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Hepatoprotective and proapoptotic effect of *Ecballium elaterium* on CCl₄-induced hepatotoxicity in rats

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ABSTRACT

Objectives: To investigate the effects of perorally administered juice on tetrachloromethane (CCl₄)-induced hepatotoxicity model in rats.
Methods: Male Wistar rats were tube-administrated silymarin, Ecballium juice at 0.2 mL/kg and 0.7 mL/kg daily for 3 consequent days, *i.e.*, 3.28 µg and 11.48 µg of cucurbitacin B per kg of body weight respectively. On the third day, liver damage was induced by intraperitoneal application of CCl₄. On the fourth day, abdominal cavity was macroscopically examined and liver samples were taken for histopathological and immunochemical evaluation. HPLC was used to determine the content of the active substance cucurbitacin B.
Results: The experiment revealed that 0.7 ml/kg juice concentration expressed the highest pro-apoptotic activity, but with prevailing negative effects. Compared with the lower concentration, there was an observable vasodilatation with consequent interstitial hemorrhages and a larger scope of inflammatory damage, which suppressed the hepatoprotective effect. In the 0.2 mL/kg concentration, there was a smaller pro-apoptotic activity but other parameters had better results, and the liver parenchyma damage was reversible.
Conclusions: No reactions confirming the potentially allergic effect on laboratory rats were observed; its hepatoprotective and anti-inflammatory effect was confirmed on a model of acute liver damage.

1. Introduction

Natural remedies for the prevention and therapy of liver diseases have been popular worldwide in the traditional medicine; however, there is a limited amount of information on their biological activity. To date, there have been many experimental studies utilizing *in vitro* or *in vivo* animal models, which provide basic information on the effect and possible toxicity, necessary for further stages of clinical evaluation. Hepatoprotective drugs used in classical medicine have their origin in plant extracts *e.g.*, silymarin from *Silybum marianum* (milk thistle).

Ecballium elaterium (L.) (*E. elaterium*) A. Rich (squirting cucumber) is a wild-growing herb from family Cucurbitacae, which is abundant in the Mediterranean. Its fruits contain black seeds and juice, which has been used in the Mediterranean regions as a natural remedy for several indications, especially as an analgesic, antipyretic and antiphlogistic [1]. In Anatolia region, fresh juice has been a favourite drug for the treatment of rhinosinusitis [2]. Anti-inflammatory effect of an aqueous extract from *E. elaterium* for this indication was confirmed in a rabbit model [3].

The juice contains a broad spectrum of biologically active substances [4]. The main active anti-inflammatory substance with a confirmed curative and preventive effect on tetrachloromethane (CCl₄)-induced blood damage is cucurbitacin B, a

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triterpene derivative isolated from fruits and seeds [5]. The decrease of bilirubin levels in blood was experimentally demonstrated in animals suffering from hepatitis [6]. The impact of cucurbitacin on the levels of free and conjugated plasma bilirubin in humans with normal or increased bilirubinemia was proved by Greige-Gerges *et al.* [7].

During a pre-clinical experiment, the hepatoprotective activity of squirting cucumber juice was confirmed on tetrachloromethane-induced liver damage model in laboratory rats [8]. However, when administering concentrated juice, there was reported several adverse effects. Based on the application route and the dose, there were reported ADRs such as mucosa irritation, pharyngeal edema, hypersalivation, dysphagia, vomiting, conjunctivitis and corneal edema and erosion [1].

2. Materials and methods

2.1. Juice preparation

The green mature fruits of *E. elaterium* were collected from El-Dabaa, located in the western desert of Egypt, in August 2011. The juice was squeezed from the fruits and then filtered twice through 0.45 µm membrane filter. This procedure yielded a clear crude fruit juice, that was conserved and stored in sterile tubes at −20 °C.

2.2. Study design

This experiment was approved by the Animal Welfare and Protection Committee of the University of Veterinary and Pharmaceutical Sciences Brno. Male Wistar rats (AnLab, Czech Republic) weighing on average (275 ± 25) g were divided into 5 groups, 10 animals each. A preventive medication was implemented for the purposes of this study. Group I was used as a negative control, group II as a positive control, which was administered only CCl_4. Groups III–V were tube-administered the tested substance in total inhalation anesthesia (Isofluran®, Nicholas Piramal India) daily for 3 consequent days-group III received standard comparative hepatoprotective drug silymarin, *i.e.*, a mixture of silybinin, silydianin and silychristin 1:1:1 (Sigma–Aldrich, Czech Republic) at 20 mg/kg *po.*; group IV was administered Ecballium juice at 0.2 mL/kg *po.*; and group V juice at 0.7 mL/kg *po.* To achieve the hepatotoxic effect of the used anesthetics and the results' comparability, even the animals from the intact group were anesthetized. On the third day of the experiment, animals from groups II–V were induced liver damage by intraperitoneal application of 30% tetrachloromethane (Sigma–Aldrich, Czech Republic) suspended in olive oil at 1 mL/kg. On the fourth day of the experiment, animals were euthanized by cervical dislocation in total inhalation anesthesia. During the consequent necropsy, abdominal cavity was visually examined and liver tissue samples were taken for histopathological and immunochemical evaluation. Tissue sections were formalin-fixed, processed by standard histological procedures and hematoxylin and eosin stained. For the immunohistochemical examination, detection of fragmented DNA (TUNEL assay) during the early and intermediate stages of apoptosis was implemented. Biochemical examination was carried out by a Dimension Sysmex RxR analyzer and data were statistically evaluated by ANOVA-Tukey-HSD multiple comparison test by Unistat 5.1 software.

2.3. HPLC analysis

The HPLC system HP 1100 series (Agilent Technologies, Germany), equipped with the ChemStation software (Agilent Technologies) and comprised a binary high-pressure pump, an online vacuum degasser, an auto-sampler, a thermostated column compartment and a multi wavelength detector set at 230 nm, was used for the chromatographic analysis. The separations were carried out on a Zorbax XDB-C18 column (50 mm × 2.1 mm i.d., 1.7 µm particle size) from Agilent Technologies. Gradient elution starting from 10% of acetonitril and 90% of aqueous formic acid (0.2%) was used. End of the gradient (pure acetonitril) was at 36 min. The flow rate of mobile phase was set at 0.3 mL/min. The injection volume was 10 µL, and the column temperature was maintained at 30 °C. The method based on external standard was used for quantification of cucurbitacin B. The content of the active substance cucurbitacin B in juice was determined by HPLC at 16.4 µg/mL, so group IV received 3.28 µg and group V 11.48 µg of cucurbitacin B per kg of body weight.

2.4. Monitored parameters

Biochemical examination included the determination of total protein, albumin, bilirubin, alkaline phosphatase, alanine aminotransferase, aspartate aminotransferase, cholesterol and triacylglycerols in blood plasma.

The following histopathological characteristics were monitored: mixed steatosis, zonal and focal necrosis, epitheloid–granulomatous reaction, portal reaction, hepatocyte degeneration, Kupffer cell activity, vasodilatation, interstitial hemorrhage, fibrosis, polynuclear and macronuclear hepatocytes and cholestasis. The morphological parameters were semi-quantitatively evaluated on the scale from 0 (no changes) to 10 (maximum changes).

3. Results

3.1. Biochemical parameters

Table 1 summarizes the results of the biochemical examination. Total protein concentration in Ecballium 0.7 mL/kg was highly significantly greater and albumin concentration significantly greater compared to the negative control. Total bilirubin concentrations in silymarin and Ecballium groups (both 0.2 mL/kg and 0.7 mL/kg) were highly significantly greater when compared with the negative control group. The catalytic activity of aspartate aminotransferase was significantly greater in the silymarin group and highly significantly greater in Ecballium 0.2 mL/kg and 0.7 mL/kg groups when compared with the control. Cholesterol and triacylglycerols concentrations in the positive control, silymarin and both Ecballium groups were highly significantly higher when compared with the control.

3.2. Histopathological evaluation

Semiquantitative histopathological evaluation of the liver parenchyma is given in Table 2. Intact control revealed damage, which corresponded to the effect of total isoflurane anesthesia, *i.e.*, dilated veins with hemorrhages, reactive reactions in portobiliary space, microvesicular steatosis in their vicinity and increased activity of Kupffer cells (Figure 1A).

Table 1

Biochemical parameters in blood plasma.

Groups	Total protein (g/L)	Albumin (g/L)	Bilirubin (μmol/L)	ALP (μkat/L)	ALT (μkat/L)	AST (μkat/L)	Cholesterol (mmol/L)	TAG (mmol/L)
Ecballium 0.2 mL/kg	62.21 ± 3.16	37.68 ± 1.27	3.22 ± 0.64**	4.23 ± 0.51	2.81 ± 1.11	11.89 ± 3.69**	1.40 ± 0.32**	0.41 ± 0.17**
Ecballium 0.7 mL/kg	66.60 ± 2.98**	39.48 ± 1.51*	2.75 ± 0.42**	4.45 ± 0.77	3.15 ± 2.69	11.19 ± 4.41**	1.49 ± 0.14**	0.64 ± 0.34**
Positive control	61.80 ± 2.65	37.34 ± 2.46	2.52 ± 0.62	3.88 ± 1.18	1.49 ± 0.62	6.99 ± 1.80	1.44 ± 0.09**	0.79 ± 0.53**
Silymarin 20 mg/kg	62.62 ± 2.50	38.33 ± 1.90	2.94 ± 1.16**	4.43 ± 0.86	3.63 ± 3.23	9.40 ± 4.61*	1.46 ± 0.32**	0.64 ± 0.40**
Negative control	58.42 ± 4.60	36.55 ± 2.44	1.33 ± 0.14	5.15 ± 0.93	1.18 ± 0.52	3.77 ± 1.00	0.92 ± 0.12	1.89 ± 0.80

Values represent the mean ± SD of 10 determinations. *$P < 0.05$, **$P < 0.01$ compared to the negative control group.

Table 2

Histopathological parameters.

Histopathological parameters	Ecballium (0.2 mL/kg)	Ecballium (0.7 mL/kg)	Positive control	Silymarin (20 mg/kg)	Negative control
Mixed steatosis	4	4	4	4	5
Zonal and focal necrosis	2	3	4	3	0
Epitheloid–granulomatous reaction	4	5	4	4	0
Portal reaction	2	4	2	2	3
Hepatocyte degeneration	2	2	4	2	0
Kupffer cell activity	7	7	3	7	3
Vasodilatation	5	7	4	4	1
Interstitial hemorrhage	0	3	0	0	0
Fibrosis	2	3	2	2	2
Polynuclear and macronuclear hepatocytes	0	0	0	0	0
Cholestasis	0	1	0	0	0

Semiquantitative evaluation of the changes in liver parenchyma (0 = no changes, 10 = maximum changes).

In the positive control group, which was administered tetrachloromethane, there were observed changes, which are typical for CCl4-induced liver damage-venous dilatation with hemostasis, necrotic hepatocytes present primarily in the area around central veins and in the foci of damaged hepatocytes with polymorphonuclear infiltration. The necrosis was accompanied by inflammatory reaction with granulomatous aggregation surrounded by perivenular macrovesicular steatosis. Portobiliary space was slightly reactive with dilated veins (Figure 1B).

In the group, which was administered a conventional hepatoprotective silymarin, there were observed reactive changes, steatotic and partially also necrotic cells in the liver parenchyma, scavenging reaction around the central vein, uncircumscribed portobiliar space, and generally greater inflammatory reaction than in groups administered the tested juice. CCl4-induced liver damage after the administration of silymarin was smaller, yet, hepatocytes necrosis, macrovesicular steatosis, granulomatous inflammatory reaction and dilated central veins were present in the perivenous area of the liver parenchyma (Figure 1C).

The group administered Ecballium at 0.2 mL/kg was histopathologically characterized by persistent inflammatory reaction manifested by granulomatous aggregates. However, there was apparent reduction of perivenular macrovesicular steatosis and necrosis of hepatocytes with regenerative processes (Figure 1D).

In the 0.7 mL/kg concentration group, there was, in comparison with the lower concentration group, a visible dilatation of central veins and larger scope of inflammatory and necrotic tissue damage, which suppressed hepatoprotective effect of the administered substance (Figure 1E).

3.3. Apoptosis evaluation (TUNEL)

In the negative control group, no apoptotic changes in hepatocytes were observed (Figure 2A). Similar results were also found in the positive control group, liver parenchyma did not contain apoptotic cells (Figure 2B). It can therefore be stated that CCl4 induces especially necrotic death of hepatocytes. In the silymarin group, there were observed positive apoptotic cells, but in a smaller amount than in the Ecballium groups (Figure 2C). Apoptosis of hepatocytes is induced by both Ecballium juice concentrations. The greatest number of apoptotic hepatocytes was found in liver of rats, which were administered the 0.7 mL/kg concentration juice (Figure 2E). In the 0.2 mL/kg group, apoptotic cells were observed in smaller amounts (Figure 2D). It can be therefore supposed that quantity of apoptotic cells probably correlates with the extract concentration.

4. Discussion

During the experiment, CCl4 was used to induce an acute liver damage. Tetrachloromethane biotransformation leads to the production of highly reactive free radical metabolites, which causes lipid peroxidation and damage to hepatocyte membranes, leading to centrilobular necrosis of hepatocytes accompanied by lipid degeneration [9]. Neutrophils cumulate around the damaged hepatocytes, and they induce inflammatory reaction through pro-inflammatory cytokines (TNF-α, IL-1 and IL-6) [10]. Based on the CCl4 concentration, there can be a different degree of liver damage [11]. Single dose administration results in a centrilobular necrosis and steatosis, long-term administration causes liver fibrosis, cirrhosis and hepatocellular carcinoma [12].

Some studies imply that tetrachloromethane induces not only necrosis, but it can also initiate hepatocytes apoptosis [13–15]. Wu et al. [15] in their study verifying the hepatoprotective effect of echinacoside reported an increased number of apoptotic hepatocytes in the group exposed to CCl4, compared
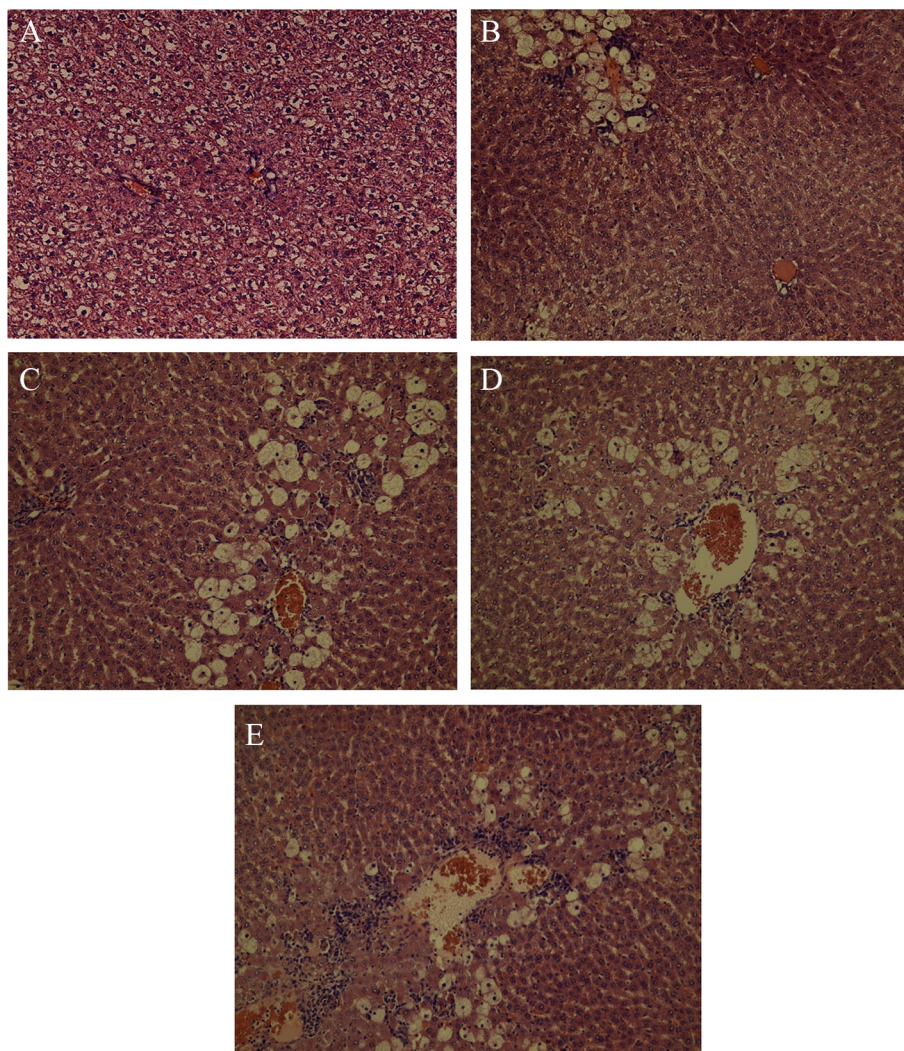
Figure 1. Histopathological result of liver parenchyma.
HE stain, magnification 200×.
A: Negative control, dilated veins with hemorrhages, reactive reactions in portobiliary space, microvesicular steatosis in their vicinity and increased activity of Kupffer cells.
B: Positive control, venous dilatation with hemostasis, necrotic hepatocytes present primarily in the area around central veins and in the foci of damaged hepatocytes with polymorphonuclear infiltration. The necrosis with inflammatory reaction and granulomatous aggregation surrounded by perivenular macrovesicular steatosis. Portobiliary space slightly reactive with dilated veins.
C: Silymarin, reactive changes, scavenging reaction around the central vein, uncircumscribed portobiliar space. Hepatocytes necrosis, macrovesicular steatosis, granulomatous inflammatory reaction and dilated central veins in the perivenous area of the liver parenchyma.
D: Ecballium 0.2 mL/kg, persistent inflammatory reaction manifested by granulomatous aggregates. Apparent reduction of perivenular macrovesicular steatosis and necrosis of hepatocytes with regenerative processes.
E: Ecballium 0.7 mL/kg, dilatation of central veins with extensive inflammatory and necrotic tissue damage.

to the intact group. Results of the study by Sun *et al.* [14] indicate that CCl_4 causes apoptosis of hepatocytes through the caspase-3 activation, which is released due to a secondary necrosis, which occurs in liver simultaneously with apoptosis.

Animals were totally anesthetized prior to the administration of tested substances. All animals were anesthetized due to isoflurane hepatotoxicity, therefore even the negative control revealed anticipated changes of the liver tissue – diffuse microvesicular steatosis as a reaction to anesthesia. Isoflurane hepatotoxicity is mediated by its metabolites, which cause inhibition of beta-oxidation and respiratory processes in the mitochondria in hepatocytes [16].

There is not enough relevant data on the hepatoprotective effect of *E. elaterium*. To date, there is only a study by Agil *et al.* [8], which focused on the hepatoprotective effect of elaterium

and cucurbitacin B isolated from the juice. The study implemented a model of CCl_4-induced hepatotoxicity, test animals were mice and laboratory rats, and silymarin was used as a comparative standard hepatoprotective substance. The study tested both prophylactic and therapeutic administration of these substances. Preventive administration of elaterium and cucurbitacin B revealed a substantial reduction of CCl_4-induced lesions. Necrosis was observed only in some hepatocytes and the steatosis stage was lower. Correspondingly, the therapeutic administration of these substances had a very favorable effect; the steatosis scope was reduced and inflammation eliminated. Liver of animals exposed only to CCl_4 revealed stage 4 steatosis and inflammatory reaction. Results imply that the hepatoprotective significance of Ecballium is probably caused by cucurbitacins,
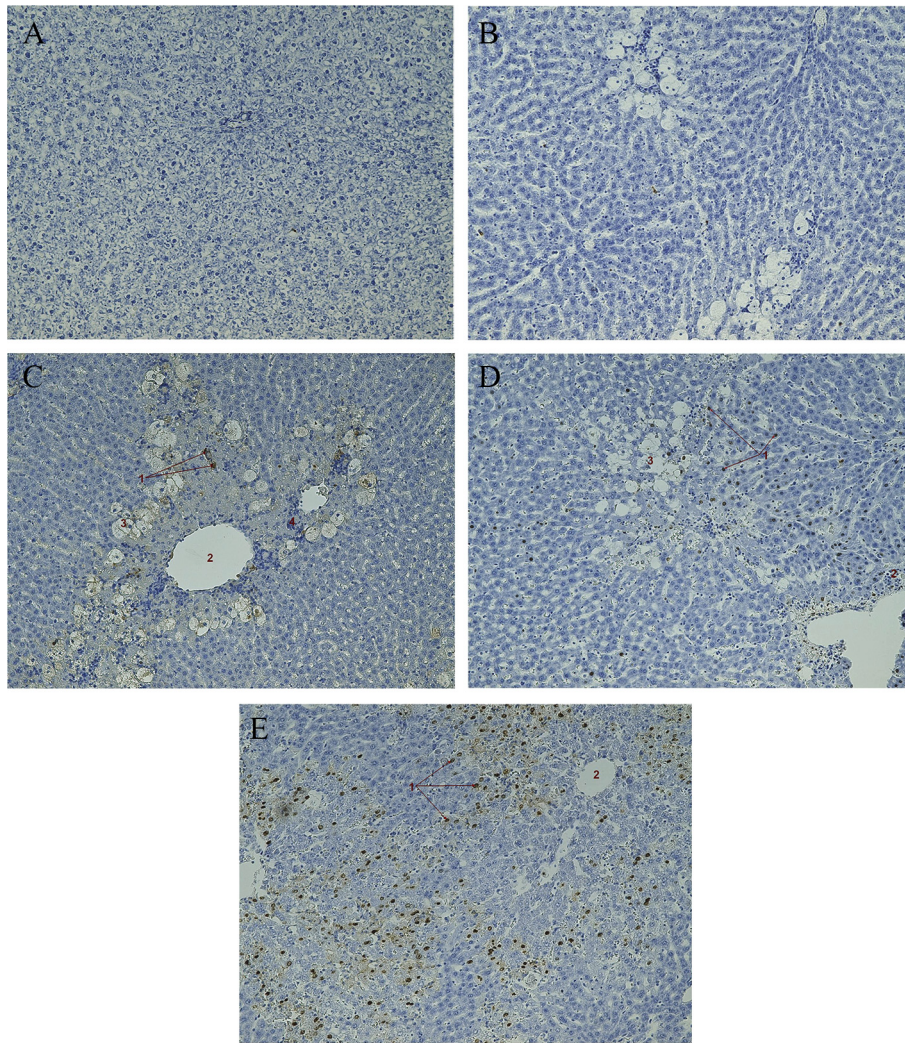
Figure 2. TUNEL result of liver parenchyma.
TUNEL, magnification 200×.
A: Negative control, no apoptotic changes in hepatocytes.
B: Positive control, necrotic hepatocytes, no apoptotic changes in hepatocytes.
C: Silymarin, apoptotic cells (1), dilated central vein (2) macrovesicular steatosis (3) with granulomatous reaction (4).
D: Ecballium 0.2 mL/kg, apoptotic cells (1), dilated central vein (2) and necrotic hepatocytes (3).
E: Ecballium 0.7 mL/kg, Apoptotic cells (1), dilated central vein (2).

especially cucurbitacin B, even though some other substances contained in the juice can participate on this effect.

Older studies by Yesilada et al. [17] identified cucurbitacin B as the main anti-inflammatory substance of Ecballium using an *in vivo* model of increased venous permeability. Other cucurbitacins in Ecballium, *i.e.*, cucurbitacin E, I and D, were found ineffective in this indication. Cucurbitacin B also revealed inhibitory activity on a model using bradykinin- and serotonin-induced edema of mice paws [18].

During the experiment, there was also evaluated the overall effect of the juice on rats' organisms and the possible development of adverse effects. Serious irritation to the mucosa is a common adverse effect of Ecballium juice, which usually occurs, if it is used undiluted. Eken et al. [19] described a case study, where a patient experienced uvular edema and nasal mucosa necrosis after an intranasal administration of undiluted juice. The absence of systemic side effects, minimum impact of standard antiallergic therapy and mucosa necrosis support the non-allergic core of the reaction. No animal showed systemic reaction after the administration of tested substances.

The direct toxic effect is also reported by a study representing a group of patients, who were exposed to the effect of undiluted juice. They experienced a serious form of mucosa irritation, which was manifested by pharynx edema, breathlessness, dysphagia, vomiting or conjunctivitis, depending on the method of administration of the undiluted juice (oral, intranasal, dermal or ocular). In the end, it was stated that the exposure to undiluted juice causes inflammatory irritation of mucosa and the anti-inflammatory effect results from the application of optimum juice concentration [1]. This implication was also confirmed by a report by Sezik and Yesilada, who stated that the degree of juice dilution represents a criterion of its toxicity [20].

As for the pro-apoptotic effect of *E. elaterium* juice, there is not enough information. To date, there have only been studies focused on the research of pro-apoptotic effect of cucurbitacin B in connection with its antitumor effect, which was successfully tested on several tumor types (*e.g.*, tumors of breast, larynx and pancreas), both individually and in combination with commercially available cytostatics [21–23]. Its effect was also confirmed in hepatocellular carcinoma (HCC). Experiments were carried

out *in vitro* on HCC cells and mice. The therapy by cucurbitacin B resulted in the termination of tumor cells growth in the S-phase of cell cycle and induction of apoptosis, evidenced by the presence of characteristic morphological changes. The effect is dose-dependent and attributed to STAT3 suppression and transcription factor regulating expression of genes, which participate in the proliferation, apoptosis suppression and angiogenesis [24,25]. Another confirmed antitumor mechanism lies in the modification of Raf-MEK-ERK signaling pathway (c-Raf suppression and ERK1/2 activation), which plays an important role in the cell proliferation, differentiation and apoptosis, and its dysregulation results in carcinogenesis. An increased c-Raf expression was observed in spontaneously developing HCC [25].

TUNEL assay methods proved pro-apoptotic activity of *E. elaterium* juice. Pro-apoptotic effect is a manifestation of its hepatoprotective effect, because hepatocytes apoptosis is not a process of a group necrosis of cells, and usually it is not accompanied by inflammatory reaction. Potentially necrotic cells become apoptotic, which results in the reduction of inflammatory reaction and the degree of liver parenchyma necrosis.

The experiment revealed that 0.7 mL/kg juice concentration expressed the highest pro-apoptotic activity, but with prevailing negative effects. Compared with the lower concentration, there was observable a significant vasodilatation with consequent interstitial hemorrhages and also a larger scope of inflammatory damage, which suppressed the hepatoprotective effect. In the 0.2 mL/kg concentration, there was a smaller pro-apoptotic activity but other parameters had better results, and the liver parenchyma damage was reversible. Based on these results, its hepatoprotective effect was confirmed on a model of acute liver damage. This effect can be beneficial in clinical practice.

Conflict of interest statement

We declare that we have no conflict of interest.

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