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Mechanism of protection of rat hepatocytes from acetaminophen-induced cellular damage by ethanol extract of *Aerva lanata*

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ABSTRACT

The aim of this study is to evaluate the protective effect of ethanol extract of *Aerva lanata* (EEAL) in preventing acetaminophen induced liver toxicity. EEAL was prepared and its hepatoprotective effect was studied in both isolated primary hepatocytes *in vitro* and in Sprague Dawley rats *in vivo*. For *in vivo* studies, the animals were grouped as Group I – Control; Group II – ACN (2 g/kg b.w.); Group III – EEAL (50 mg/kg b.w.) + ACN (2 g/kg b.w.), Group IV – EEAL (100 mg/kg b.w.) + ACN (2 g/kg b.w.). Extracellular activities of the enzymes liver aminotransferease (GOT, GPT), alkaline phosphatase (ALP) and lactate dehydrogenase (LDH) in isolated hepatocytes and rat plasma were studied colorimetrically. Expression of GST, Nrf2, COX 1 & COX2 genes in rat liver were evaluated by RT-PCR. The results showed that ACN induced down-regulation of Nrf2 and upregulation of GST gene expression, which were modulated by EEAL treatment. GOT, GPT, ALP and LDH levels were found to be lowered in both hepatocyte culture media and plasma following EEAL treatment. In addition, the medium GOT and GPT levels were diminished following EEAL treatment only. Moreover, only ALP and LDH in serum appeared to be at normal level following EEAL treatment, whereas GOT and GPT showed levels lower than control. ACN treatment increased the expression of pro-inflammatory COX 1 and COX 2 genes and the levels of these genes were reduced by EEAL treatment. EEAL pre-treated rats exposed to ACN were found to retain normal hepatic structure compared to ACN alone treated rats. From these results it can be concluded that ethanol extract of *A. lanata* possesses both anti-inflammatory and hepatoprotective activity.

KEY WORDS: *Aerva lanata*; hepatotoxicity; Nrf2; cyclooxygenases; acetaminophen

Introduction

Parenchyma and non-parenchyma cells of the liver are involved in several functions which regulate the homeostasis of our body. The presence of chemical and/or biological toxins causes severe damage to the liver leading to hepatitis and cirrhosis mediated through lipid peroxidation and other oxidative complex reactions (Kumar *et al.,* 2011). Severe lipid peroxidation induced by continuous oxidative stress triggered by oxidants is one of the major attributes to the initiation and progress of liver damage (Albano *et al.,* 1985). Hepatic injury and subsequent hepatic failure due to overdose of acetaminophen (ACN) is a serious health concern (Yoon *et al.,* 2016). Under conditions of ACN overdose, the glucuronidation and sulfation process become saturated and more extensive bioactivation of ACN to *N*-acetyl-*p*-benzoquinone imine (NAPQI) occurs, which covalently binds to produce cellular protein adducts, leading to liver failure (Jollow *et al.,* 1973). This evidence suggests that removal and/or deactivation of agents creating oxidative stress is a protective mechanism against the development of ACN hepatotoxicity. Thus, components of natural origin, i.e. detoxifying enzymes that contribute to enhance intracellular antioxidant potential are important in the protection or treatment of such injury. Though many synthetic drugs/natural preparations are now available in the market for treating liver damage, they all have been found to have some toxic side effects. Thus the development of effective drugs with lower toxicity is required. Plant derived components/products always have a potential role in the research of medicine and pharmacology. There are several plant species that are considered to have significant hepatoprotective effects in animal model (Kumar *et al.,* 2011).

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Aerva lanata (Amaranthaceae family) is a common plant found throughout the tropical region of India. Previous research on *A. lanata* showed that different parts of this plant have anti- cancer, anti-diabetic, anti-inflammatory, nephroprotective, hepatoprotective and antihelimintic properties (Ragavendran *et al.,* 2012; Nevin and Vijayammal, 2005; Anusha *et al.,* 2016). This study evaluates the hepatoprotective effect of the ethanol extract of *A. lanata* on primary hepatocytes and rat liver from toxicity induced by ACN.

Methods

Chemicals
Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), antibiotic–antimycotic solution, EZcount™ LDH cell assay kit, cell culture chamber slides and other cell culture reagents were procured from Hi-Media Laboratories, India. Taq PCR Smart Mix 2x was purchased from Orion-X, India. Verso cDNA Synthesis Kit was procured from Thermofisher Inc, USA. Oligos were synthesized by Xcelris Labs and Integrated DNA Technologies, USA.

Plant extraction
A. lanata was collected from different areas of Mahatma Gandhi University, Kottayam, Kerala, India. The plant was identified by the botanist of the Department of Botany, ST. Thomas College, Pala, Kerala, India and a voucher specimen was deposited at their herbarium (Voucher specimen No. 1503). *A. lanata* whole plant was washed and shade dried. The dried plant material was powdered and extracted with 300 ml of petroleum ether (PE; BP-60-80) using Soxhlet apparatus to remove all fatty materials. After PE extraction, the plant material was extracted with ethanol. The ethanol extract (EEAL) thus obtained was dried using a rotary evaporator, weighed and stored for further experiments.

Preliminary component identification in EEAL
The extract was analyzed for phytochemicals qualitatively for the presence of protein (xanthoproteic test) phenolic compounds (Lead acetate test), flavonoids (Alkaline reagent test), tannins (ferric chloride test), steroids, triterpenoids (Salkowski's test), saponins (Froth test), cardiac glycosides (Keller Killiani test) and alkaloids (Wagner's test) using standard procedures (Dyana and Kanchana, 2012).

In vitro anti- lipid peroxidation assay
A modified thiobarbituric acid reactive species (TBARS) assay was used to measure the lipid peroxide formed, using liver homogenate as lipid rich medium (Ohkawa *et al.,* 1979; Shabbir *et al.,* 2013). The isolated liver lobes were washed with 0.9% sodium chloride solution and dried using blotting paper. One gram of tissue was then homogenized with 10 ml of cold phosphate buffer (pH 7.4) using glass-teflon homogenizing tubes, filtered and centrifuged

at 3 000 rpm at 4 °C for 10 min. The supernatant was diluted with phosphate buffered saline (PBS) to obtain final concentration of protein equal to 8.0–15.0 mg/ml. Liver homogenates (3.0ml) were pre-treated with different concentrations of EEAL. Lipid peroxidation was initiated by adding 100 μl of 15 mM ferrous sulphate solution. After 30 min, 200 μl of this reaction mixture was mixed with 3.0 ml of 10% trichloroacetic acid (TCA). After 10 min, the tubes were centrifuged and the supernatant was separated and mixed with 3.0 ml of 0.67% thiobarbituric acid in acetic acid. The mixture was heated in a water bath at 85 °C for 30 minutes, followed by heating in boiling water bath. The intensity of the pink-colored complex formed was measured at 535 nm. The percentage of inhibition of lipid peroxidation was calculated by comparing the results of the test with those of control. The percentage of inhibition of lipid peroxidation was calculated by the formula: Inhibition (%) = [Abs (Control) − Abs(Sample)]/ Abs (Control) × 100.

Isolation of primary rat hepatocytes
Hepatocyte isolation was performed using the two-step enzymatic method (Seglen, 1976; Ateş *et al.,* 2012). The abdomens of Sprague Dawley rats were opened under general anesthesia, a cannula was inserted into the portal vein and the perfusion solution (0.9 g/100 mL NaCl; 0.04 g/100 mL KCl; 0.09 g/100 mL D-glucose; 0.21 g/100 mL NaHCO$_3$ and 2 mL HEPES – 25 mM in DMEM) was slowly infused at 37 °C. After discoloration of the liver, 30 ml of the enzyme solution (0.24 mg type 4 collagenase/mL perfusion solutions) was infused via the portal vein. After the incubation period of 30 min, the liver was cut into small pieces and filtrated through 210 μm, 70 μm and 40 μm porous membranes. The samples thus obtained were centrifuged with washing solution (0.5 g/500 mLNaCl; 0.02 g/500 mL KCl; 0.48 g/500 mL CaCl$_2$.H$_2$O; 5 mL HEPES – 19.8 mM; 1 g/500 mL bovine serum albumin (BSA) dissolved in DMEM) at 150 g for 3 min. The supernatant was discarded and the pellet obtained was washed. Separation of the dead cells was performed with 10.8 mL percoll solution (100%) and 15 mL DMEM, the resulting cell suspension was centrifuged at 2 000 rpm for 20 min. The medium layer was collected and percoll was removed by washing and centrifugation at 150 g for 3 min. The viability of isolated hepatocytes was monitored by Trypan blue method. The isolated hepatocytes were cultured in William's media at 37 °C with 5% CO$_2$. Periodic acid – Schiff (PAS) staining was performed to identify glycogen synthesis and hepatocyte function. Isolated cells were washed with PBS three times and fixed within 4% formaldehyde for 30 min. The cells were oxidized with periodic acid for 5 min, processed with Schiff's reagent for 15 min, washed with dH$_2$O for 10–15 min and observed under the inverted microscope (Olympus 1×51) (Reintoft, 1978).

Analysis of nuclear changes by DAPI staining
Chromatin changes in EEAL treated cell lines were studied by DAPI.HCl. Isolated rat hepatocytes were pre-treated

with different concentrations of EEAL and exposed to 3 mM ACN for 24 h. After the treatment period, cells were washed with phosphate buffered saline (PBS) to completely remove the growth medium. Cells were fixed for 10 min in 3.7% formaldehyde and again rinsed three times in PBS before permeabilization in 0.2% TritonX 100 for 5 min. The cells were washed and incubated with DAPI labeling solution (stock solution: 10 mg/mL in water; dilution 1:5 000 in PBS) for 5 min in the dark. The labeling solution was aspirated and cells were rinsed thrice in PBS. Morphology of the cells and nuclei were observed using a fluorescence microscope (Olympus 1×51) with the DAPI filter (Chazotte, 2011).

Animal experiments

Male Sprague Dawley rats (4 weeks old, weighing between 100 and 150 g each) were purchased from the Government Veterinary College Mannuthy, Thrissur, India. After one week of acclimation at the departmental animal house, the rats were randomly assigned to 4 groups. Group I: Normal control (administered vehicle 10%DMSO), Group II: Acetaminophen control (2 g/kg b.w.), Group III: 50 mg/kg b.w. EEAL+ Acetaminophen(2 g/kg b.w.), Group IV: 100 mg/kg b.w. EEAL + Acetaminophen (2 g/kg b.w.); (n=6). EEAL was suspended in 10% DMSO (v/v) in PBS and was given intragastrically for 14 consecutive days. After 14 days, the rats were intragastrically given 2 g/kg acetaminophen. After 24 h post acetaminophen treatment, blood was collected in tubes containing heparin as an anticoagulant and plasma was prepared to evaluate the progress of hepatic damage. All animal experiments were approved by the University Animal Ethical Committee (CPCSEA No .732/03/ac/CPCSEA dated 17/07/2009).

RNA isolation and RT-PCR analysis

Total RNA was isolated from control, treated cells and rat liver by Trizol reagent (Himedia) as described by the manufacturer's instructions. cDNA was synthesized by adding 1ng of RNA to the 5X cDNA synthesis buffer (4 μl), dNTP Mix (2 μl) RNA primer (1 μl), RT Enhancer (1μl) Verso Enzyme Mix (1 μl) and nuclease free water (20 μl) using Thermo Scientific Verso cDNA Synthesis Kit. PCR was done by using GoTaq Green master Mix in thermal cycler using the following primer sequences. GAPDH was used as an endogenous control. PCR primers for COX 1 (F-5'-CTCACAGTGCGGTCCAAC-3', R-5'-CCAGCACCTGGTACTTAAG-3'), COX 2 (F-5'-TTCAAATGAGATTGTGGAAAAAT-3' R-5'-AGATCATCTCTGCCTGAGTATCTT-3'), GST (F-5'-GCCTTCTACCCGAAGACACCT-3' R-5'-GTCAGCCTGTTCCCTACA-3', and Nrf2 (F-5'-CCAGCTGAACTCCTTAGACTCA-3', R-5'-GCTCTGC TAGGAAAGCAGAGTAAATT-3'), were designed and PCR for these genes were performed. The finished PCR product was run on 1.5% agarose (Kendell *et al.,* 2010).

Liver marker enzyme activities

To determine the effect of EEAL on liver marker enzyme activity in primary rat hepatocytes, cells were seeded on a 96 well plate and pre-treated with EEAL (10 μg/ml, 50 μg/ml, 150 μg/ml). Pure acetaminophen (final concentration 3mM) was added to the cells after 24 hours of pre-treatment with EEAL. The culture supernatants from the different experimental and control groups were collected after treatment with EEAL to estimate the levels of secreted proteins associated with normal development and functioning of the liver. Serum from rats treated with EEAL and ACN was used for biochemical analysis. To evaluate the hepatoprotective activity of EEAL in both primary hepatocytes and in rats, we analyzed the activities of glutamic oxaloacetic transaminase (GOT), glutamic pyruvic transaminase (GPT), alkaline phosphate (ALP) and LDH using commercially available kits (Agapee, India) following the manufacturers´ instructions. LDH was assessed by the kit provided by HIMEDIA, India. Protein content was estimated colorimetrically (Bradford, 1976).

Histopathology of the liver

After the experimental period, the rats were anesthetized in the animal house and the livers were removed and preserved in 10% formaldehyde. Dehydration and clearing of the tissues were performed using different concentrations of ethanol. Five micrometer thick sections were prepared and stained with hematoxylin and eosin (H/E) (Mendez *et al.,* 2005)**.** Stained sections were qualitatively evaluated using a digital microscope (Phase Contrast Fluorescence Microscope Olympus BX-43). The images were analyzed using software Q IMAGING, Micro Publisher 5.0 RTV.

Statistical analysis

Mean values ± SD were calculated for all parameters. The statistical difference was analyzed by one-way analysis of variance (ANOVA) using windows SPSS 19 and significance was calculated as *P* values. In all cases a difference was considered significant when *p*-value was <0.05. Duncan's *post-hoc* analysis was used for statistical analysis.
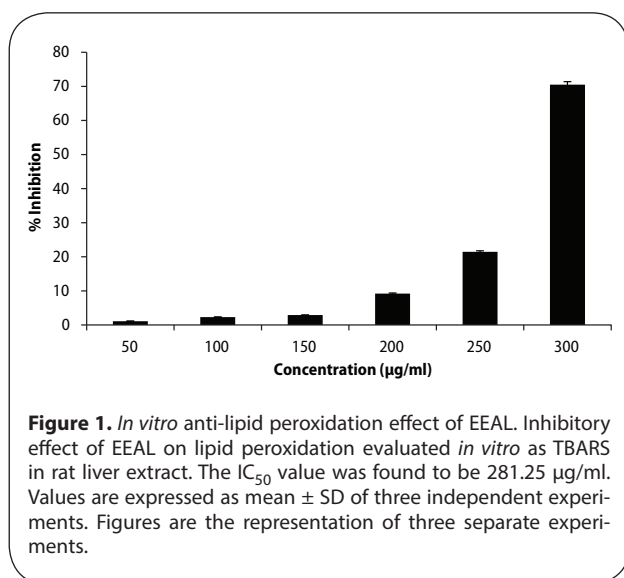
Results

EEAL prevented *in vitro* lipid peroxidation

Ethanol extraction of *A. lanata* plant gave a yield of about 5.69 g per 100 g of the plant material. Preliminary phytochemical screening was done on EEAL to identify the different classes of components present. The results indicated the presence of polyphenolic compounds, flavonoids and alkaloids in the preliminary compound identification (Table 1). Quantitative analysis showed that EEAL contained a higher amount of polyphenols (62.31±1.62 μg/100 mg) followed by flavonoids (25.12±0.75 μg/100 mg) and lower levels of alkaloids (15.20±0.43 μg/100 mg) (Table 1). The *in vitro* antioxidant activity of EEAL was studied using lipid peroxidation assays. The results showed an anti-lipid peroxidation effect of EEAL in *in vitro* conditions using rat liver extract. The IC$_{50}$ value was found to be 281.25 μg/ml (Figure 1).

Table 1. Preliminary component identification and their respective concentrations in EEAL.

Components	Presence	Concentration (µg/100mg extract)
Protein	-	NA
Phenolic Compounds	+	62.31±1.62
Flavonoids	+	25.12±0.75
Tannins	-	NA
Steroids	-	NA
Saponins	-	NA
Alkaloids	+	15.20±0.43

Results indicate the presence of polyphenols, compounds, flavonoids, cardiac glycosides and alkaloids Values are expressed as Mean ± SD of three independent experiments. NA: not applicable

Figure 1. *In vitro* anti-lipid peroxidation effect of EEAL. Inhibitory effect of EEAL on lipid peroxidation evaluated *in vitro* as TBARS in rat liver extract. The IC_{50} value was found to be 281.25 µg/ml. Values are expressed as mean ± SD of three independent experiments. Figures are the representation of three separate experiments.

Previous studies showed that the extract of *Mucuna pruriens* significantly prevented *in vitro* lipid peroxidation with an IC_{50} value of 217.25 µg/ml (Rajeshwar *et al.,* 2005), while *Leucas plukenetii* extract showed an anti-lipid peroxidation activity at 536 µg/ml (Nandy *et al.,* 2012). In comparison with these results, *A. lanata* extract has a similar anti-lipid peroxidation activity.

EEAL prevents nuclear changes induced by ACN
Rat primary hepatocytes were isolated by collagenase perfusion method. The cells were found to be highly viable and were successfully propagated in William's media. PAS staining confirmed the viability of the hepatocytes (Figure 2). The nuclear changes induced by ACN and their prevention by EEAL were studied using DAPI staining. Acetaminophen (3 mM) treatment showed significantly higher decrease in hepatocyte viability (70%), while primary rat hepatocytes pre-treated with EEAL showed significant prevention of cell toxicity. The intensification of DAPI staining in ACN treated cells pointed to apoptotic

chromatin condensation. EEAL treatment showed significant protection towards ACN exposed hepatocytes in dose dependent manner. The result indicated that EEAL had some mitogenic effects towards rat hepatocytes, since the cell number observed was much higher than that observed in control cells (Figure 3).

Liver marker enzyme activities were beneficially modulated by EEAL both *in vitro* and *in vivo*
Treatment of isolated hepatocytes with ACN showed a significant increase in the activity of LDH, ALP, GOT, GPT, while EEAL pre-treated hepatocytes when exposed to ACN showed reduced LDH ALP, GOT,GPT activity in the culture medium (Figures 4 and 5). Interestingly, the activities of GOT and GPT levels were found to be below the values of normal untreated control in hepatocytes treated with EEAL alone at a concentration of 50 and 150 µg/ml. Treatment with ACN (2 g/kg b.w.) in rats showed a significant increase in the activity of serum ALP, GPT, GOT and LDH. While pre-treatment of ACN-exposed rats with EEAL, at a concentration of 50 and 100 mg/kg b.w., resulted in reduction of upregulated ALP, GPT, GOT and LDH activities, with GOT and GPT activity below the levels of untreated control rats. Of the liver enzymes, the activity of LDH in serum of EEAL-treated rats was only mildly suppressed (Figure 6).

Gene expression analysis
PCR analysis was done to determine the expression of glutathione s transferase (GST), cyclooxygenases 1 and 2 (COX 1 and COX 2) and Nrf2 in the liver of EEAL and ACN treated rats. The inflammatory COX 1 gene was mildly overexpressed in ACN treated rats. Rat livers pre-treated with EEAL and exposed to ACN showed COX 1 gene expression downregulated compared to ACN treated animals. COX 2 gene was found to be upregulated in ACN treated rats, while EEAL pre-treatment showed a decrease in the COX 2 gene expression which was comparable with control animals. GST levels were found to be significantly upregulated in ACN treated rats. The expression levels of GST in EEAL pretreated rats were found to be normalized to those of control animals. Nrf2 levels were found to be mildly upregulated in EEAL pretreated rats exposed to ACN, while the expression levels of Nrf2 in ACN alone treated rats were found to be significantly lower (Figure 7).

Histopathological changes in the liver
Histopathological examination of the liver of ACN treated animals showed total loss of hepatic architecture, accumulation of fat globules, dilated sinusoids and centrilobular necrosis. EEAL pre-treated rats exposed to ACN were found to retain normal hepatic architecture with less damage to hepatocytes and sinusoids (Figure 8).

Discussion

ACN, a commonly used analgesics, is reported to have significant damaging effect on liver functions (Herndon
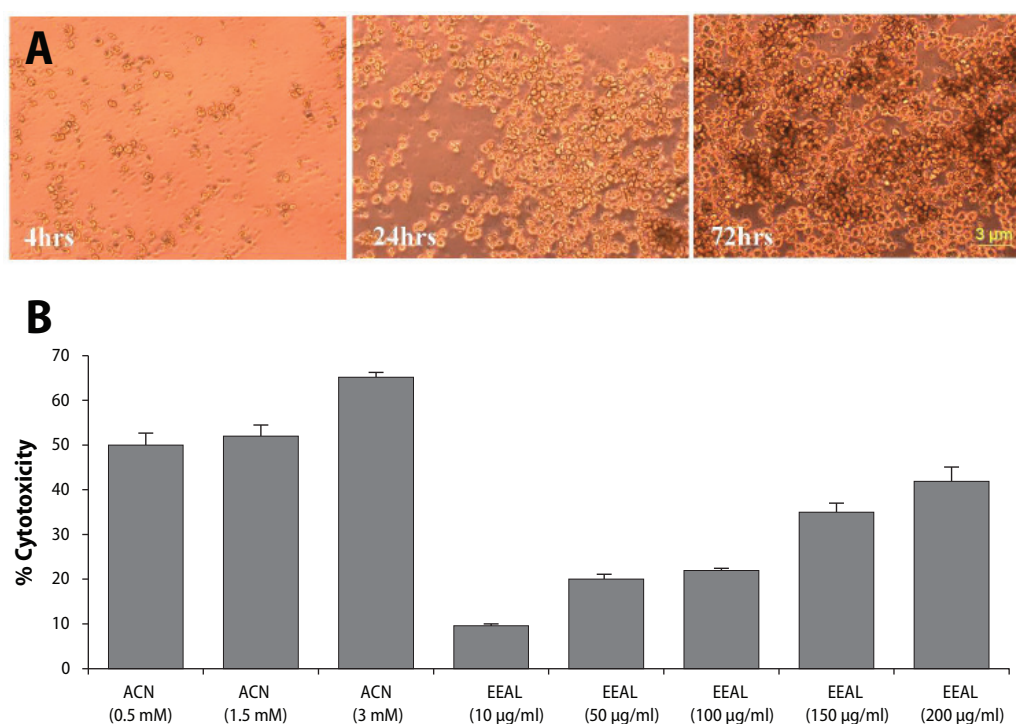
Figure 2. Isolation of rat hepatocytes and cytotoxic effect of different concentrations of EEAL and CAN. **A:** Growth progress of hepatocytes at different time intervals (10×), **B:** MTT assay of different concentrations of acetaminophen and EEAL on hepatocytes after 24 hrs. Values are expressed as Mean ± SD of three independent experiments. Figures are representation of three separate experiments. ACN – Acetaminophen; EEAL – Ethanol Extract of *A. lanata*.

et al., 2014)**.** Treatment options for toxin-induced hepatic failure due to inconsistent efficacy and toxic adverse effect made us to look into the possibility of plant-derived compounds/extracts easily accessible and non synthetic (Fogden & Neuberger, 2003; Evans, 2002)**.** In view of these observations, we selected *A. lanata* to study its hepatoprotective effect on ACN induced toxic liver and hepatocytes.

The *in vitro* antioxidant activity of EEAL was evaluated using lipid peroxidation assays. The results showed significant inhibitory efficacy of EEAL on Fe^{2+}-induced lipid peroxidation. Experiments showed that the metabolism of ACN triggers the oxidant stress due to the production of superoxide and hydrogen peroxide mediated by cytochrome P_{450} (Kunthan *et al.,* 1978; Wendel *et al.,* 1979). This was evidenced from the low lipid peroxides (LPO) in rats treated with cytochrome P450 inhibitors in ACN induced hepatotoxicity (Wendel & Feuerstein, 1981). There is a strong correlation between the oxidative damage to DNA and the formation of thiobarbituric acid reactive species. Malondialdehyde (MDA), the end product of lipid peroxidation, is a measure of membrane damage. High concentrations of substances like acetaminophen and CCl_4 cause tissue damage by initiating lipid peroxidation (Naskar *et al.,* 2009). EEAL may be interfering in the active metabolism of ACN and reduce its impact on hepatocyte damage by virtue of the antioxidant activity conferred by the biologically active components present in it.

Several phytomolecules including flavonoids, alkaloids, glycosides and saponins obtained from various plant sources have been reported as potent hepato-protective agents suppressing the initiation or propagation of the free radical chain reactions (Flora *et al.,* 1996; Hall & Cuppett, 1997). It was previously reported by several researchers that *A. lanata* is a rich source of flavonoids such as kaempferol, quercetin, isorhamnetin, etc (Saleh *et al.,* 1990). Apart from these compounds, *A. lanata* has also been reported to have β-cyanins (glycine betaine and trigonelline), sterols and carbohydrates (Zapesochnaya *et al.,* 1991; Zapesochnaya *et al.,* 1992a; Zapesochnaya *et al.,* 1992b). The results of our studies also confirmed that EEAL contains flavonoids, alkaloids and a polyphenolic class of compounds, which has to be isolated and evaluated individually for its biological effects. The results of our studies substantiate that the anti-lipid peroxidation effect of EEAL may probably be due to the presence of biologically active molecules in it.

ACN metabolism involves conversion to *N*-acetyl-*p*-benzoquinone imine (NAPQ1) and back to ACN or its conjugation with glutathione (GSH) which in turn initiate the hepatic injury leading to GSH depletion (Reid *et al.,* 2005). The expression of glutathione S transferase (GST) was found to be higher in rats treated with ACN, while
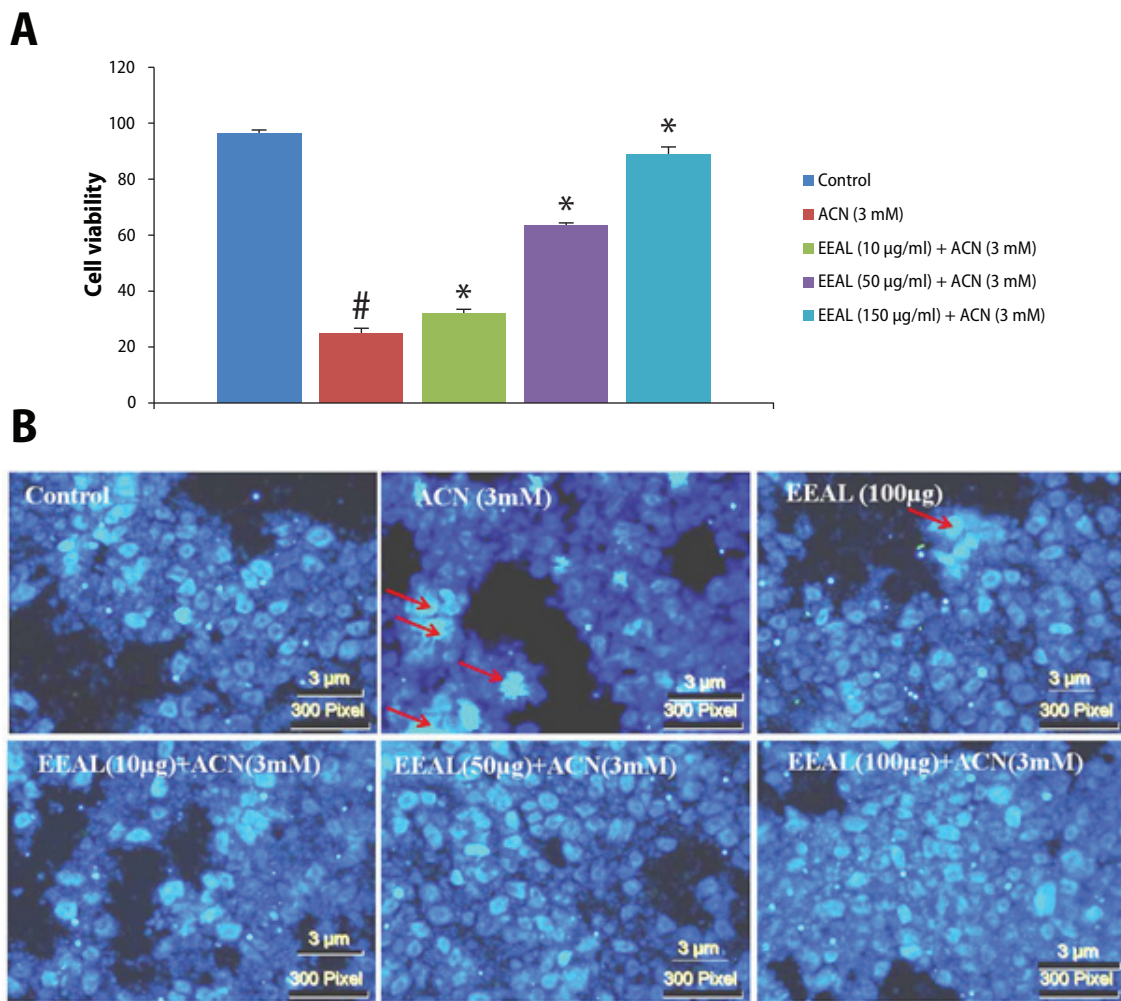
Figure 3. Prevention of ACN – induced cell death and nuclear changes in isolated rat hepatocytes by EEAL treatment. MTT assay of acetaminophen and EEAL after 24 hrs. Values are expressed as Mean ± SD of three independent experiments. (B) DAPI staining of control and treated cells (magnification 20×). Pictures are representation of three separate experiments. #Statistically significant compared to control cells ($p<0.05$), *Statistically significant compared to ACN treated cells ($p<0.05$). ACN – Acetaminophen; EEAL – Ethanol Extract of *A. lanata*. Red arrows indicate chromatin condensation.
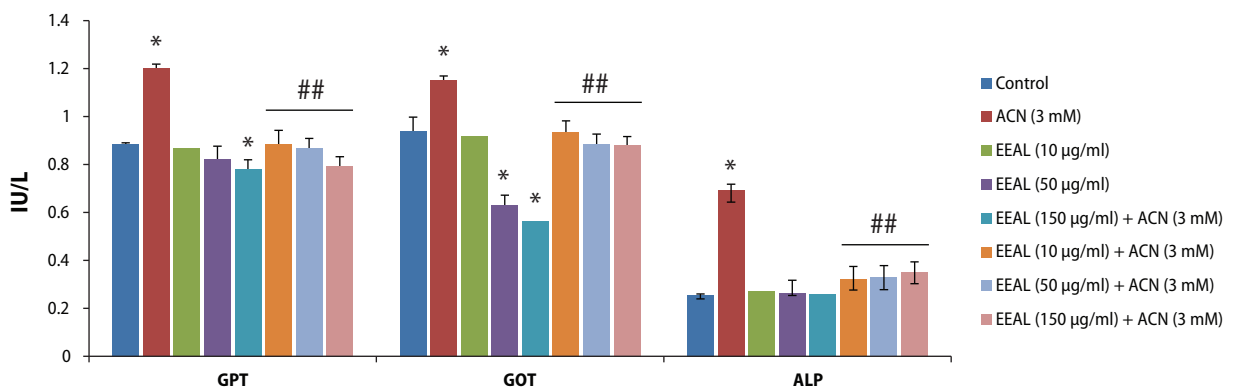
Figure 4. Effect of different concentrations of EEAL and ACN on GPT, GOT and ALP release from isolated rat hepatocytes. ACN-induced increase of GPT, GOT and ALP levels in hepatocytes media compared to the enzyme release by control and EEAL pre-treated cells. Values are expressed as Mean ± SD of three independent experiments. *Statistically significant compared to control cells ($p<0.05$), ##Statistically significant compared to ACN treated cells ($p<0.05$). GPT – Glutamine Pyruvate Transaminase; GOT – Glutamate Oxaloacetate Transaminase; ALP – Alkaline Phosphatase; ACN – Acetaminophen; EEAL – Ethanol Extract of *A. lanata*.
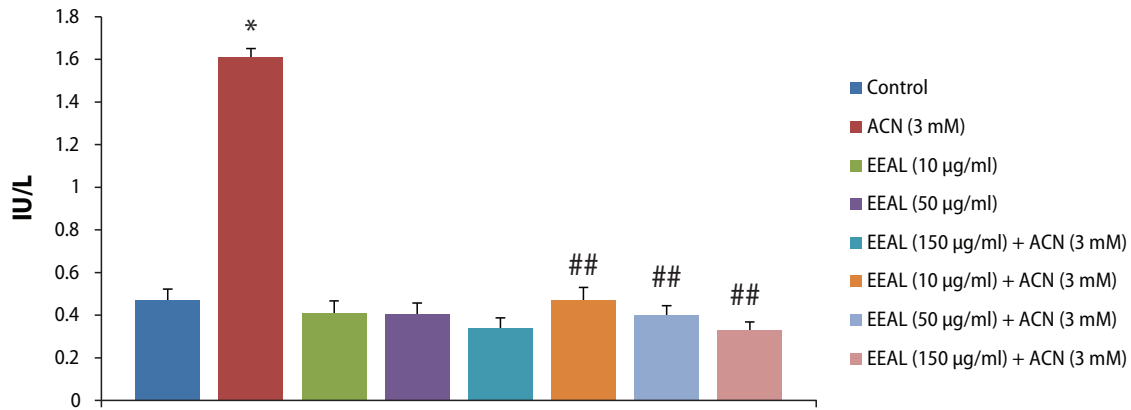
Figure 5. Effect of different concentrations of EEAL on LDH release from isolated rat hepatocytes. Values are expressed as Mean ± SD of three independent experiments. *Statistically significant compared to control cells ($p<0.05$), ##Statistically significant compared to ACN treated cells ($p<0.05$). ACN – Acetaminophen; EEAL – Ethanol Extract of *A. lanata*.
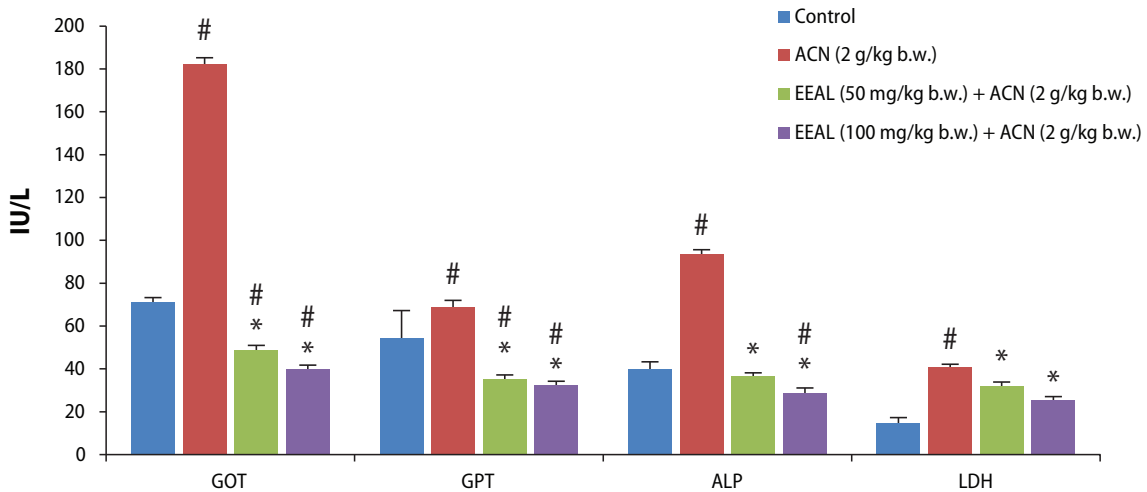

Figure 6. Levels of liver marker enzyme activities in serum of control, ACN and EEAL treated rats. ACN-induced increase of GPT, GOT and ALP serum levels compared to the serum levels of control and EEAL pre-treated rats .Values are expressed as Mean ±SD of three independent experiments. *Statistically significant compared to ACN treated rats ($p<0.05$), #Statistically significant compared to untreated control rats ($p<0.05$). ACN – Acetaminophen; EEAL – Ethanol Extract of *A. lanata*.

EEAL treated rats exposed to ACN showed a difference in the expression level with respect to the concentration of EEAL. This may be due the interference of EEAL on absorption of ACN or enhanced clearance from the circulation without affecting the normal homeostasis of the cells.

The rise in serum levels of GOT, GPT, ALT and LDH has been attributed to the damaged structural integrity of the liver, since they are cytoplasmic and released into circulation post cellular damage (Sallie *et al.*, 1991). In the present study we found that ACN elevated the activities of these marker enzymes, indicative of hepatocyte damage, while EEAL treatment restored the activity of these enzymes in rat liver. The results obtained *in vitro* using isolated hepatocytes showed the same pattern of activity.

The interesting observation we found *in vitro* was the reduction in the activities of GOT and GPT below the levels of normal cells in the culture medium of EEAL alone treated hepatocytes. This observation led us to the conclusion that EEAL may have some inhibitory effect on the production of these enzymes. Previous studies showed that it is possible that certain extracts/formulation containing pyrethroid and Neem have inhibitory effects on the activity of GOT and GPT in Khapra beetle larvae (Younes *et al.*, 2011). Studies by Tanani *et al.* (2009) also observed an inhibition of GPT activity by different solvent extracts of the wild plant *Fagonia bruguieri* and suggested that the inhibition may be due to its effect on synthesis or on functional levels of the enzyme directly or indirectly by altering the cytomorphology of the cells
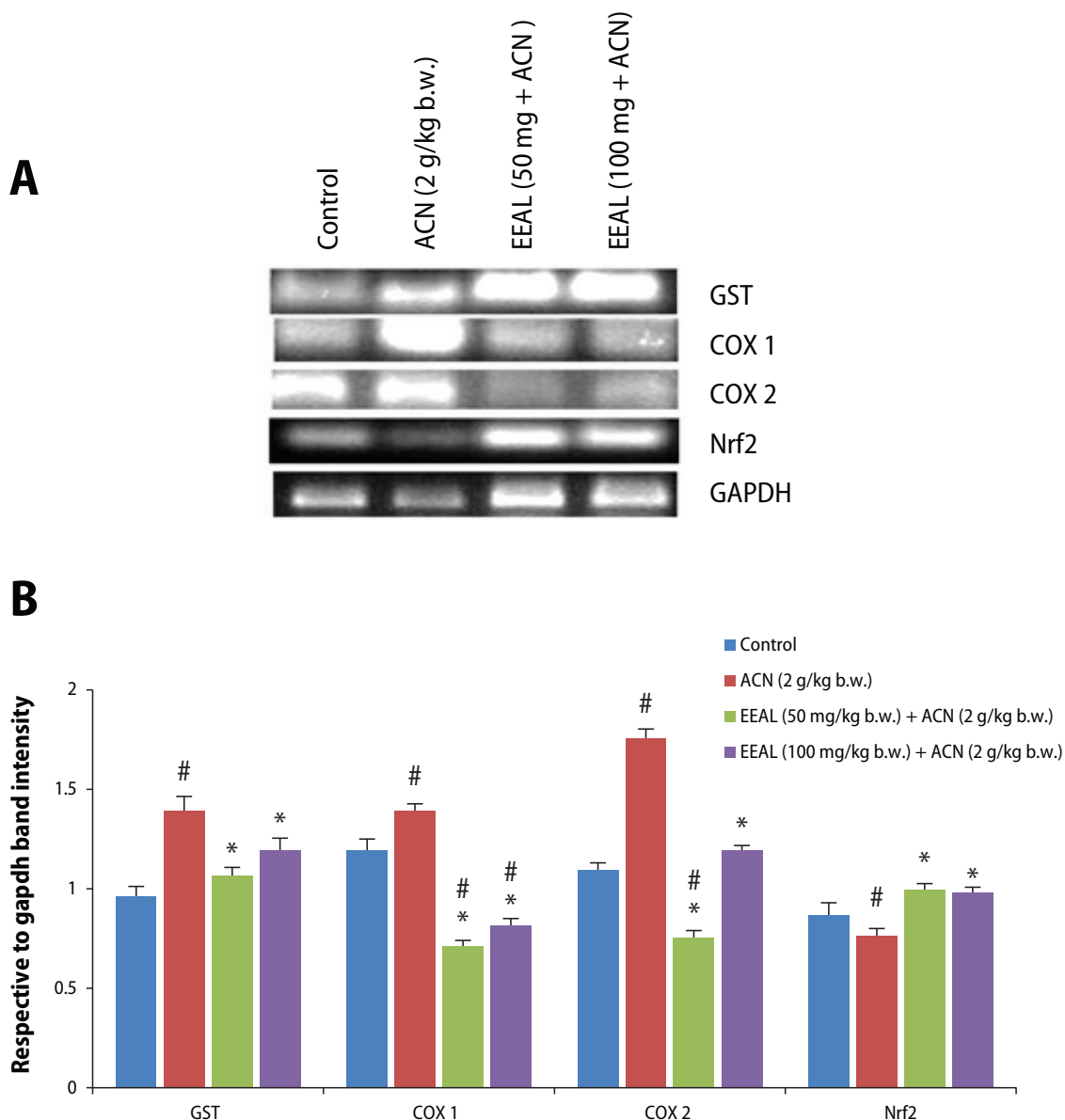
Figure 7. Expression of GST, COX1, COX 2 and Nrf2 genes in the liver of rats treated with EEAL and CAN. Expression of GST, COX 1, COX 2 and Nrf2 genes in EEAL treated rat liver. (B) Graph showing respective band intensity. EEAL dose is expressed in mg/kg body weight. Dose of ACN used was 2 g/kg body weight. ACN – Acetaminophen; EEAL – Ethanol Extract of *A. lanata*. The pictures are representation of three independent experiments. (B) Relative band intensity of gene products. Values are expressed as mean±SD of three independent experiments . #statistically significant compared to control (*p*<0.05). *statistically significant compared to ACN (*p*<0.05).

(Tananai *et al.*, 2009; Nath, 2000). We have found that EEAL did not affect the morphology of the hepatocytes, indicating the possibility of interfering in the synthesis of these marker enzymes.

Oxidative damage to biological macromolecules affects normal cellular functions and is implicated in cancer, inflammation, neurodegenerative diseases, cardiovascular diseases and aging. Eukaryotic cells have developed antioxidant defence mechanisms to neutralize reactive oxygen species (ROS) and prevent macromolecules from undergoing peroxidation. One of the most important cellular defence mechanisms against ROS and electrophilic intermediates are mediated through the

antioxidant responsive element sequence (ARE), in the promoter regions of phase II and antioxidant enzyme genes. The ARE-dependent cellular defence system is controlled by the transcription factor, transcription factor nuclear factor-erythroid 2 related factor 2 (Nrf2). Nrf2 exerts its influence on hepato-protection of compounds through ARE by enhancing GSH synthesis, bio-conjugation and clearance of toxic metabolites (Gum & Cho, 2013). Results showed a mild decrease in expression of Nrf2 in rat hepatocytes treated with ACN which was restored by pre-treatment of EEAL. These results suggest that the modulation of the antioxidant master switch Nrf2 by EEAL can contribute to the restoration

of redox homeostasis in ACN stressed hepatocytes. The Nrf2-ARE pathway is capable of inducing a phase-2 detoxification enzyme activity, promoting a disruption of (Nrf2)-Kelch-like ECH-associated protein interactions (Lau *et al.,* 2008). As a result Nrf2 translocates into the nucleus and modulates the expression of several target genes, especially HO-1 and GST, and protects the cells against oxidative stress-induced tissue. Here ACN treatment has significantly upregulated the GST expression and subsequently restored by EEAL treatment which may not be dependent on Nrf2, since the results showed down regulated levels of this transcription factor after ACN treatment. This prompts us to think about other alternative gene pathways affecting the regulation of GST expression. Such regulation can occur through another dimeric transcriptional factor as hepatic nuclear factor-1α (HNF1α), which is secreted by hepatocytes and mediates liver specific gene transcription (Park *et al.,* 2004).

ACN treatment induced a slight upregulation of COX 1 gene, while COX 2 gene expression was significantly higher in rat liver compared to untreated rats. COX 1 and COX 2 gene expression were found to be downregulated below normal levels by pre-treatment with EEAL (50 and 100 mg/kg b.w.). This effect of upregulation of COX 1 and 2 genes by ACN is uncharacteristic related to some of the previous research, which demonstrated a preferential COX-2 inhibition by ACN under different clinically relevant conditions (Hinz & Brune, 2012). Reports are also

available suggesting that ACN is a potent antipyretic and analgesic drug with very weak anti-inflammatory effects, and activities of COX enzymes in homogenates of ACN treated rat tissues showed differential inhibitory action. The findings also suggested that ACN is a weak inhibitor of both COX 1 and COX 2 and there exists a possibility of inhibition of another unidentified variant of cycloxygenase, COX 3 (Botting *et al.,* 2000). The inhibitory effect of EEAL on both COX 1 and 2 is very significant and the results can be related to previous reports suggesting that plant extracts may inhibit both COX 1 and COX 2 expressions, by interfering with the biosynthesis process (Shaikh *et al.,* 2016).

Our experiments showed that treatment of primary rat hepatocytes with ACN induced significant cell death accompanied by changes in nuclear size and chromatin condensation, while pre-treatment with EEAL prior to ACN exposure protected hepatocytes from cell death and nuclear damage. ACN induced cellular damage resulted in elevated extracellular activity of GOT, GPT, ALP and LDH in both isolated hepatocytes *in vitro* and rat liver *in vivo.* Higher levels of ALP activity in both hepatocytes and rat serum showed the extent of cell membrane damage caused by ACN since ALP is a membrane bound glycoprotein enzyme, with high concentrations in sinusoids and endothelium and is excreted into the bile and its elevation in serum occurs in hepatobiliary diseases (Swamy *et al.,* 2012). Prophylactic treatment of
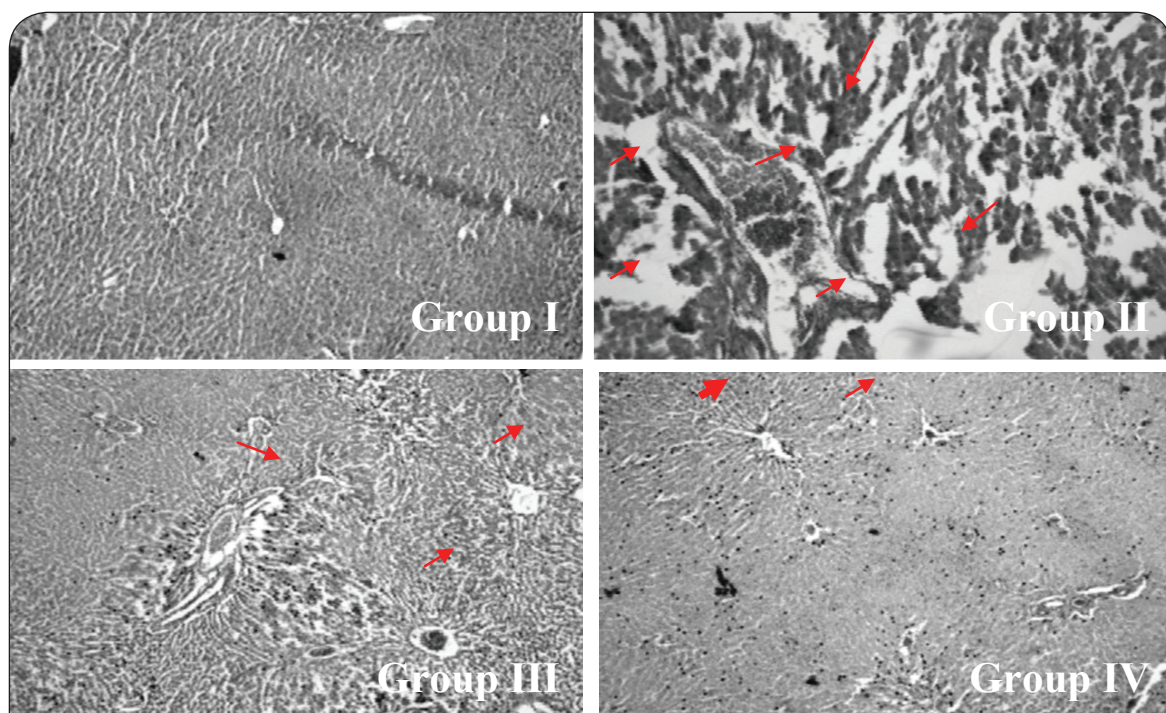
Figure 8. Histopathological changes in the liver of control and ACN exposed rats treated with EEAL. Representative microscopic photographs of histopathology of liver of Control, EEAL, EEAL and acetaminophen treated rats. Sections were routinely processed for hematoxylin and eosin staining, and were observed using a digital microscope at 10× magnification. Group I – Control; Group II – ACN (2 g/kg b.w.); Group III – EEAL (50 mg/kg b.w.)+ACN (2 g/kg BW), Group IV – EEAL (100 mg/kg b.w.) + ACN (2 g/kg b.w.).The arrows indicate loss of hepatic architecture, accumulation of fat globules, dilated sinusoids and centrilobular necrosis) ACN – Acetaminophen; EEAL – Ethanol Extract of *A. lanata.*

rat liver and isolated hepatocytes with EEAL before ACN exposure reversed the increased extracellular activities of the hepatic marker enzymes possibly by preventing the leakage of intracellular enzymes by stabilizing the cell membrane from ACN action. These molecular effects of EEAL were substantiated by histopathological findings. ACN treatment induced significant structural changes to the individual hepatocytes with enlarged sinusoids, while EEAL pre-treated rats exposed to ACN showed a normal liver architecture.

In conclusion, it was observed that the ethanol extract of _A. lanata_ has significant anti- inflammatory and hepatoprotective effects against ACN-induced liver injury, as evidenced by molecular and histopathological analysis. _A. lanata_ contains polyphenols, flavonoid and alkaloids that contribute to the exhibited activity of _A. lanata,_ which prompted us to conduct further studies to investigate the lead compound/s present in the plant and finally to carry out human clinical trials.

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