PHARMACIA

Pharmacia 70(4): 1449–1453
DOI 10.3897/pharmacia.70.e114989

Research Article

Biotechnological approaches for sustainable production of astragaloside I, II and IV from endemic species of *Astracantha aitosensis* (Ivan.) and *Astragalus membranaceus* (fisch.) by *in vitro* cultures

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Received 31 October 2023 ♦ **Accepted** 15 November 2023 ♦ **Published** 30 November 2023

Citation: Enchev P, Zarev Y, Ionkova I (2023) Biotechnological approaches for sustainable production of astragaloside I, II and IV from endemic species of *Astracantha aitosensis* (Ivan.) and *Astragalus membranaceus* (Fisch.) by i*n vitro* cultures. Pharmacia 70(4): 1449–1453. https://doi.org/10.3897/pharmacia.70.e114989

Abstract

This study investigates the bioproduction of astragalosides I, II and IV from endemic *Astracantha aitosensis* (*arnacantha*) and *Astragalus membranaceus* species, and the biotechnological methods for increased efficiency. The extracts from established *in vitro* cultures, including *A. aitosensis* callus, shoots and roots and *A. membranaceus* hairy roots, showed higher astragaloside concentrations than native roots. Specifically, *in vitro A. aitosensis* cultures produced astragaloside I and II at 0.06 and 0.10 mg/g DW, which were absent in native roots. The production of *A membranaceu*s hairy roots exceeds 8 to 15 times astragaloside I and II (0.80 and 0.90 mg/g DW) production when compared to native roots (0.10 and 0.05 mg/g DW), and around 3 times high amount related to astragaloside IV. Addressing astragaloside production challenges, this research also reveals biotechnology approaches as an alternative for sustainable production of this rare cycloartane saponins, conserving the natural habitats. A pilot reproducible *in vitro* cellular platform has been created, and protocol for specific, unconventional induction of the biosynthesis of the desired target compounds, exploiting the enzymatic system of plant cells from the unexplored plant species *A. aitosensis* has been established. Our findings clearly show the possibility of using *in vitro* cultures of *A. aitosensis* and *A. membranaceus* for biotechnology production of cycloartane type saponins.

Keywords

Astracantha aitosensis, *Astragalus membranaceus*, LC/MS analysis, cycloartane saponins, *in vitro* cultures, hairy roots

Introduction

The genus *Astragalus* stands out for its substantial medicinal properties, including immunomodulation, antiviral activity, and antitumor effects, primarily due to the presence of cycloartane-type triterpene saponins such as astragalosides. These molecules, essential for the plant`s pharmacological potential, are traditionally harvested from native plants, a practice with challenges such as inconsistent saponin content, delayed maturation of plants, and the risk of depleting natural populations, particularly the widely utilized *A. membranaceus* (Shan et al. 2019). Genus *Astragalus* within the family Fabaceae, boasts approximately 3.000 species, predominantly found in the

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mountainous locales of Southwest Asia. Among them, *A. membranaceus*, especially the *mongholicus* variety, has garnered significant attention in traditional medicinal systems, such as the Chinese Materia Medica. Specific components for genus *Astragalus*, like astragalosides, flavonoids, and polysaccharides, have been known for their potent antioxidant and therapeutic properties (Shahzad et al. 2016; Stambolov et al. 2023). Cycloartane type saponins have garnered significant attention in the medical community due to their diverse and promising pharmacological activities. Astragaloside IV has demonstrated substantial therapeutic potential in treating diabetic nephropathy by significantly reducing blood glucose levels, mitigating kidney damage, and decreasing inflammation-related gene expression in rats (Zhang et al. 2020). Furthermore, it shows promising effects against acute myocardial infarction-induced chronic heart failure by reducing inflammation and inhibiting the TLR4/MyD88/Nf-kB signaling pathway, as supported by molecular docking studies and validated in an animal model (Shi et al. 2021). Beneficial for neurological disorders, astragaloside IV has emerged as a potential neuroprotective agent for subcortical ischemic vascular dementia (SIVD). It has been shown to improve cognitive function and white matter integrity by suppressing oxidative stress and enhancing the survival of mature oligodendrocytes through the modulation of SIRT1/Nrf2 signaling (Ma et al. 2023). This finding positions astragaloside IV as a promising candidate for future therapeutic interventions in SIVD. A systematic review of numerous articles reveals increasing research interest, indicating its potential for future research in ischemia-reperfusion injury, cancer, and tumor treatment (Liu et al. 2023a). In ovarian cancer treatment, it inhibits M2 macrophage polarization within the tumor microenvironment and suppresses the HMGB1-TLR4 signaling pathway, reducing the proliferation, invasion, and migration of cancer cells (Wang et al. 2021). Furthermore, it mitigates the impact of cancer-associated fibroblasts in gastric cancer by suppressing cell growth, migration, and invasiveness through the downregulation of specific genes in these fibroblasts (Liu et al. 2023b). The other important cycloartane saponin is astragaloside II which has shown remarkable results in enhancing the sensitivity of cisplatin-resistant ovarian cancer cells to chemotherapy. It inhibits cell growth, promotes apoptosis, and regulates proteins related to drug resistance and cell cycle while inducing autophagy, potentially through the AKT/mTOR signaling pathway (Zhang et al. 2023). Considering the urgent need to enhance the production of these saponins, due to their low natural abundance and the complexities of chemical synthesis, innovative biotechnological methodologies have emerged. These techniques, such as *Agrobacterium*-mediated hairy root cultures, have proven particularly promising, demonstrating their efficiency in increasing astragaloside content, as seen in recent studies using yeast extract elicitation in hairy roots cultures (Park et al. 2021). Moreover, *in vitro* methods offer substantial advantages including controlled, sterile conditions, continuous cultivation irrespective of season, and expedited isolation of desired secondary metabolites, qualities that are incapable of being attend through conventional reliance on wild populations.

In the current study we explore the sustainable bioproduction of astragalosides I, II, and IV, utilizing *in vitro* methodologies with endemic for Bulgaria and China respectively species *A. aitosensis* and *A. membranaceus* and comparing the achieved results with the relative native production. Our investigation encompasses a variety of *in vitro* cultures, such as root, callus, and shoot cultures of *A. aitosensis*, and hairy root cultures of *A. membranaceus*. It further includes an extensive analysis comparing the astragaloside content of these cultures with their naturally occurring counterparts. The integration of these cutting-edge biotechnological approaches not only serves the rapidly increasing pharmaceutical demand but also signifies a critical stride towards preserving biodiversity. By unifying traditional knowledge with contemporary scientific innovations, this research aims to optimize sustainable saponin production, enhancing therapeutic applications worldwide.

Materials and methods

Plant material

In vitro cultures of *A. aitosensis* and *A. membranaceus* were successfully established in our laboratory. For the establishment of shoot cultures of *A. aitosensis* sterilized seeds were used. The shoots that developed from seedlings were placed in flasks containing MS culture medium. Root culture propagation was successfully accomplished using MS-Li culture medium. Root culture propagation was successfully accomplished using MS-Li culture medium. Callus cultures of *A. aitosensis* were initiated by cultivating shoot cultures on modified MS medium supplemented with kinetin (2 mg/L), IAA (5 mg/L), 2,4-D (5 mg/L), and casein (1 g/L) (G48) (Ionkova et al. 2010). The leaf segments from sterile grown *A. membranaceus* plants, were wounded with a sterile needle and *Agrobacterium rhizogenes* LBA 9402 maintained on solid YMB media and before infection grown for 48 hours at 26 °, 80 ppm in liquid YMB, being spread onto the leaves (Ionkova et al. 1997). After four days, the explants were transferred to MS medium without phytohormones, containing 500 mg/L sodium cefotaxim (Claforan) to remove the excess of bacteria. The cultures were kept in dark, at 25 °. Roots developed after 3–4 weeks of incubation. After completely removing free-living *Agrobacteria*, the hairy roots are cultivated in the usual manner. Single roots (20–30 mm long) were transferred to liquid MS medium without phytohormones. For the time course of growth and saponin production, 2–3 cm (0.5 g fresh weight) root tips were inoculated in 25 mL medium in 100 mL flasks or 50 mL in 300 mL flasks and cultivated on a gyratory shaker at 120 rpm in darkness at 25 ± 2 °C and subcultured at a four-week interval.

The native *A. aitosensis* roots and seeds were carefully collected with permission by Bulgarian ministry of environment and water and license № 949/18.08.2022, from their natural habitat (Lat. 42.726660, Lon. 27.279542) and deposited in Bulgarian Academic of Science – "Index Herbariorum" (BAS-SOM) (voucher number 178665). While native *A. membranaceus* roots were delivered by HerbaSinica Hilsdorf GmbH (Ch.-B. 160601H004).

General experimental procedures and analytical methods

All used solvents were of at least analytical grade from Fischer Scientific. The following reference substances of cycloartane saponins were used: astragaloside I (95.0%) supplied by Cayman Chemical Company, astragaloside II (99.8%) obtained from Sigma-Aldrich, and astragaloside IV (98.0%) purchased from Tokyo Chemical Industry Co. Previously optimized LC-HRESI-MS analyses was used to determine the cycloartane saponins and the same calibration model was built for quantification purposes (Enchev et al. 2023).

Extraction of plant material and following purification

The dried plant material from *in vitro* cultures of *A. aitosensis* callus, shoots, and roots, and native *A. aitosensis* roots, as well as *in vitro* hairy roots of *A. membranaceus* and native *A. membranaceus* roots were subjected to exhaustive extraction using 80% MeOH. Concentrated extracts under reduced pressure were separated by solid-phase extraction (SPE) using C18 cartridges (Sep-Pak Vac 6cc, 500 mg, Waters, Ireland) to obtain MeOH and EtOAc fractions. This process resulted in two fractions for each initial extract and aliquot of each sample was prepared for LC-HRESI-MS analysis.

Results and discussion

In vitro cultivation

The shoot cultures of *A. aitosensis* cultivated under sterile conditions achieved a biomass with a growth index (GI) of about 0.25 ± 0.03. The callus cultures from *A. aitosensis* achieved GI of approximately 1.21 ± 0.07. The growth and development of hairy roots from *A. membranaceus* were studied during a 4-week period and achieved a high final density above 17.0 g DW/L.

Identification of cycloartane saponins

During the LC-HRESI-MS analysis, astragaloside I, II and IV were identified in the negative ion mode as adducts with HCOOH. Astragaloside I was detected as protonated molecular ion with *m/z* 913.48145 $[M+FA-H]^-$ corresponding to molecular formula $C_{46}H_{73}O_{18}$ (calc. *m/z* 913.4791) and tR 9.12 min, astragaloside II produce a protonated molecular ion at *m/z* 871.47100 $[M+FA-H]^-$ with molecular formula $C_{44}H_{71}O_{17}$ (calc. *m/z* 871.4686) and tR 5.61 min, while astragaloside IV was observed as protonated molecular ion at *m/z* 829.46060 $[M+FA-H]^-$ corresponding to molecular formula $C_{42}H_{69}O_{16}$ (calc. *m/z* 829.4580) and tR 4.31 min. The compounds in samples were identified based on their spectrum, mass fragmentation patterns and *m/z* ratios compared to standards.

Quantification of cycloartane saponins

During the evaluation of astragalosides production, remarkable differences were observed between *in vitro* cultures and native samples of *A. aitosensis*. Notably, while the native roots of *A. aitosensis* lacked astragaloside I and II, they exhibited a significant astragaloside IV concentration of 0.618 mg/g DW. Conversely, the *in vitro* cultures of *A. aitosensis* not only produced astragaloside I and II, even in small amounts, but also achieved considerable concentrations of astragaloside IV, with shoots and roots achieving 0.065 mg/g DW and 0.068 mg/g DW, respectively (Fig. 1).

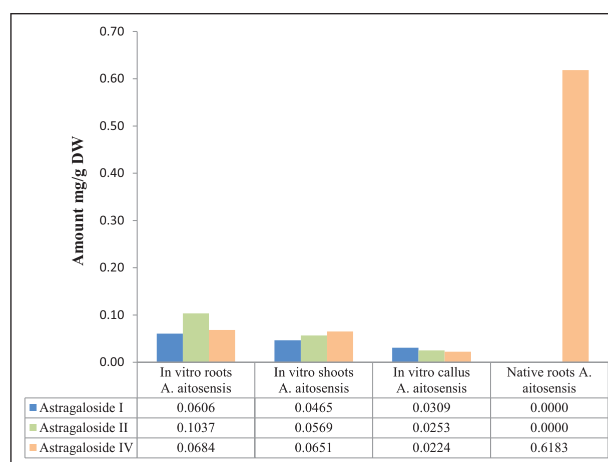

Figure 1. Amount of astragaloside I, II and IV in *in vitro* roots, shoots and callus culture from *A. aitosensis* and native roots from *A. aitosensis* represented as mg/g DW.

This deviation in metabolite content, with the *in vitro* cultures of *A. aitosensis* producing compounds absent in their native specimens, prove the transformative potential of *in vitro* cultivation and its implications for sustainable astragalosides production.

Transitioning to *A. membranaceus*, the hairy root *in vitro* cultures clearly outperformed their native counterparts for astragaloside I and II production, achieving 0.790 mg/g DW and 0.897 mg/g DW respectively, and producing a notable 0.110 mg/g DW of astragaloside IV. In comparison, the native roots of *A. membranaceus* yielded lower values for astragaloside I and II, registering 0.096 mg/g DW and 0.057 mg/g DW respectively, while still contributing 0.049 mg/g DW for astragaloside IV (Fig. 2).

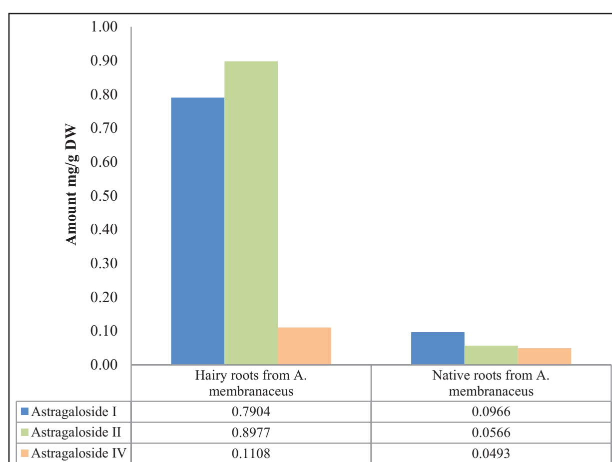

Figure 2. Content of astragaloside I, II and IV in *in vitro* hairy roots culture from *A. membranaceus*, and native roots from *A. membranaceus* represented as mg/g DW.

In summary, while the hairy roots of *A. membranaceus* stand out as abundant producers of astragaloside I and II and IV, the native roots of the endemic *A. aitosensis* remain with the highest quantity of astragaloside IV production. Nonetheless, the *in vitro* cultures, especially roots and shoots cultures of *A. aitosensis*, given their ability to produce compounds that do not present in their native origin, represent a promising and sustainable alternative for astragalosides production.

Astragalosides have proven immunomodulating, antiviral, and antitumor effects, but due to their complex structure and stereochemistry, they are still most effectively obtained from native plants (the Chinese endemic *A. membranac*eus), despite variations in the quantity and quality of the plant material, a long development period before the onset of production and excessive harvesting of endangered species for pharmaceutical purposes. Astragalosides I, II and IV characterized by their immunomodulatory action based on clinically proven effects, are highly desired in today`s global pharmaceutical market.

A pilot reproducible *in vitro* cellular platform has been created, and protocol for specific, unconventional induction of the biosynthesis of the desired target compound, exploiting the enzymatic system of plant cells from the unexplored plant species *A. aitosensis* (*arnacantha*), has been established. The method for development of *in vitro* hairy root culture of *A. membranaceus* presents the ability to produce astragalosides in 8 to 15 times increased yield (within 30–40 days) when compared to field-grown plants (3–5 years old roots).

Conclusion

The *in vitro* methodologies clearly offer an advantageous platform for the enhanced production of cycloartane saponins, minimizing the need for harvesting from wild populations. This aligns with the goals of sustainable production and conservation. In conclusion, the *in vitro* production approach holds promise for consistent and increased yield of astragalosides, offering a viable path for future therapeutic applications.

Acknowledgements

This study was financially supported by the Council of Medicinal Science at Medical University of Sofia, contract № D-203/03.08.2023.

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Supplementary material 1

LC/MS spectra of astragalosides I, II and IV within the different fractions

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Data type: pdf

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Link: https://doi.org/10.3897/pharmacia.70.e114989.suppl1