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Standardized bioactive fraction of *Phaleria macrocarpa* (Proliverenol) prevents ethanol-induced hepatotoxicity via down-regulation of NF-κB-TNFα-caspase-8 pathway

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ABSTRACT

Objective: To verify that Proliverenol has a potential ability in protecting cells from ethanol-induced hepatotoxicity.
Methods: Activity of Proliverenol against ethanol-induced apoptosis was evaluated at mRNA and protein levels in HepG2 cell exposed to Proliverenol for 1 and 3 h.
Results: Proliverenol conferred hepatoprotective activity through increasing cell survival up to 53%–69% via up-regulation of APEX1 DNA repair enzyme for 3.0–4.7 fold and down-regulating of nuclear factor-κB, tumor necrosis factorα and caspase-8 expression, allowing them to prevent 4.5–6.9 fold of alanine aminotransferase (ALT) leakage in HepG2 cells. Our finding revealed that Proliverenol repressed expression of ALT, which is significantly important as possible alternative mechanism for increased blood transaminase activities. In addition, the result also showed that caspase-8 pathway seemed to be involved in the molecular pathway rather than directly inducing mitochondrial damage.
Conclusions: The data support our hypothesis that Proliverenol has a potential ability in protecting cells from ethanol-induced hepatotoxicity. We propose that Proliverenol provides hepatoprotective activity through up-regulating expression of APEX1 that repress DNA fragmentation, and down-regulating expression of nuclear factor-κB, tumor necrosis factorα and caspase-8, which therefore repress ALT leakage and its expression.

1. Introduction

Liver is considered as one of the most vital organs that act as the center of metabolism of nutrients and excretion of waste products. Its function includes detoxification, protein synthesis, and production of biochemical compounds for digestion as well as synthesis and breakdown of small and complex molecules. These activities are necessary for normal body functions. Injury to liver cells is induced by various toxic chemicals such as antibiotics, chemotherapeutic agents, carbon tetrachloride, thioacetamide, D-galactosamine/lipopolysaccharide, microbes, and consumed alcohol [1–5]. Excessive alcohol consumption is known as the major common cause for liver disease. Despite much effort has been addressed to understand alcoholic liver disease in the past decades, there remains no effective therapy fot the disease [6]. This limitation gives considerable interest to search for useful, safe and effective hepatoprotectors of herbal origin.

Nowadays, herbal drugs have become increasingly popular in the treatment of liver diseases. *In vitro* cytotoxicity of plant extracts and bioassay guided fractions have also gained importance for drugs primary level screening. Several plant and fungi extracts have been reported to possess therapeutic properties to treat liver disease, *i.e. Sida cordifolia* [7], *Adina cordifolia* [8], *Feronia limmonia* [9], *Silybum marianum* [10], *Trigonella foenum* [11], *Phyllantus emblica*, *Camellia sinensis*, *Mangifera indica*, and *Punica granatum* [12]. These extracts showed different mechanisms in providing hepatoprotective effect. However, exploring the anti-oxidant activity of extracts through the mitochondrial pathway is the most adapted approach.

Proliverenol is a standardized bioactive fraction of *Phaleria macrocarpa* (Scheff.) Boerl (*P. macrocarpa*). In this study, we evaluated the effect of Proliverenol as a hepatoprotector in HepG2, a human hepatoma cell lines and reported here the molecular mechanism by which Proliverenol exerts its hepatoprotective action in the cultured hepatoma cells.

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2. Materials and methods

2.1. Preparation of Proliverenol

Proliverenol was bioactive fraction extracted from *P. macrocarpa* fruit. The *P. macrocarpa* fruit originated from Central Java, Indonesia. The plant has been identified by the Herbarium Bogoriense, Research Center of Biology, Indonesian Institute of Sciences with certificate No. 1261/IPH.1.02/If.8/XII/2009. Slices of *P. macrocarpa* fruit were macerated using water based solvent. The extract was filtrated, concentrated, dried in a conventional oven and then stored in a well closed container.

Identification of Proliverenol was done on a thin layer chromatography plate silica gel 60 F_{254} using ethyl acetate: aceton: formic acid: water (8:2:1:1, v/v) as the eluent. The reagent was purchased from Merck (Darmstadt, Germany). The eluent was allowed to migrate on the thin layer chromatography plate for 8 cm. The chromatogram was then observed under UV (λ = 254 nm), in which before derivatization, black band appeared at the $R_f \pm 0.6$ and $R_f \pm 0.8$; while after derivatization by 10% H_2SO_4 in water, yellow and brownish yellow bands appeared at the $R_f \pm 0.6$ and $R_f \pm 0.8$, respectively. The marker compound (band at $R_f \pm 0.6$) was quantified in Proliverenol using high-pressure liquid chromatography Waters® under UV (λ = 254 nm), in an Agilent ZORBAX Extend-C18 (4.6 mm × 250 mm, 5 μm) as a column and acetonitrile and acetic acid (pH 3.3) as mobile phase. The content of the marker compound was not less than 2.5% in Proliverenol.

2.2. Cell culture and activity of Proliverenol

HepG2 cells purchased from ATCC (Virginia, USA) were grown in minimum essential medium-α medium supplemented with 10% newborn calf serum (Gibco, Grand Island, USA), 100 μg/mL penicillin-streptomycin (Gibco, Grand Island, USA), and 1 mmol/L sodium bicarbonate (Gibco, Grand Island, USA) at 37 °C until 80% of confluence was reached. Cells were subcultured in 6-well plates in 2 mL medium or 10 cm Petri dish in 10 mL medium. After 3–5 days (about 80% of confluence), the medium were aspirated and replenished with the serum-free medium for 4 h. The cells were treated with 5% absolute ethanol and then supplemented with 168 μg/mL of Proliverenol for 1 and 3 h. RNA of the control and treated-HepG2 cells was isolated by Trizol Reagent (Invitrogen, CA, USA), and was reverse-transcribed to obtain the cDNA. The protein was isolated with lysis buffer-protease inhibitor complex (Calbiochem, Darmstadt, Germany). The cDNA was used as the template for amplification of gene fragment using PCR and real-time PCR techniques, and the protein was used to measure protein expression by western blotting. The primers used are alanine transferase (ALT) forward, 5′-CTTGCCTGCAGTTCCCTCTG-3′; ALT reverse, 5′-GCTCGAGGCCATGACTCTAC-3′; APEX1 forward, 5′-ATCTCGCGAGCAACGCGGTA; APEX1 reverse, 5′-GAAAGCAGGCCCACCCCACT-3′. All primers were synthesized by First BASE Laboratories (Singapore).

2.3. ALT assay

ALT production of the cells was assayed using ALT assay kit from Abcam (Cambridge, UK). HepG2 cells at a concentration of 1×10^6 cells per well were homogenized in 200 μL ice-cold ALT assay buffer, and then centrifuged 13000 r/min for 10 min to remove insoluble material. The positive control, standard or samples were then mixed with ALT reaction mix (ALT assay buffer, OxiRed probe, ALT enzyme mix and ALT substrate) and the absorbance was measured at 570 nm (A_1) at T_1 ($T_1 > 10$ min) then again (A_2) at T_2 after incubating the reaction at 37 °C which occurred after initial lag phase. Optical density of the color generated by oxidation of pyruvate was calculated using equation $A_2–A_1$.

2.4. Caspase-8 assay

Caspase-8 of the cells was assayed using caspase-8 human assay kit from Abcam (Cambridge, UK). HepG2 cells at a concentration of 5×10^6 cells per well were harvested and lysed with lysis buffer for 1 h at room temperature with gentle shaking and then centrifuged at 4000 r/min for 15 min. Each 50 μL of sample diluent, samples and detection antibody were loaded into the wells. After incubation at room temperature for 2 h, the liquid was removed and wells were washed using the washing buffer. Anti-rabbit IgG-HRP (100 μL) was added to the wells, which was then incubated for 1 h at room temperature and washed again. 3,3′,5,5′-Tetramethylbenzidine substrate (100 μL) was added into all wells, and incubated for 15 min at room temperature. The reaction was stopped by adding 100 μL stop solution. The absorbance was then measured at 450 nm.

2.5. Cell cycle analysis

HepG2 cells at a concentration of 1×10^4 were harvested by centrifugation for 5 min at 2500 r/min, and fixed overnight in absolute ethanol at 4 °C. The cells were then stained with 1 mL propidium iodide complex [10 mg/mL RNase, 20% NP40, 10 mg/mL propidium iodide (Merck, Darmstadt, Germany)] for 30 min in the dark room. Cell population was measured using FACSCalibur (BD Biosciences) and further analyzed using CellQuest Software (BD Biosciences).

2.6. DNA fragmentation assay

DNA fragmentation of the cells was assayed using apoptotic DNA ladder detection kit from Roche Applied Science (Illinois, USA). HepG2 cells at a concentration of 5×10^5 were harvested by centrifugation for 5 min at 2500 r/min. Cell pellet was suspended with 200 μL phosphate-buffered saline and then mixed with 200 μL of lysis buffer prior to incubation for 10 min at room temperature. Isopropanol (100 μL) was added into the tubes and the mixture was vortexed. The liquid was transferred into filter tube and centrifuged at 8000 r/min for 1 min and the supernatant was then removed. The DNA was washed with 500 μL of washing buffer. Liquid was re-transferred into a new tube and mixed with 200 μL of elution buffer. DNA fragmentation was evaluated by electrophoresis.

2.7. Anti-oxidant activity of Proliverenol

Anti-oxidant activity of Proliverenol was evaluated using 2,2-diphenyl-1-picrylhydrazyl (DPPH) assay (Sigma, MO, USA). Proliverenol was mixed with DPPH in a volumetric flask, and incubated for 30 min at room temperature. The percentage

of remaining DPPH was determined using spectrophotometer UV/vis at the absorbance of 517 nm. Ascorbic acid (8.9 μg/mL) was used as positive control.

2.8. Lipid peroxidation assay

Lipid peroxidase activity of the cells was assayed using lipid peroxidation [(malondialdehyde) MDA] assay kit from Abcam (Cambridge, UK). HepG2 cells at concentration of 1×10^6 were homogenized on ice in 300 μL MDA lysis buffer containing 3 μL butylated hydroxytoluene, and then centrifuged at 14000 r/min for 10 min to remove insoluble material. The supernatant (200 μL) from each homogenized sample was placed in a microtube. Series of standard solution or samples were added with 600 μL of thiobarbituric acid solution, incubated at 95 °C for 60 min, and cooled to room temperature in an ice bath for 10 min. Series of standard solution or samples (200 μL) were-transferred to a 96-well microplate for analysis and the absorbance was read at 520 nm.

2.9. Reactive oxygen species (ROS) assay

ROS of the cells was measured using human ELISA kit from Cusabio (Hubei, China). HepG2 cells at a concentration of 1×10^7 were stored overnight at −20 °C and freeze-thawed two times to break up the cell membranes. The cell lysates were centrifuged for 5 min at 9000 r/min and the supernatant was collected. Standard solution or samples (100 μL) were transferred into the wells and incubated for 2 h at 37 °C. The liquid was then removed from each well. Biotin antibodies (100 μL) were added to the wells for 1 h at 37 °C and mixed gently. Liquid was removed and the wells were washed three times with 200 μL washing buffer. HRP-avidin (100 μL) was added into each well and the mixture was incubated for 1 h at 37 °C, and then washed again with the washing buffer. Finally, 3,3′,5,5′-tetramethylbenzidine substrate (90 μL) was added and further incubated for 30 min at 37 °C. The reaction was then stopped by adding 50 μL of stop solutions. Optical density of each well was measured within 5 min using a microplate reader at 450 nm.

2.10. Statistical analysis

The statistical differences between the test and control samples were determined by student's *t*-test using the Stat View software package (Abacus Concepts, Piscataway, NJ, USA). Values were expressed as means ± SD for at least two independent experiments ($P < 0.05$).

3. Results

3.1. Production of liver enzyme downregulated by Proliverenol

In this study, ethanol treatment was found to induce liver cellular damage and cell death in HepG2 cells. ALT has become the most important marker of hepatotoxicity condition across species [13–16]. The result showed that treatment with 5% ethanol increased ALT production in HepG2 cells while application of Proliverenol decreased ALT expression (Figure 1). This result suggested that Proliverenol affected ALT expression at both mRNA (1.3–2.3 fold) and protein (4.5–6.8 fold) levels.

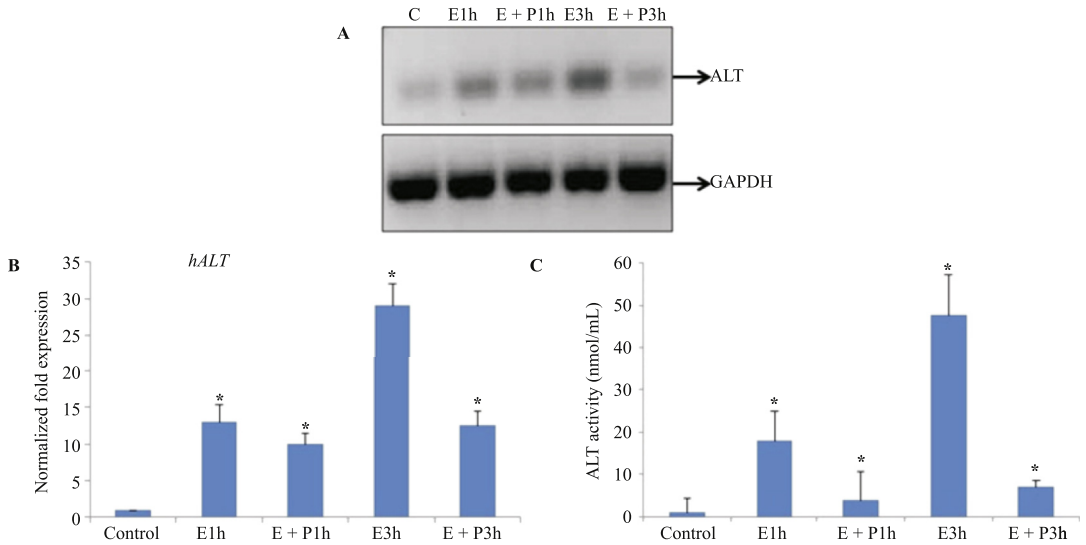

Figure 1. Proliverenol down-regulates ALT expression in ethanol-induced hepatotoxicity in HepG2 cells.
A: ALT mRNA expression level was analyzed using PCR; B: Real-time PCR analysis was performed to monitor the ALT mRNA expression level using commercially available SYBR green dye. ALT mRNA was normalized using β-actin; C: ALT protein expression level in the media was analyzed using ALT assay kit. E1h: EtOH treatment for 1 h; E + P1h: EtOH + Proliverenol treatment for 1 h; E3h: EtOH treatment for 3 h; E + P3h: EtOH + Proliverenol treatment for 3 h. Values are mean ± SD of three samples. *: $P < 0.05$ versus control. GAPDH: Glyceraldehyde 3-phosphate dehydrogenase.

3.2. Ethanol-mediated apoptosis prevented by Proliverenol through down-regulating nuclear factor-κB (NF-κB) pathway

Many regulators are involved in the inflammation-fibrosis-cancer process, and NF-κB pathway appears to have a central function in liver homeostasis [17]. This pathway also involves another signaling molecule, tumor necrosis factorα (TNFα), which initiates cell death processes. Our present study showed that Proliverenol could decrease expression of NF-κB and TNFα that was previously increased by ethanol induction (Figure 2A).

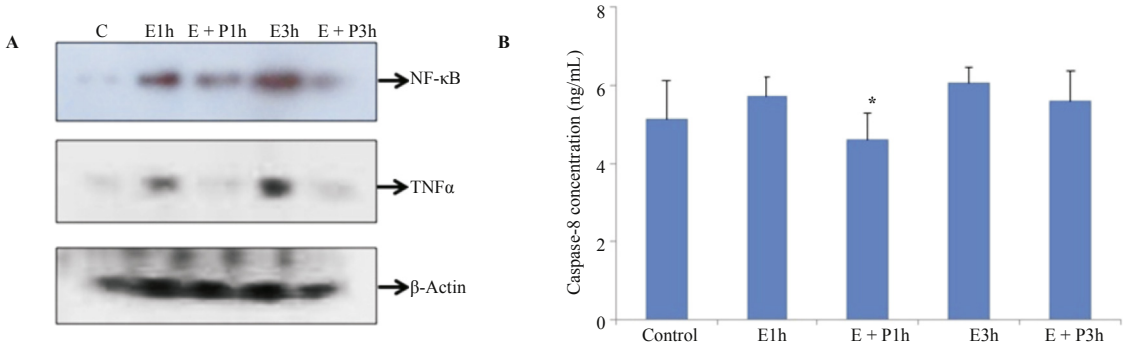

Figure 2. Proliverenol prevents ethanol-mediated apoptosis by down-regulating Fas-NF-κB pathway in HepG2 cells.
A: Down-regulation of NF-κB and TNFα. Intracellular protein was prepared and subjected to western blot analysis to determine NF-κB and TNFα expression level. β-actin was used as an internal control; B: Down-regulation of caspase-8. Values are mean ± SD of three samples. *: $P < 0.05$ versus control.

Involvement of caspase-8 and caspase-9 in prevention of ethanol-mediated cell death by Proliverenol was evaluated. Western blotting analysis of these two caspases showed that caspase-8 seemed to be significantly more involved in the processes rather than caspase-9 (data not shown). The result is in agreement with previous report on the ethanol-induced hepatotoxicity conditions leading to cell death through caspase-8 activity [1].

Furthermore, production of caspase-8 was also analyzed in the control and ethanol-Proliverenol-treated cells. The result showed that 5% ethanol treatment increased caspase-8 production in HepG2 cells and supplementation of 168 μg/mL of Proliverenol decreased its synthesis. This is in agreement with Proliverenol preventing apoptosis resulting from ethanol treatment through repression of caspase-8 production (Figure 2B).

Effect of Proliverenol on the cell cycle profile was evaluated using flow cytometer. The result showed that ethanol treatment (5%) for 1 and 3 h increased the cell cycle arrest and sub-G_1 phase of the cell, which may explain occurrence of apoptosis. Supplementation of 168 μg/mL of Proliverenol decreased sub-G_1 phase percentage from 53.4% to 69.6% (Table 1). This result confirmed that application of Proliverenol had led to protection of the cell against toxic effect of the ethanol.

Table 1
Proliverenol prevents ethanol-mediated apoptosis by decreasing sub-G_1 phase in HepG2 cells.

Treatment	Phases (%)			
	Sub-G_1	G_0/G_1	S	G_2/M
Control	9.45	50.53	9.25	30.76
EtOH 1 h	26.13	36.78	12.99	24.10
EtOH + Proliverenol 1 h	12.17	47.10	10.69	30.05
EtOH 3 h	29.86	37.71	9.67	22.77
EtOH + Proliverenol 3 h	9.08	45.70	11.46	33.76

3.3. DNA fragmentation repressed by Proliverenol through increasing APEX1 gene expression

Hepatic damage as shown by elevation of serum aminotransferase production was correlated with DNA fragmentation [18]. Occurrence of DNA fragmentation in this research was shown in the electrophoresis result. Existence of fragments other than HepG2 genome indicated that the DNA was fragmented by 5% alcohol treatment. The result confirmed that Proliverenol repressed DNA fragmentation induced by 5% ethanol in HepG2 cells (Figure 3A). Interestingly, the result

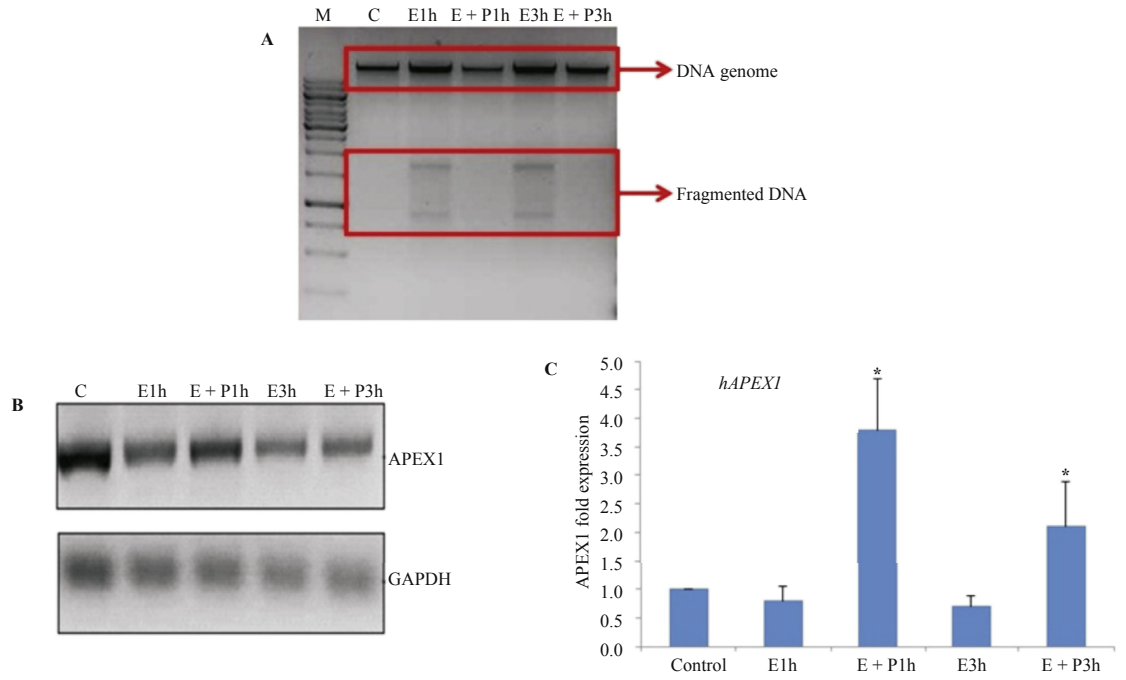

Figure 3. Proliverenol represses DNA fragmentation by increasing *APEX1* gene expression in ethanol-induced hepatotoxicity in HepG2 cells.
A: Proliverenol represses DNA fragmentation induced by ethanol treatment; B: APEX1 mRNA expression level was analyzed using PCR; C: Real-time PCR analysis was performed to monitor APEX1 mRNA expression level using commercially available SYBR green dye. ALT mRNA was normalized using GAPDH. Values are mean ± SD of three samples. *: $P < 0.05$ versus control.

also showed that ethanol-induced DNA fragmentation repeatedly produced two fragments of ~400 and ~600 bp. We hypothesized that ethanol had a specific target on fragmenting the HepG2 DNA genome.

Our previous experiment also clearly showed that expression of APEX1, an enzyme important for hepatoprotection by repairing the destructed DNA, was decreased by 5% ethanol. In contrast, treatment of Proliverenol for 1 h increased the expression of APEX1 (Figure 3B, C). This result suggested that Proliverenol may serve as hepatoprotector through up-regulation of APEX1 expression.

3.4. Anti-oxidant properties of Proliverenol

The ability of most herbal drugs in protecting liver cells was related to their anti-oxidant activities, which further improved hepatoprotection through mitochondrial pathway [5,10,19–21]. Our experiment demonstrated that Proliverenol had strong anti-oxidant activity (Figure 4) thereby capable to protect the cells. Interestingly, the result described that both ethanol and Proliverenol treatments did not significantly affect lipid peroxidation and ROS activities (Figure 5A, B). This result indicated that hepatoprotective activity of Proliverenol in HepG2 cells may not be through mitochondrial pathway (lipid peroxidase, ROS and caspase-9).

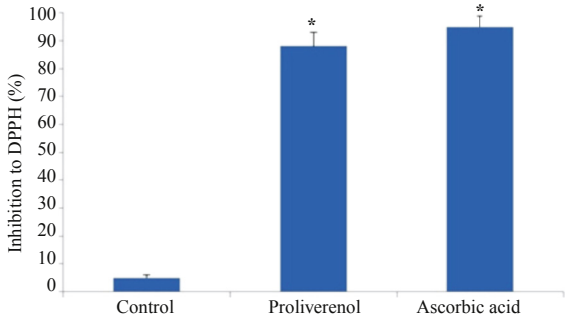

Figure 4. Anti-oxidant activity of Proliverenol compared to ascorbic acid. Anti-oxidant activity was studied with stable free radical DPPH. Values are mean ± SD of three samples. *: $P < 0.05$ versus control.

of the liver cells makes the organ as an important target of carcinogens [22–24]. Hepatotoxicity induced by several agents: ethanol, carbon tetrachloride, thioacetamide, adenosine and *tert*-butyl hydroperoxide [1,3,5,12,19,25] and the different pathways of hepatotoxicity have been reported [26].

Hepatotoxicity is associated with massive fragmentation of DNA of the liver cell, which further triggers apoptotic cascade in the liver cells [18,21,27]. The apoptotic cascade is related to several regulators, *i.e.* NF-κB and TNFα [7,23,25]. NF-κB is a central regulator of cellular stress in all cell types of the liver. Activation of NF-κB in the liver starts the transcription of genes involved in the cell cycle arrest and apoptosis [17]. On the other hand, TNFα plays a critical role in the initiation of apoptotic cascade and development of alcoholic diseases [2,28,29]. In this experiment, we found that ethanol-treated-HepG2 cells induced hepatocellular damage via DNA fragmentation, increased expression of NF-κB and TNFα at the protein level and transaminase enzymes leakage as the consequence of the cell death. Ethanol-induced stress condition leads to activation of NF-κB, which further accounts for increase of the pro-inflammatory cytokine TNFα. This cytokine then stimulates the cells to undergo apoptosis. Chronic alcohol administration was reported to increase intrahepatic mRNA level of TNFα, likely originated from increased activation of NF-κB [2,7].

In our experiment, Proliverenol was shown to block the ethanol-induced DNA fragmentation through up-regulation of APEX1 expression. This enzyme plays significant role in hepatoprotection by repairing the damaged DNA. Proliverenol appears to positively improve the DNA repair system, which therefore blocks the fragmentation, and subsequently decreases the apoptotic cascade of NF-κB and TNFα. Similar researches have also reported that DNA fragmentation in liver was repressed by hepatoprotective of *Ganoderma tsugae* in rat's liver [21] and gentiopicroside activity in mice [4].

Ethanol induced hepatotoxicity and cell death through various pathways [1,23,30]. Ethanol concentrations and incubation period could become factors that determine the pathways involved [1]. In this study, 5% ethanol treatment for 1 and 3 h was used to induce cell death. This treatment appears to affect

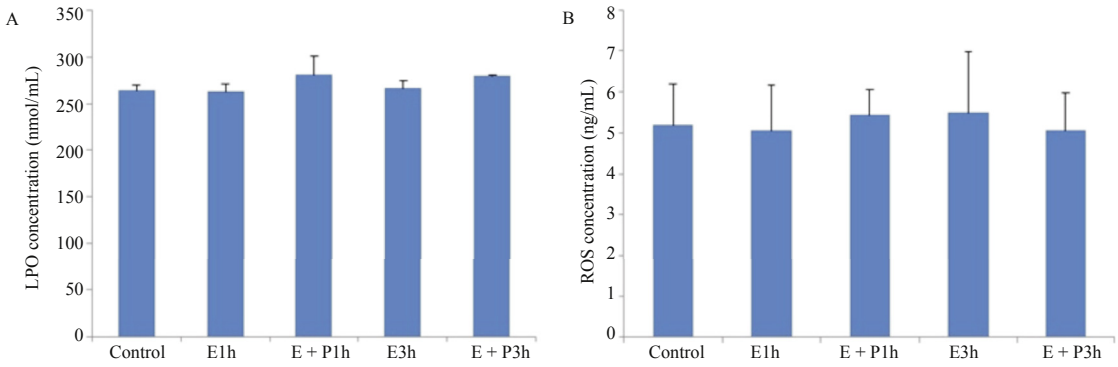

Figure 5. Effect of Proliverenol in ethanol-induced hepatotoxicity in HepG2 cells.
A: Lipid peroxidation measured by 2-thiobarbituric acid reactive substances assay and expressed as MDA level; B: ROS production. Values are mean ± SD of three samples. LPO: Lipid peroxidation.

4. Discussion

Liver has enormous regenerative capacity, but regeneration of the cells is lost by apoptotic cell death. Active proliferative response

a specific pathway of the cell death that involves caspase-8. Thus, our result reveals that this pathway is involved in the ethanol-induced hepatotoxicity condition instead of the mitochondrial pathway (lipid peroxidase, ROS, caspase-9).

Our finding also suggests important evidences that Proliverenol modulates decreasing amount of transaminase enzymes, such as ALT. Proliverenol appears to repress ALT expression at mRNA level and the protein leakage from cytoplasm into the culture medium. As ALT is the most commonly used clinical marker of hepatotoxicity, the result describes that Proliverenol positively provides potential cytoprotection on liver cells from hepatotoxicity and underlines the importance of gene regulation as possible alternative mechanism for increased blood transaminase.

In conclusion, the data obtained in the present study support our hypothesis that Proliverenol has a potential ability in protecting cells from ethanol-induced hepatotoxicity. We proposed that Proliverenol provides hepatoprotective activity through upregulating APEX1 that repress DNA fragmentation, and downregulation of NF-κB, TNFα and caspase-8, which therefore repress ALT leakage and its expression.

Conflict of interest statement

We declare that we have no conflict of interest.

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