American Journal of Plant Sciences, 2015, 6, 799-803
Published Online April 2015 in SciRes. http://www.scirp.org/journal/ajps
http://dx.doi.org/10.4236/ajps.2015.66085

Scientific Research Publishing

Protective Effects of Saponin Mixture, Isolated from *Astragalus monspessulanus* subsp. *monspessulanus* on Tert-Butyl Hydroperoxide—Induced Oxidative Stress in Isolated Rat Hepatocytes

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Received 21 January 2015; accepted 29 March 2015; published 31 March 2015

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Abstract

Saponin mixture, obtained from *Astragalus monspessulanus* subsp. *monspessulanus* (Fabaceae) was investigated for possible protective effect on *tert*-butyl hydroperoxide (t-BuOOH)-induced cytotoxicity using primary isolated rat hepatocytes. The cells were isolated by two-stepped collagenase perfusion. Liver damage was induced by one hour incubation with t-BuOOH (75 $\mu mol \cdot L^{-1}$) and discerned by decreased cell viability, increased lactate dehydrogenase (LDH) leakage into the medium, increased production of malondialdehyde (MDA) and depletion of the cell protector glutathione (GSH). Cell pre-incubation with the saponin mixture (1 mg/mL and 5 mg/mL) significantly ($p < 0.05$) ameliorated t-BuOOH-induced liver damage, judged by preserved cell viability, decreased activity of LDH, decreased MDA production and restoration of GSH. The effect was concentration-dependent, more pronounced in the highest concentration and comparable with those of silymarin, used as a positive control. The observed cytoprotective effect could be explained by the influence of the saponins on the mitochondrial function, disturbed by t-BuOOH toxic metabolites.

Keywords

***Astragalus monspessulanus*, Saponins, Hepatocytes, Antioxidant Activity, Cytoprotection**

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How to cite this paper: Kondeva-Burdina, M.S., *et al.* (2015) Protective Effects of Saponin Mixture, Isolated from *Astragalus monspessulanus* subsp. *monspessulanus* on Tert-Butyl Hydroperoxide—Induced Oxidative Stress in Isolated Rat Hepatocytes. *American Journal of Plant Sciences*, **6**, 799-803. http://dx.doi.org/10.4236/ajps.2015.66085

1. Introduction

Liver is one of the organs that are highly exposed to many potentially toxic substances due to its unique metabolism and relationship to the gastrointestinal tract. This makes the liver an important target of the toxicity of drugs, xenobiotics, and oxidative stress. Tert-butyl hydroperoxide (t-BuOH) is a toxic agent causing necrosis through inducing mitochondrial reactive oxygen formation [1]. As a prooxidant, t-BuOH was widely used in experimental toxicology as a model of liver damage. There is a large body of scientific evidence showing its effects on changes in calcium homeostasis [2], on elevation of lipid peroxidation and decrease of mitochondrial membrane potential [3] [4].

Astragalus L. (Fabaceae) is a genus distributed in Europe, Asia and North America. The pharmacological properties of *Astragalus* spp. are varied and include immunostimulant effects, anti-bacterial, antiviral properties, hepatoprotective and anti-inflammatory activity [5] [6]. These effects appear to be due mainly to the saponins in the herb plants [7] [8].

On the basis of these data, the aim of the current study was to investigate the possible hepatoprotective potential of the saponin mixture (SM) obtained from *Astragalus monspessulanus* subsp. *monspessulanus* against t-BuOH-induced liver toxicity in isolated rat hepatocytes.

2. Materials and Methods

2.1. Collection of Plant Material and Preparation of Saponins' Mixture

The overground parts of the *Astragalus monspessulanus* subsp. *monspessulanus* were collected from Rodopi Mountain, Bulgaria in May 2010. The plant was identified by Dr D. Pavlova from Faculty of Biology, Sofia University, Bulgaria where the voucher specimen has been deposited (N SO 107533). The procedure for obtaining of purified saponin fraction from the plant material was described previously [9].

2.2. Chemicals

All the reagents used were of analytical grade. Tert-butyl hydroperoxide, silymarin as well as collagenase, 1-chloro-2,4-dinitrobenzene, beta-Nicotinamide adenine dinucleotide 2-phosphate reduced tetrasodium salt (NADPH), ethylenediaminetetraacetic acid (EDTA), bovine serumalbumin (fraction V), 2,2-dinitro-5,5 dithiodibenzoic acid (DTNB) obtained from MERCK (Darmstadt, Germany); reduced glutathione (GSH), 2-thiobarbituric acid (4,6-dihydroxypyrimidine-2-thiol; TBA), trichloroacetic acid (TCA), pentobarbital sodium were purchased from Sigma Chemical Co. (Taufkirchen, Germany).

2.3. Animals

Male Wistar rats (body weight 200 - 250 g) were used. Animals were purchased from the National Breeding Center, Sofia, Bulgaria. At least 7 days of acclimatization was allowed before the commencement of the study 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. The health was monitored regularly by a veterinary physician. All performed procedures were approved by the Institutional Animal Care Committee and 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 experiments [10].

2.4. Isolation and Incubation of Hepatocytes

Rats were anesthetized with sodium pentobarbital (0.2 ml/100g). An optimized i*n situ* liver perfusion using less reagents and shorter time of cell isolation was performed [11]. The method resulted in higher amount of live and metabolically active hepatocytes. Cells were counted under the microscope (×100) and cell viability was assessed by Trypan blue exclusion (0.05%). Initial viability averaged 89%.

Liver damage was induced by one hour incubation of the isolated hepatocytes with t-BuOH at a concentration of 75 $\mu mol \cdot L^{-1}$. In order to investigate the hepatoprotective activity of the saponin mixture, isolated hepatocytes were pre-incubated for 30 min with two concentrations of the mixture (1 mg/mL and 5 mg/mL) and then incubated with t-BuOH (75 $\mu mol \cdot L^{-1}$) for one hour. The effect of saponin mixture was compared to those of

silymarin (1 mg/mL and 5 mg/mL). The following parameters were measured to assess the functional status of hepatocytes: cell viability, lactate dehydrogenase (LDH) activity, reduced glutathione (GSH) levels and malondialdehyde (MDA) quantity. Cell viability was assessed by Trypan blue exclusion method [12]. The dye was used at a final concentration of 0.05% and cells were counted under light microscope (×100). At the end of incubation, the cells were recovered via centrifugation at 400 × g at 4˚C. The supernatant was used for LDH and MDA assessment as described by Bergmeyer *et al*. [13] and Fau *et al*. [12], respectively. GSH measurement following the method used by Fau *et al*. [12] was assessed in the sediment.

2.5. Statistical Analysis

Statistical analysis was "performed using statistical programme "MEDCALC". Results are expressed as mean ± SEM for 4 experiments. The significance of the data was assessed using the nonparametric Mann-Whitney *U* test. Value of $p \leq 0.05$ was considered statistically significant. Three parallel samples were used.

3. Results

Hepatocytes' incubation with 75 µM t-BuOOH resulted in statistically significant ($p < 0.05$) reduction of cell viability by 74%, depletion of cell GSH by 72%, LDH leakage into the medium was increased five times and MDA production—6 times, compared to the control. Results are shown in **Figure 1** and **Figure 2**. Pre-incubation of the hepatocytes with the saponin mixture partially prevented the t-BuOOH-induced liver injury in a concentration-dependent manner, discerned by increased cell viability and restored LDH activity, MDA and GSH levels. The results are compared to the t-BuOOH only group (**Figure 1** and **Figure 2**).

4. Discussion

In experimental toxicology, the *in vitro* systems play an important role for the investigation of xenobiotic biotransformation and reveal the possible mechanisms of toxic stress and its protection. In the current study, we used isolated rat hepatocytes as a suitable animal replacement model to assess a possible cytoprotective effect of saponins' mixture isolated from *Astragalus monspessulanus* agains t-BuOOH-induced liver injury. The toxic

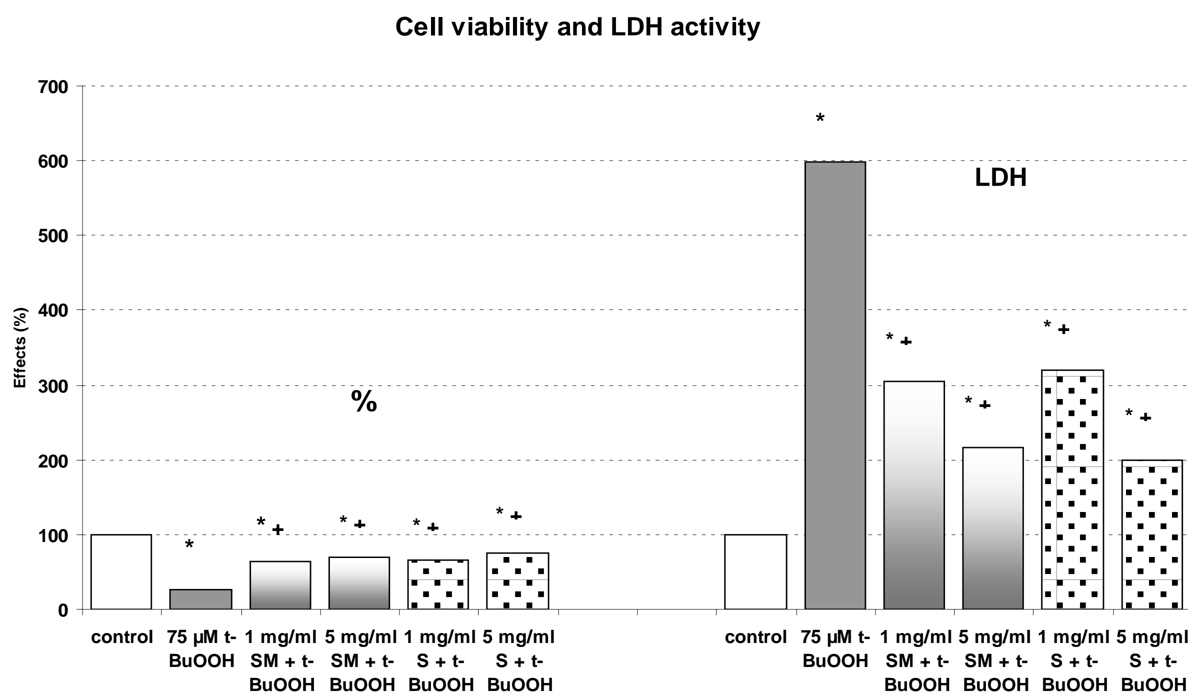


Figure 1. Effect of saponin mixture (SM) on cell viability and LDH activity on t-BuOH induced liver damage in isolated rat hepatocyets. Saponin mixture—SM; silymarin—S $^{*}p < 0.05$ vs control. $^{+}p < 0.05$ vs t-BuOH-treated group. Data are expressed as mean ± SEM of 4 different experiments (Mann-Whitney *U* test).

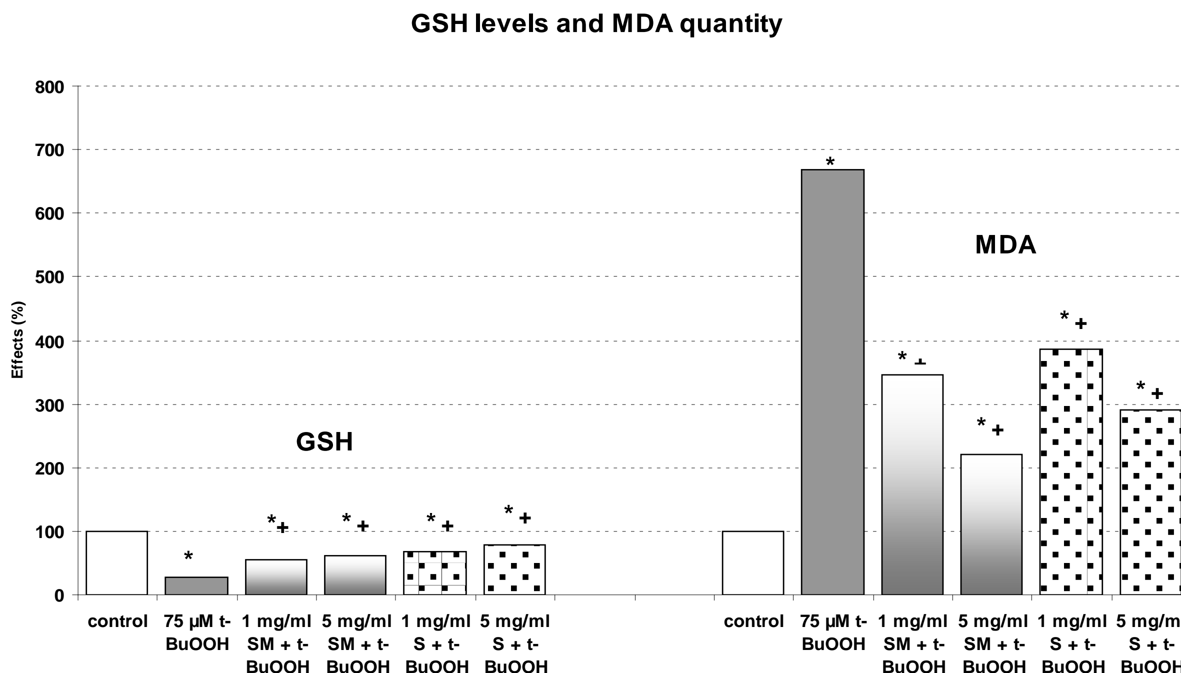
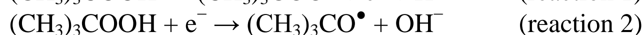


Figure 2. Effect of saponin mixture (SM) on GSH levels and MDA quantity on t-BuOH induced liver injury in isolated rat hepatocytes. Saponin mixture—SM; silymarin—S. $^{*}p < 0.05$ vs control. $^{+}p < 0.05$ vs t-BuOH-treated group. Data are expressed as mean ± SEM of 4 different experiments (Mann-Whitney *U* test).

mechanism of t-BuOH is due to its bioactivation to free radicals [14] [15]:

$(CH_3)_3COOH \rightarrow (CH_3)_3COO^{\bullet} + e^{-} + H^{+}$ (reaction 1)


$(CH_3)_3COOH + e^{-} \rightarrow (CH_3)_3CO^{\bullet} + OH^{-}$ (reaction 2)

$(CH_3)_3CO^{\bullet} \rightarrow (CH_3)_2CO + {}^{\bullet}CH_3$ (reaction 3)

In microsomal suspension, in the absence of NADPH, t-BuOH undergoes one-electron oxidation to a peroxyl radical (reaction 1), whereas in the presence of NADPH it undergoes one-electron reduction to an alkoxyl radical (reaction 2). In isolated mitochondria and intact cells, the t-BuOOH has been shown to undergo β-scission to the methyl radical (reaction 3). All these radicals cause lipid peroxidation process which explains its cytotoxic properties. t-BuOOH pro-oxidant activity was proven in our study by changes in the integrity of the hepatocytes, discerned by decreased cell viability and increased leakage of LDH into the medium, as well as by the detected increased production of MDA and decreased GSH levels.

Even though there is a wide range of drugs that are currently employed in the management of hepatic disorders, alternative approach based on the use of traditional herbal preparations and plant isolated biologically active substances has been widely implemented. A number of plants, including those of the genus *Astragalus*, have been shown to possess hepatoprotective properties by improving antioxidant status. In our study the saponin mixture, obtained from *Astragalus monspessulanus* ameliorated the liver injury induced by t-BuOH, judged by the preserved cell viability and the restored LDH activity, MDA quantity and GSH levels, in a concentration-dependent manner, as the effect was more pronounced at the highest concentration. The hepatoprotective effect of the saponins' mixture was comparable with those of silymarin. These results are in good correlation both with the literature data and our own studies. Saponins of genera Astragalus inhibit the formation of lipid peroxides in the liver [6]. The total saponins of *Astragalus membranaceus* can significantly inhibit the membrane lipid peroxidation generated by superoxide (O^{2-}), hydrogen peroxide (H_2O_2) and UV rays [7] [8]. In one of our previous studies we showed that purified saponin fraction, isolated from *Astragalus monspessulanus* had cytotoxic effect in HepG2 cell line observed at the highest concentration of 4 mg/ml [9]. In another study of ours we investigated and proved an antioxidant and hepatoprotective effects of purified saponin mixture from *Astragalus corniculatus* Bieb. in liver microsomes, isolated from spontaneously hypertensive rats and normotensive rats [16].

On the basis of our data we can conclude that under the conditions of this study, the saponin mixture obtained from *Astragalus monspessulanus* subsp. *monspessulanus* showed a hepatoprotective potential against t-BuOH-

induced liver damage in freshly isolated rat hepatocytes.

Acknowledgements

This work was supported by a Grant No. 25/2014 г. from Council of Medical Science at Medical University of Sofia.

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