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HEPATOPROTECTIVE EFFECT OF STANDARDIZED DRY EXTRACT FROM *PHYLLANTHUS EMBLICA* L. FRUITS ON ETHANOL-INDUCED HEPATOTOXICITY MODEL IN MICE AND HEPATIC STEATOSIS INHIBITION *IN VITRO*

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Summary

Hepatoprotective Effect of Standardized Dry Extract from *Phyllanthus emblica* L. Fruits on Ethanol-Induced Hepatotoxicity Model in Mice and Hepatic Steatosis Inhibition *in vitro*

The study has been undertaken to evaluate the hepatoprotective of extract from *Phyllanthus emblica* L. fruits using the ethanol-induced hepatotoxicity model in mice, as well as the effects on steatosis and lipid accumulation caused by fatty acid *via* regulation of AMPK and ACC signalling in treated HepG2 cells. The results indicated that fruits extract of *P. emblica* L. at doses of 500 and 1000 mg/kg body weight/day exhibited the hepatoprotective effect on ethanol-induced hepatic injury by reducing serum ASAT, ALAT activities, hepatic MDA content, as well as elevating liver S9 fraction SOD activities, GSH content in mouse model. In addition, the extract decreased significantly the fat accumulation and stimulated AMP-activated protein kinase (AMPK) signalling in treated HepG2 cells.

Keywords: *Phyllanthus emblica* L., *Hepatoprotective effect, Ethanol, AMPK, Lipid accumulation.*

1. Introduction

Research studies have shown that *Phyllanthus emblica* L. fruits prevent liver injuries induced by various chemicals or toxins [1],[2],[3]. Our previous study also reported that standardized dry extract from *P. emblica* L. fruits (PEF) at 500, 1000, and 2000 mg/kg/day can reduce the severity of hepatic damage on CCl_4-induced acute hepatotoxicity in mice [4]. Excessive ethanol consumption can increase liver injuries, result in alcoholic liver disease that becomes more and more popular and remains one of serious health problems. Recently, some evidence pointed out that the fruits of *P. emblica* L. can attenuate ethanol-induced oxidative stress in the liver [3],[5]. However, the protective effect of *P. emblica* L. fruits against ethanol-induced liver damage have not been investigated so far in Vietnam. On the other hand, although the hepatoprotective properties of PEF have been reported both from *in vitro* and *in vivo* studies, its mechanism of action still has been unknown. Some studies have revealed that PEF inhibits steatosis and lipid accumulation caused by fatty acid via regulation of AMP-activated protein kinase (AMPK) and acetyl-CoA carboxylase (ACC) signalling [6]. The activation of AMPK inhibits

hepatic lipogenesis mainly through inhibitory phosphorylation of ACC, a rate-controlling enzyme of fatty acid synthesis. The activation of AMPK may prevent anabolic pathways, such as lipid synthesis, and catabolic pathways, such as β-oxidation [7]. Furthermore, the inhibition of activating AMPK is also associated with the hepatic fat accumulation induced by chemical agents like ethanol. Thus, the present study investigates the therapeutic efficacy of extract of *P. emblica* L. fruits to decrease alcohol-induced hepatic damage, as well as explore the possible mechanisms of hepatoprotection.

2. Materials and methods

2.1. Standardized Dry Extract from Phyllanthus emblica L. Fruits

Phyllanthus emblica L. fruits were collected from Ba Vi district, Hanoi capital in Vietnam and authenticated by the Department of Natural Resources and Environment, National Institute of Medicinal Materials. Voucher specimens were stored at the Department of Phytochemistry - Institute of Medicinal Materials. The dry powder of *P. emblica* fruit was extracted with ethanol 50% on a rotary shaker for 24 h at room temperature. The extract was filtered, then evaporated to dryness by vacuum evaporation (under low pressure). Details of the standardized extract process described previous research that was issued under Decision No. 126/QD-VDL, February 15, 2019. The standardized extract has a moisture content of 4,7% and high extraction efficiency 32,43%.

2.2. Chemicals and reagents

The chemicals and reagents used in the present study were high-grade products from Sigma Chemical Company (St. Louis, MO, USA) unless otherwise specified. Antibodies against phosphor-AMPKα (Thr172), AMPKα, phosphor-acetyl-CoA carboxylase (ACC) (Ser79), ACC, and *β*-actin were obtained from Cell Signaling Technology (Danvers, MA, USA). Cell culture materials were purchased from Gibco (Grand Island, NY, USA).

2.3. Experimental procedure in mice

Animals

Adult male Swiss mice were obtained from CIMADE, National Institute of Hygiene and Epidemiology, weighing about 20-25 g. The animals were habituated to the laboratory animal room (Department of Pharmacology, Hanoi University of Pharmacy) for at least 7 days before any experiment manipulations. They were maintained in the animal room under controlled environmental conditions (at 24 ± 2°C, with 55-60% of humidity and 12-h light-dark cycle) with a diet provided by the National Institute of Hygiene and Epidemiology.

Experimental design

The animals were randomly divided into five groups of ten mice in each group. Group I (normal) served as normal control and was given 0,1 mL/10 g/day saline p.o. for 14 days. Group II (E) served as disease control and received ethanol 35% at dose of 3 g/kg/day, p.o. for 7 days then received ethanol 40% at dose of 4 g/kg/day, p.o. for next 7 days. Group III and IV (E+PEF) served as treatment groups and received PEF oral doses of 500 and 1000 mg/kg, respectively. Group V (E+SL) served as positive control and was given silymarin (SL) 100 mg/kg/day p.o. for 14 days. Animals in group III, IV, V received ethanol for 14 days in the same schedule as group II (animals were given PEF or silymarin one hour before receiving ethanol). On the 15th day, the last day of the period, all groups (except for the control group) received the last dose of ethanol 50% 5 g/kg/day, p.o [8]. After oral administration of the last dose for 9 hours, animals were sacrificed, blood and liver were collected for various biochemical and histopathology examinations [4].

Serum analyses: Serum was separated by centrifugation at 3000 rpm for 20 mins. Enzyme activities of alanine aminotransferase (ALAT) and aspartate aminotransferase (ASAT) were measured by the spectrophotometric method according to instructions of the kit supplier (Biosystems S.A -Barcelona, Spain), using semi-auto biochemistry analyzer TC-3300 Plus (Teco Diagnostics, USA).

Liver S9 fraction assay: Liver tissues were collected, and liver S9 fractions were immediately isolated. Superoxide dismutase (SOD), reduced glutathione (GSH), and lipid peroxidation were determined from freshly isolated mitochondria. In details, the liver samples (10% w/v) were homogenized in ice-cold phosphate buffer (100 mM, pH 7,4) then centrifuged at 10,000 rpm for 30 min at 0°C (5702R, Eppendorf, Germany). The supernatant (PMS) was subsequently collected and used for measurement of biochemistry parameters.

Hepatic lipid peroxidation level was assayed by the thiobarbituric acid reactive substance (TBARS) method. Specifically, 0.15 mL PMS was mixed with 2 mL thiobarbituric acid (0.25% in acetic acid, pH 2.4–2.6) in presence of saturated butylated hydroxytoluene (BHT). The reaction mixture was placed in a boiling water bath for 60 min. The formed colored adduct was extracted by *n*-butanol and then measured spectrophotometrically at 532 nm. The result was expressed as malondialdehyde (MDA) equivalent (nmol MDA/g tissue), using freshly prepared tetramethoxypropane as standard.

GSH level in liver tissue was estimated by a spectrophotometric method using Ellman's reagent. PMS was precipitated by the addition of one volume of 4% sulfosalicylic acid. The supernatant obtained after centrifugation (20 µl)

was mixed with 10 mM 5,5′- Dithiobis (2-nitrobenzoic acid) (20 µl) and phosphate buffer (160 µl). The absorbance of the product was immediately recorded at 412 nm. GSH content was expressed as µmol reduced GSH/g tissue.

SOD activity was assayed by xanthine method. The reaction mixture contained diluted PMS (replaced by water in control wells), 50 mM, pH 10.2 carbonate buffer, 0.1 mM EDTA, 100 mM xanthine, 0.025 mM nitrotetrazolium blue chloride (NBT) and 0.01 U/mL xanthine oxidase. The rate of reduction of NBT was measured at 560 nm for 5 min at 25°C. The result was calculated from a standard curve prepared with different concentrations of SOD and expressed as units of SOD per gram tissue (kU/g). One unit of SOD was defined as the amount of enzyme that inhibits the rate of NBT reduction by 50%.

Histopathology: The liver was fixed in a formalin solution, sectioned, and stained with haematoxylin-eosin and photographed at a magnification of x 100. Levels of hepatic injury were observed and compared between each group.

2.4. In vitro assays

Cell culture

The human hepatoblastoma cell line HepG2 was purchased from ATCC (Rockwill, MD, USA). The cells were grown in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% FBS and 1% penicillin/streptomycin at 37°C in an atmosphere containing 5% CO_2. Cell cultures were used in experiments when they reached 80-90% confluence.

MTT cell proliferation assay

Cell cytotoxicity was examined using an MTT reduction assay [9]. HepG2 cells at a density of 5 × 10^4 cells/mL were seeded in 48-well plates at 37°C in an atmosphere containing 5% CO_2 and treated with or without 3, 10, 30 and 100 µM of PEF for 24 h. After that, the cells were exposed to 10 µL MTT 2 mg/ml. At the end of the treatment, cell cytotoxicity was quantified by measuring absorption at 570 nm using a microplate reader ELISA.

Oil Red O staining

HepG2 cells were incubated with or without 3, 10, 30 and 100 µM of PEF in the presence or absence of 25 mM glucose for 24 h. Cells were stained with the Oil Red O working solution before being washed and fixed. The stained cells were observed and photographed using an Inverted System Microscope (Canzese, Germany).

Western Blot Assay

After treatment with various concentrations of PEF for 2 h, cells were collected and washed with PBS. The harvested cells were then lysed on ice for 30 min in 100- µl lysis buffer [60 mM Tris-HCl (pH 6.8) and 2% sodium dodecyl sulfate (SDS), 10% glycerol] and centrifuged at 12,000g for 30 min. Protein concentration was determined using the BCA protein assay kit (Pierce, Rockford, IL) and was subject to 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). After that, protein electrotransferred to a nitrocellulose membrane and immunoblotted with rabbit polyclonal antibodies specific for phosphor-AMPK, phosphor-ACC and β-actin as a control. The membranes were then probed with mouse peroxidase-conjugated secondary antibodies. Finally, protein bands were detected using an enhanced chemiluminescence Western blot detection kit. Band intensity was quantified by densitometry using Image J software.

2.5. Statistical analysis

All data are expressed as the mean ± standard error of the mean (SEM). Statistical analyses were performed by a one-way analysis of variance (ANOVA) followed by Dunnett's test to compare the difference. Statistical significance was set at $p < 0.05$.

3. Results

3.1. Effects of PEF administration on ethanol-induced mice hepatic injury

3.1.1. Effects of PEF administration on hepatic ASAT, ALAT:

The serum activities of ASAT, ALAT of normal, control and experimental animals are given in Table 1.

Table 1. Effect of PEF on serum ASAT, ALAT activities in mice

Group	Treatment	ASAT		ALAT	
		Activity (U/L)	Reduction	Activity (U/L)	Reduction
I	Normal	148.6 ± 37.18		67.99 ± 6.36	
II	Control (E)	238.00 ± 60.00$^{\#}$		92.10 ± 11.25$^{\#}$	
III	E + PEF 500 mg/kg	166.63 ± 33.12*	30%	80.19 ± 7.19*	13%
IV	E + PEF 1000 mg/kg	143.00 ± 28.48*	40%	64.80 ± 3.40*	30%
V	E + SL 100 mg/kg	180.90 ± 30.49*	24%	71.1 ± 3.39*	23%

Data were presented as mean ± SEM.; n=10; $^{\#}$:p < 0.05 vs. normal group; *:p < 0.05 vs. control group.

The results indicated that the group treated with ethanol significantly increased in activities of enzymes when compared to the normal group ($p < 0.05$) whereas treatment with PEF of the ethanol-administered groups significantly reduced activities of enzymes when compared to the control group. The activities of ASAT, ALAT (30% and 13%, respectively) were significantly lower in the group treated with PEF at 500 mg/kg/day when compared to the ethanol-administered group. The activities of

ASAT, ALAT (40% and 30%, respectively) were significantly lower in the group treated with PEF at 1000 mg/kg/day when compared to the ethanol-administered group.

3.1.2. Effects of PEF administration on SOD activities and GSH, MDA content in mice:

We measured MDA levels as an index of lipid peroxidation, GSH content and SOD activities as indexes of preventing oxidative stress in controls and experimental animals. The effect of PEF on lipid peroxidation, GSH content and SOD activities are shown in Table 2.

Table 2. Effect of PEF on hepatic MDA, GSH content and SOD activities in mice

Group	Treatment	MDA (mmol/g)	GSH (mmol/g)	SOD (kU/g)
I	Normal	19.33 ± 6.53	3.87 ± 1.18	322.93 ± 52.82
II	Control (E)	196.21 ± 90.01$^{\#\#}$	0.70 ± 0.36$^{\#\#}$	221.48 ± 57.46$^{\#\#}$
III	E + PEF 500 mg/kg	95.22 ± 34.55*	1.07 ± 0.53	332.88 ± 15.74**
IV	E + PEF 1000 mg/kg	47.72 ± 16.67**	2.05 ± 0.41**	354.00 ± 39.01**
V	E + SL 100 mg/kg	41.23 ± 15.34**	2.02 ± 0.64**	327.78 ± 27.62**

Data were presented as mean ± SEM.; n=10; $^{\#\#}$:p < 0.001 vs. normal group; **:p < 0.001 vs. control group.

Ethanol administration resulted a significant ($p < 0.001$) increase in MDA levels and a significant ($p < 0.001$) decrease in GSH content and SOD activities compared to normal animals. The results showed that co-administration of ethanol and PEF at both dose of 500 or 1000 mg/kg/day significantly prevented the elevation of MDA levels ($p < 0.001$), as well as improved GSH content and SOD activities significantly ($p < 0.001$) compared with ethanol alone administered group.

3.1.3. Effects of PEF administration on liver injury in Histopathological examination:

The normal group showed a normal appearance of the liver. The liver samples of ethanol-administered mice showed focal hepatocytes damage and degeneration. The administration of ethanol along with PEF showed a near-normal appearance of hepatocytes. (Fig.1).

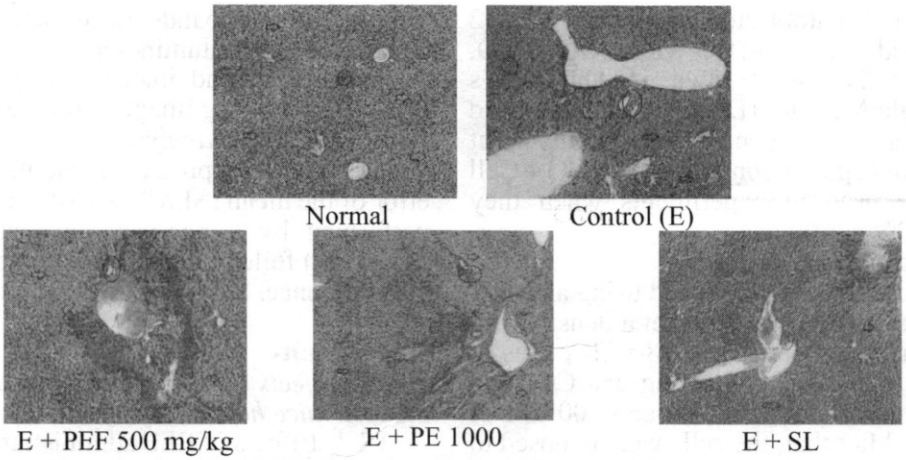

Fig. 1. Microscopic evaluation of PEF effects on ethanol induced hepatotoxicity. Liver specimens were stained with hematoxylin and eosin (H&E), x 100. Symbol ○ and ⇨ indicated inflammatory cells and degeneration.

3.2. Hepatoprotective effects of PEF in vitro

3.2.1. PEF inhibits cell viability and induces cytotoxicity in HepG2 cells:

Table 3. Effect of various concentrations of PEF on cell proliferation in HepG2 cells.

Group	Viability over control (%)
Control	100.00 ± 7.23
PEF 3 μM	99.98 ± 8.92
PEF 10 μM	89.44 ± 14.48
PEF 30 μM	86.83 ± 9.42
PEF 100 μM	79.78 ± 5.61

Data were presented as mean ± SEM; n=6.

The percent of the viability of HepG2 cells when treated with different concentrations of PEF measured as formazan formed from MTT is shown in Table 3. PEF at a concentration of up to 100 μM did not affect the cell viability. At the highest concentration (100 μM) tested in our experiment, the percent viability was reduced to around 80%. Considering these results, the noncytotoxic concentrations of PEF were selected to study the effect of PEF on lipid accumulation and phosphorylation AMPKα and ACC by Western blot analysis in HepG2 cells.

3.2.2. PEF reduces lipid accumulation in HepG2 cells:

To investigate the antisteatotic effect of PEF, HepG2 cells were exposed to various concentrations of PEF (3, 10, 30, 100 μM) in the absence or presence of a glucose mixture at a concentration of 25 mM for 24 h. Total intracellular lipid levels in HepG2 cells were measured after Oil Red O staining. The results

showed that PEF at 10, 30, 100 μM significantly decreased lipid accumulation induced by glucose in a dose-dependent manner (Fig. 3). The lipid-lowering effect of PEF was also confirmed by microscopic examination of the fluorescence of Oil Red O-stained HepG2 cells (Fig. 2).

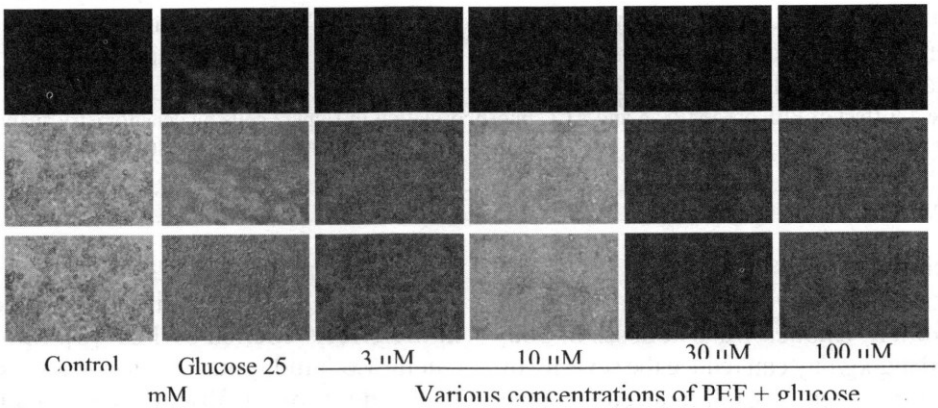

Fig. 2. Cells were exposed to 25 mM glucose together with various concentrations of PEF (3, 10, 30, 100 μM) for 24h. Intracellular lipids were stained with Oil Red O and assessed by a fluorescence spectrophotometer

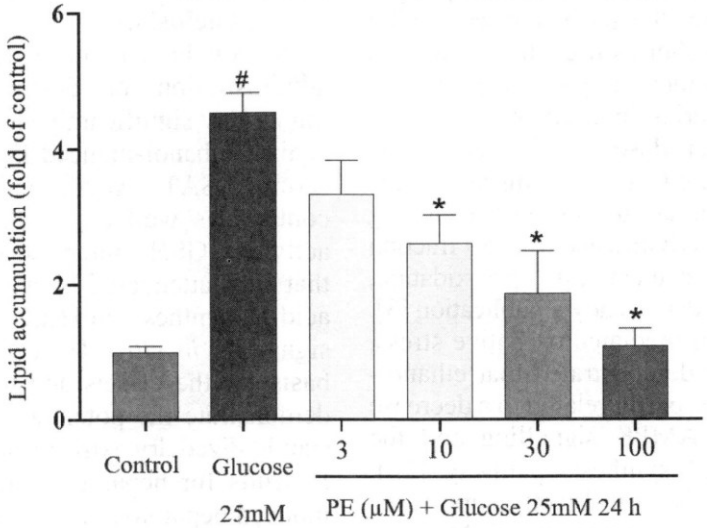

Fig. 3. The effects of PEF on Intracellular lipid accumulation were stained with Oil Red O staining in HepG2 cells. Data were presented as mean ± SEM; $^{\#}$ $p < 0.05$ vs. untreated control; * : $p<0.05$ vs. only glucose 25mM-treated cells

3.2.3. PEF activates AMPK and ACC phosphorylation in HepG2 cells:

Fig. 4. showed the effect of PEF on the phosphorylated AMPKα and ACC in HepG2 cells.

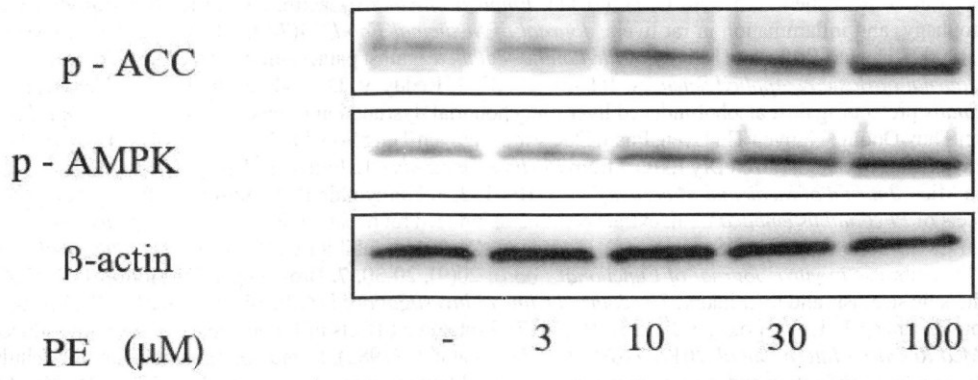

Fig.4. Effect of PEF on the phosphorylated AMPKα and ACC in HepG2 cells by Western blot analysis. β-Actin served as an internal control

The Fig. 4 revealed that PEF increased the phosphorylated levels of the AMPK protein (phosphorylation of AMPKα on Thr172) and its immediate substrate ACC (phosphorylation of ACC on Ser79) in a concentration-dependent manner in HepG2 cells (Fig. 4). Band intensity was quantified by densitometry analysis using Image J 1.49u software (Table 4). The analysis showed that the levels of phospho-AMPK α and phospho-ACC were both notably increased by PEF treatment at 10, 30 and 100 μM.

Table 4. Effect of PEF on AMPK α and ACC phosphorylation in HepG2 cells by densitometry analysis

Band intensity	Control	PE 3 μM	PE 10 μM	PE 30 μM	PE 100 μM
AMPKα/ β-Actin	1.00	0.87	1.34	1.70	2.57
ACC/ β-Actin	1.00	0.86	1.34	2.10	3.42

4. Discussion

Our previous study revealed that standardized dry exxtract from *P. embilca* fruits at doses of 500, 1000, and 2000 mg/kg/day can reduce the severity of hepatic damage on CCl_4-induced acute hepatotoxicity in mice. Several reports have shown that *P. emblica* L. fruits modulate oxidative stress and ameliorate ethanol-induced hepatoxicity [3], [5]. These findings suggested that the extract can exhibit effects on chronic alcohol-induced liver injury in mice. Our study confirmed the protective effect of PEF against liver injuries induced by ethanol in mouse model. PEF at doses of 500 and 1000 mg/kg/day administered orally improves the parameters of ethanol-induced liver injury including serum ASAT, ALAT activities and liver S9 fraction SOD activities, GSH content, lipid peroxidation, similar to Vaddi Damodara Reddy's publication [3].

Apart from ethanol-mediated oxidative stress, some evidences have demonstrated that ethanol-induced hepatoxicity is partly related to a decrease in the activation of AMPK signalling and the increase of fatty acid synthesis pathway [10]. Moreover, AMPK activation directly suppresses ACC activity and the expression of ACC by inhibiting the sterol regulatory element-binding protein (SREBP) [7]. A recent work by Chi-Cheng Lu has demonstrated that PEF at concentrations of 50, 100 and 200 μg/mL could inhibit lipid accumulation in HepG2 cells, implying that AMPK and ACC signalling have important roles in the effects elicited by PEF [6]. Similar to Chi-Cheng Lu 'study, our *in vitro* results showed that PEF at doses of 30, 50 and 100 μM attenuated lipid accumulation in HepG2 cells, as well as increased the phosphorylation of AMPKα and ACC in HepG2 cells.

5. Conclusion

In conclusion, this study revealed that PEF administration at doses of 500 and 1000 mg/kg/day significantly showed protective action against ethanol-induced liver injury by reducing serum ASAT, ALAT activities, hepatic MDA content, as well as elevating S9 fraction SOD activities, GSH content. In addition, we also found that PEF attenuated lipid accumulation and fatty acid biosynthesis in HepG2 cells through AMPK signalling *in vitro*. Hence, this study provides a basis for the effects and mechanisms of PEF to demonstrate the potential therapeutic benefits of standardized dry extract from *Phyllanthus emblica* L. fruits for hepatoprotective effect on ethanol-induced hepatotoxicity model in mice and hepatic steatosis inhibition *in vitro*.

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