A novel *Cordyceps militaris* Ferment Extract with Chinese characteristics targets on well aging

Shaojing Sun, Liangliang Wen, Yan Jin, Wenjie Qu, Hua Mao, Liang Ma, Ruiyan Wang, Xueping Guo, Zhen Lu*,

Bloomage Biotechnology Corp., Ltd

No. 678 Tianchen St., High-Tech Development Zone, Jinan, China

* Zhen Lu , Bloomage Biotechnology Corp., Ltd

No. 678 Tianchen St., High-Tech Development Zone, Jinan, China

e-mail: luzhen@bloomagefreda.com, Tele:+86 13791078877

ABSTRACT

As a Chinese characteristic herb, *Cordyceps militaris* has been widely used in food, health care products, skin care products and other fields. However, the active ingredients in *Cordyceps militaris* are difficult to be fully utilized in traditional extraction technology. For that reason, *Cordyceps Militaris* Ferment Extract (CMF) is developed by high-efficiency fermentation process using highly active *Lactobacillus* as the strain and Cordyceps militaris as the main substrate. CMF can effectively regulate the hypoxia environment of skin and improve the aging problems caused by skin hypoxia, such as inflammation, collagen loss, pigmentation and elasticity reduction.

As a switch that regulates cellular respiration, Hypoxia-inducible factors (HIF-1α) is activated under hypoxia and causes more than one hundred downstream gene responses. The expression of HIF-1α was significantly increased by 8% with 0.1% CMF, so as to activate the anti-aging pathway. Concretely, it is showed that 0.1% CMF could significantly improve the expression of collagen I protein by 23% in fibroblasts model. Meanwhile, 0.1% CMF up-regulated the expression of LC3B in cells by 15%, thus it is indicated that CMF significantly improved cellular activity and autophagy ability. Further, CMF has a very strong free radical scavenging ability, and it can remove more than 67.8% of hydroxyl free radicals at the concentration of 2% (v/v). Moreover, when the concentration was 3.0% (v/v), the inhibition rate on melanoma production was as high as 36.7%. Additionally, CMF has a certain inhibitory effect on LPS stimulated mouse macrophages to produce IL-6, TNF-α, IL-1β three inflammatory factors.

Key words: *Cordyceps militaris*; Fermentation; HIF-1α, Anti-inflammatory, anti-oxidant, well aging.

1. Induction

Cordyceps militaris, a Chinese herbal medicine, is a plant with Chinese characteristics and has broad application prospects in the field of cosmetics [1-2]. However, the current utilization of *Cordyceps militaris* is difficult to fully utilize due to traditional extraction methods [3]. In order to improve the bioavailability of *Cordyceps militaris*, the advanced fermentation technology is used to develop new products related to the *Cordyceps militaris* [4-7].

Cordyceps Militaris Ferment Extract (CMF) is obtained by high-efficiency fermentation process using highly active *Lactobacillus* as the strain and *Cordyceps militaris* as the substrate. In this study, metabolic regulation theory was used to accurately control the fermentation process by stages. By adjusting the fermentation parameters such as C/N ratio, ventilation, dissolved oxygen, pH and the proportion of medium components, the carbon metabolism pathway and nitrogen metabolism pathway are strengthened [8]. And then the active macromolecular proteins, monosaccharides and other components in *Cordyceps militaris* are converted by *Lactobacillus* into high active components such as Cordyceps polysaccharides, cordyceps acid, and amino acids [9]. In addition, *Lactobacillus* can also efficiently promote the release of cordycepin from *Cordyceps militaris* and improve the utilization rate of *Cordyceps militaris* [6-7, 10]. Realize the maximum biotransformation and enrichment of main active ingredients, such as cordycepin, Cordycepic acid, polysaccharide and amino acid [11]. CMF can increase the expression of hypoxia inducible factor HIF-1α and improve skin inflammation and skin elasticity by regulating the skin hypoxia environment [12-15].

The efficient and stable fermentation process of *Cordyceps militaris* is developed by BLOOMAGE BIOTECH through continuous screening and effective utilization of probiotic strains, combined with its own advantages in fermentation process for more than 20 years. CMF products based on advanced fermentation technology are stable,

controllable and effective. Advanced technology, mild environmental protection, non-toxic and harmless products. Therefore, CMF is in line with the trend of green and sustainable development.

2. Materials and methods

2.1. Materials, chemicals and reagents

Cordycepic acid test solution, Cordycepin quasi-quality (purity≥99.0%), Cordycepin was detected by ultraviolet light, and its maximum absorption peak at 260 nm was used to detect the content of cordycepin. Chromatographic column: CAPCELL PAK-C18 column (4.6*250 mm), mobile phase: 0.04 mol/L potassium dihydrogen phosphate and 5% acetonitrile, flow rate: 1.0 mL/min, detection wavelength: 260 nm, sample volume：1 μL, column temperature: 35℃.

2.2. Culture medium

Seed medium (1 L): Peptone 10.0 g, beef extract 10.0 g, yeast extract 5.0 g, diammonium hydrogen citrate 2.0 g, glucose 20.0 g, Tween 80 1.0 mL, sodium acetate 5.0 g, dipotassium hydrogen phosphate 2.0 g, magnesium sulfate 0.58 g, sulfuric acid Manganese 0.25 g, agar 18.0 g, distilled water 1 L, pH 6.2~6.6.

Fermentation medium (1 L): *Cordyceps militaris* powder 10 g, fructose 15 g, glucose 10 g, sodium acetate 5 g, K_2HPO_4 2 g, $MgSO_4$ 0.58 g, peptone 5 g, pH value 6.2-6.6.

2.3. Microbial fermentation

The strains stored in the glycerol tube were placed in 100 mL of MRS medium, and cultured at 37 °C for 24 h. After the OD_{600} reached 4-6, 100 mL of the seed solution was transferred to a fermenter (3.5 L/5 L) for cultivation at 37 °C. 150 r/m aeration for 4 L/min, uncontrolled pH value fermentation for 36-48 h, process monitoring pH value and cordycepin content, the residual sugar is exhausted, the fermentation is completed, 3% hexanediol and 0.08% ethylhexylglycerol are added to mix well, and purified, at last, the CMF product was prepared for analysis and functional research.

2.4. Analysis methods

2.4.1. Cordycepin

2.4.1.1. Detection method

Prepare 12 g/L cordycepin solution as the standard solution, and dilute the standard solution and the sample to an appropriate multiple before use. Record the retention time and peak area of the chromatographic peak, take the average value of the peak area of the specimen and the standard solution to calculate the corresponding concentration of cordycepin by external standard method.The content of cordycepic acid in the sample is calculated according to the following formula.

$$C_1=\frac{A_1\times C_s\times n}{A_s}$$

C_1: is the content of cordycepin in the sample (mg/L).

A_1: is the average value of peak area of cordycepin in the sample.

A_s: is the average value of the peak area of cordycepin in the standard.

C_s: is the content of cordycepin in the standard (mg/L).

n: is the dilution multiple of cordycepin in the sample.

2.4.2. Cordycepic acid

2.4.2.1. Steps.

(1) Reagent preparation.

Nash reagent, 0.1% L-rhamnose, 0.015 mmol/L sodium periodate solution and chromogenic solution were prepared according to the experimental method. Prepare 1 g/L cordyceps acid solution as the standard solution, and dilute the standard solution and the sample to be tested to an appropriate multiple before use.

2.4.2.2. Determination method.

(1) Sample testing:

Detection of cordycepic acid: 1 mL was put into a test tube with a tube, 1 mL water was added to the blank control, then 1 mL sodium periodate solution was added, in quick succession, the mixture was left at room temperature for 10 min. 2 mL 0.1% L-rhamnose was added to each test tube to remove excessive sodium periodate. After shaking and mixing, 4 mL fresh Nash reagent was added and placed in a water bath at 53℃. Keep warm for 15 min, then cool quickly to room temperature. Finally, the absorbance was measured at 412 nm.

Fructose detection: 2 mL CMF was put into the plug tube, 2 mL of water was added

to the blank control, and 4 mL of mixed solution A and B were added. The mixture was mixed and kept in the water bath at 80℃ for 30 min. After cooling, the absorption value at 650 nm was observed.

(2) Calculation:

① The fructose content a was calculated by using the fructose standard curve 2;

② The absorption value A_0 of fructose in the product was obtained by using the content a of fructose in the fructose standard curve 1 and ①.

③ The light absorption value A_1 of cordycepic acid and fructose was obtained by cordycepic acid detection method.

④ Calculate the cordycepic acid absorption value $A_X = A_1 - A_0$, and substitute A_X into the cordycepic acid standard curve to get the content of cordycepic acid.

2.5.Functional effect measurement

2.5.1. Determination of HIF-1α Expression

(1) Cell seeding:

Take HaCaT cells and culture in a T75 flask with DMEM medium. When the cell density is about 80%, they are inoculated into a 24-well plate with a cover slip, then cultured at 37°C and 5% CO_2.

(2) Sample processing:

After 24 h, the supernatant was aspirated, and the experimental group was added with complete medium containing 0.01% and 0.1% of the sample, while the control group was set to incubate for 72 h in a 37°C, 5% CO_2 incubator.

(3) Immunofluorescence detection:

Aspirate the supernatant, wash the cells twice, then fix the cells with ice methanol at -20 °C, then add HIF-1α primary antibody and incubate at 4 °C overnight, wash three times with PBS the next day, and add the corresponding secondary antibody. Incubate at room temperature in the dark for 1.5 h, stain the nuclei with DAPI, and then observe the photos under a fluorescence microscope. Finally, the fluorescence data of the photos was semi-quantitatively analyze by Image J software.

(4) Data analysis:

After the background was removed, the readings were normalized and expressed as

Means ± SEM, and then data wereanalyzed using Graphpad Prism statistical software.

2.5.2. Determination of the expression level of collagen I

The expression level determination method of collagen I refers to 2.5.1, while it is the only difference that Collagen I is the primary antibody.

2.5.3. Determination of the expression of autophagy marker LC3B

The expression level determination method of LC3B refers to 2.5.1, while the primary antibody is LC3B.

2.5.4. Determination of hydroxyl radical scavenging ability

8.8 mmol/L hydrogen peroxide, 9 mmol/L ferrous sulfate, 9 mmol/L salicylic acid ethanol solution were prepared according to the experimental method. Test method take several test tubes and add 0.2 mL of 9 mmol/L ferrous sulfate, 0.2 mL of 9 mmol/L salicylic acid ethanol solution and CMF into the pipette stock solution 3.0 mL, and finally add 8.8 mmol/L hydrogen peroxide solution 0.25 mL to start the reaction. The reaction was carried out at 37℃ for 30 minutes, and the light absorption value was measured at 510 nm with purified water as the reference. Considering the absorbance value of the sample itself, ferrous sulfate, salicylic acid ethanol solution, sample solution of different concentrations and distilled water are used as the background absorption value of the sample. •OH clearance rate is calculated according to the following formula.

(3) Result calculation

$$\text{• OH clearance rate} = 1 - \frac{Ax - Ax_0}{A_0}$$

A_0: is the absorbance of the blank control solution

Ax: is the absorbance after adding the sample solution

Ax_0: is the absorbance of the background of the hydrogen peroxide sample solution without color developer.

2.5.5. Determination of melanin content

2.5.5.1. Effect on the proliferation of B16 melanoma cells

B16 melanoma cells in logarithmic growth stage were taken to 2×10^4 cells /mL were inoculated into 96 well cell culture plates, add 100 μL of cell suspension to each cell

plate well, and place in a carbon dioxide incubator at 37°C, 5 % CO_2 routinely incubated overnight. The sample is prepared into the required concentration with complete medium containing serum, and filtered and sterilized with 0.22 μm filter membrane. Discard the old culture medium and add 1.5% CMF to the experimental group samples were added with the same amount of serum containing cell culture medium in the normal control group. After continuous culture for 24 h, the relative proliferation rate of cells was detected by WST-1 method. The relative proliferation rate (RGR) is the ratio of the absorbance of the sample group to that of the normal control group.

2.5.5.2. Melanin content detection

Take 3 mL the logarithmic growth phase B16 cells and inoculate them in a 6-well culture plate at a density of 2×10^4 cells/mL, and incubate them in a incubator at 37 °C and 5% CO_2 for 24 h. Discard the old culture medium. The model group was added with 3 mL of serum-containing medium, the experimental group was added with 3 mL of sample solution, and 60 μL of forskolin solution (2 mmol/L) was added to each well of the model group and experimental group to stimulate the production of melanin, and the culture was continued for 72 h.

The determination method of melanin content in cells is as follows: trypsin digestion, centrifugation to remove the supernatant, add 500 μL NaOH solution of 1mol /L (containing 10% DMSO) to lyse cells, heat at 80℃ for 30 min, centrifuge at 3000 r/m for 10 min, take the supernatant and add it in 96 well plate, 100 μl/well, and determine the absorbance at 450 nm, so as to obtain the total amount of melanin. The melanin content of the model group is 100%, Obtain the relative value and inhibition rate of melanin content in the experimental group. If the sample has a significant effect on the proliferation of B16 cells, the ratio of the relative value of melanin content to the corresponding proliferation rate of B16 cells is the change rate of melanin production per unit cell.

2.5.6. Determination of anti-inflammatory efficacy

The macrophage model was carried out to stimulate the production of inflammatory factors with LPS to investigate whether the sample could inhibit the secretion of inflammatory factors. Prepare the mother liquor with concentration of 500000 units/mL

with serum-free 1640 culture medium for LPS, filter and sterilize with 0.22 μm filter membrane, store it in refrigerator at -20℃, and dilute it into 10000 units /mL before use. The samples in contact with cells were prepared with LPS action solution.

Raw264.7 cells in logarithmic growth phase were seeded in 24-well plates at a density of 1 × 105/mL, cultured at 37 °C and 5% CO_2 for 24 h, and CMF samples were added, 1 mL per well, and the model group was added without sample In the negative control group, serum-free 1640 medium without LPS was added, and the culture was continued for 24 hours. The content of inflammatory factors in the culture supernatant was determined according to the instructions of the ELISA detection kit for IL-6, TNF-α and IL-1β.

3. Results

3.1.Determination of cordycepin

The liquid phase detection chromatograms of the standard and CMF samples are shown in Fig 1 and Fig 2, respectively. The retention time of sample 2 is consistent with that of the standard sample, which proves that sample 2 contains cordycepin. The concentration of cordycepin in the sample is shown in Table 1, and sample 2 is significantly higher than sample 1, it is indicated that the content of cordycepin is significantly increased after fermentation by microorganisms and the concentration of cordycepin can reach to 125 ppm.

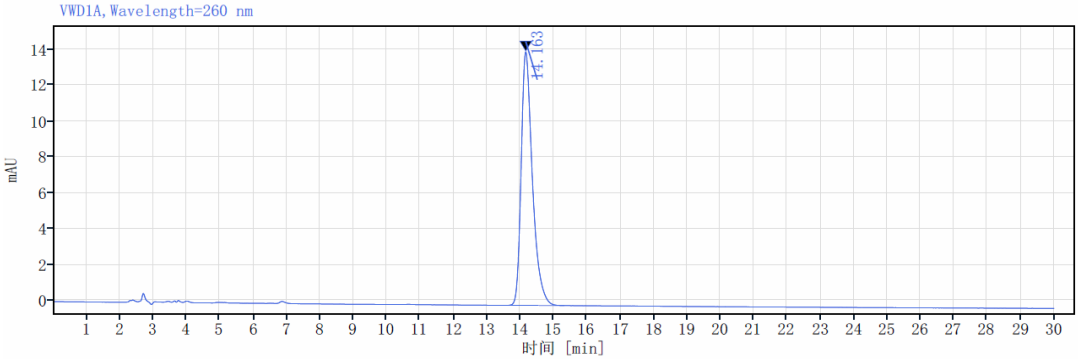

Fig.1 Chromatogram of cordycepin standard

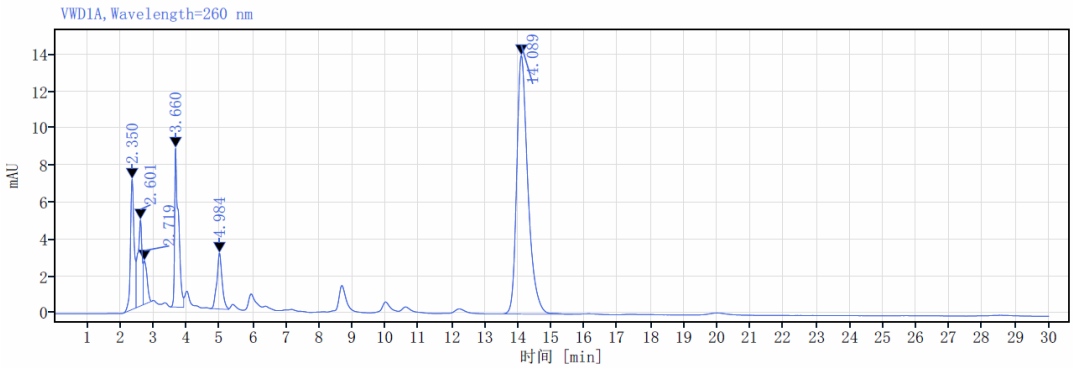

Fig.2 Chromatogram of sample 2

Table 1 content of cordycepin

Sample name	Cordycepin content/ppm
Sample 1 (before fermentation)	109
Sample 2 (after fermentation, CMF)	125

3.2.Determination of cordycepic acid

The results of the determination of cordycepic acid are shown in Table 2. The concentration of cordycepic acid in sample 1 is only 5.76 g/L, and the concentration of wormwood oxalic acid in sample 2 is 17.62 g/L, which is 3.06 times that of sample 1.

Table 2 content of cordycepic acid

Sample name	Cordycepic acid content g/L
Sample 1 (before fermentation)	5.76
Sample 2 (after fermentation, CMF)	17.62

3.3. *Determination of HIF-1α Expression*

The discovery of HIF-1α is the award-winning achievement of the 2019 Nobel Prize in Biology and Medicine. As a switch that regulates cellular respiration, HIF-1α is activated under hypoxia and causes more than one hundred downstream gene responses. The microscope images and results of the expression value of HIF-1α are shown in Figures 3 and Table 4, severally. The denser the distribution of green fluorescence sites and the higher the brightness, the higher the expression of HIF-1α. Compared with the blank control, it can be seen from Figure 1 that the expression value of HIF-1α was significantly increased by CMF. When the concentration was 0.1%, the expression level of HIF-1α could reach to 108%, thereby improving skin hypoxia deficiency and cell

viability

Table 4 Expression value of HIF-1α based onHaCaT model

	Control	Sample1（0.01% CMF）	Sample2（0.1% CMF）
Average relative fluorescence intensity (%)	100%	102%	108%*

"*" $p<0.01$

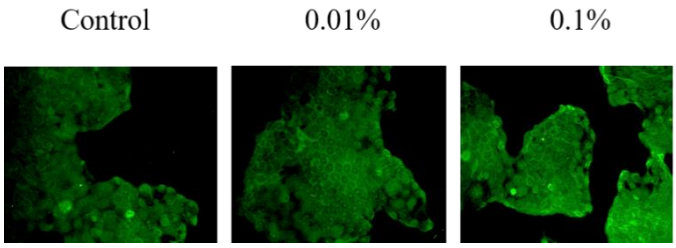

Fig 3 Expression value of HIF-1α in cells

3.4. Determination of the expression level of collagen I

The microscope images and results of the expression content of collagen I are shown in Figures 4 and Table 5, separately. The denser the distribution of green fluorescence sites and the higher the brightness, the higher the expression content of Collagen I. Compared with control, it is showed that the expression of Collagen I was up-regulated significantly by 23% with 0.1% CMF, which suggested CMF will play an important role in anti-aging by increasing expression of collagen I.

Table 5, Collagen I value in human fibroblasts FB cells model

	Control	Sample1（0.01% CMF）	Sample2（0.1% CMF）
Average relative fluorescence intensity (%)	100%	117%	123%*

"*" $p<0.01$

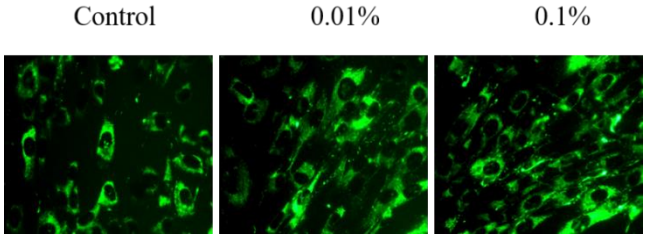

Fig 4 Expression level of collagen I in cells

3.5. Determination of the expression of autophagy marker LC3B

During autophagy, LC3B is involved in the formation of autophagosomes and is a important marker protein for autophagy occurrence. The microscope images and results of the expression content of autophagy marker LC3B are shown in Figures 5 and Table 6, respectively. The denser the distribution of green fluorescence sites and the higher the brightness, the higher the expression content of LC3B. When the concentration was 0.01% and 0.1%, the expression level of LC3B increased by 12% and 15%, severally, thus it is indicated that CMF significantly improved cellular autophagy and promoted cellular repair and regeneration, then helping skin resist damage and aging.

Table 6 LC3B content in human immortalized epidermal cells HaCaT

	Control group	Sample1（0.01%）	Sample2（0.1%）
Average relative fluorescence intensity (%)	100%	112%***	115%***

"***" $p<0.001$

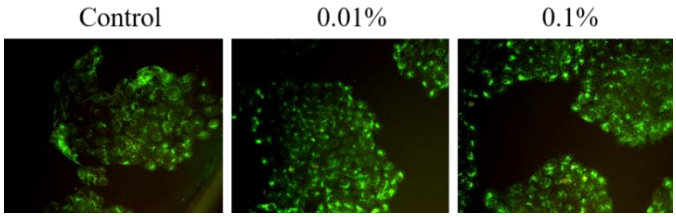

Fig5 Expression level of LC3B in cells

3.6. Evaluation of hydroxyl radical scavenging ability

The results are shown in Table 6. When the concentration of sample 2 is above 2%, it has high activity of scavenging hydroxyl radicals, the clearance of hydroxyl radicals in sample 1 is 16.45%, and the scavenger of sample 2 is 67.80%, after microbial fermentation, the ability of samples to scavenge hydroxyl radicals is significantly improved, which is 4.12 times that before fermentation.

Table 6 Effect of CMF on hydroxyl radical

（v/v）	Sample1 (before fermentation)	Sample2 (after fermentation, CMF)
20%	45.92%	100%*
10%	33.31%	100%*

5%	27.51%	88.56%*
2%	16.45%	67.80%*

"*" $p<0.01$

3.4. Evaluation of Whitening Efficacy

The results are shown in Table 7. At the concentration of 1.5% and 3%(v/v), CMF have a certain inhibitory effect on the proliferation of melanoma cells B16. The inhibitory rate of 1.5% and 3% on the total amount of melanin is18.38% and 37.73%, respectively, and 3% CMF has a more significant inhibitory effect on melanoma value.

Table 7 Effect of CMF on melanin content

Sample name	Relative proliferation rate (%)	Relative value of melanin content (%)	Inhibition rate of total melanin content (%)	Inhibition rate of melanin content per unit cell (%)
Normal control group	100	75.99	--	--
Model group	--	100	--	--
1.5% CMF	99.32	81.62	18.38***	17.83***
3.0% CMF	91.15	63.27	37.73***	30.58***

"***" $p<0.001$

3.5. Evaluation of anti-inflammatory efficacy

When the human body is attacked by germs, viruses or pathogens, it shows the inflammatory mode: the activity of pro-inflammatory cytokines increases, chemokines also increase, etc. It will causes the death of functional cells and eventually leads to tissue fibrosis and skin aging, thus the expression level of pro-inflammatory factors (such as IL-1, IL-6 and TNF-α) play a key role in regulating the inflammatory response. Under the stimulation of LPS, the secretion of IL-6, IL-1β and TNF-α by macrophages was significantly increased (Table 3). Taking IL-6 and TNF-α as the main indicators, 1.5% CMF inhibited the release of inflammatory factors IL-6, IL-1β and TNF-α, especially IL-1β with a 100% inhibition rate. Therefore, CMF can effectively reduce skin problems caused by inflammation, while sample 1 has faint inhibition effect based

on this model.

Table 8 Inhibitory effect of CMF on pro-inflammatory factors

	IL-6		IL-1β		TNF-α	
Content pg/mL	Content pg/mL	inhibition rate/%	Content pg/mL	inhibition rate/%	Content pg/mL	inhibition rate/%
Normal control group	0	--	0	--	200.4	--
Model group （LPS）	111.55	--	7.48	--	553.17	--
sample 1 （1.5%）	423.11	--	0	100**	663.01	--
1.5% CMF	98.73	11.49*	0	100**	505.25	8.66

“*” $p<0.05$， “**” $p<0.01$

4. Discussion

HIF-1α is the core of an oxygen sensing mechanism. As the human body ages, the activity of HIF -1α will also decrease, moreover, there are many downstream target genes of HIF-1α (such as LM-332) related skin aging, just like regulating inflammation, autophagy, cell viability, and skin regeneration. Therefore, it is an effective way of well aging to increase the activity of HIF-1α.

Definitely, it have been verified that CMF can up-regulated the expression of HIF-1α, thereby activating downstream signaling pathways. First, cell rejuvenation under hypoxic stress was regulate by CMF, Second, the expression value of collagen I was enhanced which will play an important role in skin anti-aging efficacy; Moreover, the expression of autophagy marker LC3B was improved ignificantly by CMF, it reveal that CMF will activate cellular autophagy and improve cellular metabolism level; Furthermore, the radical scavenging ability of CMF is presented, especially for *hydroxyl* radical, so CMF will be helpful to protect skin from oxidative stress. Additionally, it is showed a significant inhibitory effect on the melanin secretion level of B16 cells, so that the skin dullness will be improved by CMF. Last but not least, the

release of inflammatory factors IL-6, IL-1β and TNF-α was inhibited by CMF, thus inflammatory response was regulated and reduce the possibility of skin allergies. In conclusion, CMF can significantly promote the expression of HIF-1α and improve skin aging problems caused by collagen loss, pigmentation, and reduced skin elasticity caused by hypoxia.

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