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Research Article

Evaluation of methyl jasmonate as elicitor in the production of cycloartane saponins in *Astragalus aitosensis in vitro* root cultures

Preslav Enchev[1], Yancho Zarev[1], Iliana Ionkova[1]

1 *Faculty of Pharmacy, Medical University - Sofia, Sofia, Bulgaria*

Corresponding author: Yancho Zarev (zarev.yancho@gmail.com)

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Abstract

Methyl jasmonate (MeJA) treatment is known to increase the levels of plant secondary metabolites. The significant medicinal benefits of cycloartane type saponins, including immune modulation, antiviral properties, and antitumor activity, drive high global demand, making sustainable production methods economically, medically, and ecologically important. In this study the effect of MeJA as an elicitor for promoting higher accumulation of cycloartane saponins – astragaloside I, II and IV was evaluated. The experiment was conducted using biotechnological approaches for sustainable production using *in vitro* root cultures of the endemic plant species *Astragalus aitosensis*, which is known to produce rare cycloartane saponins. LC-HRESI-MS analysis was used to identify and quantify compounds. The content of astragaloside I increased significantly by 68% (118 ng/mg DW) compared to control group (70 ng/mg DW). Astragaloside II was found with 1.5 points increased amount (473 ng/mg DW), compared to control (331 ng/mg DW) and astragaloside IV production was also improved. The highest cost-efficacy concentration of the elicitor for all tested astragalosides was observed at 50 µL MeJA.

Keywords

Methyl jasmonate (MeJA), biotechnology, elicitation, cycloartane saponins, *Astragalus aitosensis*

Introduction

The genus *Astragalus* is known for its significant medicinal benefits, including immune system modulation, antiviral properties, and antitumor activity. These effects are primarily due to cycloartane-type saponins (Li et al. 2014). One well-known species, *A. membranaceus* (Fisch.) Bge., especially the roots, referred to as Radix *Astragali* (RA) or Huangqi in China and Korea, is widely used in traditional remedies to strengthen 'Qi' (vital energy). RA is extensively utilized as both medicine and food, holding great potential for pharmaceutical and nutraceutical development. The rising demand for RA has led to annual sales exceeding 10 million tons, mainly from Chinese farms. However, natural populations, especially *A. membranaceus* var. *mongholicus*, are declining sharply due to overharvesting. The cultivation process, particularly in regions like Shanxi Province, takes 6–8 years, limiting yield and making it difficult to meet global demand. Consequently, RA is now a nationally protected plant in China (Zhang et al. 2021). The demand for RA is driven by its numerous pharmacological benefits, attributed to its rich content of cycloartane-type saponins, especially astragalosides. These compounds exhibit a wide range of biological activities, including hepatoprotection, neuroprotection,

cardiotonic effects, anti-aging, anticancer, and anti-inflammatory properties. This therapeutic potential underscores the need for sustainable cultivation practices to ensure a steady supply (Lee et al. 2013). Astragaloside II has shown significant pharmacological benefits, such as immune response modulation, tissue repair, and inflammation prevention. It has been found to ameliorate podocyte injury and mitochondrial dysfunction in diabetic nephropathy by regulating the Nrf2 and PINK1 pathways, offering a potential therapeutic strategy (Su et al. 2021). In recent studies, cycloartane-type saponins demonstrated the best results among 7000 phytocompounds screened for anti-dengue virus activity. Astragaloside II showed the most significant antiviral activity against dengue virus serotypes 1 and 3, followed by astragaloside III and IV, highlighting their potential as non-toxic therapeutic agents (Indu et al. 2021). Additionally, astragaloside II significantly induces proliferation, differentiation, and mineralization in primary osteoblasts, potentially preventing postmenopausal bone loss by increasing BMP-2 expression and activating several signaling pathways (Kong et al. 2012). Similarly, astragaloside I promotes osteogenic effects by increasing ALP levels and extracellular matrix calcium in a dose-dependent manner, enhancing BMP-2, BGP, and OPG/RANKL expression, thus showing potential for treating bone diseases (Cheng et al. 2016). Astragaloside IV, on the other hand, exerts strong anti-inflammatory effects by inhibiting key inflammatory mediators such as NF-κB and reducing proinflammatory cytokine production. It also displays potent antioxidative activity by scavenging reactive oxygen species (ROS) and regulating oxidative stress through pathways like PPARγ and FoxO1. Additionally, AS-IV's antifibrotic effects have been demonstrated in reducing renal fibrosis and epithelial–mesenchymal transition (EMT), while its antitumor properties include inhibition of cancer cell proliferation, migration, and invasion by modulating EMT-related pathways such as PI3K/AKT and NF-κB. Its ability to enhance chemosensitivity further emphasizes its potential as a therapeutic agent in cancer treatment (Liang et al. 2023). These factors, the growing demand and use of cycloartane-type saponins, along with novel studies revealing more pharmacological and medical benefits, mean that there are significant population pressures on *Astragalus* species (Zhang et al. 2021). Therefore, producing these biologically active compounds sustainably is of economical, medical and ecological interest. Ensuring a steady supply while preserving natural populations requires implementing effective cultivation practices and exploring alternative sources to meet the increasing global demand for these valuable phytochemicals.

In this context, one of the biotechnological methods being applied for sustainable production is the *in vitro* cultivation of *Astragalus* species (Ionkova 2008; Shkondrov et al. 2019; Stambolov et al. 2023). This study focuses on *Astracantha arnacantha* (M. Bieb.) Podlech subsp. *aitosensis* (Ivanisch.) Réer & Podlech, a local endemic subspecies of *Astracantha arnacantha* (M. Bieb.) Podlech, emphasizing its accepted nomenclature and endemic status (Anon n.d.). The accumulation of secondary metabolites is part of the plant`s natural defense against pathogens and environmental stresses and is generated in response. Elicitors are living organisms, nonliving agents, or environmental factors that stimulate plants to produce secondary metabolites, which play a key role in plant defense mechanisms and stress responses. Elicitors activate various signal pathways within the plant, triggering the production of these compounds. Biotic and abiotic elicitors, such as fungal carbohydrates, yeast extract, chitosan, and MeJA have been shown to effectively stimulate production. Among them, MeJA is particularly effective (Ionkova 2009). Since chemical elicitors can be classified as external stress factors, the concentration and duration of exposure should be investigated for optimal production results. In this study, we utilize MeJA to enhance the production of cycloartane-type saponins in *A. aitosensis in vitro* root cultures, focusing on optimizing the concentration of MeJA for the most effective outcomes. For this purpose, to assess the efficacy of MeJA in enhancing the production of cycloartane-type saponins three different concentrations: 50, 100, and 200 µL were tested over *in vitro* root cultures of *A. aitosensis*. The quantitative assay performed by LC-HRESI-MS analysis ensured the impact of MeJa treatment across varying doses.

Materials and methods

Plant material

Astragalus aitosensis explant was collected from their natural habitat - town Aytos, Bulgaria (Lat. 42.726660, Lon. 27.279542), with a permission of Ministry of Environment and Water (№ 949/18.08.2022) and a sample was deposited in the Bulgarian Academy of Sciences – "Index Herbariorum" (SOM - 178665). Establishment and propagation of the *in vitro* root culture were successfully done in our laboratory on G-56 culture medium (Enchev et al. 2023).

General experimental procedures and analytical methods

LC-MS analysis was performed with Dionex Ultimate 3000 RSLC UHPLC-HRESI-MS system from (ThermoFisher Scientific, Bremen, Germany)), which includes a 6-channel SRD-3600 degasser, an HPG-3400RS high-pressure gradient pump, a WPS-3000TRS autosampler, and a TCC-3000RS column compartment, connected to a Thermo Scientific Q Exactive Plus mass spectrometer (ThermoFisher Scientific, Bremen, Germany). All solvents used were of at least analytical grade and sourced from Fischer Scientific (Loughborough, UK). The reference substances for cycloartane saponins included astragaloside I (95.0%) from Cayman Chemical Company (Michigan, USA), astragaloside II (99.8%) from Sigma-Aldrich (Darmstadt, Germany), and astragaloside IV (98.0%) from Tokyo Chemical Industry Co (Tokyo, Japan). To determine the cycloartane saponins, previously optimized LC-HRESI-MS analyses were employed, and the same chromatographic and spectral analysis conditions as well as calibration model were used for identification and quantification purposes (Enchev et al. 2023).

Extraction and LC-MS sample preparation

The roots after a 21-day period of *in vitro* cultivation were dried and double-extracted in centrifuge vials containing 1.5 mL 70% MeOH for 40 min using an ultrasonic bath (37 kHz, 40 °C. Each vial was then centrifuged at 120,000 rpm for 2 min, and a 1 mL was transferred into a 10 mL volumetric flask. The vials were refilled with 1 mL of 70% MeOH and subjected to a second 40 min extraction under the same ultrasonic conditions. After centrifugation, another 1 mL was transferred to the volumetric flask and filled up to 10 mL with water, 1 mL aliquot of each sample was used for LC-MS analysis.

Identification of cycloartane saponins

The amounts of astragalosides I, II and IV were detected using a LC-HRESI-MS device. The compounds were compared to reference standards, by retention times and fragmentation patterns. The standards were dissolved in 50% MeOH and diluted to 5 progressively increasing concentrations upon which was built calibration curve for quantitative assay. The amounts of astragalosides were calculated with the calibration equation formula from the curve of the standards. All the astragalosides I, II and IV were detected in the *in vitro* root cultures.

Effect of elicitor and statistical analysis

The effect of elicitation with MeJA was evaluated according to the cycloartane-type saponins accumulation. The experiment examined three concentrations of MeJA (50 µL, 100 µL, and 200 µL) on *in vitro* root cultures of *A. aitosensis*. Each MeJA concentration was tested in triplicate. Two control groups were included: one group treated with EtOH (serves to dissolve MeJA) and one group without any treatment. The data from the MeJA-treated groups were compared to the control groups to assess the effectiveness of MeJA in enhancing cycloartane-type saponin production. Statistical significance was determined using one-way ANOVA.

Results

Elicitation of in vitro root cultures of *A. aitosensis*

The application of 50 µL MeJA resulted in a significant increase in astragaloside I production, reaching 118.86 ng/mg DW compared to 70.77 ng/mg DW in the untreated group and 75.79 ng/mg DW in the EtOH treated group. However, higher concentrations of MeJA did not yield proportionally higher levels of astragaloside I. Specifically, 100 µL MeJA led to a lower production (90.86 ng/mg DW), while 200 µL MeJA increased the production from 113 ng/mg to 143.12 ng/mg DW, which is still not as efficient as the 50 µL treatment (Fig. 1).

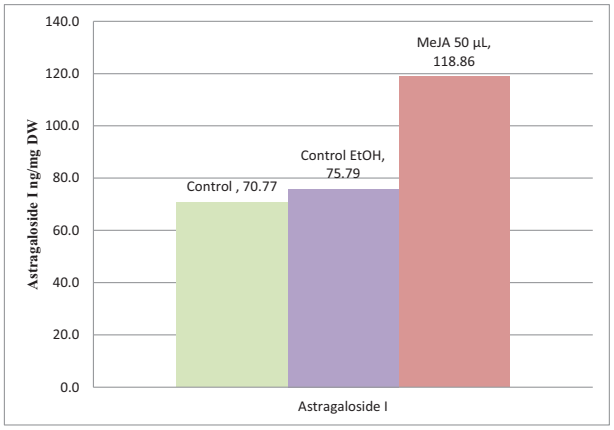

Figure 1. Astragaloside I content in *in vitro* cultures after treatment with 50 µL MeJA.

For astragaloside II, the 50 µL MeJA treatment also proved to be optimal concentration of treatment, increasing production to 473.89 ng/mg DW from 331.24 ng/mg DW in the untreated control group and 371.21 ng/mg DW in the EtOH treatment (Fig. 2). Also, it is obvious that the amounts for astragaloside II are much higher, even in the control group, and after elicitation the production becomes considerable. Bearing in mind that astragaloside II particularly is one of the most active and well-known for its pharmacological properties saponin, this makes it a priority for increased productivity.

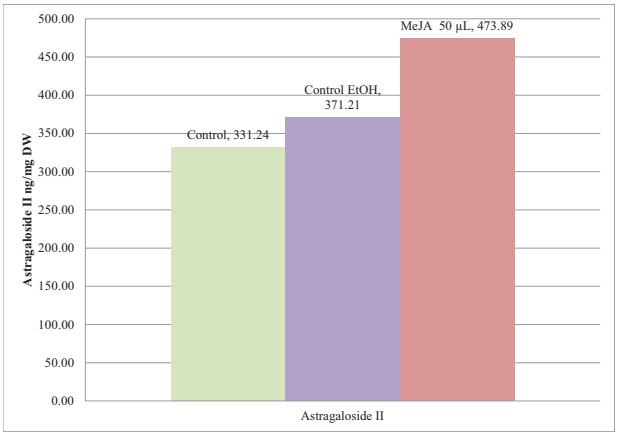

Figure 2. Astragaloside II content in *in vitro* cultures after treatment with 50 µL MeJA.

The impact of a 200 µL MeJA concentration on the production of astragaloside II in *A. aitosensis in vit*ro root cultures was considerable. The control group exhibited a baseline production of 445.24 ng/mg DW for astragaloside II. When treated with 200 µL MeJA, the astragaloside II production increased to 915.32 ng/mg DW. This increase represents a 105% enhancement in production compared to the control (Fig. 3). Interestingly, the control group treated with EtOH alone showed a slight increase in astragaloside II production to 519.20 ng/mg DW, indicating that EtOH itself may have a minor elicitation effect. However, the 200 µL MeJA treatment was far more effective, proving MeJA's potency as an elicitor. The 200 µL MeJA concentration

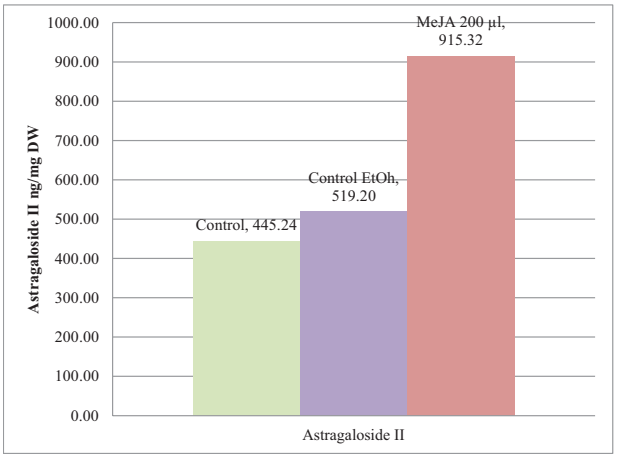

Figure 3. Astragaloside II content in *in vitro* cultures after treatment with 200 µL MeJA.

yielded the highest increase in astragaloside II production among the tested concentrations. This suggests that MeJA is highly effective in inducing the biosynthesis of this cycloartane saponin. However, it's important to balance the substantial increase in production with the economic and practical considerations of using a higher concentration of MeJA.

For astragaloside IV, the 50 µL MeJA concentration increased production to 199.02 ng/mg DW from the control 129.46 ng/mg DW. The 100 µL concentration resulted in a lower production of 152.36 ng/mg DW, while the 200 µL concentration increased it further to 225.08 ng/mg DW. These results indicate that while higher concentrations of MeJA can increase production, the 50 µL treatment provides a significant increase at a lower cost (Fig. 4).

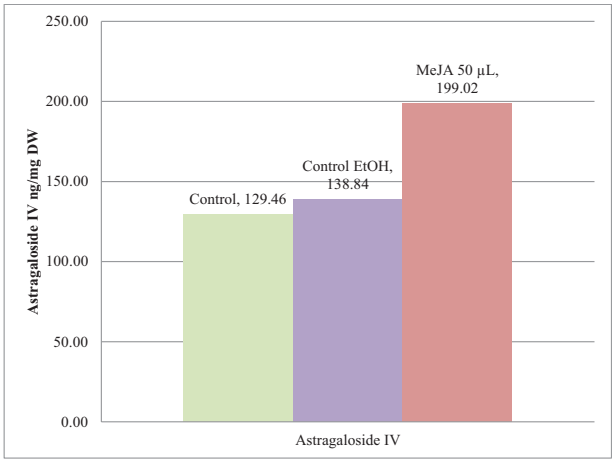

Figure 4. Astragaloside IV content, when treated with 50 µl MeJA.

The graphic analysis comparing the total saponin content, calculated as the sum of astragalosides I, II, and IV, reveals significant insights into the effectiveness of MeJA treatments (Fig. 5). The total saponin content in the control group was 531.47 ng/mg DW, which increased to 791.77 ng/mg DW with 50 µL MeJA treatment. This represents a 49% increase, indicating that even a relatively low concentration of MeJA significantly enhances saponin

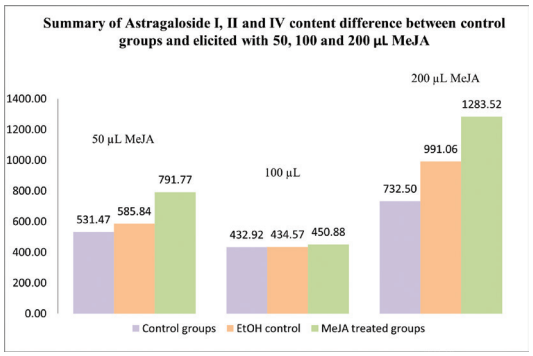

Figure 5. Effect of the elicitor over the expression of astragalosides I, II and IV as summary content.

production. For the 100 µL MeJA treatment, the total saponin content was 450.88 ng/mg DW. This slight increase compared to the control groups 432.92 ng/mg DW suggests that the intermediate concentration is less effective, potentially due to suboptimal signaling pathways activation. The 200 µL MeJA treatment resulted in the highest total saponin content at 1283.52 ng/mg DW. This nearly doubles the control groups' saponin content, showing a 142% increase. However, these high concentrations cost, and rationality must be considered against the efficiency of lower concentrations.

Discussion

The results indicate that MeJA significantly enhances the production of cycloartane-type saponins in the *in vitro* root cultures of *A. aitosensis*. Specifically, the 50 µL concentration appears to be the most efficient for increasing Astragaloside I and IV levels, offering a cost-effective strategy for increasing saponin yield. In contrast, higher concentrations such as 200 µL proved most beneficial for astragaloside II production, where a substantial 105% increase was observed. This data aligns with previous studies highlighting MeJA`s role as a potent elicitor in enhancing secondary metabolite production (Ionkova, 2009). A study by Jiao et Al. demonstrated that MeJA was the most effective among several elicitors for promoting astragaloside production in *A. membranaceus* hairy root cultures. In that study, MeJA-treated cultures produced up to 5.5 mg/g DW of total astragalosides, a 2.1-fold increase over the control (Jiao et al. 2016). This supports the findings in *A. aitosensis*, where MeJA improved the production of specific astragalosides. However, while higher concentrations of MeJA, such as 200 µL, can further improve the production, they may not be economically viable for large-scale production due to the higher costs and diminishing returns observed for astragaloside I and IV at higher doses. Similarly, Jiao et al. noted that excessive MeJA concentrations could result in metabolic stress and reduced biomass, making optimal concentration selection critical for industrial scalability (Jiao et al. 2016).

The 50 µL MeJA treatment is particularly noteworthy for its efficiency as an elicitor in increasing the combined summary of astragaloside I, II, and IV production by 49%, supporting its potential application in industrial settings where cost-effectiveness is of primary importance. Moreover, the marked increase in astragaloside II at higher MeJA concentrations may warrant further investigation into optimizing elicitor levels for specific saponins, as astragaloside II is known for its significant pharmacological properties, including antiviral and anti-inflammatory activities. The role of MeJA in enhancing secondary metabolite production can be attributed to its action as a signaling molecule that activates gene expression in biosynthetic pathways. In *A. membranaceus* cultures, MeJA treatment upregulated key genes such as MVD, IDI, FPS and SS, which are involved in the biosynthesis of astragalosides (Jiao et al. 2016). Similar mechanisms could be responsible for the observed increase in *A. aitosensis* saponins production, and further research into the genetic responses of this species could improve our understanding of how the plant metabolism works and can be manipulated for optimal metabolite production.

These findings contribute to the growing knowledge regarding the use of biotechnological approaches for the sustainable production of high-value medicinal compounds, employing the potential of *in vitro* root cultures as a viable platform for meeting the global demand for rare saponins without overharvesting natural populations of *Astragalus* species.

Conclusion

In this study the effectiveness of MeJA as an elicitor in the endemic *A. aitosensis in vitro* root culture was evaluated. In particular, its potential to increase the production of secondary metabolites and more specifically, rare cycloartane-type saponins, astragalosides I, II and IV. Different concentrations of the elicitor were tested. Saponins were found in the cultures, and their quantities were determined with chromatographic methods and analyzed.

The study demonstrates that while higher concentrations of MeJA (200 µL) significantly increase the production of cycloartane-type saponins in *A. aitosensis*, the 50 µL concentration is the most cost-effective for sustainable production. The 50 µL MeJA treatment increased the total saponin content by 49% compared to the control group. The 200 µL concentration of MeJA was particularly effective in increasing astragaloside II production, nearly doubling the content compared to the control. However, the overall efficiency and practicality of using such high concentrations must be weighed against the significant gains achieved with lower concentrations. As a conclusion, MeJA can be used as an elicitor in a low dose of 50 µl, inducing the production of cycloartane-type saponins – a valuable beneficial phytochemical with important pharmaceutical applications. The biotechnological method has proven effective and is continuously being optimized for production and cost-efficiency. By increasing the production of phytochemicals through biotechnological methods and enhancing these processes, we are contributing to the sustainable sourcing of rare substances to meet global demand, while preserving the biodiversity of endemic species.

Acknowledgements

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