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EFFECTS OF EXTRACT FROM *ASTRAGALUS GLYCYPHYLLOIDES* ON CARBON TETRACHLORIDE-INDUCED HEPATOTOXICITY IN WISTAR RATS

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ABSTRACT

The antioxidant effect of aqueous-ethanolic extract from *Astragalus glycyphylloides* (Fabaceae), and its protection against carbon tetrachloride-induced hepatotoxicity were investigated in male Wistar rats. For seven consequent days the animals were treated orally with *A. glycyphylloides* extract (EAG) (100 mg/kg). Two hours after the last administration the animals were charged with carbon tetrachloride (10%, 1,25 mL/kg p.o.). The rats were sacrificed 18 hours later by decapitation. Carbon tetrachloride, administered alone, induced significant hepatotoxicity, manifested with reduced glutathione (GSH) depletion, increased level of malondialdehide (MDA) and reduced antioxidant defense. EAG pretreatment, however, completely reversed the effect of carbon tetrachloride on GSH and MDA concentrations and antioxidant enzyme activities as catalase (CAT), superoxide dismutase (SOD), glutathione peroxidase (GPx), glutathione reductase (GR) and glutathione-S-transferase (GST). The results of this study showed an antioxidant activity of extract from *Astragalus glycyphylloides* and its protective effect against carbon tetrachloride-induced oxidative stress and hepatotoxicity.

KEY WORDS: **Oxidative stress, antioxidant effect, carbon tetrachloride, *Astragalus glycyphylloides***

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INTRODUCTION

Astragalus species are known to have different therapeutic activities and are used for medicinal purposes in many countries. Various effects of the plants have been associated with saponins, flavonoids and polysaccharides. These biologically active substances have interesting pharmacological properties as hepatoprotective, immunostimulant, cardiovascular, antiviral etc [1,2,3]. *Astragalus glycyphylloides* DC (Fabaceae) is distributed in Western Asia (Iran, Turkey), Caucasus (Armenia, Azerbaijan, Georgia, and Russia), Eastern Europe (Ukraine), Southeastern Europe (Bulgaria, Former Yugoslavia and Greece). Extracts from the plant possess antifungal and insecticidal effects [4,5]. Diverse pharmacological effects of many biologically active substances are studied by different experimental models. Carbon tetrachloride (CCl_4) is a well known hepatotoxin used as a model for investigation of hepatotoxicity and hepatoprotection. Liver injuries induced by CCl_4 are mediated through the formation of reactive intermediates such as trichloromethyl radical (CCl_3•) and its derivative trichloromethyl peroxy radical (CCl_3OO•). These free radicals are thought to react with membrane lipids leading to their peroxidation [6]. A wide range of drugs are currently employed in the management of hepatic disorders. Inhibition of free radicals is very important in terms of liver pathology. Natural plant-derived products, containing flavonoids are being investigated as a source of antioxidants as these may have great relevance in the prevention of diseases associated with oxidative stress [7,8]. In previous experiments [9] we have shown a hepatoprotective effect of other species of genus Astragalus, as *Astragalus corniculatus*. On the basis of these data, the aim of the following study was to show the antioxidant effect of extract from *Astagalus glycyphylloides* (EAG) and its possible protection against carbon tetrachloride-induced hepatotoxicity in rats. Protective activity of EAG at 100 mg/kg dose was statistically compared with positive control silymarin (100 mg/kg, po), a known hepatoprotective drug.

MATERIALS AND METHODS

1. Plant material and preparation of the extract

Astragalus glycyphylloides herbs were collected in July 2009 in Rila Mountain, Bulgaria. The plant was identified by Dr. D. Pavlova from the Department of Botany, Faculty of Biology, Sofia University, where voucher specimen has been deposited (S093817). The air-dried plant material (300 g) was powdered and extracted exhaustively with 50% EtOH in an ultrasonic bath with a working frequency of 35 kHz. The extract was filtered, concentrated under reduced pressure and lyophilized to a powder. Further phytochemical investigations of EtOH extract led to the isolation of eight known flavonoids quercetin, quercetin-3-*O*-arabinoside (avicularin), quercetin-3*O*-galactoside (hyperoside), quercetin-3-*O*-glucoside (isoquercitrin), kaempferol, isorhamnerin, isorhamnetin-3-*O*-glucoside and isorhamnetin3-*O*-arabinoside. The compounds were identified [10] on the basis of chemical and spectral analysis and compared with data reported in the literature. Additionally the flavonoids were further confirmed by co-TLC with authentic samples. This is the first report on the isolation of these compounds from *Astragalus glycyphylloides.*

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 and 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 (№А-11-1081/03.11.2011). All performed procedures were approved by the Institutional Animal Care Committee (CENIMUS) and the principles stated in the European Convention for the Protection of Vertebrate Animals used for Experimental and other Scientific Purposes (ETS 123)[11] were strictly followed throughout the experiment.

3. Chemicals

All the reagents used were of analytical grade. Carbon tetrachloride, as well as other chemicals, 1-chloro-2,4-dinitrobenzene, beta–Nicotinamide adenine dinucleotide 2`-phosphate reduced tetrasodium salt (NADPH), ethylenediaminetetraacetic acid (EDTA), bovine serum albumin (fraction V), silymarin, trichloracetic acid, thiobarbituric acid, epinephrine, reduced glutathione (GSH), glutathione reductase, oxidized glutathione (GSSG), hydrogen peroxide (H_2O_2) and cumene hydroperoxide were purchased from Sigma Chemical Co (Germany). 2,2'-dinitro-5,5'dithiodibenzoic acid (DTNB) was obtained from MERCK (Germany).

4. Design of the in vivo experiment

The animals were divided into six groups (n=6).

Group 1 – control, treated with saline

Group 2 - treated with EAG alone (100 mg kg-1/po/ 7 days) [9]

Group 3 - treated with silymarin (100 mg kg-1/po/ 7 days) [12]

Group 4 - treated with carbon tetrachloride (10% solution, 1.25 mL/kg po once) [13]

Group 5 - treated with EAG (100 mg kg-1/po/7 days), 2 hours after the last treatment administered carbon tetrachloride (10% solution, 1.25 mL/kg po., once)

Group 6 - treated with silymarin (100 mg kg-1/po/7 days), 2 hours after the last treatment administered carbon tetrachloride (10% solution, 1.25 mL/kg po., once)

The animals in all groups were sacrificed on the eight day from the beginning of the experiment. Livers were taken for assessment of biochemical parameters.

5. Preparation of liver homogenate for lipid peroxidation (LP) assessment

Lipid peroxidation was determined by measuring the rate of production of thiobarbituric acid reactive substances (TBARS) (expressed as malondialdehyde (MDA) equivalents) [14]. Briefly, one volume of homogenate was mixed with 1 mL 25 % trichloracetic 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 1.56×10^5 M^{-1} cm^{-1} and expressed in nmol/g wet tissue.

6. Preparation of liver homogenate for GSH assessment

GSH was assessed by measuring non-protein sulfhydryls after precipitation of proteins with TCA [15]. Briefly, tissues were homogenized in 5% TCA and centrifuged for 20 min at 4 000 x g. The reaction mixture contained 0.05 mL supernatant, 3 mL 0.05 M phosphate buffer (pH = 8) and 0.02 mL DTNB reagent. The absorbance was determined at 412 nm and the results expressed as nmol/g wet tissue.

7. Preparation of liver homogenates for antioxidant enzyme activity measurement

The livers were rinsed in ice-cold saline and minced with scissors. 10 % homogenates 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. The protein content of liver homogenate was measured by using bovine serum albumin as a standard [16].

i.Catalase activity (CAT)[17]

Briefly, 10 µL of homogenate were added to 1 990 µL solution (containing 6.8 µL of 30 % H_2O_2 + 1 983.2 µL 0.05 M phosphate buffer, pH=7.4). CAT activity was determined by measuring the decrease in absorbance at 240 nm. The enzyme activity was expressed as µmol/min/mg.

ii. Superoxide dismutase activity (SOD) [18]

SOD was measured following spectrophotometrically the autooxidation of epinephrine at pH=10.4, 30° C, using the molar extinction coefficient of 4.02 mM^{-1} cm^{-1}. The incubation mixture contained 50 mM $NaHCO_3$-Na_2CO_3 buffer, pH = 10.4. The raction was started by the addition of epinephrine. SOD activity is expressed as nmol of epinephrine prevented from autooxidation after addition of the sample.

iii.Gluthatione peroxidase activity (GPx) [19]

GPx was measured by NADPH oxidation, using a coupled reaction system consisting of glutathione, glutathione reductase and cumene hydroperoxide. Briefly, 100 µL of enzyme sample was incubated for 5 minutes with 1.5 mL 0.05 M phosphate buffer (pH=7.4), 100 µL 1 mM EDTA, 50 µL 1 mM GSH, 100 µL 0.2 mM NADPH and 1 unit glutathione reductase. The reaction was initiated by adding 50 µL cumene hydroperoxide (1 mg/mL) and the rate of disappearance of NADPH with time was determined by monitoring absornance at 340 nm. Results were expressed as nmol/min/mg protein.

iv.Glutathione reductase avtivity (GR) [20]

GR was mesured by following NADPH oxidation spectrophotometrically at 340 nm and using an extinction coefficient of 6.22 mM^{-1} cm^{-1}. The incubation mixture contained 0.05 M phosphate buffer, pH=7.4, 2.5 mM GSSG and 125 µM NADPH at 30°C. Results were expressed as nmol/min/mg protein.

v. Glutathione-S-transferase activity (GST) [21]

GST was measured using 1-chloro-2, 4-dinitrobenzene (CDNB) as substrate. The incubation mixture containing 1.6 mL 0.05 M phosphate buffer, 100 µL 1 mM GSH, 100 µL 1 mM EDTA and 100 µL homogenate, was incubated for 15 minutes at 37 C. After the incubation, 100 µL 1 mM CDNB was added and the increase in absorbance with time was recorded at 340 nm. The enzyme activity was expressed as nmol of CDNB-GSH conjugate formed/min/mg protein.

8. Statistical analysis

Statistical analysis was performed using statistical programme 'MEDCALC'. Results are expressed as mean ± SEM for six rats in each group. The significance of the data was assessed using the nonparametric Mann–Whitney test. Values of $P \leq 0.05$ were considered statistically significant.

RESULTS

Effect of EAG and silymarin pre-treatment on lipid peroxidation, GSH level and antioxidant profile in rats with induced CCl_4 intoxication is shown in Table I. Compared to the respective control group, CCl_4 toxicity is presented by increased amount of MDA (by 75 %, $p<0.05$), decreased (by 37%, $p<0.05$) levels of GSH, decreased activities of hepatic antioxidant enzymes, as followed: CAT (by 30%, $p<0.05$), SOD (by 30%, $p<0.05$), GPx by (54%, $p<0.05$), GR (by 56%, $p<0.05$) and GST (by 46%, $p<0.05$). EAG and silymarin pre-treatment prevented CCl_4 – induced toxicity by inhibiting lipid peroxidation and restoring the levels of cell glutathione and antioxidant enzymes. When comparing the data obtained from EAG + CCl_4 group versus CCl_4 - only group, a significant decrease in MDA quantity by 41 % ($p<0.05$) and an increases in GSH levels by 37 % ($p<0.05$), in SOD activity by 41 % ($p<0.05$), in CAT activity by 34 % ($p<0.05$), in GPx activity by 79 % ($p<0.05$), in GR activity by 87 % ($p<0.05$) and in GST activity by 20 % ($p<0.05$) were observed. EAG antioxidant activity was comparable to those of silymarin.

Table I

Effects of extract from Astragalus glycyphylloides (EAG) and silymarin (SL) pre-treatment on hepatic liver peroxidation and antioxidant profile in rats challenged with CCl_4

Groups	Control	CCl_4	Silymarin	SL+CCl_4	EAG	EAG+CCl_4
MDA[a]	0,67±0,08	1,17±0,12*	0,69±0,15	0,84±0,07*+	0.71±0.07	0.69±0.03+
GSH[a]	4.92±0.45	3,08±0,21*	4,90±0,15	4,32±0,39+	4,33±0,30	4,22±0,31+
CAT[b]	73,4±7,9	51,1±7,1*	76,4±8,2	70,1±7,2+	67,8±8,2	68,4±9,2+
SOD[b]	0,22±0,02	0,15±0,01*	0,21±0,02	0,22±0,01+	0,20±0,04	0,19±0,02+
GPx[c]	105,3±14,5	48,3±14,8*	92,5±8,6	89,9±15,2+	96,9±16,6	86,4±9,6+
GR[b]	0,21±0,03	0,09±0,01*	0,19±0,02	0,14±0,01+	0,22±0,03	0,16±0,01+
GST[b]	0,32±0,02	0,17±0,02*	0,29±0,04	0,24±0,02+	0,25±0,02	0,21±0,01+

[a]nmol/g tissue
[b]µmol /min/mg protein
[c]nmol/min/mg protein
Data are expressed as mean ± SEM of six rats.
**Significant difference from control values (Mann–Whitney test, p<0.05).*
+Significant difference from CCl_4 treated group (Mann–Whitney test, p<0.05).

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[22]. Therefore, identifying new sources of compounds having potent antioxidant and hepatoprotective activity is considered to be in great importance. The literature does not provide any information about antioxidant activity of Astragalus *glycyphylloides*. Having in mind these data we evaluated *in vivo* antioxidant and hepatoprotective potential of extract from *Astragalus glycyphylloides* (EAG) on CCl_4 model of liver toxicity and the results were compared with silymarin (as a positive control). Carbon tetrachloride is metabolized by the cytochrome P450 system to produce toxic trichloromethyl radicals, which act as free radical cascade initiators[23]. CCl_4 reactive metabolites bind covalently to microsomal and others macromolecules and initiate processes of LP. Disturbed cellular processes were most likely due to increased levels of TBARS[24], which was confirmed in this study. We found that after CCl_4 administration, the level of MDA, a marker for LP was increased and pretreatment with EAG and silymarin lowered CCl_4 elevated levels of MDA. 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 [25,26,27]. In our *in vivo* study treatment with CCl_4 reduced the levels of intracellular GSH with a reciprocal decrease in GPx activity which was significantly reversed by EAG.

Some authors[28,29] suggest that decrease in GSH, after administration of CCl_4 was most likely due to increased oxidative stress. Antioxidant protection in living tissues is performed by some enzymes such as CAT, SOD, GPx, GR, and GST[30]. Catalase and SOD play a major role as antioxidant enzymes in the mammalian system, by detoxifying hydrogen peroxide generated by various oxidases and other enzymatic and non-enzymatic auto - oxidation of compounds[31]. Glutathione peroxidase counteracts the free radicals using GSH as a substrate. As a result, GSH is oxidized to GSSG (oxidized glutathione), which in turn is reduced to GSH by glutathione reductase at the expense of NADPH, forming a redox cycle. The decrease in the activity of these enzymes is associated with accumulation of highly reactive free radicals that provoke such effects as loss of integrity and function of cell membranes [32].

There is a suggestion [33] that this reduced enzyme activity may be due to their oxidative inactivation by excessive formation of reactive oxygen species and their inhibition of gene expression. Our results showed that CCl_4 significantly reduced the activities of investigating enzymes, whereas EAG restored their activity (Table 1) and the results are comparable with the results obtained in animals pretreated with silymarin. Phytochemical analysis of EAG revealed the presence of eight known flavonoids quercetin, isoquercitrin, avicularin, hyperoside, kaempferol, isorhamnerin, isorhamnetin-3-O-glucoside and isorhamnetin-3-O-arabinoside. Many data about antioxidant effects of these phenolic compounds can be found in the literature. The increase in the activity of antioxidant enzymes and SOD from EAG was most likely due to the ability of flavonoids, available in this plant, to captures free radicals (including superoxide) [34]. 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 [35]. From the experiments and results obtained, it was suggested that EAG might reduce the formation of free oxygen species and protect antioxidant defense system in rats by increasing the activity of antioxidant enzymes and by increasing the amount of endogenous antioxidant GSH.

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