The study of mRNA expression, skin hydration, shrink pores and smooth skin function of Lens esculenta seed extract in cosmetics

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Abstract

Background:

With the increase of age, the skin become grain, and the pores become thicker. During age-related skin slackening, the pore walls slacken and widen, supported by the epidermis and the dermis. The skin microrelief, defined by the orientation and the depth of the furrows, also contributes to pore distortion and age. Pore dilation, which leading to accumulation of nucleated cells around the pores, also seems to be related to an abnormal accelerated keratinization process.

This study is to evaluation the effect of the lens esculenta seed extract (LESE) to restores the normal keratinization process, stimulate the expression of collagen I and reinforcing the pore wall support structures. And it is also researching the extract's function of reduced nucleated cells and limited pore wall slackening. The clinical efficacy shown the actives function in formulation.

Methods:

Evaluation of promoting effects on mRNA expression of genes related to skin barrier function, Keratinocytes were incubated with a test sample and gene expression levels of Transglutaminase 1 and Involucrin (IVL) were analyzed by real-time RT-PCR. Staining of nucleated cells is to assess in vivo the effect of active formulated in emulsiion on the keratinization process on the cheeks in comparison with placebo. A 8-weeks Clinical testing was conducted on 30 chinese subjects around pore and wrinkle by VISIA and Primos, and professional dermatologists evaluation.

Results:

Tested at 1% on normal human keratinocytes, Lens esculenta seed extract significantly increased the expression of mRNAs coding for transglutaminase 1 by 34% ($p<0.05$), involucrin by 27%($p<0.05$). thus, favors keratinocyte differentiation. Lens esculenta seed extract significantly reduces the number of nucleated cells located around pores by 20.8% ($p<0.05$). The sample with 3% Lens esculenta seed extract (LESE) produced good clinical improvements on skin hydration, shrink pore and tighten skin. In promos testing, the pore become shrinker after 56D treatment than before. The clinical dermatologist evaluation showed increase of 34.75% in skin fineness and smooth. The average clinical score of the skin texture in the sample area was significantly increased by 34.75%, the number of blackheads and pimples was significantly reduced by 99.10%, the grade of inflammatory acne was significantly reduced by 30.16%, and the effective rate of facial acne reduction was 77%.

Conclusion:

In summary, Lens esculenta seed extract has a significant increase of TGM-1 and IVL mRNA expression and reduce nucleated cells, which help to shrink pores. In a clinical efficacy trail the sample contain 3% Lens esculenta seed extract shows good improvement in shrink pores and skin smooth function.

Keywords: Lens esculenta seed extract; transglutaminase 1; Involucrin; shrink pore; skin smooth.

1. Introduction.

Facial pores are unfixed and dynamic structures [1]. With the increase of age, the facial pores enlarged show a visible topographic changes of skin surface [2]. Conspicuous facial pores as a cosmetic concern, which are one type of serious aesthetic defects for women. As the openings of adnexa at the skin surface, facial pores can be applied both to the orifices of the sudor parous glands, which secret sweat and eliminate toxins from the body, and to the orifices of the hair follicles.

Follicles, which is associated with a sebaceous gland, have a funnel shaped structure with a base

in the middle dermis. Sugiyama-Nakagiri's conducted a survey to elucidate that there are ethnic-dependent differences in facial pore size and epidermal architecture around facial pores in the skin of Caucasian, Asian, Hispanic, and African American women [3]. Asians had the smallest pore areas compared with other racial groups. And their report previously characterized the epidermal architecture around facial pores that correlated with the appearance of those pores [3]. However, the mechanism(s) that underlie the conspicuousness and enlarged reasons of facial pores have not been elucidated. According to Sang's [1] review, high sebum excretion, increased hair follicle volume and decreased elasticity around pores, are the 3 major clinical causes of enlarged facial pores. Other related causes to pore size, such as chronical recurrent acne, sex hormones, sun exposure and skin care regimen, et al. [4]

There have been many attempts to develop a cosmetic ingredient able to solve the enlarged facial pore. We find Lens esculenta (Lentil) Seed extract, a natural active ingredient rich in oligosaccharides, which attenuates the appearance of dilated pores responsible for uneven skin grain, decreased elasticity around pores.

In keratinization process study, we conducted a survey to elucidate the 1% LESE promoting effect on Transglutaminase 1 (GTM 1) and Involucrin (IVL) mRNA expression in keratinocyte, were analyzed by real-time RT-PCR. Transglutaminases (TGs) are Ca^{2+}-dependent enzymes that catalyze the formation of Nε-(γ-glutamyl) lysine bonds between proteins and the covalent incorporation of biogenic polyamines into proteins through N, N-bis(γ-glutamyl) bonds. The TGs are important in many biological processes including the formation of the epidermal skin barrier [5].

Involucrin is a soluble cytoplasmic protein precursor of the epidermal cornified envelope that becomes cross-linked by transglutaminase during envelope assembly. Involucrin is expressed in a range of stratified squamous epithelia, including the cornea which lacks a distinct cornified layer.

Pore enlarged and dilation seems to be related to an accelerated keratinization process refer to accumulation of nucleated cells around the pores [6]. We want to evaluate keratinization process around pores. Staining nucleated cells is to assess in vivo the effect of active formulated in emulsion on the keratinization process on the cheeks in comparison with placebo. In an 8-weeks Clinical testing, the study was conducted on 30 Chinese subjects with pore and wrinkle by VISIA and Primos, and professional dermatologists' evaluation. The skin hydration evaluated by

corneometer CM825.

In summary, Lens esculenta seed extract has a significant increase of mRNA expression which help to shrink pores. In a clinical efficacy trail the sample contain 3% Lens esculenta seed extract shows good improvement in shrink pores and skin smooth function.

2. Materials and Methods.

2.1 Materials

The test samples were taken by application of a flexible slide on which a drop of cyanoacrylate had been previously deposited and spread (3S-Biokit, ref. G03SCK Monaderm). The slide was applied at constant pressure for 30 seconds, then removed gently to pick up as many cells as possible. These samples were stored at 4°C.

Lens esculenta (Lentil) Seed extract (LESE) was provided by R&D center of Mageline Biology Tech Co., Ltd.

The test samples with 3% LESE, control sample without LESE and sunscreen were provided by Mageline Biology Tech Co., Ltd.

2.2 Assessment of the expression of transglutaminase 1 and involucrin

The objective of this study is to assess the capacity of LESE to increase the expression of mRNA coding for transglutaminase 1 and involucrin, two proteins involved in keratinocyte differentiation.

This study was carried out on normal human keratinocytes by quantitative PCR.

Normal human keratinocytes were seeded and incubated at 37°C in an atmosphere containing 5% CO2. After 24 hours, the cells were treated with LESE at 0.5% or 1.0% (v/v). They were then incubated at 37°C in an atmosphere containing 5% CO2. After 48 hours, the cells were recovered, and total RNA was extracted. The RNA was reverse-transcribed, and the complementary DNA obtained was analyzed by the quantitative PCR technique. The mRNA of β-2 microglobulin, an internal reference control, was also analyzed in parallel with the transglutaminase 1 and involucrin mRNAs.

Incorporation of fluorescence (SYBR Green) was measured continuously using a thermal cycler (iCycler–MyiQ–Bio-Rad). Ct analysis (relative quantification) was performed using the Genex

software (Bio-Rad).

2.3 Nucleated cells on the keratinization process

The objective of the study is to assess in vivo the effect of LESE formulated at 3% in emulsion on the keratinization process on the cheeks in comparison with placebo.

The study was conducted on 20 healthy male and female Caucasian volunteers aged between 22 and 67 years, average age 41±12 years, selected as having dilated pores.

The effect of LESE was studied by assessing the presence of nucleated cells around pores on samples taken from the cheeks, after appropriate staining of the cells. The assessment was performed by five trained evaluators scoring each photograph blind on a scale from 0 to 4.

Test samples were taken before and after 28 days of twice-daily applications of the products.

The location of the measurement sites and their marking at the different times must be strictly reproducible. The measurements were made on symmetrical areas of the cheeks.

All participants provided signed informed consent at the beginning of the study. The investigation was performed in accordance with the ethical principles that have their origin in the Declaration of Helsinki and applicable regulatory requirements.

The sample were taken by application of a flexible slide on which a drop of cyanoacrylate had been previously deposited and spread (3S-Biokit, ref. G03SCK Monaderm).

The slide was applied at constant pressure for 30 second, then removed gently to pick up as many cells as possible. These sample were stored at 4 ℃.

Labelling and observation of nucleated cells

The location of the measurement sites and their marking at the different times must be strictly reproducible. The measurements were made on symmetrical areas of the cheeks.

To observe the nucleated cells, the samples were labelled with crystal violet, a dye staining cell nuclei (22964100, ACROS ORGANICS).

After cell labelling, the samples were observed under a microscope in white light (IX70, Olympus) fitted with a CCD camera (DXM. 1200C, Nikon) linked with image analysis software (NIS-Elements AR, Nikon). Four representative photographs of each sample were taken.

The presence of nucleated cells was assessed on photographs by five trained evaluators using a

5-step scoring scale. Each photograph was assigned a code and randomized in order to ensure a totally blind assessment.

2.4 Clinical testing by Chinese subjects

Thirty Chinese healthy subjects, aged from 30 to 45 years old, were recruited. According to the self- assessment, the facial skin was rough and loose, and the wrinkles (crow's feet) on one side of the face were graded from 1 to 3 (according to the Japanese wrinkle classification map), the fine lines on one side of the face were graded from 1 to 3 (according to the Asian skin aging map VOL.02, p46- 47), the forehead wrinkles were graded from 1 to 3 (according to the Asian skin aging map VOL.02, p32- 33), and the nasolabial groove wrinkles on one side of the face were graded from 1 to 2 (according to the Asian skin aging map VOL.02, p46- 47). According to the atlas of Asian skin aging VOL.02 (English version), p54- 55, there were no obvious redness, skin lesions, scars, etc., in the test area, 30 people were recruited, and the effective data were 30.

According to the method of use, the Chinese subjects use sample twice daily for 8 weeks, in the morning and evening. Randomly apply an appropriate amount of test sample on one side of the face, and control sample on the other side. Apply sunscreen to the whole face every morning.

The clinical evaluation of the test area of the subject's face was carried out through the specified time of D0, D14, D28, D56, VISIA- CR and PRIMOS were collected to analyze skin wrinkles and texture, and skin elasticity and firmness were measured to evaluate whether the test samples had the effect of improving facial wrinkles and texture, and the satisfaction of the subjects during the test was recorded.

2.5 Clinical evaluation of dermatologists

The dermatologists evaluated the efficacy of the product according to the facial condition of the subjects at D0, D28 with one half-face treated with the yeast essence balance lotion containing the SRFF and the sunscreen cream, and the other half-face applied only sunscreen cream.

2.6 Statistical analysis

SPSS 19.0 software was used to compare the self-differences before and after the same part of the test and the inter group differences between the product group and the control group by paired

t-test. The statistical significance level was set as $P < 0.05$.

3. Results

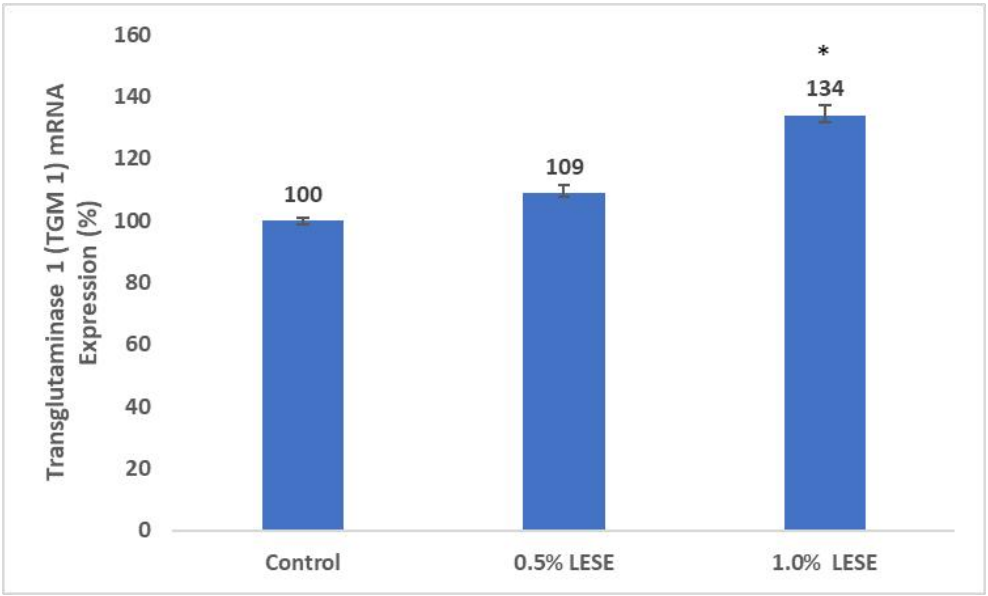
3.1 Assessment of the expression of transglutaminase 1 and involucrin

Transglutaminases (TGs) are significant enzymes for the formation of the cornified cell envelope and for the integrity of erythrocyte membranes. [7,8] TGs are responsible for the cross linking of several structural proteins including envoplakin, loricrin, periplakin, small proline-rich proteins and the previously mentioned filaggrin protein. TGM-1 are also capable of attaching and cross link lipids on the already cross-linked proteins [9].

Tested in vitro at 0, 0.5%, 1% LESE on normal human keratinocytes, 1% LESE significantly stimulates the expression of involucrin (by 27%) and transglutaminase 1 (by 34%), two markers of keratinocyte differentiation.

These results indicate that LESE can enhance the skin barrier. The higher the expression of TGM-1 and IVL mRNA, the better adhesion of the outer matrix to the skeletal structure in the cell, and the more complete cornified cell envelope, the stronger the brick wall structure of the cell, the tighter the cell and matrix link, and the better the skin barrier function.

(a)



(b)

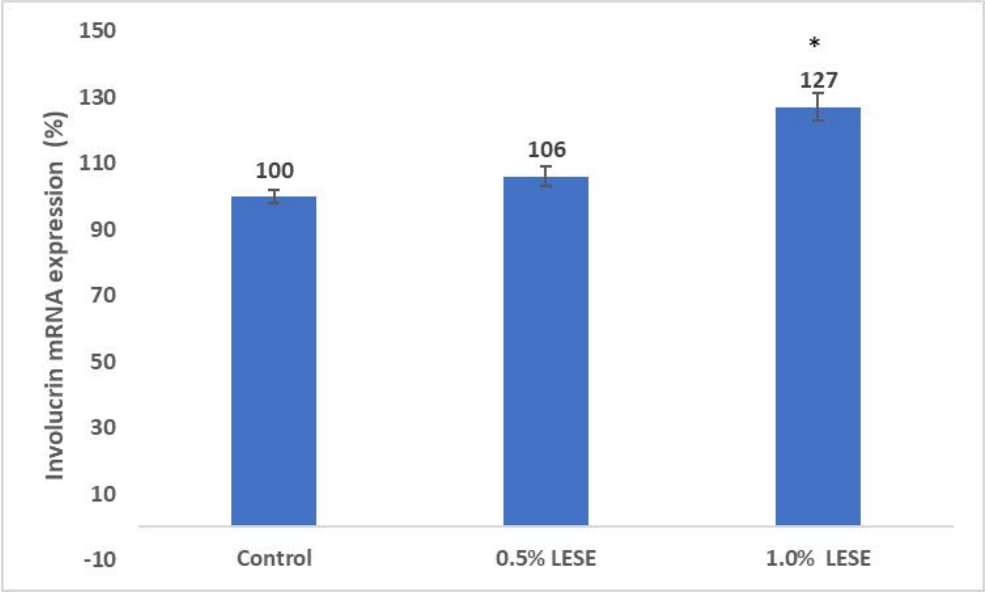


Figure 1 The mRNA expression of transglutaminase 1 (a) and involucrin (b) （* P<0.05）

3.2 The nucleated cells around pores

Compared the physiology of the corneocytes located around dilated pores with that of corneocytes from non-dilatated pores, the study show that dilated pores were frequently surrounded by nucleated cells in comparison with non-dilated pores [10]. The presence of cells is an indication of parakeratosis, that have abnormally retained their nucleus when they reach the skin surface which is accelerated and incomplete keratinization of keratinocytes to corneocytes.

This parakeratosis can be caused by various factors, such as inflammation, chronical recurrent acne, sex hormones, or abundant unsaturated fatty acids, et al.

The accumulatio of nucleated cells around the pores is the major cause for the pore dilation. To fix the pore enlarged, we need to restore the normal keratinization process that will help to reduce the appearance of dilated pores. We need an active which can help to encourage the renewal of the nucleated cells around dilated pores by new mature corneocytes.

After 28 days of twice-daily treatment and comparison in vivo testing, the sample with 3% LESE show a significant reduces the number of nucleated cells located around pores by 20.8% ($P<0.05$).

3.3 The facial pores evaluation by VISIA-CR and PRIMOS

All the Surface replicas were collected by VISIA-CR to compare facial pore number and facial pore area were shown in Figure 2. VISIA-CR captures images with standard, cross-polarized,

parallel-polarized and ultraviolet light. VISIA-CR allows visualize skin conditions and analyses skin pores on the forehead, nose, cheek and jaw under cross-polarized image photography [11].

The Change proportion = (measured value after using the sample –measured value before using the sample) / measured value before using the sample * 100%.

As figure 2 the pore number rate decreased significantly by 1.73% after 56 days daily treatment of sample with 3% LESE. The proportion of average pore area decreased significantly by 6.87% after 56 days treatment of sample with 3% LESE.

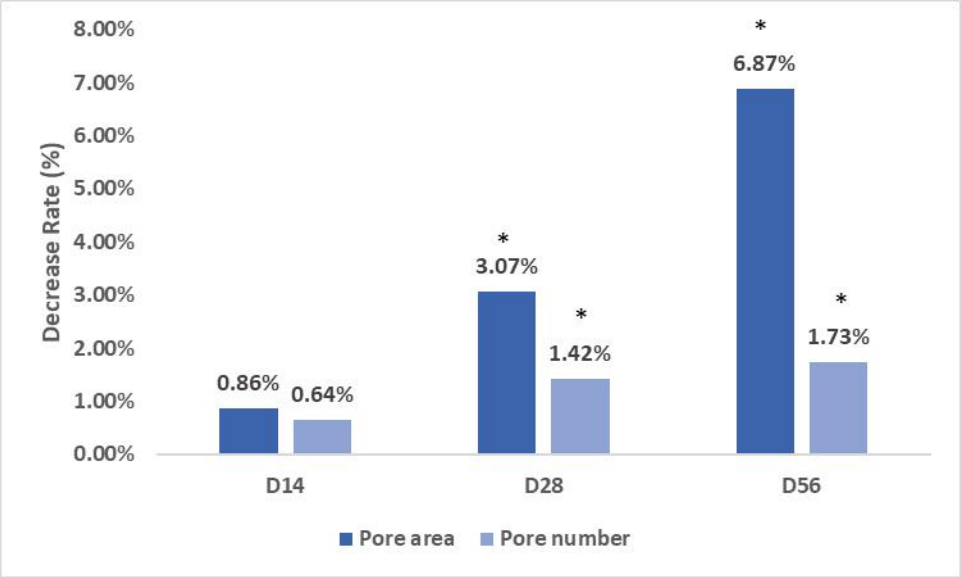
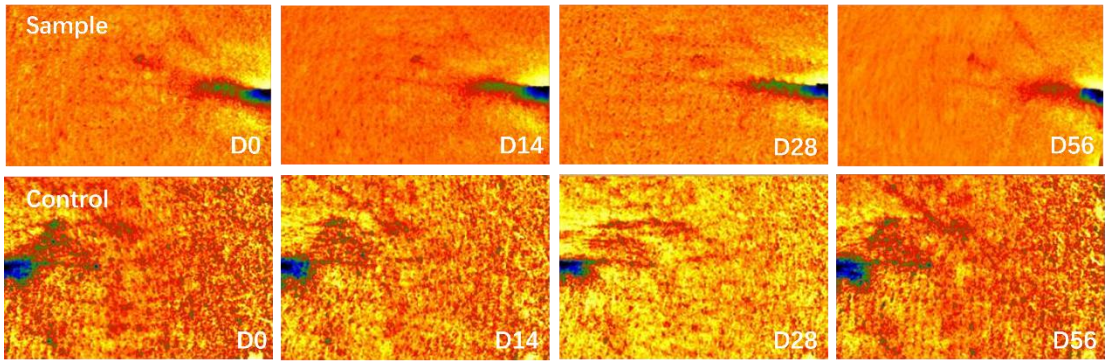


Figure 2 Decrease rate of pore number and pore area after daily application of sample with 3% LESE for 56 Days versus before use by VISIA-CR

We used another method to study the relationship between LESE and skin pores using PRIMOS analysis. The figure 3 shows the PRIMOS picture for Crow's feet (a) and Nasolabial sulcus (b) from D0, D14, D28 and D56. After the sample treatment with 3% LESE formulation, the facial pores become smaller. And control side still with enlarged facial pores.

(a)



(b)

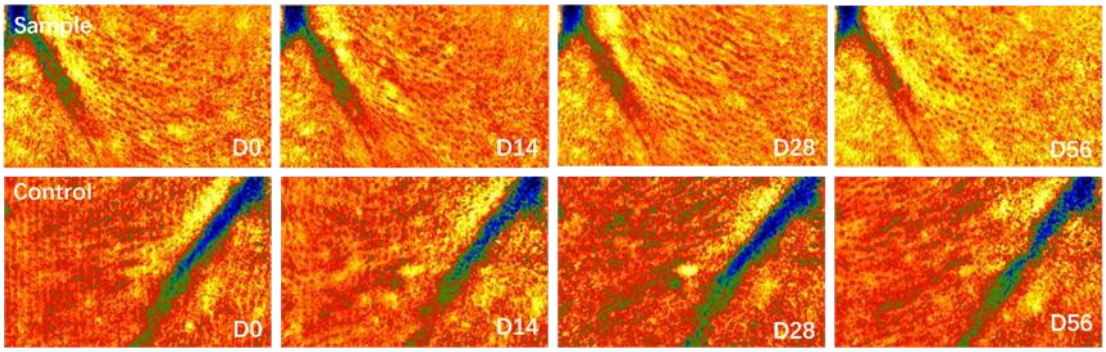


Figure 3 The PRIMOS Pictures for Crow's feet (a) and Nasolabial sulcus (b) taken using before and after 14, 28, 56 days of sample with 3% LESE

3.4 The apex nasi blackhead and acne evaluation by VISIA-CR

Blackhead is one of the common diseases in dermatology which is a type of acne. Excessive sebum secretion leads to blockage of hair follicle orifice. Under the condition of long-term exposure to the air, sebum is affected by oxidation and dust in the air to form blackhead. The occurrence factors are related to large amount of sebum secretion, pilosebaceous vasculature, daily living habits and heredity.

In order to effectively monitor and treat blackhead, we choose VISIA-CR as an accurate and reliable method to quantitatively evaluate blackhead.

As shown in Figure 4, the average area of blackhead and acne in the nasal tip area decreased significantly by 6.80% after 56 Days application of sample with 3% LESE by VISIA-CR. The proportion of the average area of blackhead and acne in the nasal tip area decreased significantly by 6.90% after 56 Days application of sample with 3% LESE.

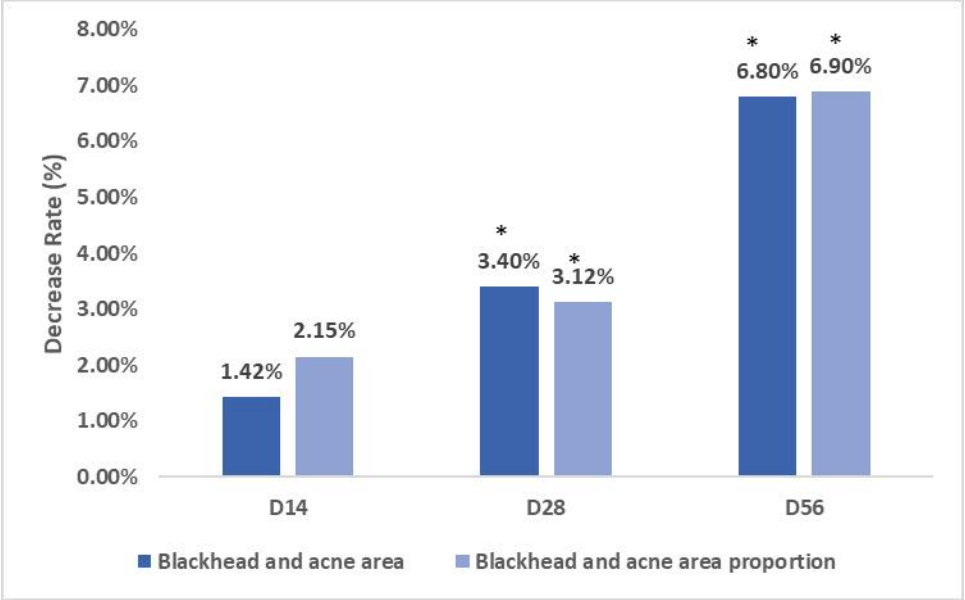


Figure 4 Decrease rate of the apex nasi blackhead and acne area after daily application of sample with 3% LESE after 56 Days by VISIA-CR

3.5 Acne volume evaluation (PRIMOS)

Acquisitions of the real 3D microtopography of the Acne volume area were taken with the PRIMOS lite system (Canfield), a three-dimensional measurement device. To ensure a good reproducibility of the acquisition conditions along the study period, the measurements were carried out with PRIMOS face device (Canfield). Moreover, during the measurements, volunteers wore a black mobcap. To determine variations of acne area parameter, one region (randomized side, left or right) in the different areas of interest were determined and evaluated with the PRIMOS software (Canfield). In general, the lower the acne area parameter, less acne the skin.

As figure 5 showed that, the Acne volume evaluation by PRIMOS. The acne volume decreased significantly by 43.45% at 28 Days treatment. The Acne volume decreased significantly by 45.76% after 56 Days treatment.

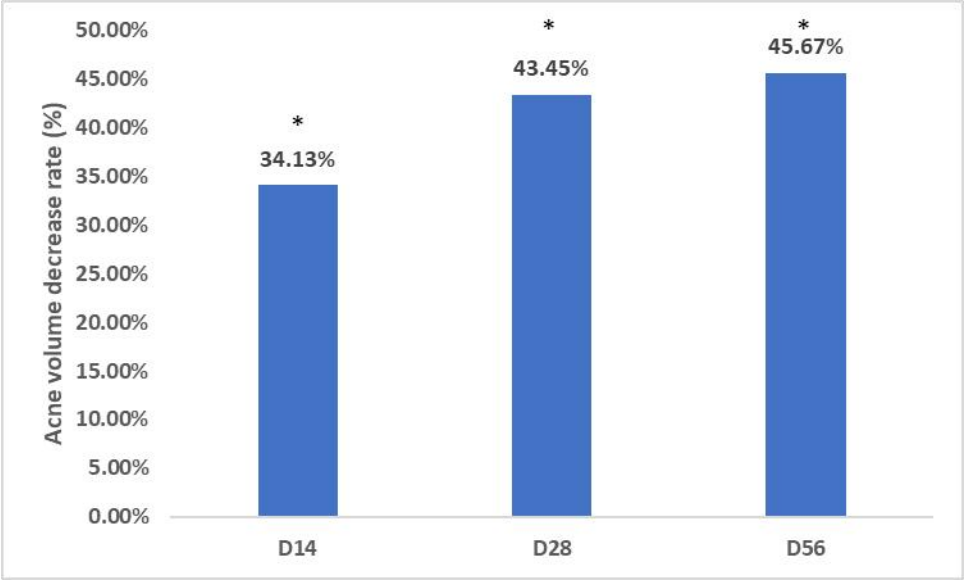


Figure 5 Decrease rate of the acne volume after daily application of sample with 3% LESE after 56 Days by PRIMOS

In our in vitro experiments, 3% LESE, was by far the most effective of substances tested. Further experiments confirmed that LESE benefit to decrease facial pores, blackhead and acne, Therefore, 3% LESE was selected for clinical studies to assess it efficacy in vivo.

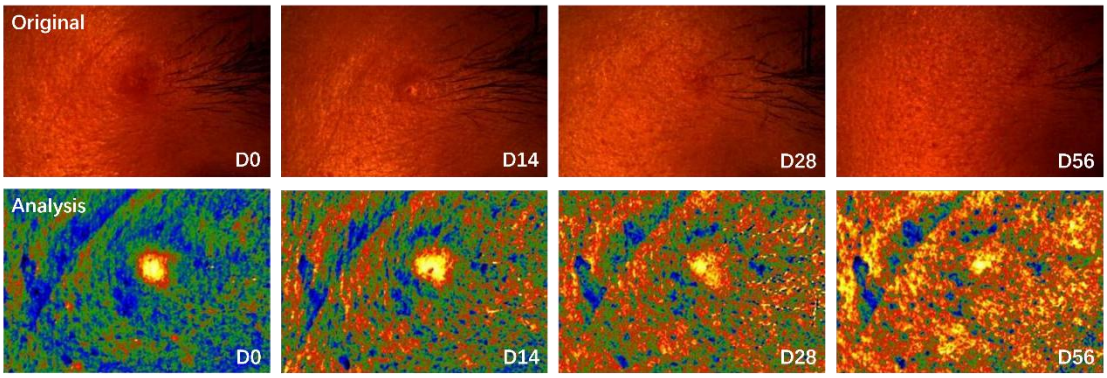


Figure 5 The acne volume original and analysis diagram by PRIMOS

3.6 **Clinical evaluation of dermatologists**

In order to evaluate the sample with 3% LESE function against skin wrinkles, facial pores, blackheads and acne. both sides use the same sunscreen in 56 Days of clinical testing.

After using the sample for 56 days, the average clinical score of the outer corner wrinkles in the sample area was significantly reduced by 23.48%, and the average clinical score of the outer corner wrinkles in the control area was significantly reduced by 7.65%; The average clinical score

of the whole face fine lines in the sample area was significantly increased by 29.86%, and the average clinical score of the whole face fine lines in the control area was significantly increased by 7.64%; The average clinical score of the skin texture in the sample area was significantly increased by 34.75%, and the average clinical score of the skin texture in the control area was significantly increased by 12.06%.

After 56 days of using the sample, the number of blackheads and pimples was significantly reduced by 99.10%, the number of papules was significantly reduced by 93.79%, the grade of inflammatory acne was significantly reduced by 30.16%, and the effective rate of facial acne reduction was 77%.

After 56 Days of sample and control treatment with the same sunscreen cream, the sample with 3% LESE has shown significant help to outer corner wrinkles, nasolabial furrow wrinkles, and the average clinical score of the whole face fine lines. The LESE have shown a significantly benefit to reduce the number of blackheads and pimples and acne.

4. Discussion.

The increase of pore volume is related to the metabolism of skin keratinocytes. Promoting the metabolic renewal of keratinocytes can help reduce pore enlargement. According to the in-vitro mRNA expression testing, Normal human keratinocytes which treated with 1% LESE showed significantly stimulate the expression of involucrin (by 27%) and transglutaminase 1 (by 34%), two markers of keratinocyte differentiation. The Promotion of the TGM-1 and IVL mRNA expression, promote the differentiation of keratinocytes at the cellular level, help to thickening and renewal of keratinocytes around pores, and benefit to narrow pores.

The accumulatio of nucleated cells around the pores is the major cause for the pore dilation. After 28 days of twice-daily treatment and comparison in vivo testing, the sample with 3% LESE show a significant reduces the number of nucleated cells located around pores by 20.8% ($P<0.05$). This ingredient can reduce the aggregation of nucleated cells, which helps to reduce pores enlargement.

In 56-Days clinical testing on Chinese subjects, the pore number rate, which evaluate by VISIA-CR, decreased significantly by 1.73% after 56 days daily treatment of sample with 3% LESE. The proportion of average pore area decreased significantly by 6.87% after 56 days treatment of sample with 3% LESE.

Clinical studies on Chinese subjects showed that the number and area of pores decreased significantly in VISIA-CR evaluation.

In the PRIMOS analysis, the facial pores become smaller. And control side still with enlarged facial pores after 56 Days treatment with the sample of 3% LESE.

The LESE can also help reduce blackheads and acne during the clinical assessment. The average area of blackhead acne in the nasal tip area decreased significantly by 6.80%, and the proportion of the average area of blackhead acne in the nasal tip area decreased significantly by 6.90%, after 56 Days application of sample with 3% LESE which evaluated by VISIA-CR. The acne volume evaluation by PRIMOS, decreased significantly by 43.45% at 28 Days treatment. The Acne volume decreased significantly by 45.76% after 56 Days treatment.

After using the sample for 56 days, the average clinical score which evaluated by dermatologists, The average clinical score of the skin texture in the sample area was significantly increased by 34.75%, the number of blackheads and pimples was significantly reduced by 99.10%, the grade of inflammatory acne was significantly reduced by 30.16%, and the effective rate of facial acne reduction was 77%.

5. Conclusion.

In summary, Lens esculenta seed extract has a significant increase of TGM-1 and IVL mRNA expression and reduce nucleated cells, which help to shrink pores. In a clinical efficacy trail the sample contain 3% Lens esculenta seed extract shows the correlations between pore size and epidermal architecture around facial pores. And 3% LESE also show significantly reduced blackheads, pimples, facial acne.

Acknowledgments.

NONE.

Conflict of Interest Statement.

NONE.

References.

1. Sang JL, Joon S, Se YJ, et al (2016) Facial Pores: Definition, Causes, and Treatment Options. Dermatol Surg 42:277-285.
2. Sugiyama-Nakagiri Y, Sugata K, Iwamura M, Ohuchi A, et al (2008) Age-related changes in the epidermal architecture around facial pores. J Dermatol Sci 50:151-4.
3. Sugiyama-Nakagiri Y, Sugata K, Hachiya A, at el (2009) Ethnic differences in the structural properties of facial skin. J Dermatol Sci 53: 135-139.
4. Kim BY, Choi JW, Park KC, et al (2013) Sebum, acne, skin elasticity, and gender difference – which is the major influencing factor for facial pores? Skin Res Technol 19: e45-53.
5. Lorand L, Graham RM (2003) Transglutaminases: crosslinking enzymes with pleiotropic functions. Nat Rev Mol Cell Biol 4: 140-156.
6. Katsuta Y, Lida T, Inomata S, et al. (2004) Improving the appearance of facial pores. Cosmetics & toiletries 119(10): 59-64.
7. Ideguchi, H., Nishimura, J., Nawata, H. et al. (1990) A genetic defect of erythrocyte band 4.2 protein associated with hereditary spherocytosis. Br. J. Haematol. 74, 347-353.
8. Matsuki, M., et al. (1998) Defective stratum corneum and early neonatal death in mice lacking the gene for transglutaminase 1 (keratinocyte transglutaminase). Proc. Natl Acad. Sci. USA 95, 1044-1049.
9. Candi E, Schmidt R, Melino G (2005) The cornified envelope: a model of cell death in the skin. Nat Rev Mol Cell Biol 6: 328-340.
10. Katsuta Y, lida T, Inomata S, et al. (2004) Improving the Appearance of Facial Pores. Cosmetic & Toiletries, 119(10):59-64.
11. Dąbrowska M, Mielcarek A, Nowak I (2018) Evaluation of sex-related changes in skin topography and structure using innovative skin testing equipment. Skin Res Technol 24: 614-620.