Effects of diosgenin, isolated from *Asparagus officinalis*... PHARMACIA, vol. 61, No. 4/2014

**EFFECTS OF DIOSGENIN, ISOLATED FROM *ASPARAGUS OFFICINALIS*, ON ISOLATED HEPATOCYTES FROM RATS, TREATED WITH PHENOBARBITAL**

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**Abstract.** Diosgenin, isolated from *Asparagus officinalis*, was investigated for its possible protective effects on carbon tetrachloride (86µM)-induced toxicity in hepatocytes, isolated from rats treated with phenobarbital. Hepatocytes were isolated by two-stepped collagenase perfusion. The effects were compared with those of silymarin. Cell incubation with carbon tetrachloride (CCL₄) led to a significant decrease in cell viability, increased LDH leakage, decrease levels of cellular glutathione and evaluation in MDA quantity. Following the induction with Phenobarbital, carbon tetrachloride, administered alone, increased its toxic effects. Pre-incubation with diosgenin (0,01 µM, 0,1 µM, 10 µM and 100 µM) significantly ameliorated, the exam parameters in the model of CCL₄-induced hepatocytes damage. The effects of diosgenin were concentration-dependent and were similar to those of silymarin (0,01 µM, 0,1 µM, 10 µM and 100 µM). Our results suggest that diosgenin, isolated from *Asparagus officinalis*, showed cytoprotective and antioxidant activity against this model of hepatotoxicity, possibly by affecting the metabolism of carbon tetrachloride.

**Key Words:** hepatocytes, diosgenin, phenobarbital, cytoprotection, antioxidant activity

**Introduction**

Diosgenin, a steroid sapogenin, can be found in some species of Dioscorea L.; *Asparagus*, Lilicaceae; *Tribulus terrestris*, Zygophyllaceae. Diosgenin is obtained by acid hydrolysis of steroid saponins – dicosin and gracillin. Diosgenin is widely used as a source for half-synthesis of sex hormones, corticosteroids and contraceptive drugs [10]. Araghiniknam et al. have found that diosgenin in vivo decreased absorption of exogenic and endogenic cholesterol [2].

Its anti-inflammatory effect is proved on experimental indomethacin-induced intestinal inflammation, as well as its spasmodic action in bilious colics [14]. It is proved that Dioscorea considerably decreases serum triglycerides, phospholipids and increases level of HDL and reduced the serum lipid peroxidation [11].

In the present study, the effects of diosgenin on isolated hepatocytes are compared to effects of silymarin (S), isolated from *Silybum marianum*, herb commonly known as Milk thistle. It is used as herbal remedy and also as a prescription drug to treat variety of liver disorders [6]. Principal active components of Milk thistle extract are flavonolignans, collectively termed Silymarin, present as three isomers, namely silibinin, silychristin and silydianin.

In our previous experiments we found that diosgenin, isolated from *Asparagus officinalis*, revealed statistically significant cytoprotective and antioxidant effects in conditions of carbon tetrachloride-induced hepatotoxicity. We suggest that these effects are connected with possible membrane stabilization and influence of carbon tetrachloride metabolism in isolated rat hepatocytes [8].

To support this thesis, the study aims investigating possible effects of diosgenin, isolated from *Asparagus officinalis*, and silymarin in conditions of carbon tetrachloride-induced toxicity on hepatocytes, isolated from rats treated with Phenobarbital.

**Materials and Methods**

**Plant material**

Roots of *Asparagus officinalis* are collected in June 2003 in the northeast region of Bulgaria, local-
ity Beljaev hill, nearby town of Beloslav. The plant is identified by Dr. D. Pavlova from the Department of Botany, Faculty of Biology, Sofia University. A voucher specimen is deposited in Herbarium of Sofia University (SO 97380).

**Extraction and isolation**

Plant material is extracted repeatedly with 80% MeOH then concentrated in vacuo. Residue is suspended in H$_2$O, and sequentially partitioned with Et$_2$O, CHCl$_3$, EtOAc and n-BuOH. Diosgenin is obtained after acid hydrolysis (5% H$_2$SO$_4$ for 4 h) of BuOH extract, followed by column chromatography on silica gel and preparative TLC. Compound is identified by physical and spectral data, comparing with literature [4, 1, 7].

**Chemicals**

In our experiments, pentobarbital sodium (Sanoﬁ, France), HEPES (Sigma Aldrich, Germany), NaCl (Merck, Germany), KCl (Merck), D-glucose (Merck), NaHCO$_3$ (Merck), KH$_2$PO$_4$ (Scharlau Chemie SA, Spain), CaCl$_2$, H$_2$O (Merck), MgSO$_4$, 7H$_2$O (Fluka AG, Germany), collagenase from *Clostridium histolyticum* type IV (Sigma Aldrich), albumin, bovine serum fraction V, minimum 98% (Sigma Aldrich), EGTA (Sigma Aldrich), 2-thiobarbituric acid (4,6-dihydroxypyrimidine-2-thiol; TBA) (Sigma Aldrich), trichloroacetic acid (TCA) (Valerus, Bulgaria), 2,2'-dinitro-5,5'-dithiodibenzoic acid (DTNB) (Merck), lactate dehydrogenase (LDH) kit (Randox, UK), carbon tetrachloride (Merck) were used.

**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. At least 7 days of acclimatization was allowed before the commencement of the study. The health was monitored regularly by a veterinarian physician. The 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 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 experiment.

**Isolation and incubation of hepatocytes**

Rats were anesthetized with sodium pentobarbital (0.2 ml/100 g). An optimized *in situ* liver perfusion using less reagents and shorter time of cell isolation was performed [9]. The method resulted in higher amount of live and metabolically active hepatocytes.

After portal catheterization, the liver was perfused with HEPES buffer (pH = 7.85) + 0.6 mM EDTA (pH = 7.85), followed by HEPES buffer (pH = 7.85), without any addition and finally HEPES buffer, containing collagenase type IV (50 mg/200 ml) and 7 mM CaCl$_2$ (pH = 7.85).

The liver was excised, minced into small pieces and hepatocytes were dispersed in Krebs-Ringer-bicarbonate (KRB) buffer (pH = 7.35) + 1% bovine serum albumin.

Cells were counted under the microscope and the viability was assessed by Trypan blue exclusion (0.05%) [5]. Initial viability averaged 89%.

Cells were diluted with KRB, to make a suspension of about 3 x 10$^6$ hepatocytes/ml. Incubations were carried out in flasks, containing 3 ml of the cell suspension (i.e. 9 x 10$^6$ hepatocytes) and were performed in a 5% CO$_2$ + 95% O$_2$ atmosphere [5].

**Lactate dehydrogenase release**

Lactate dehydrogenase release in isolated rat hepatocytes was measured as described by Bergmeyer et al. [3].

**GSH depletion**

At the end of the incubation, isolated rat hepatocytes were recovered by centrifugation at 4°C, and used to measure intracellular reduced glutathione (GSH), which was assessed by measuring non-protein sulphydryl’s after precipitation of proteins with trichloroacetic acid (TCA), followed by measurement of thiols in the supernatant with DTNB. The absorbance was measured at 412 nm [5].

**MDA assay**

Hepatocyte suspension (1 ml) was taken and added to 0.67 ml of 20% (w/v) TCA. After centrifugation, 1 ml of the supernatant was added to 0.33 ml of 0.67% (w/v) 2-thiobarbituric acid (TBA) and heated at 100°C for 30 min. The absorbance was measured at 535 nm, and the amount of TBA-reactants was calculated using a molar extinction coefficient of MDA 1.56 x 10$^5$ M$^{-1}$cm$^{-1}$ [5].
Statistical analysis

Statistical analysis was performed using statistical programme ‘MEDCALC’. Results are expressed as mean ± SEM for 6 experiments. The significance of the data was assessed using the nonparametric Mann–Whitney test. Values of $P \leq 0.05$; $P \leq 0.01$ and $P \leq 0.001$ were considered statistically significant. Three parallel samples were used.

Results

During phenobarbital induction, carbon tetrachloride, administered alone in concentration 86 µM, statistically significantly increased its own cytotoxicity. The cell viability and GSH level were decreased with 80 % and 75 %, respectively. The LDH leakage and MDA level were increased with 483 % and 169 %, respectively, compared to the control (figure 1).

In combination with CCl₄, diosgenin, isolated from *Asparagus officinalis*, statistically significant, concentration-dependent, reduced the damage caused by the hepatotoxic agent. The effects on the exam parameters are similar to those of Silymarin.

Concentration 0,01 µM diosgenin preserved cell viability by 153 %; 0,1 µM – by 227 %; 10 µM – by 253 % and 100 µM – by 327 %, compared to CCl₄. Silymarin in concentration 0,01 µM preserve cell viability with 167 %; 0,1 µM – by 220 %; 10 µM – by 240 % and 100 µM – by 253 %, compared to CCl₄ (Figure 2).

The activity of LDH was decreased by 0,01 µM diosgenin – with 41 %, by 0,1 µM – with 45 %, 10 µM – by 54 % and 100 µM – by 64 %, compared to CCl₄. Silymarin (0,01 µM) decreased LDH leakage with 76 %; 0,1 µM – by 77 %; 10 µM – by 79 % and 100 µM – by 80 %, compared to CCl₄ (Figure 3).

GSH depletion is preserved by 0,01 µM diosgenin – with 100 %, by 0,1 µM – with 120 %, 10 µM - by 180 % and 100 µM - by 220 %, compared to CCl₄. Both concentrations 0,01 µM and 0,1 µM of silymarin didn’t preserve statistically significant GSH depletion; 10 µM preserved GSH depletion by 100 % and 100 µM – by 260 %, compared to CCl₄ (Figure 4).

Concentration 0,01 µM diosgenin didn’t decreased the production of MDA, statistically significant. The production of MDA is decreased by 0,1 µM diosgenin – with 59 %, 10 µM – by 70 % and 100 µM – by 71 %, compared to CCl₄. Silymarin in concentration 0,01 µM decreased MDA level with 19 %; 0,1 µM – by 48 %; 10 µM – by 51 % and 100 µM – by 56 %, compared to CCl₄.

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.

Isolated liver cells are used as a suitable model for evaluation of the cytoprotective effects of some per-
Figure 2. Effects of diosgenin (0.01 – 100 µM) on cell viability (%), after carbon tetrachloride-induced cytotoxicity on hepatocytes, isolated from rats, treated with Phenobarbital

* P < 0.05; ** P < 0.01; *** P < 0.001 vs control

Figure 3. Effects of diosgenin (0.01 – 100 µM) on LDH leakage, in conditions of carbon tetrachloride-induced cytotoxicity on hepatocytes, isolated from rats, treated with phenobarbital

* P < 0.05; ** P < 0.01; *** P < 0.001 vs control

* P < 0.01; ** P < 0.001 vs CCl₄
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Figure 4. Effects of diosgenin (0.01 – 100 µM) on GSH depletion, in conditions of carbon tetrachloride-induced cytotoxicity on hepatocytes, isolated from rats, treated with phenobarbital

* P < 0.05; ** P < 0.01; *** P < 0.001 vs control
+ P < 0.05; ++ P < 0.01; +++ P < 0.001 vs CCl₄

Figure 5. Effects of diosgenin (0.01 – 100 µM) on MDA level, in conditions of carbon tetrachloride-induced cytotoxicity on hepatocytes, isolated from rats, treated with phenobarbital

** P < 0.01; *** P < 0.001 vs control
+++ P < 0.001 vs CCl₄
spective biologically active compounds, both newly synthesized and plant isolated.

During induction with phenobarbital, carbon tetrachloride, administered alone, increased its cytotoxicity. In model of induced with Phenobarbital carbon tetrachloride toxicity, diosgenin revealed higher statistically significant cytoprotective and antioxidant effects, compared to its effects in non-induced carbon tetrachloride intoxication.

It’s known that CCl$_4$ is bio-activated by CYP2E1, as well as CYP2B1 and possibly CYP3A, to form the trichlormethyl radical (●CCl$_3$), which initiates the chain reaction of lipid peroxidation \[13\]. In model of phenobarbital-induced carbon tetrachloride toxicity, diosgenin revealed higher statistically significant cytoprotective and antioxidant effects, compared to its effects in non-induced carbon tetrachloride intoxication. The decrease of the carbon tetrachloride-induced cytotoxicity during combination with diosgenin, possibly was connected of competition between both compounds on metabolic level, which lead to decreased production of trichloromethyl radical. This prominent cytoprotective effect of diosgenin in Phenobarbital-induced carbon tetrachloride cytotoxicity, confirmed the possible role of CYP3A isoform in the metabolism of diosgenin.

It’s known that Sylbin (the main compound in Silymarin) inactivate human CYP3A4 and CYP2C9, as the main liver glucuronosyltransferases \[14\]. On isolated rat hepatocytes, hepatoprotective effect of Silymarin in this toxicity model possibly is connected with change in the activity of some isoforms of cytochrome P450, playing role in carbon tetrachloride-induced bioactivation.

As a result of our experiments we find out that diosgenin has excessive statistically significant cytoprotective and antioxidant effect under conditions of oxidative stress, induced by carbon tetrachloride in hepatocytes, isolated from rats treated with phenobarbital. We suggest that this cytoprotective and antioxidant effects of diosgenin might be due to decrease lipid peroxidation products, prevention of GSH level and influence of some isoforms of cytochrome P450.

**Conclusion**

In our studies on carbon tetrachloride-induced toxicity in vitro, diosgenin, isolated from Asparagus officinalis, showed statistically significant, concentration-dependent, protective and antioxidant effects. Cytoprotective effects of diosgenin in isolated rat hepatocytes might be due to the influence of the metabolic bioactivation (possibly CYP3A activity) and oxidative stress.

**References**


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