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Glycyrrhiza glabra suspension cell Culture and its applications in cosmetics

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1. Introduction

Licorice (*Glycyrrhiza species*) is a valuable botanical resource rich in bioactive compounds, including flavonoids, triterpenoids, and polyphenols [1]. These components have been extensively utilized across various industries due to their diverse pharmacological and functional properties. In the pharmaceutical field, liquiritin is known for its antidepressant and hypoglycemic effects [2], while isoliquiritin promotes angiogenesis [3], and glabridin demonstrates potential in inhibiting tumor metastasis [4]. In the tobacco industry, licorice root extract is employed to enhance the flavor and sensory profile of tobacco products [5-6]. However, it is in the cosmetics industry that licorice and its active compounds have gained significant prominence, owing to their multifunctional benefits for skin health and appearance.

As a prominent flavonoid in licorice, glabridin is widely recognized for its skin-brightening properties. It effectively inhibits tyrosinase activity, reducing melanin production and addressing hyperpigmentation issues such as dark spots and uneven skin tone [7]. Beyond its depigmenting effects, glabridin exhibits potent anti-inflammatory and antioxidant properties. These attributes make it effective in soothing irritated skin, reducing redness, and protecting against environmental stressors such as UV radiation and pollution [8]. Its ability to neutralize free radicals also contributes to its anti-aging potential, helping to prevent oxidative stress-induced skin damage.

Licorice-derived compound dipotassium glycyrrhizinate is highly valued for its anti-inflammatory and moisturizing properties. It strengthens the skin barrier, improves hydration, and reduces redness, making it a key ingredient in formulations targeting sensitive or dry skin [9]. Its calming effects are particularly beneficial for alleviating conditions such as eczema and dermatitis.

Glycyrrhetinic acid is known for its ability to inhibit the enzyme 11-beta-hydroxysteroid dehydrogenase. This action helps reduce cortisol-induced skin damage, improving skin elasticity and resilience [10]. Additionally, glycyrrhetinic acid exhibits anti-inflammatory and antimicrobial properties, further enhancing its utility in skincare products.

Licorice extracts are rich in antioxidants, which play a critical role in neutralizing free radicals and preventing oxidative stress—a major contributor to premature skin aging. By mitigating oxidative damage, licorice-based ingredients help reduce the appearance of fine lines, wrinkles, and other signs of aging, making them a popular choice in anti-aging formulations [11].

The multifunctional properties of licorice-derived compounds have positioned them as versatile ingredients in the cosmetics industry. From skin brightening and anti-aging to anti-inflammatory and moisturizing effects, licorice extracts address a wide range of skincare concerns. As the demand for natural and sustainable cosmetic ingredients grows, the economic value and application prospects of licorice continue to expand.

Despite its potential, the utilization of licorice resources faces several challenges in China. The quality of cultivated licorice is inconsistent due to the chaotic genetic background and poor genetic stability of seeds. Excessive use of pesticides and heavy metal contamination in soil further degrade the quality. Overharvesting exacerbates environmental degradation and desertification. Additionally, the traditional cultivation cycle of 3-4 years leads to market price instability.

Plant cell suspension culture has emerged as a versatile tool in modern biotechnology, offering significant applications and advantages in the production of valuable secondary metabolites. In the context of *Glycyrrhiza* spp. (licorice) cultivation, suspension cultures provide a sustainable alternative to traditional agricultural methods, particularly for the synthesis of bioactive compounds such as glycyrrhizin, flavonoids, and other medicinal metabolites. Unlike whole-plant cultivation, suspension systems enable controlled, large-scale production under optimized conditions, eliminating seasonal limitations and reducing land use. Additionally, genetic stability and consistency in metabolite yield are enhanced through clonal propagation. The technology also supports downstream processing efficiency and reduces environmental impacts associated with pesticide use. These advantages position cell suspension cultures as a promising platform for industrial applications in pharmaceuticals, nutraceuticals, and functional foods, driving innovation in the sustainable utilization of medicinal plants.

The induction of Glycyrrhiza glabra suspension cell culture offers a novel approach to address these challenges. Studies have shown that Glycyrrhiza glabra suspension extract significantly outperforms traditional licorice extracts in terms of active compound content, antioxidant activity, tyrosinase inhibition, and MMP-1 inhibition.

2. Materials and Methods

2.1 Seed Treatment and Sterile Seedling Cultivation

Select plump seeds of *Glycyrrhiza glabra* and place them in a small beaker. Add 98% concentrated sulfuric acid, stirring continuously, with the amount of sulfuric acid sufficient to cover the seeds. Treat the seeds for 40 minutes. After treatment, rinse thoroughly with water, soak in 75% ethanol for 30 seconds, and then rinse 2–3 times with sterile water. Next, disinfect with 1% $HgCl_2$ for 3 minutes, followed by several rinses with sterile water. Transfer the seeds to an MS basal medium (without hormones) for dark cultivation. Once the seeds germinate, transfer them to light conditions to obtain sterile seedlings. The cultivation temperature is 25°C, with a light intensity of 1500 lx and 12 hours of daily illumination. When the seedlings reach a height of 3–4 cm, they can be used as explant materials.

2.2 Induction and Cultivation of Glycyrrhiza Callus

Cut the hypocotyls of sterile seedlings into 5 mm segments and aseptically transfer them to an MS induction medium supplemented with naphthaleneacetic acid (NAA, 1.0 mg/L) + 2,4-dinitrophenoxiacetic acid (2,4-D, 1.0 mg/L) + 6-benzylaminopurine (6-BA, 1.0 mg/L). Inoculate six explants per bottle, with 10 replicates. After one week of dark cultivation, callus begins to form at the cut ends. Transfer the explants to a cultivation environment at 25°C with 12 hours of daily illumination. After four weeks of cultivation, transfer the callus to a subculture

medium containing MS + NAA (0.5 mg/L) + 2,4-D (0.5 mg/L) + 6-BA (0.5 mg/L). Subculture every 28 days.

2.3 Suspension Culture of Glycyrrhiza Cells

Fast-growing and well-dispersed *Glycyrrhiza glabra* cells were selected using the cell mass screening method and subcultured several times before being transferred to a liquid medium. The liquid medium composition was: MS + NAA (0.5 mg/L) + 2,4-D (0.5 mg/L) + 6-BA (0.5 mg/L), with an initial pH of 5.8. The inoculation amount was 5%, and the culture was placed on a shaker for suspension culture at 120 rpm, with a temperature of 25°C and 12 hours of daily illumination (light intensity of 1500 lx). Cultures with good suspension properties were transferred to fresh medium to obtain well-dispersed cell suspensions. After more than 10 subcultures, a stable suspension cell line was established.

2.4 Extraction and Determination of Total Flavonoids in Glycyrrhiza Cells

Preparation of Standard Solutions:

Accurately weigh a dried and constant-weight flavonoid standard (rutin) and dissolve it in methanol to prepare a 0.1 mg/mL rutin standard solution. Take 0.0, 0.5, 1.0, 1.5, 2.0, 2.5, and 3.0 mL of the standard solution and place each volume into separate 10 mL test tubes. Add methanol to each tube to a final volume of 5 mL, then add 0.5 mL of 10% KOH solution. Mix thoroughly, allow to react for 5 minutes, and then dilute to 10 mL with methanol. Mix well and measure the absorbance at 410 nm using a UV-Vis spectrophotometer. Plot a standard curve using the concentration of rutin solution and the corresponding absorbance.

Sample Preparation for Total Flavonoid Determination:

Accurately weigh a certain amount of dried and constant-weight powdered glycyrrhiza cells, pass through a 100-mesh sieve, and ultrasonically extract with 30 times the volume of 50% ethanol for 1 hour. Filter the extract to obtain the sample solution, and prepare the sample for detection following the method used for the flavonoid standard.

2.5 Measure total phenolic content

Mix 0.5 mL of plant extract with 2.5 mL of diluted Folin-Ciocalteu reagent in a test tube. Add 2 mL of 7.5% sodium carbonate solution and vortex for 10 seconds. Incubate the mixture in the dark for 30 minutes at room temperature. Measure the absorbance at 765 nm using a UV-Vis spectrophotometer. Standard Curve and Calculation: Prepare a calibration curve using gallic acid solutions of known concentrations (20–100 µg/mL).Calculate the TPC by comparing the sample absorbance to the standard curve.

2.6 Measuring ROS in HaCaT cells using the fluorescent probe DCFH-DA:

Cell Culture: HaCaT cells are cultured in appropriate growth medium (usually DMEM with 10% fetal bovine serum and 1% penicillin-streptomycin) at 37°C in a humidified 5% CO_2 atmosphere.

Treatment: Cells are treated with the test sample or control.

DCFH-DA Staining: The cells are incubated with DCFH-DA, a cell-permeable fluorescent probe that is converted into a fluorescent product (DCF) upon oxidation by ROS. The DCFH-DA is typically added to the cells in a serum-free medium or phosphate-buffered saline (PBS) and incubated for a specific period (e.g., 20-30 minutes) at 37°C, away from light.

Washing: After incubation, the cells are washed with PBS to remove any unincorporated DCFH-DA.

Fluorescence Detection: The fluorescence intensity of the oxidized DCF within the cells is measured by fluorometer. The excitation wavelength is 488 nm, and the emission is detected around 525 nm.

2.7 The HET-CAM test for evaluating eye irritation

Fertilized chicken embryos are incubated for 8-10 days, and those with well-developed blood vessels are selected. The test substance is placed on the chorioallantoic membrane (CAM), and reactions such as hemorrhage, coagulation, and blood vessel lysis are observed and scored. Use sterile saline as negative and 0.1mol/L NaOH solution as positive control.

2.8 DPPH & ABTS Radical Scavenging Assay

Prepare samples with different concentration. Mix 0.1 mL of extract solution with 3.9 mL of DPPH (0.1mM methanol solution) or ABTS (methanol solution A734 is 0.7) in a test tube. Incubate for 30 (DPPH) or 6 (ABTS) minutes in the dark at room temperature. Measure the absorbance at 517nm (DPPH) or 734nm (ABTS) using photometer. Calculate the percentage inhibition using the formula:

Inhibition=(1−Absorbance of sample/Absorbance of control)×100%.

Plot the inhibition percentage against extract concentration to determine the IC50 value.

2.9 Tyrosinase inhibition test

Tyrosinase inhibition activity was evaluated using the mushroom tyrosinase assay. Briefly, the reaction mixture contained 100 µL of L-tyrosine (2.5 mM), 50 µL of enzyme solution (250 U/mL), and 50 µL of sample solution in phosphate buffer (pH 6.8). After incubation at 37°C for 10 min, absorbance was measured at 490 nm using a microplate reader. Tyrosinase inhibition was calculated as follows:

Inhibition (%) = [(Abs(control) − Abs(sample))/Abs(control)] × 100.

Kojic acid served as the positive control.

2.10 **MMP-1 inhibition test**

MMP-1 inhibition activity was assessed using a fluorometric assay. Briefly, the reaction mixture contained 50 µL of MMP-1 enzyme (1 U/mL), 50 µL of substrate solution (MCA-Pro-Leu-Gly-Leu-Dpa-Ala-Arg-NH2), and 50 µL of sample solution in assay buffer (50 mM Tris-HCl, 150 mM NaCl, 10 mM $CaCl_2$, pH 7.5). After incubation at 37°C for 30 min, fluorescence was measured at Ex/Em 320/400 nm using a fluorometer. Inhibition (%) was calculated as:

Inhibition (%) = [(Fluorescence(control) − Fluorescence(sample))/Fluorescence(control)] × 100.

IL-4 served as the positive control.

3. Results

3.1 Total Flavonoid Content

The total flavonoid content in the suspension extract was 43 mg/g, more than three times that of the control. Additionally, the glabridin content in the suspension extract was 3.59 mg/L, significantly higher than the control.

Fig.1 *Glycyrrhiza* suspension cell culture and extract powder

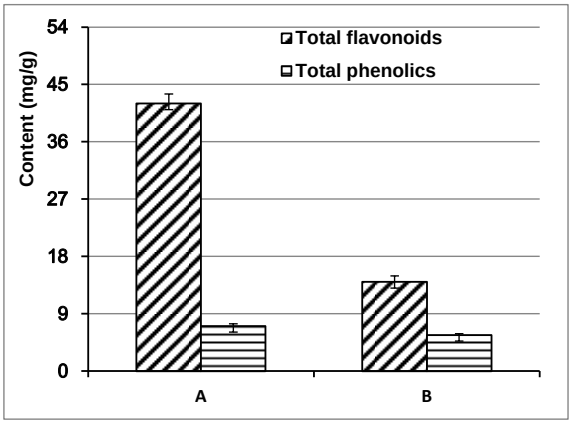

Fig. 2 Total flavonoid and total phenolic content of *Glycyrrhiza* suspension cell extract (A) and plant extract (B)

3.2. Antioxidant Activity

The IC50 values of the suspension extract for DPPH and ABTS radical scavenging were 537.00 mg/L and 202.25 mg/L, respectively, significantly lower than those of the control (Fig. 3). At a concentration of 0.0625%, the suspension extract also demonstrated significantly higher reactive oxygen species (ROS) scavenging activity in HaCaT cells compared to the control.

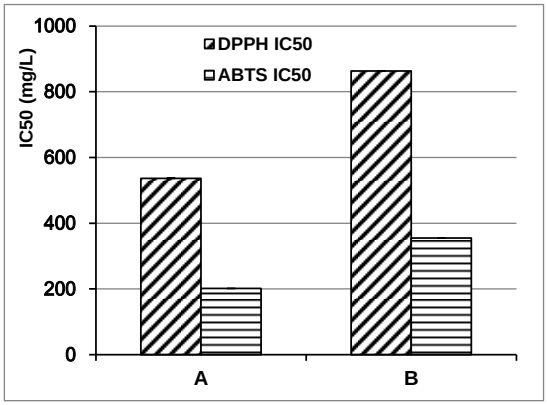

Fig. 3 IC50 for DPPH and ABTS radical scavenging

A: *Glycyrrhiza* suspension cell extract. B: *Glycyrrhiza* plant extract (B)

3.3 Eye irritation test

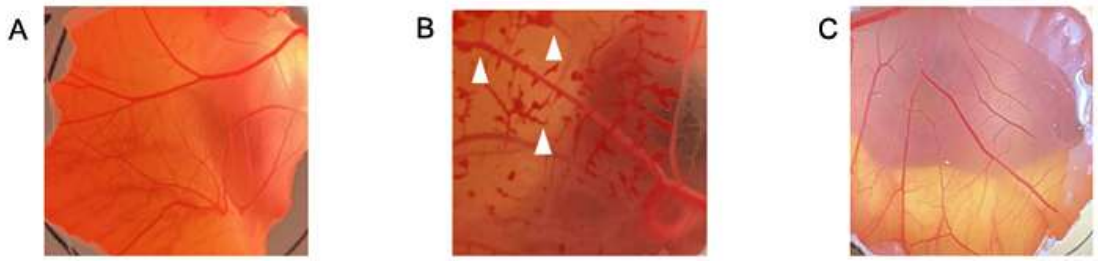

Fig. 4 HET-CAM test

A. negative control, B. positive control, C. suspension cell extract

The eye irritation potential of the Glycyrrhiza glabra suspension extract was evaluated using the chicken embryo test. At a concentration of 0.0625%, the sample showed no irritation to the chicken chorioallantoic membranes. No hemorrhage, coagulation, and blood vessel lysis were observed (Fig. 4).

3.4 Tyrosinase Inhibition

At a concentration of 625 mg/L (0.0625%), the suspension extract achieved a tyrosinase inhibition rate of 76.6 ± 3.5%, significantly higher than the control. At 0.125% concentration, it inhibited melanin synthesis in B16 cells by 62.32%, also significantly outperforming the control.

3.5 MMP-1 Inhibition

At a concentration of 0.25%, the suspension extract significantly inhibited MMP-1 synthesis in fibroblasts (Fig.5).

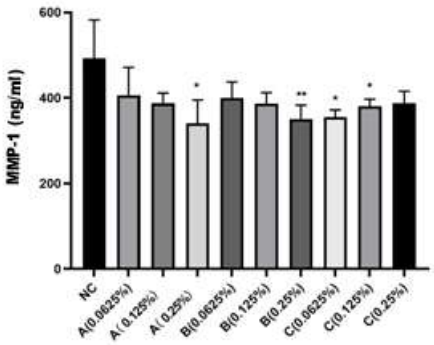

Fig.5 MMP-1 inhibition test for Glycyrrhiza glabra suspension extract

4. Discussion

Glycyrrhiza glabra suspension culture offers significant advantages over traditional plant extraction and callus culture methods. Unlike direct plant extraction, which relies on seasonal harvests and environmental factors, suspension cultures enable year-round, controlled production of bioactive compounds. This method ensures consistent yields and reduces variability caused by natural fluctuations. Compared to callus cultures, suspension systems

typically exhibit higher growth rates and metabolite productivity, as cells remain in a proliferative state. Additionally, suspension cultures allow for easier scale-up and genetic stability, minimizing the risk of somaclonal variation observed in callus systems.

The composition of glycyrrhizin and other bioactive compounds in suspension cultures may differ from those in intact plants. While plant-derived extracts often contain a broader spectrum of secondary metabolites, suspension cultures can be optimized to overproduce specific compounds through elicitation or precursor feeding, enhancing target metabolite concentrations.

In cosmetics, suspension-derived extracts offer several benefits. Their controlled production ensures standardized active ingredient profiles, improving product consistency and efficacy. Moreover, the sustainable nature of suspension cultures aligns with eco-friendly practices, appealing to environmentally conscious consumers.

Looking ahead, advancements in genetic engineering and metabolomics could further optimize suspension cultures for high-value compound production. Integration with bioreactor technologies and omics-driven strain improvement holds promise for enhancing yields and exploring novel applications in pharmaceuticals and cosmetics. Overall, *Glycyrrhiza glabra* suspension culture represents a versatile and sustainable platform for bioproduct development.

5. Conclusion

Licorice is a valuable botanical resource rich in bioactive compounds, including flavonoids, triterpenoids, and polyphenols, which have gained significant prominence in the cosmetics. Glabridin is widely recognized for its skinbrightening properties by inhibiting tyrosinase activity, reducing melanin production and addressing hyperpigmentation issues. Dipotassium glycyrrhizinate is highly valued for its anti-inflammatory and moisturizing properties. Glycyrrhetinic acid is known for inhibit the enzyme 11-beta-hydroxysteroid dehydrogenase, helps reduce cortisol-induced skin damage, improving skin elasticity and resilience. Licorice suspension cell culture offers a novel approach to alternate plant extracts. Studies have shown that suspension cell extract significantly outperforms traditional licorice extracts in terms of active compound content, antioxidant activity, tyrosinase inhibition, and MMP-1 inhibition.

The superior performance of Glycyrrhiza glabra suspension extract in terms of active compound content, antioxidant capacity, and inhibition of tyrosinase and MMP-1 highlights its potential as a sustainable solution to the challenges faced by licorice resources. Its application in cosmetics holds promising prospects for future development, particularly in addressing skin brightening, anti-aging, and inflammation-related concerns. By leveraging advanced biotechnological approaches, the cosmetics industry can harness the full potential of licorice-derived compounds to meet the growing demand for effective and natural skincare solutions.

6. References

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