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A combination of deep eutectic solvent and enzymatic degradation of polysaccharide from *Naematelia aurantiabla*: a sustainable way to enhance its skin care function properties

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Abstract: Polysaccharide is well-known as skin care ingredient for their bioactivities, including moisturization, antimicrobial, antioxidant, anti-inflammatory, anti-glycation, and anti-aging. *Naematelia aurantiabla* (NA) is a rare, valuable, edible and medicinal fungus, named as “the queen of fungus”. *Naematelia aurantiabla* polysaccharide (NAP) is one of the main bioactive components, which possesses a lot of significant benefits in the regulation of physiological health, including immunostimulating, antioxidant, antidiabetic, hypolipidemic functions and anti-titumor properties. It is reported that NAP is a high molecular weight polysaccharide around 1×10^6-3×10^6 Da, which would limit to cross cell membranes and could not be utilized efficiently, resulting in decreased biological activity. Thus, a combination of deep eutectic solvent (DES) and enzymatic degradation would be utilised to obtain low molecular weight polysaccharide of *Naematelia aurantiabla* (NALP) in this study. Compared to the conventional methods, this strategy was much more environmentally friendly and efficient. Betaine-based DES could improve the NAP extraction efficiency significantly. Subsequently, NALP would be obtained by gentle degradation of composite enzymes (cellulase, pectase, and glucoamylase). Under the optimal of a combination of DES and enzymatic degradation, the molecular weight of NALP was below 5×10^5 Da. Results showed that NALP exhibited excellent antioxidant activity by inhibiting ROS generation and enhancing superoxide dismutase (SOD) activity. Meanwhile, NALP could inhibit the expression of IL-1α, IL-6, and TNF-α, showing excellent anti-inflammatory property. Aquaporin-3 expression assay also demonstrated that NALP had moisturizing activity. Above all, NALP would be a promising skin-care ingredient applied in the comestic industry.

Key words: *Naematelia aurantiabla* polysaccharide, deep eutectic solvent, enzymatic degradation, skin care

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1. Introduction

Naematelia aurantialba (Bandoni & M. Zang) *Millanes & Wedin,* another botanical name *Tremella aurantialba* Bandoni et Zang, belonging to the so-called “jelly mushrooms” group that forms a large brain-shaped, golden basidiocarp is a species of edible fungus with high nutritional and medicinal value.[1]The fruiting bodies of *N. aurantialba* are rich in polysaccharides, proteins, amino acids, vitamins, and other nutrients. [2] The total polysaccharide content of the functional nutrients in *N. aurantialba* is as high as 72.8%.[3]*Naematelia aurantiabla* polysaccharide (NAP) is one of the main bioactive components, which possesses a lot of significant benefits in physiological regulation, including immunostimulating,[4-5] antioxidant,[6] antidiabetic,[7]and hypolipidemic functions,[8] antitumor.[9] It is reported that NAP is a high molecular weight polysaccharide around 1×10^6-3×10^6 Da, which would limit to cross cell membranes and could not be utilized efficiently, resulting in decreased biological activity.[10]

Deep eutectic solvents (DESs) generally can be prepared by mixing two or more components together, in which one acts as the hydrogen bond donor (HBD) and the other as the hydrogen bond acceptor (HBA), to form a eutectic liquid mixture at ambient temperature, [11-13] which have attracted the attention of researchers because it was recognized as a type of green and sustainable solvent in 2003. DESs show many advantageous properties, such as easy to produce, non-flammable, non/low-toxic, relatively high solubility, biocompatible and biodegradable. [14-16] These properties enable DESs to be used in various fields, such as in pharmaceutics,[17-18] synthesis of porous materials,[19] electrochemistry[20] and extraction processes.[21-22]

Deep eutectic solvents (DES) can create a favorable environment for polysaccharides and are a new generation of biodegradable solvents[23]. Few studies have been conducted on the use of DES to enhance enzymatic degradation. Therefore, a combination of deep eutectic solvent (DES) and enzymatic degradation would be utilised to obtain low molecular weight polysaccharide of *Naematelia aurantiabla* (NALP) in this study. Compared to the conventional methods, this strategy was much more environmentally friendly and efficient. Betaine-based DES could improve the NAP extraction efficiency significantly. Subsequently, NALP would be obtained by gentle degradation of composite enzymes (cellulase, pectase, and glucoamylase). Under the optimal of a combination of DES and enzymatic degradation, the molecular weight of NALP was below 5×10^5 Da.The skin care function properties of NAP and NALP were evaluated by antioxidant activity,anti-inflammatory property and moisturizing activity.The results showed that NALP would be a promising skin-care ingredient applied in the comestic industry.

2. Materials and Methods

2.1. Materials and chemicals

Naematelia aurantiabla was purchased from Shaoguan (Guangdong, China). Betaine, rhamnose, pectinase, glucoamylase, acetic acid, sodium acetate, $CaCl_2$ were purchased from Macklin Biochemical Technology Co., Ltd (Shanghai, China). Dialysis membrane (500 Da) Sinopharm Chemical Reagent Co. (Shanghai, China). HSF cells, HaCaT cells were purchased from Beina Bio(Beijing, China). BCA protein concentration determination kit, SOD Kit, ROS detection, LPS purchased from Sigma-Aldrich(USA). Enzyme-linked immunosorbent assay(ELISA) kits for IL-1α, TNF-α and IL-6 were purchased from Jingmei Biotech(Jiangsu, China). AQP-3 primary antibody, fluorescent secondary antibody were purchased from Abcam(USA). DAPI was purchased from Invitrogen(USA). Magnesium L-ascorbate 2-phosphate sesquimagnesium salt hydrate(Vc derivant), DMEM medium supplemented with 10% FBS were purchased from Thermo Fisher Scientific (Waltham，USA). Methyl thiazolyl tetrazolium(MTT)was purchased from Solarbio(Beijing,China).

2.2.The preparation of NAP and NALP

NA was purchased from Shaoguan (Guangdong, China). NA powder (100 g) was added to water (6 L), heated at 75 °C for 2 h. After centrifugation, the supernatant was filtered at 25 °C through a ceramic membrane filter column with a pore diameter of 50 nm (pressure of 0.45 MPa), and the intercepted solution was freeze-dried to obtain NAP. The extraction rate of NAP was 38.9 %, and the proportion of polysaccharides in freeze-dried powder was 96.4%.

NA powder (100 g) was added to 1.5% betaine-rhamnose(betaine-based DES) aqueous solution (6 L), and 0.5% Complex enzymes (cellulase, pectase, and glucoamylase (1:1:2)) in acetic acid/sodium acetate bufer(pH 4.5). The degradation of NA was carried out at 55 °C) for 1.5 h. After adjusting the pH to neutral, the salt and DES was removed through a dialysis membrane (500 Da), and the retained solution was freeze-dried to obtain NALP. The yield rate of NALP was 45.6 %, and the proportion of NALP in freezedried powder was 92.1 %.

2.3 Antioxidant activity assay

2.3.1 Cytotoxicity test

Conventional cell culture. Prepare the cell suspension, inoculate it into a 96-well cell culture plate, and culture for 18 to 24 hours. Discard the original culture medium in each well, add 100 uL of test samples of different concentrations to each well(0.2, 0.1, 0.05 mg/ml), and return them to the incubator for incubation with 72 soils for 1 h. Take out the culture plate, remove the original culture medium, add 100 uL of culture medium to each well, then add 20 uL of MTT solution, and incubate in the incubator for 3 to 4 hours. Remove the liquid from each well, add 100 ul DMSO to each well, place it in an oscillator for 10-15 minutes,

and then measure the absorbance at a wavelength of 570nm on an microplate reader. The cell viability was calculated using the following formula:

Cellviability(%) = $[(A_s - A_b)/(A_c - A_b)] \times 100\%$

A_s: experimental group, A_c: control group, A_b: blank group

2.3.2 Determination of SOD activity and MDA content

Normal HSF cells were inoculated into 6-well plates and cultured for 18-24 hours. Three groups of samples (NAP and NALP with 0.2, 0.1, 0.05 mg/mL) and the positive control group (PC) with different concentrations were incubated for 24 hours. The original culture media were discarded and washed once with PBS. Except for the negative control group, DMEMs containing H_2O_2 were added to the remaining groups for 2 hours, and the corresponding culture media for each group were replaced and incubated for 48 hours. Cells were collected, cell disruption was performed respectively, and the activity of superoxide dismutase (SOD) and the content of malondialdehyde (MDA) were detected according to the instructions of the kit.

2.3.3 Determination of ROS content

Conventional cell culture. Normal HSF cells were inoculated into 6-well plates and cultured for 18-24 hours. Remove the culture plates, discard the original culture medium in the Wells, expose three different concentrations of the sample groups (NAP and NALP with 0.2, 0.1, 0.05 mg/mL) to be tested and the positive control group (PC), and incubate for 24 hours. Remove the culture plates, discard the original culture medium in the Wells, wash once with PBS. Except for the negative control group, add serum-free DMEM medium containing H_2O_2 to the other groups for 2 hours, and then replace it with the corresponding medium of each group and incubate for 48 hours. Add 10 uM DCFH-DA fluorescent dye and incubate in the incubator for 30 minutes. The cells were washed twice with PBS, digested and collected. The ROS level was detected at an excitation wavelength of 488 nm and an emission wavelength of 525 nm on a flow cytometer (represented by fluorescence intensity).

2.4 Anti-inflammatory activity assay

2.4.1 Cytotoxicity detection

Under sterile conditions, the samples were prepared and diluted with serum-free medium to concentrations of 5, 2.5, 1.25mg/mL, and stored at 4℃ for future use. HaCaT cells were seeded in 96-well plates and cultured overnight under conditions of saturated humidity, 5%CO_2 and 37℃. Sample addition will be carried out the next day. 24 hours after sample addition, the cell metabolic activity was detected by the CCK-8 method, and the cell survival rate was calculated. The cell viability was calculated using the following formula:

Cellviability(%) = $[(A_s - A_b)/(A_c - A_b)] \times 100\%$

A_s: experimental group, A_c: control group, A_b: blank group

2.4.2 Anti-inflammatory activity test

HaCaT cells were inoculated in 24-well plates and cultured under conditions of saturated humidity, 5%CO_2 and 37℃. Samples were added as **Table 1** the next day. After 24 hours of sample addition, the culture supernatants of each well were collected. After centrifugation, the contents of IL-1α, IL-6 and TNF-α in the culture supernatants were detected respectively according to the instructions of the ELISA kit, and the release inhibition rate was calculated.

Table 1 Experimental grouping for Anti-inflammatory activity test

Experimental grouping	Detection model	Induction condition	Test subject	Detection index
Normal(N)	HaCaT cells	/	/	TNF-α IL-1α IL-6
Model(M)		LPS 1μg/mL	/	
Positive control(PC)		LPS 1μg/mL	Dexamethasone 100 ug/mL	
Sample		LPS 1μg/mL	NAP and NALP with 5, 2.5, 1.25 mg/mL	

Inhibition rate(%)=(1-$\frac{As}{Ai}$) ×100%

A_s: Release volume of the sample group, A_i: Release amount of the induction group

2.5 In Vitro moisturizing effects assay

Detection of AQP-3 expression: HaCaT human keratinocytes were inoculated in 24-well plates and cultured overnight under conditions of 95% humidity, 5%CO_2 and 37℃. The next day, the cells were randomly divided into the blank control group, the positive control group and the sample group. Samples were added according to **Table 2** and incubated at 95% humidity, 5%CO_2 and 37℃ for 24 hours.

Table 2 Experimental grouping for AQP-3 expression detection

Experimental grouping	Detection model	Test subject	Detection index
Model(M)	HaCaT cells	/	Expression of AQP-3
Positive control(PC)		$CaCl_2$ 100 ug/mL	
Sample		NAP and NALP with 5, 2.5, 1.25 mg/ml	

At the end of time, AQP-3 immunofluorescence staining was performed. Photos were taken and recorded under a 20×microscope. The average fluorescence intensity of AQP-3 was calculated using ImageJ software, and the increase rate of AQP-3 expression was also calculated.

Expression increase rate (%)=($\frac{Average\ fluorescence\ intensity\ of\ the\ sample\ group}{Average\ fluorescence\ intensity\ of\ the\ blank\ control\ group}$ − 1)×100%

2.6 Statistical analysis

All data were expressed as mean ± standard deviation. The r-test was used to compare among groups. A P value <0.05 was considered to have a significant difference in the results, and a P value <0.01 was considered to have an extremely significant difference in the results.

3. Results and discussion

3.1 Antioxidant activity assay

3.1.1 Cytotoxicity test of NAP and NALP

The viability of HSF cells was assessed to determine the effects of NAP and NALP (0.2, 0.1, 0.05 mg/mL). **Fig. 1-**A demonstrate that the cell viability of all treated groups was above 90 %, indicating that the hydrolysate and polysaccharide were non-toxic to HSF cells .

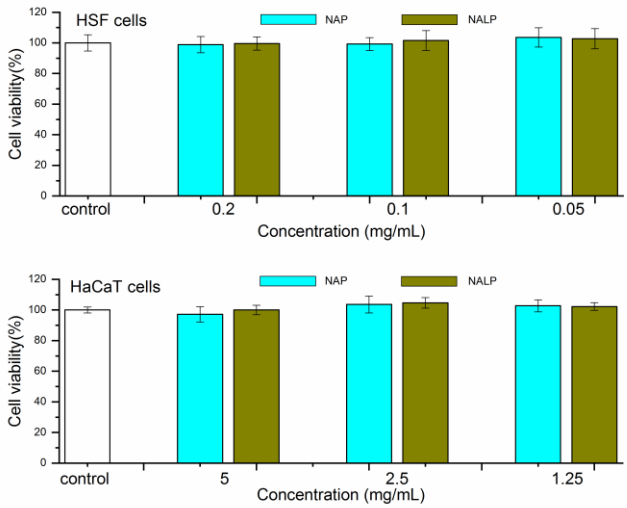

Figure. 1 Cell survival rate of control group(control), NAP and NALP at different concentrations. HSF, Cell viability of HSF cells; HaCaT, Cell viability of HaCaT cells.

3.1.2 Determination of SOD activity

Superoxide dismutase (SOD) is regarded as a substance that enhances the antioxidant defense of cells[24]. SOD can eliminate harmful substances produced by organisms during metabolism, counteract and block the damage caused by oxygen free radicals to cells, and repair damaged cells in a timely manner. This paper studies the effects of NAP and NALP on intracellular antioxidant enzymes under oxidative stress conditions.

The effects of different concentrations of NAP and NALP on the intracellular SOD activity of H_2O_2 -induced HSF cells are shown in **Fig. 2**-A. Compared with the negative control group (N) at 147.27±3.29%, the relative content of SOD in the model group (M) at 100± 6.43% was significantly decreased.Compared with the model group (M), the relative content of SOD in the positive control group (PC) was at 130.26±3.07%,significantly increased,suggesting successful modeling. Compared with the model group, the relative contents of SOD in NAP and NALP at the test concentrations of 0.20mg/mL, 0.10mg/mL and 0.05mg/mL were

significantly increased . In NAP group, the relative contents of SOD at the test concentrations of 0.20mg/mL, 0.10mg/mL and 0.05mg/mL were at 130.5±3.16%,119.72±3.48% and 116.67±4.26%; meanwhile in NAP group were at 133.98±4.56%,129.95±4.21%,123.97±5.05. It indicates that the effect of NALP against lipid peroxidation is superior to that of NAP.

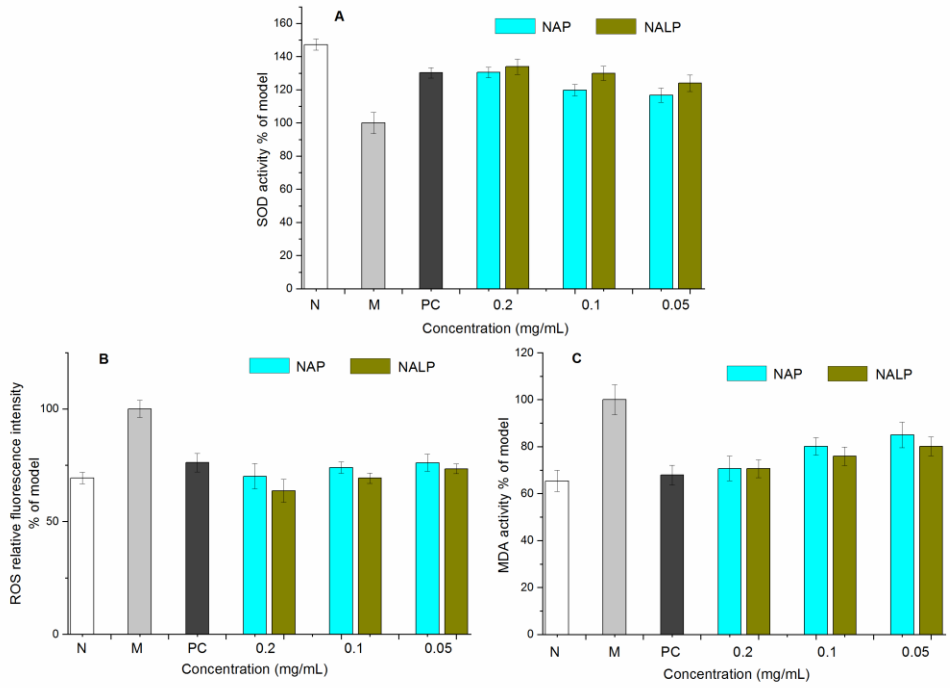

Figure 2. Determination of SOD activity , ROS and MDA content at normal group(N),model group(M),positive control group (PC) and different concentrations of NAP and NALP. A, SOD activity % of model. B,ROS relative fluorescence intensity % of model. C,MDA activity % of model.

3.1.3 Determination of ROS and MDA content.

Reactive oxygen species (ROS) are a series of reactive oxygen species clusters produced by cells during metabolic processes[25]. When cells are subjected to oxidative stress, they generate excessive ROS, which can cause various damages, aging and other lesions. The fluorescence intensity produced by DCFH-DA dye entering cells is directly proportional to the intracellular ROS level. The antioxidant effect of the test substance can be reflected by measuring the ROS level.

The effects of NAP and NALP on the MDA content in H_2O_2 -induced HSF cells are shown in **Fig. 2**-B.Compared with the control group, the ROS content of HSF cells in the model group increased after induction with H_2O_2. Compared with the model group, the addition of NAP and NALP at different mass concentrations could inhibit the ROS content to varying degrees, and there was a significant difference . It indicates that the effect of NALP against lipid peroxidation is superior to that of NAP.

Compared with the relative contents of ROS in the negative control group (N) at 69.36±2.58%, the model group (M) at 100.00±3.84% was significantly increased ; and the relative content of ROS in the positive control group(PC)was at 76.15±4.08%, which was significantly lower than the model group, suggesting successful modeling.

At the test concentrations of 0.20mg/mL, 0.10mg/mL and 0.05mg/mL, compared with the model group, the relative contents of ROS in NAP and NALP were significantly decreased. the relative contents of ROS in NAP at the respective concentrations of 0.20mg/mL, 0.10mg/mL and 0.05mg/mL were 70.10±5.6%，73.93±2.57% and 76.12±3.85%.Compared with NAP, the content of ROS secreted by HSF cells treated with NALP were 63.66±5.02%,69.30±2.33% and 73.41±2.15%, decreased significantly , indicating that the effect of NALP against lipid peroxidation was superior to that of NAP.

The content of malondialdehyde (MDA) often reflects the degree of lipid peroxidation in the body and indirectly reflects the degree of cell damage. MDA can cause lipid peroxidation and then form peroxides. The amount of its content directly affects the degree of lipid peroxidation in the body, thereby indirectly reflecting the degree of cell damage[26].

The effects of NAP and NALP on the MDA content in H_2O_2 -induced HSF cells are shown in **Fig. 2**-C. Compared with the negative control group(N), the MDA content of HSF cells in the model group (M) increased after induction with H_2O_2. The MDA content of HSF cells in the model group was at 100±6.38%, higher than the negative control group at 65.4±4.5% and the positive control group (PC) at 67.88±4.24%, suggesting successful modeling.

Compared with the model group, the addition of NAP and NALP at different concentrations could inhibit the MDA content to varying degrees. The MDA content of HSF cells NAP group at the respective concentrations of 0.20mg/mL, 0.10mg/mL and 0.05mg/mL were at 70.71±5.17%,75.88±3.99% and 80.1±4.03%;and the NALP group was at 70.6±3.83%,80.12±3.69% and 84.97±5.49%. At different concentrations, The MDA content of the NAP group was higher than the NALP group ,it indicates that the effect of NALP against lipid peroxidation is superior to that of NAP.

3.2 Anti-inflammatory activity assay

3.2.1 Cytotoxicity test of NAP and NALP

The viability of HSF cells was assessed to determine the effects of NAP and NALP (5,2.5,1.25 mg/ml). **Fig. 1**-B demonstrate that the cell viability of all treated groups was above 90 %, indicating that the hydrolysate and polysaccharide were non-toxic to HaCaT cells .

3.2.2 Effect of NAP and NALP on Inflammatory Factors in LPS-Stimulated HaCaT Cells

When pathogens and their secretions or other foreign irritants invade the skin, they can rapidly activate keratinocytes to produce responses, including activating arachidonic acid metabolism and secreting cytokines, etc[27]. Among them, IL-1α, IL-6 and TNF-α are important cytokines for evaluating anti-inflammatory activity. By detecting the release of IL-1α, IL-6 and TNF-α, the condition of keratinocytes can be reflected, thereby evaluating the anti-inflammatory effect of the samples.

As shown in **Fig. 3** ,Compared with the negative control group (N), the content of IL-1α, IL-6 and TNF-α in the model group (M) was significantly decreased ; Compared with the model group (M), the content of IL-1α, IL-6 and TNF-α in the positive control group (PC) was significantly increased , suggesting successful modeling.

Compared with the model group, NAP and NALP at concentrations of 5, 2.5, 1.25 mg/mL could significantly inhibit the release of IL-6, IL-1α and TNF-α in HaCaT cells caused by LPS . Moreover, the inhibitory ability of NALP is superior to that of NALP. Specific data as **Table 3**.

Table 3 The release of IL-1α, IL-6 and TNF-α in HSF cell at different concentrations

Experimental grouping		Concentrations (mg/mL)	IL-1α(pg/mL)	IL-6(pg/ mL)	TNF-α(pg/ mL)
Normal group(N)		/	6.62±0.15	11.88±0.22	31.03±0.34
Model group (M)		/	8.43±0.15	21.02±0.37	39.65±0.49
Positive control(PC)		/	4.94±0.50	5.53±0.06	30.15±0.77
Sample	NAP	5	4.74±0.54	11.90±0.58	13.90±0.70
		2.5	5.94±0.85	14.38±0.11	14.36±0.53
		1.25	6.87±0.53	18.55±0.04	18.11±0.57
	NALP	5	4.18±0.37	9.30±0.32	14.54±1.70
		2.5	5.38±0.26	11.95±0.54	17.34±1.70
		1.25	6.12±0.56	14.06±0.60	21.32±0.88

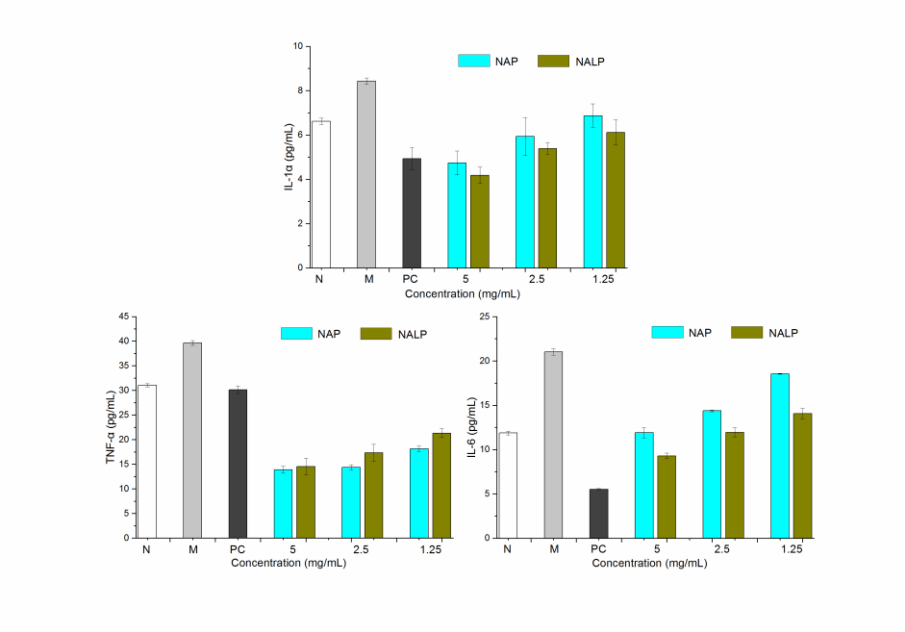

Figure 3. The release amounts of IL-1α, IL-6 and TNF-α.

3.3 In Vitro moisturizing effects assay

The moisturizing effect of the samples can be evaluated by detecting the expression of AQP-3 protein. As shown in **Fig. 4**, Compared with the model group(100±5.3%), the expression level of AQP-3 in the positive control group(192.1±6.2%) was significantly increased , suggesting successful modeling. At the test concentrations of 5 mg/mL, 2.5 mg/mL and 1.25 mg/mL, compared with the model group, the expression level of AQP-3 in NAP and NALP was significantly increased , and the expression level of AQP-3 in NALP was higher than that in the NAP group. Expression increase rate of AQP-3 in NALP group at the test concentrations of 5 mg/mL, 2.5 mg/mL and 1.25 mg/ mL were at 126.1±5.345%,74.56±5.184% and 54.84±5.89%,higher than of the NAP group at 120.3±4.712%, 65.4±6.321% and 34.3±4.514%.

Aquaporin-3 (AQP-3) is a channel protein located on the cell membrane, which can regulate the transmembrane transport of water, glycerol, etc. The level of AQP-3 protein expression is positively correlated with the strength of skin moisturizing function. The moisturizing effect of the samples can be evaluated by detecting the expression of AQP-3 protein[28].Thus, the moisturizing performance of NALP is superior to that of NAP.

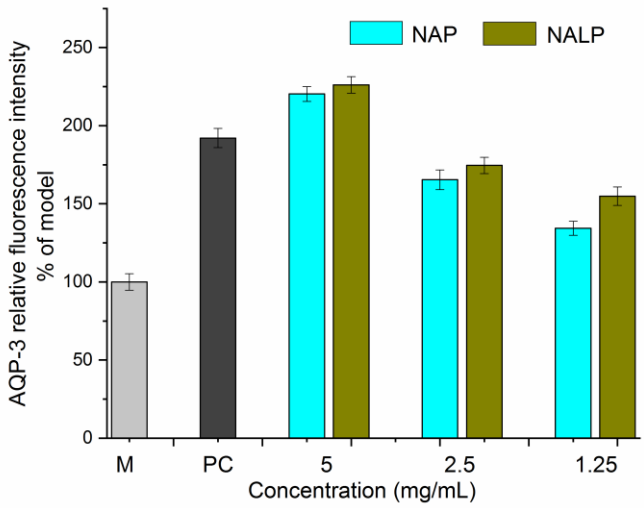

Figure 4. The expression level of AQP-3 protein.

4. Conclusion

In this study, a combination of deep eutectic solvent (DES) and enzymatic degradation would be utilised to obtain low molecular weight polysaccharide of *Naematelia aurantiabla* (NALP). Betaine-based DES could improve the NAP extraction efficiency significantly. NALP would be obtained by gentle degradation of composite enzymes (cellulase, pectase, and glucoamylase). Under the optimal of a combination of DES and enzymatic degradation, the molecular weight of NALP was below 5×10^5 Da. Results showed that NALP exhibited excellent antioxidant activity by inhibiting ROS generation and enhancing superoxide dismutase (SOD) activity. Meanwhile, NALP could inhibit the expression of IL-1α, IL-6, and TNF-α, showing excellent anti-inflammatory property. Aquaporin-3 expression assay also demonstrated that NALP had moisturizing activity. Above all, NALP would be a promising skin-care ingredient applied in the comestic industry.

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