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Acteoside ameliorates inflammatory responses through NFkB pathway in alcohol induced hepatic damage

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1. Introduction

Alcohol is one of the most psychotropic substances and its chronic consumption leads to liver damage which is a major worldwide health problem [1]. In India, alcohol has emerged to be the leading cause of liver disease related mortality in 2010–2011 [2]. Atleast 10–35% of chronic alcohol consumers develop alcoholic hepatitis. Metabolism of ingested alcohol occurs primarily in the liver, where it is converted to acetaldehyde by the enzyme alcohol dehydrogenase. Acetaldehyde is further metabolized in the mitochondria by aldehyde dehydrogenase to acetate, which is eventually metabolized in the muscles to carbon dioxide. Prolonged alcohol consumption leads to accumulation of reactive oxygen species which cause peroxidation of the cell membrane, inhibition of protein synthesis, altered cytokine production, hepatic DNA damage and eventually cell death [3,4].

NFκβ (Nuclear Factor Kappa β) is a proinflammatory transcription factor which plays a prominent role in promoting cellular stress in liver cells. The NFκβ proteins are sequestered in the cytoplasm of the cells in complex with inhibitory Iκβ proteins [5]. Chronic alcohol intake triggers the translocation of NFκβ into the nucleus by the activation of Iκβ kinase (IKK) which phosphorylates Iκβ leading to its ubiquitination and proteosomal degradation. NFκβ activates the pro-inflammatory genes by binding to their promoter region, thereby upregulating the production of proinflammatory mediators like TNF-α and IL-6 [6–8]. Therefore, an imperative approach to alleviate the liver injury caused by chronic alcohol intake is via suppression of inflammatory reactions.

Currently available treatment options for alcoholic hepatitis like corticosteroids, pentoxifylline, and *N*-acetyl cysteine may lead to serious side effects on prolonged use. Thus, it is important to look for alternative therapeutic options in the form of herbal medicines which offer potent efficacy and safety [9].

Phenylethanoid glycosides (PhGs) belong to a group of water soluble natural compounds. Structurally, they are identified by a hydroxyphenylethyl moiety linked to β-glucopyranose through glycosidic bond. They are widely distributed in medicinal plants and have been reported to be isolated from leaves, roots, and barks of plants [10]. The pharmacological studies reveal that PhGs possess anti-oxidant, antimicrobial, neuro protective, antitumor and analgesic properties [11,12]. The aim of the present study was to evaluate the potential of ACT as a novel drug to treat alcoholic hepatitis by assessing its protective effect on alcohol induced inflammation in HEPG2 and Wistar rat models. Moreover an insight into the underlying mechanism of protective effects of ACT against alcohol induced damage was also carried out in vitro and in vivo. The study revealed that ACT efficiently attenuated the alcohol induced damage to the liver by recouping the levels of serum and tissue parameters, and moreover by suppressing inflammation by the inhibition of NFκβ/Iκβ signalling pathway.

Abbreviations: ACT, acteoside; ALT, alanine transaminase; AST, aspartate transaminase; DMEM, Dulbecco's Modified Eagle's Medium; DNA, deoxyribonucleic acid; ELISA, enzyme linked immunosorbent assay; FBS, fetal bovine serum; GSH, glutathione; HPLC, high performance liquid chromatography; HEPG2, human hepatoma cell line; IKK, Iκβ kinase; IL-6, interleukin-6; LP, lipid peroxidation; MTT, (3-(4,5,dimethylthiazole-2-yl)-2,5 diphenyltetrazolium bromide; NFκβ, Nuclear Factor Kappa β; OECD, Organisation for Economic Cooperation and Development; PBS, phosphate buffer saline; PhG, phenylethanoid glycoside; TG, triglycerides; TGF-β1, transforming growth factor-beta1; TLC, thin-layer chromatography; TNF- α, tumor necrosis factor-alpha

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2. Materials and methods

2.1. Chemicals

All chemicals used were of analytical grade. The kits for the estimation of cytokines were purchased from R&D Systems, USA and those for the estimation of triglycerides and albumin from Transasia Biomedical Ltd. (ERBA Mannheim), Solan (HP), India. Dulbecco's Modified Eagle's Medium (DMEM), Phosphate Buffer Saline (PBS), Trypsin, (3-(4,5,dimethylthiazole-2-yl)-2,5 diphenyltetrazolium bromide (MTT) were purchased from SIGMA. FBS was obtained from GIBCO Invitrogen Corporation. P-NF-κβ p65, p-Iκβ and β-actin were from Cell Signalling Technology.

2.2. Isolation of acteoside

Fresh leaves of *Colebrookea oppositifolia*, procured from IIIM research farm, were shade dried and then grounded to coarse powder with the help of mixer grinder. The coarsely powdered leaves of *Colebrookea oppositifolia* were extracted with 100% ethanol (Hot Extraction; Soxhlet extraction), 50% Aqueous ethanol and 100% water respectively (Cold Extraction; Mechanical Stirring). Dried ethanol fraction was dissolved in minimum quantity of methanol: water mixture and then adsorbed on weighed quantity of silica gel (100–200 mesh) to get free flowing material. The column was first eluted with chloroform. Then column was eluted with the solvent by gradually increasing the polarity with methanol in chloroform. A total 450 fractions of 250 ml each were collected and distilled. TLC of all the fractions was done using different developing solvents. The fractions were pooled on the basis of TLC pattern. Fractions 280–360 showed one major spot on TLC. It was subjected to re-chromatography using Chloroform-methanol as eluent and one compound was isolated and was identified as ACT (Fig. 1). Standardisation was carried out by HPLC analysis (Column: RP18e, Mobile phase: MeOH: 1.5% AcOH in water (gradient), Flow rate: 0.5 ml/min, λ_{max}: 335 nm) performed on a Shimadzu (Nexera) LC-30 AD system consisting of an autosampler (SIL-30 AC); detector (SPD-M20A); communication bus module (CBM-20 A) and Labsolutions software. The percentage of ACT was found to be 20%.

2.3. Cell culture

HepG2 cells were cultured in DMEM medium supplemented with 10% FBS and penicillin streptomycin at 37 °C in a 5% humidified CO_2 atmosphere until cells reached 80% confluence. The controls of Acteoside solvents contained DMSO upto 0.04% while the medium group remained untreated.

2.4. Cell viability assay

HepG2 cells were seeded at a density of 1×10^4 cells/ml into 96 well culture plate and incubated overnight. Cells were treated with different concentrations of ACT and incubated for 48 h. MTT was then added to the cells, incubated at 37 °C for 4 h, following which the cell viability was determined by ELISA reader at 570 nm [13].

$$\text{Cell viability}(\%) = \frac{\text{ODcontrol} - \text{ODsample}}{\text{ODcontrol}} \times 100$$

2.5. Safety study

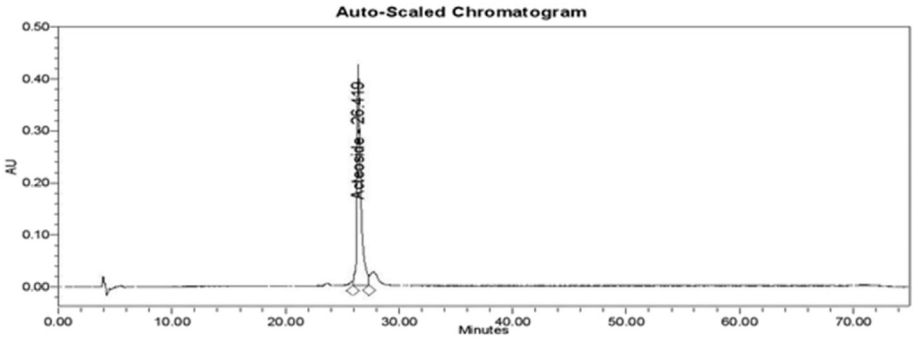
The acute toxicity study of ACT was done according to the OECD guidelines. Female Wistar rats (150–200 g), bred in the Institute's Animal House, were used for the study. Animals were housed under standard conditions (22 ± 3 °C, 50–60% relative humidity and 12 h photo period) and kept on standard rodent pellet diet (Lipton India Ltd., Bombay) and water ad libitum. The animals were kept in their cages for at least 5 days prior to dosing to allow for acclimatisation to the laboratory conditions. Different doses (5–2000 mg/kg) of ACT were given orally to different groups (3 rats/dose), while one group served as the control. The individual animals were observed continuously during the first 30 min, and then periodically during the first 24 h, and finally up to a total of 14 days to check for mortality, signs of toxicity and gross behavioral changes.

2.6. Inhibition of pro-inflammatory cytokines (TNF-α and IL-6)

The ability of ACT to inhibit the production of pro-inflammatory cytokines (TNF-α and IL-6) in HepG2 cells was determined by ELISA. HepG2 cells seeded into 96 well plate at a density of 1×10^4 cells/ml were treated with different concentrations of ACT for 1 h and then stimulated with alcohol (100 mM) for another 24 h. Silymarin was used as positive control. The culture supernatants were collected and assayed for cytokines using ELISA (R&D systems) as per the manufacturer's instructions.

2.7. Western blot analysis

HepG2 cells (0.8×10^6 cells/ml) were treated with ACT and silymarin for 1 h followed by stimulation with alcohol (100 mM) for 24 h. Following treatment, the cells were washed with ice cold PBS and the cell pellet was obtained by centrifugation at 12,000 rpm at 4 °C. The cell pellet was then resuspended in RIPA buffer (SIGMA) for 40 min over ice, after which it was centrifuged at 12,000 rpm at 4 °C to obtain whole cell lysate. Bradford's method was used for protein estimation using



	Peak Name	RT	Area (μV*sec)	% Area	Height (μV)	Amount	Units
1	Acteoside	26.419	9823158	100.00	409669	3000.000	ng

Fig. 1. HPLC profile of Acteoside (ACT).

BSA as the standard. The samples were subjected to SDS-PAGE (sodium dodecyl sulfate-polyacrylamide gel electrophoresis) and transferred to PVDF membrane. Membrane was blocked for 1 h and incubated overnight with primary antibody at recommended concentration. The membrane was then washed and subsequently incubated with peroxidase-conjugated secondary antibody. The detection of proteins was carried out by Immobilon Western (Chemiluminescent HRP substrate, Millipore) mediated chemiluminescence by ChemiDoc XRS + (BioRad).

2.8. Annexin V/PI staining

HepG2 cells were seeded into 6 well plates at a density of 0.01×10^6 cells per well and were pre-treated with ACT at 60 µM, 80 µM and 100 µM for 1 h followed by ethanol (100 mM) for 24 h. Cells were washed with PBS and were resuspended in binding buffer. Before 30 min of termination of experiment annexin antibody and PI were added into each well. The apoptotic cells were analysed by flow cytometer (FACS BD CALIBER).

2.9. Animals

In the present study, female Wistar rats weighing 150–160 g were used [14–16]. They were housed under standard conditions of 12 h photo period, 23.0 ± 2 °C, 60–70% humidity, allowed free access to food (Ashirwad India Ltd., Chandigarh, India) and water. The experimental procedures were in accordance with the Institutional Animal Ethics Committee guide lines for animal care.

2.10. Grouping

The animals were divided into 6 groups (10 animals/group). Group 1 “vehicle control” was given vehicle (distilled water) only. Group 2 was administered alcohol p.o. as per the schedule. Groups 3, 4 and 5 received ACT at doses of 20, 10 and 5 mg/kg p.o. and alcohol as per schedule. Group 6 was administered silymarin at a dose of 50 mg/kg p.o. and alcohol as per schedule.

2.11. Treatment schedule

Alcohol was administered to the animals as per the following schedule: Day 1–Day 7: 5 ml/kg p.o.; Day 8–Day 14: 7.5 ml/kg, p.o.; Day 15–Day 21: 10 ml/kg, p.o.; Day 22–Day 24: 12 ml/kg, p.o. Treatment with the ACT, silymarin and vehicle to the respective groups was given once daily, 30 min after alcohol administration from Day 8 to Day 24.

2.12. Body weight and mortality

The animals were monitored daily for mortality and the body weight was recorded weekly during the study.

2.13. Blood biochemistry

At the end of the experimental period, blood was withdrawn from retro-orbital plexus and allowed to clot for 30 min. It was then centrifuged at 3000 rpm for 10 min to obtain hemolysis free serum for biochemical analysis. The activities of serum transaminases (ALT and AST) were analysed by the method of Reitman and Frankel. Estimation of TG, albumin and cytokines were carried out by standard kits.

2.14. Liver tissue parameters

After the experiment, the animals were sacrificed by decapitation and livers were excised. Before homogenization, the livers were washed, perfused with ice cold 0.9% normal saline and weighed. 10% homogenate were prepared in chilled 10 mM Tris-HCl (pH −7.4) for the estimation of Glutathione (GSH) and Lipid peroxidation (LP) [17,18].

2.15. Overall protection

Overall protection is determined by the following formula

$$H = 1 - \frac{T - V}{C - V} \times 100$$

H = Overall percentage protection, T = Mean value of drug & hepatotoxin
C = Mean of hepatotoxin alone, V = Mean value of vehicle treated animals.

2.16. Histopathology

At the end of the experiment, rats were sacrificed and livers were excised as mentioned under heading 2.6. They were then thoroughly washed in saline, fixed in 10% formalin and embedded in paraffin wax. 4–5 µm sections of livers were prepared and stained with hematoxylin and eosin dye to be examined for histopathological changes [19,20].

2.17. Statistical analysis

Results are expressed as Mean ± SE (n = 6). The data was analysed statistically using student's *t*-test. p-Value < 0.05 was considered as criterion of significance.

3. Results

3.1. Standardisation of ACT

The HPLC protocol of ACT was developed and the HPLC profile indicated 100% purity of the isolated ACT (Fig. 1).

3.2. Effect of ACT on viability of HepG2 cells

The MTT results showed that ACT was non-toxic to HepG2 cells with viability being > 90% up to 100 µM concentration (Fig. 2).

3.3. Safety study

In acute toxicity study, the oral LD_{50} of ACT was found to be > 2000 mg/kg. Moreover no mortality, signs of toxicity or gross behavioral changes were observed, suggesting that ACT possess a reasonable margin of safety.

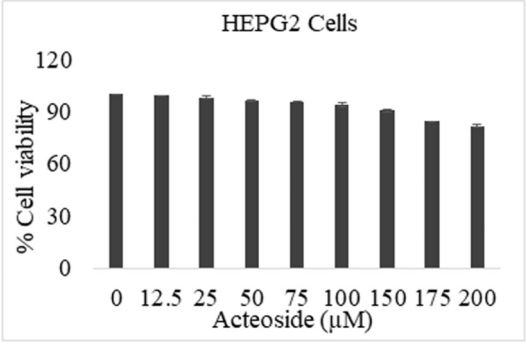


Fig. 2. Viability of HepG2 cells after 48 h treatment with ACT.

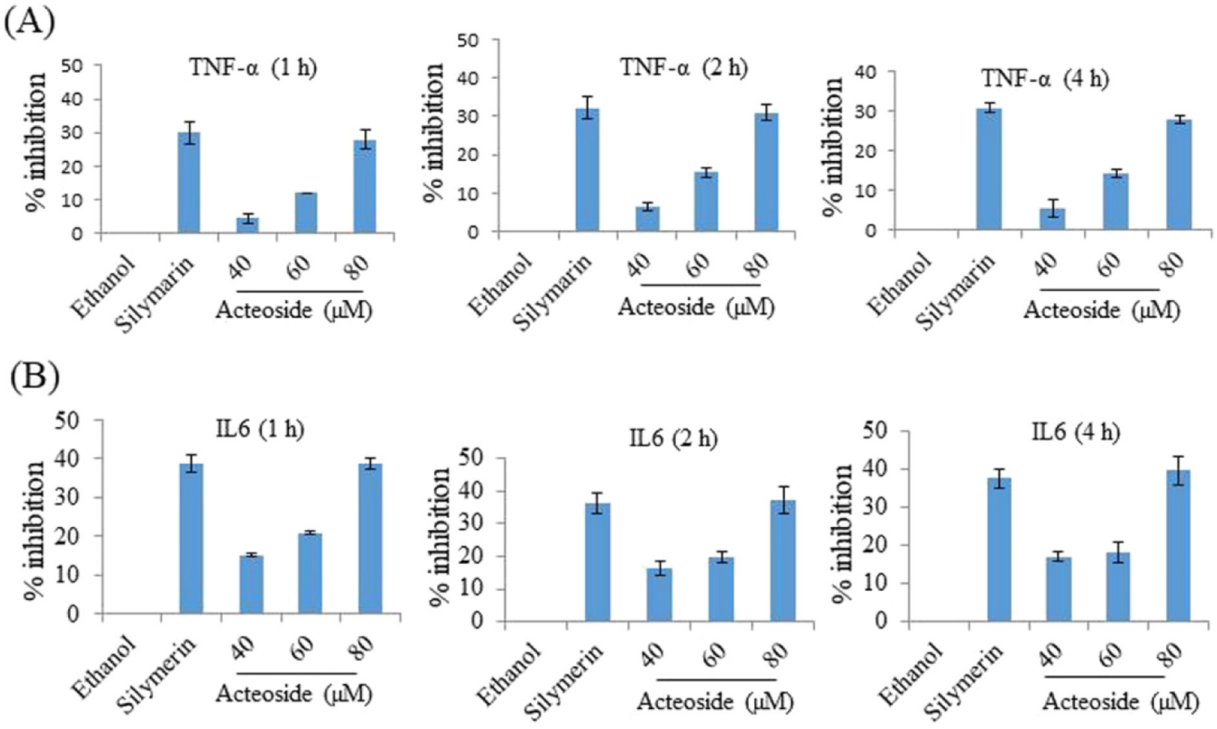


Fig. 3. Inhibition of alcohol induced TNF-α and IL-6 production by acteoside in HepG2 cells. HepG2 cells were pretreated with ACT (40, 60, 80 μM) for 1 h, 2 h and 4 h, followed by stimulation with alcohol (100 mM) for 24 h. Cytokine production was measured with ELISA. Each value represents mean ± standard deviation of three independent experiments.

3.4. Effect of ACT on alcohol induced production of proinflammatory cytokines in HepG2 cells

Excessive inflammation and hepatocellular damage in alcoholic hepatitis is caused due to the over production of pro inflammatory cytokines. ELISA assays were carried on cultured supernatants from HepG2 cells and on serum samples collected from rats treated with ethanol or without acteoside. There was a significant increase in the production of TNF-α and IL-6 in alcohol induced cells. Pre-treatment with ACT at 40, 60 and 80 μM for 1 h, 2 h and 4 h inhibited the alcohol induced production of TNF-α and IL-6, with maximum inhibition shown at 80 μM concentration. The percent inhibition of TNF-α and IL-6 by ACT was evaluated to be 28.28% and 37.91% respectively. The positive control, Silymarin (100 μM) showed 29.9% of TNF-α and 38.7% of IL-6 (Fig.3). The effect of ACT was constant at 1 h, 2 h and 4 h respectively. Thus ACT contributes to hepatoprotection by modulating proinflammatory cytokines production.

3.5. Inhibitory effect of ACT on activation of NFκβ/Iκβ signalling axis

Various immune and inflammatory responses are regulated by the key the NF-κB signalling pathway which control the expression of various proinflammatory cytokines such as IL-6 and TNF-α.As indicated above ACT potentially reduced the level of IL-6 and TNF-α but to have an insight into the molecular mechanism underlying the inhibitory effect on proinflammatory cytokines the NFKB pathway was analysed by Western Blot. HepG2 cells were exposed to ethanol, and were treated with ACT, after which the protein expression levels of NF-κB were detected by western blot analysis. Treatment with ACT at different concentrations decreased the levels of phopho (p)-NFκβ p65 in alcohol stimulated cells when compared to the p65 expression of the lysates of only ethanol-exposed cells (Fig. 4). they also downregulated the phosphorylation of Iκβ in alcohol stimulated cells (Fig. 4) in the similar pattern, thereby suggesting that ACT inhibits the activation of NFκβ.

3.6. Body weight and survival analysis

There was a significant difference observed in the average body weights of normal control and alcohol treated group. Treatment with ACT, particularly at 20 mg/kg and 10 mg/kg increased the weights towards normal (Fig. 5A).

Survival was significantly less in alcohol administered group as compared to the vehicle control. A much decreased mortality was observed in ACT treated groups as compared to alcohol intoxicated group, thereby suggesting the protective effect of ACT against alcohol induced mortality (Fig. 5B).

3.7. Serum parameters

Alcohol administration resulted in significant increase in the levels of serum marker enzymes (ALT and AST), TG and a decrease in the levels of albumin, suggesting damage to the hepatic cell membrane. Treatment with acteoside at doses of 20, 10 and 5 mg/kg dose dependently reversed the levels of these parameters, thereby depicting its protective effect.

Chronic alcohol exposure induced significant increase in the expression of pro-inflammatory cytokines like TNF-α and IL-6. Expression of TGF-β1 was also found to be elevated in alcohol administered rats, which has been implicated in the development of hepatic fibrosis. Treatment with ACT successfully suppressed the expression of cytokines, suggesting that the protective action of acteoside may be due to its anti-inflammatory property (Fig. 6). It also dose-dependently recovered the levels of TGF-β1, indicating that ACT may help to control the progression of fibrosis (Table 1).

3.8. Hepatic tissue parameters

A significant increase in the levels of lipid peroxidation and a decrease in GSH was recorded in alcohol intoxicated rats, which is an indicator of tissue oxidative stress. Treatment with ACT produced a

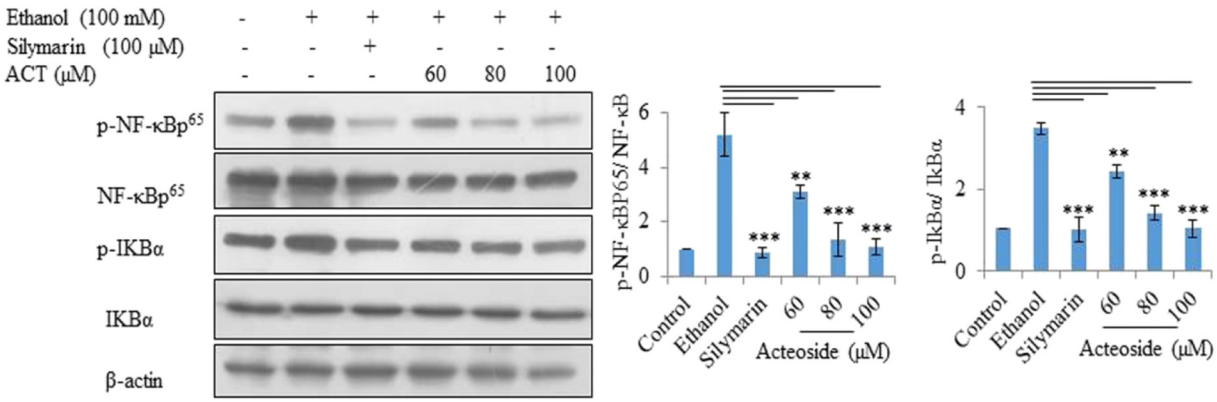


Fig. 4. Inhibition of the alcohol induced activation of NF-κβ signalling pathway. Effect on phosphorylation of NF-κβ and IKβ in a dose dependent manner was studied in comparison to silymarin (100 µM) by western blotting. The graphs shown represent the mean ± standard deviations of three independent technical repeats. Statistical significance was assessed by one-way ANOVA followed by Dunnett's test. *p < 0.05; **p < 0.01; ***p < 0.001 vs alcohol alone.

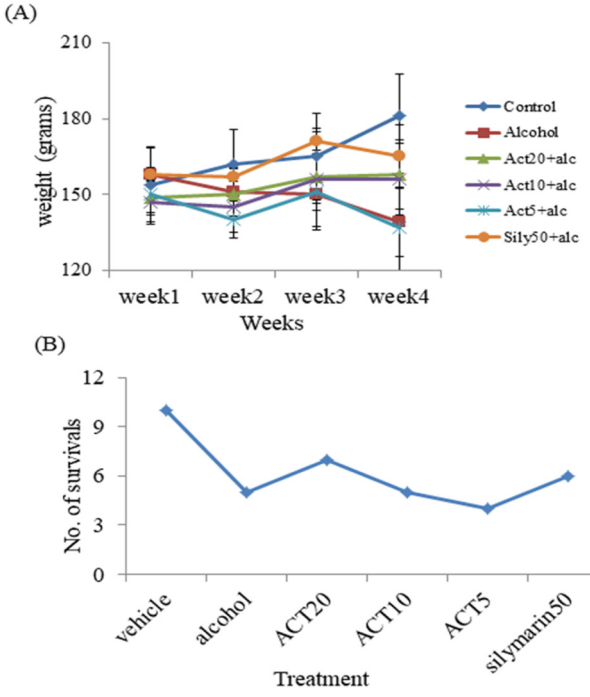


Fig. 5. (A): Weekly body weight record, values represent mean ± standard deviation (n = 10), (B): effect of ACT on mortality against alcohol toxicity.

potential decrease in lipid peroxidation and an increase in hepatic GSH in a dose dependent manner, which depicts that it possesses anti-oxidant property (Table 2).

3.9. Overall protection

Treatment with ACT at doses of 20, 10 and 5 mg/kg exhibited overeall protection of 66.65 ± 7.32%, 60.73 ± 7.89% and 44.43 ± 7.8% respectively, whereas silymarin exhibited 63.13 ± 7.77% protection (Fig. 7).

3.10. Histopathological analysis and effect of ACT on apoptosis

Microscopic examination revealed nuclear degeneration of hepatocytes, ballooning hepatocytes, extensive hepatic microsteatosis, necrosis and lymphocytic infiltration in alcohol administered rats. ACT at the dose of 20 mg/kg showed significant liver protection against alcohol induced hepatoxicity, well comparable to silymarin (Fig. 8(A–F)). It was also confirmed that ACT at a concentration of 60, 80 and 100 µM protected HepG2 cells against ethanol induced apoptosis as detected by the cleavage of caspase 3 (Fig. 8G). Furthermore, the annexin PI cells positive cells induced by ethanol was rescued by dose dependent manner. (Fig. 8H). These results clearly demonstrated that ACT rescued the cells from ethanol induced apoptotic cell death.

4. Discussion

Alcohol-induced hepatitis is one of the devastating outcomes of alcohol overconsumption. The extensive studies have shown that the

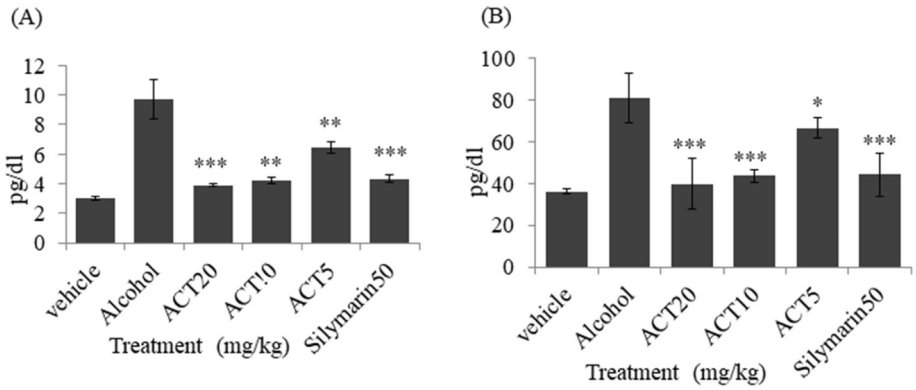


Fig. 6. Effect of ACT on alcohol induced cytokine levels in Wistar rats. A, TNF-α. B, IL-6. Values represent the mean ± standard deviation (n = 10). *: p < 0.05; **: p < 0.01; ***: p < 0.001, ns: not significant.

Table 1
Effect on serum parameters.

Treatment	Dose mg/kg	Serum parameters				Cytokines		
		ALT (units)	AST (units)	Albumin (g/dl)	TG (g/dl)	IL-6 (pg/ml)	TGF β1 (ng/ml)	TNF-α (pg/ml)
Vehicle	–	49.62 ± 2.75	54.25 ± 1.48	4.01 ± 0.098	117.09 ± 0.09	36.22 ± 1.07	64.84 ± 1.16	3.0 ± 0.15
Vehicle + alcohol	–	77.57 ± 1.53^{a,***}	85.27 ± 1.22^{a,***}	2.82 ± 0.11^{a,***}	268.54 ± 21.89^{a,***}	81.09 ± 11.81^{a,***}	98.97 ± 1.11^{a,***}	9.68 ± 1.33^{a,***}
Acteoside + alcohol	20	57.54 ± 2.75^{b,***} (71.6%)	64.44 ± 1.53^{b,***} (67.1%)	3.29 ± 0.15^{b,*} (39.49%)	173.09 ± 3.62^{b,**} (63.02%)	39.85 ± 12.32^{b,*} (91.9%)	64 ± 3.80^{b,***} (87.7%)	3.92 ± 0.1^{b,***} (86.2%)
Acteoside + alcohol	10	59.22 ± 3.88^{b,**} (65.2%)	65.32 ± 2.18^{b,***} (64.3%)	3.08 ± 0.12^{b,ns} (21.84%)	176.5 ± 4.64^{b,**} (60.7%)	43.76 ± 3.03^{b,*} (83.19%)	64.48 ± 2.89^{b,***} (81.5%)	4.2 ± 0.2^{b,**} (82.03%)
Acteoside + alcohol	5	60.96 ± 1.47^{b,***} (57.39%)	66.05 ± 2.50^{b,***} (61.9%)	2.92 ± 0.05^{b,ns} (8.40%)	191.37 ± 3.59^{b,***} (50.9%)	66.67 ± 4.94^{b,ns} (32.13%)	66.96 ± 3.45^{b,***} (79.1%)	6.42 ± 0.3^{b,*} (48.8%)
Silymarin + alcohol	50	55.8 ± 2.75^{b,***} (72.17%)	62.71 ± 1.60^{b,***} (63.89%)	3.1 ± 0.09^{b,ns} (23.52%)	173.27 ± 1.54^{b,***} (62.9%)	44.30 ± 10.30^{b,*} (81.9%)	58.09 ± 5.0^{b,***} (90.4%)	4.35 ± 0.2^{b,**} (79.6%)

Values represent the mean ± SE and within parentheses hepatoprotective activity percent of five animals in each group; ns: not significant; units: Each unit is μmol pyruvate/min/L.
[a] Compared to vehicle.
[b] Compared to vehicle + alcohol.
* $p < 0.05$.
** $p < 0.01$.
*** $p < 0.001$.

Table 2
Effect on hepatic parameters.

Treatment	Dose (mg/kg)	Hepatic parameters	
		LP	GSH
Vehicle		92.66 ± 2.75	5.03 ± 0.06**
Vehicle + alcohol		177.27 ± 10.01^{a,***}	2.85 ± 0.16^{a,***}
Acteoside + alcohol	20	149.56 ± 0.5^{b,*} (32.7%)	4.16 ± 0.14^{b,***} (60.09%)
Acteoside + alcohol	10	153.25 ± 1.82^{b,*} (28.3%)	4.15 ± 0.09^{b,***} (59.63%)
Acteoside + alcohol	5	162.45 ± 3.9^{b,ns} (17.5%)	3.95 ± 0.11^{b,***} (43.8%)
Silymarin	50	139.27 ± 2.39^{b,***} (44.9%)	3.8 ± 0.17^{b,***} (43.5%)

Values represent the mean ± SE and within parentheses hepatoprotective activity percent of five animals in each group. ns: not significant. LP: lipid peroxidation (nmol MDA/g liver); GSH: glutathione (μmol GSH/g liver).
[a] Compared to vehicle.
[b] Compared to vehicle + alcohol.
* $p < 0.05$.
** $p < 0.01$.
*** $p < 0.001$.

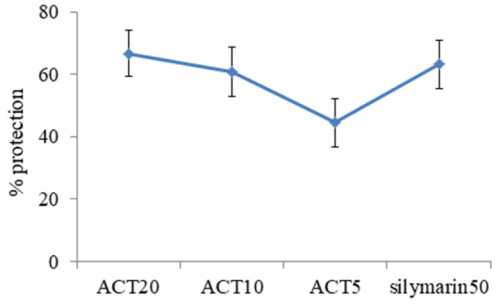
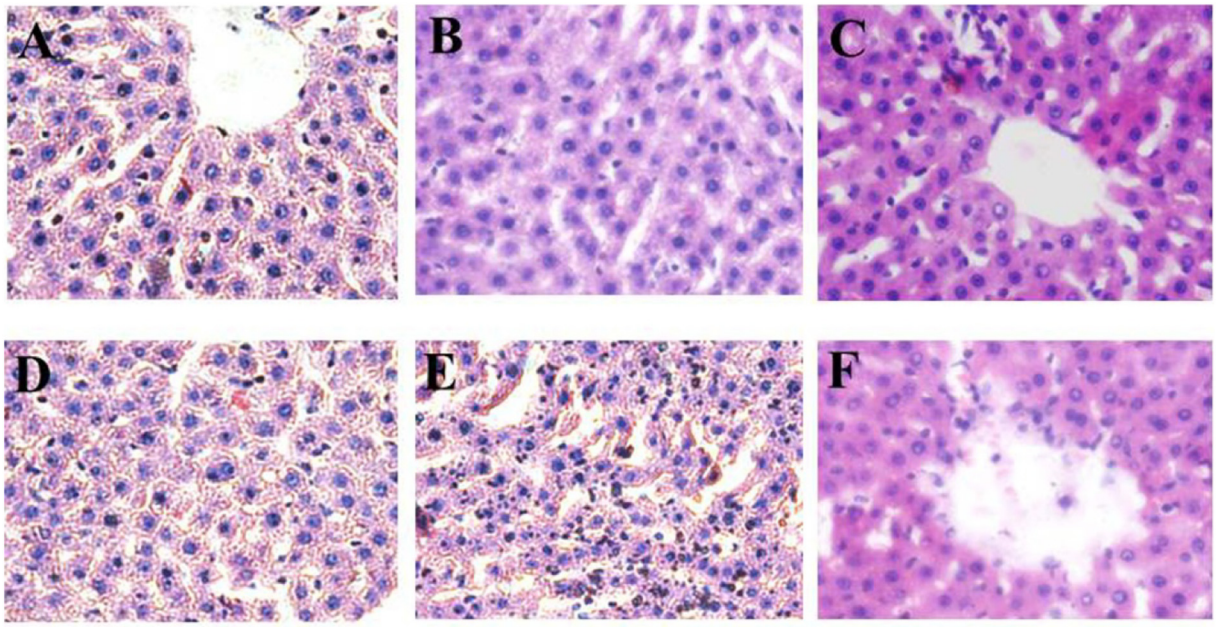
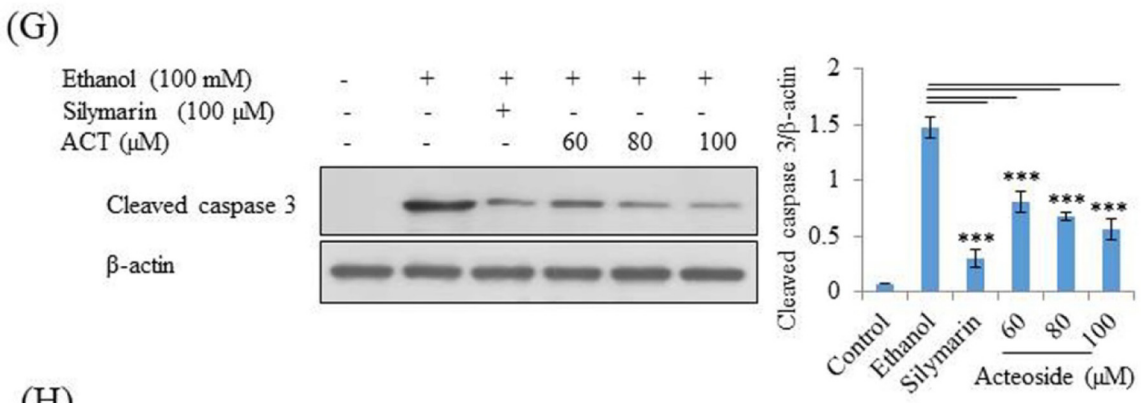


Fig. 7. Overall protective percentage.

pathogenesis of alcoholic hepatitis include oxidative damage, excessive inflammation, metabolic damage, apoptotic damage etc. [21]. About 80–90% of the ingested alcohol is metabolized in the liver, thus making it more susceptible to alcohol induced damage [22]. Chronic alcohol consumption leads to structural changes in the cell, thereby increasing the permeability of the cell membrane. This increased permeability results in leakage of serum transaminases (ALT and AST) into the blood circulation, which are key indicators of hepatic damage [23]. Inflammatory reactions and inflammatory factors can further deteriorate the oxidative stress induced cell injury. Alcohol overconsumption can cause oxidative stress and inflammation which can damage important cell organelle structure or function. Acteoside is one of the most well characterized phenyl glycosides. Acteoside is reported to possess wide range of biological activities such as Antioxidant, hepatoprotective, anti-metastatic etc. [24]. The present study demonstrated that acteoside may play an important role in protection against alcoholic hepatitis. Treatment with ACT ameliorated the increased levels of ALT and AST which may be suggestive of repair of hepatic tissue damage [25]. Abnormality in triglyceride metabolism and its accumulation in hepatocytes is another ill effect of chronic intake of alcohol which leads to their increased levels [26,27]. Studies have shown that serum albumin is also known to decrease in alcoholic hepatic damage [28]. ACT successfully recovered the abnormal changes in the levels of triglycerides and albumin in a dose dependent manner, implying protection against fatty liver and protein synthesis.

Various experimental researches have indicated an increase in the permeability of the intestinal membrane due to chronic alcohol consumption which augments the portal circulation of endotoxin [29]. This enhances the sensitivity of the kupffer cells to LPS-stimulated





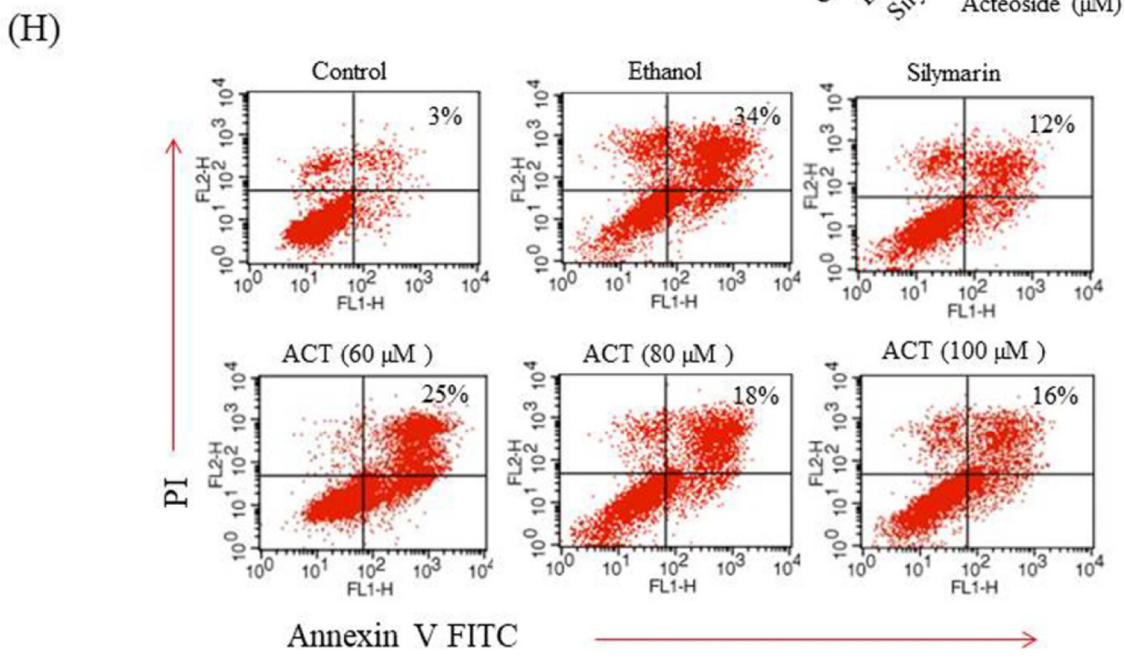


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Fig. 8. Histopathology of liver tissues. (A) Normal rat liver section: shows normal hepatocytes with well-preserved cytoplasm; (B) alcohol stimulated liver section: shows nuclear degeneration, microsteatosis, necrosis, lymphocytic infiltration; (C) ACT20 + alcohol: shows central vein with less hepatocyte degeneration and less microsteatosis; (D) ACT10 + alcohol: shows less extensive microsteatosis and scattered lymphomononuclear cell infiltrate; (E) ACT5 + alcohol: shows degeneration of hepatocytes, microvesicular steatosis, necrosis lymphomononuclear cell infiltrate; (F) silymarin50 + alcohol: shows very less steatosis, most of the hepatocytes show well preserved cytoplasm, scattered lymphomononuclear cell infiltrate; (G) Western blot analysis of cleaved caspase 3 in HepG2 cells. (H) Flow cytometer analysis of apoptotic cells induced by ethanol in HepG2 cells.

production of TNF-α, one of the major inflammatory cytokines. TNF-α is known to damage liver by causing injury. Elevated levels of TNF-α further induces the production of other cytokines like IL-6and IL 8 [30]. In the present study, ACT significantly decreased the levels of TNF-α and IL-6, both *in vitro* and *in vivo*. The damage evoked by alcohol was successfully reversed by ACT, suggesting the anti-inflammatory potential of ACT. NF-KB pathway a prototypical proinflammatory signalling pathway plays a crucial role in alcoholic hepatitis. The role of NF-kB in inflammation has long been considered as vital because of its expressive involvement in proiflammatory genes like chemokines, cytokines and adhesion molecule. Alcohol has been reported to upregulate the activation of NFκβ pathway [31]. The anti-inflammatory potential of ACT was confirmed by studying the NFκβ pathway. As shown by our study, ACT dose dependently decreased the upregulated expression of NFκβ and inhibited the phosphorylation and degradation of Iκβ, confirming that ACT protects against alcohol induced injury by inhibiting the NFκβ/Iκβ signalling pathway.

The literature shows that during alcohol metabolism in the liver, a large amount of ROS is generated through alcohol metabolism and mitochondrial pathway [32]. Large amount of toxic products and acetaldehyde is generated during alcohol metabolism which mediates toxicity [21]. During excessive alcohol exposure important cell organelles are damaged due to oxidative stress and acetaldehyde toxicity. The function and structure of various cellular organelles such as endoplasmic reticulum is disordered and mitochondrial dysfunction has been observed in alcoholic hepatitis [21].

In the liver, alcohol is primarily metabolized to acetaldehyde, which is known to upregulate the expression of TGF-β1 [33]. Kupffer cells have also been implicated to be an important source of TGF-β1. Excessive alcohol consumption stimulates TGF-β1 to activate the stellate cells, which are considered to be the major fibrogenic cells of the liver [34]. Our study clearly demonstrated the increased expression of TGF-β1 in alcohol administered rats. Treatment with ACT dose dependently ameliorated the expression of TGF-β1, depicting that ACT may be helpful in suppressing the development of fibrosis.

Chronic alcohol metabolism enhances the production of reactive oxygen species and depletes the level of antioxidants (like GSH), thereby creating a state of oxidative stress in the liver cells. The reactive oxygen species also react with the macromolecules like lipids, thereby causing peroxidation of the lipids [35]. Treatment with ACT at 20, 10 and 5 mg/kg recovered the abnormal levels of lipid peroxidation and GSH produced by alcohol administration, thereby indicating the antioxidative potential of ACT.

The biochemical findings were very well supported by the histopathological observations. Treatment with ACT showed marked recovery of the damage produced by alcohol in a dose dependent manner, hence suggesting the protective potential of ACT against alcohol induced liver injury. Apoptosis was further validated by cleaved caspase 3 and annexin V FITC which was induced by ethanol. Surprisingly, ACT rescued HepG2 cells in a dose dependent manner.

5. Conclusion

In summary, our study demonstrated that the protective effect of *Colebrookea oppositifolia* against alcoholic hepatitis may be due to the presence of acteoside – a phenylethanoid glycoside in it. The study proves that acteoside protects against alcoholic hepatitis mainly by inhibiting the production of cytokines and downregulating the NFκβ/Iκβ signalling pathway. It also helps in preserving the structural integrity of the cell membrane and possesses antioxidant properties. Thus, it can be further explored to be a safe and effective therapy for alcoholic hepatitis in future.

Conflict of interest

The authors declare that they have no conflicts of interest.

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