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ORIGINAL ARTICLE

Protective effect of betulin and betulinic acid on acetaminophen and ethanol-induced cytotoxicity and reactive oxygen species production in HepG2 cells

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Abstract: Natural triterpenoids such as ursolic and oleanolic acids have been detected to suppress enzymes which play a role in liver damage, e.g. cytochrome P450, cytochrome b5, CYP1A and CYP2A, and to increase the antioxidant substances glutathione, metallothioneins, and glutathione-S-transferase, with simultaneous protective effects on liver mitochondria.

To test the hypothesis that plant triterpenes may also induce mechanisms leading to reduced production of reactive oxygen species after induction with ethanol and acetaminophen, the cytoprotective effect of two plant triterpenes, betulin and its oxidized form, betulinic acid, was compared. Acetaminophen alone at concentrations between 25-250 µg/ml exhibited dose-dependent cytotoxicity for HepG2 cells. When a mixture of 25 µg/ml of acetaminophen was mixed with different concentrations of 5-50 mM of ethanol, an additional increase in the toxicity was observed, depending on the ethanol concentration used. Acetaminophen alone was detected as a strong inducer of superoxide anion in HepG2 cells. Moreover, the mixture of acetaminophen with ethanol induced significantly more superoxide anion than acetaminophen and ethanol alone. When betulin or betulinic acid were preincubated with HepG2 cells the level of superoxide anion and hydrogen peroxide was significantly lower than in respective controls. Betulin, in comparison to betulinic acid, was a stronger inhibitor of oxidative burst.

Key words: betulin, betulinic acid, ethanol, acetaminophen

INTRODUCTION

Herbal medicines derived from plant extracts are being increasingly utilized to treat a wide variety of diseases, despite relatively little knowledge of their modes of actions.

Triterpenes are aliphatic polycyclic compounds based on a skeleton of 30 carbon atoms, and are synthetized in plants from squalene as the precursor [1]. Triterpenes have multiple biological effects, including anti-inflammatory, antitumour and immunoregulatory activities [2].

Oleanolic acid has been described as being protective against acute hepatotoxicity carbon tetrachloride and acetaminophen [3, 4]. Several studies have demonstrated that hepatotoxicity of cadmium can be reduced by plant triterpenes such as oleanolic acid [5], ursolic acid [6] and betulin [7]. The exact mechanism of hepatoprotective activity of triterpenes has not yet been described, but it may be connected with induction of metallothioneins or mediated by unknown proteins newly synthetized in cells under triterpene induction [8]. Moreover, this protective effect seems to be, at least partially, dependent of the cytotoxic factor, as ethanol or cadmium [9, 10]. One may speculate that all the above-mentioned hepatotoxic agents may act, at least in part, by generation of reactive oxygen species. The results of experiments by Yamashita et al. [11], in which lupeol, betulin and betulinic acid were potent inhibitors of stimulus-induced superoxide anion generation in human neutrophils, seem to confirm this supposition.

Acetaminophen (APAP) overdose is the most frequent cause of acute liver failure in the USA and many European countries [12]. The initial step in toxicity is cytochrome P-450 (including CYP2E1 isoform) metabolism to the reactive metabolite N-acetyl-p-benzoqinone imine (NAPQI). At therapeutical doses, NAPQI is efficiently detoxified by glutathione (GSH). In overdose, the conjugation of reactive metabolite with GSH leads to GSH depletion and NAPQI covalently binds to proteins to form acetaminophen adducts, leading to mitochondrial dysfunction with the formation of reactive oxygen species (ROS) and peroxynitrite [13]. The oxidant stress is ultimately responsible for the opening of the mitochondrial membrane permeability transition (MPT) pore and necrotic cell death [14]. Several case reports indicate that alcoholics experienced hepatotoxicity, or have even died from liver failure due to exposure to APAP [15]. The increased susceptibility of alcoholics is supposedly due to induction of liver microsomal enzymes by ethanol with increased formation of the toxic metabolite of APAP. The antidote for APAP poisoning is N-acetyl cysteine (NAC), which is a precursor of gluthathione, so that it increases the available gluthathione for conjugation with NAPQI. It also has an anti-inflammatory and antioxidant effect. NAC increases local nitric oxide (NO) concentrations, causing a vasodilatory effect on the microcirculation that enhances oxygen delivery to the peripheral tissues [16]. It has recently been described

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that commonly used antioxidants protect hepatocytes against acetaminophen-induced cytotoxicity [17].

The protective effect of betulin and betulinic acid against ethanol-induced cytotoxicity in HepG2 cells has already been described [18]; however, the effect of betulin and betulinic acid on acetaminophen-induced cytotoxicity has not been examined.

To test the hypothesis that plant triterpenes may induce in cells a mechanism leading to reduced production of reactive oxygen species, the cytoprotective effect of two plant triterpenes, betulin and its oxidized form, betulinic acid, was compared. Except for the protective effect of triterpenes on acetaminophen-induced cytotoxicity in HepG2 cells, especially in the presence of ethanol, their influence on the acetaminophen-induced generation of superoxide anion and hydrogen peroxide in HepG2 cells was estimated.

MATERIAL AND METHODS

Cell cultures. The human hepatoma HepG2 cell line obtained from American Type Culture Collection (Manassas, VA, USA) was cultured in Eaglea's Medium (MEM), supplemented with 10% heat-inactivated foetal calf serum (FCS), 2 mM L-glutamine, 1% nonessential amino acids (NEAA), 1.5 g/L sodium bicarbonate, and 1% Antibiotic-antimycotic, pH 7.4. The cells were seeded in tissue culture plates (Falcon, Bedford, MA, USA) and incubated at 37°C in a humidified atmosphere with 5% CO_2. HepG2 cells were subcultured twice a week by trypsinization in 0.25% trypsin-EDTA solution after washing with Ca-Mg-free saline. Culture media and antibiotics were purchased from Gibco (Grand Island, NY, USA), 0.25% trypsin-EDTA, FCS and NEAA were obtained from Sigma-Aldrich (Steinheim, Germany). In some experiments, Hanks' Balanced Salt Solution (HBSS) (Sigma-Aldrich) was used.

Treatment of HepG2 cells with acetaminophen, ethanol and triterpenes (betulin and betulinic acid). HepG2 cells were grown in 96-well plastic plates (Nunc, Roskilde, Denmark), 4×10^4 cells/well. After 24 h incubation, the medium was replaced with a fresh one with an addition of different acetaminophen (0-500 μg/ml), or ethanol (5-50 mM) concentrations. Acetaminophen and ethanol were purchased from Merck (Darmstad, Germany). The cells, treated with acetaminophen or ethanol, were maintained in a humidified atmosphere with 5% of CO_2, at 37°C for 24 h. The toxicity of the substances used was determined by MTT assay, as described earlier [13, 14]. HepG2 cells were also preincubated with 10 μM betulin or 1 μM betulinic acid for 24 h followed by challenge with acetaminophen or ethanol. Such a model allowed evaluation of the possible protection against acetaminophen and ethanol by pretreatment with triterpenes. After 24h of incubation at 37°C, cell viability was measured by the MTT method.

Measurement of superoxide anion (O_2^-) production by the cytochrome c reduction assay [19]. HepG2 cells were grown in 96-well plastic plates (4×10^4 cells/well) with or without triterpenes. After 24 h incubation at 37°C, the cultures were washed twice with HBSS and an assay for the superoxide anion was performed. Briefly, HBSS (207.5 μl), 12.5 μl of cytochrome C solution in HBSS (final concentration of 75 μM), 5 μl of either SOD solution (final concentration of 60 U/ml) or 5 μl of distilled water, and 25 μl acetaminophen solution in HBSS (final concentration 25 or 100 μg/ml) with or without ethanol was added into each well on the 96-well plate. Control wells were also used, in which the cells were incubated without oxidative burst inducers. The microplate was incubated at 37°C for 60 min and transferred to the microplate reader. The absorbance values at 550 nm (the differences in OD between samples with and without SOD) were converted to nanomoles of O_2^- based on the extinction coefficient of cytochrome C: $\Delta E_{550} = 21 \times 10^3\ M^{-1}cm^{-1}$. The results were expressed as nanomoles of O_2^- per 1×10^6 cells per 60 min. All chemicals were purchased from Sigma Aldrich.

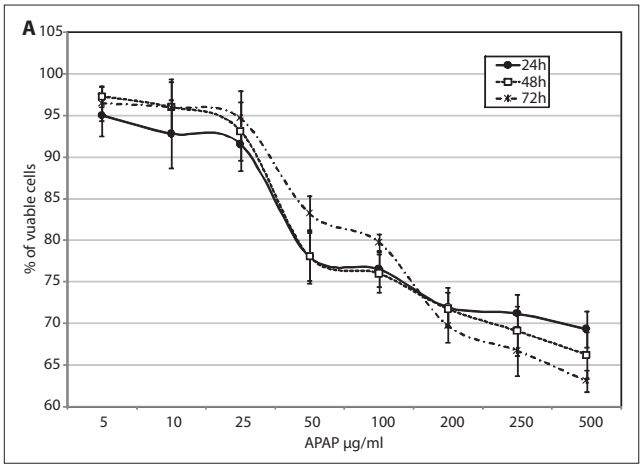
Measurement of hydrogen peroxide (H_2O_2) production [19]. HepG2 cells grown in 96-well plastic plates (4×10^4 cells/well) with or without triterpenes. After 24 h incubation at 37°C, the cultures were washed twice with Hanks' Balanced Salt Solution (HBSS), and the medium replaced with HBSS (100 μl/well), with an addition of acetaminophen solution in HBSS (final concentration 25 or 100 μg/ml) with or without ethanol for 60 min at 37°C. In control wells the cells were incubated without acetaldehyde. Those wells were washed twice with HBSS and the measurement of intracellular hydrogen peroxide was performed. The assay was based on horseradish-dependent peroxidation (HRPO) of phenol red by H_2O_2 leading to the formation of a compound that exhibited absorbance at 600 nm. Briefly, the cells were covered with 100 μl/well of the assay solution prepared on the day of the experiment, and consisted of HBSS, phenol red (Sigma, final concentration of 0.56 mM), HRPO (Serva, Heidelberg, Germany, final concentration of 20 U/ml). Additionally, 10 μl/well of 1N NaOH was added. After 3 min of incubation, the plate was read at 600 nm in the microplate reader. The results were expressed as nanomoles H_2O_2 per 10^6 cells per 60 min based on the phenol red extinction coefficient ($\Delta E_{600} = 19.8 \times 10^3\ M^{-1}cm^{-1}$).

Statistical analysis. Statistical analysis was performed using Statistica software (version 6.0). Values were expressed as mean ± S.D. The significance of differences was determined with the use of an analysis of variance, two-way ANOVA test with post hoc Tukey's test. The Wilcoxon's paired test was used for comparisons within groups. p values <0.05 were considered to be significant.

RESULTS

Toxicity of acetaminophen and the mixture of acetaminophen with ethanol in HepG2 cells. Acetaminophen (APAP) alone at concentrations between 25-500 μg/ml exhibited dose-dependent cytotoxicity for HepG2 cells (Figure 1A). When 25 μg/ml of acetaminophen was mixed with different concentrations 5-50 mM of ethanol, an additional increase in the toxicity was observed, depending on the ethanol concentration used (Figure 1B).

Induction of reactive oxygen species (ROS) in HepG2 cells after treatment with acetaminophen and ethanol. The influence of betulin and betulinic acid. Acetaminophen alone was detected as the strong inducer of superoxide anion as well as hydrogen peroxide in HepG2 cells.



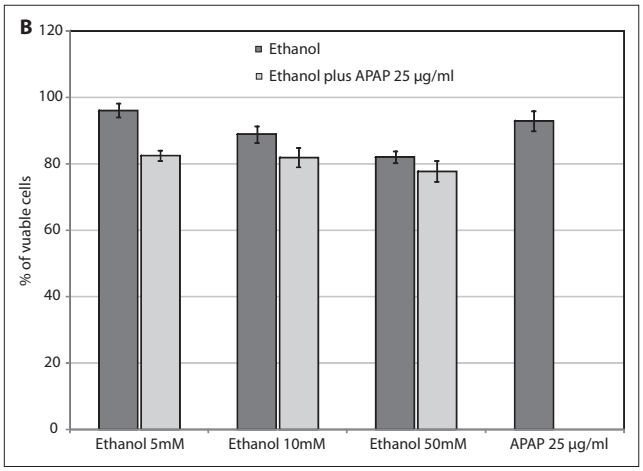
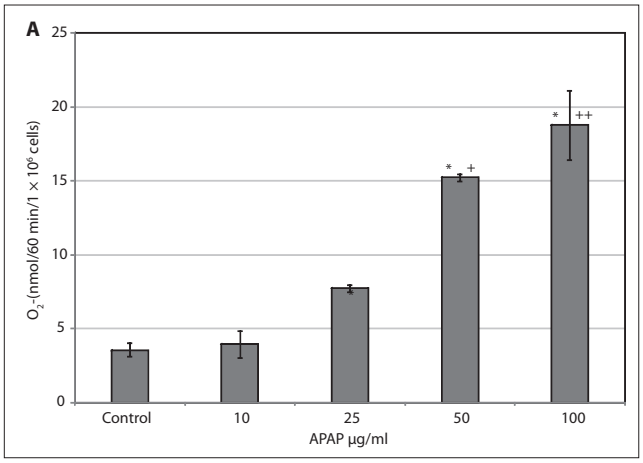


Figure 1 Influence of acetaminophen (APAP) alone (**A**) or mixed with ethanol (**B**) on HepG2 cells viability. After 24-72 h of incubation, HepG2 cells with different acetaminophen concentration (**A**) or after 24 h of incubation HepG2 cells with mixture ethanol and acetaminophen in indicated concentrations (**B**), the toxicity was determined by the MTT method. Values are means ± SD of results from 5 experiments.

The ROS production depended on the APAP concentration (Figure 2A, Figure 3A). Moreover, when 25µg/ml of acetaminophen was used together with ethanol, this mixture induced more superoxide anion than acetaminophen alone (Figure 2B). H_2O_2 production was slightly increased when a mixture of APAP and ethanol was used; however, the statistical difference was observed only after using the compound with the highest acetaminophen and ethanol concentration (Figure 3B).



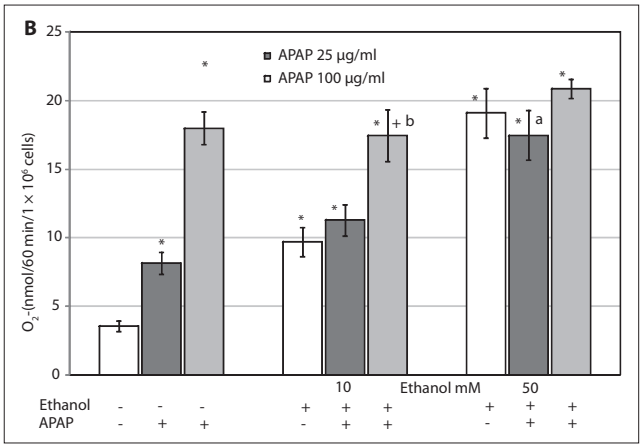
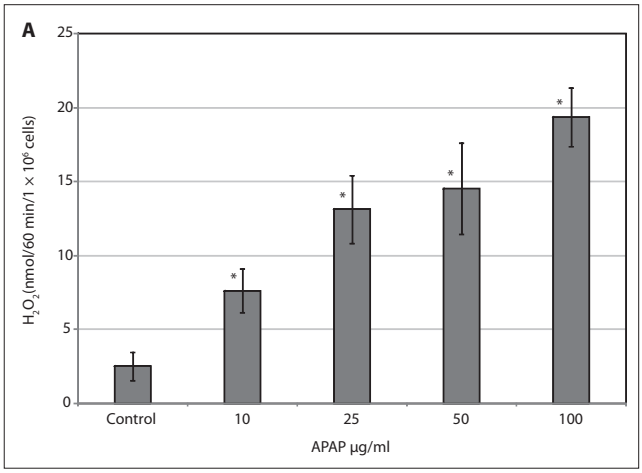


Figure 2 Superoxide anion (nmol/60 min/1 × 10^6 cells) production after acetaminophen alone (**A**) or mixed with ethanol (**B**) treatment of HepG2 cells. Results are expressed as mean ± SD of 4 independent experiments.
* Significantly different from respective control (cells incubated without acetaminophen or ethanol), p≤0.05 (Wilcoxon test).
+ Significantly different in comparison to cells treated with 25µg/ml APAP, p≤0.05.
++ Significantly different in comparison to cells treated with 50µg/ml APAP, p≤0.05.
[a] Statistically different in comparison to 25µg/ml APAP used alone, p≤0.05.
[b] Statistically different in comparison to 10mM ethanol used alone, p≤0.05 (two-way ANOVA).



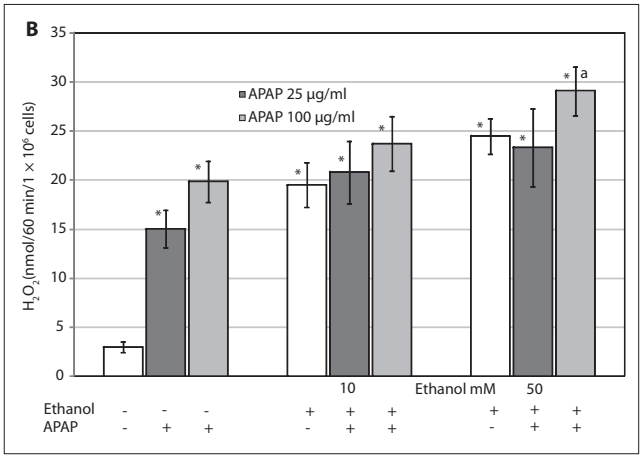


Figure 3 Hydrogen peroxide (nmol/60 min/1 × 10^6 cells) production after acetaminophen alone (**A**) or mixed with ethanol (**B**) treatment of HepG2 cells. Results are expressed as mean ± SD of 4 independent experiments.
* Significantly different from respective control (cells incubated without acetaminophen or ethanol), p≤0.05 (Wilcoxon test).
[a] Statistically different in comparison to 100µg/ml APAP used alone, p≤0.05 (two-way ANOVA).

When HepG2 cells were preincubated with betulin (1 or 10μM) or betulinic acid (1μM) the level of superoxide anion production induced with APAP or ethanol was significantly lower than in respective controls (Figure 4A). Betulin in 1μM concentration more effectively inhibited the superoxide production than in 10μM; therefore, in the next experiment this higher betulin concentration was omitted. Both betulin and betulinic acid effectively inhibited hydrogen peroxide production in HepG2 cells after acetaminophen and ethanol treatment; however, in comparison to betulinic acid, betulin was a stronger inhibitor of oxidative burst (Figure 4B).

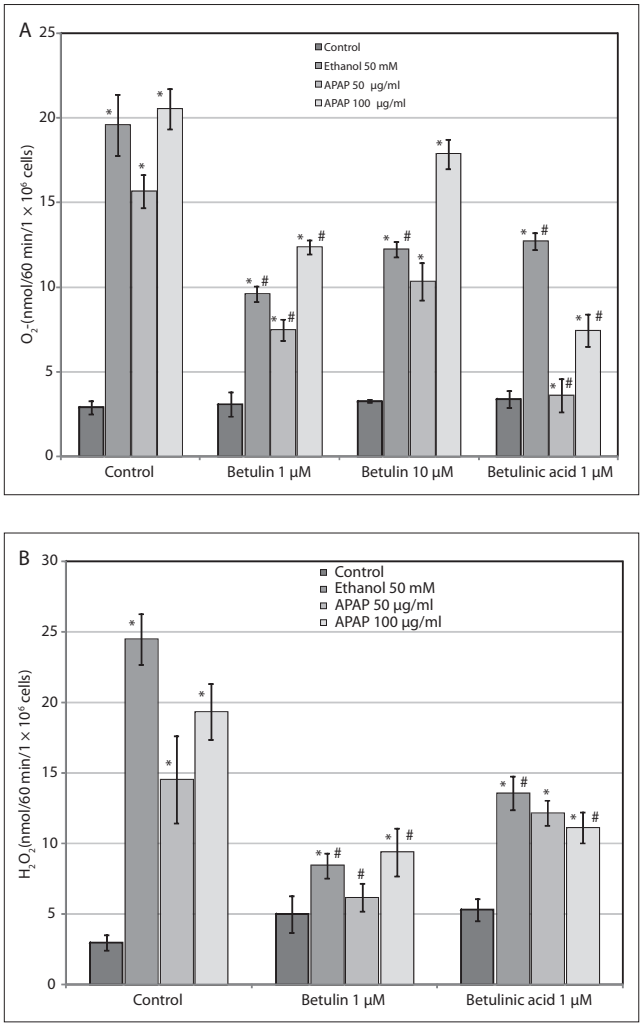


Figure 4 Preincubation of HepG2 cells for 24 h with betulin or betulinic acid inhibits ethanol- (50 mM) or acetaminophen- (50 or 100μg/ml) induced superoxide anion (**A**) or hydrogen peroxide (**B**) production.
Results are expressed as mean ± SD of four independent experiments.
*Significantly different from respective control (cells incubated without ethanol, acetaminophen and a triterpene or treated only with a triterpene), p≤0.05.
#Statistically significant in comparison to cells treated with ethanol or acetaminophen alone, p≤0.01. The triterpenes significantly changed the ethanol effect, p≤0.01 (two-way ANOVA).

DISCUSSION

It is well established that in hepatocytes, ethanol is predominantly metabolized by cytosolic alcohol dehydrogenase (ADH) to acetaldehyde, which is further metabolized to acetate by the mitochondrial aldehyde dehydrogenase (ALDH) in the presence of NAD^+ as a cofactor. However, ethanol can be metabolized to acetaldehyde and subsequently to acetate by a microsomal ethanol-inducible cytochrome P4502E1 or peroxisomal catalase. The metabolic disturbances associated with ethanol oxidation and oxidative stress are the most important in hepatic injury. Some of these alterations depend on changes in the redox state due to NADH generation via the ADH pathway. Furthermore, the induction of cytochrome P4502E1 contributes to acetaldehyde generation, protein adduct formation, enzyme inactivation, decreased DNA repair, reduced liver glutathione and lipid peroxidation [20].

As shown in Figure 2B, ethanol induced in a dose-dependent manner superoxide anion production in HepG2 cells. Triterpenes alone at the concentration used in this experiment did not induce significant levels of superoxide anion. However, when betulin or betulinic acid were present in the culture medium 24 h before addition of ethanol, they caused a significant decrease in the amounts of superoxide anion, as well as hydrogen peroxide produced by HepG2 cells.

In the present work, the production of ROS was enhanced when cells were exposed to acetaminophen (APAP) and ethanol. The part of ROS can be a product of acetaminophen cytotoxicity, but in part was due to ethanol metabolism. In vivo it was established [21] that liver damage induced by a acetaminophen occurs when the ability of the cells to detoxify the toxic metabolite N-acetyl-benzoquinone-imine is exceeded. Ethanol seems to enhance the toxicity of acetaminophen by induction of cytochrome CYP2E1. Furthermore, in human beings, depletion of GSH as the result of inhibition of GSH synthesis by ethanol enhances susceptibility to acetaminophen toxicity [15]. It has been detected that acetaminophen toxicity in mice is accompanied by an increase in another ROS; this means NO synthesis and the formation of nitrotyrosine-protein adducts [22, 23].

Very strong evidences that both ethanol and acetaminophen can be metabolized by CYP2E1 was produced by the experiments of Cheung et al. in 2005 [24], who observed that in CYP2E1 humanized transgenic mice, after treatment with acetaminophen there was a significant elevation in serum alanine aminotransferase level, an increase in hepatocyte necrosis, and a decrease in P450 levels. It is also known that CYP2E1 enzyme metabolizes and activates a wide array of toxicological substances, including ethanol, acetaminophen, acetone, benzene, halothane or dimethylhydrasine [24]. In all of these substrates, metabolism is connected with the generation of ROS, which participates in cell injury. We therefore suspected that in our experiment the toxicity observed in HepG2 cells after acetaminophen treatment, especially with the mixture of acetaminophen and ethanol, and production of superoxide anion, as well as hydrogen peroxide, were connected mainly with the metabolism of both substances via cytochrome P450, particularly by its isoform CYP2E1. 200μg/ml is a sufficient acetaminophen concentration in sera for antifever effect, which is not toxic for the liver. This medicine concentration in sera is achieved 4 h after taking the recommended doses of acetaminophen [25]. However, as we show in our research, even smaller doses of acetaminophen, especially mixed with ethanol, cause hepatic oxidative stress.

Both betulin and betulinic acid have been described as being inhibitors of ROS production in human neutrophils [11]. Moreover, they were also described as inhibiting ROS production by ethanol-activated hepatic stellate cells. The mechanism of triterpene action on stellate cells was connected

with their influence on cellular signaling mechanisms, including JNK, p38 MAPK and NFκB pathways [data not yet published]. We therefore can speculated that similar mechanisms are also involved in the protective effect of triterpenes on acetaminophen and ethanol activated HepG2 cells. Confirmation of these supposition needs further examination.

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