Effect of Purified Saponin Mixture from *Astragalus corniculatus* on Toxicity Models in Isolated Rat Hepatocytes

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Abstract

A purified saponin mixture (PSM) isolated from *Astragalus corniculatus* Bieb. (Fabaceae) was investigated for its protective effect in two models of toxicity, carbon tetrachloride (CCl₄) and tert-butyl hydroperoxide (t-BuOOH), using isolated rat hepatocytes. CCl₄ undergoes dehalogenation in the liver endoplasmic reticulum. This process leads to trichlormethyl radical (CCl₃) formation, initiation of lipid peroxidation, and measurable toxic effects on the hepatocytes. Oxidative damage is widely recognized as being involved in the development of many pathological conditions. In our experiment, t-BuOOH was used as a model of oxidative stress. The hepatocytes were incubated with the PSM alone (0.01–100 µM) and along with CCl₄ (86 µM) and t-BuOOH (75 µM). As a sign of cytotoxicity, cell viability was used. CCl₄ and t-BuOOH significantly decreased hepatocyte viability. Our data indicate that PSM showed lower toxic effects compared to CCl₄ and t-BuOOH and in combination exerted statistically significant protection of cell viability against the toxic agents.

Keywords: Isolated hepatocytes, cell viability, carbon tetrachloride toxicity, tert-butyl hydroperoxide toxicity, *Astragalus corniculatus*, saponins.

Introduction

Chemical studies on *Astragalus* species reported the presence of triterpenoid saponins, which exhibited a wide range of biological properties, including immunostimulant, hepatoprotective, antiviral, cardiotonic and analgesic activities (Rios & Waterman, 1997; Verotta & El-Sebakhy, 2001). In previous publications the protective effect of a purified saponin mixture (PSM) from *Astragalus corniculatus* Bieb. (Fabaceae) against myeloid Graffi tumor in hamsters, expressed by decrease of the tumor transplantation, on tumor growth inhibition and mortality percentage reduction (Krasteva et al., 2004) was reported. The immunostimulating activity on the blood PMNs and pMos, of PSM, in Graffi-tumor bearing hamsters was also reported (Toshkova et al., 2007). Phytochemical investigations of PSM led to the isolation of three new oleanane-type triterpene saponins (Krasteva et al., 2006, 2007).

CCl₄ is widely used as a model of experimental toxicity in rats. Its toxic effects that led to liver injury are mainly due to a cytochrome P450-dependent biotransformation of CCl₄ to free radicals. They initiate the process of lipid peroxidation, which is often the cause of inhibition of enzyme activity (Ferreira et al., 2003). The experimental toxicity induced by CCl₄ is widely used as a model of liver injury in rats.

The cellular system of energy supply localized in mitochondria is another target of many hepatotoxic substances causing oxidative stress and is one of the most important mechanisms through which hepatotoxic factors induced apoptotic and necrotic processes (Kroemer et al., 1998). Tert-Butyl hydroperoxide (t-BuOOH) is a chemical with pro-oxidant activity. It has been used as a model of toxicity on which metabolism of different compounds have been elucidated.

Based on the information available, the objective of the following study was to investigate the possible protective effect of a purified saponin mixture, extracted from *Astragalus corniculatus*, using models of CCl₄- and t-BuOOH-induced toxicity in isolated rat hepatocytes.

Materials and Methods

Chemicals

Thin-layer chromatographic study was carried out on Kieselgel 60 F₂₅₄ (0.24 mm thick, Merck, Germany) plates.
The spots were visualized by spraying with anisaldehyde/conc H2SO4, followed by heating at 110°C. Column chromatography was carried out with Sephadex LH-20 (Pharmacia, Sweden) and silica gel 60 (70–230 mesh, Merck, Germany).

In our experiments, pentobarbital sodium (Sanofi, France); HEPES (Sigma Aldrich, Germany); NaCl, KCl, d-glucose, NaHCO3 and CaCl2·2H2O (Merck, Germany); KH2PO4 (Scharlau Chemie Sa, Spain); MgSO4·7H2O (Fluka AG, Germany); collagenase from Clostridium histolyticum type IV (Sigma Aldrich); EGTA (Sigma Aldrich); carbon tetrachloride (Merck, Germany); tert-butyl hydroperoxide (Sigma, Germany) and Trypan blue (Merck, Germany) were used.

Plant material

Astragalus corniculatus was collected in July 1999 in Northern 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 (SO95265).

Preparation of PSM

The air-dried powdered aerial parts of Astragalus corniculatus were extracted with 50% EtOH. The extract was filtered, concentrated and successively treated with CHCl3 and EtOAc, respectively. The aqueous residue was dissolved in MeOH and precipitated in Me2CO yielding crude saponin mixture. Part of this was purified by column chromatography on silica gel and Sephadex LH-20 to afford several fractions, one of which containing mixture of three saponins (1, 2 and 3). This fraction was used for the pharmacological study.

The saponins were further isolated from the fraction by preparative thin-layer chromatography (TLC) on silica gel and identified as new oleanane-type triterpene saponins: 3β-O-[O-4-oxo-pentopyranosyl (1→2)-β-D-glucopyranosyl]-21α-hydroxy-olean-12-ene-28-oic acid; 21α-hydroxyolean-12-ene-28-oic acid 3β-4-oxo-pentopyranoside and 19α-hydroxy-olean-12-ene-28, 21β-olide 3-β-D-xylopyranoside (Figure 1). The structures of the isolated compounds were elucidated by chemical and spectral methods. Details of the isolating and identifying of the saponins have been published previously (Krasteva et al., 2006, 2007).

Animals

Male Wistar rats (body weight 200–250 g) were used. Rats were housed in plexiglass cages (3 per cage) in a 12/12 light/dark cycle, temperature 20 ± 2°C. Food and water were provided ad libitum. Animals were purchased from the National Breeding Center, Sofia, Bulgaria. All performed procedures were approved by the Institutional Animal Care Committee and were in accordance with European Union Guidelines for animal experimentation.

Isolation and incubation of hepatocytes

Rats were anesthetized with sodium pentobarbital (0.2 mL/100 g). In situ liver perfusion and cell isolation were performed as described by Fau et al. (1992), with modifications (Mitcheva et al., 2006). After portal catheterization, the liver was perfused with 100 mL HEPES buffer (pH = 7.85) + 0.6 mM EDTA, followed by 200 mL HEPES buffer (pH = 7.85), without any addition, and finally 200 mL HEPES buffer containing collagenase type IV (50 mg/200 mL) and 7 mM CaCl2 (pH = 7.85). The liver was excised, minced into small pieces and hepatocytes were dispersed in 60 mL Krebs-Ringer-bicarbonate (KRB) buffer (pH = 7.35) + 1% bovine serum albumin. After filtration, the hepatocytes were centrifuged and washed out with KRB buffer. Cells were counted under the microscope and cell viability was assessed by Trypan blue exclusion (0.05%) (Fau et al., 1992). Initial viability averaged 89%. Cells were diluted with KRB to make a suspension of about 3 × 10⁶ hepatocytes/mL. Incubations, performed in a 5% CO2 + 95% O2 atmosphere, were carried out in 25 mL Erlenmeyer flasks. Each flask contained 3 mL of the cell suspension (i.e., 9 × 10⁶ hepatocytes/mL). The cells were incubated in vitro with the toxic agents CCl4 (86 µM) and t-BuOOH (75 µM) alone and after pre-incubation with PSM (100–0.1 µM).

Statistical analysis

Statistical analysis was performed by applying Student’s t-test, with P < 0.05 considered statistically significant. All results (n = 4) are expressed as mean ± SD.

Results

In the first series of experiments hepatocytes were incubated with PSM in four decreasing concentrations (100–0.1 µM). The results are shown in Table 1. The values were compared with control hepatocytes. The data indicate that cell viability was decreased by PSM in the concentration-dependent manner. The most prominent effect was observed at concentration 100 µM – cell viability was decreased by 32% (p < 0.01).

The effects of the toxic agents CCl4 and t-BuOOH on cell viability are shown in Tables 2 and 3, respectively. CCl4 decreased cell viability by 61% (p < 0.01). After cell incubation with t-BuOOH viability was reduced by 77% (p < 0.01). Results were compared to the control hepatocytes.
The effect of PSM in CCl₄ and t-BuOOH models of toxicity is shown in Tables 2 and 3, respectively. The values were compared to hepatocytes incubated with the toxic agents. PSM statistically significantly reduced the damage caused by the hepatotoxins and preserved cell viability in a concentration dependent manner. The most visible was the effect of PSM at the highest concentration 100 µM.

**Discussion**

Isolated hepatocytes provide the opportunity to evaluate the effects by direct interactions of the studied compounds with endogenous factors. Hepatocytes are a convenient *in vitro* model to investigate xenobiotic biotransformation and to elucidate possible mechanisms of toxic stress and its protection. Isolated liver cells are used as a suitable model for evaluation of the cytoprotective effects of some prospective biologically active compounds, both newly synthesized and plant isolated.

It has been recognized for some time the protective and antioxidant effects of different *Astragalus* species. Zhang et al. (1992a) have reported the antioxidant effect of *Astragalus membranaceus* extract and its component cycloastragenol-xyllosyl-glucoside on lipid peroxidation, *in vivo/in vitro*. In their study, Hong et al. (1994) discussed
in vitro antioxidant effect of water extract from Astragalus on rat heart mitochondria. Rate et al. (1998) carried out randomized study including 40 adult patients with chronic hepatitis C being treated with Astragalus mixture for 6 months. A significant improvement of liver enzymes was observed. There are data in the literature about 43 patients with myocardial infarction treated with Astragalus (Rios & Waterman, 1997). The treatment led to reduction of the both chemical agents (CCl4 and t-BuOOH), we could discuss a possible influence of PSM on the metabolism, taking place in hepatocytes. Regarding the toxic mechanisms of the both chemical agents (CCl4 and t-BuOOH), we could suggest that Astragalus corniculatus interferes on the level of cytochrome P-450 system, related to the metabolism of CCl4 and on the mitochondrial level, related to the t-BuOOH biotransformation.

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References


