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The antioxidants are not enough. *Malus sylvestris* (L.) Mill. extract enhances the carbon tetrachloride liver toxicity in albino rats.

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Summary

Liver toxicosis induced by CCl_4 exposure is a canonical model for steatohepatitis. Antioxidants are frequently used for hepatoprotection but sometimes they have no beneficial effect based on the prooxidant properties or matrix toxicity. Four experimental groups (Control, Extract, CCl_4 and CCl_4 + Extract) of albino rats were used in order to evaluate the effect of the hydroglycerin alcoholic *Malus sylvestris* (L.) Mill. extract in CCl_4-induced steatohepatitis. Blood transaminases and TNFα were increased after CCl_4 administration and cell-mediated inflammatory response was enhanced in the same way with transaminases and TNFα. Extract administration after CCl_4 exposure decreased the cell-mediated inflammatory response and transaminases activity and increased TNFα. Liver histopathological evaluation revealed that the extract administration stimulates steatohepatitis compared to CCl_4 and that is the origin of the decreased transaminases - high liver injuries from which transaminases does not pass in blood flow. Liver injuries were stimulated by the extract matrix and the antioxidants did not exert a hepatoprotective effect.

Introduction

The liver is involved in a wide variety of biochemical pathways which can be summarized into several major categories such as carbohydrate, fat, protein and xenobiotic metabolism (Pandit *et al*., 2012). The liver is considered the most affected organ in drug toxicity due to the fact that it is placed between the absorption site and the systemic circulation and is one of the major sites of transformation and elimination of xenobiotic compounds (Dey *et al*., 2013; Russmann *et al*., 2009). Many drugs or other chemical substances present in the environment can cause chemical-driven liver damage (hepatotoxicity) (Pandit *et al*., 2012). Carbon tetrachloride (CCl_4) is one of the most representative halogenated compound in chemistry and is commonly encountered in the environment (Malaguarnera *et al*., 2012).
The classic toxicity of CCl_4 is to induce liver injury, steatosis and liver fibrosis (Dong *et al*., 2016; Sun *et al*., 2010; Masuda, 2006; Bishayi *et al*., 2002). CCl_4 causes severe oxidative stress, increased protein carbonyl groups, lipid peroxidation, glutathione (GSH) depletion, disturbance of calcium homeostasis (Dong *et al*., 2016; Ritesh *et al*., 2015; Masuda, 2006). Biochemical alterations are prone to appear before the histological damage is evident (Lutz *et al*., 2003). CCl_4 is metabolized in the microsomes through oxidation to phosgene ($COCl_2$) and through reductive dehalogenation catalyzed by cytochrome P-450 2E1, P-450 2B1 and P-450 2B2 to a very reactive product, trichlorometyl radical (-CCl_3) (Ji *et al*., 2014; Lutz *et al*., 2003). This radical is extremely aggressive and can covalently bind to lipids causing fatty degeneration (steatosis), nucleic acids, functioning as a hepatocellular carcinoma initiator, proteins which lead to their decrease,

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and also can react with oxygen (O_2) to form a highly reactive species, the trichlorometylperoxy radical (-CCl_3OO) (Lutz *et al*., 2003; Zhu *et al*., 1999). The former radical initiates lipid peroxidation of polyunsaturated fatty acids associated with phospholipids. On molecular level, carbon tetrachloride, activates tumor necrosis factor α (TNF-α) and transforming growth factor α/β (TGF-α/β) leading to liver fibrosis. The hepatotoxic dose of CCl_4 also increases serum levels of aspartate aminotransferase (AST), alanine aminotransferase (ALT) and interleukin 6 (IL-6) synthesis, an inflammatory acute phase response factor, which antagonizes TNF-α in order to protect against oxidative stress (Streetz *et al*., 2001).

In the present paper was studied the effect of hydro glycerin alcoholic *M. sylvestris* (L.) Mill. extract on modulating the hepatoprotective and immunostimulatory functions in rats intoxicated with carbon tetrachloride. *M. sylvestris* or European crab apple is a species of the genus *Malus* in the family *Rosaceae*. It is rich in bioactive polyphenolic compounds (flavonoids, anthocyanins and tannins) which act as active antioxidant substances. These antioxidants have the ability to scavenge for reactive oxygen species (ROS) which cause lipid peroxidation (Stojiljković *et al*., 2018, 2016 a, b; Parola and Robino, 2001; Guyot *et al*., 1997).

Due to the fact that this extract contains ethanol and glycerin it is important to know how these molecules interact with CCl_4. Even if the liver was already subjected to CCl_4 toxicosis, the key question to be asked is: would anyone expect to observe a hepatic improvement done by *M. sylvestris* extract due to the fact that this extract is known to have hepatoprotective effects according to Stojiljković *et al*., (2016 a)?

Materials and methods

Extract and reagents

Standardized *M. sylvestris* buds extract was provided from PlantExtrakt Cluj-Napoca and synthetic composition analysis revealed that the extract contains chlorogenic acid, galic acid, potassium, proteins, sterols and amino acids such as aspartic acid, glutamic acid and isoleucine. The reagents included in standard assay packets with colorimetric and kinetic methods for AST, and ALT were obtained from BioMaxima S.A., Lublin, Poland. ELISA kit for TNFα was purchased from ELAB-Science, China. Neutral formalin solutions were purchased from Chemical Company S.A., Iasi, Romania. All other chemicals and solvents used in the study were of analytical grade.

Animals

Adult (3-month-old) Wistar female rats weighing 180-200 g were provided *ad libitum* access to standard rat chow and water. Animals were maintained in a light/temperature controlled room with a light/dark cycle of 12/12 h under 22^0C constant temperature. Rats were housed 8/cage and all rats in the same cage corresponded to one of the experimental groups. Animal care and procedures were carried out in accordance to the European Communities Council Directive 2010/63/UE. Animals were divided in four groups: Control (C), Extract (E), CCl_4 and CCL_4 + E.

Carbon tetrachloride-induced hepatotoxicity and extract administration

Carbon tetrachloride was administred in a dose of 700 µL/kg b.w. during 7 days by enteral route, diluted with vegetal oil in final volume of 1 mL according to OECD reglementations. The extract was administered by enteral route in 200 mg dried substance/kg. b.w., daily during seven days. In animals from CCl_4+E, the extract was administered immediately after carbon tetrachloride administration.

Biochemical, morphopatological and hematological analyses

At the end of the experiment, the animals were deeply anesthetized with ketamine-xylazine (ketamine: 50mg/kg; xylazine: 10 mg/kg body weight, i.p.). Blood samples from retro-orbital plexus were collected in EDTA anticoagulant tubes and immediately submitted for hematological analysis. Complete Blood Counts were performed using a differential Abacus Junior Vet automatic analyzer (Diatron Messtechnik).

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For blood biochemistry samples were drown on clot activated vacutainers and the serum was separated by centrifugation and analyzed for the determination of AST and ALT according to Farcaș *et al.,* (2018). Haematoxylin and eosin (H&E) staining was performed, as described previously (Toma *et al.,* 2017). Briefly, following deparaffinization in xylene (Lachner Chemicals, Czech Republic) twice for 5 min and hydration in 100% ethanol twice for 5 min, 95% ethanol for 3 min and 70% ethanol for 3 min, the liver sections at 5 µm thickness were stained by H&E protocol according to Roman *et al.,* (2014). The slides were then rinsed in distilled water and differentiated in 95% ethanol for 10 min. Slides were then dehydrated and mounted in synthetic medium. Light microscopy was performed with an Optika B-383LD2 microscope.

Statistics

All results are expressed as mean ± SD. Comparisons between multiple groups were made using a one-way ANOVA followed by Bonferroni`s post-hoc test. $P < 0.05$ was considered statistically significant. Bonferroni`s post-hoc test was considered statistically significant at $P < 0.05$ and was interpreted as follows: $*P < 0.05$, $**P < 0.01$, $***P < 0.001$ when comparisons were made with C group and # $P < 0.05$, ## $P < 0.01$, ### $P < 0.001$ when comparisons were made with CCl_4 group.

Results and discussions

Carbon tetrachloride is a canonic agent for inducing steatosis, inflammation and liver disregulation and is a frequently used biological model for steatohepatitis. After carbon tetrachloride administration, ALT (130 ± 13 U/L) (**Fig.1A**) and AST (380 ± 43 U/L) (**Fig. 1B**) were significantly increased compared to C group ($P < 0.001$) which marked 36 ± 7.2 U/L for ALT and 150 ± 21 U/L for AST. Extract administration significantly reduced ($P < 0.01$) the blood transaminases activity compared to CCl_4 group (58 ± 7.5 U/L for ALT and 160 ± 15 U/L for AST) and the activity was near Control level. Increased blood transaminases activity demonstrated that CCl_4 induced hepatocellular dysfunction marked by high cell membrane permeability and necrosis. These facts were also suggested by increased number of leucocytes (WBC) (**Fig. 2**) and lymphocytes (LYM) (**Fig. 3**) and high level of blood TNFα (**Fig. 4**). Both WBC and LYM were increased ($P < 0.001$) after CCl_4 administration ($17 \pm 1.22*10^9$ leucocytes/L and $11.5 \pm 1.33*10^9$ lymphocytes/L) compared to Control ($7.63 \pm 3.31*10^9$ leucocytes/L and $36 \pm 7.2*10^9$ lymphocytes/L). The extract administration did not show significant changes when was independent administrated but CCl_4+E revealed significant decreasing ($P < 0.01$) of the WBC ($9.04 \pm 2.63*10^9$/L) and LYM ($6.10 \pm 1.43*10^9$/L). Exponential increasing of TNFα suggested that the route of the CCl_4-induced liver inflammation is mediated by TNFα/NF-κB signaling. In C, TNFα was 13.75 ± 3.5 pg/mL, after independent extract administration (E group), 15 ± 2.58 pg/mL, in CCl_4 group, 36.25 ± 6.13 pg/mL and TNFα concentration in CCl_4+E group was 49 ± 6.83 pg/mL. Apparently, the extract demonstrated the beneficial effect on the CCL_4 - intoxicated liver. Histological evaluation in C group (**Fig. 5**), revealed normal structures without any pathological signs. The liver from the E group (**Fig.6**) also revealed normal histological features with normal shape and distribution of the hepatocytes, slight dilation of the sinusoids and low inflammation. However, histopathological evaluation showed that extract administration enhances the lipid degeneration and inflammation of the liver. Centro-lobular steatosis after CCl_4 exposure (**Fig. 7**) associated with high number of the inflammatory cells, outline the histological features of the CCl_4-induced steatohepatitis. Further, the extract administration considerably increased the steatosis and tissue-specific inflammatory reaction (**Fig. 8**) *via* Kupffer cells proliferation and increasing number of infiltrative lymphocytes.

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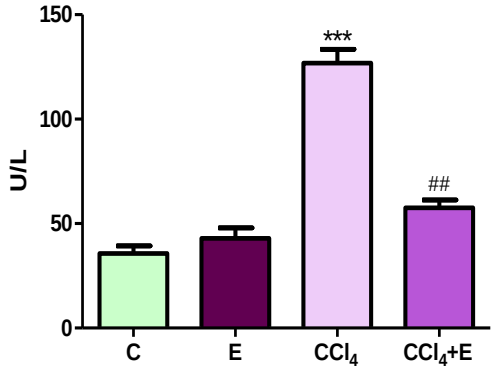

Fig. 1 A ALT variations in C and experimental groups.

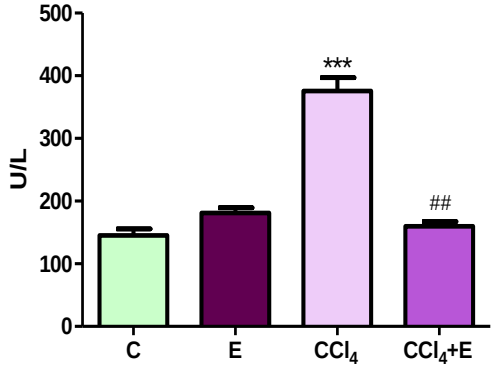

Fig. 1 B AST variations in C and experimental groups.

Carbon tetrachloride increased blood ALT and AST activity and the extract administration apparently normalized the transaminases blood level. However, histopathological evaluation revealed that ALT and AST decreased after extract administration as a result of high liver degeneration which reduced the transaminases passing from liver in blood flow. Values are expressed as mean ± SD. (*) is for comparison with C group and (#) is for comparison to CCl_4 group. $P < 0.05$ was considered statistically significant.

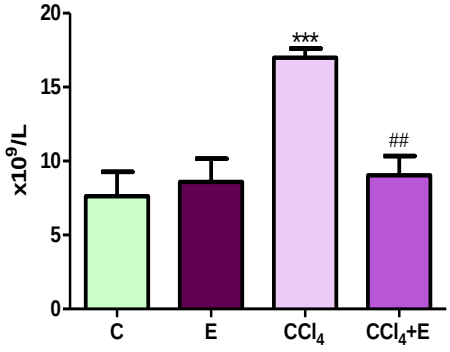

Fig. 2 White blood cells (WBC) counting in rats exposed to CCl4 and in other experimental groups.

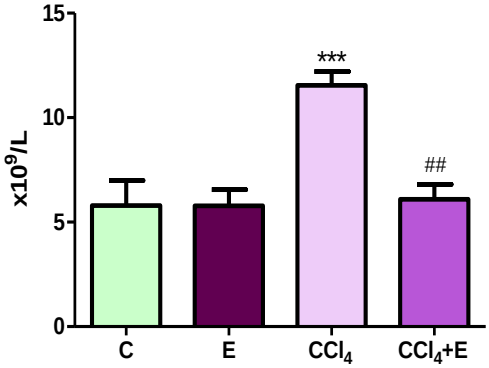

Fig. 3 Lymphocytes (LYM) number in Control and experimental groups.

Cell-mediated inflammatory response after carbon tetrachloride exposure and *M. sylvestris* administration revealed that carbon tetrachloride induced leukocytosis but the extract administration led to normal values of WBC and LYM. The cell-mediated anti-inflammatory effect of the extract may be evaluated in future researches. Values are expressed as mean ± SD. (*) is for comparison with C group and (#) is for comparison to CCl_4 group. $P < 0.05$ was considered statistically significant.

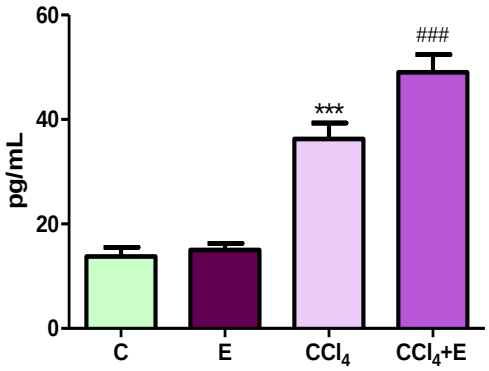

Fig. 4. TNFα variations in Control and experimental groups.

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This figure depicts that cell-mediated anti-inflammatory response is not associated with humoral-mediated inflammatory response. TNFα increasing after extract administration is related with histological features (Fig. 8) which confirmed the high degree of the steatosis in CCl_4+E compared to CCl_4 (Fig. 7). Values are expressed as mean ± SD. (*) is for comparison with C group and (#) is for comparison to CCl_4 group. $P < 0.05$ was considered statistically significant.

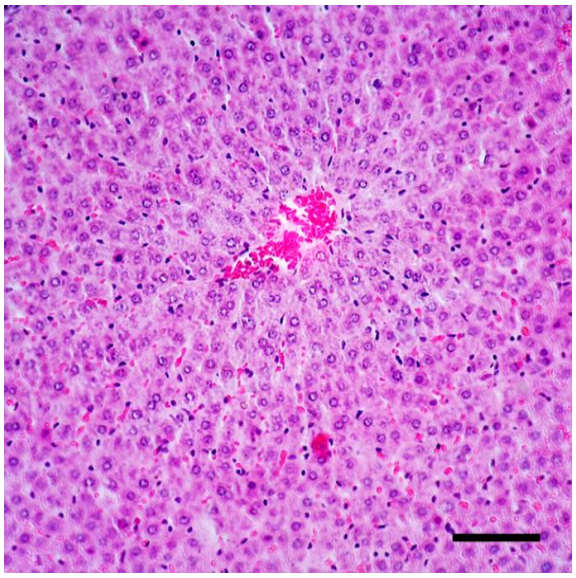

Fig. 5 Liver section in Control group. H&E, x200, scale bar = 20 µm.

In control group, normal histological appearance of the centrolobular area which contains erythrocytes and normal peripheral Kupffer cells was observed. Hepatocytes have normal nucleus shape, without any pathological changes.

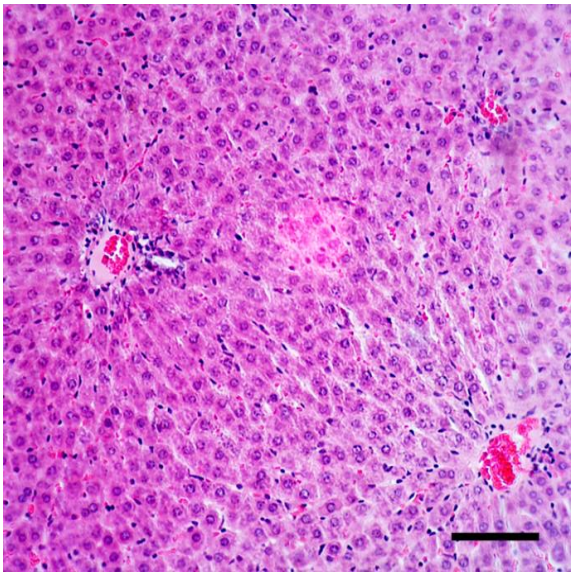

Fig. 6 Histological aspect of the liver in E group, after 7 days of the treatment. H&E, x200, scale bar = 20 µm.

The *M. sylvestris* extract did not induced prominent histological changes but a slight inflammation was detected. However, the biochemical and hematological evaluation has not detected pathological changes related to liver function.

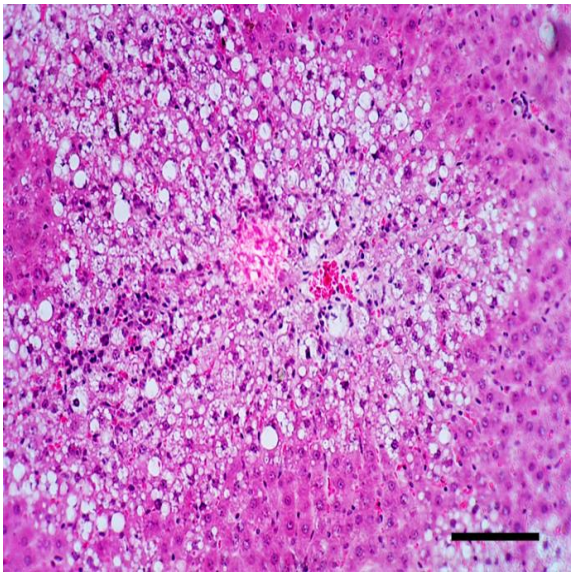

Fig. 7 CCl_4 induced steatohepatitis with centrolobular propensity. H&E, x200, scale bar = 20 µm.

Hepatocytes with prominent lipid degeneration, Kupffer cells proliferation, hepatocytes necrosis is related to blood biochemical (transaminases, TNFα) and cellular (WBC, LYM) variations, the well-

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known histological features of CCl_4-induced liver toxicity.

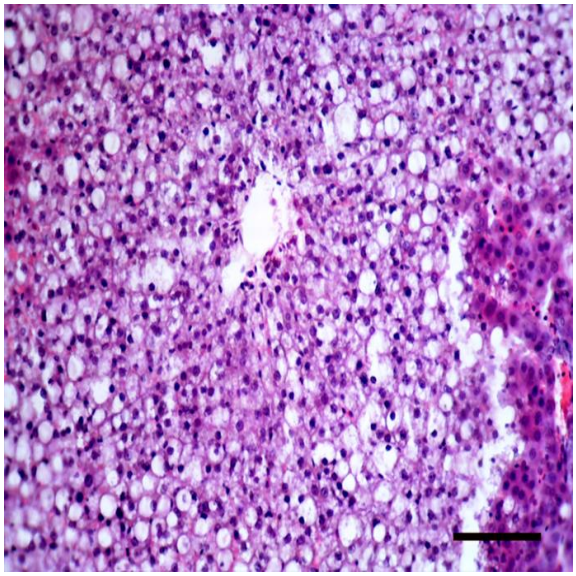

Fig. 8 Liver section in CCl_4+E group. H&E, x200, scale bar = 20 µm.

Co-administration of CCl_4 and Extract induced very prominent steatohepatitis which was not restricted in centrolobular area as in CCl_4 group. Lipid degeneration was extended from centrolobular area to portal system and liver parenchyma.

Discussion

During the last 50 years, the biological model for steatosis and liver inflammation presented in our experiment was used and improved in a large body of papers (Xiang *et al*., 2017; Dong *et al*., 2016; Itoh *et al*., 2010; Masuda, 2006; Janbaz *et al*., 2002; Sipes *et al*., 1991; Hove and Hardin, 1951). Carbon tetrachloride diluted in vegetal oil and administered in rats or mice by gavages or injections has induced hepatotoxicity, kidney failure, anemia, local necrosis of the gastro-intestinal tract and also neuronal damages (Kader, 2017; Rusu *et al*., 2005). Moreover, some studies noticed that carbon tetrachloride induced liver, kidney and brain toxicity in pups whose mothers were exposed to CCl_4 and that demonstrates the passing of the CCl_4 metabolites through placenta and then in milk composition (Chen *et al*., 2017). Alongside, the pleiotropic effects of the carbon tetrachloride were used in order to investigate the actions of different compounds with biological or synthesis origin for the purpose of establishing new therapeutically properties in hepatotoxicity, kidney failure or cerebral damages conditions according to Janbaz *et al*., (2002). However, some studies which tested plant extracts with complex mixture of antioxidants such as chlorogenic acid, galic acid, luteolin, rutin or vitamin A, did not suggested the extract matrix toxicity.

Our results demonstrated that carbon tetrachloride has induced inflammation by activating TNFα signaling and increasing leucocytes/lymphocytes reaction in accordance with Mitazaki *et al*., (2018) which also obtained similar results in ovarectomized rats. The extract administration in many studies improved liver function by decreasing AST and ALT activity, down regulation of TNFα and normalized blood triglycerides concentration (Stojiljković *et al*., 2016 a). When morphopatological evaluation of the liver does not associate with biochemical and/or hematological parameters, the risk of wrong conclusion is imminent. Our results demonstrated that antioxidants from *M. sylvestris* extract were not enough to counteract the invasive actions of the carbon tetrachloride metabolites. In other experiments (Mihailović *et al*., 2018), a simple alcoholic extract of *M. sylvestris* fruits exerted antioxidant effects in an independent manner of extract matrix (ethanol). Based on the presented results, the same antioxidant mixture but in a different matrix composition (ethanol and glycerin) stimulated the hepatotoxicity of the carbon tetrachloride probably by glycerin contribution in free fatty acids esterification. The level of cellular polyunsaturated fatty acids affects the level of lipid peroxidation and is likely to be proportional with the level of lipid droplets (containing triglycerides) accumulated in the cell according to Ding *et al*., (2010). Independent administration of CCl_4 has induced centrolobular steatosis, liver inflammation and hepatocytes necrosis. More prominent steatohepatitis, than in CCl_4 group, was seen after CCl_4 exposure and Extract administration. Liver degeneration was enhanced in rats exposed to CCl_4+Extract compared to CCl_4 group and that is the reason of transaminases decreasing after extract administration. AST and ALT activity were

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not related to liver-improved function, even if AST and ALT were near Control values.

Our experimental data suggested that glycerin-ethanol extracts are not indicated in non-alcoholic steatohepatitis and antioxidants from *M. sylvestris* were not efficient in order to protect liver tissue against CCl_4 toxicity. However, the extract decreased the cell-mediated immune response in the context of liver degeneration and inflammation and maintained the TNFα signaling which appeared as the main inflammation pathway of the CCl_4-induced steatohepatitis according to Mitazaki *et al*., (2018).

Conclusions

Glycero-alcoholic *Malus sylvestris* (L.) Mill. extract is rich in antioxidants, but our study has proven the main disadvantage of its matrix. Ethanol and glycerin together alongside with liver toxicity induced by CCl_4 metabolites lead to an enhanced liver degeneration, which cannot be reversed by the antioxidants within the extract.

Acknowledgements

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