

IDENTIFICATION AND PRODUCTION OF PHENOLIC NICOTIFLORIN IN *ASTRAGALUS CHRYSOCHLORUS* CALLUS

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Abstract

Astragalus chrysochlorus Boiss. & Kotschy (2n = 16) is one of the rare Turkish endemic species and it is listed in the Red Data Book of Turkish Plants as endangered. This species has been used traditionally for its wound healing properties and a crude ethanol extract prepared from the roots exhibits antioxidant and cytotoxic activities. In this study, a detailed phytochemical analysis was performed on *A. chrysochlorus* calli that resulted in the isolation of a major constituent. The purified molecule's structure elucidation was completed by spectral methods [nuclear magnetic resonance (NMR) and mass spectrometry (MS)], which revealed a rarely encountered flavonoid in the *Astragalus* genus, nicotiflorin. In order to increase nicotiflorin content in the callus cultures, the effects of culturing time and elicitor treatment were investigated. The HPLC analyses showed that the maximal production of nicotiflorin occurred with long-term cultured (13 years old) callus as 4775 µg/g dry weight (DW), whereas it was 132 µg/g DW for short-term cultured (2 months old) ones. Then, the 24 h treatment of the yeast extract that was used as biotic elicitor had negative effect on the production of nicotiflorin. The data obtained from this study could be significant for the mass production of nicotiflorin from long term *in vitro* cultured *A. chrysochlorus* callus.

Rezumat

Astragalus chrysochlorus Boiss. & Kotschy (2n = 16) este una dintre speciile endemice din Turcia și este încadrată în lista roșie ca fiind pe cale de dispariție. Această specie a fost folosită în mod tradițional pentru proprietățile sale de vindecare a rănilor, iar un extract etanolic a fost preparat din rădăcini și prezintă activitate antioxidantă și citotoxică. În acest studiu s-a efectuat o analiză fitochimică detaliată pe *A. chrysochlorus* calli care a dus la izolarea unui constituent major. Elucidarea moleculei purificate a fost completată prin metode spectrale (rezonanță magnetică nucleară (NMR) și spectrometrie de masă (MS)), care au evidențiat flavonoidul nicotiflorin. Pentru a crește conținutul de nicotiflorin în culturile de calus, s-au investigat efectele timpului de cultivare și tratamentul elictor. Analizele HPLC au arătat că producția maximă de nicotiflorin a apărut la calusul de lungă durată (13 ani), 4775 µg/g DW, în timp ce acesta a fost de 132 µg/g DW pentru culturile de scurtă durată (cu vârsta de 2 luni).

Keywords: *Astragalus*, nicotiflorin, kaempferol 3-O-rutinoside, flavonoid, NMR, HPLC

Introduction

Phytochemicals are the richest resources for human consumption because of their various applications. By using phytochemicals, new and novel products for the treatment and prevention of serious diseases could be improved [1].

Plant secondary metabolites have significant functions in plant growth, development and physiology. The effective components of medicinal plants are usually the secondary metabolites and the synthesis of them is affected by a variety of factors, such as biotic and abiotic effectors. Also, secondary metabolites help plants to survive, defence and compete with others [2]. Thus, the production of the majority of plant phenolic compounds could be induced by the applications of abiotic and biotic elicitors. Therefore, elicitor

application is usually preferred to trigger the production of rare phytochemical sources [3].

Nicotiflorin exists in a few plants, thus there are limited number of studies conducted [3]. Nevertheless, nicotiflorin isolation has been reported from *Heteropappus altaicus* and *H. Biennis* [4], *Solidago canadensis* [5], *Ficaria verna* [6], *Clitoria ternatea* [7], *Staphylea bumalda* [8], *Trigonotis peduncularis* [9], *Acalypha indica* [10], *Carthamus tinctorius* [11], *Camellia sinensis* [3], *Caragana bungei* [12], *Solanum campaniforme* [13], *Osyris wightiana* [14], *Ampelopsis heterophylla* [15], amaranth [16] and *Aspergillus awamori* [17]. Although it is not common among *Astragalus* species, nicotiflorin isolation has been reported only from *Astragalus verrucosus* [18] and *Astragalus cruciatus* [19].

Flavonoids are a group of polyphenolic secondary metabolites found in plants and have a variety of biological activities [20]. The bioassay results demonstrated that flavonoid nicotiflorin has a neuro-protective effects [21, 22], inhibitory effect on adipogenesis [23], protective effects on reducing memory dysfunction [11] and a potent inhibitor of α -glucosidase [24]. Also, it has been shown that exerted hACAT1 (human Acyl CoA: cholesterol transferase 1) inhibitory [9], *in vitro* anti-glycation [14] and anti-oxidant activities [25].

Astragalus is used in traditional Chinese medicine because it possesses many biological activities. It has also been proven effective for clinical treatment of many diseases [26]. The well-known components of *Astragalus membranaceus*, are flavonoids, triterpene saponins, and polysaccharides. The phenyl-propanoid derivated flavonoids are associated with antioxidant and cytotoxic activities of these plants [27]. A vast number of studies were made about *Astragalus* species and its components; however, there is still so much to learn about that. A Turkish endemic, *Astragalus chrysochlorus* Boiss. and Kotschy has cytotoxic effects and selenium accumulation capacity [28-30]. To investigate other potential biological activities of nicotiflorin, there is need to find alternative source of nicotiflorin. Therefore, in the present study, the purified molecule's structure elucidation was completed by spectral methods (NMR and MS). HPLC analysis of different time regime and elicitor application on *in vitro* cultures of *A. chrysochlorus* was carried out to estimate the amount of the bioactive compound nicotiflorin.

Materials and Methods

Plant material and Callus Induction. Long-term (13 years old) and short-term (2 months old) *in vitro* cultured *Astragalus chrysochlorus* callus tissues were used as previous described in literature [31]. Divided hypocotyl explants (~0.5 cm) from 15-day old *in vitro*-germinated seedlings were used for callus induction in the presence of 0.5 mg/L of 2,4-dichlorophenoxyacetic acid (2,4-D). Fifteen explants were oriented in a horizontal position on the surface of Petri dish media and incubated in a growth chamber (Hereaus, Vötsch, No: 440/0026/86) with fluorescent light ($1400 \text{ Em}^{-2} \text{ s}^{-1}$) and 16 h/day photoperiods at $25 \pm 2^\circ\text{C}$. Five replicates were used per treatment and subcultures were done at 3 week intervals and then harvested. Harvested callus material was frozen at -80°C and then lyophilized.

Extraction of Plant Material for NMR and MS Measurements. The lyophilized and powdered plant material of *A. chrysochlorus* (callus tissues; 29 g) was extracted with MeOH (2x5 L) at room temperature for 12 hours. After filtration, the solvent was removed by rotary evaporation to afford 8.431 g of

crude extract. The MeOH extract was subjected to *vacuum* liquid chromatography (VLC) on reversed-phase material (Lichroprep RP-18, 25 - 40 μm , 20 g) employing H_2O (100 mL), H_2O -MeOH (85:25, 500 mL; 25:75, 300 mL), and MeOH (800 mL) to give ten main fractions (A1-A10). TLC analyses were performed to control these fractions. After TLC, selected fractions were applied to open column chromatography using silica gel (100 g) as stationary phase. Elution was carried out with CHCl_3 -MeOH mixtures (95:5, 1000 mL; 90:10, 400 mL; 85:15, 1000 mL; 80:20, 500 mL; 70:30, 1000 mL) to other fractions (B).

Initiation of Cell Suspension Cultures and Elicitor Treatment. *A. chrysochlorus* primary cell suspension cultures were propagated in the dark as previously described by Cakir O. *et al.* [32]. The actively grown 21-day-old friable calli were used to establish cell suspension cultures. The callus (~1.0 g) was cut into small pieces and cultured in 50 mL MS medium (3% sucrose and 1 mg/L 2,4-D in 100 mL Erlenmeyer flask) in 250 mL Erlenmeyer flask for the establishment of primary cell suspension cultures. The cultures were placed on an orbital shaker (Gerhardtthermoshake), at 120 rpm and incubated at $25 \pm 1^\circ\text{C}$. Primary cell suspension cultures were subcultured at 10 day intervals.

For the induction experiments, 5 mL culture (with 1 mL packet cell volume) of the primary cell suspension culture was subcultured in 25 mL fresh MS medium [33]. Elicitor yeast extract was prepared by ethanol precipitation as described by Chen and Chen [34]. 10 g/L concentration of yeast extract was added on the 13th day (first day of log phase) of cell culture. Water added control samples were taken and analysed as well. Collecting of the biomass by filtering was performed 24th hour of elicitor treatment. All experiments were done in triplicate. Filtered suspension cell cultures were frozen at -80°C and then lyophilized.

Experimental Design. To test the effect of length of culturing time and elicitor treatment on nicotiflorin accumulation in *A. chrysochlorus* callus, two types of experiments were performed. In the first type of experiment, long-term cultured and well adapted callus cultures and short-term cultured callus were collected. No elicitor was added to the callus cultures. Collecting of the biomass was performed 3 weeks interval. Triplicate Petri dishes were used in all experiments. After the preparation of HPLC analysis, the nicotiflorin content were quantified. In the second type of experiment, cell suspension cultures which were originated from long-term and short-term cultured callus, exposed to yeast extract as an elicitor. After both experiment, accumulated biomass was lyophilized.

HPLC Sample Preparation. 100 mg of *Astragalus chrysochlorus* powder extract obtained from samples

which are mentioned in Table I, were sonicated four times with 5 mL methanol. The clear extracts were diluted with methanol to 30 mL. Prior to HPLC analysis, the samples were filtered with a 0.45 μm PTFE (polytetrafluoro-ethylene) filter to remove non-dissolved particles.

HPLC Conditions. HPLC-DAD (diode array detection) analyses were performed with an Agilent 110 series equipped with a quaternary pump, an auto-sampler. As analytical column a *ThermoODS Hypersil C-18* column (250 mm x 4.06 mm, particle size 5 μm) was used. An acetonitrile/water and acetic acid mixture (75:25 v/v) served as isocratic mobile phase. The total run time was 30 minutes, at a flow rate of 0.8 mL/min. A sample of 10 μL was injected (partial loop injection) each run. The retention time was 6.5 min for nicotiflorin.

Calibration Curves. The standard stock solutions were prepared with methanol (1000 $\mu\text{g/mL}$ nicotiflorin). Six additional lower concentrated standard solutions (1000, 500, 250, 100, 50 and 10 $\mu\text{g/mL}$) were prepared by diluting the standard stock solutions with methanol. The solutions were stored at 4°C. Concentrations and peak areas were log transformed and the calibration curve was prepared using these values. Regression coefficients were 0.99972 for nicotiflorin (kaempferol 3-O-rutinoside). Specificity was determined by calculation of peak purity facilitated by the photo-diode array detector (PDA).

Results and Discussion

Metabolic content analysis. The lyophilized callus material of *Astragalus chrysochlorus* was extracted with MeOH. After filtration and solvent remove, crude extract was obtained. The MeOH extract was subjected to *vacuum* liquid chromatography (VLC) on reversed-phase material employing H_2O , H_2O -MeOH and MeOH to give ten main fractions (A1-A10). After TLC assays, eight fractions (A3-A10) were found to be rich in flavonoids. These fractions (1.34 g) were applied to open column chromatography using silica gel as stationary phase. Elution was carried out with CHCl_3 -MeOH mixtures to give fifty-nine fractions (B). Compound 1 was obtained from fraction B11-B20 (98.4 mg). Specifications of compound 1: ^1H -NMR (methanol- d_4 , 400 MHz), δ 8.03 (2H, d, J = 8.3 Hz, H-3', H-5'), 6.97 (2H, d, J = 8.3 Hz, H-2', 6'), 6.35 (1H, d, J = 2.1 Hz, H-8), 6.16 (1H, d, J = 1.8 Hz, H-6), 5.09 (1H, d, J = 7.2 Hz, glc H-1), 4.51 (1H, d, J = 1.4 Hz, rha H-1), 3.80 (1H, dd, J = 1.0, 10.4 Hz, glc H-6), 3.38 (1H, dd, J = 4.7, 10.8 Hz, glc H-6), 1.12 (3H, d, J = 6.2 Hz, rha H-6); ^{13}C -NMR (methanol- d_4 , 100 MHz), δ 177.9 (C-4), 164.5 (C-7), 162.0 (C-5), 160.0 (C-4'), 158.0 (C-9), 157.0 (C-2), 134.1 (C-3), 131.1 (C-2', C-6'), 121.3 (C-1'), 114.7 (C-3', C-5'), 104.2 (C-10), 103.3 (glc C-1), 100.9 (rha C-1), 98.6 (C-6), 93.6 (C-8), 76.7

(glc C-3), 75.7 (glc C-5), 74.3 (glc C-2), 72.5 (rha C-4), 70.9 (rha C-3), 70.6 (rha C-2), 70.0 (glc C-4), 68.3 (rha C-5), 67.2 (glc C-6), 16.5 (rha C-6).

Successive purification of the flavonoid rich fraction on silica gel and RP (C-18) columns yielded a pure metabolite. Compound 1 (Figure 1) was obtained as a yellow amorphous powder. Its molecular formula was determined as $\text{C}_{27}\text{H}_{30}\text{O}_{15}$ by LC-MS, ^1H - and ^{13}C -NMR data. The ^1H -NMR resonances at δ 8.03 (d, J = 8.3 Hz, 2H) and δ 6.97 (d, J = 8.3 Hz, 2H), together with those at δ 6.35 (d, J = 2.1 Hz, 1H) and δ 6.16 (d, J = 1.8 Hz, 1H) revealed 1,4-disubstituted and 1,2,3,5-tetrasubstituted aromatic rings, respectively, implying a flavonoid skeleton. Additionally, in the ^1H -NMR spectrum, two anomeric protons observed at δ 5.09 (d, J = 7.2 Hz) and δ 4.51 (d, J = 1.4 Hz) suggested a flavonoid diglycoside framework. Detailed inspection of the ^1H - and ^{13}C -NMR signals and comparison of the obtained data with those in the literature verified compound 1 as kaempferol-3-O-rutinoside (nicotiflorin) [12, 23] which is uncommon in *Astragalus* genus [18].

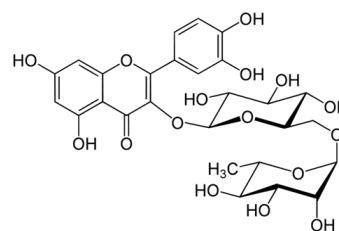


Figure 1.

Molecular structure of nicotiflorin (kaempferol 3-O-rutinoside)

Effects of culture time and elicitation on nicotiflorin production

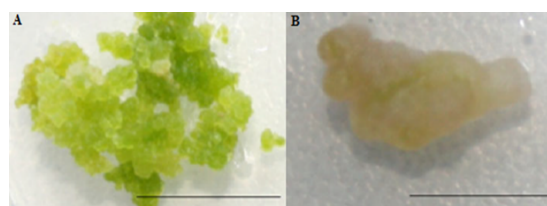


Figure 2.

Astragalus chrysochlorus callus cultures. A: Long-term cultured callus; B: Short-term cultured callus [31]. Bar: 1 cm

Obtainment of the secondary metabolites *via* callus culture has the advantage of low cost and less environmental impact [35]. But there is a strong association between callus yield and culture adaptation. Within the presented work, *A. chrysochlorus* callus and suspension cultures were tested with or without elicitor yeast extract applied conditions to enhance nicotiflorin yield. Very few amount of nicotiflorin production was observed on the tested conditions

except long-term cultured callus which was cultured for 13 years (subcultured 20 days intervals) and

well adapted to tissue culture conditions (Figure 2; Table I).

Table I

Nicotiflorin production profile of the tested conditions given as dry weight (DW)

Sample Origin (10 mg/ml)	Nicotiflorin (350 nm)
LT cultured callus, non-elicited	4775 µg/g
ST cultured callus, non-elicited	132 µg/g
LT cultured callus derived CSC control	8.5 µg/g
LT cultured callus derived CSC elicited	10 µg/g
ST cultured callus derived CSC control	140 µg/g
ST cultured callus derived CSC elicited	10 µg/g

LT: Long-Term; ST: Short-Term; CSC: Cell Suspension Culture

When considering of the adaptation of cells, minor physical or chemical changes can cause significant differences. In some cases, cells not even continue to live. But the adapted ones, survive by gaining new advantageous features. Binh *et al* [36] reported that rice (*Oryza sativa* L.) cells, grown under a continuous stress NaCl, produced a homogeneous mass of dry, compact, nodular callus with high regeneration potential. As regards, different salt stress regimes induced different advantageous features in the cultured cells [36]. On potato cultures, salinity reduced the growth rate and increased lipid peroxidation in salt-stressed line, which remained unaltered in the adapted line. Na⁺ and Cl⁻ content increased due to salinity in both lines, but the adapted line displayed greater K⁺/Na⁺ ratio then the stressed one. Electron microscopy showed that neither the structural integrity of the cells nor the membrane structure was affected by salinity, but plastids from adapted cells had higher starch content [37]. Another example for the adaptation is about the investigations on five years maintained transgenic *Catharanthus roseus* plants. They were showed that alkaloid vindoline accumulation of the transgenic plants were two times more compared to non-transformed ones. Their alkaloid profile was almost constant during 5 years [39].

In this study, suspension cells originated from both long-term and short-term cultured calli were negatively affected by 24 hour elicitation. However, abiotic elicitor copper sulphate treatment on cell cultures of *Digitalis lanata* was induced the flavonoid production over 10 times more compared to the control [38]. The results obtained from this study showed that 24 h treatment of yeast extract decreased the production of nicotiflorin in *Astragalus chrysochlorus* suspension cultures according to callus culture. It was concluded that other stressors, biotic or abiotic, could be applied to increase the production of this valuable compound in *A. chrysochlorus*.

Conclusions

In this study, the nicotiflorin content of *Astragalus chrysochlorus* is reported for the first time. Long-term cultured *A. chrysochlorus* calli are also very productive

in terms of secondary metabolite nicotiflorin and could be consider as an ideal source for the biotechnological production of nicotiflorin.

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