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Astragalus glycyphyllos and *Astragalus glycyphylloides* Derived Polysaccharides Possessing *in vitro* Antioxidant Properties

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Authors' contributions

This work was carried out in collaboration between all authors. Authors AS, PZ and IK collected the plant material, isolated and provided the investigated polysaccharide complexes. Authors MKB, VV, RS and VT designed the research. Authors MKB and RS performed the experiments. Authors MKB, VV, RS and VT analyzed the data. Authors MKB and VV wrote the paper. All authors read and approved the final manuscript.

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ABSTRACT

Aim: To evaluate and compare the effects of polysaccharides, isolated from n-butanol extracts of *Astragalus glycyphyllos* (PS1) and *Astragalus glycyphylloides* (PS2) in model of non-enzyme- and enzyme-induced lipid peroxidation (LPO) in isolated rat liver microsomes.
Place and Duration of Study: Department of Pharmacognosy and Laboratory of Drug Metabolism and Drug Toxicity, Department of Pharmacology, Pharmacotherapy and Toxicology, between

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Methods: In non enzyme-induced LPO, the microsomes were incubated with a solution of iron sulphate and ascorbinic acid (Fe^{2+}/AA). The enzyme-induced lipid peroxidation was performed by incubating rat liver microsomes with carbon tetrachloride (CCl_4) in the presence of NADPH. The effects of PS1 and PS2 were evaluated after 20 min of incubation at the following concentrations: 60 µg/mL; 6 µg/mL; 0.6 µg/mL. The production of malondialdehyde (MDA), a biomarker of lipid peroxidation was measured. Silymarin (60-0.6 µg/mL) was used as a positive control.
Results: The results of our study showed that in non-enzyme induced LPO model, both PS1 and PS2 exerted comparable, concentration-dependent antioxidant activity. At the highest concentration, which was the most potent as well, the formation of MDA was significantly decreased by 45% ($P<0.05$) by PS1 and by 40% ($P<0.05$) by PS2. In enzyme-induced LPO model, the PS1 showed slightly more potent antioxidant activity at the highest tested concentration, discerned by MDA decrease by 35% ($P<0.05$), in comparison to a decrease of 29% ($P<0.05$) by PS2 at the same concentration level. The antioxidant activity of both polysaccharides, in the both LPO models, however, was lower in comparison to silymarin which at 60 µg/mL decreased the MDA production by 53-55% ($P<0.05$) in both models.
Conclusion: On the basis of our results we conclude that the investigated two polysaccharide mixtures, PS1 and PS2 possess antioxidant properties in *in vitro* models of Fe^{2+}/AA and CCl_4/NADPH lipid peroxidation, induced in isolated liver microsomes.

Keywords: Astragalus polysaccharides; oxidative stress; lipid peroxidation; microsomes; malondialdehyde.

1. INTRODUCTION

The liver, as an organ actively involved in compounds' metabolism, is a frequent target of a number of toxicants, some of which cause damage through inducing lipid peroxidation and decreasing activity of the antioxidant self-defense system of hepatocytes.

Oxidative stress and related lipid peroxidation are regarded as one of the main biological mechanisms responsible for the structural and functional alterations observed in different levels (subcellular, cellular, tissue, organ) leading to development of different liver toxicity phenotypes.

The reactive oxygen species (ROS): superoxide anion ($O_2^{-\bullet}$), perhydroxyl radical ($HO_2^{\bullet}$), hydroxyl radical ($^{\bullet}OH$) etc., generated during the lipid peroxidation process as by-products of cellular metabolism, primarily in the mitochondria [1] are thought to be the major ones responsible for the observed pathophysiological changes. The free radicals themselves are considered to exert some very local effects due to their short lifespan [2]. However, the breakdown products of the lipid peroxides which possess prolonged half-life and the ability to diffuse from their site of formation may serve as "oxidative stress second messengers" [3]. Those breakdown products, mostly aldehydes: malondialdehyde, hexanal, 4-hydroxynonenal are used as biomarkers of lipid peroxidation [4].

Since it has been confirmed that oxidative stress and lipid peroxidation are involved in various serious pathological states including steatosis, hepatic necrosis and fibrosis [5], hypertension and diabetes [6], inflammation [7], atherosclerosis, neurodegenerative diseases, and cancer [8], a variety of supplements containing biologically active compounds or herbal extracts have got a lot of attention. A number of plants, including those of the genus *Astragalus*, have been shown to possess a number of pharmacological properties varied from immunostimulant effects, anti-bacterial and antiviral properties to hepatoprotective, anti-inflammatory activity, cardiovascular tonic effects etc. [9-11]. It has also been proved that *Astragalus membrenaceus* exerts a powerful antioxidant potential and one of the identified mechanisms is by increasing the superoxide dismutase activity [12]. The antioxidant properties of the *Astragalus* spp. are mainly attributed to the saponins as one of the main constituents [13,14]. In our previous work we have investigated the hepatoprotective and antioxidant properties of purified saponins' mixture (PSM), isolated from *Astragalus corniculatus* Bieb., using different *in vitro* models of liver injury and oxidative stress. PSM proved to possess a hepatoprotective effect against chemical injury, induced by CCl_4 and tert-butylhydroperoxide (t-BuOOH), assessed in isolated rat hepatocytes [15] as well as an antioxidant activity in a model of non-enzyme and enzyme-induced lipid

peroxidation, in liver micrososmes from normotensive and spontaneously hypertensive rats (SHR) [16].

Along with saponins, the other main constituents in *Astragalus* spp. are polysaccharides, flavonoids, amino acids, and trace elements [17]. One of the most studied *Astragalus polysaccharides* (APS) are those isolated from *Astragalus membranaceus.* They have been investigated especially with respect to their immunopotentiating properties [18], their ability to counteract the side effects of chemotherapeutic drugs, and their anticancer properties [19]. Furthermore Li et al. [20] proved the mitochondrial protection potential and anti-aging activity of APS. In an *in vitro* model of mitochondrial induced lipid peroxidation, the authors showed that APS can protect mitochondria by scavenging ROS, inhibiting LPO and mitochondrial swelling, and increasing the activities of antioxidant enzymes. The APS were identified as heteropolysaccharides, most of them being (1→4)-*α*-glucans with *α*-(1→6) linked branches comprising different monosaccharides [21]. These polysaccharides could be isolated from other *Astragalus* species, incl. *A. glycyphylloides* and *A. glycyphyllos*.

Astragalus glycyphyllos (Liquorice milk vetch, Wild liquorice) and *Astragalus glycyphylloides* DC (Fabaceae) are perennial, herbaceous flowering plants, native to Europe and widely distributed in Bulgarian flora [9]. A defatted total extract of *A. glycyphyllos* showed hepatoprotective and antioxidant activity against CCl_4-induced liver injury in male Wistar rats, discerned by decreasing the MDA production and bringing back to the control level the GSH quantity and the glutathione reductase (GR), glutathione peroxidase (GPx) and glutathione-S-transferase (GST) activities [22]. In another study, the hepatic damage induced by CCl_4 in isolated rat hepatocytes was ameliorated by an ethanolic extract from *Astrgalus glycyphylloides* [23].

The aim of this study was to investigate and compare the effects of two polysaccharide fractions, isolated from n-BuOH extracts of overground parts of *Astragalus glycyphyllos* (PS1) and *Astragalus glycyphylloides* (PS2) in models of non-enzyme- and enzyme-induced lipid peroxidation (LPO) in isolated rat liver microsomes and to compare them with the effect of silymarin.

2. MATERIALS AND METHODS

2.1 Plant Material and Isolation of Polysaccharides

The overground parts of *Astragalus glycyphyllos* L. (Fabaceae) were collected from Vitosha Mountain, Bulgaria in August 2014. *Astragalus glycyphylloides* DC. (Fabaceae) aerial parts were collected in July 2012 in Rila Mountain, Bulgaria. The species were identified by Dr D. Pavlova from Faculty of Biology, Sofia University, Bulgaria, where voucher specimens were deposited (SO-107613, *A. glycyphyllos* and SO-093817, *A. glycyphylloides*).

The air-dried plant material of both species (400 g of *A. glycyphyllos* and 300 g of *A. glycyphylloides*) was powdered in laboratory mill and exhaustively extracted with 80% methanol *via* percolation. The resulting percolates were evaporated *in vacuo* in order to eliminate the solvent. The dry residues were suspended in water and successively extracted with dichloromethane, ethylacetate and n-butanol. Then the n-butanol extracts (10.2 g of *A. glycyphyllos* and 12.3 g of *A. glycyphylloides*) were dried and dissolved in water. The water solutions were mixed with ice-cold absolute ethanol in proportion 1:4 and stirred vigorously. The resulting suspensions were centrifuged at 5000 rpm for 30 min. The supernatant was discarded and the precipitates were washed several times with ice-cold ethanol, again centrifuged and finally dried in a desiccator over anhydrous $CaCl_2$. The presence of polysaccharides in the resulting dried amorphous powders was proved by Molisch's test [24]. The polysaccharide content was expressed as percentage (%) of the mass of the corresponding dry n-butanol extract from which the polysaccharide was obtained. It was established to be 19.64% w/w for the n-BuOH extract from *A. glycyphylloides* and 12.31% w/w for the n-BuOH extract of *A. glycyphyllos.* The method used for obtaining the polysaccharide fractions completely eliminates the possibility other high-molecular weight compounds (proteins, etc.) to be isolated alongside [21].

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℃ ± 2℃ 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-day acclimatization was allowed before the commencement of the study and their health was monitored regularly by a certified veterinary physician. All performed procedures were approved by the Institutional Animal Care Committee (within the frame of the scientific project 3D/2015) and the principles stated in the European Convention for the Protection of Vertebrate Animals used for Experimental and other Scientific Purposes (ETS 123) [25] were strictly followed throughout the experiment.

2.3 Chemicals and Reagents

All reagents used were of analytical grade. Silymarin, as well as the other chemicals: 2-thiobarbituric acid, trichloroacetic acid, nicotineamideadenine dinucleotidephosphate (reduced) (NADPH), ascorbic acid and bovine serum albumine (fraction V), were purchased from Sigma Chemical Co. (Germany, Darmstadt). Carbon tetrachloride (CCl_4), 2,2`-dinitro-5,5`-dithiodibenzoic acid (DTNB) were obtained from Merck (Germany, Darmstadt).

2.4 Preparation of Liver Microsomes

Liver microsomes were isolated from untreated rats, as described by [26]. Briefly, the livers of the animals, sacrificed by cervical decapitation were removed after perfusion with ice-cold KCl 1.15% and then homogenized with ice-cold 0.1 M Tris potassium buffer, pH 7.5 (1:4), using a glass homogenizer with a Teflons pestle. The prepared liver homogenate was centrifuged at 10000 x g for 20 min to obtain the postmitochondrial fraction which was then centrifuged at 105000 x g for 60 min to obtain microsomal fraction. The microsomal pellets were resuspended in 0.1M potassium phosphate buffer, pH 7.4, containing 20% glycerol. The content of microsomal protein was determined by the method of Lowry [27] using bovine serum albumin as a standard and adjusted to 1 mg protein/mL.

2.5 NADPH-dependent CCl_4-induced Lipid Peroxidation *in vitro* (CCl_4/NADPH)

The microsomes were preincubated with three concentrations (60 µg/ml; 6 µg/mL; 0.6 µg/mL) of the investigated polysaccharides of *A. glycyphyllos* (PS1) and *A. glycyphylloides* (PS2) and silymarin, used as a positive control [28]. The pre-incubation was performed at 37°C for 15 min. At the minute 15, the enzyme-induced LPO was started with 20 mM CCl_4 in the presence of 1 mM NADPH [29]. The reaction was stopped with mixture of trichloroacetic acid (TCA) 25% and thiobarbituric acid (TBA) 0.67% 20 minutes after initiating the LPO reaction and MDA quantity was assessed.

2.6 Ascorbat-iron Induced Lipid Peroxidation (Fe^{2+}/AA)

The microsomes were preincubated with PS1 and PS2 (60 µg/mL; 6 µg/mL; 0.6 µg/mL) and silymarin, at the same concentrations, at 37°C for 15 min. The reaction was started with a solution of iron sulphate 20 mM and ascorbic acid 0.5 mM [30]. The reaction was stopped with mixture of TCA 25% and TBA 0.67% at 20 min after LPO initiation and MDA quantity was assessed.

2.7 MDA Assay

Lipid peroxidation was determined by measuring the rate of production of thiobarbituric acid reactive substances (TBARS) (expressed as malondialdehyde (MDA) equivalents, as described by [31]. The absorbance was measured at 535 nm using a Spectro UV-VIS Split spectrophotometer. MDA concentration was calculated using a molar extinction coefficient of $1.56 \times 10^5 M^{-1}$ cm^{-1} and expressed in nmol/mg protein.

2.8 Statistical Analysis

Statistical program “MEDCALC” was used for analysis of the data. The results are expressed as mean ± SEM of four animals per group. Three parallel samples for each group were measured. The significance of the data was assessed using the non-parametric Mann-Whitney *U* test. Values of $P \leq 0.05$ were considered statistically significant.

3. RESULTS

3.1 Ascorbat-iron Induced Lipid Peroxidation (Fe^{2+}/AA)

The effect of PS1 and PS2 on MDA quantity assessed in Fe^{2+}/AA LPO model is shown in Table 1. Microsomal incubation with Fe^{2+}/AA mixture led to nearly 2-fold increase in MDA

level. The addition of the polysaccharides in three consequently decreasing concentrations (60, 6, 0.6 μg/mL) in the reaction mixture prior to initiation of LPO, reduced the formation of MDA in a concentration-dependent manner. The most prominent effect was observed at the highest tested concentration. Compared to the Fe^{2+}/AA group, the MDA production measured at 60 μg/mL, was reduced by 45% (P<0.05) after pre-incubation with PS1 and by 40% (P<0.05) after pre-incubation with PS2. Compared with each other, the potency of the effect of both polysaccharide fractions was similar, however, compared to silymarin their effect appeared to be less potent.

Table 1. Effect of PS1 and PS2 on MDA measured in Fe^{2+}/AA model

Group	MDA nmol/mg
Control	0.69 ± 0.08
Fe^{2+}/AA	1.95 ± 0.32[a]
60 μg/mL PS1 + Fe^{2+}/AA	1.07 ± 0.2[a,b]
6 μg/mL PS1 + Fe^{2+}/AA	1.31 ± 0.1[a,b]
0.6 μg/mL PS1 + Fe^{2+}/AA	1.35 ± 0.2[a,b]
60 μg/mL PS2 + Fe^{2+}/AA	1.16 ± 0.3[a,b]
6 μg/mL PS2 + Fe^{2+}/AA	1.37 ± 0.2[a,b]
0.6 μg/mL PS2 + Fe^{2+}/AA	1.52 ± 0.3[a,b]
60 μg/mL SM + Fe^{2+}/AA	0.88 ± 0.03[a,b]
6 μg/mL SM + Fe^{2+}/AA	1.03 ± 0.08[a,b]
0.6 μg/mL SM + Fe^{2+}/AA	1.19 ± 0.09[a,b]

Data are expressed as mean ± SEM of four different experiments. [a]Significant difference from control (Mann–Whitney U test, P<0.05); [b] Significant difference from Fe^{2+}/AA group (Mann–Whitney U test, P<0.05); Polysaccharides from A. glycyphyllos (PS1); Polysaccharides from A. glycyphylloides (PS2); Silymarin (SM)

3.2 NADPH-dependent CCl_4-induced Lipid Peroxidation *in vitro* (CCl_4/NADPH)

In Table 2 the effect of PS1 and PS2 on MDA quantity assessed in CCl_4/NADPH LPO model is shown. Incubation of the microsomes with CCl_4/NADPH mixture resulted in significant increase in MDA quantity by 41% (P<0.05). The pre-incubation with PS1 and PS2 (60, 6, 0.6 μg/mL) again showed concentration-dependent antioxidant activity. In this model, however, the effect of PS1 was slightly more potent than the effect of PS2. At 60 μg/mL MDA production was reduced by 35% (P<0.05) by PS1 and by 29% (P<0.05) by PS2. The effects were compared to the CCl_4/NADPH group. In comparison to the both polysaccharide fractions, silymarin showed stronger antioxidant activity at the highest concentration: MDA production was decreased by 55% (P<0.05), compared to CCl_4/NADH group. In the mid and in the lowest concentrations, however, the effect of both PS1 and PS2 were commensurable with the effect of silymarin.

Table 2. Effect of PS1 and PS2 in CCl_4/NADPH model

Group	MDA nmol/mg
Control	0.69 ± 0.05
CCl_4/NADPH	0.97 ± 0.99
60 μg/mL PS1 + CCl_4/NADPH	0.67 ± 0.09[b]
6 μg/mL PS1 + CCl_4/NADPH	0.70 ± 0.06[b]
0.6 μg/mL PS1 + CCl_4/NADPH	0.78 ± 0.03[a,b]
60 μg/mL PS2 + CCl_4/NADPH	0.68 ± 0.08[b]
6 μg/mL PS2 + CCl_4/NADPH	0.74 ± 0.08[b]
0.6 μg/mL PS2 + CCl_4/NADPH	0.78 ± 0.04[a,b]
60 μg/mL SM + CCl_4/NADPH	0.45 ± 0.05[a,b]
6 μg/mL SM + CCl_4/NADPH	0.68 ± 0.04[a,b]
0.6 μg/mL SM + CCl_4/NADPH	0.75 ± 0.07[b]

Data are expressed as mean ± SEM of four different experiments. [a]Significant difference from control (Mann–Whitney U test, P<0.05); [b] Significant difference from CCl_4/NADPH group (Mann–Whitney U test, P<0.05); Polysaccharides from A. glycyphyllos (PS1); Polysaccharides from A. glycyphylloides (PS2); Silymarin (SM)

4. DISCUSSION

Oxidative breakdown of biological phospholipids, a result of lipid peroxidation process, occurs in most cellular membranes including mitochondria, microsomes, peroxisomes and plasma membrane [32]. The toxicity of lipid peroxidation products in mammals generally involves neurotoxicity, hepatotoxicity and nephrotoxicity [33].

Reactive oxygen species (ROS) are primarily produced in the mitochondria and in the endoplasmic reticulum of hepatocytes *via* the cytochrome P450 enzymes [34] which makes the liver one of the main target organs subjective to oxidative stress. Chronic liver diseases are nearly always characterized by increased oxidative stress, regardless of the cause of the liver disorder.

Natural products that are found in vegetables, fruits, plant extracts, herbs, insects, and animals, have been traditionally used for treating liver diseases [35]. Many of those products have been clinically tested and proved as potent

hepatoprotective agents and are used against commonly occurring liver diseases. One of these products is silymarin which is considered as reference drug. It is the main constituent of *Silybum marianum* (milk thistle). Chemically, silymarin is a mixture of flavonolignans that consists mainly of silibin, silydianin and silychristin and has been widely used as a therapeutic agent for a variety of acute and chronic liver diseases [36]. Along with silymarin, however, identifying new sources of compounds with potent antioxidant and hepatoprotective activity is considered to be of great importance for the treatment of drug-induced liver injuries. A number of plants, including those of the genus *Astragalus*, have been shown to possess hepatoprotective properties exerted by ROS scavenging and/or by improving the antioxidant status of the cells [16,37,38]. Flavonoids and saponins are one of the main constituents in *Astragalus* spp. considered to be instrumental for the antioxidant properties of representatives of this genus [13,14]. Along with them, the polysaccharides which are also found in different representatives of *Astragalus spp*. are also shown to possess a variety of positive effects including immunopotentiating, anti-aging and antioxidant properties [19,20]. The most studied *Astragalus polysaccharides* (APS) are those isolated from *A. membranaceus.* However, there is little information about the biological properties of polysaccharides isolated from other *Astragalus* spp. and no data at all on Bulgarian species.

Therefore the objectives of this study was to evaluate and compare the antioxidant properties of two polysaccharides, isolated from *A. glycyphyllos* (PS1) and *A. glycyphylloides* (PS2), using *in vitro* models of enzyme and non-enzyme lipid peroxidation, induced in rat liver microsomes.

The extent of lipid peroxidation was measured by thiobarbituric acid reactive substances (TBARS), expressed as MDA equivalents. Our results showed that the preincubation of the reaction mixture with PS1 and PS2 resulted in lower MDA formation, both in Fe^{2+}/AA and CCl_4/NADPH lipid peroxidation models. The effect was concentration-dependent and most pronounced at the highest concentration level (see Tables 1 and 2). It is worth noting that compared with each other, the potency of the effect of both polysaccharides was similar in the model of non-enzyme induced LPO. In the model of enzyme-induced LPO, however, the effect of PS1 measured at the highest tested concentration (60 μg/mL) was slightly more potent than the effect of PS2 at the same concentration (see Table 2). In addition, again at the highest concentration, the effect of both polysaccharide fractions, compared to silymarin appeared to be less potent but in the mid and in the lowest concentrations (6 and 0.6 μg/mL) the antioxidant effect was commensurable with those of silymarin.

The results of our study are in good correlation with the available literature information related to the *in vitro* and *in vivo* antioxidant properties of polysaccharides isolated from plants, bacteria and fungi [39]. The *in vivo* antioxidant activity of the plant polysaccharides appears to be due to an increase of the endogenous antioxidant enzymes such as superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPx), reduction of MDA levels and stabilization the cell membranes [40-43].

The main mechanism underlining the antioxidant activity of polysaccharides *in vitro* appears to be attributed to their free radical-scavenging activity. The polysaccharides present in Guarana powder [44] and the Litchi polysaccharides isolated from the pulp tissue of *Litchi chinensis* Sonn. [45], exhibited a strong capacity for scavenging 1,1-diphenyl-2-picryl-hydrazyl (DPPH) radical.

5. CONCLUSION

Taking together all the available information and on the basis of our results we conclude that the investigated two polysaccharides, isolated from n-BuOH extracts of *A. glycyphyllos* (PS1) and *A. glycyphylloides* (PS2) possess antioxidant properties in *in vitro* models of Fe^{2+}/AA and CCl_4/NADPH lipid peroxidation, induced in isolated liver microsomes. The antioxidant activity of PS1 and PS2, discerned by a significant decrease in MDA production induced by the pro-oxidant mixtures, was concentration-dependent and similar to those of silymarin. The possible antioxidant mechanism of the evaluated polysaccharide fractions might be due to free radical scavenging properties resulting in membrane protection from oxygen radical-mediated damage. It should to be noticed, however, that due to the relatively limited information on their *in vitro* antioxidant activity, further studies are required in order to clarify the exact antioxidant mechanisms of the polysaccharides at the molecular and higher level.

CONSENT

It is not applicable.

ETHICAL APPROVAL

All performed procedures were approved by the Institutional Animal Care Committee (KENIMUS) within the frame of the scientific project 3-D/2015. 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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