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Study on the Soothing and Repairing Effects of *Artemisia Annua* Combined with *Centella Asiatica* Extract

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Abstract: *The soothing and repairing effects of Artemisia annua and Centella asiatica extract composition were investigated in cell, zebrafish, and human clinical experiments. The effects of the composition on the secretion of TNF-α from RAW264.7 (mouse macrophages) were studied in cell experiments, and the effects of the composition on the proliferation of HCEC (human corneal epithelial cells) and HaCaT (human immortalized epidermal cells) were also studied. The repairing effect of the composition was verified by zebrafish experiment. In the human clinical experiment, the changes in skin transdermal water loss TEWL value and skin redness a* in volunteers before and after using the gel of the composition were tested. The results showed that the composition had no significant stimulating effect on HCEC, but significantly inhibited the secretion of TNF-α by RAW cells. The composition significantly promoted the migration of HaCaT cells and the repairing of zebrafish tail fins. The human clinical experiment showed that the rate of change of skin transdermal water loss TEWL value of the gel was -17.95% (P<0.001), and skin redness was significantly reduced (P<0.01). In conclusion, the combination of Artemisia annua and Centella asiatica extract has soothing and repairing effects.*

Keywords: *Artemisia annua; Centella asiatica; Sooth and repair; Cells; Zebrafish; Human clinical trial*

1. Introduction

Artemisia annua is an annual herb belonging to the dicotyledonous family, Asteraceae, and Artemisia genus and is widely distributed. In ancient times, it could be used for clearing heat, relieving summer heat, resisting malaria, etc. It could also be applied to the skin as an external drug, and can also be used as a spice, containing rich volatile oil. It contains artemisinin, artemisinic acid, artemisinol, flavonoids and so on[1-5]. In 1967, a national antimalarial research project was initiated in China, and more than 380 herbal extracts were evaluated by Chinese scholar Tu Youyou for their antimalarial activities, and *Artemisia annua* was found to be the most active herb[6-7]. It is well known that artemisinin is an important active ingredient in the fight against malaria[8-11]. In recent years, with the deepening of research, artemisinin has been found to have anti-inflammatory and antibacterial biological activities[12-14] and has been gradually explored and applied by the cosmetic industry[15-16], while the soothing and oil-controlling effects of *Artemisia annua* extract need further study, and the mechanism of action is still unclear.

Centella asiatica, also known as Asian water cotyledon, tiger grass, horseshoe grass, etc., belongs to the Umbelliferae family of herbs. It was first found in Asia and is mainly distributed in tropical areas of China, India, Pakistan, Madagascar, equatorial Africa, Central America, and Oceania[17]. The main components of Asiaticoside include asiaticoside, hydroxy Asiaticoside, Asiaticoside and hydroxy cistoxalic acid[18-19]. Among them, Asiaticoside is considered to be the main ingredient that exerts its effect[20]. Three thousand years ago, in places such as China and India, the herb was used as a "cure-all", an anti-inflammatory agent to improve the healing of minor wounds, burns, and hypertrophic wounds. It has also been recommended as an antipyretic, diuretic, rheumatic, antibacterial, antiviral, and used to treat venous insufficiency, improve cognition, relieve anxiety, and as an anticancer agent[21]. In recent years, *Centella asiatica* extract has also been used as an ingredient in cosmetics, which can resist photoaging and light lines[22]. It has a good antibacterial effect and can achieve anti-inflammatory effects by raising and repairing the skin barrier[23-25], so it can be made into acne products and cosmetics for

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post-sun repair. At the same time, triterpenoid compounds of *Centella asiatica* can also promote collagen synthesis, smooth fine lines, and maintain skin elasticity[26-27].

Inflammation is a defensive immune response triggered by bacterial or viral infection, noxious stimulation, or cell injury. In damaged tissues, it leads to the activation of immune cells such as macrophages, monocytes and neutrophils, which produce pro-inflammatory factors such as tumor necrosis factor TNF-α and interleukin IL-6[28], thus triggering the body's inflammatory response. The stratum corneum of the skin is like a brick wall, in which the keratinocytes or "bricks" are surrounded by intercellular lipid lamellae that act like "mortar" to maintain the integrity of the stratum corneum and the skin permeability barrier[29-30]. While repair is the repairing of damaged skin with cosmetics to restore the barrier function of the stratum corneum after skin damage. As consumer demand for soothing and repairing skincare products continues to increase, the study of the soothing and repairing effects of naturally extracted plants has become a hot spot in the cosmetic industry[31]. The evaluation methods of soothing and repairing effects are mainly divided into in vitro methods and in vivo methods. In vitro methods include cell biology and zebrafish experiment methods, while in vivo methods include human efficacy evaluation and so on. The cell biology method evaluates the soothing effect of the sample by detecting the changes of TNF-α and other inflammatory factors in the cell. It also included cell migration to reflect the repairing effect of the sample and zebrafish tail fin repairing experiment. In the human efficacy evaluation method, professional evaluators evaluate and measure the repair effect after using cosmetics[25].

At present, more and more people are paying attention to their appearance, and the market demand for soothing and repairing cosmetics is increasing. In recent years, *Artemisia annua* and *Centella asiatica* extract have also been used as ingredients in cosmetics, and the soothing and repairing effects of their composition need to be further investigated. In this study, the effects of *Artemisia annua* and *Centella asiatica* extract composition on the proliferation toxicity of human corneal epithelial cells (HCEC) and TNF-α secretion in mouse mononuclear macrophages (RAW264.7) were investigated. The effects of the composition at different concentrations on the proliferation and migration of human immortalized epidermal cells (HaCaT) and zebrafish tail fin repair were measured. In vivo experiment, the gel of the composition was prepared, and the changes of transdermal skin water loss and skin redness a* value of volunteers were detected to verify the soothing and repairing effects of the composition and to provide theoretical guidance and data support for the development and use of *Artemisia annua* and *Centella asiatica* extract cosmetics.

2. Materials and Methods

2.1. Materials

Reagents: Potassium glycyrrhizinate, anhydrous sodium acetate, anhydrous sodium carbonate, acetylacetone, p-dimethylaminobenzaldehyde, sodium hyaluronate, purchased from Shanghai Maclin Biochemical Technology Co., LTD. Methylthiazolyl tetrazolium(MTT), vitamin C, dimethyl sulfoxide(DMSO), purchased from Sigma-Aldrich Corporation. Anhydrous ethanol, anhydrous calcium chloride, purchased from Tianjin Damao Chemical Reagent Co., LTD. All reagents were of analytical grade. Phosphate buffered saline(PBS), fetal bovine serum(FBS), purchased from Gibco Life Technologies, USA. Cell culture medium, purchased from Hyclone, USA. *Artemisia annua* extract, and *Centella asiatica* extract, purchased from Yunnan Eng Biotechnology Co., LTD. RAW264.7, HCEC, HaCaT, purchased from Beina Biotechnology Co., LTD. AB strain zebrafish, purchased from Hangzhou Hunter Biotechnology Co., LTD. Experimental equipment: DH-160HI CO2 incubator, Shanghai Santn Instrument Co., LTD. Super clean workbench, Suzhou Antai Instrument Co., LTD. Multiskan Sky, Thermo Fisher Scientific, USA. Thermostatic water bath, Shanghai Yiheng Technology Co., LTD. Tewameter® TM Hex transdermal water loss tester, CL400 skin redness tester, CK, Germany.

2.2. Stimulation Test of Human Corneal Epithelial Cells (HCEC)

When the cell density reaches or exceeds 80%, adjust the cell density to $1*10^4$ per well according to the counting result, inoculate the cells into 96-well plates, and return them to the incubator (37℃, 5% CO_2) for incubation. In the experiment, a control group and experimental groups were established. The samples in the experimental groups were subjected to 6 concentration gradients obtained by half dilution of *Artemisia annua* (1%) and *Centella asiatica* (0.1%) extract. In addition, a positive control group was established using 0.05% sodium dodecyl sulfate (SDS), and three replicate wells were included for each

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condition. After incubation for 24 hours, samples prepared with PBS (100 μL) were added to each well of the experimental group and incubated for an additional 5 minutes. After this treatment period, the original medium was removed, washed three times with PBS, and a CCK-8 working solution (100 μL) was added to each well. The culture plate was then returned to the incubator for an additional 2 hours before the absorbance was measured at a wavelength of 450 nm. Cell viability was calculated.

2.3. TNF-α Inhibition in RAW 267.4 Cells

RAW264.7 cells that had reached approximately 80% confluence were aseptically transferred to a centrifuge tube for collection. After enumeration using a cell counting plate, the appropriate sample concentration was selected based on the results of the cell viability assay. Dexamethasone (100 μg/mL) was used as a positive control for the experiment. Lipopolysaccharide (1 μg/mL) was added one hour after the addition of the test sample. After 24 hours of incubation, the cell supernatant was collected and centrifuged at 1000 rpm for 10 minutes. The resulting supernatant was then transferred to a centrifuge tube for determination of TNF-α concentration using a mouse tumor necrosis factor-α kit that was equilibrated at room temperature for 30 minutes before use.

2.4. Proliferation Experiment of HaCaT Cells

When the cell growth was stable, the cells were inoculated into 96-well plates at $1*10^4$ per well and incubated in the incubator. In the experiment, the blank control group and the experimental groups were set up. Five concentration gradients were set up for the samples, and 3 multiple wells were set up. According to the experimental design, the subjects were assigned different concentrations of the basal medium. After 24 hours, the samples were added according to the experimental groups. After 24 hours of treatment, the 96-well plate was removed, 20 μL MTT working fluid (5 mg/mL) was added to each well, and the plate was returned to the incubator for 4 hours, then the liquid in the well was removed and 100 μL DMSO was added to each well again. Absorbance (OD value) was measured at a wavelength of 490 nm after shaking for 10 minutes.

2.5. Repair Experiment of Zebrafish Tail Fin

Fish were bred according to the male: female ratio of 1:2, and eggs were collected and incubated overnight in a 28℃ incubator. A blank control group (no tail fin cut) and experimental groups (tail fin cut with a scalpel) were established. The experimental groups included a model group and sample groups, each with 10 larvae of 3dpf. The appropriate sample concentration was selected as the test concentration and the sample solution to be tested was configured. After dosing, the 6-well plate was placed in the incubator and incubated for 48 hours without light. After dosing, the fish was photographed using the methylcellulose-loaded slide. The tail fin area of fish was measured using ImageJ, and the repair rate of the tail fin area was calculated.

2.6. Methods of Human Clinical Trials

2.6.1. Sample Preparation

In the human clinical studies, the sample used was a cosmetic liquid gel formulated by the laboratory using the composition of *Artemisia annua* and *Centella asiatica* extract. The formulation is shown in Table 1, with ARISTOFLEX AVC as the thickening agent, phenoxyethanol as the preservative, and glycerin as the humectant. Once these three ingredients were thoroughly mixed, 1% *Artemisinin annua* and 0.1% *Centella asiatica* extract were added.

Table 1: Grouping of test samples.

Group	Sample name	Character	Production date	Shelf life
Sample group	1% *Artemisinin extract* +0.1% *Centella asiatica* extract +0.6%ARISTOFLEX AVC+0.4% phenoxyethanol +2% glycerin + deionized water to 100%	gels	2023.10.25	Three years
Control group	/	/	/	/

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2.6.2. Stability Test

The prepared gel was packaged in a clear glass bottle with a sample size of 10 mL per bottle. The heat resistance test was set at 40℃ and the cold resistance test was set at -10℃, while the normal temperature test was sealed and placed at normal temperature. An optical aging test was performed under incandescent light. After sealing and for three months, the changes in appearance were observed.

2.6.3. Test Method of Human Efficacy

Tests were conducted on 26 volunteers aged 18-60 with no injuries on the inner arm. A sample group and a control group were established, and the test area on the inner arm of each subject was randomly assigned. The forearm of the above subjects was torn 20 times with medical tape to induce skin barrier dysfunction, and 5 mg/mL of histamine was added to create a model for 10 minutes (marked erythema). One area was used with the test product and the other was left blank as a negative control area. The skin transdermal water loss rate (TEWL) and skin redness a* values of the forearm were measured before the tape was torn, immediately after product application (i.e., immediately after histamine), and 2 hours after product application. The short-term repairing effect of the product on the skin barrier damage caused by the skin irritation induced by the tear of the tape was evaluated by the difference of the skin indices before and after the application of the gel.

2.7. Data Analysis

SPSS 22.0 was used for data analysis. Before statistical analysis, the parameters were tested for normal distribution. If the data were normally distributed, the analysis was performed by *t*-test. If the data were not normally distributed, the rank sum test was used for analysis. $P<0.05$ indicated that the sample was significant.

3. Results and Discussion

3.1. Effects of the Composition on HCEC Cells

As shown in Table 2, the survival rate of cells in the positive group after SDS stimulation decreased significantly ($P < 0.01$), and the mean survival rate was 13.69%, indicating that the stimulation conditions were effective. The survival rate of HCEC cells was more than 80%, and the highest survival rate was 89.58% at 0.125%+0.0125% ($P < 0.05$), indicating that the composition is almost non-irritating. Ruszymah et al.[32] showed that the extract of *Centella asiatica* extract could promote the proliferation and migration of rabbit corneal epithelial cells, indicating that *Centella asiatica* extract has certain soothing and repairing effects. In this experiment, the combination of *Artemisia annua* and *Centella asiatica* extract also obtained soothing and non-irritating results.

Table 2: Effects of Artemisia annua and Centella asiatica composition on HCEC cell viability.

Group	HCEC survival rate (%)	*P* values with the blank group	*P* values with the positive group
Blank control group	98.81 ± 3.61	/	0.005**
Positive group	13.69 ± 7.31	0.005**	/
1%+0.1%	86.31 ± 2.87	0.015*	0.005**
0.5%+0.05%	85.42 ± 4.12	0.059	0.004**
0.25%+0.025%	89.29 ± 2.68	0.004**	0.006**
0.125%+0.0125%	89.58 ± 1.36	0.048*	0.003**
0.0625%+0.00625%	81.55 ± 1.03	0.022*	0.003**
0.03125%+0.003125%	88.69 ± 1.36	0.041*	0.003**

Note: Compared with the blank group or the positive group, "*": $P<0.05$, indicating a significant difference, "**" : $P<0.01$, indicating a very significant difference.

3.2. Effects of the Composition on TNF-α Levels

Induction of RAW264.7 cells to secrete tumor necrosis factor TNF-α and other inflammatory factors by lipopolysaccharide is a classic model of inflammatory pathways, and if the drug can inhibit the secretion of TNF-α by RAW264.7, it indicates that this drug has a soothing and anti-inflammatory effect[33-35]. As shown in Figure 1, the concentration of TNF-α in the model group was significantly

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increased after lipopolysaccharide stimulation ($P < 0.01$), which proved that the experiment was effective. At the concentrations of 1%+0.1%, 0.5%+0.05%, and 0.1%+0.01%, the *Artemisia annua* and *Centella asiatica* extract composition could significantly inhibit the TNF-α secreted by RAW264.7 induced by lipopolysaccharide. The inhibition rates were 69.85%, 62.69%, and 54.06%, respectively.

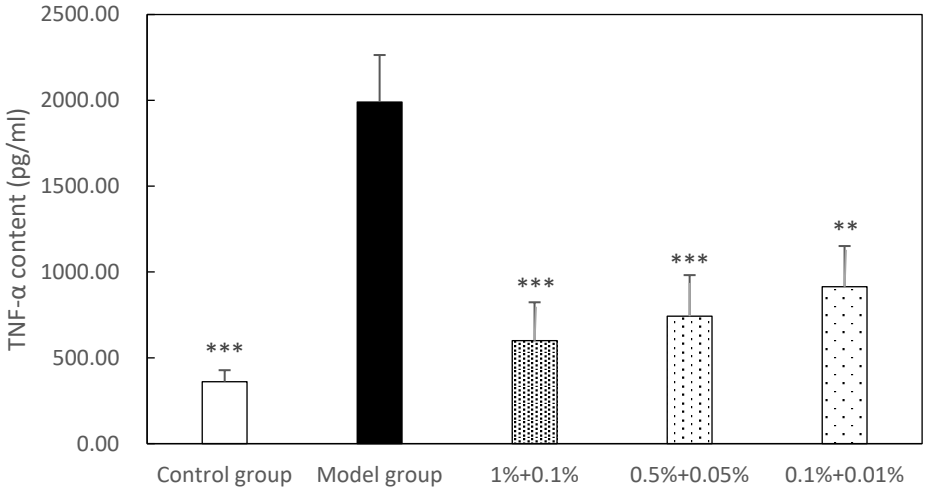

Figure 1: Effect of Artemisia annua and Centella asiatica on TNF-α levels.

3.3. Effects of the Composition on HaCaT Cell Proliferation

Cell proliferation is one of the important physiological functions of living cells and an important life characteristic of living organisms, indicating cell vitality and state. Suitable for screening materials and formulations that promote cuticle thickening, skin barrier stability and health. When the cell proliferation rate of the sample group was higher than that of the negative group, it was considered that the tested substance at this concentration could promote the proliferation of keratinocytes and promote the proliferation and metabolism of the stratum corneum, and it was concluded that the tested substance had a repairing effect. The survival rate is shown in Figure 2. Compared with the control group, the value-added rates of the composition at 1%+0.1% and 0.25%+0.025% were 105.54% and 101.86%, respectively, indicating that the composition could promote the proliferation of HaCaT cells, indicating that the composition of *Artemisia annua* and *Centella asiatica* had a certain repairing effect, which is consistent with the findings of Diniz et al.[36] and Wan et al.[37].

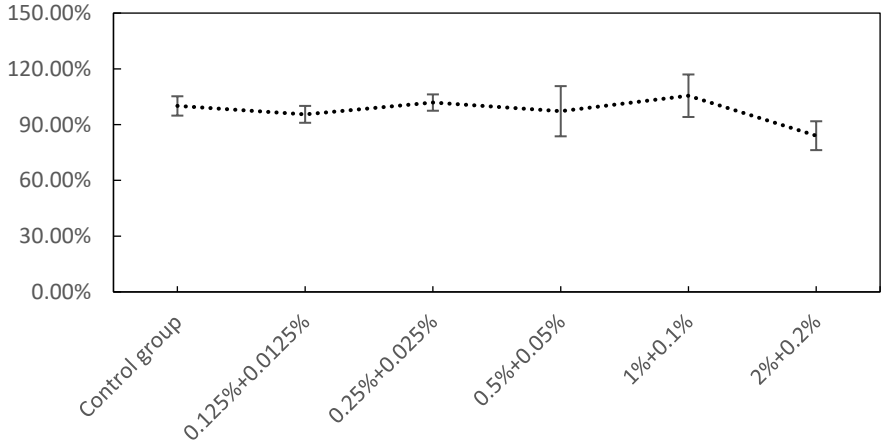

Figure 2: Effect of the composition on HaCaT cell proliferation.

3.4. Repair Experiment of Zebrafish Tail Fin

Zebrafish have the remarkable ability to fully regenerate a lost appendage, faithfully restoring its size, shape, and tissue patterning. Studies over the past several decades have identified mechanisms underlying the formation, spatial organization, and regenerative growth of the blastema, a pool of

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proliferative progenitor cells that closely resemble key steps and principles of human wound healing and can be used to evaluate the repair efficacy of drugs[38-40]. Results showed that the tail fin area of the negative group was significantly lower than that of the blank group (Table 3). At 0.5%+0.05% and 0.1%+0.01% of *Artemisia annua* and *Centella asiatica* extract composition, there were significant differences compared with the negative group (P<0.05), and the repair rates of tail fins were 30.47% and 30.95%, respectively.

Table 3: Comparison of zebrafish tail fin repair area.

	Tail fin area	Repair rate	*P*-value
Negative group	69665.17±2409.71	/	/
Blank control group	108024.89±12486.51	100.00%	0.001**
0.5%+0.05%	81351.50±6072.50	30.47%	0.003**
0.1%+0.01%	81538.00±7205.13	30.95%	0.013*

3.5. Results of Human Clinical Trials

The stability test results showed that no precipitation was observed in the heat resistance test, the cold resistance test, the normal temperature test and the photoaging test, no precipitation was observed and the original appearance was maintained.

3.5.1. Results of skin transdermal water loss TEWL value[41]

The smaller the TEWL value of normal skin transdermal water loss, the better the barrier effect of the skin and the higher the water content of the skin. Conversely, the higher the TEWL, the more damaged the skin barrier and the lower the skin moisture content. As can be seen from Table 4, after tape tearing and histamine stimulation, the skin barrier was damaged, the skin TEWL value significantly increased, and the skin TEWL value significantly decreased 2 hours after sample coating compared with the initial value (P<0.001). After 2 hours of application, the rate of change of TEWL value in the sample group was significantly lower than that in the blank group (P<0.001), indicating that the application of the sample group had a significant effect on reducing the transdermal water loss after 2 hours.

Table 4: Results of skin transdermal water loss TEWL value.

	Group	Histamine before	Histamine after	2 hours
Mean value	Sample group	8.14 ±1.40	20.64 ±7.96	7.74 ±1.83
	Blank group	8.29 ±1.84	23.20 ±8.28	8.86 ±5.49
Rate of change	Sample group	/	153.60%***	-62.50%***
	Blank group	/	179.90%***	-61.79%***

Note: Self-change rate was compared before and after histamine and after 2 hours of coating; significance between groups was the comparison of the 2-hour change between groups. ***: P<0.001, indicating a highly significant difference.

3.5.2. Skin redness a value*

The results in Table 5 showed that after repeated tape tearing and histamine application, the skin showed obvious erythema accompanied by a significant increase in the a* value, which indicates skin redness. The a* value of skin redness was measured at 0 hours after modeling and 2 hours after sample application. Notably, the a* value at 2 hours in the sample group showed a significant reduction compared to its initial value (at 0 hours) after modeling (P<0.05). Furthermore, the rate of change of the redness a* value in the sample group was significantly lower than that observed in the blank group (P<0.01), suggesting that after 2 hours of use, there was a significant decrease in skin redness as indicated by the reduced a* value in the sample group, thus demonstrating its soothing effect.

Table 5: Results of skin redness a value.*

	Group	Histamine before	Histamine after	2 hours
Mean value	Sample group	8.09 ±1.03	10.63 ±1.73	8.16 ±1.07
	Blank group	8.28 ±1.05	11.30 ±1.41	8.79 ±0.94
Rate of change	Sample group	/	31.41%***	-23.26%***
	Blank group	/	36.43%***	-22.24%***

Note: Self-change rate was compared before and after histamine, and after 2 hours of coating. Significance between groups was the comparison of the 2-hour change between groups. **: P<0.01, ***: P<0.001, indicating a highly significant difference.

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4. Conclusion

The results of the HCEC experiment showed that the composition of *Artemisia annua* and *Centella asiatica* extract had no irritation, and significantly inhibited the secretion of TNF-α at the concentrations, which proved that the composition had a certain soothing effect. The results of HaCaT cells and zebrafish experiments showed that the composition had certain repairing effects. Human clinical experiment results showed that the composition had an obvious repairing effect as well as a soothing effect. In conclusion, the composition of *Artemisia annua* and *Centella asiatica* extract has certain soothing and repairing effects on the skin, and its application in the cosmetic industry will have a good development prospect.

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