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Chemical analysis and anti-inflammatory comparison of the cell culture of *Glycyrrhiza* with its field cultivated variety

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ABSTRACT

Suspended cells of *Glycyrrhiza* (CG) possessed a similar content of flavonoids and a lower content of triterpenes, when compared with its field cultivated equivalent (NG). Xylene-induced ear oedema and ovalbumin-induced mouse paw oedema were applied, to compare the effects of CG and NG on the acute inflammatory response. Extracts of the cell culture of *Glycyrrhiza* possessed a similar anti-inflammatory effect to those of NG, through the enhancement of the SOD activity of plasma and liver tissues. The use of a cell culture of liquorice instead of field cultivation would be potentially profitable.

1. Introduction

The roots and rhizomes of liquorice (*Glycyrrhiza*) species have long been used worldwide as an important agricultural commodity. This species contains a large number of active components, including flavonoids, isoflavonoids, chalcones and triterpene saponins (Asl & Hosseinzadeh, 2008). Flavonoids, secondary metabolites of the plant, are especially used for anti-oxidative (Li et al., 2011), anti-inflammation (Kim et al., 2008), and skin-whitening properties. Triterpenes, also main active compounds in liquorice, exhibit anticancer, antiviral, anti-inflammatory (Schrofelbauer et al., 2009) and immunoregulative (Cao et al., 2006) properties.

In recent years, liquorice has been increasingly used as a health additive formulated into a variety of commercial products, such as drugs, foods, drinks and cosmetics, which are marketed in Asia as well as many other countries around the world (Iida et al., 2007). Generally, almost all commercial liquorice root is collected from farms. However, demand for liquorice is high and supply is limited. Cell culture has been exploited as an alternative for more efficient and controllable production of liquorice and its active metabolites (Wang, Qi, & Wang, 2010). The flavonoid productivity of the cells which are cultured for 3 years is higher than that of the 3-year-old plant, which suggests that flavonoid production by cell cultures of liquorice is potentially profitable (Yang, He, Yu, Ji, & Wang, 2009).

Until now, there are no reports regarding the comparison of chemical and pharmacological properties between cell-cultured and field-cultivated products. In this research, we compared the compositions of triterpenes and flavonoids in cultured cells and field cultivated liquorice, in relation to the observed anti-inflammatory effects.

2. Materials and methods

2.1. Plant material

Native roots and seeds of *Glycyrrhiza uralensis* were supplied by Beijing Institute of Shizhen Chinese Herbal Medicine.

2.1.1. Callus induction and proliferation

Seed surfaces were sterilised with 70% EtOH for 30 s, immersed in 5% NaOCl solution for 20 min and rinsed three times with sterile

Abbreviations: 6-BA, 6-benzylaminopurine; CG, crude extract of cell cultural *Glycyrrhiza*; MS, Murashige and Skoog; NAA, naphthaleneacetic acid; NG, Crude extract of field cultivated *Glycyrrhiza*; SOD, superoxide dismutase; t_R, retention time.

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distilled water. Disinfected seeds were inoculated into Murashige and Skoog (MS) solid medium containing 3% sucrose (Murashige & Skoog, 1962). Each conical flask (100 mL) was cultured at 23 ± 2 °C with a light intensity of 2500–3000 lx in a biochemical incubator. After 1 week of culture, seedlings were observed. The middle tissue of hypocotyls were cut into sections of 5 mm and inoculated into MS solid medium containing 1.0 mg L^{-1} 2,4-dichlorophenoxyacetic acid (2,4-D), 0.2 mg L^{-1} 6-benzylaminopurine (6-BA) and 3% sucrose. Each Petri dish (9 cm in diameter and 1.5 cm in height) was sealed and cultured at 23 ± 2 °C in the dark. After 4 weeks of culture, calli were induced, which proliferated under the same conditions for a further 4 weeks.

2.1.2. Shake flask culture of suspension cells

Suspension cells were initiated from callus and cultivated in 1-L conical flasks with 400 mL 3/4MS medium supplemented with 1.0 mg L^{-1} 2,4-D, 1.0 mg L^{-1} 1-napthaleneacetic acid (NAA), 0.2 mg L^{-1} 6-BA and 3% w/v sucrose. Suspension cells were cultured on a rotary shaker (120 rpm) at 23 ± 2 °C under dark/light conditions (illumination (7:00–19:00)). Subculture was routinely conducted at 3-week intervals.

2.2. Sample preparation

After 18 days culture, cell suspensions were filtered and washed several times with distilled water. The fresh cells were dried at 50 ± 2 °C for 2 days and then ground into powder. The powders (30 g) were extracted twice with 750 mL 50% methanol containing 0.3% ammonia for 1 h at 65 °C (Huang, Zhou, & Song, 2011a). Following filtration, the extract was evaporated to dryness.

Dried, native roots (40 g) of *G. uralensis* were also ground into powder. The powder (30 g) was then extracted using the same procedure as for the cell suspensions.

2.3. Analyses of total flavonoids and triterpenes

2.3.1. Determination of total flavonoids

NG and CG (0.5 g) were dissolved in methanol in separate 250-mL volumetric flasks. After mixing 1 mL of each solution with 1 mL of 10% KOH for 5 min with shaking, absorbance was read at 400 nm against distilled water blank on a spectrophotometer (Tianjin, China). Rutin was chosen as a standard. Using a five-point standard curve (20–100 μg), the levels of total flavonoids contents in NG and CG were determined in triplicate, respectively. The data were expressed as milligram rutin equivalents/100 mg lyophilised powder (NG and CG). The regression equation was $y = 0.0013x - 0.0024$ (y, the absorption of the mixture; x, flavonoids concentration of samples (%)).

2.3.2. Determination of total triterpenes

A colorimetric method with vanillin–acetic acid system was performed for the quantification of total triterpenes. Briefly, the crude triterpenes extract was dissolved in methanol to a designed concentration (1 mg/mL) and 2 mL of the solution were added to a 10-mL tube. After the solution was evaporated to dryness in a water bath, 0.2 mL of freshly-prepared 5% (w/v) vanillin–acetic acid solution and 0.8 mL perchloric acid were added, mixed and incubated at 55 °C for 15 min. The tubes were taken out and cooled in running water. Then 5.0 mL acetic acid were added and absorbance of the solution was measured at 595 nm. Glycyrrhizin was chosen as a standard. Using a five-point standard curve (50–250 μg), the total triterpene contents in NG and CG were determined in triplicate, respectively. The data were expressed as milligram glycyrrhizin equivalents/100 mg lyophilised powder (NG and CG). The regression equation was $y = 0.0007x - 0.0073$ (y, the absorption of the mixture; x, triterpenes concentration of samples (%)).

2.4. HPLC analysis of the main compounds

2.4.1. Calibrations

Each standard was accurately weighed: 2 mg were dissolved in 10 mL of methanol and diluted with methanol to an appropriate concentration. The mixed solution of eight standards was prepared in methanol and stored in at 4 °C until required for analysis.

Validation of this analytical method was performed in accordance with International Conference on Harmonization (ICH) guidelines. The method was validated in terms of linearity, limit of detection and quantification, precision and accuracy. The regression equation is shown in Table 1.

2.4.2. Sample preparation for HPLC analysis

Extracts of NG and CG (each 20 mg/mL) were analysed using an Agilent 1100 high-performance liquid chromatograph (Agilent, Santa Clara, CA). Chromatographic separations were performed on a Promosil RP-C_{18} column (4.6 × 250 mm, 5 μm, Agela, China). The analytical column temperature was kept at 30 °C; acetonitrile (A) and 0.04% formic acid (B) under gradient conditions (0–4 min, 20% B; 4–20 min, 20–38% B; 20–25 min, 38–55% B) was the mobile phase at a flow rate of 1 mL min^{-1}. The wavelength of UV detector was 260 nm. The injection volume was 20 μL.

2.5. Animals

Male Kun-Ming mice (China), weighing 23–28 g were used in all experiments. Animals were obtained from the Institute of Laboratory Animal of Chinese Academy of Medical Sciences, Beijing, China. The animals were fed a standard rodent diet with free access to water, and were housed in rooms maintained at 25 ± 1 °C with a 12 h light/dark cycle following international recommendations. The Animal Ethics Committees of the Faculty of Medicine approved all experimental protocols, in accordance with 'Principles of Laboratory Animal Care and Use in Research' (Ministry of Health, Beijing, China).

Table 1
Linearity of calibration curve for eight standards and their mean contents in two samples (mg/g, $n = 3$).

Peaks	Standards	t_R[a] [min]	Regression equation[b]	R^2	Field	Cell
1	Liquiritin	8.20	$Y = 1.376x + 93.64$	0.9987	4.051	2.384
2	Isoliquiritin	14.4	$Y = 1.062x - 64.78$	0.9977	1.439	1.903
3	Liquiritigenin	18.3	$Y = 2.690x - 182.6$	0.9972	0.229	1.653
4	Licochalcone A	22.5	$Y = 2.508x - 173.0$	0.9966	0.251	0.778
5	Glycyrrhizin	24.2	$Y = 0.9129x - 55.69$	0.9961	3.702	1.936
6	Isoliquiritigenin	25.7	$Y = 2.266x - 149.4$	0.9964	0.278	0.765
7	Glabridin	32.6	$Y = 1.575x - 74.69$	0.9965	1.321	0.715
8	Glycyrrhetinic acid	38.1	$Y = 1.350x - 99.47$	0.9942	0.988	0.235

[a] t_R, Retention time.
[b] Y, Peak area; x, amount injected (ng).