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Original article

Determination of cardiac glycosides and total phenols in different generations of *Securigera securidaca* suspension culture

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Abstract

Background and objectives: The seeds of *Securigera securidaca* (L.) Deg. & Dorf. (Fabaceae) are used as anti-diabetic remedy in Iranian folk medicine. The aim of the present study was to establish the callus and suspension culture of *S. securidaca* seeds for the first time and to determine the major secondary metabolites including cardiac glycosides and total phenols. **Methods:** The culture of *S. securidaca* from seeds was initiated in hormone-supplemented MS medium containing 1 and 0.1 ppm 2, 4-D solution for solid and suspension cultures, respectively, sucrose and vitamins (B_1, B_2, B_6, Folic acid, Biotin, Nicotinamide and Ca pantothenate) at 25 °C and 12 h photoperiods. The cardiac glycosides were determined based on the calibration curve of securidaside which was isolated from the seeds extract of *S. securidaca*. Total phenolic compounds of different generations of suspension culture were determined using Folin Ciocalteu reagent. **Results:** Callus culture of *S. securidaca* was grown light cream to pale yellow in color and soft in texture while the cells of suspension culture grew cream to yellow with isolated cells and small aggregates. The production of cardiac glycosides in the 7^{th} generation were more than the seeds extract ($p<0.05$) and from the 8^{th} generation it reached to a steady state. The amount of phenolic contents were elevated and reached to its peak during the 8^{th} generation but it was much lower than the seeds extract. **Conclusion:** *In vitro* culture of *S. securidaca* showed possibility for production of cardiac glycosides more than the seeds extract.

Keywords: cardiac glycosides, phenolic compounds, plant cell culture, securidaside, *Securigera securidaca*

Introduction

Securigera is a genus of Fabaceae (Papilionaceae) which comprises about six species in the world. *S. securidaca* (L.) Deg. & Dorf. is one of three species which grow in Iran [1].

The people in the south of Iran have used the seeds of *S. securidaca* as a remedy to treat diabetes. There are several reports which confirm the therapeutic and preventive effects of the seeds against hyperglycemia and oxidative stress. According to previous studies, administration of the seeds extract has increased food consumption, body weight, and glycogen content of the liver and alteration of catalase enzyme activity in diabetic animals. The effective compounds of *S. securidaca* have reduced blood glucose by

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inducing insulin-like effects or increasing insulin release [2-7]; besides, *S. securidaca* seeds have shown some other pharmacological effects like anti-hyperlipidemic, chronotropic, diuretic and gasteroprotective properties [8-10].

The phytochemical analysis of *S. securidaca* has indicated the presence of flavonoids, alkaloids, saponins, tannins, cardiac glycosides and coumarins in the seeds [3,8,11,12]. Although there were much data about pharmacological effects of *S. securidaca*, no comprehensive knowledge on its tissue culture was available. Plant cell culture technologies were introduced at the end of the 1960's as a possible tool for studying and producing plant secondary metabolites [13]; thus, the present study deals with the successful establishment of the callus and suspension cultures of *S. securidaca* with a view to study quantity alterations of cardiac glycosides and total phenolic compounds as the major secondary metabolites in the different generations of suspension culture.

Experimental

Plant material

The seeds of *S. securidaca* were collected in September 2014 from south of Fars province, Iran. A voucher specimen of the plant was deposited at the Herbarium of Faculty of Pharmacy, Tehran University of Medical Sciences (6740-TEH), Tehran, Iran.

Chemicals

Murashige and Skoog (MS) medium, 2, 4-dichlorophenoxyacetic acid (2, 4-D) and plant agar were obtained from Duchefa Biochemie (Netherland). Vitamins were offered by Osveh Pharmaceutical Company (Iran). Picric acid (Sigma-Aldrich, Germany), Folin-Ciocateus reagent, NaOH and $NaHCO_3$ (Merck, Germany) were provided.

Initiation of calli and solid culture

The callus culture was produced from seedling of *S. securidaca*. First, the surface of the seeds were washed with water and soap three times and then sterilized in 1.25 % (w/v) aqueous calcium hypochlorite for 20 min. Then, 4-5 seeds were aseptically transferred to a glass jar containing 20 mL Murashige and Skog (MS) medium supplemented with 5 mL/L of 1 ppm 2, 4-D solution and 10 mL/L of the vitamin stock solution including vitamins B_1, B_2, B_6, folic acid, biotin, nicotinamide and calcium pantothenate (0.1, 0.05, 0.2, 0.05, 0.1, 0.2 and 0.1 g/L, respectively). After obtaining the profit seedlings, the sterile hypocotyls apical buds were cut with a sterile scalpel and then aseptically transferred to another jar for callus production.

The jars were incubated for 8 weeks in dark at 25±2 °C .The young and healthy callus were subcultured monthly by transferring to the same freshly prepared medium.

Suspension culture

Under aseptic conditions, calli from the 4^{th} generation were transferred to 500 mL flasks containing 100 mL liquid MS medium supplemented with 0.5 mL/L of 1 ppm 2, 4-D and 10 mL/L of vitamins stock solutions. The experiments were carried out at room temperature in light/dark cycle on a rotary shaker operated at 100 rpm. Subcultures to fresh medium were carried out every 3 weeks intervals.

Preparation of medium

Murashige and Skoog (MS) medium (4.4 g), sucrose (30 g) and de-ionized water were employed for preparation of one liter of solid and liquid mediums and plant agar (10 g) for solid medium. The pH was adjusted to 6 using 1 N KOH or 1% HCl. Finally, 20 mL of the solid medium and 100 mL of liquid medium were poured into containers, then autoclaved at 121°C / 1.8 bars for 15 min.

Seeds extraction

The powder of dried seeds of *S. securidaca* (1250 g) was macerated with 80% methanol at room temperature and the solvent was replaced every 48 h (3×8 L). The total extract was concentrated under vacuum.

Cells extraction

The cells were separated from supernatant in each generation of suspension culture and dried at room temperature. Each sample was dried, powdered and extracted by 80% methanol for 24 h. The marc was macerated as above once more. The cell extracts were concentrated in rotary evaporator at low temperature.

Determination of cardiac glycosides

Cardiac glycosides of each generation of suspension culture were quantitatively determined according to Solich *et al*. by some modifications [14]. For determination of cardiac glycosides, a 10% extract of each generation and total extract of seeds were mixed with 10 mL freshly prepared Baljet's reagent (95 mL of 1% picric acid + 5 mL of 10% NaOH). After an hour, the mixture was diluted with 20 mL distilled water and the absorbance was measured at 495 nm by Shimadzu UV/VIS spectrophotometer model 160A (Kyoto, Japan). For preparation of the standard curve, 10 mL of different concentrations (12.5-100 mg/L) of securidaside were prepared. Securidaside was isolated from *Securigera securidaca* extract [15] (figure 1). Total glycosides from triple replicates were expressed as mg of securidaside per g of dried extracts.

Determination of total phenolic compounds

Folin-Ciocalteu assay was carried out for determination of total phenols in the cells [16].

The methanol solution of each sample (0.2 mL, 100 µg/mL) was mixed with folin-ciocalteu reagent (2 mL, 1:10 diluted with distilled water). After 5 min, saturated $NaHCO_3$ solution (1.5 mL, 60 g/L distilled water) was added. The mixtures were allowed to stand for 90 min at room temperature, and then absorption of the solutions was measured at 725 nm using spectrophotometer. The same procedure was repeated for different concentrations of gallic acid solutions (0–100 mg/mL) as standard and the curve was plotted. The total phenolic contents were expressed as mg of gallic acid equivalents (GAE) per g of dried extracts.

Statistical analysis

The results were presented as mean±SD. Statistical significance between groups was evaluated by Student's t-test and $p<0.05$ was considered significant.

Results and Discussion

Callus culture grew light cream to pale yellow in color and soft in textures. After four weeks some parts of calli became yellow to brown, friable and harder.

The suspension culture cells grew cream to yellow with isolated cells and small aggregates. The cells were fine and friable till the end of the experiments.

The formation of cardiac glycosides was followed up by TLC in the cell suspension culture of different generations. Cardiac glycosides were just detected in cells and there were not any cardiac glycosides in supernatants. The quantitative amount of total cardiac glycosides has been shown in table 1 based on the calibration curve of securidaside (y= 2.285x-0.012, r^2=0.984).

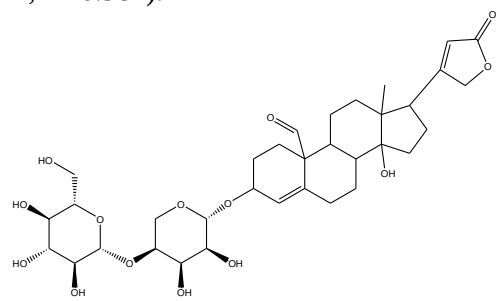

Figure 1. Structure of securidaside; securigenin-3-O-β-D-glucopyranosyl-(1→4)-β-D-xylopyranoside

In previous studies, securidaside has shown remarkable Na^+, K^+-ATPase inhibitory activity in the range 10^{-9} to 10^{-6} mol/L. The activity was found between those of ouabain and digitoxin [17]. It has caused increase in the amplitude of the heart contraction and bradycardia in a dose of 0.5 to 1 mg/kg, and has decreased the aerial pressure and respiration in the dose of 0.2-0.25 mg/kg in mice. LD_{50} of securidaside in white mouse has been found to be 25 mg/kg [18].

Securidaside has shown cytotoxicity against 9-KB and P-388 cells [14].

The formation of cardiac glycosides started at the end of the 6th generation and reached its peak during the 7th generation, then it reached to a steady state (figure 2). Statistical analysis showed that the amount of cardiac glycosides in the 7th generation of suspension culture was more than the seeds of plant (p<0.05), so cardiac glycosides formation increased in suspension culture.

The 7th to 9th generations had produced green calli more than other generations. There have been some reports that suggested a direct relationship between cardiac glycosides formation and greening. Eisenbeib *et al*. have demonstrated that cardenolides accumulated in shoot cultures of *Digitalis lanata* Ehrh. when cultivated in light condition and by transferring to permanent dark, the cardenolide content gradually decreased and reached to non-detectable levels. Shimomura *et al*. have reported that light enhanced cardenolide accumulation in root cultures [19]; however, there were reports about the existence of cardiac glycosides in tissue which was cultured in the dark. Hagimori *et al*. and Ohlesson *et al*. reported that dark-grown shoots of *D. purpurea* and tissue culture of *D. lanata* contained cardenolides [20,21].

Another factor which showed considerable effects on cardenolides production in tissue culture was absence of some minerals in the media. Sahin *et al*. reported that elimination of calcium, magnesium or both enhanced the accumulation of cardenolides in callus cultures of endemic *Digitalis* species of Turkey [22].

Total phenolic contents of the plant extracts were determined using a colorimetric assay based on reduction of Folin-Ciocalteu reagent. The calibration curve of gallic acid was plotted (y=0.007x, r^2=0.999) and table 1 showed the total phenol content of different generations in terms of gallic acid equivalents. The highest amount of total phenolic content was observed in the 8th generation (65.71±1.24 mg GAE per g of extract) (figure 2).

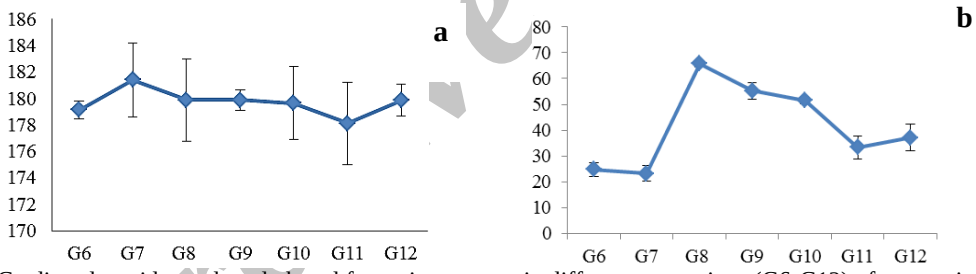

Figure 2. Cardiac glycosides and total phenol formation process in different generations (G6-G12) of suspension culture of *S. securidaca*; a: total cardiac glycosides as mg securidaside/g extract; b: total phenolic content as mg GAE/ g extract

Table 1. Amounts of cardiac glycosides and total phenolic content in different generations of suspension culture of *Securigera securidaca*

Extracts	Dry Weight of cells (g)	Total extract percentage	Cardiac glycosides (mg securidaside/ g extract)	CG/EC (%)	Total Phenolic content (mg GAE/ g extract)	PC/EC (%)
G6	13.16	24.70	179.14±0.67	17.91	24.76±2.61	2.47
G7	20.52	7.63	181.40±2.78	18.14	23.27±3.09	2.32
G8	12.55	37.27	179.87±3.10	15.57	65.71±1.24	5.69
G9	15.41	53.04	179.87±0.75	18.00	55.24±3.30	5.52
G10	5.74	43.58	179.65±2.79	17.96	51.43±1.10	5.14
G11	5.13	12.69	178.12±3.10	17.81	33.33±4.51	3.33
G12	4.71	31.79	179.86±1.23	17.98	37.14±5.07	3.71
Seeds Extract	322.64	18.02	176.47±1.15	17.64	147.14±2.46	25.96

Results are expressed as Mean±SD of three determination; G: germination; GAE: gallic acid equivalent; EC: Extractable compounds; CG: Cardiac Glycosides; PC: Phenolic Content

Phenolic contents in tissue culture was so lower than seeds extract, so it seems that culture condition was not proper for production of phenolic compounds in tissue culture. There were various factors which enhanced the total phenolics in tissue cultures. El-Beltagi *et al*. demonstrated that high doses of gamma irradiation positively enhanced accumulation of total phenolics and total flavonoids in rosemary callus culture [23]. In another investigation, Abohatem and his colleges observed low rate of culture transfers (every 15 or 20 days) tended to increase the phenolic contents and peroxidase activities in plant tissue culture of date palm (*Phoenix dactylifera* L.) [24]. Lim *et al*. showed elicitation of *Orthosiphon stamineus* cell suspension culture with chitosan enhanced phenolic compounds biosynthesis and antioxidant activity [25]. Cai *et al*. demonstrated that increasing of minor elements like Co, Ag and Cd were most effective to stimulate the phenolic acid production [26].

The enhancement of phenolic compounds of *S. securidaca* is important because of the isolated flavonoids from the seeds extract showed considerable cytotoxic effects on cancer cell lines (T47D, Caco-2 and HT-29) in comparison to methotrexate and no toxicity to NIH/3T3 normal cells [27].

Finally, it could be concluded that the high cardiac glycosides producing capacity of the culture was due to the nature of the explants/seeds used for culture development or because of type of medium and conditions used for cultivation of the cells. So tissue culture is a good suggestion for production and increasing the cardiac glycosides of *S. securidaca* seeds.

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Declaration of interest

The authors declare that there is no conflict of interest. The authors alone are responsible for the content of the paper.

References

[1] Recshinger KH. *Securigera securidaca (Legominoseae).* In: Recshinger KH, Ed. *Flora Iranica*. Graz: Akademische Druck-u, 1984.

[2] Ghitasi I, Nikbakht MR, Sadeghi H, Sabzali V, Sabzali S, Shahrani M. The hypoglycemic effects of a hydro-alcoholic extract from *Securigera securidaca* seeds on induced diabetic in male rats. *J Shahrekord Univ Med Sci*. 2007; 8(4): 68-73.

[3] Hosseinzade H, Ramezani M, Danaei AR. Antihyperglycaemic effect and acute toxicity of *Securigera securidaca* L. seed extract in mice. *Phytother Res*. 2002; 16(8): 745-747.

[4] Porchezhian E, Ansari SH. Effect of *Securigera securidaca* on blood glucose levels of normal and alloxan-induced diabetic rats. *Pharma Biol*. 2001; 39(1): 62-64.

[5] Pouramir M, Shahaboddin ME, Moghadamnia AA, Parastouei K. To study the effects of *Securigera securidaca* (L.) seed against alloxan-induced hyperglycemia. *J Med Plants Res*. 2011; 5(14): 3188-3191.

[6] Roostazadeh A, Firoozrai M, Shabani M. Effect of aqueous seed extract of *Securigera Securidaca* on erythrocytes catalase activity in type 1 diabetic rats. *Qom Univ Med Sci J*. 2008; 1(4): 9-14.

[7] Zahedi-Asl S, Marahel H, Zare B. Study on the effects of chloroformic extract of *Securigera Securidaca* on serum glucose level and liver glycogen content of mice. *J Kerman Univ Med Sci*. 2005; 12(1): 32-38.

[8] Ali AA, Mohamed MH, Kamel MS, Fouad MA, Spring O. Studies on *Securigera securidaca* (L.) Deg. et Dorfl. (Fabaceae) seeds, an antidiabetic egyptian folk medicine. *Pharmazie*. 1998; 53(10): 710-715.

[9] Garjani AR, Fatahiazad F, Zakher A, Allaf Akbari N, Azarmie YA. The effect of total

extract of *Securigera securidaca* L. seeds on serum lipid profiles, antioxidant status, and vascular function in hypercholesterolemic rats. *J Ethnopharmacol*. 2009; 126(3): 525-532.

[10] Mard SA, Bahari Z, Eshaghi N, Farbood Y. Antiulcerogenic effect of *Securigera securidaca* L. seed extract on various experimental gastric ulcer models in rats. *Pakistan J Biol Sci*. 2008; 11(23): 2619-2623.

[11] Komissarenko AN, Kovalev VN. Hydroxy coumarins and flavones of *Securigera securidaca*. *Chem Nat Compd*. 1987; 23(2): 252.

[12] Zamula VV, Maksyutina NP, Kolesnikov DG. Cardenolides of *Securigera securidaca*. *Khim Prir Soedin*. 1965; 1(3): 153-156.

[13] Vanisree M, Lee CY, Lo SF, Nalawade SM, Lin CY, Tsay HS. Studies on the production of some important secondary metabolites from medicinal plants by plant tissue cultures. *Bot Bull Acad Sinica*. 2004; 45(1): 1-22.

[14] Solich P, Sedliakova V, Karlicek R. Spectrophotometric determination of cardiac glycosides by flow-injection analysis. *Anal Chim Acta*. 1992; 269(2): 199-203.

[15] Tofighi Z. Phytochemical investigation of *Securigera securidaca* (L.) Deg. & Dorf. Ph.D. thesis. Facaulty of Pharmacy, Tehran University of Medical Sciences, Tehran, Iran, 2013.

[16] Singleton VL, Orthofer R. Lamuela-Raventos RM. Analysis of total phenols and other oxidation substrates and antioxidants by means of Folin-Ciocalteu reagent. *Method Enzymol*. 1999; 299(1): 152-178.

[17] Mraz M, Opletal L, Sovova M, Drasar P, Havel M. Inhibition of Na^+, K^+-ATPase by the glycosides from *Coronilla varia*. *Planta Med*. 1992; 58(5): 467-468.

[18] Viqar Uddin A, Anwer B. *Spectroscopic data of steroid glycosides: cardenolides and pregnans*. USA: Springer, 2006.

[19] Eisenbeib M, Kreis W, Reinhard E. Cardenolide biosynthesis in light- and dark-grown *Digitalis lanata* shoot cultures. *Plant Physiol Bioch*. 1999; 37(1): 13-23.

[20] Hagimori M, Matsumoto T, Obi Y. Studies on the production of *Digitalis* cardenolides by plant tissue culture. III Effects of nutrients on digitoxin formation by shoot-forming cultures of *Digitalis purpurea* L. grown in liquid media. *Plant Cell Physiol*. 1982; 23(7): 1205-1211.

[21] Ohlsson AB, Bjork L, Gatenbeck S. Effect of light on cardenolide production by *Digitalis lanata* tissue cultures. *Phytochemistry*. 1983; 22(11): 2447-2450.

[22] Sahin G, Verma SK, Gurel E. Calcium and magnesium elimination enhances accumulation of cardenolides in callus cultures of endemic *Digitalis* species of Turkey. *Plant Physiol Bioch*. 2013; 73: 139-143.

[23] El-Beltagi HS, Ahmed OK, El-Desouky W. Effect of low doses γ-irradiation on oxidative stress and secondary metabolites production of rosemary (*Rosmarinus officinalis* L.) callus culture. *Radiat Phys Chem*. 2011; 80(9): 968-976.

[24] Abohatem M, Zouine J, El Hadrami I. Low concentrations of BAP and high rate of subcultures improve the establishment and multiplication of somatic embryos in date palm suspension cultures by limiting oxidative browning associated with high levels of total phenols and peroxidase activities. *Sci Hortic-Amsterdam*. 2011; 130(1): 344-348.

[25] Lim FL, Yamb MF, Asmawi MZ, Chan LK. Elicitation of orthosiphon stamineus cell suspension culture for enhancement of phenolic compounds biosynthesis and antioxidant activity. *Ind Crop Prod*. 2013; 50: 436-442.

[26] Cai Z, Kastell A, Speiser C, Smetanska I. Enhanced resveratrol production in *Vitis vinifera* cell suspension cultures by heavy

metals without loss of cell viability. *Appl Biochem Biotech*. 2013; 171(2): 330-340.

[27] Tofighi Z, Asgharian P, Goodarzi S, Hadjiakhoondi A, Ostad SN, Yassa N. Potent cytotoxic flavonoids from Iranian *Securigera securidaca*. *Med Chem Res*. 2014; 23(4): 1718-1724.