



**“1+1>2” :Double emulsion simultaneously encapsulate both lipid-soluble and water-soluble components derived from *Haematococcus pluvialis* enhancing antioxidant and anti-aging efficacy**

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## 1. Introduction

*Haematococcus pluvialis* (*H. pluvialis*) is a widely distributed green alga in nature, containing nutrients such as *H. pluvialis* proteins, *H. pluvialis* polysaccharides (Hpp), and astaxanthin (Ast), which are widely used in the food, pharmaceutical, and cosmetic industries <sup>[1]</sup>. Ast is a potent antioxidant that endows *H. pluvialis* with strong antioxidant capacity, making it a promising candidate in antioxidant and anti-aging research<sup>[2]</sup>. In particular, polysaccharides derived from microalgae are known to have the ability to modulate the activity of human immune cells and may induce anti-inflammatory and anticancer effects<sup>[3]</sup>. Research on the application of the highly active Ast component has been quite mature. However, the combined application of various substances extracted from *H. pluvialis* has been overlooked. The various components in the mixture have very important application value for antioxidant and anti-aging effects in the human body.

After extracting the nutrients from *H. pluvialis*, they are extremely unstable when stored in a natural environment. Therefore, certain stabilization systems need to be employed to enhance their stability. Currently, methods such as emulsions, microcapsules, liposomes, and nanoparticles are effective for stabilizing these active substances. The construction of emulsions has certain advantages in promoting the transdermal absorption of active substances in human skin. The water-in-oil-in-water (W/O/W) double emulsion is a uniform dispersion in which small droplets of the oil phase are encapsulated by an inner water phase, and this primary emulsion is then further encapsulated by an outer water phase, simultaneously encapsulating incompatible water-soluble and lipid-soluble active substances<sup>[4]</sup>.

However, there was currently a lack of research on simultaneously encapsulating water-soluble and lipid-soluble active substances from *H. pluvialis*. Moreover, this study provided new ideas for developing active substances from novel raw material sources. The system could promote the antioxidant effects of encapsulated active substances in the skin and facilitate their absorption, thereby offering new perspectives for the development of skincare and other functional products. It also provided a reference for solving the problem of simultaneous encapsulation of incompatible active substances in biotechnology.

## 2. Materials and Methods

### 2.1. Materials

*H. pluvialis* powder was purchased from Yunnan Boxin Biotechnology Co., Ltd. (Yunnan, China). Ast ester (99%) was obtained from Chengdu Plant Standardization Pure Biotechnology Co., Ltd. Polyglyceryl-10 laurate was obtained from Guangzhou Kesalu Commercial Co., Ltd. Polyglycerol-2 diglycidyl stearate ester was obtained from BASF (China) Co., Ltd. Aristoflex® Silk (Sodium polyacryloyldimethyl taurate) was obtained from Guangzhou Own Snow Chemical Co., Ltd. Hydroxypropyl beta-cyclodextrin was obtained from Shandong Binzhou Zhiyuan Biotechnology Co., Ltd. The raw materials related to the cell experiments were purchased from Shanghai Biyuntian Biotechnology Co., Ltd. All other ingredients were used as purchased.

### 2.2. Extract of Hpp and Ast from *H. pluvialis*

*H. pluvialis* and anhydrous ethanol were weighed at a solid-to-liquid ratio of 1:20, subjected to ultrasonic extraction at room temperature, and then transferred for stirring extraction at 45°C for 1.5 h. After that, the extracting solution was centrifuged at 5000 r/min for 10 min. The supernatant was the active extract of *H. pluvialis*, while the precipitate was the residue of *H. pluvialis*. The supernatant was transferred to a rotary evaporator, and ethanol was removed at 45°C for 60 min to obtain the crude extract of *H. pluvialis* oil (mainly containing Ast). The oil component was dissolved in capric triglyceride. The residue was combined with 2% (wt) NaOH solution at a solid-to-liquid ratio of 1:25, heated in an 80°C water bath for 1 h, and then centrifuged after cooling. The supernatant was subjected to ethanol precipitation for 24 h and then centrifuged to obtain the polysaccharide extract (Hpp).

### 2.3. Preparation of double emulsion

A two-step method was adopted to prepare a W/O/W (water-in-oil-in-water) double emulsion. Initially, a 1:1 mixture of 5% astaxanthin oil and 5% Hpp solution was prepared, and 12% of this mixture (based on the total formulation) was used. Then, 3 wt% of polyglycerol-2 dipolyhydroxystearate was added, and the mixture was homogenized at 12,000 rpm for 5 min to obtain the initial water-in-oil (W/O) emulsion. Further, 32.5% of a 0.5% cyclodextrin solution and 2.5% of polyglycerol-10 laurate were added to the w/o emulsion, and the resulting mixture was homogenized at 12,000 rpm for 5 min to obtain a W/O/W primary emulsion. Subsequently, 50% Aristoflex® Silk was added, and the mixture was homogenized using a high-pressure homogenizer (KS-KBS, Cabaus Nanotechnology (Shanghai) Co., Ltd., China) at a pressure of 20 bar for 5 min to obtain the final water-in-oil-in-water (W/O/W)

emulsion. Based on the ratio of Hpp to Ast in the emulsions, six types of emulsions were prepared and named: (2:1), (1:1), (1:2), (2:0), (0:2), and Control (containing neither Hpp nor Ast).

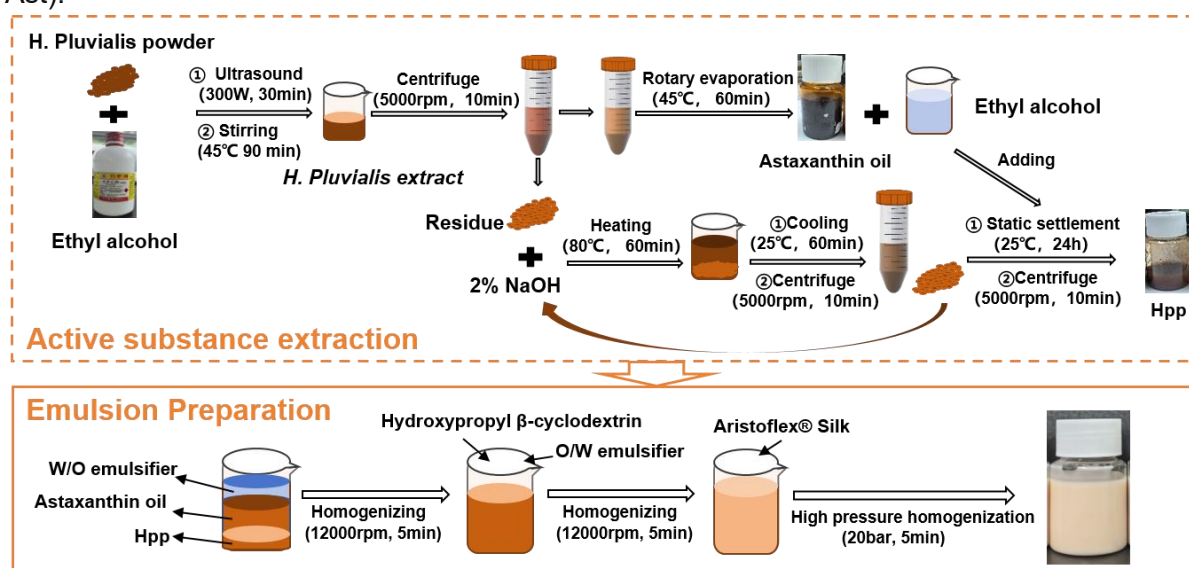


**Figure 1.** The flowchart for extracting Ast and Hpp from *H. pluvialis*, and the process of preparing the emulsion.

## 2.4. Characterization of double emulsion

### 2.4.1. Particle size, polydispersity index and zeta potential

Particle size, polydispersity index, and zeta potential of the W/O/W double emulsion were measured using a nanoparticle size analyzer (Zetasizer Nano ZEN, Malvern Panalytical, UK). Before the test, the emulsion was diluted with distilled water at a ratio of 1:500 (v/v) to avoid multiple scattering effects. The distilled water was adjusted to the same pH value as the sample using sodium hydroxide or hydrochloric acid before use. Each emulsion was measured in triplicate in parallel.

### 2.4.2. Structural characterization

To examine the morphology of the emulsions, a 1 mg/mL Nile red solution was mixed with the emulsion and agitated for 2 h in darkness. The microstructure of the emulsions was then observed using a confocal laser scanning microscope (CLSM, Zeiss LSM980 with Airyscan2, Germany) equipped with 10× and 50× magnification lenses under the excitation light of 488 nm.

### 2.4.3. Ast encapsulation rate

The Ast standard solution was prepared in anhydrous ethanol at a concentration of 0.1 mg/mL. 1.0, 2.0, 3.0, 4.0, 5.0, and 6.0 mL of the emulsion solution were each diluted to 100 mL with anhydrous ethanol, and the absorbance was measured at 474 nm. 1 mL of freshly prepared Ast emulsion was thoroughly mixed with 5 mL of anhydrous ethanol and centrifuged at 8000 rpm for 5 min. Then, the absorbance value of the emulsion was measured using a spectrophotometer (L6S, Shanghai Yile Analytical Instruments Co., Ltd., China) at a

wavelength of 474 nm. The Ast concentration was calculated based on the standard curve, and the encapsulation rate was then calculated using the following Eq. (1):

$$E_{Ast}(\%) = m_1/m_2 \times 100\% \quad (1)$$

Where  $m_1$  represented the Ast content measured in the emulsion, and  $m_2$  was the total amount of Ast added.

#### 2.4.4. The stability of emulsion

Each emulsion was stored in the dark at temperatures of 4 °C, 25 °C, and 40 °C. The color variations of the emulsion were assessed using a colorimeter (YL4660, China). Subsequently, three primary parameters were documented: luminosity (L), red-green chromaticity (a), and yellow-blue chromaticity (b). The total color difference ( $\Delta E$ ) was computed using Eq. (2):

$$\Delta E = \sqrt{(L^* - L)^2 + (a^* - a)^2 + (b^* - b)^2} \quad (2)$$

where  $L^*$ ,  $a^*$ , and  $b^*$  are the color values of the white plate ( $L^*=40.12$ ,  $a^*=-0.52$ ,  $b^*=-0.45$ ).

#### 2.5. Antioxidant activity measurement

**DPPH:** Various volumes of the double emulsion (0.1, 0.2, 0.4, 0.6, 0.8, 1.0, and 1.2 mL) were each diluted with anhydrous ethanol to a final volume of 2 mL. Subsequently, 200  $\mu$ L of the emulsion was combined with an equal volume of a DPPH ethanol aqueous solution (0.04 mg/mL). Following a 30 min incubation period in the dark, the mixture was centrifuged. Then, 200  $\mu$ L of the supernatant was extracted, and its absorbance was measured at a wavelength of 517 nm. The DPPH scavenging rate was calculated using Eq. (3):

$$\text{DPPH scavenging rate (\%)} = [A_1/(A_1 - A_2)] \times 100 \quad (3)$$

where  $A_1$  represented the blank absorption value, and  $A_2$  was the absorption value of the emulsions.

**ABTS:** At an absorbance of 734 nm, the ABTS solution was diluted with anhydrous ethanol to an absorbance within the range of  $0.7 \pm 0.02$ . Then, 100  $\mu$ L of the emulsion solution and 100  $\mu$ L of the ABTS solution were added to the 96-well plate as the experimental group ( $A_1$ ). For the control group ( $A_2$ ), 100  $\mu$ L of anhydrous ethanol was used instead of the ABTS solution. For the standard group ( $A_0$ ), 100  $\mu$ L of ABTS solution and 100  $\mu$ L of anhydrous ethanol were added. The reaction was carried out in the dark for 30 minutes. Then, the absorbance was measured using a microplate reader at a wavelength of 734 nm. The above experiment was repeated three times. The clearance rate was calculated using Eq. (4):

$$\text{ABTS scavenging rate (\%)} = [1 - (A_1 - A_2)/A_0] \times 100 \quad (4)$$

#### 2.6. The anti-inflammatory and anti-aging effect

##### 2.6.1. Anti-inflammatory

RAW264.7 cells were treated with emulsions for 24 h. Supernatant IL-1 $\alpha$ , IL-6, and TNF- $\alpha$  were measured by ELISA. The relative amount of inflammatory factors (IL-1 $\alpha$ /IL-6/TNF- $\alpha$ ) was calculated using Eq. (5)

$$\text{inflammatory factor relative amount (\%)} = C_1/C_0 \times 100\% \quad (5)$$

where  $C_1$  represented the inflammatory factor content of emulsions handle,  $C_0$  was the inflammatory factor of negative control (The basic culture medium containing lipopolysaccharide).

### 2.6.2. Relative content of MMP-1

Keratinocytes were treated with emulsion, exposed to UVB (90 mJ/cm<sup>2</sup>), and cultured for 4 h. Relative content of MMP-1 was according to Eq.(6):

$$\text{Relative content of MMP-1} = C_1/C_2 \times 100\% \quad (6)$$

where  $C_1$  represented the content of MMP-1 in cells treated with emulsion, and  $C_2$  represents the MMP-1 content of the negative control treatment cells.

### 2.6.3. Elastase inhibition rate

Five groups were established: emulsion groups (5% emulsion concentration), emulsion substrate groups, solvent groups, and solvent substrate groups. Each group consisted of three parallel tests. Different reagents were added, and the mixtures were shaken evenly, incubated at 25 °C for 15 min, and then the absorbance was measured at 410 nm using a microplate reader. The elastase inhibition rate was calculated according to Eq.(7):

$$\text{Elastase inhibition rate (\%)} = [1 - (C - D) / (A - B)] \times 100\% \quad (7)$$

where A represented the absorbance of the solution without emulsion, B was the absorbance of the solution without emulsion and elastase, C was the absorbance of the solution containing emulsion and elastase, and D was the absorbance of the solution containing emulsion but without elastase.

### 2.6.4. Human fibroblast $\beta$ - galactosidase staining

Human fibroblast cells were seeded in a 96-well plate. After 24 h, the basic culture medium containing different concentrations of the emulsion samples was added. After another 24 h, the OD<sub>490</sub> nm value was measured using the MTT method, and the effect of the test samples on the viability of human fibroblast cells was analyzed using a t-test. Then, human fibroblasts were cultured in a 6-well plate for 24 h. Subsequently,  $\beta$ -galactosidase fixative solution was added, and the cells were fixed at room temperature for 15 min. Next, the staining working solution was added, and the cells were incubated at 37 °C. Finally, the stained cells were observed under an optical microscope.

## 3. Results

### 3.1. Physical properties and encapsulation efficiency of double emulsion

As listed in Table 1, the emulsion with a 1:1 ratio had the smallest particle size (227.19 ± 0.72 nm) and the highest  $E_{Ast}$  (87.13 ± 0.25%), with the standard curve equation for Ast being  $y = 0.023x - 0.016$ . At a 2:1 ratio of Hpp to Ast, the polydispersity index was the lowest (0.21 ± 0.01). A high proportion of Hpp solution was present within the Ast oil droplets, causing the emulsion droplets to become irregular and thereby enhancing the dispersibility. The apparent viscosity was positively correlated with the oil content. The apparent viscosity of the emulsion gradually increased as the oil content increased. The 1:2 ratio of Hpp to Ast resulted in an emulsion viscosity of up to 3698.6 Pa·s, **and** the addition of HPP increased the viscosity of the emulsion, thereby enhancing its stability.

**Table 1.** Physical properties and encapsulation rate ( $E_{Ast}$ ) of emulsions.

| Name | Physical property |     |                |           | $E_{Ast}(\%)$ |
|------|-------------------|-----|----------------|-----------|---------------|
|      | Particle size     | PDI | Zeta-potential | Viscosity |               |

|         | (nm)                        |                           | (mV)                        | (Pa.s)                      |                            |
|---------|-----------------------------|---------------------------|-----------------------------|-----------------------------|----------------------------|
| Control | 199.11 ± 0.46 <sup>a</sup>  | 0.34 ± 0.01 <sup>a</sup>  | -20.00 ± 1.93 <sup>bc</sup> | 1256 ± 21 <sup>b</sup>      | /                          |
| (2:1)   | 364.62 ± 2.25 <sup>ab</sup> | 0.21 ± 0.01 <sup>a</sup>  | -48.70 ± 2.22 <sup>a</sup>  | 213 ± 6.1 <sup>c</sup>      | 47.21 ± 0.20 <sup>ab</sup> |
| (1:1)   | 227.19 ± 0.72 <sup>c</sup>  | 0.45 ± 0.01 <sup>ab</sup> | -44.26 ± 0.65 <sup>ab</sup> | 2520.3 0± 9.6 <sup>ab</sup> | 87.13 ± 0.25 <sup>a</sup>  |
| (1:2)   | 1173.47 ± 2.91 <sup>a</sup> | 0.52 ± 0.01 <sup>c</sup>  | -46.56 ± 1.79 <sup>bc</sup> | 3698.6 ± 9.7 <sup>cd</sup>  | 68.50 ± 0.27 <sup>b</sup>  |
| (2:0)   | 828.85 ± 1.40 <sup>bc</sup> | 0.51 ± 0.02 <sup>ab</sup> | -48.63 ± 1.90 <sup>ab</sup> | 2636± 13 <sup>a</sup>       | /                          |
| (0:2)   | 649.38 ± 0.59 <sup>ab</sup> | 0.47 ± 0.02 <sup>a</sup>  | -45.32 ± 3.04 <sup>d</sup>  | 2625 ± 17 <sup>ab</sup>     | 75.22 ± 0.19 <sup>b</sup>  |

Note: Values in table are mean standard deviation. Different letters in the same column indicated that the difference was statistically significant ( $P < 0.05$ ).

### 3.2. Microstructure of double emulsion

As shown in Figure 2, the oil phase of the green fluorescent part contained a large amount of Hpp, which appeared as small black dots. These Hpp particles, dispersed within the Ast oil and encapsulated by the cyclodextrin aqueous solution, were observed as black spots, confirming a water-in-oil-in-water emulsion structure.

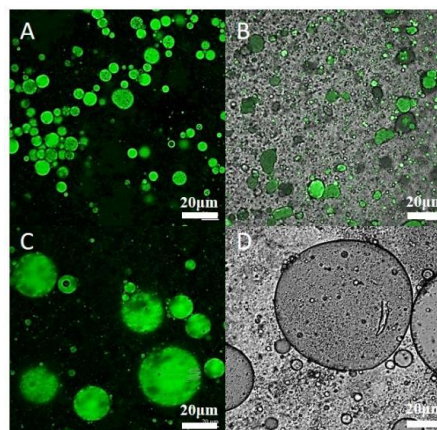


**Figure 2.** CLSM image (A、B: magnification of ×20 ; C、D: magnification of ×50) of the (1:1) emulsion.

### 3.3. Stability of double emulsion

As shown in Figure 3, the centrifugal stability and temperature stability of the emulsion indicated the stability period of the emulsion in encapsulating active substances. When the six emulsions were centrifuged at 5000 rpm for 10 min, it could be observed that the (1:1) emulsion did not exhibit stratification or release of active substances compared to the other emulsions (Figure 3a). When the (2:1) emulsion containing a large amount of Hpp was subjected to high temperature, the crystalline region was disrupted, resulting in gelation, flocculation, and stratification of the active substances (Figure 3c). Moreover, at 40 °C, the color difference of the (1:1) emulsion changed the least, while that of the (2:1) emulsion

changed the most (Figure 3b). In general, this revealed that the (1:1) emulsion had better stability.

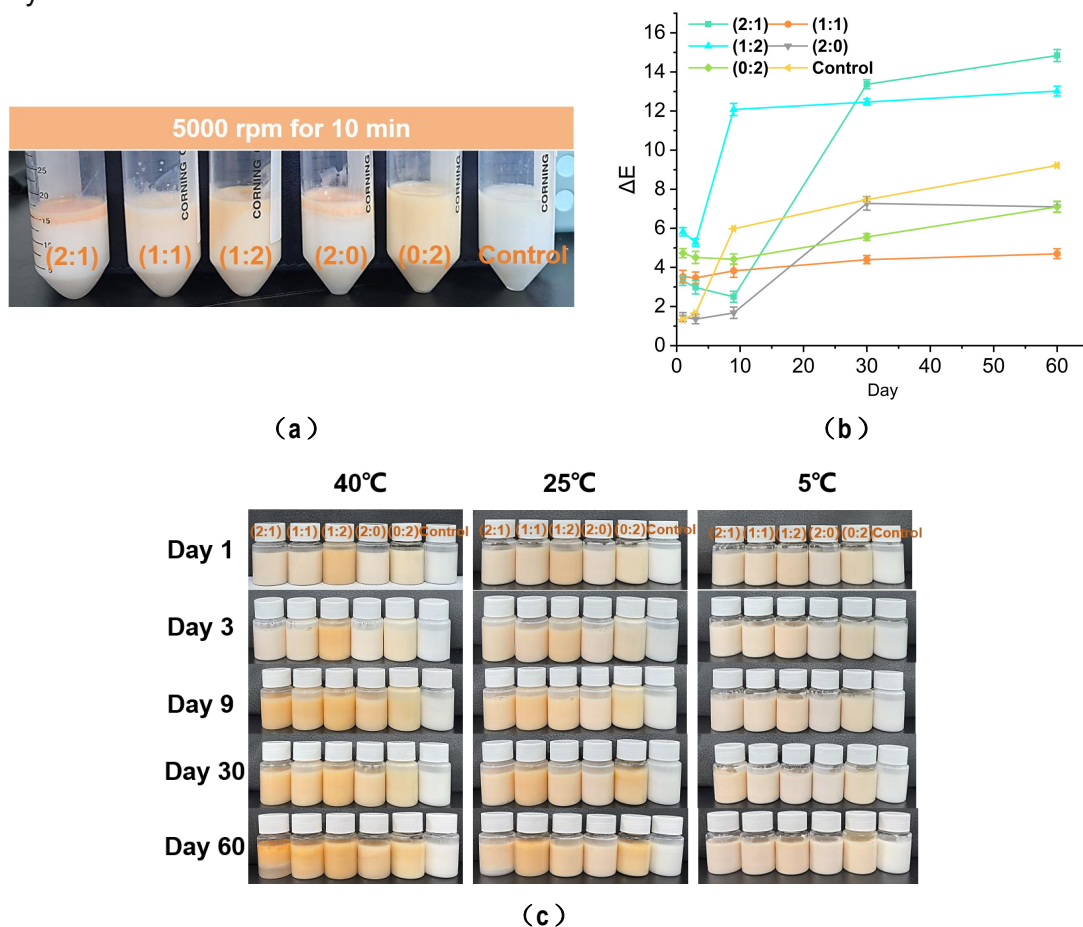


**Figure 3.** (a) Centrifugal stability. (b)  $\Delta E$  values after 60 days of storage at 40 °C ( $P < 0.05$ ). (c) Stability images after 60 days of storage at 40 °C, 25 °C, and 5 °C .

### 3.4. The antioxidant activity of double emulsion

As shown in Figure 4, the ratio of Ast to Hpp at 1:1 had the highest DPPH radical scavenging rate (83.60%) and ABTS scavenging rate (90.21%). Compared to the control emulsion, both emulsions containing Ast and Hpp had certain antioxidant capabilities. Interestingly, the Ast that played a major role in previous studies did not make the emulsion more potent in antioxidant capacity, simply due to its high concentration in the double-layer emulsion.

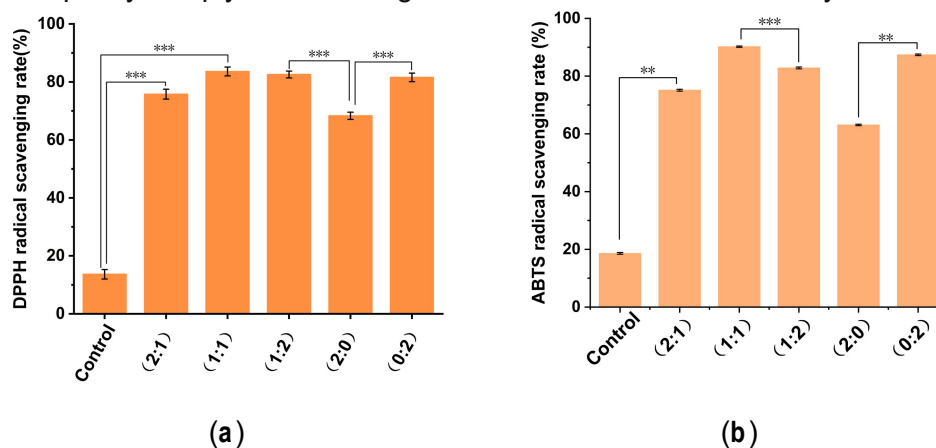


**Figure 4.** (a) The DPPH radical scavenging rate and (b) ABTS scavenging rate of emulsions (\*\* $p < 0.05$ , \*\*\* $P < 0.01$ ).

### 3.5. The anti-inflammatory and anti-aging effects of double emulsion

The double emulsion composed of a 1:1 ratio of Hpp and Ast had the strongest inhibitory effect on skin inflammatory factors exposed to UVB radiation. The relative contents of IL-6 and TNF- $\alpha$  were the lowest, and the IL-1 $\alpha$  content was significantly lower than that of the control emulsion (Figure 5a). The relative content of MMP-1 was 15.98% lower than that of the control group, demonstrating a significant ability to inhibit cellular MMP-1 content (Figure 5b). At a 5% emulsion concentration, the (1:1) emulsion exhibited an elastase inhibition rate of 11.7% (Figure 5c). At an emulsion concentration of 0.5%, cell viability was relatively high. In human fibroblast  $\beta$ -galactosidase staining treated with the (1:1) emulsion, the blue areas were fewer, and the area of the blue regions was 44.16% smaller compared to the negative control (Figure 5d). Overall, these results indicate that the double emulsion with a 1:1 ratio of astaxanthin and polysaccharides has excellent soothing and anti-aging effects.

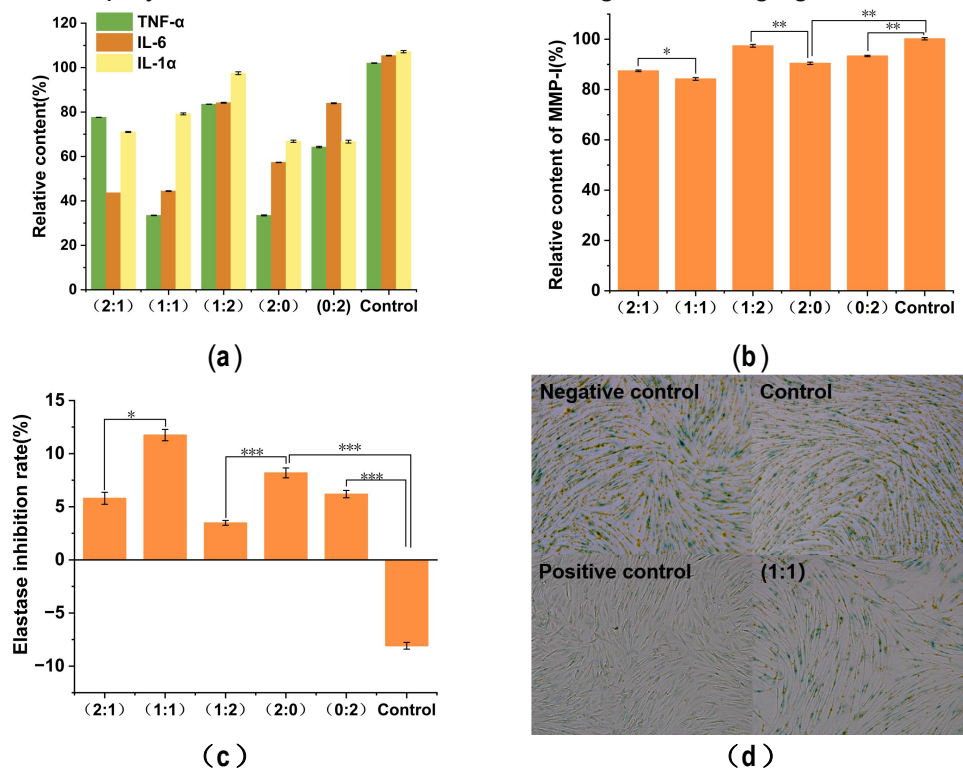


**Figure 5.** Effect of emulsion (5% concentration) on (a) cellular inflammatory factors (IL-1 $\alpha$ , IL-6, TNF- $\alpha$ ) and (b) relative content of MMP-1. (c) Elastase inhibition rate and (d) human fibroblast  $\beta$ -galactosidase staining (50 $\times$ ) under 0.05% emulsion (\* $P < 0.05$ , 0.05 < \*\*\* $P < 0.01$ , \*\*\* $P < 0.01$ ).

## 4. Discussion

Skin aging is associated with a variety of internal and external factors, among which ultraviolet (UV) radiation plays a particularly crucial role by promoting the degradation of collagen and elastin via overexpressed matrix metalloproteinases. Elastase is a key molecule connecting UV radiation, inflammatory response, and skin structural damage. It is mainly

synthesized and secreted by fibroblasts and can degrade elastin in the skin, leading to skin aging<sup>[5, 6]</sup>. Natural products offer potential solutions for anti-aging and antioxidant effects and provide an economical and safe treatment option for skin diseases with fewer side effects.

The double emulsion system proposed in this study effectively encapsulates and stabilizes the natural active components. The thermodynamic instability is mainly attributed to the interfacial behavior of the bicontinuous emulsion, which stems from its unique double-membrane, three-phase structure. Hpp can prevent aggregation of the oil phase by forming a specific spatial structure<sup>[9]</sup>. This research indicated that Ast interacted with Hpp in different ways. Hydrogen bonds and hydrophobic interactions jointly drive these interactions, exposing more hydrophilic groups on the surface of Hpp, thereby enhancing the hydrophilicity and wettability of Hpp and improving the stability of the emulsion<sup>[10, 11]</sup>.

In recent years, studies have been conducted on the antioxidant and anti-aging effects of Ast and Hpp from *H. pluvialis*. These active substances have been shown to inhibit UVB- or UVC-induced inflammatory cytokine generation and apoptosis in the HaCaT cell line, alleviate skin inflammatory responses, and inhibit elastase and matrix metalloproteinases<sup>[7, 8]</sup>. This research further explored the synergistic effect of Ast and Hpp. The antioxidant and anti-aging effects of the emulsions were in the order: (1:1) > (1:2) > (2:1) > (0:2) > (2:0), achieving a “1 + 1 > 2” effect. The water-in-oil-in-water emulsion in this study not only fully utilizes the extracts of *H. pluvialis* but also exhibits excellent stability, significantly inhibiting skin inflammation and ultraviolet radiation-induced aging. The Hpp will be systematically analyzed and verified in future studies and are expected to be efficiently applied in the cosmetics industry.

## 5. Conclusion

In the current study, two active substances Hpp and astaxanthin were successfully extracted from *Haematococcus pluvialis*, thus making full use of plant resources. Furthermore, an aqueous-in-oil-in-water encapsulation system was used to simultaneously encapsulate the oil-soluble active substance (astaxanthin) and the water-soluble active substance (Hpp). At a 1:1 ratio, astaxanthin and polysaccharides showed good emulsion dispersibility, smaller particle size, and stability. Their antioxidant and anti-aging effects were significant, far superior to those of emulsions containing twice the amount of Hpp or Ast. This effectively saved astaxanthin resources and expanded the application of Hpp in the cosmetics field.

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