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DETERMINING THE ABILITY OF ASTAXANTHIN EXTRACTED FROM *HAEMATOCOCCUS PLUVIALIS* ALGAE ON PROTECTING SKIN FROM PHOTOAGING ON MOUSE MODEL

Specilization: Biotechnology

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SUMMARY OF DOCTORAL DISSERTATION

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INTRODUCTION

Important of subject

Astaxanthin is a carotenoid with the formula 3,3'-dihydroxy-β-carotene-4,4'-dione. Astaxanthin has the highest antioxidant activity today (100 times more than α-tocopherol and 10 times more than compounds such as zeaxanthin, lutein, canthaxanthin, and β-carotene). Therefore, astaxanthin is widely used in medicine to treat diseases caused by oxidation, such as cardiovascular diseases, joint problems, and diabetes.

Reactive oxygen species (ROS) are one of the leading causes of skin aging. The amount of ROS in the skin increases with the physiological aging process as well as being affected by external factors such as ultraviolet rays (UV rays) and dust. These ROS disrupt skin cells (fibroblasts, keratinocytes) and molecules in the skin matrix, such as collagen and elastin, and hinder the synthesis of these molecules. AST is a potent antioxidant capable of scavenging these free radicals, thereby inhibiting the skin aging process and rejuvenating aging skin. In cosmetics, astaxanthin is used in two forms: topical lotions and oral supplements. Although many commercial products are available, research on the use of astaxanthin in cosmetics is limited. The studies conducted so far are fragmented, and inconsistent, and have been carried out on a small scale. Therefore, there is currently no definitive recommendation on the most effective use of astaxanthin in the field of cosmetology.

Studies on astaxanthin have been conducted in Vietnam, but the results are still limited, especially in terms of applied studies.

Overall Objective: *Evaluation of the anti-aging effect of astaxanthin-rich Haematococcus pluvialis extract on cell and mouse models*

Specific objective

- Successfully induce astaxanthin synthesis from Haematococcus pluvialis.
- Assess the efficacy of the astaxanthin-rich extract in protecting cells from oxidative stress under in vitro conditions.
- Evaluate the skin protection effect of the astaxanthin-rich extract against UV rays.

1. BACKGROUND

1.1. *Overview of microalgae H. pluvialis*

This section provides an introduction to the morphological characteristics and life cycle of H. pluvialis, including the transitional conditions between the different stages of the microalgae life cycle.

1.2. Introduction of AST

This section introduces AST: structure, synthesis pathway, properties

1.3. The sources of AST

This section introduces the current AST sources such as: *H. pluvialis*, salmon, and fungi.

1.4. Anti-aging mechanism of AST

This section explores the anti-aging properties of AST and its mechanisms, including its applications in anti-inflammatory processes, DNA repair, and cell protection.

1.5. Structure of the skin

This section provides an overview of the structure and composition of the skin, including its three-layer structure and the presence of fibroblast cells, epidermal cells, and keratinocytes.

1.6. Skin aging

This section focuses on UV-induced skin aging, discussing the effects of UV rays on skin cells and the skin structure, as well as providing an introduction to cellular aging.

1.7. Cellular senescence

2. MATERIAL-METHOD

2.1. Experimental design: Experimental method.

2.2. Fresh sample

- Algae H. pluvialis: strain LC (HP-C), Institute of Biotechnology (IBT), Vietnam Academy of Science and Technology (VAST)
- Human fibroblast cells (hF): frozen hF cells (Passage 8).
- *Mus musculus* var. *Albino*: female, weighing from 20-25 g, about 4-6 weeks old, provided by the Institute Pasteur, Ho Chi Minh City

2.3. Chemicals

- Medium for the algae cultivation: BG-11, cell culture medium: DMEM/F12 supplemented with 10% FBS.

2.4. Overall experimental diagram

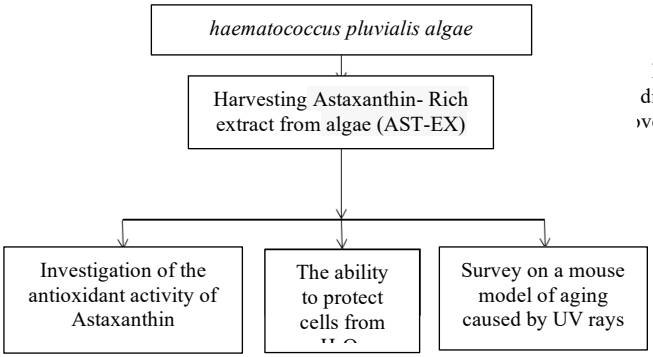
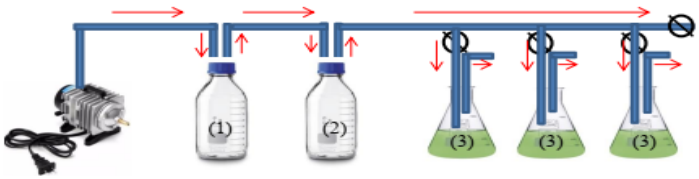


Figure 2. 1: schematic diagram representing the overall experimental works

2.5. Research method

2.5.1. Design of algae cultivation system



Direction of air flow: → Damper:
(1): dust air filter cotton bottle, (2): water bottle, (3): algae cultivation bottle

Figure 2. 2. System of Cultivating Algae

Algae Cultivating system is installed according to the diagram of Figure 2.2 with room temperature at 25°C, long LED light.

2.5.2. Method cultivating algae in the 1000 ml culture bottle

HP-C algae were inoculated into a 1000 ml culture bottle containing 500 ml of BG-11 medium with an initial density of 10x104 cells/ml and cultured at 25°C, 3000 lux (3 klux) light conditions. light:dark 12h:12h, mild aeration conditions 2L/min, gently shake 3 times/day, culture for 15 days.

2.5.3. Method of inducing HP-C algae to synthesize AST by high light intensity

Algae were inoculated into culture bottles with an initial density of 50x104 cells/ml. Algae were illuminated at 10 klux with a 24:0 h light:dark cycle. After 24 days, the algae were collected and the concentration and total AST were assessed.

2.5.4. Method for extraction AST

Table 2. 2: Experimental set-up for extraction of AST-EX from HP-C algae

	TN 1	TN 2	TN 3
S1: Freeze drying	No	Yes	Yes
S2: Treated to HCl 2N	No	No	Yes
S3: Homogenization in chloroform/methanol	Yes	Yes	Yes

Collect biomass of algal cysts and dry them according to each experimental group.

HCl treatment step: 50 mg of biomass was placed in 5 ml of 2N HCl solution for 1 min, centrifuged 3 times to remove HCl, then the biomass was freeze-dried at 4oC for 1 day.

Homogenization step in solvent: Weigh 50 mg of algae biomass in each group, add 10 ml of dichloromethane/methanol solution, and carry out mechanical homogenization at 10000 rpm for 10 minutes to collect the solution, the residue is separated. and centrifuged to a transparent extract.

2.5.5. Method for quantitative AST

Dried algae powder was mixed with methanol and saponified with 0.107 M NaOH in absolute methanol with a volume ratio of 1 ml of NaOH: 5 ml of solution, aerated to 5 ml, incubated in the dark at 4oC, for 6 hours. Then, AST was assessed by HPLC system.

2.5.6. Method for obtaining AST-rich algae extract of H. pluvialis (AST-EX)

Purpose: Collection of Haematococcus pluvialis Algal Extract

Principle: The extraction procedure for collecting the algae H. pluvialis extract is determined based on the optimal process evaluated in section 2.5.7.

The Haematococcus pluvialis extract, rich in astaxanthin (AST), is referred to as AST-EX and is characterized by its total AST concentration. For example, AST-EX 5 µg/ml indicates an extract of H. pluvialis with a total AST concentration of 5 µg/ml.

2.5.7. FRAP method

Prepare FRAP solution: acetate buffer 300 mmol/l, pH 3.6. TPTZ 10 mmol/l mixed in HCl 40 mmol/l. FeCl3 20 mmol/l. Add 190 µl of FRAP solution to plate -96 well, add 10 µl of AST or Trolox extract. The mixture was incubated against light for 2 h, and absorbance was measured at 593 nm. Oxidation capacity of AST will be calculated according to FRAP value: FRAP value = $A_0/A_1 \times N$, where A_0, A_1: OD value of the sample, $FeSO_4$ at 593 nm.

2.5.8. ABTS method

Prepare a solution of ABTS+• with an OD753 value of 0.7 ± 0.02.

Prepare AST solutions of 12.5 concentrations; 25; 50; 100 and 150 µg/ml. Prepare 1 mM trolox to a concentration range of 0.05; 0.1; 0.15; 0.2 and 0.3 mM. Calculation formula: % Inhibition = [(A0 - Am)/A0]

× 100% and IC50 = (50 - b)/a, where, A0 is the OD753 value of ethanol and ABTS+•. TEAC (mmol/g) = (IC50Trolox/ IC50ASX).

2.5.9. Method to evaluate the toxicity of AST-EX on human Fibroblast

hF cells were inoculated onto a 96-well plate at an initial density of 8x103 cells/well. After 1 day, replace the culture medium with the medium according to the experimental groups. After 1 or 2 days of culture, observe cell shape by microscopy and conduct MTT method to evaluate cytotoxicity. The percentage of viable cells was evaluated according to the following formula: % live cells = OD_{590SP}/OD_{590NC} x 100

Experimental groups: 6 groups and 2 variables including AST-EX concentration and incubation time according to the items below:

• Concentrations of AST-EX 0.5, 1, 5, 10 µg/ml and commercial AST 10 µg/ml

• AST-EX incubation time: 1 day or 2 days

2.5.10. Method to evaluate the proliferation of fibroblasts in medium completed AST-EX

hF cells were cultured on 96-well plates at an initial density of 1x103 cells/well and grown in medium with a concentration of AST-EX 05-10 µg/ml. Conduct MTT method to evaluate cell proliferation after 11 days.

2.5.11. Scratch-wound assay to access cell migration

hF cells were inoculated onto a 96-well plate at an initial density of 10 × 103 cells/well. After 1 day, use a white tip to make an incision between the cell layers and change the medium according to experimental design. AST-EX concentration: 0.5, 1, 1.5, 5, 10 µg/ml. Negative control: The culture medium contained 0.2% DMSO. Recording images of moving cells after 3 hours, 12 hours and 24 hours, image processing using ImageJ software.

2.5.12. MTT assay for cell viability

The hF cells were inoculated in plate 96 at a density suitable for the experiment and incubated at 37oC, 5% CO2. Remove the old medium, add 50 µl of MTT Reagent and 50 µl of culture medium to each well, and incubate at 37°C for 3 h. Then, dissolve the crystals with 50 µl of MTT Solvent, shake the plate well for 15 min under dark conditions. Measure optical density at 590 nm

2.5.13. Methods of cellular senescence induction using hydrogen peroxide (H_2O_2)

Cells were cultured in 24-well plates at a density of 3x104 cells/well and cultured for 1 day. After 1 day, remove the medium, wash with PBS, treat cell wells at concentrations of 100, 150, 175, 200 µM for 90 minutes, 120 minutes depending on the experiment. Cells were then cultured and aging marker expression assessed to determine the optimal procedure.

2.5.14. Senescence-associated β-galactosidase staining assay

Senescence-associate β-galactosidase activity was detected using a β-galactosidase staining kit (ab102534, Abcam, US).

2.5.15. Method to evaluate the ability of AST-EX to protect cells against the aging factor H_2O_2.

The experiment was divided into a total of 8 groups based on AST-EX concentration. The group treated with AST-EX concentrations of 0.5, 1, 1.5, 5, 10 µg/ml, before treatment with 150 µM H2O2 for 90 minutes (symbols are AST-EX0.5, AST, respectively. -EX1, AST-EX1,5, AST-EX5, AST-EX10) and treatment group with commercial AST concentration of 10 µg/ml (symbol TM10)

2.5.16. Hoestch and phalloidin staining assay to evaluate cell shape

Cells were fixed with paraformaldehyde and stained with Hoestch, phalloidin according to the manufacturer's instructions

2.5.17. Method to evaluate cellular mRNA expression by real-time PCR

Collecting total RNA using the Total RNA PurificATION-EXion Kit

Perform qPCR reaction according to SyGreen 1-Step Lo-ROX kit

The expression was evaluated using the $2^{-\Delta\Delta Ct}$ formula.

Table 2. 4. Primer sequence

hMMP1-F	CAGAGATGAAGTCCGGTTTTTC
hMMP1-R	GGGGTATCCGTGTAGCACAT

hMMP3-F	CAAAACATATTTCTTTGTAGAGGACAA
hMMP3-R	TTCAGCTATTTGCTTGGGAA
$hp16^{INK4a}$ -F	TGAGCACTCACGCCCTAAGC
$hp16^{INK4a}$-R	TAGCAGTGTGACTCAAGAGAAGCC
hp21-F	GAGCACTGCCCAACAACAC
hp21-R	ATGGCGGGAGGTAGACTGA
hCOL1A1-F	AGCAGGCAAACCTGGTGAAC
hCOL1A1-R	AACCTCTCTCGCCTCTTGCT
hELN-F	TGTCCATCCTCCACCCCTCT
hELN-R	CCAGGAACTCCACCAGGAAT
hGAPDH-F	GAAGGTGAAGGTCGGAGT
hGAPDH-R	GAAGATGGTGATGGGATTTC

2.5.18. WESTERN BLOT method evaluates the expression of CDK4, CDK6 and cyclin D1

Collect total protein by cell lysis in Optiblot LDS Sample Buffer. Then, proteins were electrophoresed with Precast Gel SDS-PAGE 4-12% for 2 h at 50V, PVDF membrane hybridization and incubation of anti-CDK4, anti-CDK6, anti-cyclin D1, anti-actin antibodies. Then secondary antibody staining and identification by X-ray film.

2.5.19. Methods for design a model of skin aging on mice (In vivo)

Mices were provided by the Pasteur Institute, Ho Chi Minh City, shaving and UV irradiation according to the process of increasing intensity 1-6 MED over 8 weeks. Morphological changes in mouse skin were recorded weekly and recorded by imaging, HE histological slices on the last day of the experiment.

2.5.20. Method to evaluate the ability to protect mice skin from UVB rays

Powdered AST is mixed with organic sacha inchi oil for cosmetic use. The obtained AST was applied directly to the mouse skin according to the experimental set-up.

Experimental set-up: mices were divided into 6 groups as shown in Table 2.5. Each experiment was conducted with 4 ones

Table 2. 5. Experimental set-up of the effects of AST on mouse skin

Lot symbol	quantity and sex	Treatment method	Time
ĐC	4	UV irradiation according to the aging process 2.5.19	8 weeks
Sacha	4	Apply sacha inchi oil before each UVB exposure according to the 2.5.19 . procedure	8 weeks
AST-EX5	4	Apply AST-EX 5 µg.ml (algae extract with total AST concentration of 5 ug/ml) before each UVB irradiation according to procedure 2.5.19	8 weeks
AST-EX10	4	Apply AST-EX 10 µg.ml (algae extract with total AST concentration 10 ug/ml) before each UVB irradiation according to procedure 2.5.19	8 weeks
AST-EX20	4	Apply AST-EX 20 µg.ml (algae extract with total AST concentration 20 ug/ml) before each UVB irradiation according to procedure 2.5.19	8 weeks
AST-EX200	4	Apply AST-EX 20 µg.ml (algae extract with total AST concentration 200 ug/ml) before each UVB irradiation according to procedure 2.5.19	8 weeks

Evaluation criteria on research subjects: surface observation, histological staining.

3. RESULTS AND DISCUSSION

3.1. *H. pluvialis* cultivating culture and AST . induction

Growth Stage: The HP-C algae are green and have a size of 20 ± 3 µm. During the mature stage, the algae follicles turn completely red and have a size of 32.61 ± 4.57 µm. At this stage, the maximum astaxanthin (AST) concentration reached 3.09% of the dry biomass.

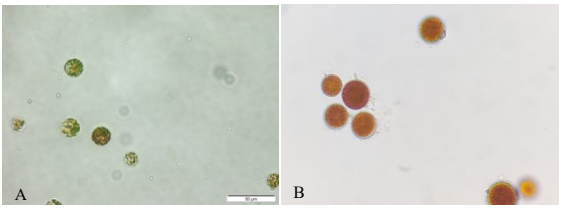
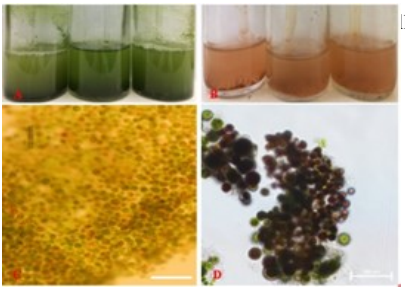


Figure 3. 1. Life cycle of HP-C algae used in the study (x40).



licle. Scale bar: 50 µm.

Figure 3. 2. HP-C culture results
A: proliferation culture in BG-11 medium after 26 days. B: Synthetic induction of AST in light of 10 klux. C: HP-C algae under the conditions of proliferation culture, D: algae HP-C under conditions of AST

3.2. Extraction of AST by solvent

The comparison between TN3 and TN1 shows that the freeze-drying method is effective in supporting the extraction of AST from algae

	EX 1	EX 2	EX 3
Total AST concentration obtained (% from algae)	$1,73 \pm 0,13^{a}$	$3,09 \pm 0,19^{b}$	$3,01 \pm 0,25^{b}$
Rate of free AST (% of total AST)	$4,50 \pm 0,27^{a}$	$4,43 \pm 0,40^{a}$	$4,37 \pm 0,50^{a}$

Table 3. 1. Summary results of AST extraction according to the procedure EX1, EX2, EX3.

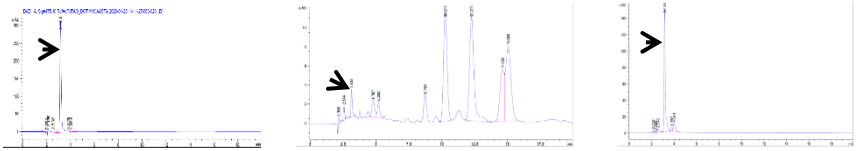


Figure 3.3 Results HPLC of *Haematococcus pluvialis* extraction

The extraction procedure by combining lyophilization and assimilation in solvent was used for all of the following experiments.

3.3. Antioxidant FRAP

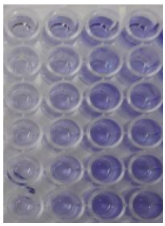


Figure 3. 5. FRAP result

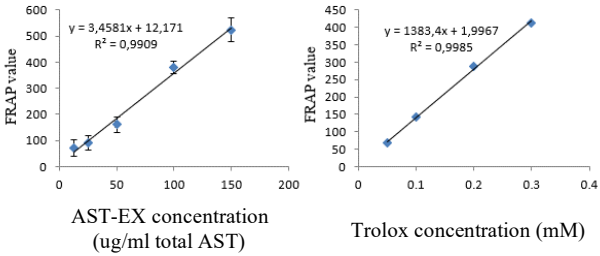


Figure 3. 6. FRAP value graph

3.4. Antioxidant ABTS

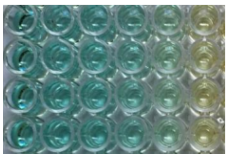


Figure 3. 7: ABTS result

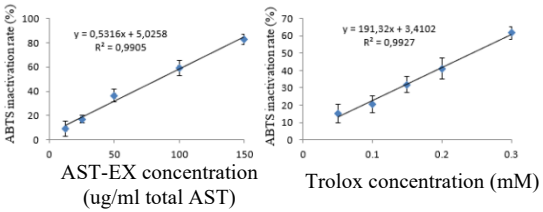


Figure 3. 8. value graph $ABTS^{+\bullet}$

3.5. In vitro toxicity

According to ISO 10993-5, AST-EX concentrations of 0.5-10 µg/ml are not cytotoxic.

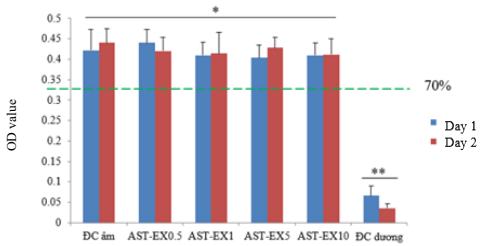


Figure 3. 9. Graph showing the OD_{495} value obtained from the AST-EX toxicity test

Table 3. 2. RBG ratios of AST-EX at different concentrations

	Day 1	Day 2
Negative Control	100%	100%
AST-EX 0.5	104,27 ± 7,64%[a]	95,45 ± 7,87%[a]
AST-EX 1	97,16 ± 7,49%[a]	94,09 ± 11,91%[a]
AST-EX 5	95,73 ± 7,12%[a]	97,27 ± 6,00%[a]
AST-EX 10	97,16 ± 6,87%[a]	93,64 ± 8,79%[a]
Positive Control	15,64 ± 5,73%[b]	8,53 ± 2,42%[b]

3.6. Proliferation

These results confirm that AST-EX 05-1.5 µg/ml has no adverse effect on hF cell proliferation.

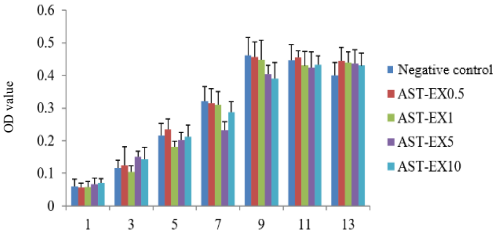


Figure 3. 10. Graph showing hF cell growth in medium completed AST-EX after 13 hours

	DT (hour)
Negative Control	49,3 ± 3,1[a]
AST-EX0.5	47,8 ± 2,5[a]
AST-EX1	49,2 ± 3,2[a]
AST-EX1.5	50,2 ± 3,3[a]
AST-EX5	55,2 ± 2,1[ab]
AST-EX10	58,8 ± 4,7[b]

Table 3. 3. Proliferation time of hF cells in experimental groups

3.7. Migration

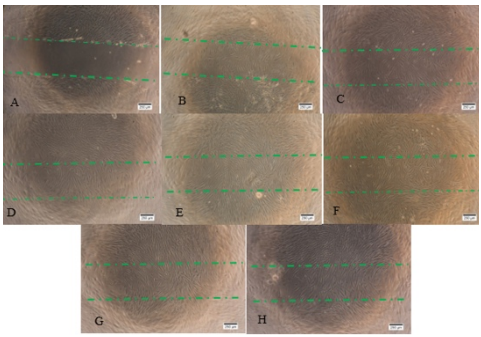


Figure 3. 11. Results of hF migration test under AST-EX 0.5-10 g/ml

Statistical processing results showed that there was no difference between groups at the same time of 3 hours, 12 hours, or 24 hours. The above results showed that AST-EX 0.5-10 µg/ml did not affect the cell migration process.

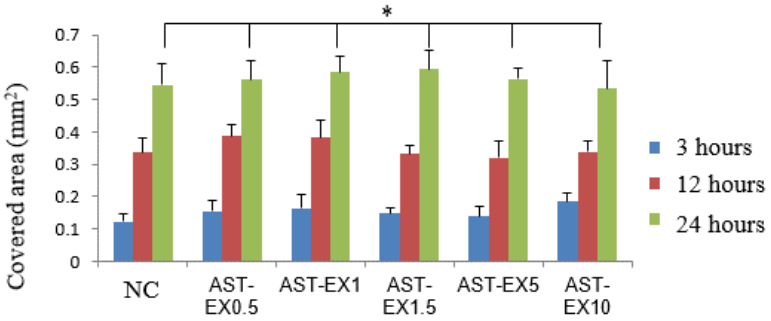


Figure 3. 12. Diagram showing the area covered by cells in the migration assay.

Table 3. 4. Results of evaluating the effectiveness of supporting hF migration after 24 hours (% of area compared to wound area)

	3 hours	12 hours	24 hours
Negative control	16,3 ± 3,1[a]	44,9 ± 6,0[a]	72,3 ± 8,9[a]
AST-EX0.5	20,8 ± 4,5[a]	51.5 ± 5,1[a]	74,8 ± 7,9[a]
AST-EX1	21,9 ± 5,9[a]	51,1 ± 7,3[a]	77,9 ± 6,4[a]
AST-EX1.5	19,7 ± 3,5[a]	44,1 ± 3,5[a]	79,2 ± 7,6[a]
AST-EX5	18,4 ± 6,5[a]	42,8 ± 6,5[a]	75,2 ± 4,3[a]
AST-EX10	24,8 ± 4,1[a]	45,2 ± 4,1[a]	71,2 ± 11,2[a]

3.8. Investigation of the process of inducing cell senescence by H_2O_2

3.8.1. Cell growth result

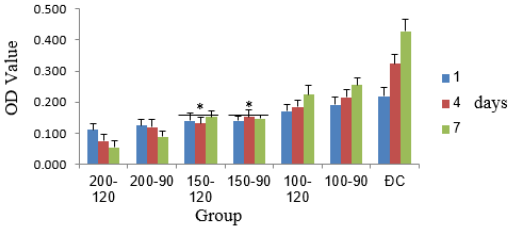


Figure 3. 13. Graph showing the increase in OD values in groups after 7 days

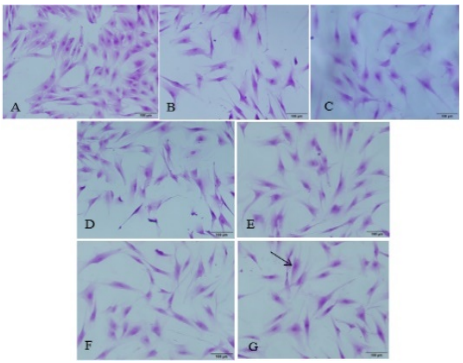


Figure 3. 14. Change of hF cell shape when treated with H2O2 on 4th day (x50).

A, B, C, D, E, F, G: cells of the control groups, 100-90, 100-120, 150-90, 150-120, 200-90, 200-120. Scale bar: 100 m, arrow: enlarged cells

3.8.2. Survey results on SA-β-Gal expression

Table 3. 5. The percentage of cells positive for SA-gal when treated with H2O2 (a, b, c: statistical difference, $p<0.05$

Group	control	100-90	100-120	150-90	150-120	200-90	200-120
SA-gal positivity rate	4,60 ± 1,52[a]	47,14 ± 8,48[b]	51,44 ± 11,79[b]	65,25 ± 8,97[c]	70,73 ± 8,31[c]	72,03 ± 6,73[c]	74,99 ± 7,29[c]

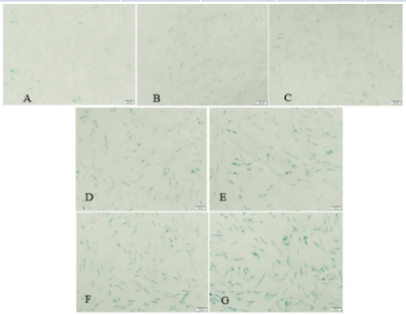


Figure 3. 15. Results of SA-gal staining of hF cells treated with H_2O_2 at different concentrations. (x100).
A, B, C, D, E, F, G: cells groups: Control, 100-90, 100-120, 150-90, 150-120, 200-90, 200-120. Scale bar: 100 µm

Therefore, the concentration of 150 µM, the treatment time of 90 minutes and 120 minutes are all suitable for the set criteria.

3.9. Investigation of AST-EX's ability to protect fibroblast cells from the effects of H_2O_2

3.9.1. Results of cell area assessment

This result shows that H_2O_2 changes cell shape: cells flatten, expand cytoplasm and nucleus, while AST-EX 1-10 µg/ml limits this effect (Figure 3.26 and Table 3.26). 3.10).

Table 3. 6. Cell area and nucleus area in AST-EX and H_2O_2 treatment groups (a, b, c d: statistical difference in the same row, $p<0.05$)

	Control	H_2O_2	AST-EX0.5	AST-EX1	AST-EX1.5	AST-EX5	AST-EX10	TM10
Cell Area	2430,9 ± 212,6^a	10527,25 ± 3798,1^b	3919,2 ± 1172,2^c	3102,7 ± 1172,2^d	3065,8 ± 393,8^d	3193,2 ± 393,8^d	3153,1 ± 393,8^d	3868,9 ± 345,8^{cd}
Nucleus Area	224,8 ± 21.4^a	383,3 ± 93,7^b	293,9 ± 27,1^c	255,4 ± 42,0^a	216,3 ± 43,5^a	246,2 ± 41,6^a	251,1 ± 38,4^a	285,2 ± 24,5^c
Rate of cell/nucleus	11,8 ± 2.1	17,1 ± 1,6	13,2 ± 4,0	12,3 ± 2,6	14,9 ± 4,3	13,2 ± 2,0	12,8 ± 1,9	13,6 ± 4,4

3.9.2. The results of the assessment of cell proliferation

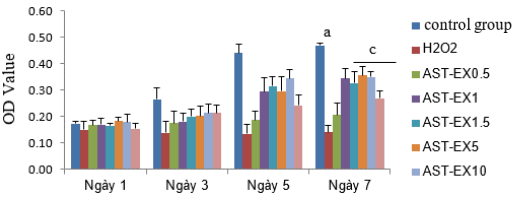


Figure 3. 17. Graph showing cell proliferation treated with AST-EX and H_2O_2 through days

Table 3.7. Doubling time of AST-EX and H_2O_2 treated cells

	Doubling time
Negative control	68,08 ± 2,06^a
H_2O_2	Không tăng trưởng
AST-EX0.5	572,51 ± 258,18^b
AST-EX1.0	198,57 ± 46,68^c
AST-EX1.5	199,88 ± 64,36^c
AST-EX5	215,09 ± 57,29^c
AST-EX10	213,23 ± 50,01^c
TM10	269,53 ± 102,78^c

This showed that H_2O_2 inhibited cell proliferation, cells in the TM group still maintained the ability to proliferate although lower than in the AST-EX group.

3.9.3. *Results of evaluating SA-gal expression*

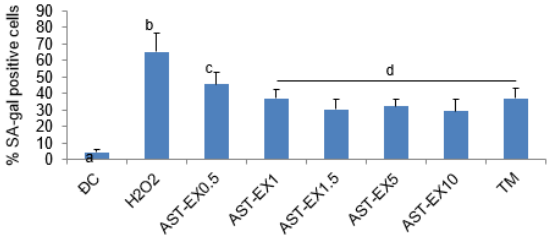


Figure 3.18. The histogram shows the percentage of cells that are positive for SA-gal

This result partly demonstrates the ability of AST-EX to protect cells from oxidative stress agent H_2O_2.

3.9.4. *Results of evaluating the expression of aging markers p53, p2, p16 by realtime-PCR*

The results showed that AST-EX concentration of 1, 1.5 g/ml reduced the expression of aging markers p53, p21, p16 (Figure 3.23).

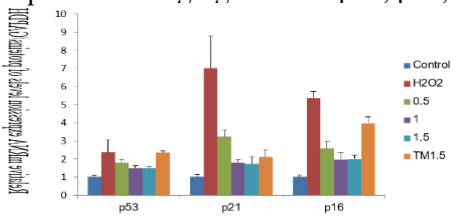


Figure 3. 20. Graph showing the expression of aging markers p53, p16, p21 in groups of cells treated with AST-EX and H_2O_2

Table 3. 9. Relative mRNA expression of p53, p21, p16 genes in the cytoprotective experiment of AST-EX (a, b, c, d: is the statistical difference in the same line), $p<0.05$)

	Control	H_2O_2	AST-EX0.5	AST-EX1	AST-EX1.5	TM1.5
MMP3	$1,0 \pm 0,2^{a}$	$4,7 \pm 1,0^{b}$	$2,3 \pm 0,3^{cd}$	$2,1 \pm 0,4^{c}$	$1,6 \pm 0,1^{c}$	$2,8 \pm 0,2^{d}$
MMP1	$1,0 \pm 0,2^{a}$	$5,0 \pm 0,4^{b}$	$3,3 \pm 0,3^{c}$	$1,7 \pm 0,2^{d}$	$1,2 \pm 0,1^{ad}$	$3,0 \pm 0,2^{c}$
COL1A1	$1,0 \pm 0,3^{a}$	$0,3 \pm 0,1^{b}$	$0,7 \pm 0,3^{b}$	$1,7 \pm 0,4^{a}$	$1,2 \pm 0,4^{a}$	$1,4 \pm 0,5^{a}$
Elastin	$1,0 \pm 0,1^{ab}$	$0,1 \pm 0,0^{c}$	$0,1 \pm 0,0^{c}$	$1,2 \pm 0,3^{a}$	$0,8 \pm 0,1^{b}$	$1,0 \pm 0,1^{ab}$

3.9.5. *Results of evaluating the expression of cell function at the transcriptional level*

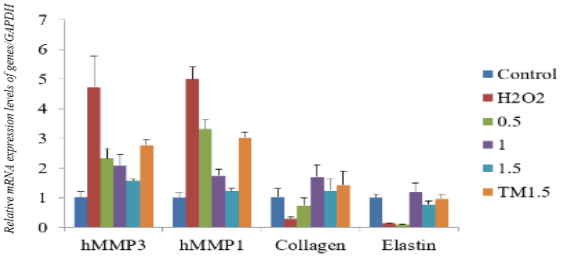


Figure 3. 21. Graph showing the expression of hMMP3, hMMP1, collagen, elastin cells treated with AST-EX

Meanwhile, cells in the AST-EX group had an increase of 2 MMP3, MMP1 mRNA compared to the control group but decreased compared with the H_2O_2 group (Figure 3.24). For collagen and elastin, the H_2O_2 cell expression decreased compared with the control group (reduced to 29% and 13% compared to the control group respectively).

3.9.6. *Results of evaluating the expression of cell function at the protein level*

Cells in the AST-EX1 group showed an increase in cylin D1 expression (1.5 times higher than the control group) and a decrease compared with the cells in the H2O2 group.

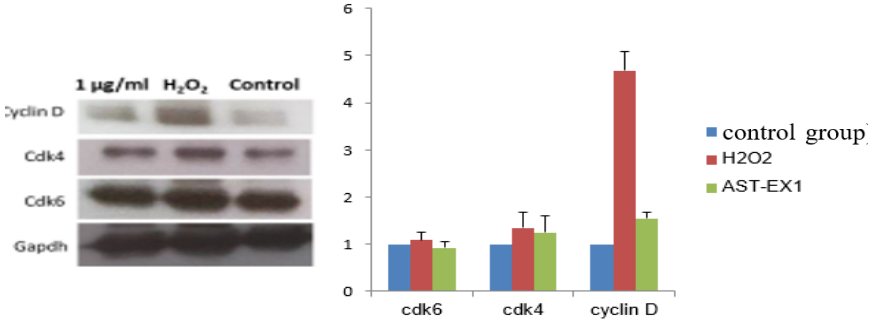


Figure 3. 22. Histogram showing the expression of CDK4, CDK6, cyclin D1 of hF cells

Table 3. 10. Results of relative expression of CDK4, CDK6 and cyclin D proteins (a,b: statistical difference in the same column, p<0.005)

	CDK6	CDK4	cyclin D
Control	1± 0,2[a]	1 ± 0,1[a]	1 ± 0,1[a]
H_2O_2	1,1 ± 02[a]	1,3 ± 0,2[a]	4,7 ± 0,4[b]
AST-EX1	0,9 ± 0,2[a]	1,2 ± 0,2[a]	1,5 ± 0,3[a]

The results show that AST-EX reduces the harmful effects of H_2O_2 on fibroblast cells, the concentration of 1 µg/ml is the minimum concentration that shows the cytoprotective effect.

3.10. Results of evaluating the ability to protect mouse skin from aging due to UV rays

3.11.1 Model of skin aging caused by UV rays

At week 6, wrinkles appeared across the back of the mouse, these marks were more obvious when the mouse moved. Weeks 7-8, the skin of the mouse is stretched, the skin is less elastic, so the crack becomes indistinct.

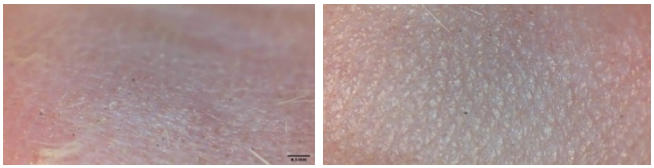


Figure 3.24. Results of mouse skin analysis after uv irradiation
A: control group, B: 8 weeks after UVB irradiation

Trichrome staining results showed that the epidermal thickness of the AST-EX group was reduced compared with the UV irradiation or oil applied group.

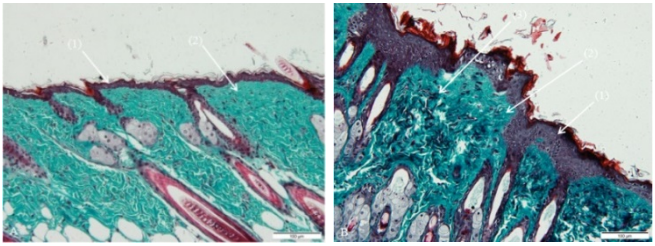


Figure 3.25. Results of trichrome staining of mouse skin samples
A: Control mouse skin sample, B: 8-week UV irradiated mouse skin sample. (1): epidermis, (2): dermis, (3): abnormal collagen structure

Table 3. 11. Summary of the results of the skin aging on mouse model caused by UVB rays. (a,b: statistical difference, $p<0.05$)

	Control group	UVB irradiation group
Skin wrinkles point	$0,5 \pm 0,5^a$	$4,67 \pm 0,47^b$
Skin laxity point	$0,25 \pm 0,43^a$	$2,5 \pm 0,5^b$
Epidermal thickness (µm)	$21,3 \pm 0,8^a$	$65,8 \pm 2,7^b$
Inflammatory cell infiltration	Not detected	Yes
The appearance of abnormal collagen structures	Not detected	Yes

3.11.2 Result of AST-EX's ability to protect skin from UV damage

Table 3. 12. Summary of results for skin wrinkling and epidermal thickness. (a, b,c,d,e,f: statistical difference in the same line, $p < 0.05$)

	Control	UVB	Sacha	AST-EX5	AST-EX10	AST-EX20	AST-EX200
Skin wrinkles point	$0,5 \pm 0,5$	$4,5 \pm 0,5$	$3,5 \pm 0,5$	$3,25 \pm 0,43$	$3,0 \pm 0,7$	$2,5 \pm 0,5$	$2,25 \pm 0,43$
Skin laxity point	$0,25 \pm 0,43$	$2,5 \pm 0,5$	$2,25 \pm 0,43$	$1,75 \pm 0,42$	$1,5 \pm 0,5$	$1,0 \pm 0,7$	$0,75 \pm 0,43$
Epidermal thickness (µm)	$21,4 \pm 6,7^a$	$58,9 \pm 20,9^b$	$54,7 \pm 12,9^b$	$47,3 \pm 10,0^c$	$41,7 \pm 9,8^d$	$33,5 \pm 9,6^e$	$28,9 \pm 7,3^e$
Inflammatory cell infiltration	+	+++++	++++	++	++	+	+
The appearance of abnormal collagen structures	+	+++++	++++	+++	+++	++	++

Dermatoscopy results show that AST-EX 10, 20, 20 has good effect in protecting the surface of the mouse skin.

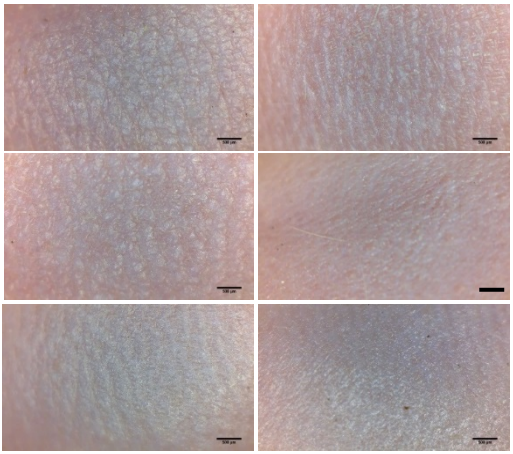


Figure 3. 27. Results of dermatoscopy after 8 weeks of UVB irradiation.

A: UVB irradiation group, B: Sacha inchi oil-treated mice, C, D, E, F: AST-EX 5, 10, 20 mice, 200 g/ml

Trichrome staining results showed that, after 8 weeks of UV irradiation, there was an increase in the thickness of the epidermis, the infiltration of inflammatory cells, the formation of abnormal collagen structures (Figures 3.28, 3.29).

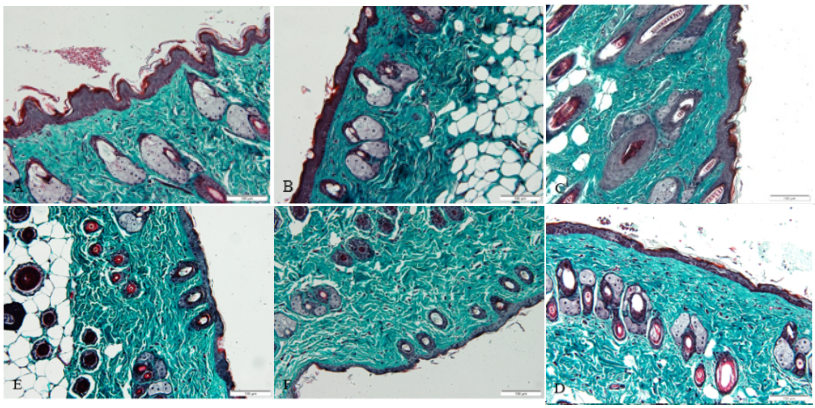


Figure 3. 28. Trichrome staining of mouse skin samples after 8 weeks of UVB irradiation.

A: UVB irradiation group, B: Sacha inchi oil-treated mice, C, D, E, F: AST-EX 5, 10, 20 mice, 200 g/ml

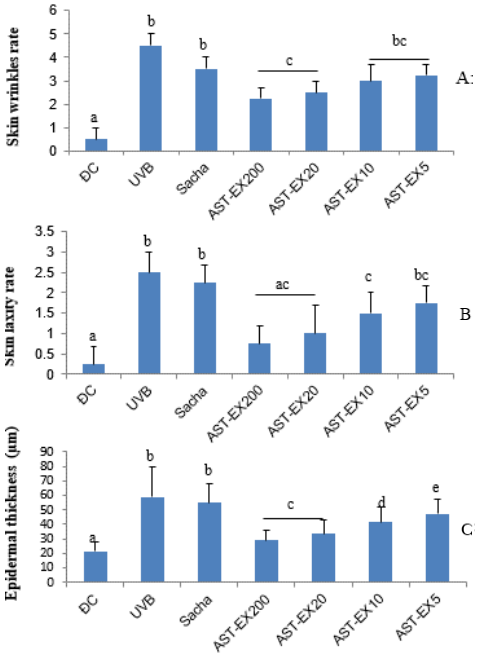


Figure 3. 29. Graph showing the results of AST-EX's anti-aging mouse skin test.
A: Histogram shows skin wrinkling, B: Histogram shows skin flaccidity, C: histogram shows epidermal thickness

4. CONCLUSION

From the results obtained from the study, we would like to conclude the following:

- Successfully induced AST from HP-C algae with the highest concentration of 3.1% dry weight.
- The AST-rich extract has the ability to protect cells from H_2O_2 agents and the concentration of 1 g/ml is the minimum concentration that has a function.

- AST-rich extract, when used in topical formulation, protected the mouse's skin from UV rays, the concentration of 20 µg/ml giving the best results.

NEW CONTRIBUTIONS OF THE THESIS

Research novelties:

- Investigate some properties of local algae *H. pluvialis* isolated in Vietnam
- Research shows that astanxanthin-rich extract from microalgae has the ability to protect cells from strong stress oxidative agent H_2O_2 in vitro and this ability is higher than pure AST. This is the basis for using AST-rich extracts for humans
- The study showed that the optimal concentration when using AST-rich extract was 20 µg/ml.

LIST OF PUBLICATIONS

Quan Minh To, Nhan Dinh Tran, Truc Thi Thanh Vo, Thao Thu Huynh, Dieu Quang Tran, Trinh Nguyen Ai Ta1, Bien Dinh Lai, Dung Hoang Nguyen, Long Thanh Le, Determining the ability of AST from H. pluvialis on the protection of skin in the mouse model, Journal of Applied Biology & Biotechnology Vol. 9(04), pp. 85-92

Quan Minh To, Dieu Quang Tran, Tuyet Thi Vy Le, Ha Le Bao Tran, Long Thanh Le, The ability of AST-rich *H. pluvialis* algal extract on preventing harmful effects of H_2O_2 on human fibroblast cells *in vitro,* European Journal of Molecular & Clinical Medicine, Volume 08, Issue 03, 2021, 1809-1919.