



## Prevention of cadmium-induced toxicity in liver-derived cells by the combination preparation Hepeel®

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### ABSTRACT

Cadmium is a heavy metal of considerable environmental concern that causes liver damage. This study examined the possible prevention of cadmium toxicity in human HepG2 cells and primary rat hepatocytes by Hepeel®, a combined preparation of tinctures from seven different plants. Hepeel® prevented cadmium chloride (CdCl<sub>2</sub>)-induced cell death in both HepG2 cells and hepatocytes, and also reduced the loss of glutathione, lipid peroxidation, nuclear fragmentation, caspase activation and release of mitochondrial cytochrome C. To compare their relative efficacy, the seven constituent plant tinctures of Hepeel® were also separately tested. The tinctures China and Nux moschata, which exert solely anti-oxidative effects, failed to reduce cytotoxicity, and only protected against loss of glutathione and lipid peroxidation. In contrast, the tinctures Carduus marianus and Chelidonium, demonstrated anti-apoptotic effects, and protected HepG2 cells and primary hepatocytes against CdCl<sub>2</sub>-induced cell death. These results demonstrate how the effectiveness of Hepeel® is determined by the synergistic features of its constituent tinctures. Furthermore, we conclude that cadmium toxicity in the liver is mainly due to stimulation of the intrinsic apoptotic pathway, but may be intensified by increased oxidative stress.

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### 1. Introduction

Environmental exposure to fluctuating concentrations of heavy metals poses an enormous challenge for biological organisms. Toxic metals cause a vast array of adverse effects, including neurotoxicity, hepatotoxicity, immunotoxicity and carcinogenicity (Waalkes et al., 2000; Godt et al., 2006). Due to the global dispersion of heavy metals and their extensive use in modern society, some human exposure to toxic metals is inevitable. This ongoing prevalence of metal exposure necessitates protective measures at the environmental, social and individual level.

Cadmium is one of the most common toxic heavy metals, due to its primary accumulation in the liver and kidney (Godt et al., 2006). Cadmium causes hepatic, renal, skeletal, respiratory, and vascular disorders in humans (Nordberg, 1992; Waalkes et al., 2000), and it may also affect Leydig cells of the testes and hepatocytes and stellate cells of the liver (Koizumi et al., 1992; Dudley and Klaassen, 1984; Fariss, 1991; Souza et al., 2004a,b). Furthermore, cadmium is a potent carcinogen (Godt et al., 2006).

There is growing evidence that oxidative stress (Sarkar et al., 1995) via reactive oxygen species (ROS) generation and mitochondrial damage are among the basic mechanisms of cadmium

toxicity (Sarkar et al., 1995; Koizumi et al., 1994; Rikans and Yamano, 2000). Recently, apoptotic mechanisms involving caspase-dependent and caspase-independent pathways were described for cultured hepatocytes and livers exposed to cadmium *in situ* (Habeebu et al., 1998; Aydin et al., 2003; Pham et al., 2006; Oh and Lim, 2006; Li and Lim, 2007; Lasfer et al., 2008). However, despite much progress in research, the relative contribution of oxidative stress and apoptotic mechanisms to cadmium toxicity is still unclear.

The combination preparation Hepeel® is frequently used to stimulate liver function and improve antioxidant function in acute and chronic diseases, such as cholangitis and cholecystitis (Gebhardt, 2003). Hepeel® also demonstrates several other protective features, such as induction of glutathione-S-transferase activity (Gebhardt, 2003). These findings prompted the present investigation of the hepatoprotective potential of Hepeel®, and its seven constituent plant tinctures, against cadmium-induced hepatocellular damage. To thoroughly examine this, and to provide comparative experimental data for two different cell types, we used the human hepatoblastoma cell line HepG2 and primary rat hepatocytes. Exposure to Hepeel® largely prevented cell death, and oxidative and apoptotic pathomechanisms were differentially affected by the constituent tinctures. The combined anti-oxidative and anti-apoptotic properties of Hepeel® and its constituent tinctures support its overall protective effect against cadmium-induced toxicity in liver cells.

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## 2. Materials and methods

### 2.1. Materials

Hepeel<sup>®</sup> tinctures were prepared from seven different plants, according to procedures 3a and 4a of the German Homeopathic Pharmacopoea (HAB, 2000), and were provided by the Biologische Heilmittel Heel GmbH (Baden-Baden, Germany). The following seven constituent tinctures were used: (1) *Chelidonium*, prepared from *Chelidonium majus* L. (Ch-B 007009, 10<sup>-2</sup> dilution), (2) *Carduus marianus*, prepared from *Silybum marianum* L. (Ch-B 007034, 10<sup>-2</sup> dilution), (3) *Veratrum*, prepared from *Veratrum album* L. (Ch-B 007050, 10<sup>-3</sup> dilution), (4) *Colocynthis*, prepared from *Citrullus colocynthis* L. (Ch-B 007058, 10<sup>-3</sup> dilution), (5) *Lycopodium*, prepared from *Lycopodium clavatum* L. (Ch-B 007001, 10<sup>-3</sup> dilution), (6) *Nux moschata*, prepared from *Myristica fragrans*, Houtt (Ch-B 007026, 10<sup>-3</sup> dilution), and (7) *China* prepared from *Cinchona pubescens*, Vahl (Ch-B 007018, 10<sup>-3</sup> dilution).

The commercially available formulation of Hepeel<sup>®</sup> is a combination of all tinctures at the dilutions given above, with the addition of Phosphorus, a 10<sup>-4</sup> dilution of yellow phosphor. Hepeel<sup>®</sup> was supplied in sterile ampoules by Biologische Heilmittel Heel GmbH. The relative volume composition of 1.1 ml Hepeel<sup>®</sup> injection solution is: *Chelidonium* (*Chelidonium majus*, 10<sup>-3</sup> dilution) 1.1 µl, *Carduus marianus* (*Silybum marianum*, 10<sup>-1</sup> dilution) 0.55 µl, *Veratrum* (*Veratrum album*, 10<sup>-5</sup> dilution) 2.2 µl, *Colocynthis* (*Citrullus colocynthis*, 10<sup>-5</sup> dilution) 3.3 µl, *Lycopodium* (*Lycopodium clavatum*, 10<sup>-2</sup> dilution) 1.1 µl, *Nux moschata* (*Myristica fragrans*, 10<sup>-1</sup> dilution) 1.1 µl, *China* (*Cinchona pubescens*, 10<sup>-2</sup> dilution) 1.1 µl and Phosphorus (10<sup>-2</sup> dilution) 0.55 µl.

Dichlorodihydrofluorescein diacetate (DCFH) was purchased from Sigma (Daisenhofen, Germany). All other chemicals were from Roche Diagnostics (Mannheim, Germany), Merck (Darmstadt, Germany), Roth (Karlsruhe, Germany) or Sigma (Daisenhofen, Germany). Cell culture plates with tissue culture quality were from Techno Plastic Products AG (Trasadingen, Switzerland).

### 2.2. Culture of HepG2 cells

HepG2 hepatoblastoma cells were cultured in 1 × Dulbecco's Modified Eagle's Medium (DMEM) (Gibco, Eggenstein, Germany) supplemented with 2 mM glutamine, 10% foetal calf serum, 40 U/ml streptomycin and 50 U/ml penicillin, as previously described (Gebhardt, 2003). Cells were passaged weekly, when confluent. Cell stocks (passages 31 till 40) were kept frozen in liquid nitrogen. Frozen cells were thawed, cultured for one week, and passaged at least once before use. Confluent HepG2 cell cultures were used for all experiments.

### 2.3. Preparation and culture of rat hepatocytes

Sprague–Dawley rats were bred and maintained at the Medizinisch-Experimentelles Zentrum at the University of Leipzig, according to local ethical rules for animal care. They were kept on normal maintenance diet V1534 (Sniff, Soest, Germany) and tap water, ad libitum. Primary hepatocyte cultures were prepared from the livers of male rats (260–310 g) with collagenase perfusion, as previously described (Gebhardt, 1997). Cells were cultured in Williams medium E (Lonza, Verviers, Belgium) on collagen-coated plastic plates, at a uniform cell density of 125,000 cells/cm<sup>2</sup>. During the first 2 h, culture medium was supplemented with 10% calf serum, and serum-free medium was used thereafter. The medium volume was maintained at 100 µl/cm<sup>2</sup> of plating area. Additional details of cell culture have been reported elsewhere (Gebhardt, 1997; Gebhardt et al., 1994). For toxicity experiments, incubation in various agents usually started 2 h after plating.

### 2.4. Induced toxicity with cadmium chloride

The optimal concentration range of CdCl<sub>2</sub>-induced cytotoxic effects was different for each cell type. For HepG2 cells, culture medium was supplemented with concentrations ranging from 3 to 8 µM. For primary rat hepatocytes, optimal concentrations ranged from 2 to 6 µM. The highest CdCl<sub>2</sub> concentrations caused the greatest cell death in each cell type. In HepG2 cells, 8 µM CdCl<sub>2</sub> caused about 95% cell death, within 30 h of incubation. In hepatocytes, 6 µM CdCl<sub>2</sub> caused 72% cell death within 24 h of incubation.

### 2.5. Preparation of Hepeel<sup>®</sup> and tinctures

To prepare a working dilution of each tested compound, one part Hepeel<sup>®</sup> or tincture was mixed with 9 parts (v/v) of serum-free Williams Medium E, and gently shaken for 20 min at room temperature. This working solution of effective 0.1 dilution was used for further dilutions with Williams Medium E as specified in figure legends. Appropriate controls replaced each tincture or Hepeel<sup>®</sup> with equal volumes of ethanol.

### 2.6. Determination of cytotoxicity

Cytotoxicity of the tested compounds was determined using the colorimetric MTT-assay (MTT, 3-[4,5-dimethylthiazol-2-yl]-2,5-dephenyl tetrazolium bromide), as previously described (Gebhardt, 1997).

### 2.7. Determination of lipid peroxidation and ROS production

Malondialdehyde (MDA) measurements were used to quantify lipid peroxidation (Gebhardt, 1997). Briefly, HepG2 cells or rat hepatocytes seeded on 60 mm Petri dishes were incubated with or without CdCl<sub>2</sub> (3 or 4 µM) for 60 min after 30 h and 24 h of cultivation, respectively. In order to enhance oxidative stress, some plates were simultaneously exposed to *t*-butyl hydroperoxide (*t*-BHP; final concentration 1.5 mM). Thereafter, cells were washed with 0.9% NaCl, resuspended, and scraped into 1 ml of 50 mM potassium phosphate buffer (pH 7.4), then homogenised by sonication for 10 s (15% of maximum power, Sonopuls HD 2200, Bandelin electronic, Berlin, Germany). MDA was determined by thiobarbituric acid (TBA) assay (Esterbauer and Cheeseman, 1990; Gebhardt, 1997). The protein content of homogenates was measured following the procedure of Lowry et al. (1951).

Measurement of intracellular ROS was accomplished by using the DCFH assay (Wang and Joseph, 1999). HepG2 cells or rat hepatocytes cultured overnight in collagen-coated 96-well black flat bottom plates were washed 3 times with Krebs-Ringer-HEPES (KRH) solution pH 7.2 (Pavlica and Gebhardt, 2005). Cells were preloaded with 0.1 mM DCFH in either DMEM (HepG2 cells) or Williams Medium E (rat hepatocytes) for 30 min, then washed 3 times with KRH buffer. Cells were then treated simultaneously with CdCl<sub>2</sub> (3 µM) and the test compound diluted 1:10 with different starting dilutions indicated in Table 3 for an additional 30 min. Fluorescence (485/520 nm, Spectrofluor, TECAN) was recorded every min for up to 30 min, while temperature was maintained at 37 °C. Percentage increase in fluorescence units/well was calculated by the formula:  $F_{30} - F_0 / F_0 \times 100$ , where  $F_0$  = fluorescence at time 0 min, and  $F_{30}$  = fluorescence at time 30 min (Pavlica and Gebhardt, 2005).

### 2.8. Determination of cellular glutathione content

To measure glutathione (GSH) content, cells were cultured in 6-well plates for 30 h (HepG2 cells) or 24 h (primary hepatocytes). Test compounds were added 2 h after plating, along with the first change of medium. At the end of the incubation period, cells were washed and scraped into HEPES buffered isolation medium as previously described (Pavlica and Gebhardt, 2005). Determination of total GSH content was performed according to method of Gebhardt and Fausel (1997).

### 2.9. Detection of apoptotic nuclei with DAPI

The blue nucleic acid dye DAPI (4',6-Diamidino-2-phenylindole) was dissolved in methanol at 5 mg/ml, and stored as stock solution. Cells were washed twice with potassium phosphate buffer (PBS) and fixed with ice-cold methanol. Thereafter, a working solution of DAPI (1 µg/ml) in methanol was added, and cell nuclei were stained for 15 min at 37 °C. Destaining was achieved by replacing methanolic DAPI with pure methanol, followed by two rounds of washing with PBS.

### 2.10. Determination of caspase activity

Measurement of caspase-3 activity was based on the cleavage of a colorimetric substrate determined by the increase in absorbance at 405 nm. The assay was performed according to the instructions of the manufacturer (caspase-3 activity assay kit; Oncogene, Bad Soden, Germany) and adapted for HepG2 cells as described by Ohuchida et al. (2004). Recombinant caspase-3 was used for assay calibration.

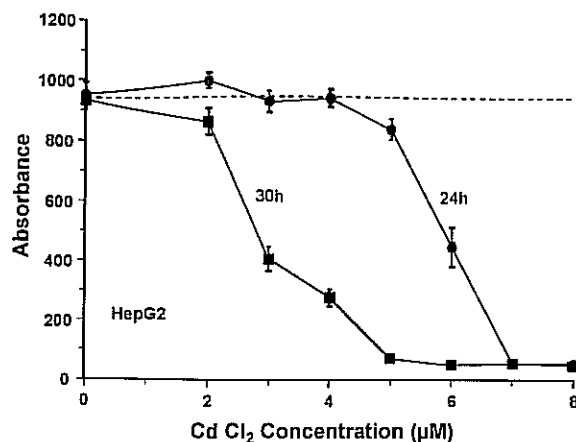
Caspase-Glo<sup>™</sup> 3/7 assay (Promega, Mannheim, Germany) is based on the cleavage of the proluminescent substrate by caspase 3/7 activity in the sample. A luminescent signal is generated via luciferase, and the assay was performed as previously described (Pavlica and Gebhardt, 2005). Briefly, 24 h (hepatocytes) or 30 h (HepG2 cells) after exposing the cells to medium containing Hepeel<sup>®</sup> or each tincture, the medium was removed. Then 0.1 ml of fresh medium was added to each well, together with 0.1 ml of the Caspase-Glo<sup>™</sup> 3/7 reagent solution reconstituted according to the recommendations of the supplier. The well volume was shaken for 30 s, then incubated for 1 h at room temperature. After additional shaking for 20 s, luminescence was measured using a Multilabel-Reader Mithras LB 940.

### 2.11. Preparation of cellular fractions and Western blot analysis

To measure cytochrome C release, cellular extracts were prepared by lysing the cells in 10 mM Tris-HCl buffer (pH 7.4) containing 2 mM EDTA, 1 µM β-mercaptoethanol, 1 µM leupeptin, 100 µM PMSF (phenylmethylsulfonyl fluoride), and 250 mM sucrose. Cells were homogenised by repeated passaging through a 26-gauge needle, and were centrifuged at 14,000 × g × 20 min at 4 °C. Cytosolic supernatants and pellets containing mitochondria were diluted in order maintain equal concentrations of mitochondrial protein, then used for Western blot analysis as previously described (Haupt et al., 2000). Cytochrome C was detected with anti-cytochrome C (A-8) antibody (sc-13156, Santa Cruz Biotechnology Inc., Heidelberg, Germany) followed by alkaline phosphatase-conjugated secondary antibody.

### 2.12. Statistical evaluation

Data were analysed for significance with a Student's *t*-test for comparisons between two groups. Data are presented as mean ± standard deviation (SD) of three to four measures, except when stated otherwise.



**Fig. 1.** The effect of different CdCl<sub>2</sub> concentrations and exposure times on the viability of HepG2 cells, determined by MTT assay. HepG2 cells were cultured for 24 h (circles) or 30 h (squares), in the presence of CdCl<sub>2</sub>. Each absorbance value is given as a mean  $\pm$  SD of triplicate measures. Mean control level is indicated by the dashed horizontal line. The EC<sub>50</sub>-values for CdCl<sub>2</sub>-induced toxicity amounted to 5.9  $\mu$ M after 24 h of cultivation, and 2.8  $\mu$ M after 30 h of cultivation.

### 3. Results

#### 3.1. Cytotoxicity of cadmium chloride on hepatocellular populations

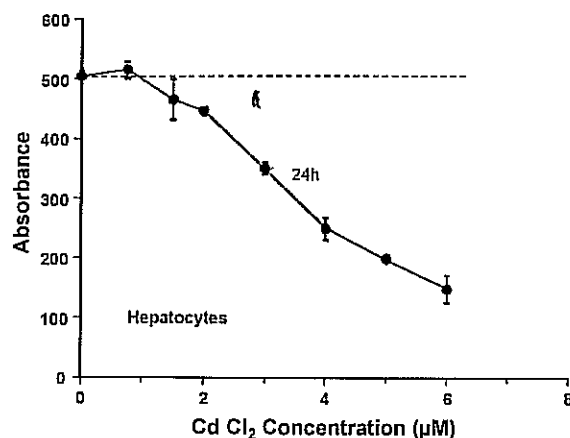
The cytotoxic effect of CdCl<sub>2</sub> on HepG2 cells was concentration- and time-dependent. Within the first 24 h of exposure, HepG2 cells tolerated up to 5  $\mu$ M CdCl<sub>2</sub>, but quickly died at higher concentrations (Fig. 1). At 7  $\mu$ M CdCl<sub>2</sub>, almost all cells were dead or had detached from the substratum. At 5  $\mu$ M CdCl<sub>2</sub> or below, no visible alterations in cell morphology and nuclei were detectable after 24 h (data not shown). However, deterioration was seen at 5  $\mu$ M CdCl<sub>2</sub> when cultivation was continued for another 6 h (Fig. 1). At that time, cadmium-induced cytotoxicity was already apparent at lower concentrations. The first signs of cytotoxic influence were detected above 2  $\mu$ M, and almost all cells died at a concentration of 5  $\mu$ M, as determined by MTT reduction to less than 10% of controls. The EC<sub>50</sub>-value for CdCl<sub>2</sub>-induced cytotoxicity in HepG2 cells was 5.9  $\mu$ M at 24 h, and 2.8  $\mu$ M at 30 h of cultivation.

Rat hepatocytes were even more sensitive to cadmium, and cytotoxicity was more prominent than in HepG2 cells, at all culture times. At 24 h after addition of CdCl<sub>2</sub>, MTT reduction was already decreased in a concentration-dependent manner, above 2  $\mu$ M doses (Fig. 2). At 6  $\mu$ M, absorbance was reduced by approximately 70%. The EC<sub>50</sub>-value for CdCl<sub>2</sub>-induced toxicity was 3.7  $\mu$ M. After 30 h, absorbance measurements in the MTT assay had further dropped, and were lower than those of HepG2 cells at all concentrations of cadmium (data not shown). Therefore, all subsequent measurements of cell viability were performed in HepG2 cells at 30 h of cultivation, and in rat hepatocytes at 24 h of cultivation.

#### 3.2. Protection against cadmium cytotoxicity by Hepeel® and constituent tinctures

In the presence of Hepeel®, cadmium cytotoxicity was reduced in both cell types. In HepG2 cells at 30 h of culture, Hepeel® application resulted in the gradual increase of viability from 32% (control) to 53%, as dilutions changed from 10<sup>-3</sup> to 10<sup>-1</sup> (Fig. 3A). At the 10<sup>-2</sup> dilution, the increase in viability was significant ( $P < 0.01$ ).

Among the constituent tinctures, only *Carduus marianus* and *Chelidonium* were effective at reducing cadmium cytotoxicity (Table 1). Within the range of 10<sup>-5</sup> to 10<sup>-3</sup> dilutions, *Carduus marianus* caused increased cell viability in a concentration-dependent



**Fig. 2.** The effect of different concentrations of CdCl<sub>2</sub> on the viability of rat hepatocytes, determined by MTT assay. Rat hepatocytes were cultured for 24 h (circles) in the presence of CdCl<sub>2</sub>. Each absorbance value is given as a mean  $\pm$  SD of triplicate measures. Mean control level is indicated by the dashed horizontal line. The EC<sub>50</sub>-value for CdCl<sub>2</sub>-induced toxicity is 3.7  $\mu$ M.

manner, to values between 60 and 70% (Fig. 3B). *Chelidonium* application resulted in maximal values slightly above 60% (Fig. 3C). Also, the cell sensitivity was slightly higher with *Carduus marianus*, as significant differences were seen starting at the 2.5  $\times 10^{-5}$  dilution, whereas with *Chelidonium* significant differences were not apparent until the more concentrated dilutions of 10<sup>-4</sup> and lower.

Similar results were obtained with rat hepatocytes after 24 h of cultivation. The 10<sup>-1</sup> dilution of Hepeel® increased viability from 68% to almost 88%. At the same 10<sup>-1</sup> dilution, *Carduus marianus* reached 95% and *Chelidonium* reached 87% viability (Table 1). As for HepG2 cells, the other constituents of Hepeel® did not reduce cytotoxicity (Table 1).

#### 3.3. Cadmium-induced lipid peroxidation and ROS

Exposure of HepG2 cells to CdCl<sub>2</sub> for 24 h did not change the basal rate of lipid peroxidation, as evidenced by the unchanged cellular production of malondialdehyde (MDA) compared to control measures (Table 2). However, when challenged with 1.5 mM *t*-BHP, HepG2 cells exposed to 3  $\mu$ M CdCl<sub>2</sub> responded with a 2.1-fold increase, and those exposed to 4  $\mu$ M responded with a 2.3-fold increase of MDA, compared to control cells not exposed to cad-

**Table 1**  
Protective potency of different plant tinctures and Hepeel® against CdCl<sub>2</sub>-induced cytotoxicity in HepG2 cells and rat hepatocytes.

Material	Starting dilution	Viability (% untreated control) <sup>a</sup>	
		HepG2 cells	Hepatocytes
CdCl <sub>2</sub> only (3 $\mu$ M) <sup>b</sup>		32.1 $\pm$ 3.0	68.5 $\pm$ 2.2
<i>Chelidonium</i>	10 <sup>-2</sup>	62.6 $\pm$ 7.1*	87.4 $\pm$ 2.3*
	10 <sup>-3</sup>	48.4 $\pm$ 4.5*	77.8 $\pm$ 1.9*
<i>Carduus marianus</i>	10 <sup>-2</sup>	61.3 $\pm$ 4.5*	95.7 $\pm$ 1.8*
	10 <sup>-3</sup>	51.6 $\pm$ 7.7*	83.1 $\pm$ 2.0*
<i>Veratrum</i>	10 <sup>-3</sup>	41.4 $\pm$ 9.9	71.2 $\pm$ 1.6
<i>Lycopodium</i>	10 <sup>-3</sup>	43.3 $\pm$ 15.6	72.3 $\pm$ 2.1
<i>Colocynthis</i>	10 <sup>-3</sup>	31.3 $\pm$ 8.3	67.8 $\pm$ 2.4
<i>Nux moschata</i>	10 <sup>-3</sup>	35.6 $\pm$ 3.9	70.4 $\pm$ 1.5
<i>China</i>	10 <sup>-3</sup>	36.2 $\pm$ 3.9	71.5 $\pm$ 2.2
Hepeel®		52.6 $\pm$ 7.1*	88.1 $\pm$ 2.4*

<sup>a</sup> Control values without CdCl<sub>2</sub> were set 100%.

<sup>b</sup> Note that CdCl<sub>2</sub> is also present in all conditions listed below (Hepeel® or each constituent tincture).

\* Significantly different from respective controls ( $P < 0.01$ ).

**Table 2**  
Production of MDA by HepG2 cells exposed to CdCl<sub>2</sub> and *t*-BHP.

Exposure	Concentration (μM)	Production of MDA (% control)	Increase in DCF fluorescence (arb. units)
None		100 ± 6	15.4 ± 3.5
CdCl <sub>2</sub>	3	109 ± 8	17.3 ± 4.2
	4	111 ± 9	16.6 ± 3.4
<i>t</i> -BHP	1500	246 ± 22*	41.2 ± 4.8*
CdCl <sub>2</sub> + <i>t</i> -BHP	3 + 1500	508 ± 46 <sup>†</sup> **	84.7 ± 7.6 <sup>†</sup> **
	4 + 1500	572 ± 61 <sup>†</sup> **	93.1 ± 8.3 <sup>†</sup> **

\* Significantly different from respective controls ( $P < 0.01$ ).

\*\* Significantly different from *t*-BHP alone ( $P < 0.01$ ).

**Table 3**  
Production of MDA by HepG2 cells exposed to CdCl<sub>2</sub> and *t*-BHP when treated with Hepeel® or its constituent plant tinctures.

Material	Starting dilution	Production of MDA (% control) <sup>a</sup>		Increase in DCF fluorescence (arb. units)	
		Exposure		Exposure	
		CdCl <sub>2</sub>	CdCl <sub>2</sub> + <i>t</i> -BHP	CdCl <sub>2</sub>	CdCl <sub>2</sub> + <i>t</i> -BHP
None		110 ± 8	512 ± 43	17.8 ± 4.1	82.9 ± 7.3
Chelidonium	10 <sup>-2</sup>	96 ± 11	488 ± 35	16.5 ± 3.6	83.4 ± 12.2
	10 <sup>-3</sup>	94 ± 8	497 ± 41	16.7 ± 5.2	81.7 ± 8.6
Carduus marianus	10 <sup>-2</sup>	76 ± 12*	233 ± 31*	5.8 ± 4.0*	28.2 ± 6.2*
	10 <sup>-3</sup>	82 ± 10*	249 ± 27*	7.8 ± 3.6*	44.9 ± 7.8*
Veratrum	10 <sup>-3</sup>	102 ± 9	506 ± 45	17.0 ± 4.4	85.4 ± 5.1
Lycopodium	10 <sup>-3</sup>	98 ± 13	523 ± 52	16.7 ± 3.8	80.6 ± 6.5
Colocynthis	10 <sup>-3</sup>	116 ± 18	509 ± 31	16.3 ± 4.6	78.9 ± 5.3
Nux moschata	10 <sup>-3</sup>	85 ± 9*	372 ± 36*	8.7 ± 2.8*	47.6 ± 5.9*
China	10 <sup>-3</sup>	80 ± 7*	354 ± 41*	10.2 ± 3.4*	56.8 ± 4.1*
Hepeel®		83 ± 8*	311 ± 34*	13.5 ± 3.8*	68.7 ± 5.5*

<sup>a</sup> Control values without CdCl<sub>2</sub> (3 μM) were set at 100%.

\* Significantly different from respective controls ( $P < 0.01$ ).

mium. Likewise, ROS production of HepG2 cells detected by DCFH fluorescence was stimulated by CdCl<sub>2</sub> only in the presence of *t*-BHP (Table 2). The relative increase in ROS production was comparable to that for lipid peroxidation.

As shown in Table 3, Hepeel® significantly reduced *t*-BHP-induced MDA production in both untreated HepG2 (control) cells and HepG2 cells exposed to CdCl<sub>2</sub> for 24 h. Among all tinctures, Carduus marianus was the most effective (Table 3). Dilutions up to 10<sup>-4</sup> significantly reduced MDA production. Chelidonium, Veratrum, Colocynthis, and Lycopodium tinctures were not effective in this assay, while China seemed almost as equally potent as Carduus marianus. Nux moschata was slightly less effective, but also contributed to the anti-oxidative response. These results were almost mirrored in measures of ROS production (Table 3), which indicated that the anti-oxidative potential of Hepeel® and its constituents was very broad.

The results for rat hepatocytes were different. In these cells, CdCl<sub>2</sub> led to an increase of MDA production of 55%, and an increase in ROS production of 32%, compared to control hepatocytes unexposed to cadmium. However, as in HepG2 cells, the sensitivity to *t*-BHP in the presence of cadmium was also increased approximately 2-fold, from 155% to 302% for MDA, and from 132% to 273% for ROS. The following agents significantly counteracted the impact of CdCl<sub>2</sub>, as apparent via the following reduction in MDA measures; Hepeel® preparation (31%), Carduus marianus (36%), China (18%), and Nux moschata (16%). All other tinctures were ineffective at reducing the CdCl<sub>2</sub>-induced MDA production (data not shown).

### 3.4. Cadmium-induced loss of GSH

A moderate drop in cellular GSH (19 ± 5%) was observed in HepG2 cells in response to exposure to CdCl<sub>2</sub> at a concentration of 3 μM (Table 4). This value is in accordance with an EC<sub>50</sub>-value

of approximately 4.5 μM. This loss was considerably enhanced (55 ± 4%) when cells were additionally exposed to *t*-BHP. Only Hepeel® and the tinctures Carduus marianus, China and Nux moschata were able to significantly counteract the influence of CdCl<sub>2</sub>, with or without additional *t*-BHP (Table 4). When used alone, Hepeel® and Carduus marianus were able to completely restore cellular GSH content.

### 3.5. Cadmium-induced apoptosis

Cadmium toxicity via apoptosis was measured by DAPI-staining in two different ways; counting of fragmented nuclei and monitoring of cell death. Within 30 h of 3 or 4 μM CdCl<sub>2</sub> exposure, apoptotic fragmentation in HepG2 cell nuclei was apparent after DAPI staining, and total cell numbers were decreased (Fig. 4). Specifically, the percentage of apoptotic nuclei increased from less than 0.1% (controls) to about 8% in the presence of 3 μM CdCl<sub>2</sub> (Table 5). At earlier time points, such as 24 h, the proportion of fragmented nuclei was

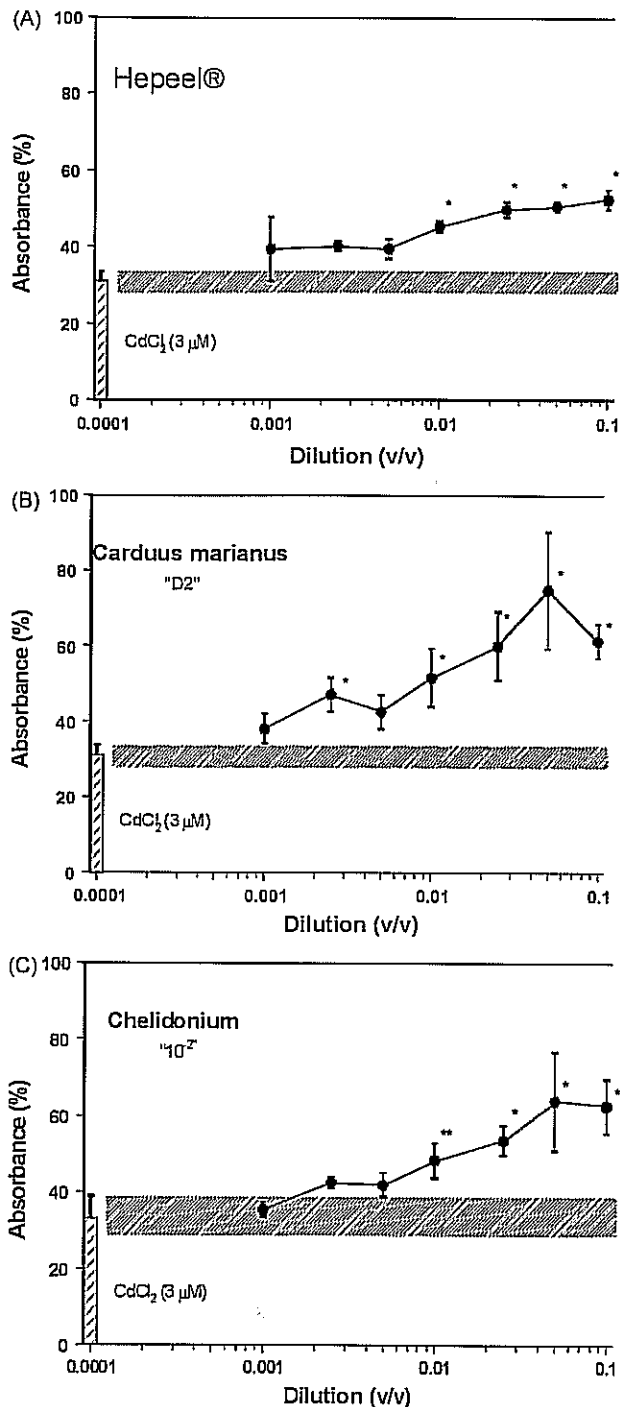
**Table 4**  
Cellular content of GSH of HepG2 cells exposed to CdCl<sub>2</sub> and *t*-BHP.

Exposure	Starting dilution	Content of GSH (% control) <sup>a</sup>	
		CdCl <sub>2</sub>	CdCl <sub>2</sub> + <i>t</i> -BHP
None		81 ± 5	45 ± 6*
Hepeel®		96 ± 7*	74 ± 6*
Chelidonium	10 <sup>-2</sup>	76 ± 6	41 ± 5
Carduus marianus	10 <sup>-2</sup>	102 ± 4*	83 ± 7*
Colocynthis	10 <sup>-3</sup>	83 ± 5	48 ± 4
Nux moschata	10 <sup>-3</sup>	94 ± 6*	75 ± 8*
China	10 <sup>-3</sup>	91 ± 4*	68 ± 5*

<sup>a</sup> Control values without CdCl<sub>2</sub> (3 μM) were set at 100%.

\* Significantly different from respective controls ( $P < 0.01$ ).

\*\* Significantly different from CdCl<sub>2</sub> alone ( $P < 0.01$ ).



**Fig. 3.** The effect of different treatment dilutions on the viability of HepG2 cells exposed to 3  $\mu\text{M}$  CdCl<sub>2</sub>. The viability with the combination preparation Hepeel® is shown in A. The viability with the tincture Carduus marianus is shown in B. The viability with the tincture Chelidonium is shown in C. For all panels, absorbance values were normalised to absorbance for untreated cells (100%, horizontal line across top) while absorbance of control cells exposed to 3  $\mu\text{M}$  CdCl<sub>2</sub> is shown as a bar  $\pm$  SD on the lower left. The grey horizontal bar indicates the control value across the graph, for visual comparison to all other measured values. Symbol (\*) indicates significant difference from CdCl<sub>2</sub>-treated controls,  $P < 0.01$ ; (\*\*),  $P < 0.05$ .

lower. Apparently, the low frequency of apoptotic nuclei was due to non-synchronised cell cycle of the HepG2 cells. This view was supported by the observation that cells partially synchronised by storage in the cold (4 °C) responded to CdCl<sub>2</sub> with a far higher fraction of apoptotic nuclei (not shown). At concentrations of 5 or 6  $\mu\text{M}$ , signs of apoptosis were hardly detectable under the microscope

**Table 5**

The proportion of fragmented nuclei induced by CdCl<sub>2</sub> in HepG2 cells and hepatocytes after treatment with Hepeel® or two of its constituent tinctures.

Condition	Fraction of fragmented nuclei (%) <sup>a</sup>	
	HepG2 (30 h)	Hepatocytes (24 h)
None	0.08 $\pm$ 0.09	0.02 $\pm$ 0.05
Cadmium <sup>b</sup>	8.34 $\pm$ 3.17*	42.66 $\pm$ 11.85*
Hepeel®	0.07 $\pm$ 0.08	0.02 $\pm$ 0.06
Cadmium + Hepeel®	4.58 $\pm$ 1.03**	4.21 $\pm$ 0.74**
Cadmium + Carduus marianus	5.16 $\pm$ 0.94**	7.50 $\pm$ 2.27**
Cadmium + Chelidonium	n.d.	11.02 $\pm$ 2.53**

n.d., not determined.

<sup>a</sup> Values represent means  $\pm$  SD from counting numbers of fragmented and total nuclei from 20 microscopic fields.

<sup>b</sup> Concentrations of CdCl<sub>2</sub> were 3  $\mu\text{M}$  and 4  $\mu\text{M}$  for HepG2 cells and hepatocytes, respectively.

\* Significantly different from respective controls ( $P < 0.01$ ).

\*\* Significantly different from cadmium alone ( $P < 0.01$ ).

(not shown), since many HepG2 cells detached or decomposed completely within 30 h of CdCl<sub>2</sub> exposure.

Apoptotic response was even larger in rat hepatocytes (Fig. 5). The peak of apoptosis occurred approximately 24 h after to CdCl<sub>2</sub> exposure. At 3  $\mu\text{M}$  CdCl<sub>2</sub>, the proportion of apoptotic nuclei increased from almost zero (control, as in Fig. 5A) to about 21% (not shown). In the presence of 4  $\mu\text{M}$  CdCl<sub>2</sub>, the percentage of fragmented nuclei was greater than 40%, the highest value observed (Table 5). At 6  $\mu\text{M}$  CdCl<sub>2</sub>, many apoptotic cells had already detached, and thus the proportion of fragmented nuclei appeared lower (Fig. 5C).

Addition of Hepeel® to the culture medium considerably reduced the apoptotic response at all concentrations of CdCl<sub>2</sub> in HepG2 cells and hepatocytes (Table 5). This influence was particularly pronounced in hepatocytes exposed to 4  $\mu\text{M}$  CdCl<sub>2</sub>, wherein the proportion of apoptotic nuclei was diminished from 42% to 4% (Table 5). A similar but less pronounced effect of Hepeel® could be observed in the presence of 6  $\mu\text{M}$  CdCl<sub>2</sub> (cf. Fig. 5D).

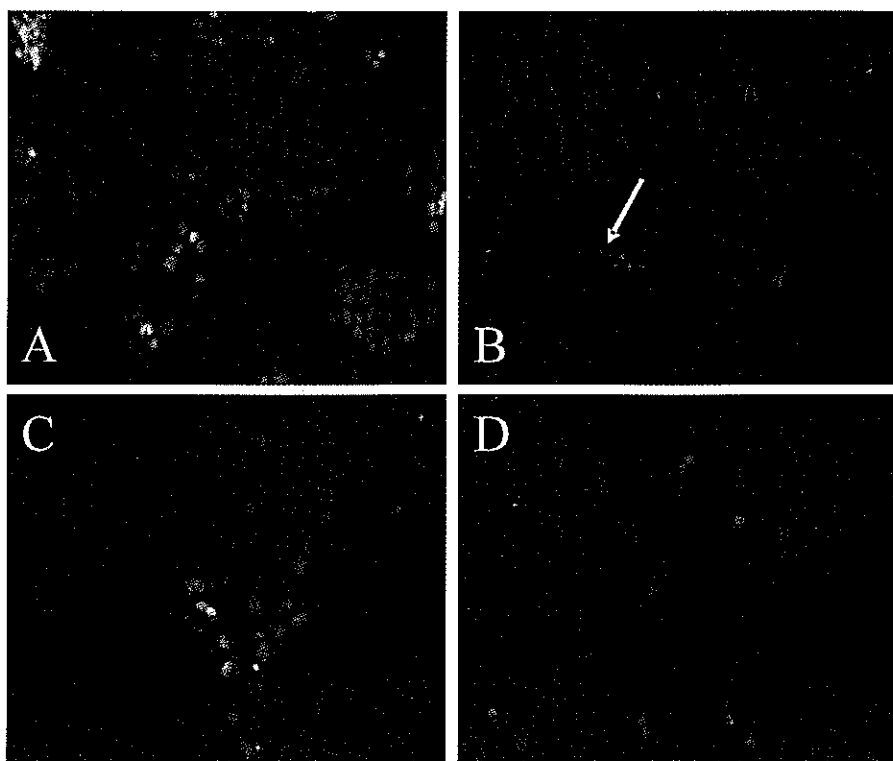
Similar to the results seen in the MTT assays, the co-application of either Carduus marianus or Chelidonium with CdCl<sub>2</sub> effectively reduced the number of apoptotic nuclei, and enhanced cell survival (Fig. 4C and D). In the presence of 4  $\mu\text{M}$  CdCl<sub>2</sub> and 10<sup>-4</sup> final tincture dilutions, the proportion of fragmented nuclei in hepatocytes was 7% for Carduus marianus and 11% for Chelidonium (Table 5).

### 3.6. Cadmium-induced activation of caspases

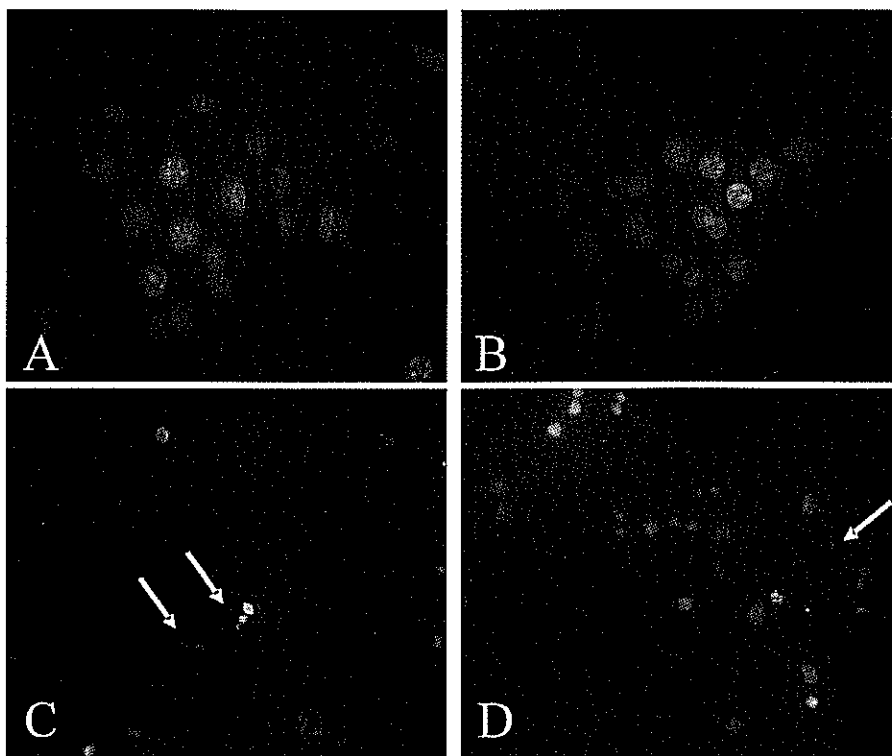
Results for caspase-3 activity measurements were similar to those for apoptosis. In HepG2 cells, 3  $\mu\text{M}$  CdCl<sub>2</sub> induced a significant increase in caspase-3 activity within 24 h (Table 6), with a 1.8-fold increase in caspase 3 and a 2.5-fold increase of caspases activity as measured by caspase 3/7 assay. Simultaneous addition of Carduus marianus at a 10<sup>-4</sup> dilution to the culture medium resulted in a decrease of caspase-3 activity to about 1.3-fold, and the 10<sup>-3</sup> dilution decreased caspase-3 activity to about 1.2-fold, relative to the vehicle-treated controls. Chelidonium was slightly less effective, but still reduced caspase-3 activity significantly in both assays (Table 6). A similar result was obtained for the Hepeel® 10<sup>-1</sup> dilution, which reduced CdCl<sub>2</sub>-induced caspase activity in both assays by approximately 40% (Table 6). Aside from Carduus marianus and Chelidonium, none of the other constituent tinctures was effective. An example of an ineffective tincture is shown with China, in Table 6.

### 3.7. Cadmium-induced release of cytochrome C

The release of cytochrome C from mitochondria of HepG2 cells was significantly higher in the presence of 3  $\mu\text{M}$  CdCl<sub>2</sub> than in unex-



**Fig. 4.** Photomicrographs of DAPI-stained HepG2 cells. HepG2 cells were cultured without (A), or with (B–D)  $3 \mu\text{M CdCl}_2$ . HepG2 cells were simultaneously treated with  $3 \mu\text{M CdCl}_2$  and *Carduus marianus* ( $10^{-3}$  dilution) (C) or *Chelidonium* ( $10^{-3}$  dilution) (D). The relatively higher number of cells in the bottom row compared to panel B reflects the protective influence of co-applied tinctures (C and D). Magnification:  $20\times$ .



**Fig. 5.** The effect of Hepeel® on apoptotic nuclear fragmentation induced by the highest tested concentration of  $\text{CdCl}_2$  in cultured rat hepatocytes. Hepatocytes cultured without Hepeel® (A and C) or with Hepeel® ( $10^{-1}$ ) (B and D) were exposed to vehicle (A and B) or  $6 \mu\text{M CdCl}_2$  (C and D), and stained by DAPI after 24 h. Cell counts showed that the proportion of fragmented nuclei (yellow arrows) was considerably increased with  $\text{CdCl}_2$  exposure, yet it was relatively lower with application of Hepeel®. Magnification:  $40\times$  (A and B);  $20\times$  (C and D).

**Table 6**  
Inhibition of caspase-3 activation in CdCl<sub>2</sub>-exposed HepG2 cells by Hepeel® or single plant tinctures.

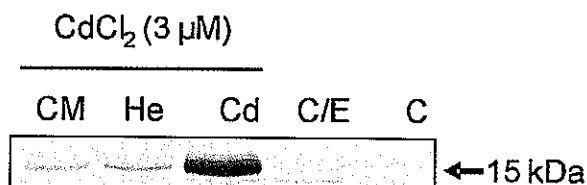
Material	Starting dilution	Caspase activity (% untreated control) <sup>a</sup>	
		Caspase-3 assay	Caspase 3/7 assay
CdCl <sub>2</sub> only (3 μM)		178 ± 21	254 ± 19
Chelidonium	10 <sup>-2</sup>	143 ± 11*	196 ± 16*
	10 <sup>-3</sup>	154 ± 14*	212 ± 18*
Carduus marianus	10 <sup>-2</sup>	116 ± 12*	148 ± 23*
	10 <sup>-3</sup>	130 ± 14*	166 ± 21*
China Hepeel®	10 <sup>-2</sup>	172 ± 13	241 ± 20
		149 ± 10*	187 ± 18*

n.d. = not determined.

<sup>a</sup> Control values without CdCl<sub>2</sub> were set at 100%.

<sup>b</sup> Note that CdCl<sub>2</sub> is also present in all conditions listed below (Hepeel® or each constituent tincture).

\* Significantly different from respective controls ( $P < 0.01$ ).



**Fig. 6.** Western blot showing the relative release of mitochondrial cytochrome C in treated and untreated HepG2 cells. Cells were cultured for 30 h with different agents (Carduus marianus = CM, Hepeel® = He, CdCl<sub>2</sub> = Cd, medium with solvent (C/E), and normal medium = C) with or without 3 μM CdCl<sub>2</sub> (horizontal line). Hepeel® dilution was 10<sup>-1</sup> and Carduus marianus dilution was 10<sup>-3</sup>. Cytosolic extracts were diluted in order to contain equal concentrations of mitochondrial protein, prior to detection of cytochrome C as described in Section 2.

posed cells, which showed almost no release (Fig. 6). Densitometric analysis revealed a 27-fold increase in cytochrome C in cadmium-treated versus vehicle control cells. Hepeel® (10<sup>-1</sup>) reduced the release of cytochrome C by about 5-fold, and Carduus marianus (10<sup>-3</sup>) reduced it by 7-fold (Fig. 6). Chelidonium was almost as effective as Carduus marianus, while treatment with China showed no effect (data not shown).

#### 4. Discussion

Our results demonstrate a strong protective effect of the combination preparation Hepeel® and several of its constituent plant tinctures on the cultured human hepatoblastoma cell line HepG2 and rat hepatocytes. We showed that cadmium-induced hepatocellular damage is effectively counteracted by these agents. To gain insight into potential mechanisms of this protective effect, we focussed on two aspects: oxidative stress, and occurrence of apoptosis.

There are conflicting reports in the literature about oxidative stress during cadmium cytotoxicity. While some authors report that cadmium cytotoxicity is due to, or at least associated with, increased oxidative stress and lipid peroxidation (Dudley and Klaassen, 1984; Fariss, 1991; Rikans and Yamano, 2000; Souza et al., 2004a,b; Koyu et al., 2006), other authors could not detect enhanced lipid peroxidation in response to cadmium exposure *in vivo* and *in vitro* (Harvey and Klaassen, 1983; Aydin et al., 2003).

There are several ways in which our results do not fully support the assumption that oxidative stress is the sole mediator of CdCl<sub>2</sub>-induced toxicity. First, the significant increase in the formation of ROS in response to CdCl<sub>2</sub>, which corroborates the findings of Oh and Lim (2006), was efficiently counteracted by some of the plant tinctures studied (China and Nux moschata), which were found

to exert strong anti-oxidative effects (Gebhardt, 2003). However, these tinctures did not reduce cadmium-induced cytotoxicity in our experiments. Second, our observation that Chelidonium protected against cadmium-induced cytotoxicity, but did not diminish ROS production, makes it unlikely that ROS are major mediators of cadmium toxicity in HepG2 cells or rat hepatocytes. Similar reasoning applies to the loss of GSH which is also counteracted by China and Nux moschata, but not by Chelidonium. Third, CdCl<sub>2</sub> was not able to stimulate lipid peroxidation in HepG2 cells without the presence of *t*-BHP. Thus, lipid peroxidation seems to play a minor role in this form of cytotoxicity. However, our findings illustrate that exposure to cadmium rendered the cells more vulnerable to peroxide exposure.

In turn, it remains possible that lower intracellular concentrations of GSH may aggravate some effects of cadmium. For instance, inactivation of critical sulfhydryl groups of essential proteins by cadmium (Li et al., 1994; Kim et al., 2003) may occur more frequently in the presence of a diminished content of GSH.

Concerning the occurrence of apoptosis in response to cadmium exposure our results are consistent with findings in mouse and rat liver (Habeebu et al., 1998; Pourahmad et al., 2001; Li and Lim, 2007) as well as human hepatocytes (Lasfer et al., 2008), and corroborates similar conclusions based on the observation of DNA laddering and other markers of apoptosis in response to cadmium exposure in HepG2 cells (Aydin et al., 2003; Oh and Lim, 2006).

The finding that HepG2 cells were somewhat less sensitive than hepatocytes to cadmium may at least partially be due to the fact that HepG2 cells are non-synchronously proliferating cells, while primary hepatocytes are quiescent. Consequently, hepatocytes cultures are a more homogeneous cell population. Furthermore, HepG2 cells originate from hepatoblastomas, which as cancer cells are generally less sensitive to oxidative stress than normal hepatocytes. Another reason for decreased sensitivity in HepG2 cells may be that the cadmium-induced loss of GSH in HepG2 cells was less marked than in rat hepatocytes.

Our results with DAPI staining also showed that treatment with Hepeel® and the single plant tinctures, which protected against cadmium toxicity, reduced the number of apoptotic nuclei. Furthermore, these agents also inhibited the activation of pro-apoptotic caspases and the release of mitochondrial cytochrome C. Therefore, these results strongly suggest that the most effective single tinctures, Carduus marianus and Chelidonium, are able to counteract intracellular processes other than oxidative stress, such as events leading to caspase activation and subsequent apoptosis, in response to cadmium. Silibinin is an active substance in the Carduus marianus tincture, and is known to exert an anti-apoptotic influence in other systems (Singh and Agarwal, 2004; Pook et al., 2006). Thus, silibinin may contribute to the protective effects of the tincture. However, direct anti-apoptotic properties of Chelidonium have not yet been described. Interestingly, alkaloids derived from Chelidonium such as chelerythrine and sanguinarine interact with the cytoskeleton (Slaninova et al., 2001), and of those, the alkaloid chelerythrine is an inhibitor of protein kinase C (Herbert et al., 1999). In addition, chelerythrine has recently been described as an inhibitor of BclXL function, which may help explain the pro-apoptotic effect observed with Chelidonium (Chan et al., 2003). In fact, detailed studies on the molecular interactions of chelerythrine revealed binding sites distinct for the BH3 (Bcl-2 homology 3) binding cleft (Zhang et al., 2006). This finding raises the possibility of alternate mechanisms favouring interactions of pro-survival members of the Bcl-2 family. In light of these findings, the concentration of chelerythrine in our experiments is much lower than the EC<sub>50</sub> value for its pro-apoptotic effect (Chan et al., 2003; Malikova et al., 2006). Thus, at low concentrations anti-apoptotic influences of chelerythrine and sanguinarine seem to predominate.

Therefore, our results strongly suggest that the protective function of Hepeel<sup>®</sup> against cadmium-induced cytotoxicity results from the synergistic actions of its composite tinctures. The decisive anti-apoptotic influence of Hepeel<sup>®</sup> may be supported by its anti-oxidative features that help stabilise cellular GSH content, and consequently the sulfhydryl status of cellular proteins. Further studies are needed to discern whether this protective effect is specific to cadmium toxicity in hepatocytes, or can be generalised to other toxins and cell populations.

In conclusion, Hepeel<sup>®</sup> efficiently antagonised cytotoxic and apoptotic effects of the heavy metal cadmium in hepatocyte cell populations. This protective function is likely based on anti-apoptotic influence distinct from anti-oxidative function, but may be rendered more efficient by the synergistic effects of both. These observations add to the list of beneficial effects recently reported with this preparation (Gebhardt, 2003), and support the possible therapeutic use of Hepeel<sup>®</sup>, particularly for cases of heavy metal poisoning.

### Conflict of interest

None.

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