EUROPEAN JOURNAL OF BIOMEDICAL AND PHARMACEUTICAL SCIENCES
http://www.ejbps.com

ISSN 2349-8870
Volume: 7
Issue: 10
01-06
Year: 2020

DETERMINING ABILITY OF ASTAXANTHIN EXTRACTED FROM HAEMATOCOCCUS PLUVIALIS ON STRESS-INDUCED HUMAN FIBROBLAST

Quan To Minh*[1], Nhan Tran Dinh[1], Dieu Tran Quang[1], Huy Nguyen Dinh Huy[1], Truc Vo Thi Thanh[2], Thao Huynh Thu[2] and Bien Lai Dinh[2]

[1]University of Science, Vietnam National University of Ho Chi Minh City.
[2]Ho Chi Minh City of Food Industry.

***Corresponding Author: Quan To Minh**
University of Science, Vietnam National University of Ho Chi Minh City.

Article Received on 21/07/2020 **Article Revised on 11/08/2020** **Article Accepted on 31/08/2020**

ABSTRACT
Astaxanthin (ASX) is one of the powerful antioxidants, which has been highly effective against oxidative species. However, the use of this natural pigment in topical skincare products in the protection of skin from oxidative stress has yet to understand thoroughly. This study concerns the assessment of ASX on protecting human dermal fibroblast (hF) against H_2O_2. ASX was extracted from *Haematococcus pluvialis* using Sacha Inchi oil (eASX) and concentration determined by HPLC was 240.6 ± 21.11 µg/ml. hF was pre-treated with eASX (0.1 and 0.5 µg/ml) or commercial astaxanthin (comASX) for 24 hours before being senescence induced by incubating with 175 µM H_2O_2 for 90 minutes. In comparison to the control group, ASX groups showed the smaller cell nucleus area and lower senescence β-galactosidase (SA b-gal) expression. The cell nucleus area of hF in control group, eASX groups with the concentration of 0.1 and 0.5 µg/ml and comASX group were 305.5 ± 29.5µm², 281.8 ± 31.2 µm, 276.5 ± 38.1 µm² and 270.6 ± 26.9 µm², respectively. The percentage of SA b-gal-expressed hF in these groups was 58.9 ± 3.5%, 31.6 ± 4.6%, 29.4 ± 2.1% and 28.2 ± 3.9%, respectively. In conclusion, eASX has been shown to have the protection effective on hF against the senescence effect of H_2O_2.

KEYWORDS: antioxidants, Astaxanthin, *Haematococcus pluvialis*, hydrogen peroxide.

INTRODUCTION
Astaxanthin (ASX), recently, has been acknowledged as one of the most effective antioxidants. Utilizing the two polar groups at both ends and the non-polar center in the structure, ASX easily attaches to the cell or organelle membrane. Hence, ASX is able to protect cells from the react with both exogenous and endogenous oxidative radicals. Currently, ASX was consumed orally as a form of nutrient, however, its application on cosmetic field has been neglected.[1,2] For skin rejuvenation and protection against harmful environmental factors, ASX is commonly absorbed through oral route. Skin is largest organ, which main function is preventing the damage from environemtal agents, such as dust and bacteria. One of the most skin-damaging element is ultraviolet light (UV) emitted from the Sun. Depends on the wavelength, UV can be subdivied into three subtypes: UVA (320-400 nm), UVB (280-320 nm), and UVC (200-280 nm).[3] The skin damaged by UV produces ROS within its cell, thus destroying many dermal major molecules, such as collagen and elastin. These modification leads to the senescence or apoptosis inducing in the main skin cells like fibroblast and epithelium.[3-5] Hence, UV ray contacted skin becomes dark and dry, possesses cracks. ASX and other antioxidants have been applied to protect skin from UV light.[6,7] In this research, ASX was used to protect fibroblast against the effect of H_2O_2, which is a strong oxygen species. Aftermath, we aimed to assess the protective ability of ASX product in topical skincare product.

MATERIALS AND METHODS
Extraction of astaxanthin
Astaxanthin was extracted according to Sarada (2006) with some modifications.[8] Astaxanthin-contained *Haematococcus pluvialis* was freeze-drying and stored in -20°C before use. Freeze-dried algae were unfreezed, treated by HCl 2M for 2 min, ground into powder. The ground agal biomass was mixed with the Sacha Inchi oil, the mixture was kept heating and stirred for 60 min. The suspension was centrifuged by 3,000 rpm for 10 min to obtain supernatant. These steps were repeated until algal biomass turned white. ASX concentration was evaluated by HPLC.[9]

In vitro cytotoxicity assay
Fibroblast cell was cultured from human skin (hF). hFs were seeded on 96-well plate at the density of 5×10^3 cells/well. After 24 hours, the medium was replaced by new medium contained ASX extracted from the algae

(eASX) (0, 0.1, 0.5 µg/ml) or commercial astaxanthin (comASX) (5 wells/group). After 1 day, cytotoxicity was evaluated by WST-1 assay.[10]

***In vitro* evaluation of protective ability of ASX against H_2O_2-induced oxidative stress**

H_2O_2-induced oxidative stress in fibroblast was performed according to Yokoyama (2013) with some modifications.[11] hFs were seeded on 96-wells plate at the density of 10×10^3 cells/well. After 1 day, cells were pretreated with eASX (0, 0.1, 0.5 µg/ml) or comASX (0.5 µg/ml) for 1 day. Next day, these cells were challenged by 175 µM H_2O_2 dissolved in DMEM/F12 5% FBS for 90 minutes. Then, the medium was changed by culture medium (DMEM/F12 10% FBS). After 4 days, cell viability, morphology, nucleus area and senescence-associated b-galactosidase (SA b-gal) expression were determined by cell counting, Giemsa stain and senescence staining kit.

RESULTS

Astaxanthin extraction in plant solvent result

After suspending *H. pluvialis* with Sacha Inchi oil, the solution became dark red, the biomass lost its color and turned transparent (Figure 1). Based on HPLC method, the concentration of ASX (eASX) in Sacha Inchi was 240.6 ± 21.1 µg/ml (n=5) and ratio of free ASX was 4.2%.

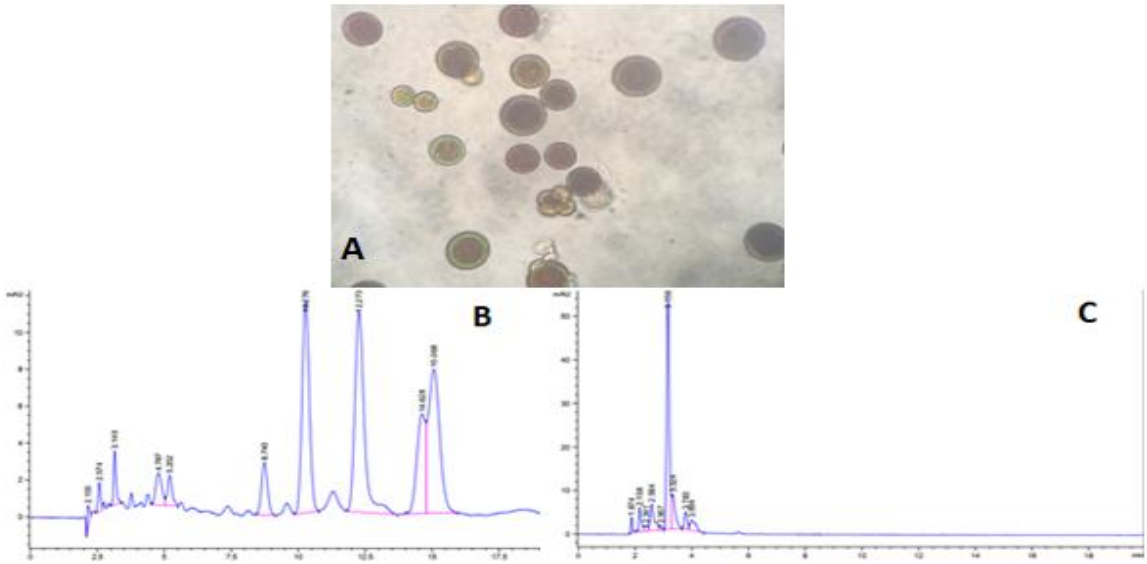

Fig 1: Astaxanthin from *H. pluvialis*. A: ASX-induced algae. B: HPLC of astaxanthin solvent before saponification. C: after saponification.

Result of cytotoxicity test *in vitro*

hF cells were seeded on the wells and cultured in the medium contained eASX (0.1, 0.5 µg/ml) or comASX (0.5 µg/ml) or control medium (0 µg/ml) (n=3). After 1 day, cell viability was determined by WST-1 assay. After 1 day, hFs maintained their morphology with only a few rounded and detached cells (Figure 2). The result of WST-1 assay showed that OD value in eASX and com. ASX was as high as control (Table 1).

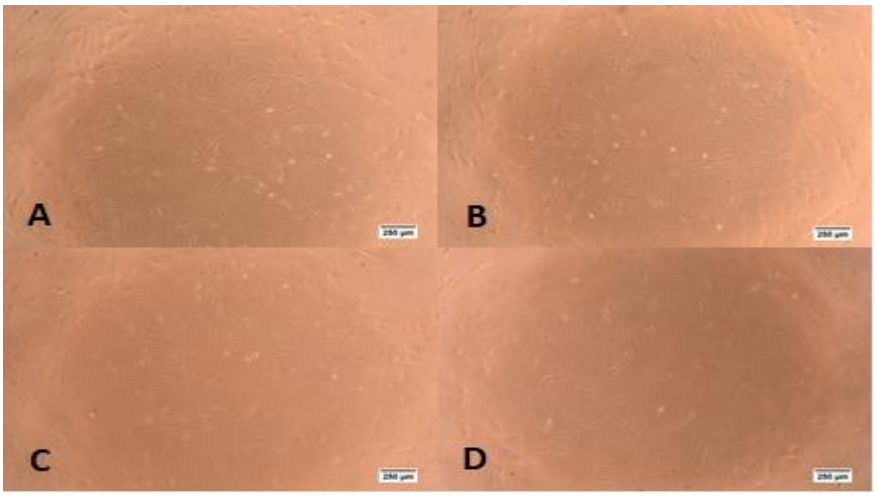

Fig 2: Fibroblast cells after 1 day treatment with ASX.

The obtained results displayed that eASX with concentration from 0.1 - 0.5 µg/ml was non-toxic to fibroblasts. Therefore, ASX concentration of 0.1 µg/ml and 0.5 µg/ml was used in later experiments.

Table 1: OD value from WST-1 assay.

ASX concentration	0 µg/ml (control)	0.1 µg/ml (eASX)	0.5 µg/ml (eASX)	0.5 µg/ml (com. ASX)
OD value	0.35 ± 0.03	0.37 ± 0.02	0.38 ± 0.05	0.33 ± 0.03

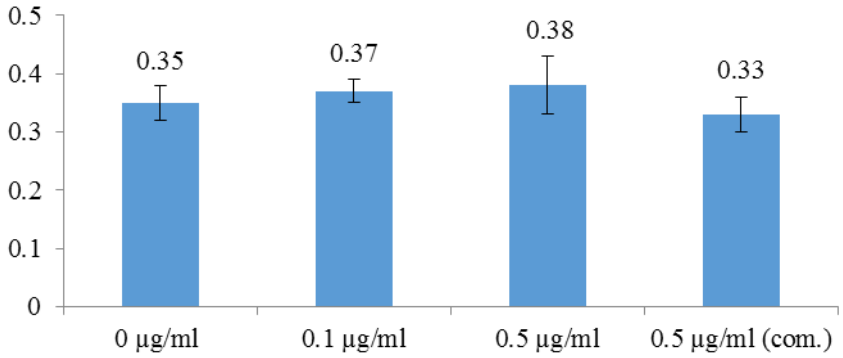

Fig 3: The column graph express OD value in cytotoxicity test.

Result of *in vitro* evaluation of protection activity of ASX

hF was pre-treated with ASX 0.1 – 0.5 µg/ml 1 day before being challenged with H_2O_2 175 µM for 90 min. In this experiment, cells were cultured in 5 groups: normal group (culture medium), H_2O_2 group (treated with H_2O_2), extracted ASX groups (pretreated with eASX 0.1 or 0.5 µg/ml and treated by H_2O_2) and commercial group (pretreated with comASX 0.5 µg/ml and treated by H_2O_2).

Viability test showed that H_2O_2 induced cell death. After treatment, a lot of cells detached and suspened in medium. For the next 4 days, up to 50% of cells lost. Moreover, flat cells with large nucleus appeared and predominated in flasks (Figure 4, 5 and Table 3). In ASX groups (eASX and com.), cell viability was higher than H_2O_2 group, there were fewer large cells (about 28-32%). This result implied ASX could increase cell viability.

Survial cells in these groups were stained with β-galactosidase staining kit, which was used to detect β-galactosidase in senescence cell. The results showed that cells in H_2O_2 group expressed more SA-b-gal than other groups. In ASX groups, the ratio of SA-b-gal expressed cells decreased (statistically different).

Based on these results, we concluded that ASX protected fibroblast cells from oxidative stress of H_2O_2: cell viability was higher, less change in morphology and low SA-b-gal expression.

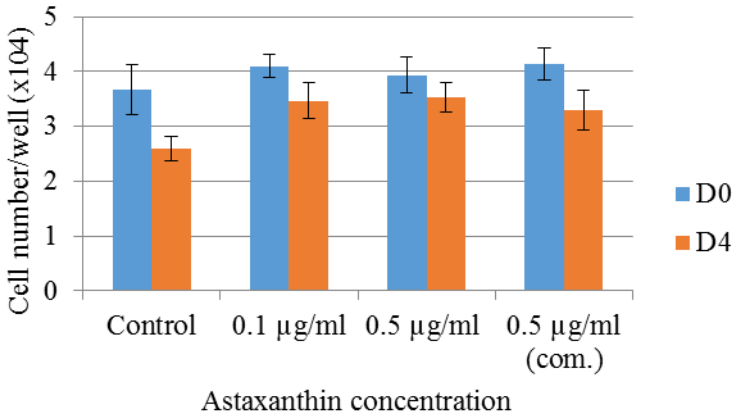

Fig 4: Cell number in wells 4 days after treatment.

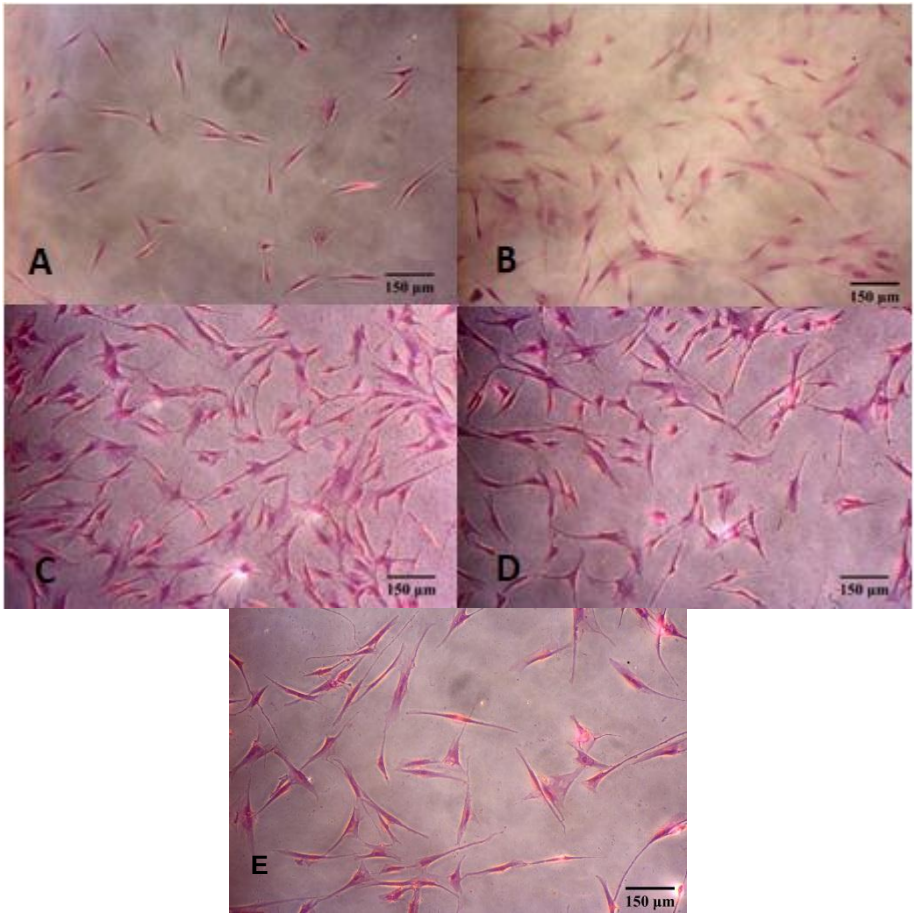

Fig 5: Giemsa stain of fibroblast cells 4 days after treatment. A: normal cells, B: H_2O_2 group, C: eASX 0.1 µg/ml, D: eASX 0.5 µg/ml, E: comASX 0.5 µg/ml.

Table 3: The result of nucleus area and ratio of SA b-gal-positive cells.

Astaxanthin concentration	Cell nucleus median (µm²)	β-galactosidase expression
Normal	255.0 ± 16.8	3.1 ± 0.5%
H_2O_2 treatment	305.5 ± 29.5	58.9 ± 3.5%
eASX 0.1 µg/ml	281.8 ± 31.2	31.6 ± 4.6%
eASX 0.5 µg/ml	276.5 ± 38.1	29.4 ± 2.1%
comASX 0.5 µg/ml	270.6 ± 26.9	28.2 ± 3.9%

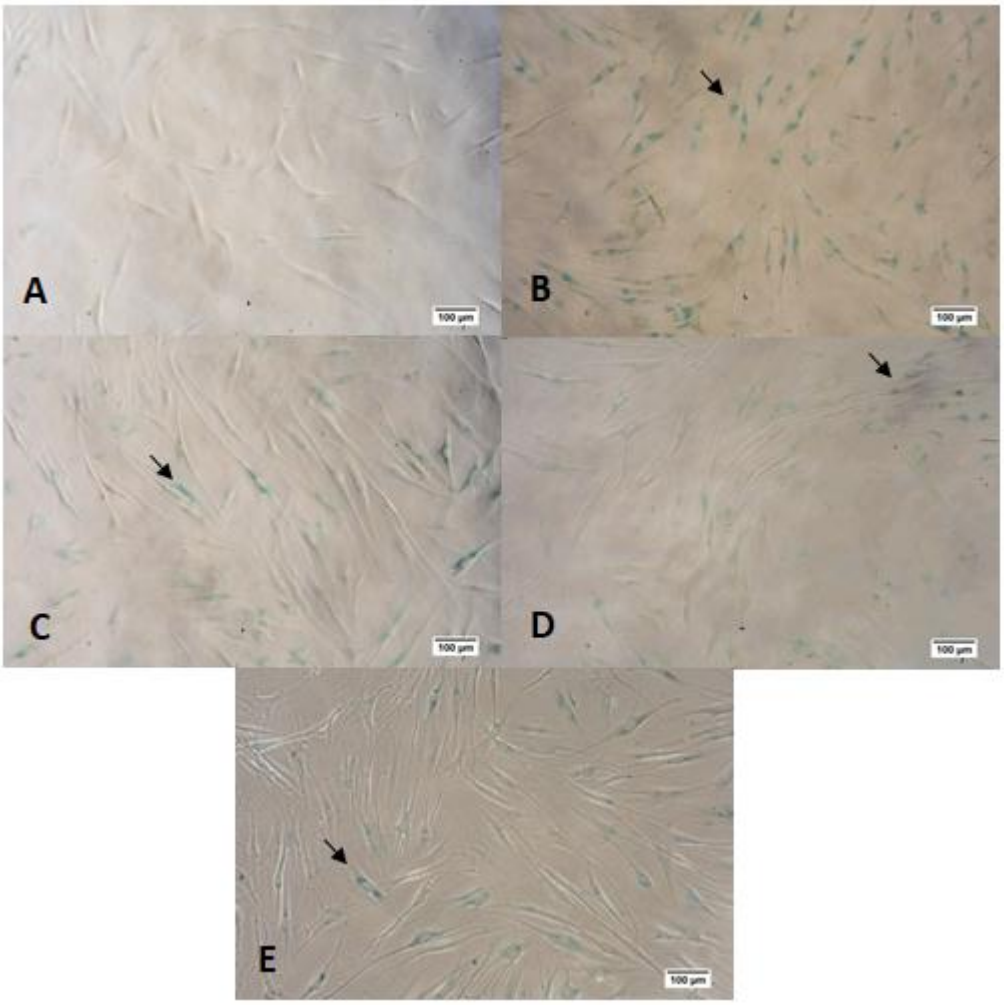

Fig 6: Results of SA-b-gal stain. A: normal cells, B: H_2O_2 group, C: eASX 0.1 µg/ml, D: eASX 0.5 µg/ml, E: comASX 0.5 µg/ml.

DISCUSSIONS AND CONCLUSIONS

ASX is one of the most effective antioxidants recently. The presence of two polar groups at both ends and the non-polar center, ASX easily attaches to the cell or organelle membrane such as mitochondrial, nucleus membrane.[12] Thus, ASX protects cells from the impact of oxidative radicals created inside or outside of the cell. Currently, ASX was employed as many forms of nutrient, research focus on cosmetic application has been neglected.[1,2] In this research, ASX was extracted in the plant oil solvent as it would be applied to be a component of skin-applied cosmetic. Sacha Inchi oil contains various factors, such as vitamin E (8.99 ± 0.16 mg/100g), a-linolenic (50,8%) and linoleic (33,4%) acids, which make it suitable for skin moisturing.[13,14] Since the aim of the product is skin-applied, biological aspects, including effects of ASX on fibroblast and the protection ability of ASX against harmful elements like oxidative radicals (*in vitro*), need to be detemined. In *Haematococcus pluvialis*, ASX exists on free (5%), monoester (70%) and diester (25%) form.[15] Today, the price of ASX is still considered high (1000 – 2000 USD/kg). One of the major causes is the cost of de-esterification process, which de-esterifies ester form and produces free astaxanthin. In order to reduce the production cost, in this research, astanxanthin was used in crude form and its acitivity was compared to commercial free ASX (Sigma). Results of extraction and de-esterification showed solvent contained ASX in both form: free and ester and free ASX accounted for 4.2% of total ASX. This solution was used for later experiments.

Fibroblast is the main cell of dermis of skin. In the dermis layer, fibroblasts proliferate and synthesize proteins like collagen and elastin to maintain the function and mechanic property of skin. When a wound appears, the surrounding fibroblasts migrate to the section, proliferate and construct a new skin region.[16] The sun UV-light is one of the most harmful environmental factors. When being exposed to UV, skin produces ROS, which destroys protein like collagen and elastin, and fibroblast.[17] ROS reduces cell viability and causes premature of cell senescences. Thus, the quantity and quality of proteins in the skin reduce significantly, which leads to the signs of senescence on skin, such as thinner layer, lacking elasticity and wrinkles.[18] In cytotoxicity test, fibroblast was incubated in culture medium containing ASX (eASX 0.1, 0.5 µg/ml or comASX 0.5

µg/ml), in comparison to the cells in control medium. If ASX was toxic to skin, the amount of cell would reduce gradually in time and OD value in WST-1 assay would decrease. The obtained outcome confirmed that both eASX and comASX were non-toxic to fibroblast. In this research, the stress inducing factor used *in vitro* on fibroblasts was H_2O_2. H_2O_2 is one of oxidizing agents created by the effect of UV on skin. Moreove, H_2O_2 is a strong oxidative stress frequently used in many anti-aging researches on skin. H_2O_2 affects the cells, which leads to cell death, or the appearance of many senescence signs, such as expansion of cell morphology and nucleus area, expression of SA b-gal (gold standard marker for cell senescence).[19,20] The results showed that H_2O_2 triggered cell death (50% cells died at day four after treatment), flattened survival cells, increased nucleus area 20% and rised ratio of SA b-gal-positive cells to nearly 60%. The cell protection characteristic of ASX was tested on fibroblast against the oxidative stress of H_2O_2. The cells were cultured with the media contained ASX for 24 hours before treating with H_2O_2. The results showed that ASX-pretreated cells resisted harmful effects of H_2O_2. Compared to H_2O_2 group, ASX-pretreated cells had higher viability, smaller nucleus area and the lower number of SA b-gal-positive cells. There was no statistical difference between ASX groups in these aspects (eASX 0.1, 0.5 and comASX 0.5 µg/ml). To be concluded, eASX (4.2% and 95.8% in free and ester form) had protective ability as high as comASX (over 97% in free form) and eASX 0.1-0.5 µg/ml was suitable to be utilized in topical skincare products.

CONCLUSION

The experiment was conducted with the purpose of evaluating the effect of ASX in skin-applying cosmetic product. The results showed that ASX 0.1-0.5 µg/ml was non-toxic, protected cell from H_2O_2 *in vitro*.

ACKNOWLEDGEMENTS

This research is funded by Vietnam National University HoChiMinh City (VNU-HCM) under grant number C2018-18-20.

REFERENCES

1. Davinelli, S., M. Nielsen, and G. Scapagnini, *Astaxanthin in Skin Health, Repair, and Disease: A Comprehensive Review.* Nutrients, 2018; 10(4).
2. Higuera-Ciapara, I., L. Félix-Valenzuela, and F.M. Goycoolea, *Astaxanthin: A Review of its Chemistry and Applications.* Critical Reviews in Food Science and Nutrition, 2006; 46(2): 185-196.
3. Kim, M. and H.J. Park, *Molecular Mechanisms of Skin Aging and Rejuvenation*, in *Molecular Mechanisms of the Aging Process and Rejuvenation*, 2016.
4. Bosset, S., et al., *Photoageing shows histological features of chronic skin inflammation without clinical and molecular abnormalities.* Br J Dermatol, 2003; 149(4): 826-35.
5. Rabe, J.H., et al., *Photoaging: mechanisms and repair.* J Am Acad Dermatol, 2006; 55(1): 1-19.
6. Komatsu, T., et al., *Preventive effect of dietary astaxanthin on UVA-induced skin photoaging in hairless mice.* PLoS One, 2017; 12(2): e0171178.
7. Meephansan, J., et al., *Effect of astaxanthin on cutaneous wound healing.* Clin Cosmet Investig Dermatol, 2017; 10: 259-265.
8. Sarada, R., *An Efficient Method for Extraction of Astaxanthin from Green Alga Haematococcus pluvialis.* Journal of Agricultural and Food Chemistry, 2006; 54: 7585-7588.
9. Su, F., et al., *Hydrolytic efficiency and isomerization during de-esterification of natural astaxanthin esters by saponification and enzymolysis.* Electronic Journal of Biotechnology, 2018; 34: 37-42.
10. Chintong, S., et al., *In Vitro Antioxidant, Antityrosinase, and Cytotoxic Activities of Astaxanthin from Shrimp Waste.* Antioxidants (Basel, Switzerland), 2019; 8(5): 128.
11. Yokozawa, T., A. Satoh, and Y.J. Kim, *Modulation of oxidative stress by proanthocyanidin in H2O2-exposed human diploid fibroblast cells.* Biosci Biotechnol Biochem, 2013. 77(10): 2056-60.
12. Bosch, R., et al., *Mechanisms of Photoaging and Cutaneous Photocarcinogenesis, and Photoprotective Strategies with Phytochemicals.* Antioxidants (Basel, Switzerland), 2015; 4(2): 248-268.
13. Gutiérrez, L.F., L.M. Rosada, and Á. Jiménez, *Chemical composition of Sacha Inchi (Plukenetia volubilis L.) seeds and characteristics of their lipid fraction.* Grasas y Aceites, 2011; 62: 1. DO - 10.3989/gya044510, 2011.
14. Souza, A., et al., *Sacha inchi as potential source of essential fatty acids and tocopherols: Multivariate study of nut and shell.* Acta Scientiarum - Technology, 2013; 35: 757-763.
15. Galarza, J.I., et al., *Deesterification of astaxanthin and intermediate esters from Haematococcus pluvialis subjected to stress.* Biotechnol Rep (Amst), 2019; 23: e00351.
16. desJardins-Park, H.E., D.S. Foster, and M.T. Longaker, *Fibroblasts and wound healing: an update.* Regenerative Medicine, 2018; 13(5): 491-495.
17. Kulka, M., *Mechanisms and Treatment of Photoaging and Photodamage.* 2013.
18. Ichihashi, M. and H. Ando, *Photoaging of the skin.* ANTI-AGING MEDICINE, 2009; 6(6): 46-59.
19. Kiyoshima, T., et al., *Oxidative stress caused by a low concentration of hydrogen peroxide induces senescence-like changes in mouse gingival fibroblasts.* Int J Mol Med, 2012; 30(5): 1007-12.
20. Gille, J.J.P. and H. Joenje, *Cell culture models for oxidative stress: superoxide and hydrogen peroxide versus normobaric hyperoxia.* Mutation Research/DNAging, 1992; 275(3): 405-414.