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EFFECT OF A PURIFIED SAPONINS' MIXTURE FROM *ASTRAGALUS GLYCYPHYLLOIDES*, ADMINISTERED ALONE, ON ISOLATED RAT BRAIN SYNAPTOSOMES AND HEPATOCYTES

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ABSTRACT

Purified saponins' mixture (PSM), obtained from *Astragalus glycyphylloides* (family Fabaceae) was examined for possible toxic effects on isolated rat brain synaptosomes and hepatocytes when administered alone. Synaptosomes and hepatocytes were incubated with PSM at three different concentrations: 60 μg/ml; 6 μg/ml; 0.6 μg/ml. The effects of PSM were compared to those of silymarin, in the same concentrations. The main parameters, characterizing functional and metabolic status of synaptosomes and hepatocytes were investigated: viability (by Trypan blue – for hepatocytes and MTT-test – for synaptosomes), lactate dehydrogenase activity (LDH), level of reduced glutathione (GSH) and production of malondialdehyde (MDA). It was found that administered alone the PSM did not reveal any statistically significant toxic effects on both isolated rat brain synaptosomes and hepatocytes, compared to silymarin.

Key words: *Astragalus glycyphylloides*, saponins' mixture, synaptosomes, hepatocytes, toxicity.

Introduction

Since millennia, plants have been used in traditional medicine worldwide to treat different conditions and illnesses. Such examples are the species of genus *Astragalus* L. (Fabaceae) (Bratkov et al., 2016).

The wide variety of secondary metabolites – flavonoids, saponins, polysaccharides and others, presented in the plants from *Astragalus* genus make them an interesting object for investigation.

Astragalus species are known to have numerous and varied biological activities, exerting immune modulating, antimicrobial, antiviral, tumour inhibiting, anti-inflammatory, hepatoprotective, cardio-vascular, etc. (Rios & Waterman, 1997).

However, the lack of information on one of their relatives, *Astragalus glycyphylloides*, makes this species a target for further investigation.

The present study investigates the effects of purified saponins' mixture (PMS), isolated from *Astragalus glycyphylloides*, administered alone, on isolated rat brain synaptosomes and hepatocytes.

Materials and methods

Plant material. The overground parts of *Astragalus glycyphylloides* DC. (Fabaceae) were collected in July 2016 from Rila Mountain, Bulgaria. The species was identified by Dr D. Pavlova from Faculty of Biology, Sofia University, Bulgaria, where a voucher specimen was deposited (SO-093817).

Obtaining of purified saponins' mixture (PSM). The dry plant material was powdered in laboratory mill and exhaustively extracted with 80% methanol *via* percolation. The resulting percolates were evaporated in vacuo to eliminate the solvent. The dry residue was suspended in

water, successively and exhaustively extracted with dichloromethane, ethyl acetate and n-butanol. The n-butanol extract was subjected to column chromatography (CC) over Diaion HP-20, eluting with H_2O-MeOH (100:0 → 0:100, v/v) to give ten main fractions (I-X). Fraction IX was analysed by LC-HREISMS and a total saponin content of 60% w/w was detected. There was one main saponin, with a molecular mass of 623 u.

Chemicals. Pentobarbital Sodium (Sanofi, France), HEPES (Sigma Aldrich, Germany), NaCl (Merck, Germany), KCl (Merck, Germany), D-Glucose (Merck, Germany), $NaHCO_3$ (Merck, Germany), KH_2PO_4 (Scharlau Chemie SA, Spain), $CaCl_2.2H_2O$ (Merck, Germany), $MgSO_4.7H_2O$ (Fluka AG, Germany), Collagenase from *Clostridium histolyticum* type IV (Sigma Aldrich, Germany), Albumin, Bovine serum fraction V, minimum 98% (Sigma Aldrich, Germany), EGTA (Sigma Aldrich, Germany), 2-Thiobarbituric acid (4,6-dihydroxypyrimidine-2-thiol; TBA) (Sigma Aldrich, Germany), Trichloroacetic acid (TCA) (Sigma Aldrich, Germany), 2,2'-dinitro-5,5'-dithiodibenzoic acid (DTNB) (Merck, Germany), Lactate dehydrogenase (LDH) kit (Randox, UK), D(+)Sucrose (Fluka AG, Germany), $MgCl_2x6H_2O$ (Merck, Germany), Percoll (Sigma Aldrich, Germany), NaH_2PO_4 (Merck, Germany), 6-hydroxydopamine (Merck, Germany), MTT (3-[4,5-dimethylthiazol-2-yl]-2,5diphenyl-tetrazolium bromide) (Sigma Aldrich, Germany), were used.

Animals. Male Wistar rats (body weight 200 – 250 g) were used. The rats were housed in plexiglass cages (3 per cage) in a 12/12 light/dark cycle, under standard laboratory conditions (ambient temperature 20°C ± 2°C and humidity 72% ± 4%) with free access to water and standard pelleted rat food 53-3, produced according ISO 9001:2008.

Animals were purchased from the National Breeding Centre, Sofia, Bulgaria. At least 7 days of acclimatization was allowed before the commencement of the study. A veterinary physician monitored the health regularly. The vivarium (certificate of registration of farm № 0072/01.08.2007) was inspected by the Bulgarian Drug Agency in order to check the husbandry conditions (№ A-11-1081/03.11.2011). The Institutional Animal Care Committee approved all performed procedures, the principles stated in the European Convention for the Protection of Vertebrate Animals used for Experimental and other Scientific Purposes were strictly followed throughout the experiment.

Isolation and incubation of hepatocytes. Rats were anesthetized with sodium pentobarbital (0.2 ml/100 g body weight). *In situ* liver perfusion and cell isolation were performed as described by Fau et al. (1992), with modifications (Mitcheva et al., 2006). The modifications consist of: usage of less collagenase and shorter time of cell isolation. The method provided in higher amount of live and metabolically active hepatocytes.

After portal catheterization, the liver was perfused with HEPES buffer + 0.6 mM EDTA and HEPES buffer, containing collagenase type IV and 7 mM $CaCl_2$. The liver was excised, minced into small pieces and hepatocytes were dispersed in Krebs-Ringer-bicarbonate (KRB) buffer (pH = 7.35) + 1% bovine serum albumin.

Cells were counted under the microscope and the viability was assessed by Trypan blue exclusion (0.05%) (Fau et al., 1992). The initial viability averaged 89%.

Cells were diluted with KRB, to make a suspension of about 3 x 10^6 hepatocytes/ml. Incubations were carried out in flasks, containing 3 ml of the cell suspension (i.e. 9 x 10^6 hepatocytes) and were performed in a 5% CO_2 + 95% O_2 atmosphere carbogen (Fau et al., 1992).

Lactate dehydrogenase (LDH) release. Lactate dehydrogenase release in isolated rat hepatocytes was measured spectrophotometrically at 314 nm by using LDH kit (Mitcheva et al., 2006).

Depletion of reduced glutathione (GSH). At the end of the incubation, isolated rat hepatocytes were recovered by centrifugation at 4 °C, and used to measure intracellular reduced glutathione (GSH), which was assessed by measuring non-protein sulfhydryls after precipitation of proteins with trichloroacetic acid (TCA), followed by measurement of thiols in the supernatant with DTNB. The absorbance was measured at 412 nm (Fau et al., 1992).

Assay of malondialdehyde (MDA) production. Hepatocyte suspension (1 ml) was taken and added to 0.67 ml of 20% (w/v) TCA. After centrifugation, 1 ml of the supernatant was added to 0.33 ml of 0.67% (w/v) 2-thiobarbituric acid (TBA) and heated at 100 °C for 30 min. The absorbance was measured at 535 nm, and the amount of TBA-reactants was calculated using a molar extinction coefficient of MDA 1.56 x 10^5 $M^{-1}cm^{-1}$ (Fau et al., 1992).

Isolation and incubation of synaptosomes. Synaptosomes were prepared by brains from adult male Wistar rats, as previously described by Taupin et al. (1994) with small modifications (Kondeva-Burdina et al., 2014). The brains were homogenized in 10 vol. of cold Buffer 1 (5 mM HEPES and 0.32 M sucrose). The synaptosomes were isolated by gradient centrifugation using Percoll reagent. Synaptosomes were re-suspended and incubated in Buffer 2, containing: 290 mM NaCl, 0.95 mM $MgCl_2.6H_2O$, 10 mM KCl, 2.4 mM $CaCl_2.H_2O$, 2.1 mM NaH_2PO_4, 44 mM HEPES, 13 mM D-glucose. Incubations were performed in a 5% CO_2 + 95% O_2 atmosphere carbogen.

The content of synaptosomal protein was determined according to the method of Lowry et al. (1951) using serum albumin as a standard.

Synaptosomes' viability measured by MTT-test. After incubation with the compounds, synaptosomes were treated with MTT solution (0.5 mg/ml) for 1 h in 37 °C. Then they were centrifuged at 15 000 x *g* for 1 min. The formed formazan crystals were dissolved in DMSO. The extinction was measured spectrophotometrically at $\lambda = 580$ nm (Mungarro-Menchaca et al., 2002).

***GSH level in synaptosomes*.** GSH was determined with the Ellman reagent (DTNB), which forms colour complexes with –SH group at pH = 8 with maximum absorbance at 412 nm (Robyt et al., 1971).

Statistical analysis. Statistical analysis was performed using statistical programme 'MEDCALC'. Results were expressed as mean ± SEM for six experiments. The significance of the data was assessed using the nonparametric Mann-Whitney U test. Values of $p \leq 0.05$ and $p \leq 0.01$ were considered statistically significant. Three parallel samples were used.

Results

In experimental toxicology, *in vitro* systems play an important role for the investigation of xenobiotic biotransformation and reveal the possible mechanisms of toxic stress and its protection.

Effects of PSM on isolated rat brain synaptosomes

Administered alone, PMS at three concentration levels did not reveal statistically significant neurotoxic effects on rat brain synaptosomes. Both PSM and silymarin did not change the main parameters, which functionally characterize synaptosomes: their viability (measured by MTT-test) and level of reduced glutathione (GSH) (Figures 1 and 2).

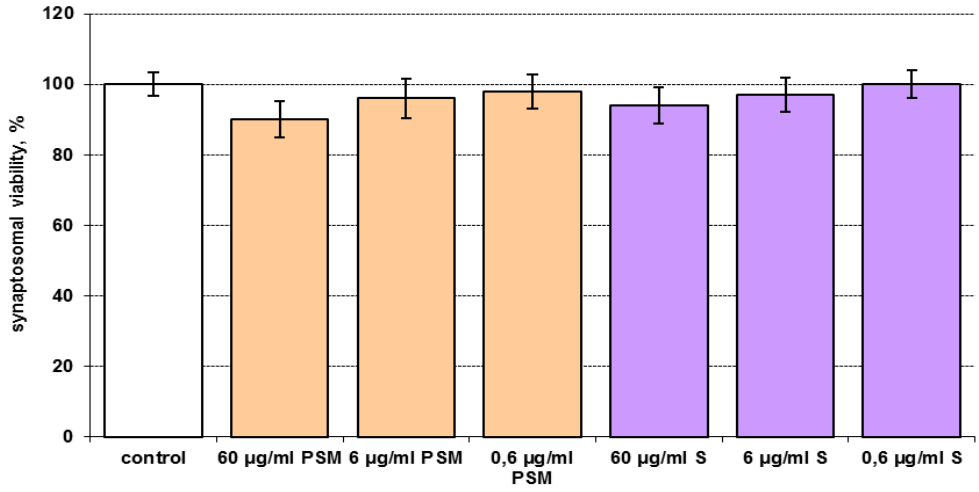

Figure 1: Effects of PSM and silymarin (at concentrations 60 µg/ml; 6 µg/ml; 0.6 µg/ml) on synaptosomal viability

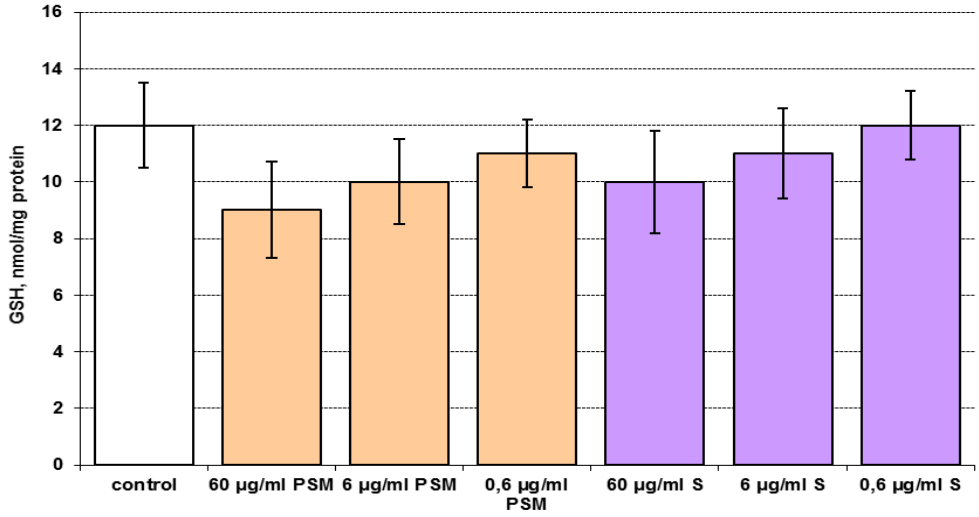

Figure 2: Effects of PSM and silymarin (at concentrations 60 µg/ml; 6 µg/ml; 0.6 µg/ml) on GSH level

Effects of PSM on isolated rat hepatocytes

Administered alone, PMS at three concentrations did not reveal statistically significant hepatotoxic effects on isolated rat hepatocytes. The PSM and silymarin did not change the main parameters, which characterized functional and metabolic status of hepatocytes: cell viability (measured by Trypan blue exclusion), LDH leakage, GSH level and production of MDA (Figures 3, 4, 5 and 6).

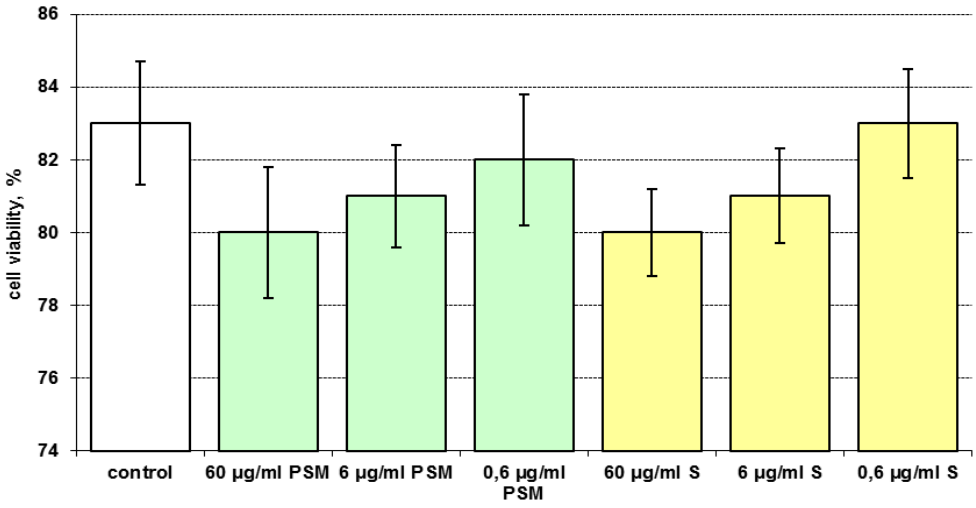

Figure 3: Effects of PSM and silymarin (at concentrations 60 µg/ml; 6 µg/ml; 0.6 µg/ml) on cell viability

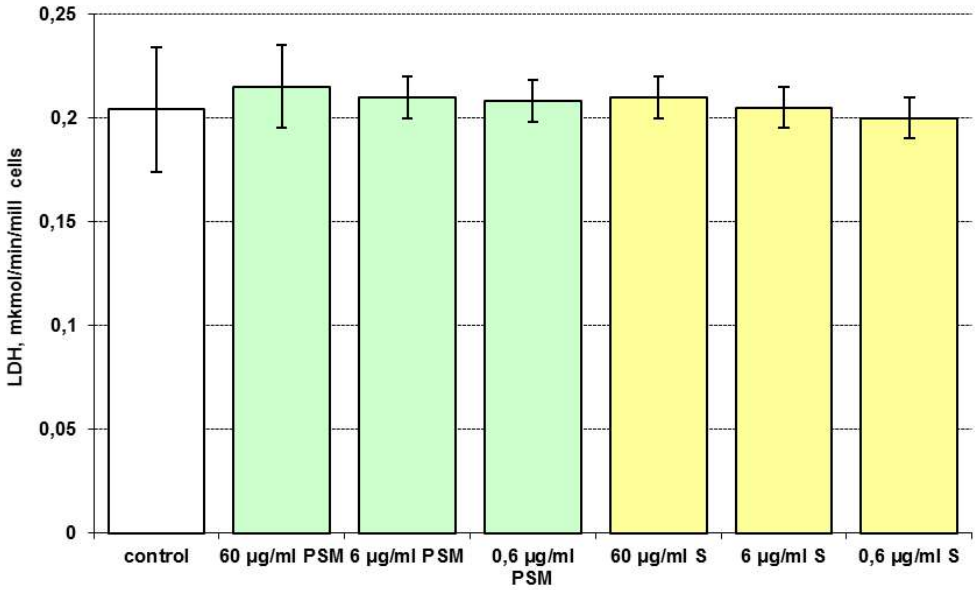

Figure 4: Effects of PSM and silymarin (at concentrations 60 µg/ml; 6 µg/ml; 0.6 µg/ml) on LDH leakage

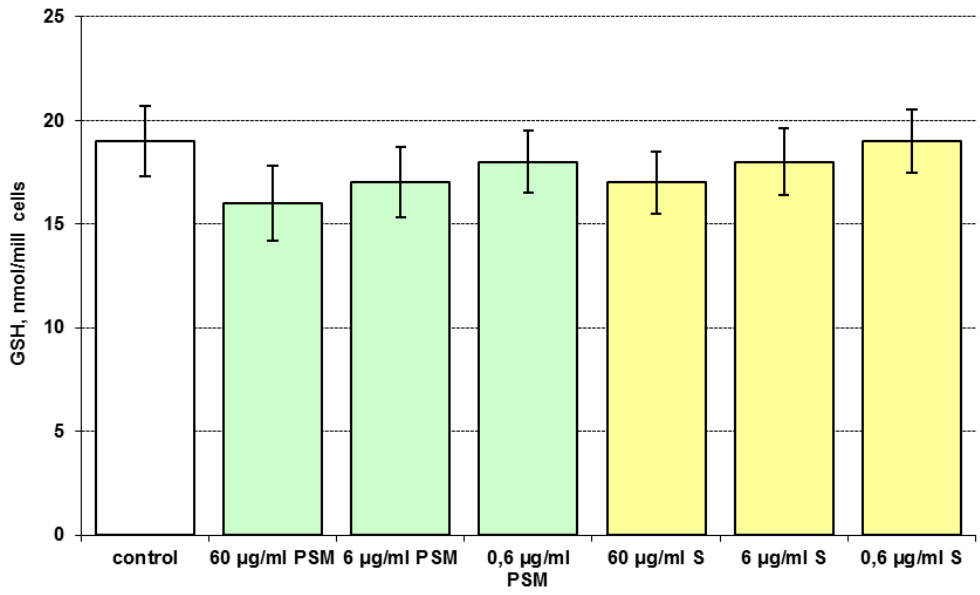

Figure 5: Effects of PSM and silymarin (at concentrations 60 µg/ml; 6 µg/ml; 0.6 µg/ml) on GSH level

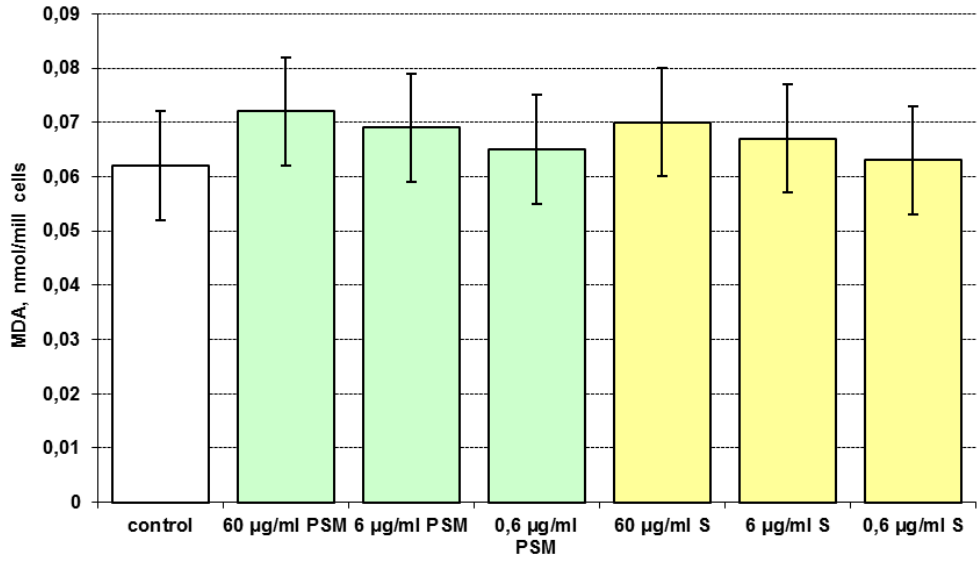

Figure 6: Effects of PSM and silymarin (at concentrations 60 µg/ml; 6 µg/ml; 0.6 µg/ml) on MDA production

Discussion

Saponins are naturally occurring structurally and functionally diverse phytochemicals that are widely distributed in plants. Although saponins are one of the largest classes of natural plant products, their biological functions, own metabolism (in plants, as well as in animals) and distribution in plants are not completely understood. Some authors found that the enzymes, which metabolize saponins in animal cells, are: one isoform of cytochrome P450 (CYP716A12) and uridin-glucorunyl transferase (UGT73F3), as well as dioxygenase and transaminase (Moses et al., 2014). According to these literature data, we suggest that the investigated saponins' mixture, which was found not toxic on synaptosomes and hepatocytes, was metabolized to non-toxic products, which were directly eliminated.

Conclusion

Our study proved that purified saponins' mixture, isolated from *Astragalus glycyphylloides*, administered alone, did not reveal any statistically significant toxic effects on rat brain synaptosomes and isolated hepatocytes.

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