Production of saponins from in vitro cultures of Astragalus glycyphyllos and their antineoplastic activity

Aleksandar Shkondrov\textsuperscript{a}, Ilina Krasteva\textsuperscript{a}, Iliana Ionkova\textsuperscript{a}, Pavlinka Popova\textsuperscript{a}, Yancho Zarev\textsuperscript{a}, Rositsa Mihaylova\textsuperscript{a} and Spiro Konstantinov\textsuperscript{b}

\textsuperscript{a}Department of Pharmacognosy, Faculty of Pharmacy, Medical University of Sofia, Sofia, Bulgaria; \textsuperscript{b}Department of Pharmacology, Pharmacotherapy and Toxicology, Faculty of Pharmacy, Medical University of Sofia, Sofia, Bulgaria

**ABSTRACT**

In the last decade the need of plant-derived cytotoxic compounds exceeds the possibilities for obtaining them from naturally grown sources. In vitro plant biotechnology offers a unique and often invaluable alternative for production of complex biologically active substances without harming the flora. *Astragalus glycyphyllos* L. (Fabaceae) is a plant native to Bulgaria that has been reported to contain triterpenoid saponins and flavonoids. It is used in folk medicine as an antihypertensive, diuretic, anti-inflammatory, anti-tumour, laxative, expectorant agent, etc. The aim was to study the saponin content of *A. glycyphyllos* cultures grown in vitro and their antiproliferative activity. Three types of cultures were developed: callus, shoot and suspension cultures. Murashige and Skoog’s, as well as other media, supplemented with various concentrations and combinations of plant growth regulators and different photoperiods were used. In all cultures the saponin content was determined by a novel liquid chromatography–mass spectrometry (LC–MS) method. Compared to the wild grown species, in vitro shoot cultures accumulated double the amount of the main saponin (225.00 ng/mg dw) found in the plant. Saponin-rich fractions obtained from shoot cultures were tested for cytotoxicity in a panel of various malignant human cells and half-maximal inhibitory concentration (IC\textsubscript{50}) values were determined. Interestingly, the saponin-rich fractions showed higher efficacy against urinary bladder cancer cells with constitutive high expression level of the xenobiotic pump gp170 (MDR1). In vitro cultures of *A. glycyphyllos* could serve as an alternative way for production of saponins, with promising antineoplastic activity, which deserves further detailed characterization.

**Introduction**

*Astragalus glycyphyllos* L. (Fabaceae) (wild liquorice, liquorice milk vetch) is a herbaceous, flowering perennial plant, native to Europe and widely distributed in Bulgarian flora [1]. In Bulgarian folk medicine, the species has been extensively used as a diuretic, to treat inflammations, menstrual pain, high blood pressure, etc. [2]. There are two main groups of secondary metabolites in *A. glycyphyllos*: flavonoids [3] and triterpenoid saponins [4]. The latter group include both cycloartane-type saponins: askendoside C and F [5] and 17(R),20(R)-3β,6α,16β-trihydroxy-cycloartanyl-23-carboxylic acid 16-lactone 3-O-β-D-glucopyranoside [6]; and oleanane-type saponins: soyasapogenol B and 3β,22β,24-trihydroxy-olean-12-ene-19-one [7,8]. Cycloartane saponins possess numerous pharmacological activities [4], incl. cytotoxicity, and are capable of modulating the immune response [9]. Many studies have proved the cytotoxic effects of *Astragalus*-derived triterpenoids and the most commonly explored ones were isolated from *A. membranaceus* (Fisch.) Bunge [10]. Nevertheless, many species from the genus have unexplored potential in anticancer therapy. Such a species is *A. glycyphyllos*, for which only volatiles from its aerial parts have been reported to exert cytotoxic activity on a panel of human tumour cells [11]. The medicinal interest in *A. glycyphyllos* and its continuous depletion are the main reasons to apply in vitro techniques for triterpenoid saponin production. Production of these compounds from plant tissue cultures is of importance, as the content in the intact plant is relatively low [12]. The quality and quantity of the secondary metabolites derived

**Keywords**

*Astragalus glycyphyllos*; in vitro plant cultures; saponins; antitumour activity; multidrug resistance overcoming
from their natural habitats as well as from cultivated plants is dependent on the environmental conditions, application of pesticides, diseases etc. In vitro plant cultures have been considered to be the most efficient technology for crop improvement as well as for alternative production of valuable therapeutic secondary metabolites [13,14]. Biotechnology offers a unique alternative for sustainable production of secondary metabolites without harming the flora and eliminates the risk of variations, increased quantity of compounds, and may lead to finding novel pharmacologically significant molecules [15–17]. Astragalus-derived saponins produced in plant cell cultures were reported previously, many with higher quantity than those from wild grown plants [4,12]. The aims of this study were: (i) to investigate the biotechnological possibilities to increase the yield of saponins of Astragalus glycyphyllos by in vitro cultivation; (ii) to quantify the saponin content using a suitable analytical method; (iii) to explore the cytotoxic effects of saponin-rich fractions of different origin on various human malignant tumour cell lines.

Materials and methods

Plant material

Astragalus glycyphyllos was collected from Vitosha Mountain, Bulgaria, in June (herbs) and August (seeds) 2018. The species was identified by Dr. D. Pavlova from the Faculty of Biology, Sofia University, where a voucher specimen was deposited (no. SO-107613).

In vitro cultivation

Seeds were surface-sterilized and incubated using modified Murashige and Skoog (MS) medium (DoH), supplemented with 1 g/L casein and 20 g/L sucrose. To produce shoot cultures, explants of 3-week-old seedlings were cultivated on MS medium [18]. Callus cultures were initiated from shoot explants, cultivated on modified MS medium (1 g/L casein, 2 mg/L kinetin, 0.02 mg/L indole-3-acetic acid (IAA) and 0.1 mg/L 2,4-dichlorophenoxyacetic acid – G48 medium) as well as modified MS medium (1 g/L casein, 2 mg/L kinetin, 1 mg/L IAA – GS6 medium), both in the dark and light. The period of cultivation was 3 weeks. When callus was cultivated on liquid G48 medium without agar-agar on a rotary shaker (90 rpm) in light, suspension cultures were derived. The period of cultivation was 2 weeks.

Obtaining of purified saponin fractions from wild grown and in vitro shoots

Aerial parts of wild grown plants (200 g) were defatted with dichloromethane and then extracted by percolation with 80% methanol (MeOH; 1200 mL). After evaporation, the dried extract was separated by column chromatography (CC) on Diaion® HP-20 (Supelco, USA) (H2O/MeOH gradient) and 50 fractions (100 mL) were collected. Fractions 1–3, rich in cycloartane saponin (S1) (Supplemental Figure S1), previously isolated [6] were used for pharmacological tests. The same procedure for extraction and purification was performed on shoots (4.82 g). Fifty fractions (20 mL) were collected and seven main subfractions were obtained. Fractions 4–6 contained S1 and were used for pharmacological investigation.

Quantitative analysis of saponins

A sample (200 mg) of the plant material was extracted with 80% MeOH (2 × 4 mL) in reflux (30 min each). The extracts were filtered combined and the volume adjusted to 10.0 mL with 80% MeOH in a volumetric flask. An aliquot of 1 μL was injected to the ultra-high-performance liquid chromatography (UHPLC) system. UHPLC high resolution electrospray ionization mass spectrometry (UHPLC-HRESI/MS) analysis was performed using a Q Exactive™ Plus Orbitrap mass spectrometer with a heated electrospray ionization (HESI) ion source (ThermoFisher Scientific, Bremen, Germany) in ultra-high resolution mode (70,000, at m/z 200) and a UHPLC system (Dionex UltiMate 3000 RSLC, ThermoFisher Scientific, Bremen, Germany). The operating conditions of the HESI source ionization device were: 3.5 kV voltage and 320°C capillary temperature, 25 units of carrier gas flow and 5 units of dry gas flow. All other detector parameters were set in such a way as to obtain the most intense signal from [M-H]−. Nitrogen was used to atomize the samples. UHPLC separations were performed on a Kromasil® C18 column (1.9 μm, 2.1 × 50 mm, Akzo Nobel, Sweden) at 30°C. A mobile phase of 0.1% HCOOH (A) and MeCN + 0.1% HCOOH (B) with a flow rate of 0.3 mL/min was used. Gradient elution was performed as follows: 10% B for 0.5 min, then increase to 30% B for 7 min, isocratic with 30% B for 1.5 min, increase to 95% B for 3.5 min, isocratic with 95% B for 2 min, then return to 10% B for 0.1 min and 4 min equilibration at 10% B prior to the next injection. The reference used was 17(R),20(R)-3β,6α,16β-trihydroxyocta23-carboxylic acid 16-lactone 3-O-β-D-glucopyranoside (S1) (Supplemental Figure S1). The saponin was isolated...
(99.8% purity) and structurally elucidated by nuclear magnetic resonance (NMR) and MS as reported before [6]. The HRESIMS spectrum of S1 showed an adduct \([M + HCOO]^-\) at \(m/z = 623.3441\) (calcd. 623.3432), which is stable, always present and suitable as an ion marker to perform quantitation. Full scan MS–SIM was used with a set range of \(m/z\) 621.4 to 625.4. Standard solutions of S1 were prepared in MeOH as follows: 12.8; 64; 128; 320; 640 and 1280 ng/mL. One microlitre of each solution was injected in the UHPLC-MS system in triplicate to obtain the calibration curve. The program Thermo Scientific Xcalibur®, Version 4.2.28.14 was used for data collection, to construct the calibration curve and to calculate the results.

**Antitumour activity in vitro**

**Cell lines, cultivation conditions and stock solutions**

The following cell lines were used: T-24 (bladder carcinoma), CAL-29 (bladder transitional cell carcinoma); MJ (cutaneous T-cell lymphoma of the Mycosis fungoides type); and HUT-78 (cutaneous T-cell lymphoma of the Sézary syndrome type). All tumour cell lines were purchased from DSMZ GmbH (Braunschweig, Germany) and cultured under standard conditions. Stock solutions of saponin fractions were prepared in dimethylsulphoxide (DMSO) and subjected to serial dilutions with nutrition medium to produce the desired concentration.

**MTT assay and half-maximal inhibitory concentration (IC\(_{50}\)) determination**

The cytotoxic and antiproliferative activity of the saponins' fractions were investigated via the standard MTT test. The study was conducted through a well-known procedure [19] with slight modifications [20].

**Western blot analysis of the MDR1 expression level in urinary bladder cancer cells**

Equal aliquots of urothelial cancer cells \((2 \times 10^6\) cells\) were subjected to immunoblot analysis of the content of the multidrug resistance protein gp170 (MDR1). Exponentially growing T-24 and CAL-29 cells were harvested after trypsinization and frozen as dry pellets at \(-85 \, ^\circ\text{C}\) until western blot analysis. Cell lysates were prepared and their protein content was measured as previously described [21]. Lysates were subjected to denaturing polyacrylamide gel electrophoresis in 4–20% Precise Protein Gels (Thermo Scientific, Rockford, IL, USA), adhering to the manufacturer’s recommendations. The separated proteins were transferred to polyvinylidene difluoride membranes, which were thereafter processed as described previously [21]. The following antibodies from Santa Cruz Biotechnology (Heidelberg, Germany) were used: mouse monoclonal antibodies against MDR1 (s.c. 55510) and a horseradish peroxidase (HRP)-conjugated donkey anti-mouse antibody (sc-2314). Chemiluminescence was recorded using different exposure of X-ray films.

**Data analysis**

Data were analyzed with the program GraphPad Prizm v. 6.01. To evaluate the statistical significance, Student-Fischer t-test was conducted. Values of \(p \leq 0.05\) were considered as statistically significant. IC\(_{50}\) values were calculated by non-linear regression (GraphPad Prizm).

**Results and discussion**

**In vitro cultivation**

Shoot cultures (Supplemental Figure S2) were cultivated for a period of 4 weeks and reached growth index (GI) of 1.17 ± 0.04. Calli grown on G48 medium reached GI = 0.82 ± 0.03 in light and GI = 0.53 ± 0.04 in the dark. Callus cultures grown on G56 medium had GI = 0.76 ± 0.04 under light regimen and GI = 0.30 ± 0.03 in the dark. Suspension cultures cultivated on liquid G48 medium in light reached GI = 0.97 ± 0.02. The results from in vitro cultivation correlate with data reported before [12,13].

**Method validation**

During the UHPLC separation optimization, mobile phases similar to those reported previously [22] were used. The separation was three times faster compared to the conventional HPLC, and approx. 80% less solvent was consumed. The MS parameters like ionization mode, capillary and cone voltages, collision energy, etc. were optimized previously as part of the structural elucidation of S1 [6]. The present UHPLC-MS method does not require any type of sample purification. The reference compound S1 (Supplemental Figure S1) maintained a retention time of 10.26 ± 0.02 min. The equation \(y = 259139 + 23206.4 \times r^2 = 0.9997\) was obtained. The HRESIMS spectrum of the standard in the established \(t_R\) showed an adduct \([M + HCOO]^-\) at \(m/z = 623.3441\) (Supplemental Figure S3), consistent with the literature [6].

The method was validated as recommended by The International Council for Harmonisation of Technical
Requirements for Pharmaceuticals for Human Use (ICH) [23]. Specificity in respect of reagents was examined on blank solution except the saponin. There were no peaks in the chromatogram of this solution with \( t_R \) (S1). The limit of detection, based on three times the signal-to-noise ratio, was calculated as 0.022 pmol by repeatedly injecting 1 \( \mu \)L portions of diluted standard solution. The linearity was studied by injecting standard solutions with saponin levels ranging from 0.022 to 2.21 pmol; the correspondence between the area of the peaks and concentrations in ng/mL was proportional in the intervals with \( r^2 > 0.999 \). The precision and accuracy of the method were evaluated by spiking extracts from wild-grown \( A. \) glycyphyllos with standards at final concentrations of 37 and 540 ng/mL. Method precision, expressed as relative standard deviation (RSD %), was estimated by measuring six replicates of each concentration. The precision was 4%, and the accuracy was 1.1%. Six solutions containing the saponin were analyzed for repeatability. The standard deviation (SD) (%) was found to be \( \pm 1.0\% \) for these.

**Quantitation of saponins in plant samples**

Significant differences between the tested samples were found (Table 1). They were neither consistent with the type of the culture, nor with the level of cellular differentiation of each culture, except for the shoots, which produced two times more of the target compound than the wild grown herb. The highest quantity of saponin S1 was determined within shoot cultures \((231.15 \pm 0.22 \text{ ng/mg dw})\). This yield of S1 exceeded almost twice the amount derived from natural habitats of \( A. \) glycyphyllos \((103.77 \pm 0.04 \text{ ng/mg dw})\). In contrast to shoot cultures, the average amount achieved in callus on G48 medium under light was only \( 6.43 \pm 0.12 \text{ ng/mg dw} \). Despite the weak growth within the callus cultivated on G56 (light regimen) the average amount of saponin S1 was higher \((26.37 \pm 0.01 \text{ ng/mg dw})\). Darkness resulted in suppressed growth within callus cultivated on both medium G48 and G56 followed by decreased yield of S1 \((0.25 \pm 0.11 \text{ ng/mg dw})\) for G48 medium, whereas for G56 the S1 content was below the limit of detection (LOD) (Table 1). For that reason, established suspension cultures were grown under a light regimen of cultivation on G48, where the S1 content \((5.26 \pm 0.11 \text{ ng/mg dw})\) was comparable to that in the callus. The highest growth index was reached within shoot cultures and the lowest within callus cultures grown on G56 medium under the dark regimen of cultivation, which correlated with the quantity of S1. The higher saponin content of S1 in shoots could be explained with the highest level of organization and tissue differentiation in those cultures than in undifferentiated callus and unorganized suspension types [12,13].

**Cytotoxic activity in vitro**

The antineoplastic activity in vitro of the saponin-containing fractions 1-3 and 4-6 obtained from wild-grown and cultivated \( A. \) glycyphyllos, respectively, was tested in a panel of human tumour cell lines of different origin and characteristics using a standard MTT-based protocol for assessment of cell viability. The results of the conducted experiments are summarized in Table 2 and Supplemental Figure S4. As evident from the MTT-bioassay data, both fractions inhibited tumour cell growth in a dose-dependent manner.

However, according to the calculated IC\(_{50}\) values (Table 2), the fraction obtained from the in vitro shoot cultures (4-6) showed relatively superior cytotoxic activity as compared to that of the wild-grown species (1-3) in all of the screened tumour cell lines. Furthermore, the same fraction was found to be more effective against both lymphoma cell lines MJ and HUT-78 with closely ranging IC\(_{50}\) values of 74.5 and 77.8 \( \mu \)g/mL, respectively. The cell line that proved to be least responsive towards the 1-3 saponin-containing fraction was the bladder carcinoma cell line T-24 \((\text{IC}_{50} = 168.4 \mu \text{g/mL})\), whereas HUT-78 lymphoma cells were ranked as most chemo-sensitive \((\text{IC}_{50} = 87.6 \mu \text{g/mL})\). As opposed to 4-6, no similarity was observed in the cytotoxicity data of the 1-3 fraction in regard to tumour cell origin. Noteworthy, the estimated IC\(_{50}\)

**Table 1. Content of saponin S1 in wild and in vitro cultivated samples**

| Culture | S1, ng/mg dw ± SD |
|---------|-------------------|
| Callus, G48, hv | 6.43 ± 0.12 |
| Callus, G48, dark | 0.25 ± 0.01 |
| Callus, G56, hv | 26.37 ± 0.01 |
| Callus, G56, dark | < LOD\(^a\) |
| Shoots, MS | 231.15 ± 0.22 |
| Susp., G48, hv | 5.26 ± 0.11 |
| Wild | 103.77 ± 0.04 |

\(^a\)Under LOD (limit of detection).

**Table 2. In vitro cytotoxicity (mean IC\(_{50}\) values, [\(\mu\text{g/mL}\) ± SD] of the saponin-rich fractions 1-3 and 4-6 against T-24, CAL-29, MJ and HUT-78 tumour cell lines**

| Cell line/fraction | T-24\(^a\) | CAL-29\(^a\) | MJ\(^b\) | HUT-78\(^c\) |
|--------------------|-----------|-----------|--------|-----------|
| 1-3                | 168.4 ± 13.1 | 105.6 ± 11.5 | 126.3 ± 15.2 | 87.6 ± 7.4 |
| 4-6                | 124.8 ± 12.7 | 90.2 ± 7.1 | 74.5 ± 6.4 | 77.8 ± 4.3 |

\(^a\)Urinary bladder carcinoma.
\(^b\)Cutaneous T-cell lymphoma of the Mycosis fungoides (cutaneous) type.
\(^c\)Cutaneous T-cell lymphoma of the Sézary syndrome (leukaemic) type.
values were substantially lower in CAL-29 urinary bladder cells as compared to the cell line T-24. Both cell lines were found to express the xenobiotic pump gp170 (MDR1) as evidenced by western blotting (see Supplemental Figure S5). CAL-29 cells were found to express much higher levels of MDR-1 as compared to T-24 cells.

These findings corroborate well with the above reported data on the estimated saponin content of both fractions, whereby the concentration of S1 in the cultivated plants was found to be twice as high as compared to that in the wild-grown counterpart (Table 2). There was a different trend in the dose-response patterns, however, as seen in the column graphs of CAL-29, MJ and HUT-78 cells, by which fraction 1-3, which was poorer in the S1 saponin, paradoxically exhibited slightly more pronounced inhibitory effects at the lowest exposure levels (6.25 and 12.5 µg/mL). Nonetheless, raising the saponin concentration in the smaller serial dilutions of this fraction (25, 50 and 100 µg/mL) did not result in higher and more sustainable antineoplastic efficacy, as was the case with the 4-6 fraction. Furthermore, there was no twofold variation in the correspondent equieffective concentrations of both fractions, as is to be expected due to their S1 content. These results indicate that components other than the target cycloartane derivatives, and/or synergism with the hitherto described secondary metabolites may affect the antitumour properties of both fractions.

Finally, the greater antiproliferative effects of fraction 4-6 in the T-cell lymphoma lines (MJ, HUT-78) growing in suspension could be related to the larger membrane surface of these cultures as compared to the adherent ones (CAL-29 and T-24), which provides the amphiphilic saponins a greater accessibility to the phospholipid bilayers and favours changes in membrane fluidity and permeability. Moreover, of the two bladder carcinoma cell lines (T-24 and CAL-29), the CAL-29 cells which overexpress the MDR1 efflux pump, were slightly more chemosensitive. It could therefore be concluded that the expression levels of the MDR1 transporter do not affect the cytotoxic activity of either the 4-6 fraction, or the 1-3 fraction.

Our experimental findings about the antitumour activity of saponins are in line with many published data about *A. hamosus* [24,25], *A. suberi* [26], *A. membranaceus* [27]. In addition, apoptosis induction was detected in hepatocellular carcinoma HEP-G2 cells after treatment with *A. membranaceus* saponins [28,29]. Apart from its pro-apoptotic and anti-proliferative activities, a purified saponin mixture derived from the plant reduced the invasiveness and angiogenesis of human gastric adenocarcinoma cells [30]. For the first time evidence was obtained for overcoming the multidrug resistance phenotype by the investigated *Astragalus* saponin mixtures. Our *in vitro* findings could not be explained by immunomodulation because of the limitation of the tumour models used. Some of the other pharmacological activities such as antimicrobial and antifungal activity, as well as wound healing enhancement of the compounds from *Astragalus* could be beneficial in the case of cutaneous T-cell lymphoma and urinary bladder cancer cells.

**Conclusions**

A novel method was developed and applied for analysis of the saponin content in samples, obtained from wild grown and *in vitro* cultivated *A. glycyphyllum*. Shoot cultures produced twice the quantity of the cycloartane saponin S1 than wild species. The cytotoxicity of the saponin fractions was examined on a panel of human malignant cell lines and considerable activity was proved. These results clearly demonstrate the potential of *in vitro* cultivation as a source of pharmaceutically important metabolites such as cytotoxic triterpenoid saponins. This could be of special interest for future biotechnological approaches.

**Disclosure statement**

No potential conflict of interest was reported by the author(s).

**Funding**

This study was supported by the Ministry of Education and Science of the Republic of Bulgaria, program no. DO1-217/30.11.2018.

**References**

[1] Valev SA. Astragalus L. In: Yordanov D, editor. *Flora repubrice popularis bulgaricea*. Sofia: Aedibus Academiae Scientiarum Bulgaricae; 1976. p. 167.

[2] Nikolov SD, editor. Specialized encyclopedia of medicinal plants in Bulgaria. Sofia: Labour Publishing House; 2006.

[3] Krasteva I, Shkondrov A, Ionkova I. Advances in phytochemistry, pharmacology and biotechnology of Bulgarian Astragalus species. Phytochem Rev. 2016; 15(4):567–590.

[4] Ionkova I, Shkondrov A, Krasteva I, et al. Recent progress in phytochemistry, pharmacology and biotechnology of Astragalus saponins. Phytochem Rev. 2014; 13(2):343–374.
Linnek J, Mitaine-Offer AC, Miyamoto T, et al. Two cycloartane-type glycosides from the roots of *Astragalus glycyphyllos*. Planta Med. 2008;74(09):P8141.

Shkondrov A, Krasteva I, Bucar F, et al. A new tetra cyclic saponin from *Astragalus glycyphyllos* L. and its neuroprotective and hMAO-B inhibiting activity. Nat Prod Res. 1. 2018. DOI 10.1080/14786419.2018.1491040

Elenga PA, Nikolov S, Panova D. Triterpene glycosides from *Astragalus glycyphyllos* L. - a new natural compound of the overground parts. Pharmazie. 1987;42:422–423.

Elenga PA, Nikolov S, Panova D. Triterpene glycosides and sterols from *Astragalus glycyphyllos* L. Pharmazie. 1986;41:41–42.

Lacaille-Dubois MA. Saponins as immunoadjuvants and immunostimulants In: Wagner H, editor. Immunomodulatory agents from plants. Basel: Springer; 1999. p. 243–272.

Jung Y, Jerng U, Lee S. A systematic review of anti-cancer effects of Radix Astragali. Chin J Integr Med. 2016;22(3):225–236.

Momkév G, Krasteva I, Platikanov S, et al. Cytotoxic activity of volatiles from four *Astragalus* species. C R Acad Bulg Sci. 2007;60:1023–1026.

Ionkova I. *Astragalus* species (milk vetch): In vitro culture and the production of saponins, astragaline, and other biologically active compounds In: Bajaj PS, editor. Biotechnology in agriculture and forestry 33. Berlin: Springer; 1995. p. 97–138.

Ionkova I. Pharmaceutically significant biologically active compounds from sources with optimized phyto-chemical potential [doctor of science]. Sofia: Medical University; 2007.

Raskin I. Plants and pharmaceuticals in the 21st century In: Vasil IK, editor. Plant biotechnology 2002 and beyond. Heidelberg: Springer; 2003. p. 83–95.

Alfermann AW, Petersen M. Natural product formation by plant cell biotechnology. Plant Cell Tiss Organ Cult. 1995;43(2):199–205.

Cragg GM, Newman DJ. Natural products: A continuing source of novel drug leads. Biochim Biophys Acta-Gen Subj. 2013;1830(6):3670–3695.

Vervoort R. Pharmacognosy in the new millennium: leadfinding and biotechnology. J Pharm Pharmacol. 2000;52(3):253–262.