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Biotransformation to Produce the Anticancer Compound Colchicoside using cell suspension cultures of *Astragalus vesicarius* plant species

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This paper discusses the biotechnological process affected by means of plant suspension cultures, for production of colchicoside, the 3-*O*-glucosyl derivative of 3-*O*-demethylcolchicine. Colchicoside can be considered as an antitumoural prodrug which is activated after oral administration and may have more beneficial effects and a better toxicity profile (because of a slow-release effect) than colchicine. We have developed a green and efficient biotechnological method using colchicine, as a precursor, derived from its natural source *G. superba* seeds. Plant suspension cultures of *Astragalus vesicarius* were used to design a practical biotechnological platform to replace a methyl group at C-3 regiospecifically by a glycosyl moiety in colchicine. Using different concentrations of a colchicine-rich extract, the maximum enzymatic potential of *Astragalus vesicarius* suspension cells was achieved. According to quantitative HPLC-UV analysis, levels of 9.35 *µ*mol/g DW colchicoside were achieved. This is the first report of region-specific glycosylation at C-3 of the aromatic ring A of the colchicine using plant suspension cultures.

Keywords: *Astragalus vesicarius*, Biotechnology, Colchicoside, Colchicine, Suspension cultures.

Colchicine (a non-heterocyclic alkaloid), present in many species of *Colchicum* (e.g. *C. autumnale*, *C. luteum* and *C. speciosum*), as well as the genera *Androcymbium*, *Bulbocodium*, *Camptorrhiza*, *Dipidax*, *Gloriosa*, *Iphigenia*, *Littonia*, *Merendera*, *Ornithoglossum* and *Sandersonia* is well known to relief gout. The present study focuses on the glycoside colchicoside, which is the 3-*O*-glucoside of 3-*O*-demethylcolchicine. It has attracted clinical attention because it exhibits a low cytotoxicity. Nevertheless, acting as a prodrug, colchicoside has been shown to exhibit antitumoral activity *in vivo* in an animal model for pancreatic cancer [1,2].

Plant cell suspension cultures exhibit a vast biochemical potential for the production of specific secondary metabolites. They can serve as tools for the *in vivo* production of secondary metabolites as well as for the biotransformation of foreign substrates. Plant cell culture mediated biotransformation is now increasingly employed by synthetic chemists for the structural modification of various organic compounds [3]. Biotransformation is an *in vitro* technique in which chemical conversions of an exogenously supplied substance can be catalyzed by microorganisms, cells or their enzymes. The biotransformation reactions catalyzed by cultured plant cells include oxidation, reduction, hydroxylation, esterification, hydrolysis, methylation, glycosylation, and isomerization [4,5]. Biotransformation is an area of biotechnology that has received considerable attention, because a pharmaceutically less important precursor can be converted to the compound of interest. Recently, the biotransformation of colchicinoids to their 3-*O*-glucosyl derivatives using *Bacillus megaterium* has been reported [6].

In this work we describe the capacity of suspension cultures of *Astragalus vesicarius* to glycosylate colchicine, derived from its natural source *Gloriosa superba* seeds, regiospecifically at C-3. This biotransformation process is based on preliminary, regioselective demethylation of the C-3 methoxy group of colchicine, and a subsequent glycosylation of the demethyl derivative at the same site. *In vitro* glycosylation of colchicine to its 3-*O*-demethylglucoside has so far been investigated with different biotechnology tools, especially using microbial strains such as *Bacillus aryabhattai* [7], but here we describe a protocol using a plant suspension culture. Poulev *et al*. [8] have obtained only unspecific demethylation of colchicine using a *Colchicum variegatum* culture yielding a mixture of 3-demethylcolchicine and 2-demethylcolchicine. Regiospecific demethylation at C-3 of colchicine was achieved using selected bacterial microorganisms. Glycosylation of exogenous thio-colchicine by plant cell suspension cultures of *Centella asiatica* reported by Solet *et al*. [9] resulted into monoglycosydated derivatives at C-2 and at C-3, which is not very selective. So far, according to the best of our knowledge, region-specific demethylation of the C-3 methoxy group of colchicine, and a subsequent glycosylation of the demethyl derivative at the same site, using plant suspension cultures was not achieved. Plant suspension cultures of *A. vesicarius*, which normally do not produce this type of non-heterocyclic alkaloids, have shown to be a suitable platform for bioproduction of colchicoside [10].

The ^{1}H-NMR data and HRESIMS (*m/z* [M + H$^+$] calcd for $C_{27}H_{33}NO_{11}$: 548.2126; measured: 548.2127) of the peak trapped at Rt 10.84' – 11.38' using LC-SPE-NMR were in agreement with the reported assignments for 3-de-*O*-methylcolchicine-3-*O*-*β*-D-glucopyranoside, or colchicoside [11].

After 14 days of cultivation, suspension cultures of *A. vesicarius* from the control (untreated) group reached a growth of 278.72 g/L. In all of the treated groups, a significantly decreased growth

compared to the control was observed (Figure 1). This might be due to the high colchicine concentration and its associated cytotoxicity, by restraining the microtubules and inhibiting gene expression of cytokinesis [12]. Inhibition of cell growth was also noted by Solet *et al*., when thiocolchicine was added to suspension cultures of *C. asiatica* [9].

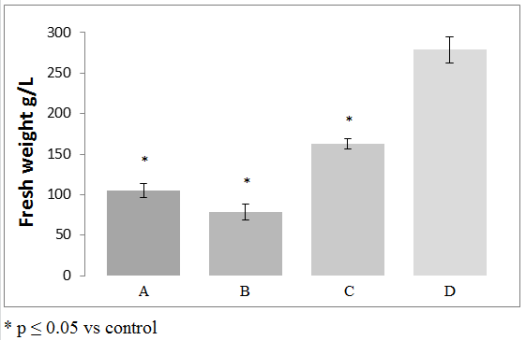

Figure 1. Growth of *A. vesicarius* suspension cultures treated with different colchicine concentrations: A – 1.04 g/L; B – 2.08 g/L; C – 3.09 g/L and D – untreated control. Values are expressed as means ± S.E.M. of 11 samples in each of the treated groups, and 6 in the control group.

Despite the suppressed growth, glycosylation of the substrate was observed in each of the treated groups. After 14 days of incubation, the colchicoside level reached 7.71 *µ*mol/g DW (RSD% 33.03) in group A, 9.35 *µ*mol/g DW (RSD% 39.85) in group B, and 8.13 *µ*mol/g DW (RSD% 27.44) in group C. The amount of colchicine taken up by the cells correlates with the increasing concentration of applied substrate. However, there is no significant difference in the amount of colchicoside produced in the three groups ($p>0.1$) (Figure 2). The maximum enzymatic potential for glycosylation of the suspension cells seems to be reached in each group. The accumulation rate of colchicoside in the cells is not dose dependent within the applied concentration range of colchicine substrate. The absence of colchicoside in the medium as determined by HPLC-UV analysis indicates it is a metabolite that is accumulated intra-cellularly.

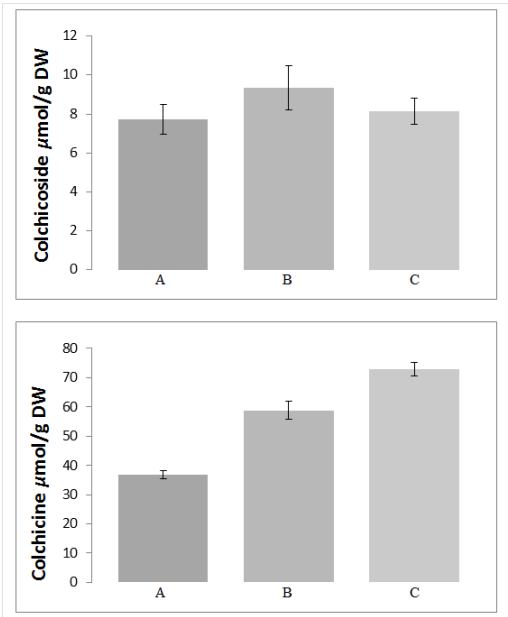

Figure 2. Amount of substrate and glycosylated derivative – colchicoside at the end of 14 days incubation of suspension cultures of *A. vesicarius* treated with different colchicine concentrations: A – 1.04 g/L; B – 2.08 g/L; C – 3.09 g/L. Values are expressed as means ± S.E.M. of 11 samples in each of the treated groups.

In conclusion, we have demonstrated that plant suspension cells of *A. vesicarius* have the capability to convert colchicine to its 3-*O*-glucosyl analogue – colchicoside. Nowadays, the main source of this monoglycosylated alkaloid remains its natural source – *G. superba* seeds (4.26 *µ*mol/g). Using a biotechnology approach for biotransformation, we propose an alternative method for bioproduction of colchicoside (9.35 *µ*mol/g DW). Future experiments should focus on the scale-up the described protocol in bioreactor systems, where the rate of biotransformation can be monitored, and where growth suppression can be overcome. In this way, the yield of important secondary metabolites may be improved.

Experimental

Plant material and culture conditions: In order to select a plant cell culture with efficient transformation capacity we screened three species (*Astragalus vesicarius*, *Linum thracicum* and *Gloriosa superba*) for colchicine glycosylation under identical conditions (data not shown). Plant cell suspension cultures of *A. vesicarius* were grown on modified Murashige and Skoog's (MS) medium [13] supplemented with 0.2 mg IAA/L (indole-3-acetic acid), 0.1 mg 2,4-D/L (2,4-dichlorophenoxy acetic acid), 2 mg kinetin/L (Kn) and 1.0 g casein/L, incubated on a rotary shaker (120 rpm) at 25 °C, pH 5.7. For the purpose of the experiment, suspension cultures were cultivated in 100 mL Erlenmeyer flasks (30 mL of fresh medium and about 3 g cells, FW) in dark conditions, because of the well-known light sensitivity of colchicine [14].

Preparation of the substrate: The seeds of *G. superba* were purchased from Gaurav International Inc. - India (phytosanitary certificate PSC #52PC). For the purpose of the experiment a colchicine rich fraction was obtained. *G. superba* seeds (900 g) were exhaustively extracted with 70% ethanol (~30 L). After each portion of fresh solvent, seeds were sonicated for 40 min. The extract was dried under reduced pressure, defatted with 3 L *n*-hexane (liquid / liquid partition) and the residue (142 g) was applied onto a flash column filled with MCI gel (160 g). Gradient elution from water to pure methanol was performed and fractions eluted with 30% to 60% methanol were combined into sub-fraction C. Sub-fraction C was applied to Sephadex LH-20 (200 g) and elution with methanol was performed. Fractions of 15 mL were collected. Based on analysis, fractions 15 − 26 were combined and applied onto a flash column filled with 80 g silica gel (70-230 mesh). Elution with a mixture of ethyl acetate / cyclohexane / methanol / ammonium hydroxide (70/15/10/5) was used to yield a colchicine-rich fraction (6.02 g).

Biotransformation procedure: The colchicine-rich fraction was dissolved in sterile water and without filtering under aseptic conditions it was added to suspensions in three concentrations (1.36 g/L, 2.72 g/L and 4.05 g/L). According to HPLC analysis, the colchicine content in this fraction was found to be 76.42% (RSD 1.29%, n=5), which corresponds to a colchicine concentration of 1.04 g/L (group A), 2.08 g/L (group B) and 3.09 g/L (group C), added to the medium on the first day of incubation. Each concentration level consisted of eleven randomized samples and the results are the average of the eleven replicates. In addition six controls were cultivated without addition of substrate. After 14 days of incubation, cells were separated from the medium with filter paper. The growth was measured as difference between initial and final FW of cells per volume of cell suspension culture.

Analytical procedure, product isolation and identification: The quantitative analysis of biotransformation precursor and product was performed by HPLC-UV. The same method was used in earlier studies to analyze colchicine and colchicoside from *Gloriosa superba*

seeds [15]. Briefly, about 100 mg of dried suspension samples were extracted for 30 min with 25 mL 80% methanol in a round bottom flask, heated under reflux. The solution was filtered through a filter paper and the extraction procedure was repeated twice, adding fresh solvent to the plant material every time. The combined filtrate was evaporated till dryness under reduced pressure. The dried extract was dissolved in 100% methanol and transferred into a volumetric flask of 5.0 mL. The solution was filtered through a Nylon syringe filter (0.25 μm) and HPLC analysis was performed to determine the amount of colchicoside, while to determine the amount of colchicine 25 times dilution with methanol was performed. Colchicine and its analogue colchicoside were quantified by HPLC under the following conditions: analytical column Phenomenex Luna, 5 μm particle size, C18 100 Å (250 x 4.6 mm) with a flow rate of 1.0 mL/min and UV detection performed at 245 nm. As a mobile phase (A) water and (B) acetonitrile were used. A linear gradient was applied: 0 min 10% B; 5 min 10% B; 25 min 40% B; 35 min 100%; 40 min 100% B. Rt (min): colchicoside (14.3'), colchicine (24.9'). The controls were analyzed following the same method.

In order to isolate colchicoside, a random sample obtained during the quantitative analysis was subjected to liquid chromatography - solid phase extraction - nuclear magnetic resonance spectroscopy (LC-SPE-NMR) consisting of an Agilent 1200 series HPLC system, with degasser, quaternary pump, automatic injection sampler and an UV/VIS variable wavelength detector (Agilent Technologies, Eindhoven, The Netherlands). Chromatographic conditions were previously optimized to provide appropriate separation. The system was operated with a Xbridge column, 5 μm particle size, C18 (250 mm x 4.6 mm, Waters, Ireland). As a mobile phase (A) water + trifluoroacetic acid 0.03% (v/v) and (B) acetonitrile were used. Water for HPLC was prepared by a Milli-Q system, Millipore (Bedford, MA, USA). Acetonitrile and trifluoroacetic acid were supplied by Acros Organics (Geel, Belgium). A linear gradient of 42 min in total was applied: 0 to 5 min 10% B, 25 min 40% B, 35 min 100% B, 37 min 100% B and equilibrated to initial conditions for 5 min. The flow rate was 1.0 mL/min and UV detection was performed at 254 nm. The injection volume within the sequence was 40 μL (5 mg/mL). Samples were collected with a Bruker/Spark Solid Phase Extraction system on 10 x 2 mm HySphere Resin GP cartridges, mean particle size 12 μm and eluted for subsequent NMR analysis using a Gilson Liquid Handler 215 with methanol-*d4* (99.8% D) from Sigma-Aldrich (Steinheim, Germany). NMR spectra were recorded on a Bruker DRX-400 NMR instrument (Rheinstetten, Germany) equipped with 3 mm inverse broad-band (BBI) probe, operating at 400 MHz for ^{1}H and at 100 MHz for ^{13}C. The spectra were processed with Topspin version 1.3. Mass spectra were recorded using a Thermo Scientific Q Exactive plus quadrupole - Orbitrap mass spectrometer coupled with a UPLC Dionex Ultimate 3 000 RSLC system equipped with a RP-18 Kinetex column (2.10 mm x 100 mm, 2.6 μm, Phenomenex Corporation, Torrence, CA, USA). MS grade solvents acetonitrile and water were used (Fischer). Gradient mixing (1.4 min 10% acetonitrile; 7 min 40% acetonitrile; 10 min 100% acetonitrile) of filtered and degassed acetonitrile/water solution of formic acid 0.1% (v/v); column temperature 30 °C; a flow rate of about 300 μl/min was used during the analysis. MS/MS data for colchicoside were collected in positive ion mode by selected ion monitoring (SIM) of the transition from *m/z* 546 to 550.

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