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Research Article

In vitro activity of two cycloartane-type triterpenoid saponins from *Astragalus glycyphyllos* on liver microsomes, hepatocytes, and isoforms of cytochrome P450

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Abstract

Two cycloartane-type saponins 3-*O*-[α-L-rhamnopyranosyl-(1→2)]-β-D-xylopyranosyl]-24-*O*-α-L-arabinopyranosyl-3β,6α,16β,24(*R*),25-pentahydroxycycloartane and Astrachrysoside A, obtained from the aerial parts of *A. glycyphyllos*, were tested *in vitro* for their possible hepatoprotective and antioxidant effects. Three models were used: isolated liver microsomes (non-enzyme-induced lipid peroxidation) and hepatocytes (*t*-BuOOH-induced oxidative stress and CCl_4-induced metabolic bioactivation). Both compounds (50 μM) exhibited statistically significant activity in all of the models, compared to the control. The saponins` hepatoprotective activity was even more pronounced in the metabolic bioactivation model. In addition, the compounds (1 μM) were evaluated for possible inhibitory activity on three isoforms of cytochrome P450. Both saponins exhibited noticeable inhibition on CYP3A4, mild inhibition oF CYP2D6, and no activity on the CYP1A2 isoform was observed.

Keywords

Astragalus glycyphyllos, cycloartane saponins, liver microsomes, hepatocytes, cytochrome P450

Introduction

Liver diseases can be considered a global threat to public health as they account for approximately two million deaths per year worldwide (Asrani et al. 2019). These may include different types of cancer, hepatitis, metabolic diseases, and drug-induced hepatotoxicity (Wohlfarth and Efferth 2009). The application of various biologically active compounds in liver disease treatment has gained considerable popularity in recent decades (Zhang et al. 2013), with triterpenoid saponins being the focus of a lot of recent research (Kokanova-Nedialkova et al. 2021; Wang et al. 2021; Yang et al. 2021; He et al. 2022). Isolated hepatocytes and liver microsomes are commonly used in many *in vitro* studies on hepatoprotective activity, as glutathione (GSH) and malondialdehyde (MDA) levels are indicative of the antioxidant effect of the tested compound (Kondeva-Burdina et al. 2024). The most widely used model for

hepatotoxicity involves CCl_4, a molecule that undergoes bioactivation in the hepatocytes. The reactive metabolites produced lead to organ insufficiency, renal failure, and encephalopathy (Racknagel et al. 1989).

The cytochrome P450 (CYP450) is a superfamily of enzymes that consists of more than 55 isoforms that take part in the metabolism of numerous xenobiotics (Angelov et al. 2022). The majority (95%) of the reactions are catalyzed by CYP1A2, CYP2C9, CYP2C19, CYP3A4, and CYP2D6 (Guengerich, 2008). A prerequisite for all novel drug candidates is the evaluation of their possible activation/inhibition effect on the isoforms of CYP450 (Iizaka et al. 2021).

Astragalus glycyphyllos L. (Fabaceae) is a perennial medicinal plant, widely distributed in Bulgarian flora. The plant is known to accumulate mainly two types of secondary metabolites: flavonoids and triterpenoid saponins, the latter being predominantly of the cycloartane-type (Stambolov et al. 2023a). Several extracts and isolated compounds from the species have been tested for various pharmacological activities. A purified extract from the aerial parts of the species was tested *in vivo* in a model of CCl_4-induced liver damage in male Wistar rats (100 mg/kg) and exhibited a significant hepatoprotective effect, compared to silymarin (Shkondrov et al. 2015). Three cycloartane-type triterpenoid saponins (3-*O*-[*α*-L-rhamnopyranosyl-(1→2)]-*β*-D-xylopyranosyl]-24-*O*-*α*-L-arabinopyranosyl-3*β*,6*α*,16*β*,24(*R*),25-pentahydroxy-20*R*-cycloartane, astrachrysoside A, and 17(*R*),20(*R*)-3*β*,6*α*,16*β*-trihydroxycycloartanyl-23-carboxylic acid 16-lactone 3-*O*-*β*-D-glucopyranoside) have been recently isolated from the plant and tested for their possible *in vitro* neuroprotective and human recombinant monoamine oxidase type B (*h*MAO-B)-inhibiting activity. All compounds showed a statistically significant dose-dependent effect on subcellular fractions and a mild inhibition on *h*MAO-B, compared to selegiline (Shkondrov et al. 2020; Stambolov et al. 2023b). The *in vitro* anti-coronavirus effect of a methanol extract (DEAG), a purified saponins' mixture (PSM), and two isolated saponins from *A. glycyphyllos* was also studied. It was found that the PSM and DEAG inhibited 100% of viral replication while individual saponins showed no effect (Hinkov et al. 2023). These studies emphasize the possible protective and antioxidant properties of cycloartane-type triterpenoid saponins from *A. glycyphyllos*. Nevertheless, to date, neither the hepatoprotective effects of the former pure saponins from the species on hepatocytes or liver microsomes nor their CYP450 activities have been studied.

The aim of this research was to evaluate the possible *in vitro* hepatoprotective and antioxidant activity of two cycloartane-type saponins, isolated from *A. glycyphyllos*, on hepatocytes and liver microsomes, and to determine the compounds` inhibitory potential on selected isoforms of cytochrome P450.

Materials and methods

Saponins

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. From the air-dried plant material, a defatted extract was obtained, following the procedure reported before (Stambolov et al. 2023b). Using a slightly modified procedure compared to the previous report regarding quantities, the saponins were isolated from the defatted extract of the aerial parts of *A. glycyphyllos* by means of repetitive column and flash chromatography. The compounds were acquired in quantities of 3 mg for S1 and 2 mg for S2, allowing the current study. The identity of the compounds was confirmed by extensive spectral (HRESIMS and NMR) analysis and comparison to the literature (Stambolov et al. 2023b). Compound S1 was identified as 3-*O*-[*α*-L-rhamnopyranosyl-(1→2)]-*β*-D-xylopyranosyl]-24-*O*-*α*-L-arabinopyranosyl-3*β*,6*α*,16*β*,24(*R*),25-pentahydroxy-20*R*-cycloartane, and saponin S2 was elucidated as Astrachrysoside A (Fig. 1).

In vitro pharmacological evaluation

Fifteen Wistar rats (male, 200-250 g) (Akimoto-Takano et al. 2005) were purchased from the National Breeding Centre of the Bulgarian Academy of Sciences in Slivnitsa, Bulgaria. The experiments were conducted in accordance with Regulation № 15 on the protection and humane treatment of experimental animals (State Gazette, issue 17, 2006) and the European standards for working with experimental

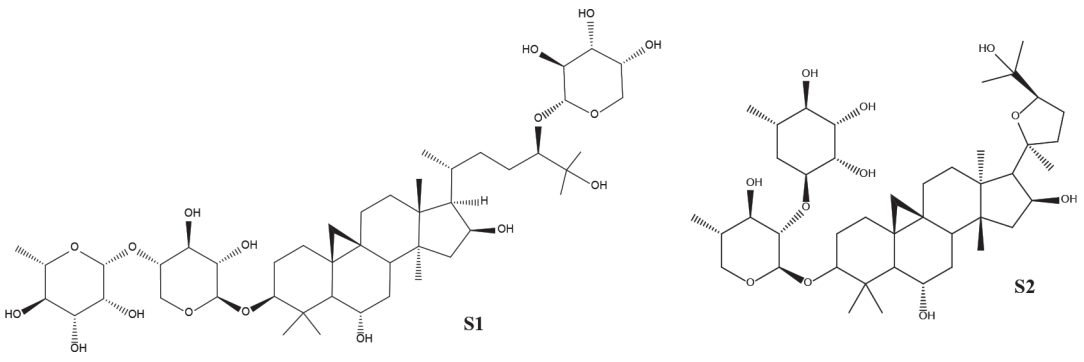

Figure 1. The investigated saponins from *A. glycyphyllos*.

animals. They were also approved by the Bulgarian Food Safety Agency with permit № 323, valid until 22.12.2026.

Microsomes (protein content 1 mg/ml) were isolated from rat liver homogenate *via* differential centrifugation (Mansuy et al. 1986). The microsomal protein was evaluated using the method of Lowry et al. (1951), while MDA was measured according to Mansuy et al. (1986).

Preparation of primary hepatocyte suspension by *in situ*, two-step collagenase perfusion (Fau et al. 1992) with modifications (Mitcheva et al. 2006) was performed. The hepatocytes' viability was determined (Fau et al. 1992).

Liver microsomes and hepatocytes were incubated for 1 h only with the saponins (50 μM). Then, a combination with the toxic agent was tested. The microsomes and the hepatocytes were firstly pre-incubated for 30 min with the saponins (in the same concentration as alone) and then subsequently incubated with the toxic agent (either *t*-BuOOH or Fe^{2+}/AA) for 1 h.

On the isolated rat hepatocytes, levels of lactate dehydrogenase (LDH), malondialdehyde, and reduced glutathione (GSH) were assessed spectrophotometrically. The activity of LDH was assessed by a kinetic method. Briefly, the hepatocytes were incubated with the substances (50 μM) for 1.5 h. The enzyme was determined in the supernatant, which was left after the centrifugation of the hepatocytes at 400 × g for 4 min. An LDH kit (Anticel, USA) was used. The extinction was determined spectrophotometrically (340 nm) at 30 s, 1, 2, and 3 min. The level of reduced glutathione (GSH) and the production of malondialdehyde (MDA) were measured, following the procedures by Fau et al. (1992).

A fluorimetric method for determining the activity of some isoforms of human recombinant cytochrome P450 with the following inhibitor screening kits (ISK) was used: CYP1A2 ISK (substrate 3-cyano-7-hydroxycoumarin, inhibitor α-naphtoflavone); CYP3A4 ISK (substrate resofurin, inhibitor ketoconazole); CYP2D6 ISK (substrate 3-[2-(N,N-diethyl-N-methylammonium)ethyl]-7-methoxy-4-methylcoumarin, inhibitor quinidine). The saponins were incubated (1 μM, DMSO solution) with the isoforms for 2 h.

The statistical analysis of the results was performed using the statistical program "MedCalc" v. 18 (MedCalc Software Ltd., Ostend, Belgium). Each experiment was performed in triplicate, and the values are represented as the mean of three (n = 3). A Mann–Whitney non-parametric test was used to examine the statistical significance of the results. When $p < 0.05$, $p < 0.01$, or $p < 0.001$, the differences between groups were accepted as statistically significant.

Results and discussion

Evaluation of the effects of the saponins (50 µM) on isolated rat liver microsomes

Administered alone, both saponins (50 μM) did not exhibit statistically significant hepatotoxic and pro-oxidant effects on rat liver microsomes (Fig. 2). Administered alone, Fe^{2+}/AA increased statistically significant MDA production by 150%, compared to the control (untreated microsomes) (Fig. 2).

In conditions of non-enzyme (Fe^{2+}/AA)-induced lipid peroxidation in rat liver microsomes, both saponins S1 and S2 decreased statistically significant the MDA production by 82% and 80%, respectively, vs. the toxic agent (Fig. 2). A good, statistically significant antioxidant effect was observed.

Evaluation of the effects of the saponins (50 µM) on isolated rat hepatocytes

Both saponins (50 μM) did not exhibit statistically significant hepatotoxic and pro-oxidant effects on isolated rat hepatocytes when administered alone (Fig. 3).

Administered alone, *t*-BuOOH decreased statistically significant cell viability by 60% and the level of GSH by 50% in comparison to the control (untreated rat hepatocytes) (Fig. 4).

The other two parameters that were negatively affected by *t*-BuOOH were the release of the enzyme LDH and the production of MDA. They were both increased statistically significant by 150% and 200%, respectively, compared to the control (untreated rat hepatocytes) (Fig. 5).

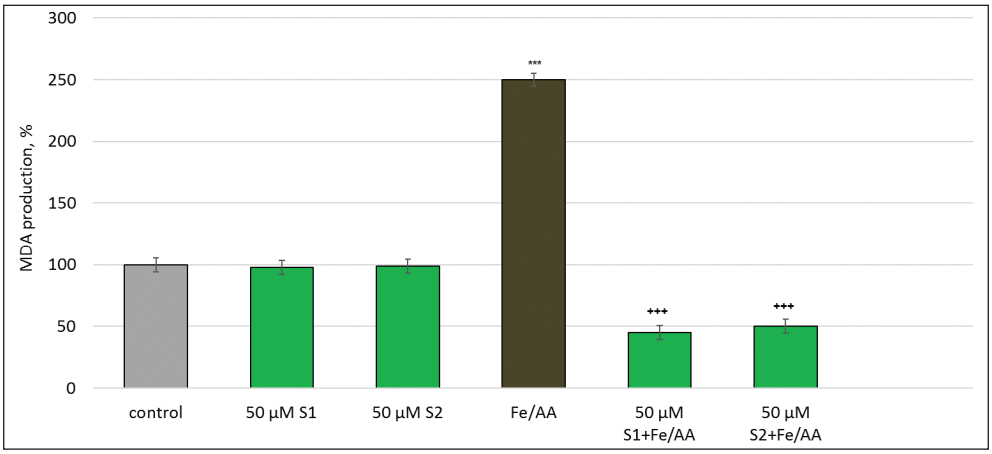

Figure 2. Effects of S1 and S2, administered alone and in conditions of non-enzyme (Fe^{2+}/AA)-induced lipid peroxidation, on MDA production in isolated rat liver microsomes; *** $p < 0.001$ *vs.* control (untreated microsomes); $^{+++}$ $p < 0.001$ *vs.* Fe^{2+}/AA.

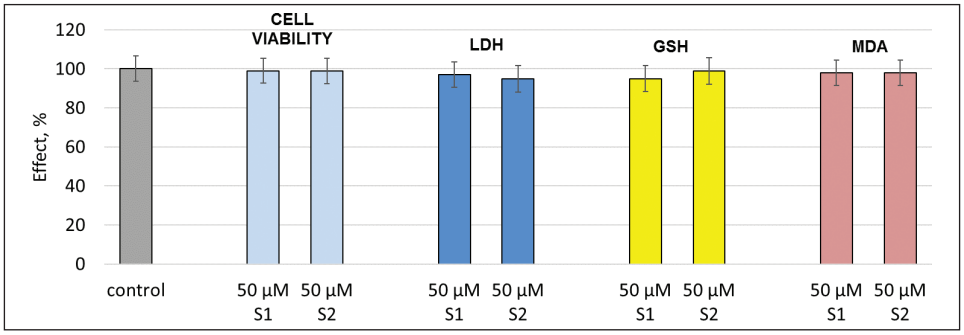

Figure 3. Effects of S1 and S2, administered alone, on main parameters characterizing the functional-metabolic status of isolated rat hepatocytes.

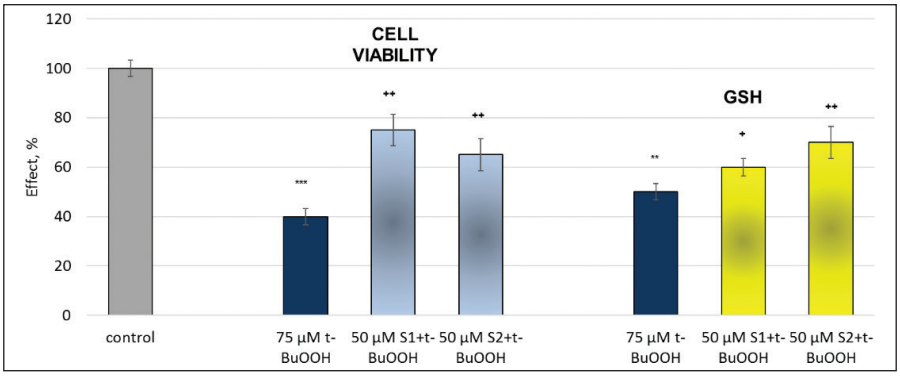

Figure 4. Effects of S1 and S2 (50 μM) in conditions of *t*-BuOOH-induced oxidative stress on cell viability and GSH level in isolated rat hepatocytes; *** $p < 0.001$ *vs* control (untreated rat hepatocytes); $^{+}p < 0.05$; $^{++}p < 0.01$ *vs. t*-BuOOH.

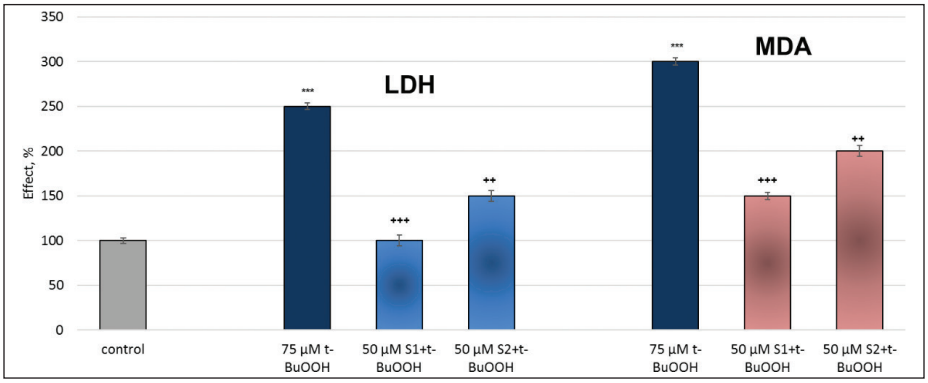

Figure 5. Effects of S1 and S2 (50 μM) in conditions of *t*-BuOOH-induced oxidative stress on LDH release and MDA level in isolated rat hepatocytes; *** $p < 0.001$ *vs.* control (untreated rat hepatocytes); $^{++}p < 0.01$; $^{+++}p < 0.001$ *vs. t*-BuOOH.

On hepatocytes, in a model of oxidative stress induced by 75 μM *tert*-butyl hydroperoxide, both saponins (50 μM) showed statistically significant hepatoprotective and antioxidant effects compared to pure *t*-BuOOH (Figs 4, 5). Saponins S1 and S2 preserved hepatocytes' viability, respectively, by 88% and 63%, and GSH level by 20% and 40%, respectively, compared to the toxic agent (*t*-BuOOH) (Fig. 4). Saponins S1 and S2 decreased statistically significant LDH release by 60% and 40% and MDA production by 50% and 33%, respectively, compared to the toxic agent *t*-BuOOH (Fig. 5).

Administered alone, carbon tetrachloride CCl_4 (86 μM) decreased statistically significant cell viability by 65% and GSH level by 60% in comparison to the control (untreated hepatocytes) (Fig. 6).

Administered alone, CCl_4 increased LDH release by 250% and MDA level by 300%, compared to the control (untreated hepatocytes) (Fig. 7).

On hepatocytes, in a model of metabolic bioactivation induced by 86 μM CCl_4, both compounds showed pronounced, statistically significant hepatoprotective and antioxidant effects compared to the pure CCl_4 (Figs 6, 7). Saponins S1 and S2 preserved statistically significant hepatocytes' viability by 129% and 100% and GSH level by 75% and 100%, respectively, compared to the toxic agent (CCl_4) (Fig. 6). Both compounds decreased statistically significant LHD release and MDA production by 74% and 78%, respectively, compared to the toxic agent—pure CCl_4 (Fig. 7). The protective effects of the saponins were better pronounced in the CCl_4 model than their effects in the *t*-BuOOH model.

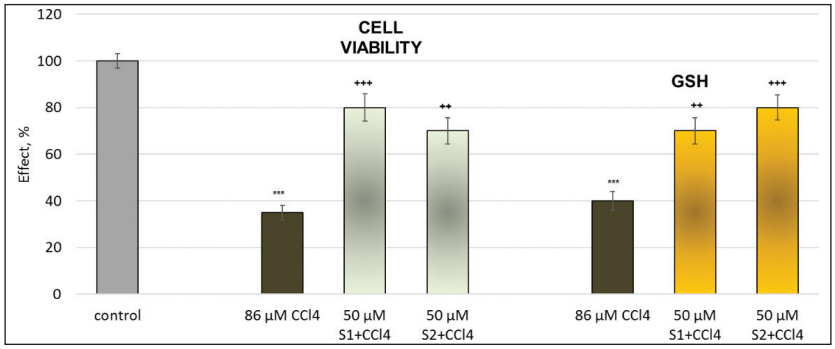

Figure 6. Effects of S1 and S2 (50 μM) in conditions of CCl_4-induced hepatotoxicity on cell viability and GSH level in isolated rat hepatocytes; *** $p < 0.001$ *vs.* control (untreated hepatocytes); $^{++}$ $p < 0.01$; $^{+++}$ $p < 0.001$ *vs.* CCl_4.

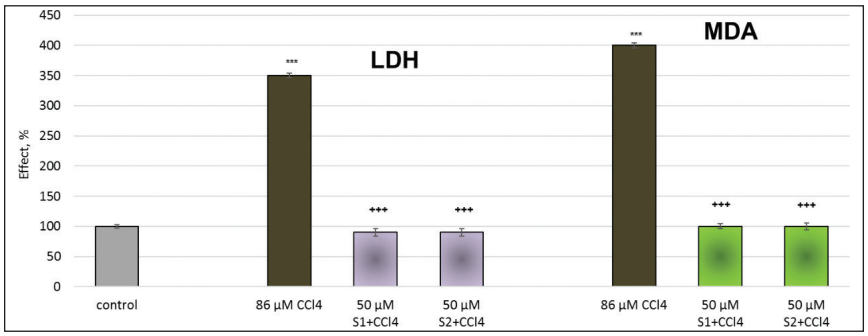

Figure 7. Effects of S1 and S2 (50 μM) in conditions of CCl_4-induced hepatotoxicity on LDH release and MDA level in isolated rat hepatocytes; *** $p < 0.001$ *vs.* control (untreated hepatocytes); $^{+++}$ $p < 0.001$ *vs.* CCl_4.

Evaluation of the effects of the saponins on cytochrome P450 isoforms' activity

The effects of S1 and S2 were evaluated on three isoforms of cytochrome P450—CYP1A2, CYP2D6, and CYP3A4 (Fig. 8).

On the CYP1A2 isoform, neither saponin showed a significant inhibitory effect. Only the classical inhibitor α-naphtoisoflavone inhibited the enzyme statistically significantly by 50% compared to the control (untreated CYP1A2). On isoform CYP2D6, which is characterized by genetic polymorphism (Guengerich, 2008), both saponins exhibited a weak inhibitory effect, reducing the enzyme activity by 20% for S1 and by 25% for S2, while the classical inhibitor quinidine reduced the enzyme activity by 55%, compared to control. On the CYP3A4 isoform, both saponins showed good statistically significant inhibition, reducing the activity by 40% and 35%, respectively, compared to the classical inhibitor ketoconazole, which showed an inhibition of 55% compared to the control (untreated CYP3A4) (Fig. 8).

The results from the present study correlate with what is known in the literature about the protective effects of various biologically active substances isolated from species of the genus *Astragalus* (Salehi et al. 2021). One of the classic models of oxidative stress is *t*-BuOOH, which, undergoing mainly mitochondrial and to a lesser extent microsomal metabolism, causes overproduction of free radicals, leading to the initiation of lipid peroxidation and a decrease in the level of reduced glutathione (O`-Donnell and Burkit 1994). In this model, saponins S1 and S2 exhibited statistically significant antioxidant and hepatoprotective effects on isolated hepatocytes. These results are in accordance with published data on the same activity of a PSM from *A. corniculatus* and *A. monspessulanus* subsp. *monspessulanus* (Mitcheva et al. 2008; Kondeva-Burdina et al. 2015).

As a fat-soluble compound, CCl_4 is distributed throughout the body, and chronic administration may lead to liver cirrhosis and tumors, as well as kidney damage. The administration of low doses of CCl_4 leads to fatty degeneration and destruction of cytochrome P450, which is observed mainly in the centrilobular zone of the liver. The toxic agent is bioactivated by several isoforms of cytochrome P450 (CYP1A2, CYP2E1, CYP2B1/B2, and CYP3A4), in which process a trichloromethyl radical is formed that leads to the initiation of the lipid peroxidation process (Weber et al. 2003). In this model of metabolic bioactivation, both saponins exhibited a more pronounced effect than the model of oxidative stress. This could be considered as one of the possible mechanisms for the hepatoprotection of *Astragalus* saponins, together with amelioration in α-smooth muscle actin, cytokeratin 19, and activation of the farnesoid X receptor (Zhang et al. 2024).

In a previous research study, two oleanane triterpenoid saponins (cauloside C and D) were tested for their inhibition on different isoforms of cytochrome P450 (CYP2C19, CYP3A4, CYP2D6, and CYP1A2). The saponins (at a concentration of 100 μM) showed inhibition only on isoform CYP3A4 while the other enzymes were not affected (Magdula et al. 2009). Our findings comply with these results, as the most pronounced inhibitory effect of

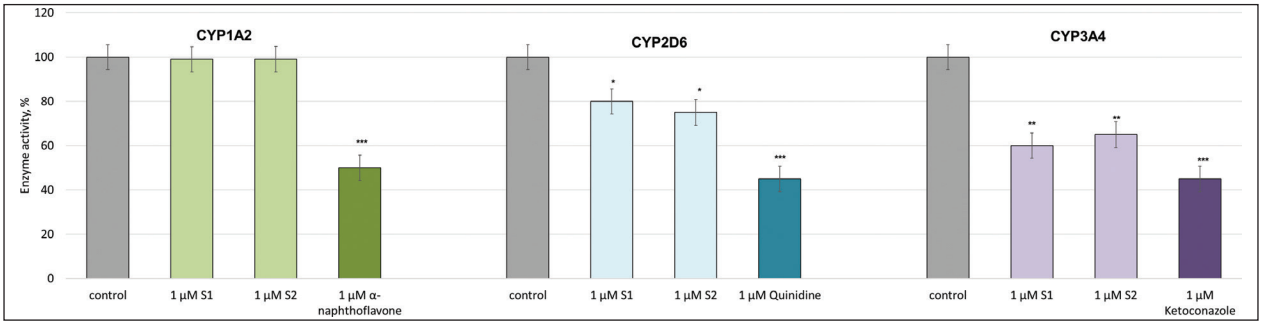

Figure 8. Effects of S1 and S2 (1 μM) on enzyme activity of CYP1A2, CYP2D6, and CYP3A4; * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$ vs. control (untreated CYP1A2, CYP2D6, and CYP3A4).

S1 and S2 was observed on CYP3A4, while a weak activity on CYP2D6 was also exhibited. The latter could potentially serve as a marker for differentiation of oleanane-from-cycloartane-type saponins regarding their activity in *in vitro* conditions.

Our findings highlight the importance of *Astragalus* species as medicinal plants and triterpenoid saponins as lead molecules for potential treatment for liver disease. In addition, the effects on isoforms of cytochrome P450 should attract attention towards possible herb-drug interactions.

Conclusion

Two cycloartane triterpenoid saponins, obtained from the aerial parts of *A. glycyphyllos*, showed statistically significant *in vitro* hepatoprotective and antioxidant effects on isolated liver microsomes in a model of non-enzyme-induced lipid peroxidation and on hepatocytes in a model of *t*-BuOOH-induced oxidative stress. Both compounds exhibited the same protective effects in a model of CCl_4-induced metabolic bioactivation. When tested for possible inhibitory activity on isoforms of cytochrome P450, both saponins exhibited well-pronounced inhibition on CYP3A4 and mild inhibition on CYP2D6, while no activity on CYP1A2 was observed.

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Additional information

Conflict of interest

The authors have declared that no competing interests exist.

Ethical statements

The authors declared that no clinical trials were used in the present study.

The authors declared that no experiments on humans or human tissues were performed for the present study.

The authors declared that no informed consent was obtained from the humans, donors or donors' representatives participating in the study.

The authors declared that no experiments on animals were performed for the present study.

The authors declared that no commercially available immortalised human and animal cell lines were used in the present study.

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Data availability

All of the data that support the findings of this study are available in the main text.

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