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# The ability of astaxanthin-rich *Haematococcus pluvialis* algal extract on preventing harmful effects of $H_2O_2$ on human fibroblast cells *in vitro*

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***ABSTRACT***
***This study aimed to determine the ability of astaxanthin-rich Haematococcus pluvialis algal extract (AE) on preventing adverse effects of $H_2O_2$ on human skin fibroblast cells. A Haematococcus pluvialis strain obtained from Vietnam was used as a source of astaxanthin. hF cells were cultured in DMEM/F12 medium supplemented with AE 0, 10, 25, 50 µg/ml for 7 days. WST-1 assay results showed that AE 10-50 µg/ml was not poisonous and didn't have any negative effects on cell proliferation. hF cells were pre-treated with AE (0, 10, 25, 50 µg/ml) for 24 hours prior to $H_2O_2$ treatment (200 µM, 120 minutes). The results showed that AE protected hF cells: reduction of G0/G1 phase cells in cell populations (16.08 – 21.52%), senescence-associated β-galactosidase – positive cells (28.9 – 40.8%), and maintenance of cell proliferation, nuclear area. AE 10 µg/ml is the optimal concentration in this study. In conclusion, AE has been shown to have the ability to protect fibroblasts from the adverse effects of $H_2O_2$.***

***Keywords: Astaxanthin, Haematococcus pluvialis, cell senescence, skin aging, hydrogen peroxide.***

## 1. INTRODUCTION

*Haematoccocus pluvialis* (Chlorophyceae, Volvocales, Haematococcaseae) is unicellular freshwater microalgae. It was considered to be the best natural commercially source of astaxanthin, a red xanthophyll carotenoid [1, 2]. The life cycle of H. pluvialis is composed of 4 distinguished stages: macrozooids, palmella, hematocysts and microzooids (Figure 1). Macrozooids are spherical or pear shape motile green cells with two equal flagella. Macrozooid cells predominate under favorable culture conditions. When exposed to unfavorable, the cells begin losing their flagella, expanding cell size, thickening cell wall, and synthesizing secondary carotenoids (mainly astaxanthin)[3-6].

Astaxanthin (3,3 -dihydroxy-β,β -carotene-4,4 –dione), consisting of the two β-ionone–type rings at both ends and one polyene chain, is one of the most effective antioxidants [7, 8]. Its potential antioxidant is more potent than vitamin C, β-carotene, canthaxanthin, zeaxanthin, α-tocopherol [1, 7, 9]. In the cosmetic field, astaxanthin was used to neutralize or quench reactive oxygen species (ROS) produced inside or outside the cells [10, 11]. ROS can be generated by cellular metabolic processes or environmental stress such as ultraviolet radiation, heat exposure...[12, 13] ROS can cause irreversible damage to DNA, the extracellular matrix (collagen, elastin, …), and up-regulate proteolytic enzymes (MMP-1, MMP-3, MMP-9, NEP). These modifications lead to cell death, senescence**,** and aging skin [14-17]. Astaxanthin was proved to prevent DNA damage, reduce the expression and activity of MMP-1 and NEP in UV – induced fibroblast *in vitro* [18, 19]. However, the protective ability of astaxanthin against ROS on fibroblast is limited. So, in this study, we aim to extract astaxanthin from a new Vietnamese *H. pluviaslis* strain (astaxanthin-rich algal extract, AE) and evaluate its effectiveness on cellular protection against $H_2O_2$.

## 2. MATERIALS AND METHODS

### 2.1.Algal extraction

The Vietnamese H. pluvialis algae with ASX concentration of 2.9 % dry mass was used in this experiment. The biomass of H. pluvialis was provided from our previous study. Astaxanthin was extracted according to Sarada (2006) with some modifications [20]. The mature H.pluvialis cyst cells were treated by HCl 2M for 2 minutes, rinsed 2 times by distilled water, freeze-dried, and stored at -20$^o$C. Then, the algal cells were ground**,** and extracted in acetone with a ratio of 1 mg biomass: 20 ml acetone for 2 hours. Astaxanthin concentration was evaluated by HPLC [21].

### 2.2. *In vitro* proliferative induction test

Human dermal fibroblast cells (hF) at passage 4 were used in this experiment. The cells were maintained in cell culture medium (CC medium) containing DMEM/F12, 10% fetal bovine serum (FBS), 1% antibiotics in 37$^o$C, 5% $CO_2$. The hF cells were seeded into a 96-wells plate at a density of $2x10^3$ cells/well. After 24 hours, the cells were cultured in CC medium supplemented with algal extracts 5, 25, or 50 μg/ml (n=9). The final DMSO concentration, which was used to dissolve AST, was 0.5%. CC medium or CC medium containing 0.5%

DMSO was used as blank or control. After 1, 4, or 7 days of culture, WST-1 assay was conducted to determine proliferation in each group [22]. OD values were recorded at wavelength 440 nm.

### 2.3. *In vitro* evaluation of the protective ability of AST against $H_2O_2$-induced oxidative stress

The protective ability of AE against $H_2O_2$-induced oxidative stress was tested on hF cells. hF cells were treated in turn by AE and by $H_2O_2$ for 2 hours. The experiment was divided into 5 groups: control, H2O2, AE 5, AE 25, and AE 50 as table 1. hF cells at passage 10, approximately 80-85% confluence were pre-treated with CC medium supplemented with AE (0, 5, 25, or 50 μg/ml) for 1 day. One day later, the cells were treated by 200 μM $H_2O_2$ for 2 hours [23]. Then, the cells were seeded on 96-well plates ($10x10^3$ viable cells/well) and cultured in CC medium to check the cell's proliferative ability, cell phase, nuclear area and senescence-associated b-galactosidase expression (SA-gal). The proliferative ability was tested by WST-1 assay on day 1, 4, or 7 after seeding. On day 1, the cell phase ratio, the nuclear area was tested by DAPI staining and calculated by Cytell microscope. The SA-gal expression was checked by a senescence staining kit (Sigma) on day 2.

### 2.4. Statistical analysis

Statistical analysis was performed using one-way ANOVA where $p<0.05$ was considered statistically significant.

## 3. RESULTS

### 3.1. Algal extraction

The red mature cyst cells (aplanosphores) have round shapes with average diameter of 64 ± 15.1 μm. After extraction, astaxanthin was dissolved into the solvent, moved outside and biomass, including ruptured cell walls, turned to white. Based on the HPLC method, astaxanthin recovery was 93.5 ± 1.8% and accounted for 9.8 ± 2.6% of AE powder's weight (Figure 2).

### 3.2. Proliferative induction test *in vitro*

hF cells were cultivated in medium supplemented with a range of algal extraction (0, 5, 25 or 50 μg/ml) (n=9) for 7 days. OD values of the WST-1 assay were shown in figure 3. The OD values of all groups on the same day were not statistically different (day 1, 4, or 7) ($p>0.05$). There were increases in OD values between experiment days of all groups from day 1 to day 7. Moreover, the Giemsa staining showed that there was no change in cell morphology between these groups (Figure 5). It was concluded that algal extract was not poisonous to cells and didn't affect cell proliferation.

### 3.3. *In vitro* evaluation of protection activity of AE

$H_2O_2$ is a powerful ROS which make cell death or cell senescence. In this study, hF cells were pre-treated with AE before contacting to H2O2. The results were shown in table 2 and figure 5-8.

The results were shown in table 2 and figure 5-8. In the $H_2O_2$ group, after one day, the ratio of G0/G1 phase increased significantly from 5.72 ± 0.63% (control group) to 85.93 ± 4.03% of cells ($p<0.05$). From day 1 to day 7, OD values of WST-1 assay stayed constant ($p>0.05$). Moreover, there was the enlargement of cell nuclear size (246.2 ± 5.49$\mu m^2$) and cell size

(data not shown) as compared to control. The percentage of SA-gal-expressed cells (a biomarker of cell senescence) in control group, 51.5 ± 6.09%, is the highest in this experiment ($p < 0.05$). It was concluded that treatment of 200 μM H2O2 for 2 hours triggered These results showed that $H_2O_2$ inhibited cell proliferation, increased of nuclear area, cell size, and triggered SA b-Gal expression. They are signs of premature cellular senescence.
In experiment groups, the cells were sequentially pre-treated with AE (5, 25, 50 μg/ml) for 24 hours and exposed to $H_2O_2$. The ratio of G0/G1 groups in these groups were lower than $H_2O_2$ group and equal to control ($p<0.05$) (59.03 - 66.07%). The WST-1 assay showed that OD values in these group increased steadily from day 1 to day 7 ($p<0.05$) although they were smaller than control ($p<0.05$). DAPI staining showed nuclear area in all AE groups was smaller than $H_2O_2$ and lager than control ($p<0.05$). The ratio of SA-gal-positive cells was lower than the control group ($p<0.05$). Based on these results, it was concluded that AE suppressed the negative effects of $H_2O_2$ on hF cells in a dose-dependent manner. AE 25 μg/ml was the best concentration on protecting hF cells from oxidative stress.

## 4. DISCUSSIONS AND CONCLUSIONS

ROS is one of the main reasons for skin aging. It can oxidize and alter DNA, protein and active skin aging-related signalling pathways: MAPK pathway [24, 25]. In many studies, $H_2O_2$, a powerful oxidizing agent, has been the most widespread inducer of cellular senescence *in vitro*, which shares hallmarks of physiological senescence: cell cycle arrest, senescence-associated b-galactosidase activity, increase of cell size [23, 24, 26, 27].
Astaxanthin is a powerful antioxidant that can neutralize or quench free radicals such as singlet oxygen, hydroxyl peroxide… Due to its unique structure: polar - nonpolar – polar, astaxanthin can permeate and protect cell membranes from free radicals [8, 28]. Many kinds of research prove the astaxanthin's effects on protecting fibroblast against UV irradiation *in vitro* [18, 19, 29]; however, little research checks on its ability to prevent oxidative stress into fibroblast [23, 30]. *Haematococcus pluvialis* is one of the best natural sources of astaxanthin that contains the maximum astaxanthin concentration of 5% dry weight [4, 10]. In Vietnam, Dang Thi Diem Hong first isolated a *H. pluvialis* strain in the North of Vietnam. This strain can reach the concentration of 0.95 $x10^6$ cells/ml in a modified RM medium after 15 days of inoculation [31]. Our previous study showed that astaxanthin occupied 2.9% of dry weight and existed in 2 different forms: free (4.4%) and esterified, in which ester form is the predominant part (data were not shown). Moreover, astaxanthin is a potent antioxidant and its TEAC value was 0.126 mmol Trolox/g extract, closed to Zuluaga's in ABTS assay [27].
In this study, we continued to investigate its protection ability on human skin fibroblast. AE was extracted according to our previous study. Astaxanthin accounted for 9.8% of algal extract mass. When exposed to CC medium containing AE in ranges of 10-50 μg/ml, hF cells still proliferated from day 1 to day 7 ($p<0.05$) and remained their normal elongate shape. After treated by $H_2O_2$, hF cells expressed some features of replicative senescence: most cells were arrested in G0/G1 phase (85.93 ± 4.03%), without growth (OD values of WST-1 of day 1, 4, 7 were similar, $p>0.05$) and increases in the nuclear area, accelerated expression of SA-gal **[26, 32]**. So, it can be concluded that $H_2O_2$ caused oxidative stress on hF cells with some signs which are similar to cellular senescence's. In AE groups, cells were pre-treated with AE

concentrations of 10-50 µg/ml and exposed to $H_2O_2$ in turn. The results showed that AE 10-50 µg/ml suppressed $H_2O_2$-induced oxidative stress: maintained cell growth, reduced of SA-gal, retained cellular morphology. So, we conclude that AE 10-50 µg/ml could have preventive effects against $H_2O_2$-induced oxidative stress on fibroblasts *in vitro* and the effectiveness of these groups were equivalent.

## 5. CONCLUSION

AE, extracted from the Vietnamese *H. pluvialis*, inhibited harmful impacts from $H_2O_2$ *in vitro*.

## 6. ACKNOWLEDGEMENTS

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## 7. CONFLICT OF INTEREST

Authors declared that they do not have any conflicts of interest.

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**Table 1:** Groups in protective ability of AE against $H_2O_2$ treatment

| | **Control** | **$H_2O_2$** | **AE 10** | **AE 25** | **AE 50** |
|---|---|---|---|---|---|
| CC medium (0.5% DMSO) | Yes | Yes | Yes | Yes | Yes |
| AE concentration | 0 µg/ml | 0 µg/ml | 10 µg/ml | 25 µg/ml | 50 µg/ml |
| $H_2O_2$ treatment | None | Yes | Yes | µg/ml | µg/ml |

**Table 2**. Results of protective test of AE. [a, b, c, d]: statistically significant in the same column.

| **Groups** | **The ratio of G0/G1 phase (%)** | **Cell nuclear area (µm$^2$)** | **The ratio of SA-gal-expressed cell (%)** |
|---|---|---|---|
| Con. | 60.59 ± 5.69[a] | 205.8 ± 7.4[a] | 2.54 ± 1.27[a] |
| $H_2O_2$ | 85.93 ± 4.03[b] | 246.2 ± 5.5[b] | 51.58 ± 6.09[b] |
| AE 10 | 66.07 ± 7.18[a] | 232.2 ± 5.0[c] | 37.62 ± 4.62[c] |
| AE 25 | 64.33 ± 3.92[a] | 224.8 ± 3.3[c] | 27.22 ± 3.54[cd] |
| AE 50 | 59.03 ± 3.92[a] | 229 ± 3.2[c] | 31.78 ± 4.50[d] |

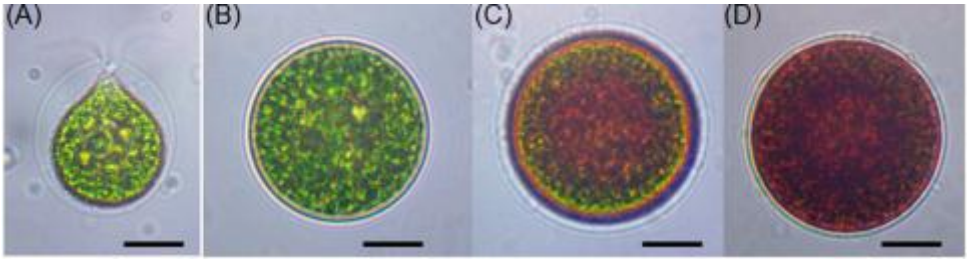
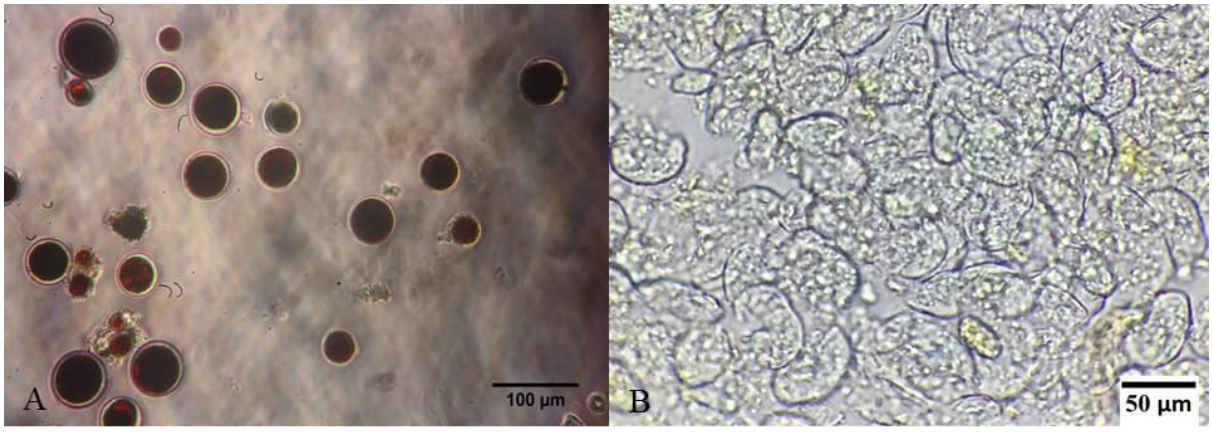



Figure 1. Stages of Haematococcus pluvialis [4]. A: Macrozooid, B: Pamella, C: hematocyst.



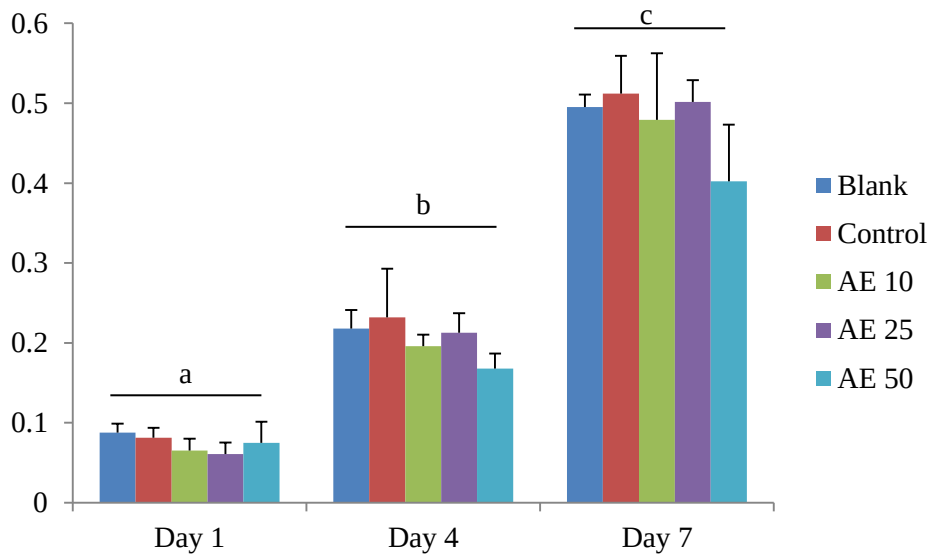



Figure 3. OD values of WST-1 assay in proliferation test. [a,b,c]: statistically different.

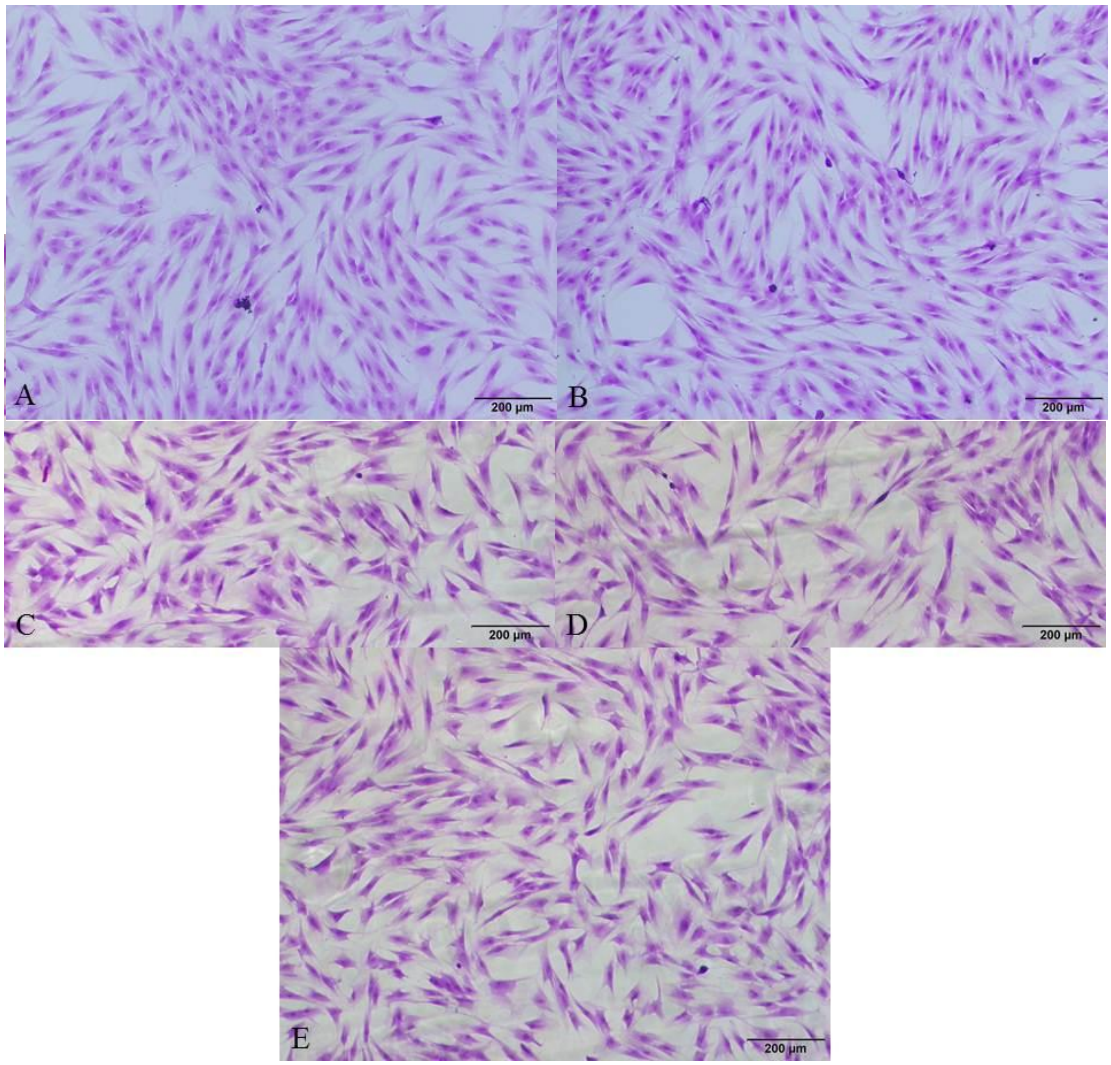



**Figure 4. Giemsa staining results of hF cells at day 7 in proliferative test.** A, B, C, D, E: con., $H_2O_2$, AE 5, AE 25, AE 50 (x100).

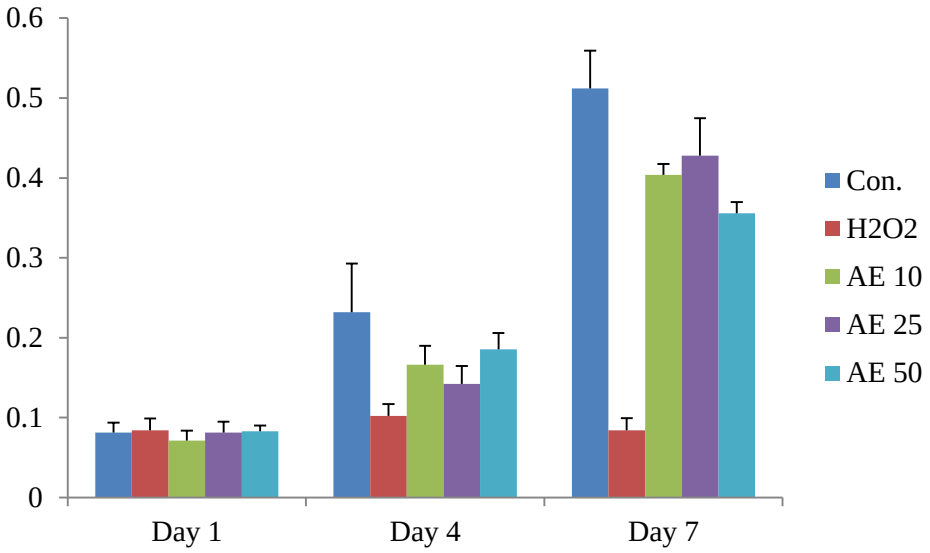
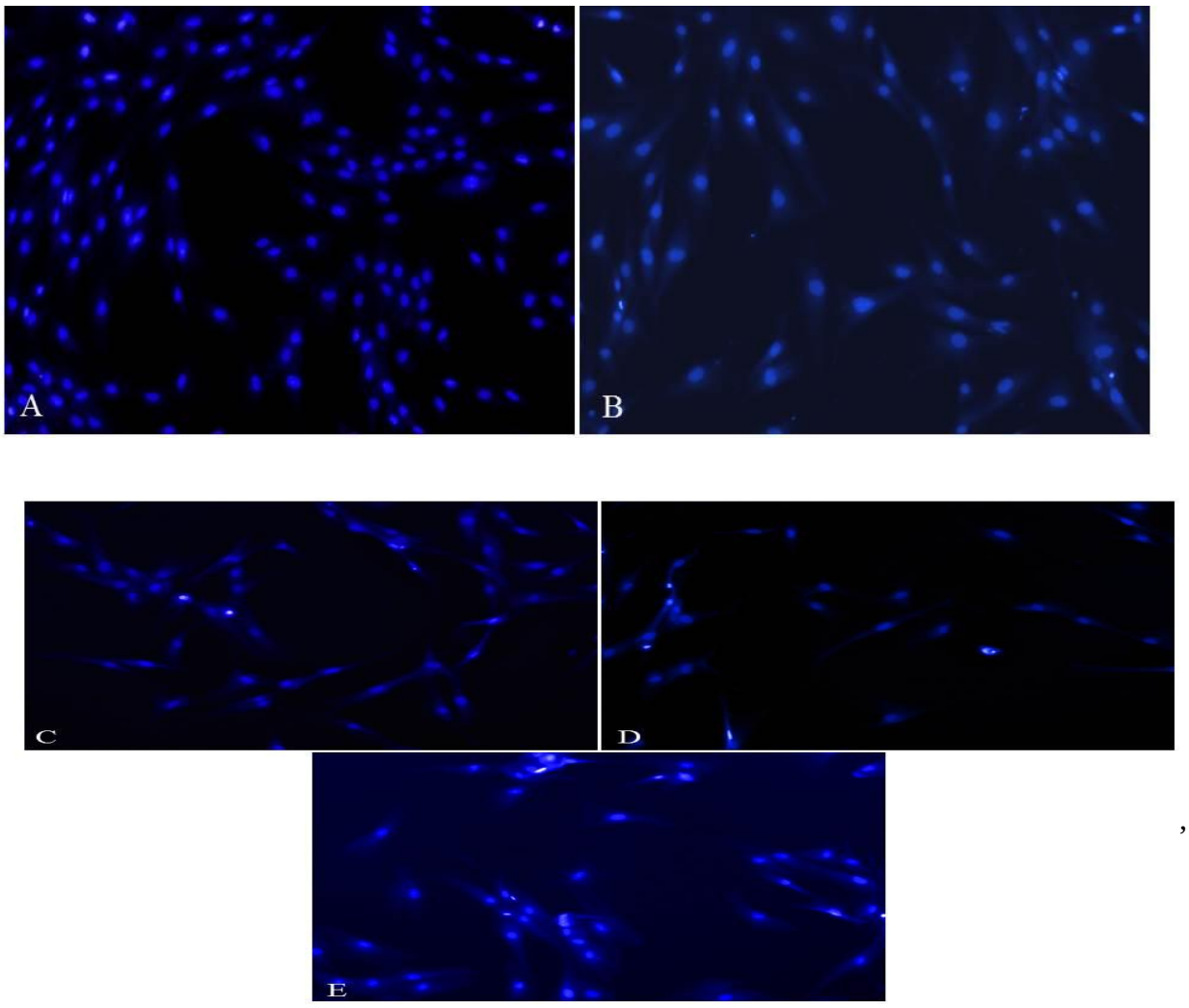



Figure 5. OD values of all groups in protective ability of algal extract



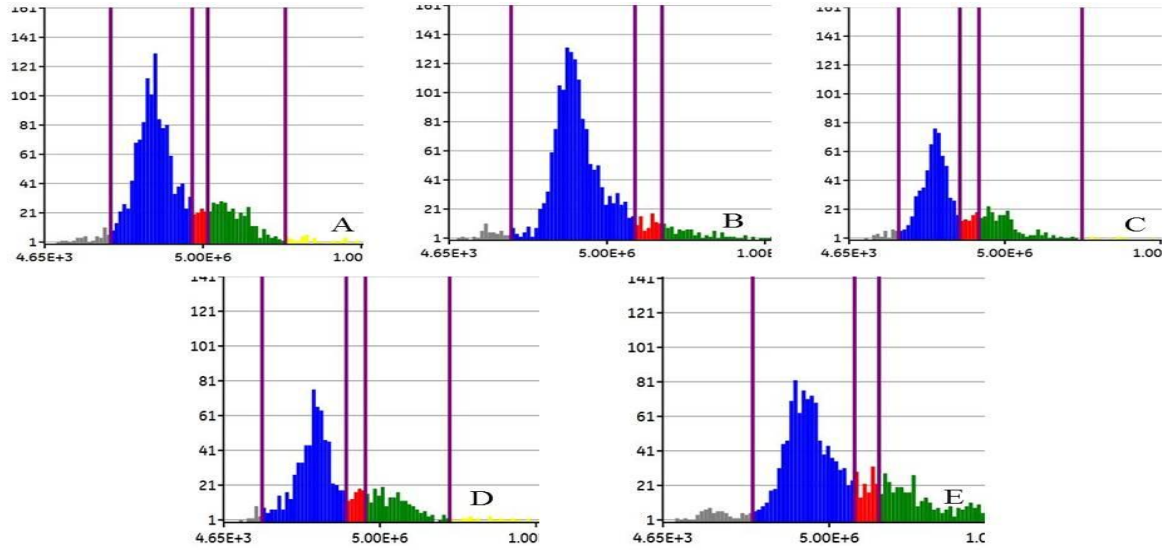



Figure 7. Cell phase ratio determined by Cytell. A, B, C, D, E: con., $H_2O_2$, AE 5, AE 25, AE 50 (x100).

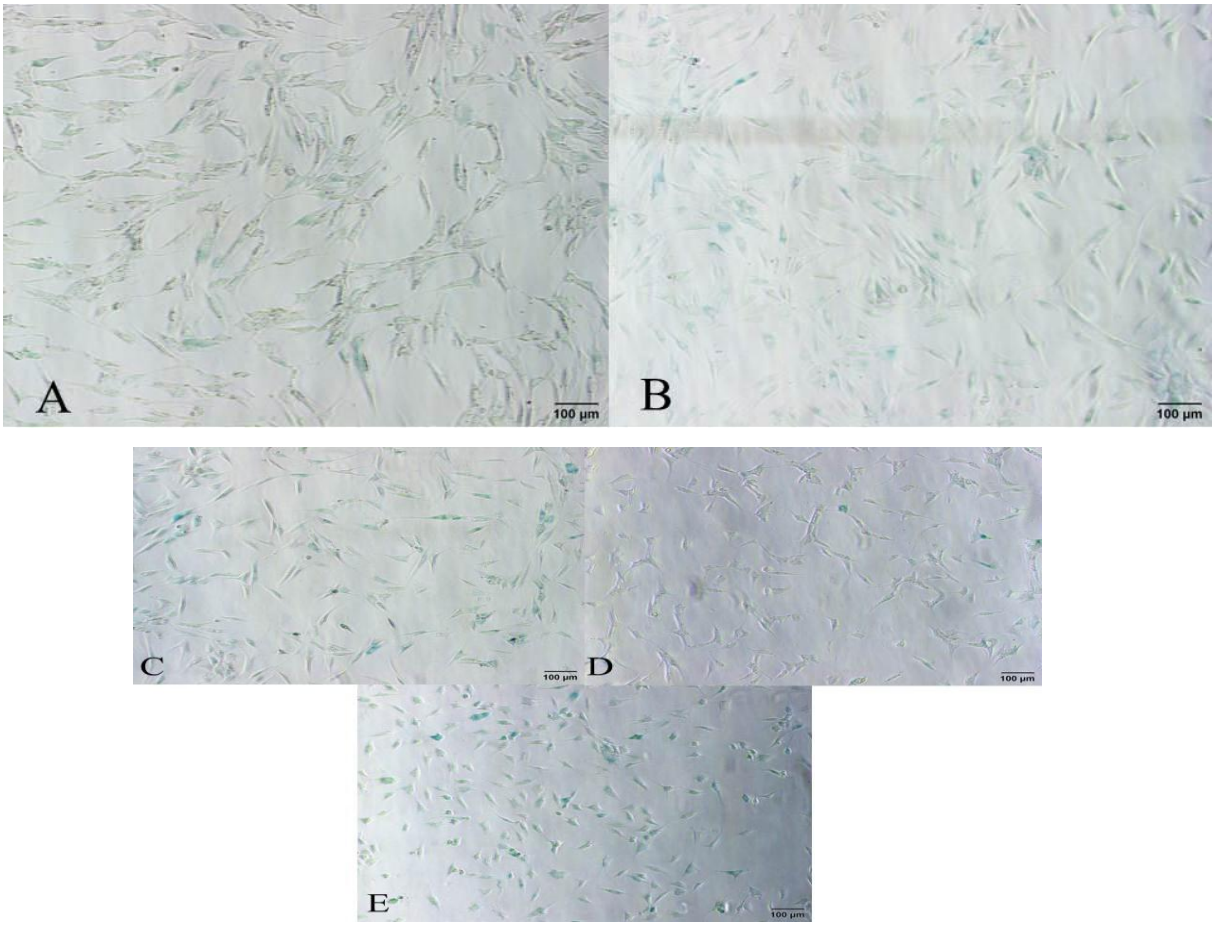



Figure 8. SA-b Gal expression of the cells. A, B, C, D, E: con., $H_2O_2$, AE 5, AE 25, AE 50 (x100).