice erythrocyte membrane. According to Vaya et al., hypercholesteremia alters the structure and fluidity of erythrocyte.

It is possible that increased lipid concentrations induced the alterations in lipid composition of the erythrocyte membrane and leads to increased erythrocyte fragility. The accretion of lipid content might occur due to the leakage of cellular content such as haemoglobin. Additionally, we showed that the deformability of RBC in hypercholesteremia animals was associated with the decrease of Ca$^{2+}$ and Mg$^{2+}$ ATPase activities it may be probable that in the elevated content of cholesterol and the oxidation of lipids directly in membranes plays chief role in RBC alteration.

Several studies demonstrated that the cholesterol-rich diet induces an accumulation of cholesterol in immune cells such as macrophages, leading to the induction of an inflammatory response. Our results demonstrated that...
HCD causes serious biochemical perturbations highlighted by the increase in serum albumin and globulin levels. The reduce in their concentrations may be related to the imbalance between the rate of synthesis and degradation of the protein. These findings could also be explained by the injury, inflammation, and/or erythrocytes destruction. The pretreatment with MECG in HCD group led in a protective effect against biochemical perturbation of unhealthy animals, which was corroborated by the significant increase of protein fraction. We suggest that active compounds present in MECG may exert anti-inflammatory activity, possibly by inhibition of inflammatory mediators and attenuation of cell migration and COX activity. In fact, Bensaad et al. provide that acid gallic possess an anti-inflammatory activity by reducing the COX-2 proteins expression. High blood cholesterol levels are one of the essential reasons underlying the production of ROS. Our data provide an insight into the interplay of hypercholesterolemia to oxidative stress. In fact, HCD has intense effects on the antioxidant status. As a result, we showed a high level of TBARS, suggesting that lipid peroxidation damage may be one of the mechanisms of HCD caused erythrocytes damage. Several investigations found that lipid peroxidation is supposed to be initiated by the generation of adverse free radicals which damage membrane function.

On the other hand, the inexorable production of ROS through HCD exposure was related with the important levels of AOPP which is known as a biomarker for protein oxidation. The corresponding statistical decrease in the TBARS and AOPP levels of the unhealthy animals and its normalizing with MECG supplementation suggest their role in free radicals scavenging action. Regarding the antioxidants enzymes, our results showed that a high cholesterol diet caused the impairment of the SOD and GPx activities in mice erythrocytes. These findings are in accordance Singh et al. Oral administration of Chaetomorpha gracilis methanolic extract during HCD exposure was capable to alleviate the injury of antioxidants enzymes defence. Concerning non enzymatic antioxidant defences, Vitamin C level is a susceptible marker of the redox status in cells. HCD leads to a decline of these antioxidants. However, pre-treatment with MECG enhanced the levels of the Vitamin C defence system status to a noteworthy level. The preventive effect of MECG led in a protective effect against erythrocytes oxidative stress, which was corroborated by the increase of antioxidants enzymes activities. Our study suggests that this MECG dose was sufficient to promote the antioxidants defence in treated group compared to hypercholesterolemia treated mice. In regard to antioxidant proprieties in vitro, it was shown that our extract posses high radicals scavenging activities (DPPH, ABTS and TAC). Thus, the chemical composition of the methanolic extract of Chaetomorpha gracilis was investigated by HPLC. This phytochemical study has led to the identification of six major flavonoids: gallic acid, rutin, quercetin, apigenin, kaempferol and naringenin. Flavonoids are well known as chain-breaking antioxidants which donate hydrogen to free radicals which results in the inhibition of lipid peroxidation. Hence, in our study, the defence offered by the alga against hypercholesterolemia could be attributed to the presence of the above-mentioned antioxidants.

5 Conclusion
The results obtained in this study noticeably indicate that Chaetomorpha gracilis methanolic extract is able to alleviate the hypercholesterolemia disorders in mice fed with high cholesterol diet for four weeks. It also diminished the oxidant status by decline lipid peroxidation and the protein oxidation and enhancing the antioxidants activities in the RBC. Our data indicate that MECG supplementation of diet during thirty days was great to decrease inflammatory markers, provides the haematological parameters and enhancing the morphology RBC impairment. Overall, our study confirmed that the protective effect of the extract was due to its strong antioxidant activity.

Acknowledgment
The present work was supported by the Tunisia Ministry of Higher Education an Scientific Research, Tunis, Tunisia through the Laboratory of Environmental Physiopathology, Valorisation of Bioactive Molecules and Mathematical Modeling, the Faculty of Sciences of Sfax, Tunisia.

Conflicts of Interest
The authors declare that there is no conflict of interest.

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