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FORMATION OF GLYCYRRHIZIN BY *IN VITRO* CULTURES OF *Glycyrrhiza glabra*

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Glycyrrhiza glabra L., Papilionaceae (Fabaceae), a perennial plant native to Iran, has numerous pharmacological effects including detoxication, antioxidant, antiulcer, anti-inflammatory, and antiviral properties [1, 2]. The most important constituent of licorice is glycyrrhizin, which occurs (up to 14%) as potassium and calcium salts in the roots of the plants cultivated in Europe and the Middle East [1, 3].

Glycyrrhizin is a sweetening agent with 50 times the sweetness of sucrose and a pronounced licorice taste; it is used in tobacco products and in drugs. Glycyrrhizin has anti-inflammatory activity and is used in the form of licorice juice as a cough mixture and expectorant [3].

Plant cell cultures are promising potential alternative sources for the production of high-value secondary metabolites of industrial importance. There are many reports on the formation of flavonoids by cultures of licorice [4–6]. Biotransformation of monoterpene aldehydes and related compounds, carvone and bornyl acetate, was carried out by cell suspension culture of *Glycyrrhiza glabra* L. The cells reduced the most saturated and unsaturated terpene aldehydes and aromatic and related aldehydes to the corresponding primary alcohols [7].

However, a few studies have been done on the production of glycyrrhetinic acid and glycyrrhizin by *in vitro* cultures [8, 9].

The aim of our work was to investigate the formation of glycyrrhizin, the active ingredient of licorice by using *in vitro* cultures of *G. glabra* var. *glandulifera.*

Licorice is mostly employed in industry for its aromatic and sweetener properties and in the pharmaceutical industry for the anti-inflammatory and antiulcerative activity of glycyrrhizin, an oleanane-type triterpene saponin [8]. *Glycyrrhiza glabra* calli culture was induced from seedlings in medium A or B. Transfer and subculture of the callus (ca. 3 years) to the same medium resulted in slow growing and soft calli producing glycyrrhizin, the main component of licorice. Transfer and subculture of the callus tissue of medium A to the same liquid media (ca. 1 year) led to slow-growing cell suspension cultures. Induction of cell metabolism enzymes in cultures is achieved by slow-growing or even geriatric cells in or near the stationary phase [9]. Higher levels of glycyrrhizin are produced in the first generation of calli tissues (2.71±0.77%) compare to the second generation (2.29±0.30%). Furthermore, glycyrrhizin levels were examined in the 3rd (3.12%), 5th (3.25%), or 13th (2.29%) generations. Glycyrrhetinic acid appeared in all cell generations. Glycyrrhizin detection in the cells showed gradual loss of glycyrrhizin. The production of glycyrrhizin has been reported from calli tissues (1.84%) and differentiated calli [8], while the absence of glycyrrhizin in undifferentiated cultured cells is partly due to interruption of the reactions leading to the synthesis of glycyrrhizin from a triterpenoid intermediate due to their inability to perform some (or all) of the specific oxidation and glycosylation reactions in the final part of the biosynthetic pathway. In such cultures have been reported other triterpenoids, betulinic acid and soyasaponins, intermediates of the glycyrrhizin biosynthesis pathway, and decreasing glycyrrhizin production potency by subcultivation [10, 11].

Plant Material. *Glycyrrhiza glabra* L. var. *glandulifera* Rgl. et Herd. seeds were collected (June 1997) from plants growing wild in the Isfahan University of Medical Sciences area. Plant voucher specimens are maintained in the Shiraz Faculty of Pharmacy Herbarium.

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Cultures. Seeds were immersed in water for 10 min, surface-sterilized in 30% H_2O_2 for 3 min, and rinsed two times in sterile deionized H_2O. Sterilized seeds were laid on autoclaved agar (0.8%) and kept in darkness; after 11 days, the seedlings were transferred for callus initiation on Murashige and Skoog (MS) basal medium [12] supplemented with sucrose (3%) and containing various concentrations of plant growth regulators (naphthaleneacetic acid: NAA, 2,4-dichlorophenoxyacetic acid: 2, 4-D, and kinetin: kn) solidified with agar (1.2%) at pH 5.7 before autoclaving. The growth and shape of calli in different MS media were examined and the highest level of callus growth was observed in the medium containing 2,4-D (1 mg/L) + kn (0.2 mg/L), medium A, or NAA (1 mg/L) + 2,4-D (0.5 mg/L) + kn (0.5 mg/L), medium B. The medium was sterilized in an autoclave at 121°C for 20 min. Callus culture were subcultured every four weeks. Suspension cultures were initiated by transfer of callus tissue into 50 mL liquid medium B (250 mL Erlenmeyer flasks) as above on a rotary shaker (100 rpm) and subcultured every 9 weeks. Callus and suspension cultures were grown under a 16/8 h light/dark photoperiod with light radiation at ca. 5000 lux provided by cool white fluorescence tubes and maintained at 25±2°C. Cell suspension growth estimation was carried out by measuring cell sedimentation for 10 min in a cell growth cycle [13].

TLC Analysis and Determination. Dried callus or cell suspension samples (1 g) were refluxed for 1 hour with 10 mL 50% MeOH or 70% EtOH. The resultant extracts were spotted on precoated silica gel plates (Merck) and chromatographed with glycyrrhizin or glycyrrhetinic acid as reference samples, in saturated chambers containing CH_3Cl_3–MeOH–H_2O (64:50:10), $CHCl_3$–HOAc glacial–MeOH–H_2O (60:32:12:8) or EtOAc–HOAc glacial–HCO_2H–H_2O (100:11:11:26) solvent mixtures for detection of glycyrrhizin (R_f values: 0.60, 0.51, and 0.65, respectively) or EtOAc–EtOH–H_2O–ammonia (65:25:9:1) solvent mixture for detection of glycyrrhetinic acid (R_f value:0.56). The dried plates were then sprayed with 5% H_2SO_4 in ethanol followed by 1% vanillin in ethanol reagent and kept at 110°C for 10 min [14]. Two individual zones were detected and identified using reference samples. Glycyrrhizin was determined in callus samples by a colorimetric method [15].

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