



Study to Evaluate the Antioxidant Activity of *Astragalus glycyphyllos* Extract in Carbon Tetrachloride-Induced Oxidative Stress in Rats

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Authors' contributions

This work was carried out in collaboration between all authors. Author AS obtained the purified extract and managed the literature search. Author MKB designed the study. Authors RS and VV performed the experiments and the statistical analysis. Author IK wrote the first draft of the manuscript. All authors read and approved the final manuscript.

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Short Communication

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ABSTRACT

Aim: To investigate the possible antioxidant and hepatoprotective effects of purified extract obtained from aerial parts of *Astragalus glycyphyllos* (EAG) using *in vivo* model of CCl₄-induced liver damage in male Wistar rats.

Study Design: A total of 36 animals were randomly allocated in six experimental groups, each consisted of six animals: Control rats (Group 1), rats challenged orally with CCl₄ (10% solution in olive oil) (Group 2), rats treated for 7 days with silymarin (100 mg.kg⁻¹, 0.5 mL/100 g) alone (Group 3) and challenged with CCl₄ after 7-day pre-treatment with silymarin (Group 4); animals in groups 5 and 6 were either treated with EAG (100 mg.kg⁻¹, 0.5 mL/100 g) alone or challenged with CCl₄ after 7-day pre-treatment with the extract.

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Methodology: On day eight of the experiment the animals in all groups were sacrificed and livers were removed and the following biochemical markers of oxidative stress: MDA quantity, GSH levels, GPX, GR and GST were measured spectrophotometrically.

Results: It was found that EAG has statistically significant effect on hepatic antioxidant defense system in *in vivo* model of carbon tetrachloride liver damage in rats. A hepatoprotective effect comparable to silymarin can be supposed.

Conclusion: The results indicate that EAG has similar significant protective effect against CCl₄-induced hepatotoxicity in rats as silymarin. This may be explained with the antioxidant and hepatoprotective properties of its bioactive compounds.

Keywords: *Astragalus glycyphyllos*; antioxidant activity; CCl₄ hepatotoxicity.

1. INTRODUCTION

Astragalus L. (*Fabaceae*) is a genus distributed widely throughout the temperate regions of the world, located principally in Europe, Asia and North America. The pharmacological properties of *Astragalus* spp. are varied and include immunostimulant effects, antibacterial and antiviral properties, the ability to promote nucleic acid synthesis in the liver, hepatoprotective, antiinflammatory activity, cardiovascular tonic effects such as hypotensive and vasodilatory action [1-3]. The herb has also been found to increase superoxide dismutase activity, thus acting as a powerful antioxidant [4]. These effects appear to be due mainly to the saponins in the herb [5,6]. Saponins of genus *Astragalus* inhibit the formation of lipid peroxides in the liver. In our laboratory the hepatoprotective effect of purified saponin mixture (PSM), isolated from *Astragalus corniculatus* Bieb. was tested against chemical injury, induced by CCl₄ and tert-butylhydroperoxide (t-BuOOH) in isolated rat hepatocytes [7] and in different *in vivo/in vitro* models of toxicity [8]. Flavonoids and saponins are the two main groups of biologically active compounds isolated from *Astragalus glycyphyllos* (Liquorice milk vetch, Wild liquorice) [9-11], which is a perennial, herbaceous flowering plant, native to Europe [1]. There is little information about pharmacological activity of *Astragalus glycyphyllos* which makes this species an object for further investigation. On the basis of this information the aim of our study was to investigate the possible beneficial effects of defatted total extract of *A. glycyphyllos* using *in vivo* model of CCl₄-induced liver damage in rats and to compare its effects with the well-known hepatoprotector and antioxidant silymarin.

2. MATERIALS AND METHODS

2.1 Plant Material and Preparation of Defatted Total Extract

The overground parts of *Astragalus glycyphyllos* were collected from Vitosha Mountain, Bulgaria in August, 2013. The species was identified by Dr D. Pavlova from Faculty of Biology, Sofia University, Bulgaria, where a voucher specimen was deposited (SO-107613).

The air-dried was powdered in laboratory mill (100 g) and exhaustively extracted with 80% methanol via percolation (5x500 mL). The percolate (2.5 L) was concentrated on a rotary evaporator in order to eliminate the solvent. The dry residue was suspended in water (500 mL) and exhaustively extracted in a separating funnel with dichloromethane (5x200 mL). Then the resulting defatted extract was dried on a rotary evaporator, pulverized and used for the experiment.

2.2 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 Center, Sofia, Bulgaria. A minimum of 7 days acclimatization was allowed before the commencement of the study. Their health was monitored regularly by a veterinary physician. 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). All performed procedures were approved by the Institutional Animal Care Committee (KENIMUS). The principles stated in the European Convention for the Protection of Vertebrate Animals used for Experimental and other Scientific Purposes (ETS 123) (Council of Europe, 1991) [12] were strictly followed throughout the experiment.

2.3 Chemicals

All reagents used were of analytical grade. Methanol, carbon tetrachloride, silymarin as well as, 1-chloro-2,4-dinitrobenzene (CDNB), beta-Nicotinamide adenine dinucleotide 2-phosphate reduced tetrasodium salt (NADPH), ethylenediaminetetraacetic acid (EDTA), bovine serum albumin (fraction V), reduced glutathione (GSH), oxidized glutathione (GSSG), glutathione reductase (GR) and cumene hydroperoxide were purchased from Sigma Chemical Co. (Taufkirchen, Germany). 2, 2-Dinitro-5, 5 dithiodibenzoic acid (DTNB), thiobarbituric acid and trichloroacetic acid were obtained from Merck (Darmstadt, Germany).

2.4 Design of the Experiment

Thirty six animals were randomly divided into six groups (n=6):

- Group 1 – control, treated with physiological saline by oral gavage (0.5 mL/100 g)
- Group 2 – challenged with CCl₄ (10% solution in olive oil, 1.25 mL.kg⁻¹ orally)
- Group 3 – treated with silymarin (100 mg.kg⁻¹/po, 0.5 mL/100 g for 7 days) [13]
- Group 4 – treated with silymarin (100 mg.kg⁻¹/po/7 days) and 90 min after the last treatment challenged with CCl₄ (10% solution, 1.25 mL.kg⁻¹po)
- Group 5 – treated with extract of *Astragalus glycyphyllos* (EAG) (100 mg.kg⁻¹/po/, 0.5 mL/100 g/ 7 days)
- Group 6 – treated with EAG (100 mg.kg⁻¹/po/7 days) and 90 min after the last treatment challenged with CCl₄ (10% solution, 1.25 mL.kg⁻¹po).

On day eight of the experiment the animals in all groups were sacrificed and livers were removed rapidly, perfused with ice-cold saline solution (0.9% NaCl), blotted dry, weighed and divided into pieces (stored on ice) as follows: 1 g each for assessment of MDA quantity, glutathione

(GSH) levels and antioxidant enzyme activities. Tissues were homogenized in ice-cold appropriate buffers using a glass homogenizer (PX-OX 2000).

2.5 Biomarkers of Oxidative Damage

Malone dialdehyde (MDA) quantity, reduced glutathione (GSH) levels, glutathione peroxidase (GPx), glutathione reductase (GR) and glutathione-S-transferase (GST) activities were measured in liver homogenate.

Lipid peroxidation was determined by measuring the rate of production of thiobarbituric acid reactive substances (TBARS) (expressed as MDA equivalents) described by Polizio and Peña [14]. Briefly, livers were homogenized with 0.1 M phosphate buffer, pH=7.4 (1:3). Homogenates were mixed with 1 mL 25% trichloroacetic acid (TCA) and 1 mL 0.67% thiobarbituric acid (TBA). Samples were then mixed thoroughly, heated for 20 min in a boiling water bath, cooled and centrifuged at 4000 rpm for 20 min. The absorbance of supernatant was measured at 535 nm against a blank that contained all the reagents except the tissue homogenate. MDA concentration was calculated using a molar extinction coefficient of 1.56x10⁵ M⁻¹cm⁻¹ and expressed in nmol.g⁻¹ wet tissue.

GSH was assessed by measuring of non-protein sulfhydryls after precipitation of proteins with trichloroacetic acid (TCA) followed by measurement of thiols in the supernatant by the DTNB reagent [15]. Briefly, livers were homogenized in 5% TCA (1:3) and centrifuged for 20 min at 4 000xg. The reaction mixture contained 0.05 mL supernatant, 3 mL 0.05 M phosphate buffer (pH=8) and 0.02 mL DTNB reagent. GSH level was expressed as nmol/g wet tissue.

Liver homogenates (10%) for antioxidant enzymes assessment were prepared in 0.05 M phosphate buffer (pH=7.4), centrifuged at 7,000 x g and the supernatant was used for antioxidant enzymes assay. Analyses were performed in triplicate and the average values were taken. Protein content was measured by the Lowry method [16].

Glutathione peroxidase activity (GPx) was measured by NADPH oxidation, using a coupled reaction system consisting of glutathione, glutathione reductase and cumene hydroperoxide [17]. Briefly, 100 µL of enzyme

sample was incubated for 5 minutes with 1.5 mL 0.05 M phosphate buffer (pH=7.4), 100 μ L 1 mM EDTA, 50 μ L 1 mM GSH, 100 μ L 0.2 mM NADPH and 1 unit glutathione reductase. The reaction was initiated by adding 50 μ L cumene hydroperoxide (1 mg/mL) and the rate of disappearance of NADPH with time was determined by monitoring absorbance at 340 nm. Results were expressed as nmol/min/mg protein using the molar extinction coefficient of 6.22 mM⁻¹cm⁻¹.

Glutathione reductase activity (GR) was measured spectrophotometrically at 340 nm according to the method of Pinto et al. [18] by following NADPH oxidation and using an extinction coefficient of 6.22 mM⁻¹cm⁻¹. The incubation mixture contained 0.05 M phosphate buffer, pH=7.4, 2.5 mM GSSG and 125 μ M NADPH at 30°C. The enzyme activity is expressed in nmol/min/mg glutathione reductase activity (GR).

Glutathione-S-transferase activity (GST) was measured using 1-chloro-2,4-dinitrobenzene (CDNB) as substrate described by Habig et al. [19]. The incubation mixture containing 1.6 mL 0.05 M phosphate buffer, 100 μ L 1 mM GSH, 100 μ L 1 mM EDTA and 100 μ L homogenate was incubated for 15 minutes at 37°C. After the incubation, 100 μ L 1 mM CDNB was added and the increase in absorbance with time was recorded at 340 nm. Enzyme activity is measured using an extinction coefficient of 9.6 \times 10³/M⁻¹/cm⁻¹ and is expressed as nmol of CDNB-GSH conjugate formed/minute/mg protein.

2.6 Statistical Analysis

Statistical analysis was performed using statistical program 'MEDCALC'. Results were expressed as mean \pm SEM, for six rats in each group. The experimental groups were compared using the Kruskal-Wallis variance analysis test and a post-hoc analysis using Mann-Whitney *U* test was performed. Values of *P*≤0.05 were considered statistically significant.

3. RESULTS AND DISCUSSION

Hepatoprotective and antioxidant effects of flavonoids are well known both in experimental and in clinical practice. Silymarin, a mixture of flavonolignans, derived from the fruits of *Silybum marianum* (milk thistle) is one of the most commonly used in medical practice hepatoprotective and antioxidant drug [20]. Identifying new sources of compounds having

potent antioxidant and hepatoprotective activity is considered to be in great importance.

In the current study the *in vivo* antioxidant effects of total defatted extract isolated from *Astragalus glycyphyllos* (EAG) on CCl₄-induced oxidative liver damage in rats was investigated. In experimental toxicology CCl₄ is used as a model compound to induce hepatotoxicity [21]. The basic mechanism of its toxicity is bioactivation by several cytochrome P 450 isoforms – CYP2E1, CYP2B1 and possibly CYP3A4 - to form the trichloromethyl radical (\cdot CCl₃), which initiates the chain reaction of lipid peroxidation [22]. The trichloromethyl radical (CCl₃ \cdot) and its derivative trichloromethylperoxy radical (CCl₃OO \cdot) react with the unsaturated fatty acids in the cellular membranes disturbing a number of cellular processes and leading to a loss of cell protection. The results of our study confirmed the pro-oxidant and hepatotoxic effect of CCl₄, discerned by statistically significant (*P*<0.05) increase by 48% in MDA production (Fig. 1) and decrease in GSH levels by 54% (Fig. 2). GSH is a low molecular weight endogenous antioxidant thiol and an important component in the cells of living organisms, protecting them from the harmful effects of free radicals, peroxides and other toxic agents. It supports normal structure and function of the cell through its redox properties. GSH, present in abundance in the liver acts either by directly scavenging the free radicals or by acting as a substrate to GPx and GST during the detoxification of hydrogen peroxides, lipid peroxides and electrophiles as well as by preventing oxidation of SH groups of proteins [23]. In support to this information, in the current study a significant (*P*<0.05) decrease in the activity of GSH-related antioxidant enzymes was observed, i.e. GPx – by 55%, GR – by 33% and GST – by 61%. The results are shown in Fig. 3, Fig. 4 and Fig. 5, respectively. EAG and silymarin pre-treatment prevented CCl₄-induced oxidative stress by inhibiting lipid peroxidation and restoring the GSH levels and enzyme antioxidant defense. In the combination group (EAG + CCl₄), EAG pre-treatment resulted in a decrease in MDA production (24%, *P*<0.05), and in an increase in GSH levels (36%, *P*<0.05), in GPx activity (83%, *P*<0.05), in GR activity (68%, *P*<0.05) and in GST activity (51%, *P*<0.05). The results were compared to CCl₄ only group. EAG antioxidant properties were comparable to those of silymarin. The results showing the hepatoprotective effect of the studied extract, isolated from *Astragalus glycyphyllos* are in good correlation with the results obtained from other

studies investigated the biological properties of extracts, isolated from other *Astragalus* species [24,25]. The main compounds in EAG are flavonoids, saponins and polysaccharides. The increase in the activity of antioxidant enzymes from EAG was most likely due to the ability of flavonoids, available in this plant, to capture free radicals [26]. This suggests that the EAG may

play an important role in preventing initiation and propagation of the lipid peroxidation process by scavenging the free radicals via the GSH, GSH-redox system and others antioxidant enzymes. However, isolation and identification of the exact biologically active compounds, alongside additional studies on their biological properties is an object of further investigation.

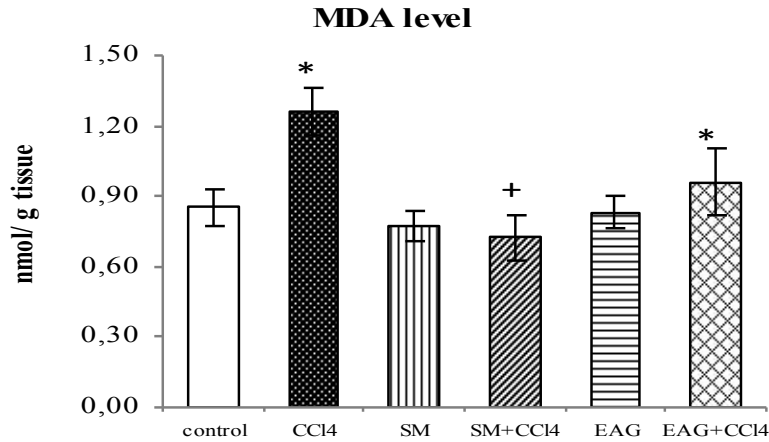


Fig. 1. Effect of defatted extract of *A. glycyphyllos* on thiobarbituric acid reactive substances (as MDA level) in rat liver homogenate

CCl₄ – carbon tetrachloride, *SM* – silymarin, *EAG* – *A. glycyphyllos* extract

**P*<0.05 vs control; +*P*<0.05 vs *CCl₄* group; Mean ± S.E.M = Mean values ± Standard error of means of six animals (Mann-Whitney U test)

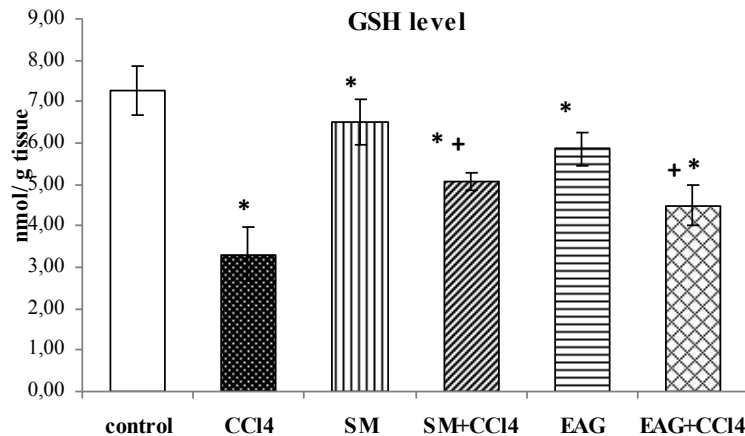


Fig. 2. Effect of defatted extract of *A. glycyphyllos* on reduced glutathione (GSH) level in rat liver homogenate

CCl₄ – carbon tetrachloride, *SM* – silymarin, *EAG* – *A. glycyphyllos* extract.

**P*<0.05 vs control; +*P*<0.05 vs *CCl₄* group; Mean ± S.E.M = Mean values ± Standard error of means of six animals-(Mann-Whitney U test)

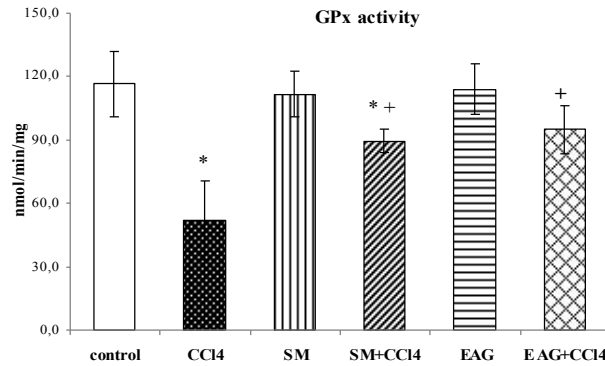


Fig. 3. Effect of defatted extract of *A. glycyphyllos* on glutathione peroxidase (GPx) activity in rat liver homogenate

CCl₄ – carbon tetrachloride, *SM* – silymarin, *EAG* – *A. glycyphyllos* extract
 **P*<0.05 vs control; +*P*<0.05 vs *CCl₄* group; Mean ± S.E.M = Mean values ± Standard error of means of six animals. (Mann-Whitney U test)

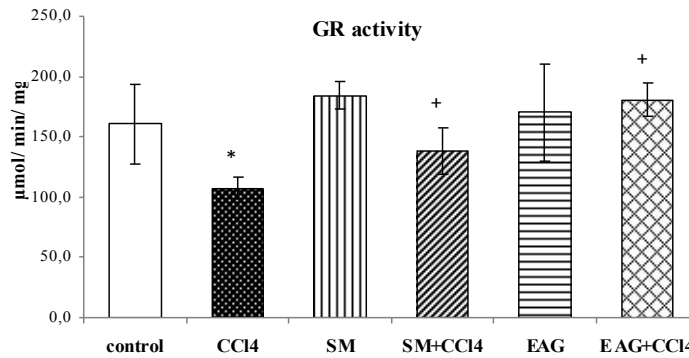


Fig. 4. Effect of defatted extract of *A. glycyphyllos* on glutathione reductase (GR) activity in rat liver homogenate

CCl₄ – carbon tetrachloride, *SM* – silymarin, *EAG* – *A. glycyphyllos* extract
 **P*<0.05 vs control; +*P*<0.05 vs *CCl₄* group; Mean ± S.E.M = Mean values ± Standard error of means of six animals. (Mann-Whitney U test)

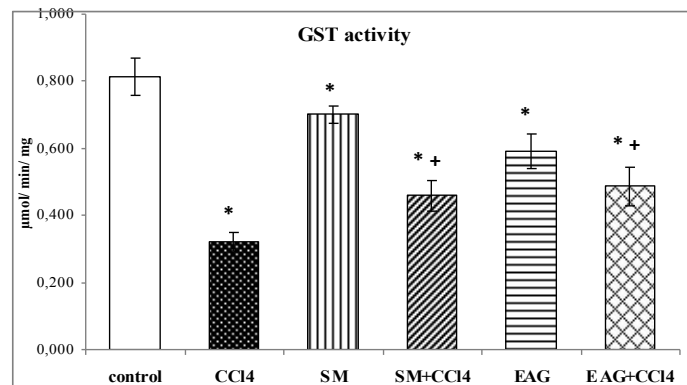


Fig. 5. Effect of defatted extract of *A. glycyphyllos* on glutathione-S-transferase (GST) activity in rat liver homogenate

CCl₄ – carbon tetrachloride, *SM* – silymarin, *EAG* – *A. glycyphyllos* extract
 **P*<0.05 vs control; +*P*<0.05 vs *CCl₄* group; Mean ± S.E.M = Mean values ± Standard error of means of six animals. (Mann-Whitney U test)

4. CONCLUSION

The results indicate that EAG has similar significant protective effect against CCl₄-induced hepatotoxicity in rats as silymarin. This may be explained with the antioxidant and hepatoprotective properties of its bioactive compounds.

CONSENT

It is not applicable.

ETHICAL APPROVAL

All performed procedures were approved by the Institutional Animal Care Committee (KENIMUS). The principles stated in the European Convention for the Protection of Vertebrate Animals used for Experimental and other Scientific Purposes (ETS 123) (Council of Europe, 1991) were strictly followed throughout the experiment.

All authors hereby declare that "Principles of laboratory animal care" (NIH publication No. 85-23, revised 1985) were followed, as well as specific national laws where applicable. All experiments have been examined and approved by the appropriate ethics committee.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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