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Development of a new sustainable cosmetic ingredient using *Saussurea laniceps* callus originated from Himalaya

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1. Introduction

Plant tissue culture technology is used to obtain callus or regenerated tissues of rare and endangered plants, which can protect natural resources from destruction [1]. Callus extracts from tissue culture have been widely used in cosmetic products because of their active ingredients and potential skin efficacy [2]. *Saussurea laniceps* Hand. -Mazz. is a traditional Tibetan medicinal snow lotus plant growing in areas with an altitude of 3200-5280 m distributed in the *Pan-Himalaya* [3]. It is mainly rich in flavonoids, phenylpropanoids, coumarins, polysaccharides, terpenoids and their derivatives, and has the effect of scavenging free radicals, anti-aging, anti-inflammatory and analgesic [4,5]. Although *Saussurea laniceps* is still a wild species and its collection is difficult, owing to the significant medicinal value of *Saussurea laniceps*, it has suffered severe damage caused by extensive human collection and utilization. This situation ultimately leads to *Saussurea laniceps* being classified as a rare plant species. These greatly limits its development and application. Currently, the chemical compositions and pharmacological effects of Saussurea laniceps have been investigated [6]. However, research focusing on the callus culture of *Saussurea laniceps* and its application in skincare products remains relatively scarce.

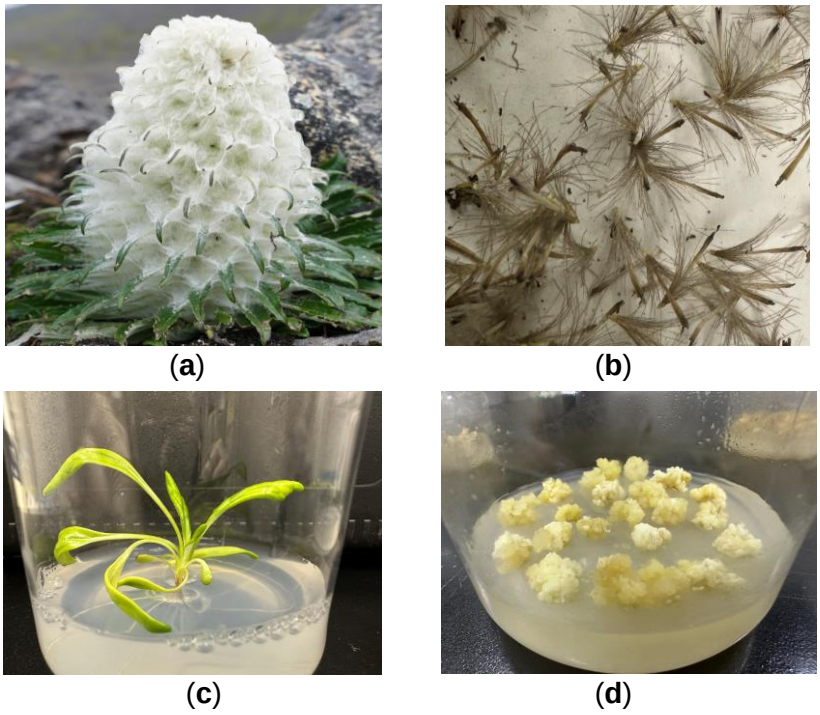
The purpose of this study is to obtain *Saussurea laniceps* callus from a sustainable source by using plant tissue culture technology, and to explore its active ingredients and potential efficacy for skin applications.

2. Materials and Methods

2.1 *Saussurea laniceps* callus culture and extract

The seeds of *Saussurea laniceps* plant (Figure 1a) were collected in Himalyan regions in China with permission on research purpose. Firstly, *Saussurea laniceps* seeds (Figure 1b) were germinated and then culture on the MS medium. Secondly, the young leaves, roots, stems of *Saussurea laniceps* seedling (Figure 1c) as an explants were cut into 0.5 to 1 cm lengths were cultured on the basic Murashige and Skoog (MS) containing 3% sucrose, 0.3%-0.35% gelrite, and different auxins and cytokinins for callus induction. Finally, the callus (Fig.1d) was proliferated. Data on the frequency of callus induction and proliferation were recorded after 4 weeks of culture: Induction frequency = (Number of inducted Callus / Total number of explants)×100; Multiplication rates = 0 day fresh weight of callus / 4 week fresh weight of callus. The pH of all

mediums was adjusted to 5.8 ± 0.1 using NaOH or HCl solution before autoclaving at 121℃ for 20 min. And the callus was grown under at (20±1) °C.
The *Saussurea laniceps* callus were harvested and ground up in distilled water, following ultrasonic extraction for 3-5 h. After centrifuge and filtration, the filtrate was dried by freeze drier and the *Saussurea laniceps* callus extract (SLCE) powder was obtained for further assessment.



(**a**) (**b**) (**c**) (**d**)

Figure 1. Culture of *Saussurea laniceps.* (a) Plant, (b) Seed, (c) Seedling, (d) Callus

2.2 Determination of syringin of *Saussurea laniceps* callus extract
The HPLC method for determining syringin in *Saussurea laniceps* callus extract: Use a C18 column, with a mobile phase of acetonitrile - water (adjustable ratio), flow rate of 1.0 mL/min, detection at 265 nm, and column temperature of 30 °C. Prepare test and reference solutions, conduct a system suitability test, plot a standard curve, and calculate the content based on peak areas.

2.3 Human dermal fibroblasts cell assay
Human dermal fibroblasts (HDF) cells at a density of 6000 cells per well were seeded in a 96-well plate for 24 h with DMEM plus 10% fetal bovine serum (FBS). After starving for overnight, the cells were treated at different concentrations of SLCE for 24 h. After the treatment of SLCE, the medium was removed followed by the addition of 200 µL of 0.5 mg/mL 3 – (4,5) – dimethylthiahiazo (z–y1) – 3, 5-di-phenytetrazoliumromide (MTT) and incubated for 4 h. Removing the medium, 100 µL of dimethylsulfoxide (DMSO) was added and dissolved for 5-10 min. The wavelength absorbance was measured at 550 nm using a Spectrophotometer.
Cell viability was obtained using the following formula:
Cell viability (%) = (the absorbance of treated cells / the absorbance of control cells) x 100

2.3.1 UVB irradiation damage recovery
HDF cells were seeded in 96-well plates, at a density of 6000 per well were incubated in a 96-well plate for 24 h. And then final concentrations of 0.01% -0.05% SLCE were treated for 24 h. Romove the medium, cells were treated by 50 mJ/cm^2 UVB. Final concentrations of 0.01% -0.05% SLCE were treated for 24 h again. Not treated with SLCE but treated with 50 mJ/cm^2 UVB (NT-UVB) was used as a control group. Cell viability was detected by MTT assay as described.

2.3.2 Enzyme-linked immunosorbent assay (ELISA) for Human type I collagen (Col-I), bone morphogenetic protein 1 (BMP1)
HDF cells were seeded in 96-well plates at a density of 6000 per well and were incubated for 24 h in DMEM plus 10% FBS. After starving for 16 h, the cells were treated with 0.005% -0.05% SLCE and 0.025% syringin for 24 h. 0.5 ug/ml ergothioneine was tested as a positive control. After the treatment, the amount of secreted type I collagen (Col-I) and bone morphogenetic protein 1 (BMP1) were measured by ELISA Kit (Bio-Swamp,China) following the protocol provided by the manufacturer, respectively.

2.4 3D full-thickness skin model culturing
The 3D full-thickness reconstructed skin model was constructed with normal human epidermal keratinocytes (NHEK) and normal human dermal fibroblasts (NHDF). UV stresses were applied to the skin equivalent samples. 5 days after being raised to the air–liquid interface, skin substitutes were exposed to four consecutive doses of 35 J/cm^2 UVA irradiations. Meanwhile, SLCE was respectively applied to skin substitutes surface at 0.005% and 0.01% by 4 times once a day, 100 μg/mL VC and 7μg/mL VE as a positive control. After the last irradiation and treaded with SLCE, the skin substitutes was cultured for another 24 h. The harvested skin equivalent samples were immediately fixed in 4%neutral buffered formalin for 24 h, and frozen at −80°C embedded in paraffin compound for histological and immunohistological analysis, respectively.The 3D full-thickness reconstructed skin model were analyzed by Immunohistochemistry. The expression of collagen Type I determined by immunohistochemical staining and image analysis.

2.5 Statistical Analysis
All data are presented as mean values ± standard deviations. Statistical significance in the data was assessed running Student's t-test. Each set of data relates to a comparison versus untreated/unstressed control. Statistical significant differences are indicated by asterisks as follows: * $p < 0.05$, **p < 0.01 and ***$p < 0.001$; # $p < 0.05$, ## $p < 0.01$ and ### $p < 0.001$.

3. Results

3.1 *Saussurea laniceps* callus induction and proliferation
Saussurea laniceps (SL) seeds were germinated and the young leaves, roots, stems or flowers of SL were cultured on the MS medium with different plant regulators for callus induction. The result showed that the highest germination rate of the untreated seeds of Saussurea laniceps (SL) is 20% (Table 1).The SL callus was induced by MS medium containing 0.5 mg/L of 6-BA and 0.3 mg/L NAA, which was the highest induction frequency by 93.1% (Table 2).
The callus with better shape was used for further proliferation culture to obtain embryogenic callus. In order to obtain callus with high proliferation capacity, the proliferation ability of SL callus was studied by the application of different basic medium- MS, SH, B5 containing different plant regulators and nutritious substances . It was found that the higher multiplication rate

(4.1-4.2) was achieved in MS medium containing 0.3-0.5 mg/L of 6-BA, 0.5 mg/L NAA and Potato powder 30 g/L. (Table 3).

The *Saussurea laniceps* callus was then extracted using ultrasonic-assisted deionized water extraction, followed by lyophilization, yielding *Saussurea laniceps* callus extract (SLCE) powder for subsequent research. Ultrasonic extraction combined with lyophilization achieved an extraction rate of 5% for SLCE. Additionally, various extraction methods were employed to enrich syringin.

Table 1. The germination rate of *Saussurea laniceps* seeds

NO.	Type of seed pretreatment	Germination rate(%)
1	Untreated	20.00
2	Gibberellin 25℃ 24h	5.00
3	Deionized water 4℃ 24h	5.26

Table 2. Callus induction of *Saussurea laniceps* as influenced by different medium and plant grow regulators.

NO.	Medium and plant growth regulators	Induction frequency(%)
1	MS+ 6-BA 1.0 mg/L + NAA 2.0 mg/L	87.0
2	MS+ 6-BA 1.5 mg/L + NAA 2.0 mg/L	93.1
3	MS+ 2,4-D 1.0 mg/L + NAA 1.0 mg/L	80.6
4	MS+ 6-BA 0.1 mg/L + NAA 2.0 mg/L	90.0
5	MS+ 6-BA 0.5 mg/L + NAA 0.3 mg/L	95.8
6	1/2MS+ 2,4-D 1.0 mg/L + NAA 1.0 mg/L	82.5
7	1/2MS+ 2,4-D 2.0 mg/L + NAA 1.0 mg/L	76.3
8	1/2MS+ 2,4-D 0.5 mg/L + NAA 0.2 mg/L	68.9
9	MS+ 2,4-D 0.5 mg/L + NAA 0.5 mg/L	58.3

Table 3. Callus proliferation ability of *Saussurea laniceps* as influenced by different basic medium.

NO.	Medium and plant growth regulators	Multiplication rate	looseness
1	MS+ 6-BA 1.0 mg/L + NAA 1.0 mg/L	3.1	++
2	MS+6-BA 0 .5mg/L+NAA 1 .5 mg/L	3.1	++
3	MS+ 6-BA 0.5 mg/L + NAA 0.5 mg/L	3.2	+
4	B5+ 6-BA 0.5 mg/L + NAA 0.5 mg/L	2.0	-
5	SH +6-BA 0.5 mg/L + NAA 0.5 mg/L +Inositol 100 mg/L	2.1	-
6	MS + mT 0.5 mg/L + NAA 0.5 mg/L	2.7	+
7	MS + 6-BA 0.5 mg/L + NAA 0.5 mg/L + Coconut Juice 100 mL/L	2.0	-
8	MS + 6-BA 0.5 mg/L + NAA 0.5 mg/L + Banana powder 30 g/L	2.3	+
9	MS + 6-BA 0.5 mg/L + NAA 0.5 mg/L +Potato powder 5 g/L	3.7	-

10	MS + 6-BA 0.5 mg/L + NAA 0.5 mg/L +Potato powder 15 g/L	3.8	++
11	MS + 6-BA 0.5 mg/L + NAA 0.5 mg/L +Potato powder 30 g/L	4.1	++
12	MS + 6-BA 0.3 mg/L + NAA 0.5 mg/L	3.4	+
13	MS + 6-BA 0.3 mg/L + NAA 0.5 mg/L+Potato powder 30 g/L	4.2	++

Remarks: The more "+" signs for the looseness degree indicate a more crispy and loose texture, while the more "-" signs signify a more compact texture.

3.2 The active constituents of *Saussurea laniceps* callus extract

The syringin content in the extract of *Saussurea laniceps* callus was analyzed by HPLC, as shown in Figure 2. The analysis revealed that the syringin content of SLCE was (2.08±0.21) % (Table 4).

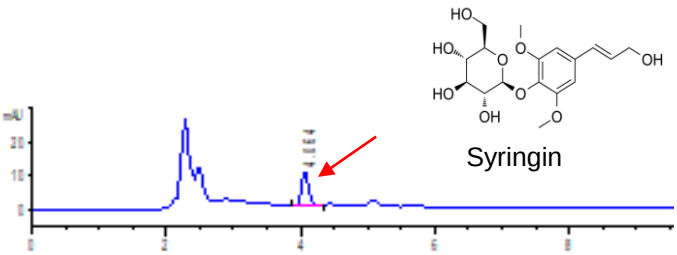


Figure 2. HPLC Chromatograms of syringin in *Saussurea laniceps* callus extract.

Table 4. The total flavonoid content, total polyphenol content and polysaccharide and Syringin content of *Saussurea laniceps* callus extract.

No.	Active constituents	Content % (w/w)
1	Syringin	2.08±0.21

3.3 Cell viability in HDF cells

We carried out MTT assay to observe HDF cell viability after treatment of SLCE. As the result, the cell viability of HDF cells was increased from 118% (0.0025% SLCE) to 228% (0.05% SLCE) as the concentration of PSCE increasing (Figure 3a). In addition, It was confirmed that the PSCE not only could promote cell proliferation but also showed excellent effect on UVB damage recovery in HDF cells (Figure 3b).

The results demonstrated that SLCE may have potential on anti-aging and skin repairing effects.

3.4 Type I collagen and bone morphogenetic protein1 synthesis in HDF cells

ELISA assay was employed to determine the content of type I collagen (Col-I) and bone morphogenetic protein 1 (BMP1) in HDF cells after treating with 0.005%,0.025% and 0.05% SLCE and 0.025% syringin, with 0.5 ug/ml ergothioneine serving as a positive control. Our results

demonstrated that 0.005%,0.025% and 0.05% SLCE, 0.025% Syringin could significantly increase Col-I synthesis by 47%, 60%,16% and 34% respectively compared with NT(Figure 3c). Meanwhile,0.005%, 0.025% and 0.05% SLCE, 0.025% syringin could also significantly increase BMP1 synthesis by 68%, 73%, 195% and 48%, respectively (Figure 3d). These results indicated that SLCE may have excellent firming and anti-wrinkle effect. What's more, syringin may be one of the crucial active ingredient of SLCE.

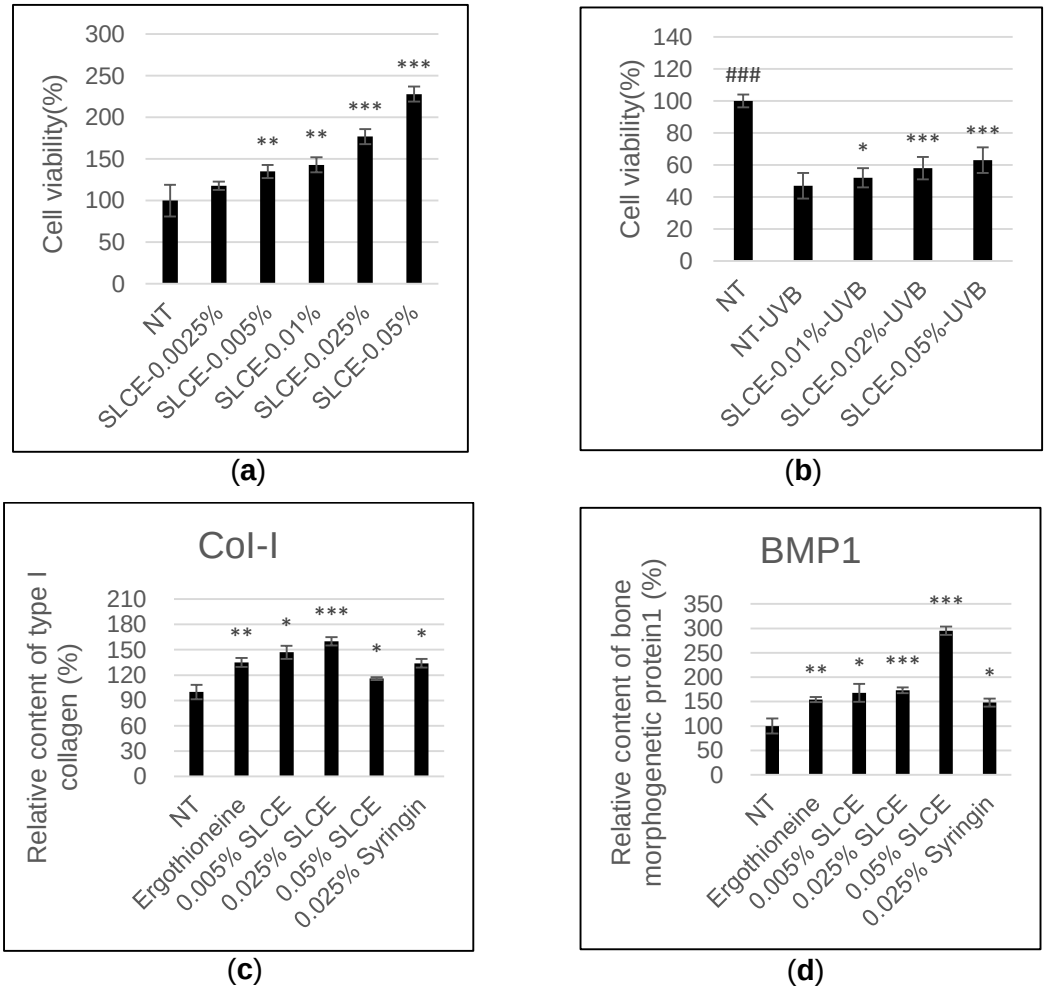
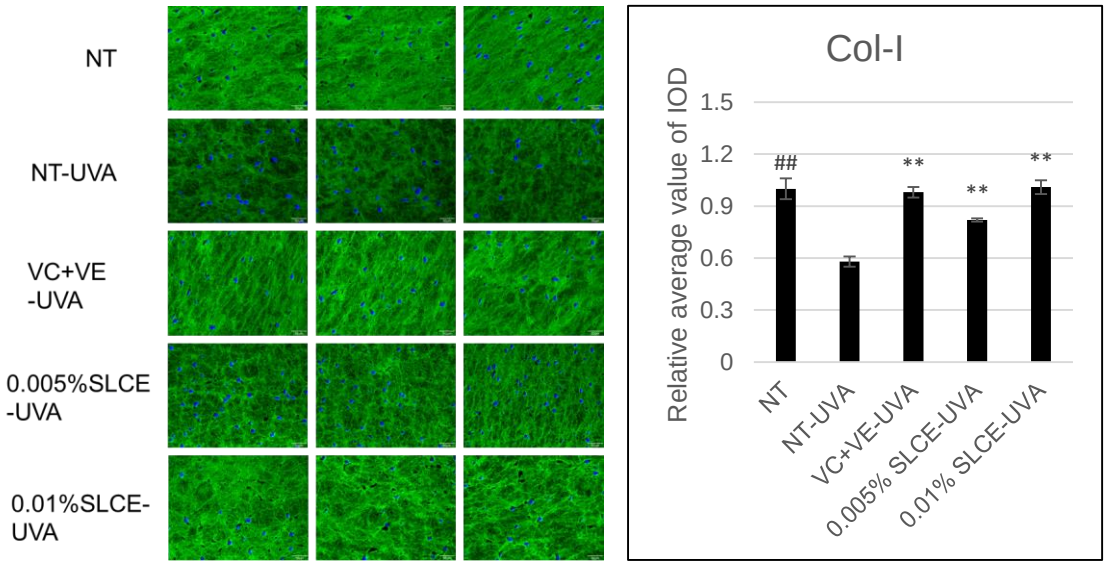


Figure 3. Effect of SLCE on cell viability, type I collagen (Col-I), bone morphogenetic protein 1 (BMP1) production in HDF cells. NT, not treated. SLCE, *Saussurea laniceps* callus extract. (a) Cell viability; (b) Cell viability (UVB); (c) Effect of SLCE on type I collagen (Col-I) production; (d) Effect of SLCE on bone morphogenetic protein 1 (BMP1) production.

3.5 Type I Collagen analysis in 3D skin model

In the UVA-exposed 3D skin model, UV radiation strongly impacted extracellular matrix-related markers by inhibiting type I collagen expression. 0.005% and 0.01% *Saussurea laniceps* callus extract (SLCE) treatment significantly counteracted these damages increasing type I collagen (Col-I) by 41.38% and 74.14%, respectively (Figure 4). The results demonstrated that SLCE may have potential on anti-photoaging and anti-wrinkle effects.



(**a**) (**b**)

Figure 4. Immunodetection (a) and image analysis (b) of type I collagen expression in the 3D full-thickness skin models unexposed to UV radiation and not treated(NT), exposed to UVA radiations and not treated(NT-UVA), exposed to UVA radiations and treated with 0.005% and 0.01% *Saussurea laniceps* callus extract. NT, not treated. SLCE, *Saussurea laniceps* callus extract.

4. Discussion

Saussurea laniceps grows in the high-altitude regions of the Himalayas and has been exposed to strong ultraviolet rays and harsh environmental conditions. Maybe this is one of the vital reasons why *Saussurea laniceps* has a lower germination rate of not more than 20%.Syringin is a common constituent of snow lotus.Previous reports have indicated that syringin exhibits nerve-regulating, anti-inflammatory, and antioxidant properties [7,8]. In this study, Syringin was enriched with different extraction methods. In vitro assays utilizing human dermal fibroblast (HDF) cells demonstrated that both the SLCE and syringin significantly promoted the secretion of type I collagen and bone morphogenetic protein 1. These results indicated that SLCE may have excellent firming and anti-wrinkle effects. Moreover, syringin is likely to be one of the key active ingredients of SLCE. The anti-inflammatory, moisturizing and whitening of SLCE, and other potential bioactivities of SLCE, as well as the related clinical trials for human validation, require further in-depth study.

5. Conclusion

The *Saussurea laniceps* callus was successfully induced and then cultured on the proliferation medium. Furthermore, ultrasonic extraction followed by lyophilization resulted in an extraction rate of 5% for the *Saussurea laniceps* callus extract (SLCE). Analysis of active constituents revealed that the SLCE contained (2.08±0.21) % syringin. In vitro HDF cell assays showed

that SLCE could promote cell viability of human dermal fibroblasts (HDF) cells, and protect cells from UVB damage. Meanwhile, both the SLCE and syringing could significantly enhanced the secretion of type I collagen protein (Col-I) and bone morphogenetic protein 1 (BMP1).Compare with the model group, SLCE could promote the synthesis of type I collagen protein (Col-I) in 3D full-thickness skin model.

These findings significantly propel the development of sustainable processing technology for Saussurea laniceps callus. Envisioned for application in anti-aging cosmetics, this technology has the potential to revolutionize natural ingredient-based skincare, setting new benchmarks for the industry.

Acknowledgments

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Conflict of Interest Statement

NONE.

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