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Based on *In Vitro* and 3D Epidermal Skin Model: The Efficacy of *Bletilla Striata* Extract for Skin Care

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Abstract

Introduction: *Bletilla Striata* (Thunb. ex A. Murray) Rchb. f.) Is a native herb of the orchidaceae family. Modern pharmacological activity studies have shown that *Bletilla striata* mainly has pharmacological activities such as promoting blood circulation and hemostasis, promoting wound healing anticancer, anti-tumor disease-resistant pathogenic microorganisms, protecting mucosa, anti-ulcer, whitening and anti-oxygen, anti-aging, and immune regulation, which have been widely recognized and applied. Research on the efficacy of plant extracts mainly adopts *in vitro* testing methods, such as cell experiment and 3D skin model experiment.

Objective: To evaluate the soothing effect of *Bletilla striata* extracton UVB-stimulated keratinocytes (HaCaT) and its barrier repair efficacy in Skinovo-Epi skin model.

Methods: The soothing and barrier repairing effects of *Bletilla striata* extract were studied *in vitro*. The cytotoxicity of the sample was measured by MTT assay, and cytotoxicity to keratinocytes was evaluated by cell survival rate. The soothing effect was evaluated by detecting changes in inflammatory cytokines (IL-6, IL-1 β , IL-8, TNF- α) secreted by UVB-stimulated keratinocytes with the sample treatment. Barrier repair efficacy was assessed in a Skinovo-Epi skin model by measuring changes in FLG (Filaggrin) and LOR (Loricrin) protein levels after topical application of the sample.

Results: The sample showed low cytotoxicity on keratinocytes. The sample significantly reduced UVB-stimulated secretion of inflammatory cytokines in keratinocytes. Topical application of the sample increased FLG and LOR protein levels in the Skinovo-Epi skin model.

Conclusions: *Bletilla striata* extract exhibits soothing and barrier repair activities *in vitro. Bletilla striata* extract reduces UVB-stimulated inflammation in keratinocytes and improves skin barrier function. The data provide novel experimental evidence supporting the innovative application of *Bletilla striata* for skin disorders. *Bletilla striata* has potential as a therapeutic herb for sensitive and damaged skin conditions.

Keywords: Bletilla striata extract; In vitro; 3D epidermal skin model; Skin care; Efficacy

Introduction

The skin is the largest organ of the human body. The skin is the first barrier against external attacks and stimuli. However, air pollution, ultraviolet exposure, and mechanical damage cause skin problems. These include roughness, sensitivity, and fragility, which bring us serious troubles. The industry is now about to research and develop safe, effective, and natural skin care products. Polysaccharide is a natural macromolecular polymer. It has a molecular weight of tens of thousands or even millions. It is widely present in living organisms. It carries important biological information in life activities. This information includes nucleic acid and protein [1]. In recent years, more and more plant polysaccharides have been isolated and identified. Studies have found that these plant polysaccharides have different biological activities. These include anti-tumor, immunomodulatory, antioxidant, and anti-inflammatory effects [2-4]. Therefore, more and more plant polysaccharides are used as raw materials for skin prote Plant polysaccharides and fungal polysaccharides offer good skin protection. They prevent skin water loss and maintain the



cuticle epidermal barrier. They also inhibit melanin production [5]. Cosmetics, which has a good and broad development prospect.

Bletilla striata (Thunb. ex A. Murray) Rchb. f.) is a native herb of the orchidaceae family. Modern pharmacological activity studies have shown that *Bletilla striata* mainly has pharmacological activities such as promoting blood circulation and hemostasis, promoting wound healing [6,7], anticancer, anti-tumor [8], disease-resistant pathogenic microorganisms, protecting mucosa, anti-ulcer, whitening and antioxygen, anti-aging, and immune regulation, which have been widely recognized and applied [9].

Based on *in vitro* method and 3D skin model, this paper systematically studied the effect of *Bletilla striata* extract on skin care. MTT cytotoxicity assay was used to evaluate the toxicity of the extract to keratinocytes (HaCaT). Based on the *in vitro* evaluation model of soothing effect, UVB was used to stimulate keratinocytes to produce inflammatory factors, and ELISA was used to evaluate the anti-inflammatory soothing effect of the extract. Based on a 3D epidermal skin model (Skinovo-Epi), the effect of the extract on skin barrier repair was evaluated by detecting changes in the content of FLG (Filaggrin) and LOR (Loricrin) proteins in the skin model.

Materials

Test System

The cells used in this test were human keratinocytes (HaCaT), and the model used in this test was the epidermal skin model (Skinovo-Epi).

Main Reagents

Bletilla striata extract (Guizhou Gui'an Precision Medicine Co. Ltd.); DMEM medium (VivaCell); maintenance medium (Skinovo); fetal bovine serum (Gibco); PBS (VivaCell); MTT (Sigma); DMSO (Chinese medicine); trypsin (Gibco); 4% paraformaldehyde (Sigma); vitamins C (CNW); Vitamin E (CNW); WY-14643(Pirinixic Acid).

Main Instruments

CO₂ incubator (Thermo, 160i); Biosafety cabinet (SujingAntai, BSC-1604IIA2); inverted microscope (Leica, DMi8); enzyme label instrument (Tecan, Spark); floor UV treatment instrument (Sigmago, SS-03AB).

Methods

Cytotoxicity test

Cell inoculation: The cells were inoculated into 96-well plates at the inoculation density of 1×10^4 cells/well, and incubated overnight in an incubator (37°, 5% CO₂).

Experimental grouping: The experiment was set up as zero setting group, control group, positive control group and sample group. In the sample group, 8 concentration gradients were set, and 3 repeat holes were set under each concentration gradient.

Liquid dispensing: Prepare sample working liquid according to the table 1.

Adding samples: Different solutions were added to the corresponding holes when the cell coverage rate in the 96-well plate reached 40%-60%. In control group, 200 μ L culture solution containing 10% PBS was added to each well. In the positive control group, 200 μ L culture solution containing 10% DMSO was added to each well. In each well of the sample group, 200 μ L culture solutions containing the corresponding concentration of samples were added. In the zero setting groups, there was no cell inoculation, and only 200 μ L

cell culture medium was added. The 96-well plates were then cultured in an incubator (37°C, 5% CO_2).

Detection: After incubation for 24 h, the supernatant was discarded, MTT working solution (0.5 mg/mL) was added, and the cells were incubated at 37°C in the dark for 2 h. After incubation, the supernatant was discarded and 100 μ L DMSO was added to each well, and OD values were read at 490 nm.

Cell relative viability calculation: The calculation is based on the following formula.

Detection of inflammatory factors

Cell inoculation: Cells were inoculated into 24-well plates at the inoculation density of 1×105 cells/well, and incubated overnight in an incubator (37°C, 5% CO₂).

Experimental grouping: Blank control group, negative control group, positive control group and sample group were set up in the experiment. Three concentration gradients were set in the sample group (Table 2).

Liquid dispensing: Prepare working liquid of different concentration according to the table1.

Adding samples: Different solutions were added to the corresponding holes when the cell coverage rate in the 24-well plate reached 40%-60%. The blank control group and the negative control group were added with 1 mL cell culture solution per well. In the positive control group, 1 mL culture medium containing 100 μ g/mL vitamin C and 7 μ g/mL vitamin E was added to each well; in each well of the sample group, 1 mL culture solution containing the corresponding concentration of the tested substance was added.

Radiation: Twenty-four hours after adding samples, the negative control group, the positive control group and the sample group received a total dose of 300 mJ/cm² of UVB radiation, while the blank control group was placed in the same environment (UVB radiation dose was 0 mJ/cm²).

Collection of cell supernatant: After incubation and culture for 24 h, the cell culture supernatant was collected in EP tube and frozen in the refrigerator at -80°C.

IL-1\beta content detection: According to the operating instructions of Human IL-1 β ELISA kit; IL-6 content detection: According to the operating instructions of Human IL-6 ELISA kit; TNF- α content detection: According to the operating instructions of Human TNF- α ELISA kit; IL-8.

Skin barrier protein test

Administration procedure

Model resuscitation: The skin model was transferred to a 24-well plate (0.9 mL model culture solution was added in advance) with 3 repeated holes in each group. The test group number was marked on the 24-well plate and incubated overnight in an incubator (37° C, 5% CO₂).

Administration: According to the experimental design in table 3, the sample was evenly applied to the surface of the skin injury model. After administration, the model was incubated in a CO_2 incubator (37°C, 5% CO_2) for 24 h.

Sample collection: After incubation, the residual test objects on the surface of the model were cleaned with a washing bottle containing sterile PBS solution, and the residual liquid inside and outside of the model was gently wiped with a sterile cotton swab. The model was



Table 1: Test concentrations setting table.

				Concentration	s setting (% V/V)			
Sample name	1	2	3	4	5	6	7	8	
Bletilla striata extract	10.000	5.000	2.500	1.250	0.625	0.313	0.156	0.078	

Table 2: Experimental design.

Groups	Sample concentrations (V/V)	Incentive condition	Test indexes	Test model	Test method
Blank control (BC)	/	/			
Negative control	/		IL-1β		
(NC)			IL-6		
Positive control	100 μg/mL Vc and 7 μg/	UVB irradiation	TNF-α	HaCaT	ELISA
(PC)	mL ve	300 mJ/cm ²	IL-8		
Bletilla striata extract	0.078%, 0.156%, 0.313%				

Table 3: Experimental design.

Groups	Sample concentrations (V/V)	Incentive condition	Test indexes	Test model	Test method
Blank control (BC)	/	/			
Negative control (NC)	/	/	FLG	Epidermal skin model (Skinovo-Epi)	Immunofluorescence
Positive control (PC)	WY-14643 (50 μM)	0.20% SDS	LOR		
<i>Bletilla striata</i> extract	0.78% (V/V)				

fixed in 4% paraformaldehyde, and subsequent immunofluorescence detection was performed.

Immunofluorescence detection

Baking sheet dewaxing: The paraffin slices are placed in a 7°C baking machine and baked for 4 hours.

Dewaxing hydration: The slices were soaked in xylene for 10 min, then soaked for 10 min after replacing xylene, soaked in anhydrous ethanol for 5 min, soaked in 95% ethanol for 5 min, and soaked in 75% ethanol for 5 min. Wash the slices with PBS buffer 3 times for 5 min each time.

Antigen repair: Put the paraffin slices into 0.01 M sodium citrate antigen repair solution, use high pressure repair, and remove the sections after cooling. Wash them with PBS buffer solution 3 times, 5 min/time.

Block peroxidase: Each slice was added with 1 drop of $3\% H_2O_2$ and incubated at room temperature for 30 min to block endogenous peroxidase activity. Wash each slice with PBS buffer solution 3 times, 5 min/time.

Serum blocking: Serum homologous to the second antibody was added to the slices and closed at 37°C for 60 min without washing.

Primary antibody incubation: Add primary antibody working solution to slices and incubate overnight at 4°C. Wash the slices with PBS buffer solution 3 times, 5 min/ time.

Secondary antibody incubation: Add the secondary antibody working liquid to the slices and incubate them at room temperature for 1 h. Wash the slices with PBS buffer solution 3 times, 5 min/ time.

Nuclear staining: After the second antibody incubation, Wash the slices with PBS buffer 3 times, 5 min/time. Remove the PBS solution attached to the slide, add 100 μ L Hochest 33342 working solution to each slice, and incubate them at room temperature for 5 min.

Images acquisition: Wash the slices with PBS buffer solution 3 times, 5 min/time. Wipe off the PBS solution with absorbent paper and seal the slices with a drop of anti-quench agent. Fluorescence microscope images were taken within 24 hours.

Results

Cytotoxicity Test Results

• Eight dosages were set for the sample and cytotoxicity test was carried out. The MTT test results were shown in table 4.

• With the 8 concentrations of the sample as the horizontal coordinate and the relative cell activity value as the vertical coordinate, the relative cell activity diagram was drawn (Figure 1).



Table 4: MTT Test Results.

Cell viability (%)	Control	Positive control (PC)			Concer	ntration gradi	ent (% V/V)			
	/	10% DMSO	10.000	5.000	2.500	1.250	0.625	0.313	0.156	0.078
Mean	100.00	26.95	46.67	51.61	57.62	66.89	80.87	87.99	96.98	101.54
SD	5.33	2.28	0.85	2.34	1.35	4.24	0.53	0.92	1.01	3.27





• Therefore, according to the results of MTT, when the sample concentration was less than or equal to 0.625% (m/V), it was not toxic to keratinocytes.

Results of IL-1ß content detection

• Statistical analysis was performed by t-test method. The Negative Control (NC) group was compared with the blank control (BC) group, and the significance is represented by #, p-value <0.05 was represented by #, and p-value <0.01 was represented by ##. The sample group and the Positive Control (PC) group were compared with the Negative Control (NC) group and the significance is represented by* p-value <0.05 was represented by*, and p-value <0.01 was represented by*

• Compared with the Blank Control (BC) group, the secretion of keratinocyte inflammatory factor IL-1 β in the Negative Control (NC) group was significantly increased (p<0.01), indicating that the stimulation conditions of this experiment are effective.

• Compared with the Negative Control (NC) group, vitamin C and vitamin E in the Positive Control (PC) group significantly reduced the secretion of keratinocyte inflammatory factor IL-1 β (*p*<0.01), indicating that the positive control test of this experiment is effective.

• Compared with the Negative Control (NC) group, the secretion of keratinocyte inflammatory factor IL-1 β was significantly decreased at 0.078%, 0.156% and 0.313% (V/V) sample concentrations (p<0.01), indicating that the tested sample has soothing effect at these concentrations.

Results of TNF-a content detection

• Based on the experimental method, the cell supernatant was collected and the TNF- α content was detected. The detection results and the change trend were shown in figure 3.

• Statistical analysis was performed by t-test method. The Negative Control (NC) group was compared with the Blank Control (BC) group, and the significance is represented by #, p-value <0.05 was represented by #, and p-value <0.01 was represented by ##. The sample group and the Positive Control (PC) group were compared with the Negative Control (NC) group, and the significance is represented by* p-value <0.05 was represented by*, and p-value <0.01 was represented by*

• Compared with the Blank Control (BC) group, the secretion of keratinocyte inflammatory factor TNF- α in the Negative Control (NC) group was significantly increased (p<0.01), indicating that the stimulation conditions of this experiment are effective.

• Compared with the Negative Control (NC) group, vitamin C and vitamin E in the Positive Control (PC) group significantly reduced the secretion of keratinocyte inflammatory factor TNF- α (*p*<0.01), indicating that the positive control test of this experiment is effective.

• Compared with the Negative Control (NC) group, the secretion of keratinocyte inflammatory factor TNF- α was significantly decreased at 0.078%, 0.156% and 0.313% (V/V) sample concentrations (p<0.01), indicating that the tested sample has soothing effect at these concentrations.

Results of IL-6 content detection

• Based on the experimental method, the cell supernatant was collected and the IL-6 content was detected. The detection results and the change trend were shown in figure 4.



• Statistical analysis was performed by t-test method. The Negative Control (NC) group was compared with the Blank Control (BC) group, and the significance is represented by #, p-value <0.05 was represented by #, and p-value < 0.01 was represented by ##. The sample group and the Positive Control (PC) group were compared with the Negative Control (NC) group, and the significance is represented by* p-value <0.05 was represented by*, and p-value <0.01 was represented by*

• Compared with the Blank Control (BC) group, the secretion of keratinocyte inflammatory factor IL-6 in the Negative Control (NC) group was significantly increased (p<0.01), indicating that the stimulation conditions of this experiment are effective.

• Compared with the Negative Control (NC) group, vitamin C and vitamin E in the Positive Control (PC) group significantly reduced the secretion of keratinocyte inflammatory factorIL-6 (p<0.01), indicating that the positive control test of this experiment is effective.

• Compared with the Negative Control (NC) group, the secretion of keratinocyte inflammatory factor IL-6 was significantly decreased at 0.078%, 0.156% and 0.313% (V/V) sample concentrations (p<0.01), indicating that the tested sample has soothing effect at these concentrations.

Results of IL-8 content detection

• Based on the experimental method, the cell supernatant was collected and the IL-8 content was detected. The detection results and the change trend were shown in figure 5.

• Statistical analysis was performed by t-test method. The Negative Control (NC) group was compared with the Blank Control (BC) group, and the significance is represented by #, p-value <0.05 was represented by #, and p-value <0.01 was represented by ##. The sample group and the Positive Control (PC) group were compared with the Negative Control (NC) group, and the significance is represented by* p-value <0.05 was represented by*, and p-value <0.01 was represented by*

• Compared with the Blank Control (BC) group, the secretion of keratinocyte inflammatory factor IL-8 in the Negative Control (NC) group was significantly increased (p<0.01), indicating that the stimulation conditions of this experiment are effective.

• Compared with the Negative Control (NC) group, vitamin C and vitamin E in the Positive Control (PC) group significantly reduced the secretion of keratinocyte inflammatory factor IL-8 (p<0.01), indicating that the positive control test of this experiment is effective.

• Compared with the Negative Control (NC) group, the secretion of keratinocyte inflammatory factor IL-8 was significantly decreased at 0.078%, 0.156% and 0.313% (V/V) sample concentrations (p<0.01), indicating that the tested sample has soothing effect at these concentrations.

FLG Test Results

• FLG immunofluorescence detection was performed after the skin model was cultured. The summary of the picture results was shown in figure 6 (green fluorescence represented FLG, blue fluorescence represented nucleus), and the relative IOD values were shown in figure 7.

• Statistical analysis was performed by t-test method. The Negative Control (NC) group was compared with the Blank Control (BC) group, and the significance is represented by #, p-value <0.05 was









Figure 5: Change trend of IL-8 content.



Groups	Duplicate hole-1	Duplicate hole-2	Duplicate hole-3
Blank control (BC)			
Negative control (NC)			
Positive control (PC) (WY -14643)			an a
Bletilla striataextract- 0.78%			la glad da ta Giorre Jaket Alder

Figure 6: Summary of FLG protein immunofluorescence staining results.



represented by #, and p-value <0.01 was represented by ##. The Positive Control (PC) group and the sample group were compared with the Negative Control (NC) group, and the significance is represented by* p-value <0.05 was represented by*, and p-value <0.01 was represented by**.

• Under the test conditions, the FLG protein content in the Negative Control (NC) group was significantly decreased compared with that in the Blank Control (BC) group (p<0.05), indicating that the negative control test was effective.

• Compared with the Negative Control (NC) group, the FLG protein content in the Positive Control (PC) group was significantly increased (p< 0.01), indicating that the positive control detection was effective.







• Compared with the Negative Control (NC) group, the content of FLG protein in the model was significantly increased after adding the *Bletilla striata extract* with concentration of 0.78% (V/V) (p<0.05).

LOR Test Results

• LOR immunofluorescence detection was performed after the skin model was cultured. The summary of the picture results was shown in figure 8 (green fluorescence represented LOR, blue fluorescence represented nucleus), and the relative IOD values were shown in figure 9.

• Statistical analysis was performed by t-test method. The Negative Control (NC) group was compared with the Blank Control (BC) group, and the significance is represented by #, p-value <0.05 was represented by #, and p-value <0.01 was represented by ##. The Positive

Control (PC) group and the sample group were compared with the Negative Control (NC) group, and the significance is represented by* p-value <0.05 was represented by*, and p-value <0.01 was represented by**.

• Under the test conditions, the LOR protein content in the Negative Control (NC) group was significantly decreased compared with that in the Blank Control (BC) group (p<0.01), indicating that the negative control test was effective.

• Compared with the Negative Control (NC) group, the LOR protein content in the Positive Control (PC) group was significantly increased (p < 0.05), indicating that the positive control detection was effective.

• Compared with the Negative Control (NC) group, the content of LOR protein in the model was significantly increased after



adding the *Bletilla striata extract* with concentration of 0.78% (V/V) (p<0.05).

Conclusions

Wound inflammation is caused by an injury to the skin or mucosal tissue. It's an inflammatory reaction. It is a natural defense response of skin tissue to injury or infection. During the inflammatory phase, the body aims to remove dead tissue, pathogens, and other harmful substances. It also stimulates fibroblasts for differentiation and repair [9].

In the study of skin inflammation, researchers use UVB irradiation. This stimulates keratinocytes. This causes them to produce a variety of inflammatory factors, such as IL-1 β , IL-6, and TNF- α , and IL-8. Researchers study how these factors change during skin inflammation's development and resolution. This helps them understand the relevant mechanisms.

Interleukin-1 (IL-1) is an important pre-inflammatory cytokine. Mononuclear macrophages and skin fibroblasts produce it. There are two subtypes of IL-1, IL-1 α and IL-1 β . They have no significant difference in biological activity. Yet, IL-1 β is the main expression form of IL-1 [10].

Tumor necrosis factor- α (TNF- α) is a key cytokine in the inflammatory response. It plays an important role in the early inflammation. Mononuclear macrophages and NK cells produce TNF- α . It promotes the activation of inflammatory cells. It induces the release of other inflammatory factors. It also regulates the immune response. TNF- α can work with IL-1 β to further enhance the inflammatory response. At the same time, TNF- α can also induce the synthesis and release of IL-6. This further amplifies the inflammatory response. TNF- α can also promote the activation of endothelial cells. It can also induce the synthesis and release of IL-8 [11]. Interleukin-6 (IL-6) is a cytokine in the chemokine family. It can regulate T cell activity in chronic inflammatory response [12]. Interleukin-8 (IL-8) is another important chemokine. It attracts white blood cells (such as neutrophils) to the site of inflammation. This affects the skin's inflammatory response.

Previous studies have shown that *Bletilla striata* extract can reduce the secretion of IL-1 β , TNF- α , IL-6, and other cytokines. This happens by affecting the expression of JNK and P38 MAPK genes, and various proteins. This action reduces the inflammatory response [12].

We conducted experimental tests to further investigate the soothing effect of the *Bletilla striata* extract on skin inflammation. We used a UVB-stimulated keratinocyte (HaCaT) model. The tests involved stimulating keratinocytes with UVB. This induced the production of inflammatory factors.

The researchers found that, at concentrations of 0.078%, 0.156%, and 0.313% (V/V), the *Bletilla striata* extract reduced the secretion of keratinocyte inflammatory factors IL-1 β , IL-6, TNF- α , and IL-8 (p<0.01). The results indicated that *Bletilla striata* extract could relieve inflammation symptoms. It did so by inhibiting the secretion of inflammatory factors like IL-1 β , IL-6, and TNF- α , and IL-8. This played a soothing role in skin inflammation.

We also found that the *Bletilla striata* extract could promote the expression of FLG and LOR proteins. FLG (Filaggrin) is a protein secreted by keratinocytes. It can produce natural moisturizing factors. FLG plays an important role in maintaining skin barrier function [13]. LOR is the basic component of the differentiated keratinocyte envelope [14].

Researchers based this study on a 3D epidermal skin model. After adding *Bletilla striata* extract at a concentration of 0.78% (V/V), the model's FLG and LOR protein content increased compared with the NC group (p<0.05). This suggests the extract could further affect skin keratinization. It might do this by promoting FLG and LOR protein expression. Thus, the *Bletilla striata* extract could repair the skin barrier.

In summary, the *Bletilla striata* extract soothes skin inflammation. It does this by inhibiting the secretion of inflammatory factors and promoting skin barrier repair. These results provide a strong experimental basis. This basis supports further developing the application of *Bletilla striata*. It treats skin inflammation.

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