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Research Article

Activity of an oleanane-type tritrepenoid saponin from *A. glycyphyllos* on human recombinant MAO enzymes

Aleksandar Shkondrov[1], Magdalena Kondeva-Burdina[2], Ivan Stambolov[1], Ilina Krasteva[1]

1 *Department of Pharmacognosy, Faculty of Pharmacy, Medical University of Sofia, 2 Dunav St., 1000 Sofia, Bulgaria*
2 *Laboratory of Drug Metabolism and Drug Toxicity, Department of Pharmacology, Pharmacotherapy and Toxicology, Faculty of Pharmacy, Medical University of Sofia, 2 Dunav St., 1000 Sofia, Bulgaria*

Corresponding author: Ilina Krasteva (krasteva.ilina@abv.bg)

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Abstract

For centuries, plants have been a leading point in the identification of potential therapeutic agents. Monoamine oxidase inhibition is a key mechanism in the treatment of various neurological and psychiatric diseases. Some triterpenoid saponins are reported to inhibit this enzyme. An extract from the aerial parts of *Astragalus glycyphyllos* was purified and separated by chromatographic techniques, which led to the isolation of one triterpene saponin. Its structure was analysed by ultra-high-performance liquid chromatography coupled with high-resolution electrospray ionisation mass spectrometry. The compound was subjected to a pharmacological study where human recombinant monoamine oxidase enzymes type A and B (*h*MAOA and *h*MAOB) were used. On the activity of *h*MAOA, the saponin had no effect, but on *h*MAOB, it exhibited statistically significant inhibition in comparison to the control, Selegiline. The compound could have potential in other models, so further investigations are required.

Keywords

Saponin, oleanane-type, *Astragalus glycyphyllos*, MAO enzyme inhibition

Introduction

In the modern world, neurodegenerative disorders are a leading cause of lower quality of life. The complex pathophysiology of this group of age-related diseases is believed to be strongly connected with oxidative stress and the dysfunction of various brain enzymes. Alzheimer's and Parkinson's diseases are the most common neurodegenerative disorders (Mateev et al. 2023). Alzheimer's disease is defined as a progressive and persistent degeneration of the brain tissue, leading to cognitive impairment and changes in personality (Guarino et al. 2018). Patients have high levels of MAOB in the pyramidal neurons (Schedin-Weiss et al. 2017), so there is potential for MAOB inhibitors as agents to postpone the progression of this neurodegenerative disorder. Parkinson's disease is caused by the depletion of dopamine in the *substantia nigra*, located in the midbrain. This nucleus is involved in the body`s locomotion and reward perception, so patients with Parkinson's are experiencing at least some sort of motor dysfunction, depression, and sleeping problems (Gordon and Woodruff 2017; Raza et al. 2019). The monoamine oxidase enzyme is known to have two isoforms in humans, and inhibitors of their enzyme activity are used as medicinal products in numerous psychiatric and neurological diseases. MAOA inhibitors, such as Chlorogyline, are drugs used to treat

depression. MAOB inhibitors (Selegiline) are used in the therapy of Parkinson's disease. Induction of MAO enzyme activity is believed to produce free radicals, which are the root cause of neurodegenerative diseases. Although both MAOA and MAOB isoforms are present in the brain, they have a different distribution (Westlund et al. 1985), and in certain regions the amount of the B-isoform is higher (Kalaria et al. 1988; Willoughby et al. 1988; Riachi and Harik 1992). In humans, MAOB expression has been shown to increase with aging as well as with the progression of neurodegenerative diseases (Ilieva et al. 2020). MAOA and MAOB are of considerable pharmacological interest due to their catabolic role in monoamines' neurotransmission in the brain. The desalination of serotonin is selectively catalysed by MAOA and irreversibly inhibited by Chlorogyline. MAOB catalyses the deamination of phenylethylamine and is irreversibly inhibited by low Selegiline. Both isoforms of MAO catalyse the oxidation of dopamine, adrenalin, and noradrenalin (Waldmeier 1987; Tipton et al. 2004; Youdim and Bakhle 2006; Youdim et al. 2006). The increased MAOB activity is one of the causes of Alzheimer's disease (AD). It was suggested that the quantity of the pathogenic amyloid β-peptide (Aβ) is regulated by MAOB (Petzer et al. 2009; Schedin-Weiss et al. 2017).

Triterpenoid saponins are a large class of plant-derived secondary metabolites which possess many pharmacological effects: immunomodulatory, cytotoxic, adaptogenic, antiviral, antioxidant, antitumor, etc. (Ionkova et al. 2014; Georgieva et al. 2021; Shkondrov et al. 2023). For some saponins, there is data on their neuroprotective effects (Sun et al. 2015). Species of genus *Astragalus* (Fabaceae) are a well-established source of both tetracyclic and pentacyclic triterpenoid saponins (Krasteva et al. 2016). Bulgarian plants from this genus have been reported to exert neuroprotective activity, as proven in different models (Simeonova et al. 2019; Kondeva-Burdina et al. 2019).

In continuation of our efforts to reveal the pharmacological potential of Bulgarian *Astragalus* plants, the aim was to isolate saponins from *A. glycyphyllos* and examine their possible MAO-inhibiting effects.

Materials and methods

Isolation

The overground parts of *A. glycyphyllos* were harvested in July 2021 from Vitosha Mt., Bulgaria. Prof. D. Pavlova (Department of Botany, Faculty of Biology, Sofia University) identified the plant, and a voucher specimen (SO-107613) is kept in the Herbarium of the same university for further reference. The air-dried plant material (200 g) was powdered (3 mm) and then extracted with dichloromethane (6 × 2 L) using percolation to remove the lipophilic constituents. The defatted plant substance was then aired and exhaustively extracted with 80% MeOH (24 × 3 L) using percolation. The obtained extract was filtered, concentrated under vacuum, and then lyophilized to produce a dry extract (42 g). The extract was separated over a Diaion HP-20 (4.7 × 45 cm) column, eluting with H_2O: MeOH (0→100%). Seven main fractions were collected (I–VII). After the TLC analysis (Silica gel plates, EtOAC:HCOOH:AcOH:H_2O = 32:3:2:6, anisaldehyde/ c. H_2SO_4, 104 °C, 10 min), fraction VI was found to be rich in saponins. It was chromatographed on a silica gel cartridge using flash chromatography with CH_2Cl_2:MeOH:H_2O (step gradient 8:2:0.2→7:3:0.3) to obtain 21 subfractions. Subfraction 12, which contained a main compound (TLC), was further separated with CH_2Cl_2:MeOH:H_2O (step gradient 9:1:0→5:6:1) using flash chromatography on a silica gel cartridge and a compound, named Sg, was obtained.

LC-MS analysis of the saponin

A Q Exactive Plus Orbitrap mass spectrometer with a heated electrospray ionisation (HESI) ion source (ThermoFisher Scientific, Bremen, Germany) coupled with a UHPLC system (Dionex UltiMate 3000 RSLC, ThermoFisher Scientific, Bremen, Germany) was used. The full scan MS was set at: resolution 70 000 (at *m/z* 200), AGC target $3e^6$, max IT 100 ms, scan range 250 to 1700 *m/z*. The MS^2 conditions were: resolution 17 500 (at m/z 200), AGC target $1e^5$, max IT 50 ms, mass range *m/z* 250 to 1750, isolation window 2.0 *m/z* and (N)CE 20. The ionization device (HESI source) was operating at +3.5 or -2.5 kV spray voltage and 320 °C capillary and probe temperature, 38 arbitrary units (a.u., as set by the Extactive Tune software) of sheath gas and 12 a.u. of auxiliary gas (both N_2); S-Lens RF level 50.0. UHPLC separations were performed on a Kromasil C18 column (1.9 µm, 2.1 × 50 mm, Akzo Nobel, Sweden) at 40 °C. The mobile phase was (A) H_2O + 0.1% HCOOH and (B) MeCN + 0.1% HCOOH, and the flow rate was 0.3 mL/min. The elution was as follows: 10% B for 1.5 min, increase to 30% B for 1 min, isocratic with 30% B for 0.5 min, increase to 95% B for 9 min, isocratic with 95% B for 2 min, return to 10% B for 0.1 min. Identification was supported by MS^2 experiments, which revealed the aglycone part of the molecule as well as the successive loss of monosaccharides of the sugar moiety. The fragmentation pattern was compared to literature data. The software Xcalibur, Version 4.2 (Thermo Scientific) was used for data collection and processing.

Measurement of monoamine oxidase activity

Monoamine oxidase activity assay of both recombinant human MAOA (*h*MAOA) and MAOB (*h*MAOB) was performed using a fluorimetric method with small modifications (Held and Buehrer 2003; Dezsi and Vecsei 2017; Kasabova-Angelova et al. 2020) by Amplex UltraRed reagent (Yagi 1976) to detect hydrogen peroxide (or peroxidase activity) in biological samples (or enzymes). In the presence of peroxidase, Amplex Red reacted 1:1

stoichiometrically with hydrogen peroxide to produce a red phosphorescent oxidation product (resorufin). Due to the high extinction coefficient (58,000 ± 5,000 $cm^{-1}.M^{-1}$), the analysis could be performed fluorometrically or spectrophotometrically. The reaction was used to detect small amounts of hydrogen peroxide (on the order of 10 pM, in a volume of 100 µL). Pure MAOA or MAOB working solution in reaction buffer, MAOA or MAOB working solution containing hydrogen peroxide, and pure reaction buffer were used as controls.

The substances (the saponin Sg at 0.050, 0.250, 0.500, 075 and 1 µM, or Chlorgyline or Selegiline, 1 µM), together with *h*MAOA or *h*MAOB, were put in a 96-well plate (8 samples for each substance), after which the plate was placed in an incubator for 30 min (in the dark, at 37 °C). At the end of the incubation period, the reaction was initiated by adding to each well on the 96-well plate a 50 µL a Mix Solution containing solutions of Amplex Red reagent, Horseradish Peroxidase (HRP) and tyramine as substrates for the enzymes, in a reaction buffer. As the reaction was continuous, fluorescence was monitored every 30 min (0, 30, 60, 90, 120 and 150 min, respectively) to scrutinize kinetics in the dark as the reaction mixture was shaken at a constant temperature of 37 °C. Fluorimetric reading was performed on a Synergy 2 Microplate Reader at two different wavelengths (λ = 570 and 690 nm).

Statistical analysis

The enzyme activity was expressed as a normalized percent of the untreated control set as 100% and the results were expressed as mean values and standard deviations (± SD) (Graph Pad Prizm, v. 6). Statistical analysis was performed by one-way analysis of variance (ANOVA) with the *post hoc* multiple comparisons procedure (Dunnet's test) to assess the statistical differences in the normal distribution. Values of $P < 0.05$, $P < 0.01$ and $P < 0.001$ were considered statistically significant.

Results and discussion

HRESIMS determination of the isolated compound

From a defatted extract of the aerial parts of *A. glycyphyllos*, using repetitive column and flash chromatography over different sorbents, one compound (Sg) was isolated as a white powder (3 mg) with a purity of 98.2% (UHPLC-MS) (Fig. 1).

From its chromatographic properties, the coloration in TLC analysis, the MS data, it was proven that Sg is a triterpenoid saponin. The structural elucidation was performed by means of HRESIMS and an analysis of the fragmentation pattern. In the negative mode, a deprotonated molecular ion was observed $[M-H]^-$ *m/z* 941.5115, $C_{48}H_{77}O_{18}^-$, and in the positive, a protonated one ($[M+H]^+$ *m/z* 943.5261, $C_{48}H_{79}O_{18}^+$), which is consistent with a molecular formula of $C_{48}H_{78}O_{18}$ (942.5188) (Fig. 2). When these ions were subjected to MS^2 analysis, a successive loss of monosaccharides was noted. Firstly, a methylpentose (Stobiecki 2000) unit (*m/z* 146.0579, $C_6H_{10}O_4$) was cleft: $[M-H-(mepent)]^-$, *m/z* 795.4536, $C_{42}H_{67}O_{14}^-$, and $[M+H-(mepent)]^+$, *m/z* 797.4682, $C_{42}H_{69}O_{14}^+$. The second loss was of a hexose (Stobiecki 2000) unit (*m/z* 162.0528, $C_6H_{10}O_5$), resulting in the following ions: $[M-H-(mepent+hex)]^-$, *m/z* 633.4008, $C_{36}H_{57}O_9^-$, and $[M+H-(mepent+hex)]^+$, *m/z* 635.4154, $C_{36}H_{59}O_9^+$. At the third cleavage, a hexuronic acid (Hvattum and Ekeberg 2003) moiety (*m/z* 176.0321, $C_6H_8O_6$) was lost: $[M-H-(mepent+hex+hexUA)]^-$, *m/z* 457.3687, $C_{30}H_{49}O_3^-$, and $[M+H-(mepent+hex+hexUA)]^+$, *m/z* 459.3833, $C_{30}H_{51}O_3^+$ (Fig. 2).

The final fragment was the sapogenin of compound Sg, and the values were consistent with soyasapogenol B ($C_{30}H_{50}O_3$, 458.3759) (Zhang and Popovich 2010). From the MS analysis, it can be concluded that Sg is a trisaccharide of soyasapogenol B, with a sugar chain/s consisting of a methylpentose, a hexose, and a hexuronic acid.

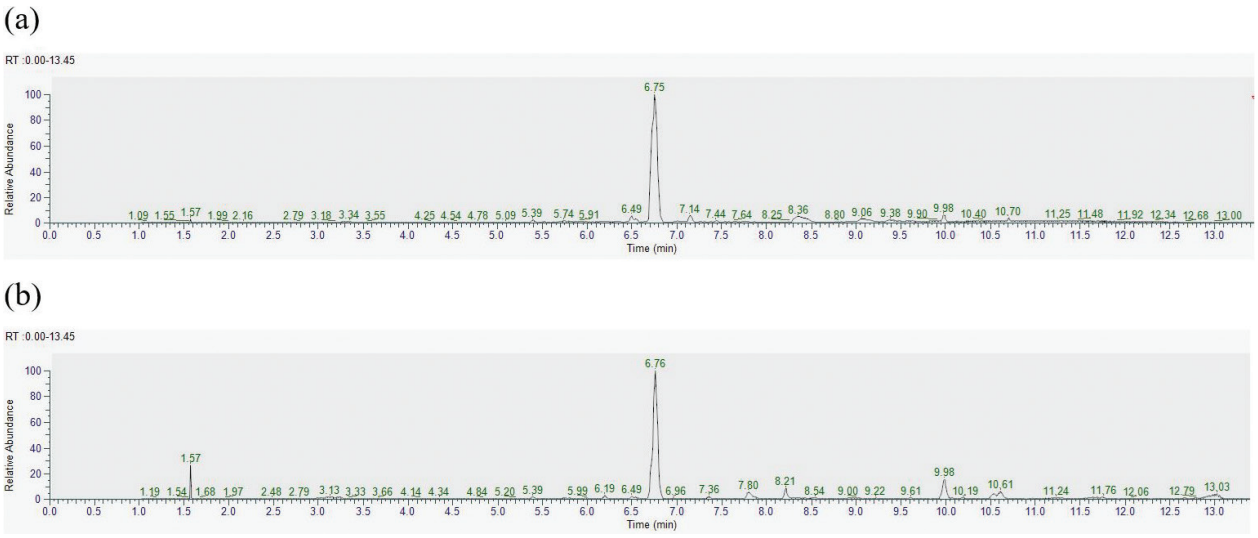

Figure 1. Chromatograms of Sg in the negative (**a**) and positive (**b**) ionization modes.

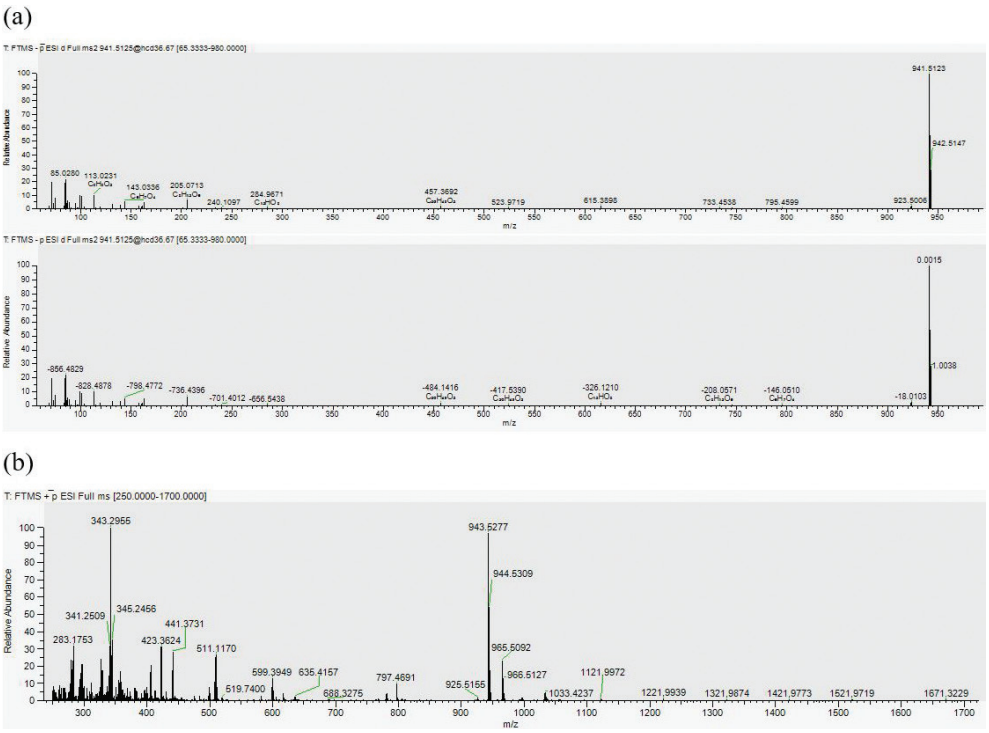

Figure 2. HRESIMS spectrum of compound Sg in the (**a**) negative and (**b**) positive modes.

Further spectral analysis of the isolated compound is necessary to determine the place and order of attachment of the sugars. Nevertheless, the aim here was to explore its pharmacological potential.

On the activity of the *h*MAOA enzyme, Sg did not reveal a statistically significant inhibitory effect. In this study, only the classical MAOA inhibitor, Chlorogyline, decreased statistically significant enzyme activity as follows: 0.050 μM – by 15%; 0.250 μM – by 30%; 0.500 μM – by 35%; 0.750 μM – by 45%; and 1 μM – by 50%, compared to the control (pure, non-treated *h*MAOA) (Fig. 3).

On *h*MAOB activity, Sg exhibited a statistically significant inhibitory effect only at concentrations of 0.750 μM and 1 μM. At a concentration of 0.750 μM, the saponin inhibited the enzyme by 25%, and at 1 μM by 35%, compared to the control (pure *h*MAOB). The effects of Sg at these concentrations were close to those of Selegiline. This classical MAOB inhibitor statistically significantly reduced the enzyme activity as follows: 0.050 μM by 20%; 0.250 μM by 35%; 0.500 μM by 40%; 0.750 μM by 50%; and 1 μM by 55% compared to the control (pure *h*MAOB) (Fig. 4).

MAO-induced oxidative stress is a potential risk factor for neuronal loss in aging and is associated with age-related neurodegenerative disorders, including Parkinson's disease (PD). It is known that mitochondrial DNA is damaged by increased levels of oxidative products due to increased MAO activity (Hauptmann et al. 1996). In a mouse model, the leading pathogenetic cause of PD is reported to be the upregulation of MAOB in astrocytes (Mallajosyula et al. 2008, 2012). Inhibition of MAO increases the content of amines in the brain, resulting in antidepressant effects and improved neuronal activity (Youdim et al. 2006; Fišar 2016). In this study, the isolated saponin exhibited a concentration-dependent inhibiting effect on *h*MAOB activity.

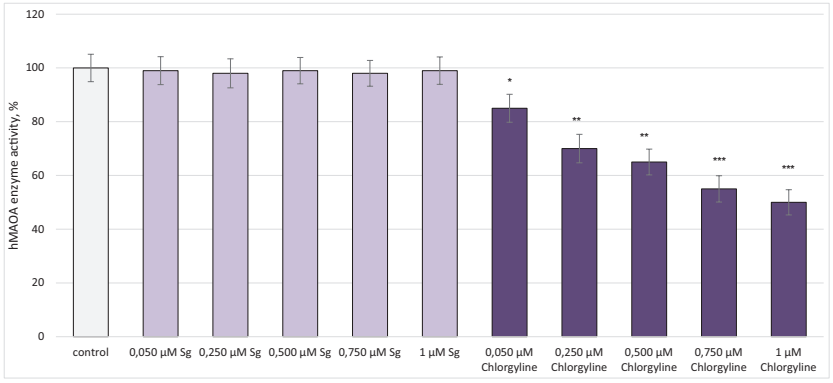

Figure 3. Effect of Sg and Chlorgyline (at concentrations of 0.050, 0.250, 0.500, 0.750, and 1 μM) on the activity (%) of human recombinant MAOA enzyme (*h*MAOA). * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$ *vs.* control (pure *h*MAOA).

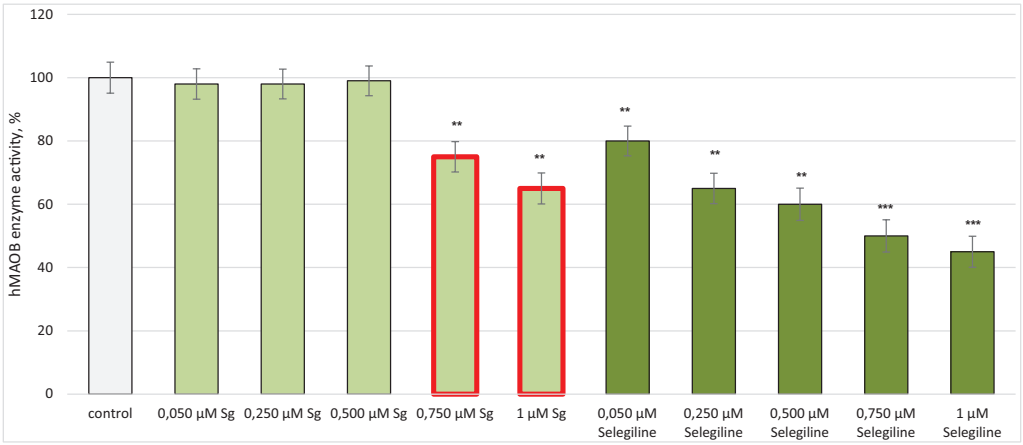

Figure 4. Effect of Sg and Selegiline (at concentrations of 0.050, 0.250, 0.500, 0.750, and 1 µM) on human recombinant MAOA enzyme (*h*MAOB) activity. ** $P < 0.01$; *** $P < 0.001$ *vs.* control (pure *h*MAOB).

From a pharmacological point of view, the identification of novel possible compounds acting as selective MAO inhibitors is of great importance. Thus, the research interest worldwide is high. It is known that the selective MAOB inhibitors Selegiline and Rasagiline have shown a protective action on neuronal cells in both *in vitro* and *in vivo* models (Ebadi et al. 2006; Youdim et al. 2006; Makoto Naoi and Inaba-Hasegawa 2013). These drugs have been approved for many years as adjunctive therapy in PD, preserving dopamine levels in the brain and thus postponing the need for L-DOPA. Another function of MAO is the deamination of GABA, which is proven in very high concentrations *in vitro* (Goldberg and Pollard 2014).

The results of the current study are consistent with the literature. There are other reports on the *h*MAOB inhibiting properties of cycloartane-type triterpenoid saponins from *A. glycyphyllos* (Stambolov et al. 2023a, b; Shkondrov et al. 2020). Unlike these, this is the first instance of an oleanane-type saponin, isolated from *Astragalus* species, exhibiting these effects.

Our findings support the importance of plant-derived secondary metabolites such as triterpene saponins as lead molecules for the possible treatment of neurodegenerative diseases. Further analysis is needed to deepen our knowledge of these valuable molecules.

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