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Effect of azelaic acid attenuates ethanol-induced mitochondrial dysfunction, oxidative stress, and apoptosis in human Chang liver cells

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Abstract

The intake of alcohol is a distressing global health problem and ethanol drunkenness has been categorized by hepatic toxicity and oxidative stress resulted in alcoholic liver disease (ALD). Ethanol is a lipophilic substance which promptly distributes to the cell membrane triggering damage to proteins, carbohydrates, lipids and nucleic acids. Oxidative stress plays an important role in ethanol-induced hepatotoxicity, and therefore antioxidants are developing as an important tool in inhibiting alcoholic liver diseases. This study was performed to evaluate the effect of azelaic acid on ethanol-induced toxicity. Azelaic acid is a naturally occurring saturated dicarboxylic acid and is known to possess profound anti-inflammatory and antioxidative effects. Ethanol (30 mM) treatment to Chang liver cells exhibited loss of cell membrane integrity, increased ROS generation, induced mitochondrial dysfunction, and DNA damage, increased the levels of lipid peroxidation, decreased the levels of antioxidants and increased the levels of proapoptotic markers such as Bax, caspase -8,-9 and -3 as compared to the control. In contrast, all there above parameters were regressed when the cells were treated with azelaic acid (500 µM). These findings suggesting that the marked hepatoprotective efficacy of azelaic acid against ethanol-induced liver cell damage.

Keywords: Ethanol (EtOH), Azelaic acid (AzA), Mitochondrial dysfunction, Chang liver cells, Alcoholic liver disease, Hepatotoxicity.

Introduction

Alcoholic liver disease (ALD) is one of the prominent causes of indisposition and impermanence worldwide (Browning et al. 2004). ALD encompasses a broad spectrum of diseases and morphological changes include alcoholic fatty liver or steatosis, alcoholic hepatitis, alcoholic cirrhosis, and finally hepatocellular carcinoma (Tome et al. 2004).

Chronic ethanol ingestion can contribute to some of the major factors, including alterations in the redox ratio, accretion of acetaldehyde, increased lipid peroxidation, formation to 1-hydroxyethyl radical, depletion of GSH levels, mitochondrial dysfunction and lipid amassing in the hepatic tissue, all of which has been associated in the pathogenesis of ALD (Caro et al. 2004; French et al.1997).

There are several signaling pathways are rehabilitated during the pathogenesis of alcoholic liver toxicity. The pro-inflammatory cytokines such as TNF-α accompanying

damage and repair progressions are elevated in ALD. The TNF- α exposed cells overproduce ROS from the respiratory chain (Hennet et al.1993) and it prompts the opening of the mitochondrial permeability transition pore (MPT) (Bradham et al.1998). An escalation in the mitochondrial membrane permeabilization indications to cytochrome c release, activation of caspases that leads to stimulation of apoptosis (Kaviarasan et al. 2008). The non-proteins localized in the mitochondria (*e.g.* Bim, Bak, and Bcl-2), are also rapidly upregulated. Cytochrome c binds to Apaf-1 and activates caspases-9, and subsequently downstream caspases that magnify the death process(Petronilli et al. 2001).

Azelaic acid is a saturated 9 carbon atom dicarboxylic acid, which is extensively present in grains such as wheat, rye, barley, oat seeds and sorghum. It has been reported to show antileukemic (Pan et al. 2017), antitumor (Marsden et al. 1983) and antidiabetic possessions Besides, it is also documented to be hepatoprotective to type 2 diabetic mice(Muthulakshmi et al. 2013). However, till to date, there is no report showing the role of azelaic acid on alcohol-induced hepatotoxicity. Therefore, our present study was formulated to scrutinize the hepatoprotective effects of azelaic acid against alcohol induced toxicity in Chang liver cells.

Materials and methods

Chemicals

3-(4, 5-dimethylthiazol-2-yl) -2,5-diphenyl tetrazolium bromide (MTT), Dimethyl sulfoxide (DMSO) 2', 7'-Dichlorofluorescein diacetate (DCFHDA), rhodamine -123 (Rh-123), Hoechst 33258, and azelaic acid were purchased from Sigma Chemicals Co. (St. Louis, USA). Primary monoclonal antibodies such as anti-tumor necrosis factor - α (TNF-α), anti-caspases -3, -8, and -9, anti-Bcl-2-associated X protein (Bax), anti-B cell lymphoma-2 (Bcl-2) and β actin were obtained from Cell Signaling Co (Beverly, MA, USA). All other chemicals and solvents were of analytical grade.

Cell culture

Human Chang liver cell line was obtained from the National Center for Cell Science (NCCS), Pune, India. The cells were routinely grown in DMEM growth medium complemented with 10% fetal bovine serum, 100 µg/ml penicillin and 100 µg/ml streptomycin. Cells were incubated in 25 cm^2 tissue culture flasks at 37° C in a humidified atmosphere (5% CO_2).

Cytotoxicity assay

The viability of Chang liver cells was assessed by 3-(4,5-dimethylthiazol-2-yl) -2,5-diphenyl tetrazolium bromide (MTT) assay by the method of (Mosmann 1983). The cells were seeded in a 96-well microtitre plate (3x 10^3 cells/well) and then incubated at 5% CO_2 for 24 hours. Cells were treated with 30 mM of ethanol with varying concentrations of azelaic acid (15.625-2000 µM) for 24, 48 and 72 h.

After 72 h of exposure to the additives, 50 µl of MTT (1 mg/ml) was added to each well, and the cells were incubated in the dark at 37° C for an additional 4h. Thereafter, the medium was removed, the formazan crystals were dissolved in 200 µl of dimethyl sulphoxide, and the absorbance was measured at 570 nm.

Assay of lactate dehydrogenase (LDH)

The LDH assay was performed by the method of (King 1965) by determining by the release of LDH into the culture medium. Chang liver cells were seeded in a 24-well plate at the density of 2×10^5 cells/well in 3 mL of culture medium. After exposure for 72 h, cells were collected and washed twice with PBS. The total LDH activity was determined after the cells were disturbed thoroughly by sonication using 0.2 M Tris-HCl, 6.6 mM NADH and 30mM sodium pyruvate. The LDH activity was determined spectrophotometrically at 340 nm.

Hoechst 33258 staining

The azelaic acid on ethanol-treated cells was stained with the fluorescent DNA binding dye Hoechst 33258, to detect the nuclear fragmentation by the method of (Ribble et al. 2005). Chang liver cells were seeded in a 24 well plate at the density of 1×10^5 cells/well in 3 mL of culture medium. After exposure for 72 h, cells were collected and washed twice with phosphate buffered saline. Subsequently, the cells were incubated in Hoechst 33258 stains having a final concentration of 10 µg /ml at room temperature for 30 min. Nuclear morphology was then detected under a TS 100 fluorescence microscope.

DCFH-DA staining

Intracellular ROS generation was determined using DCFH-DA probe on fluorescence spectrophotometer by the method of (Halliwell et al. 2004). In the presence of an oxidant, DCFH-DA is renewed to the highly fluorescent dichlorofluorescein. Next, the cells were treated with ethanol and subsequently with azelaic acid for 24, 48 h and 72 h. Cells were then rinsed with PBS and incubated with 10 µM DCFH-DA in PBS for 30 min at 37°C in dark. The fluorescence readings were then normalized to the corresponding cell number to give a relative value of DCF fluorescence units. Percentage changes in ros production of the treated were determined by comparing to the untreated control.

Alteration of mitochondrial membrane potential

The changes in mitochondrial membrane potential were investigated using with rhodamine 123 followed by the method of (Scaduto et al. 1999). Cells were seeded in a 6-well plate (1×10^5cells/well) for 24 h, later they were treated with ethanol and after 48 h prior to the treatment with azelaic acid, cells were incubated with 10 µl rhodamine 123 (1 mg/ml) for 30 min in the dark at 37°C. Cells were then rinsed with PBS, fluorescence was observed under the fluorescence microscope using a blue filter (450-490 nm). Polarized mitochondrion emits orange-red fluorescence. Fluorescent intensity was dignified at 535 nm using spectrofluorometer.

Estimation of lipid peroxidation

Exposure of ethanol to azelaic acid treated Chang liver cells in T75 flask were trypsinized and washed with PBS. Cells were seeded in a 24-well plate at a density of $1X10^5$ cells/well in 3 ml of complete medium. After 24 h, the cells were treated with 1 ml of 0.5M KCl in 10 mM Tris-HCl, mixed and then added 0.5 ml of 30 % trichloroacetic acid (TCA) and 0.5 ml of 52 mM thiobarbituric acid and heated in a water bath at 90°C for 30 min. After cooling, the mixture was centrifuged at 3000x g for 10 min. The absorbance of the supernatant was dignified at 532nm. The concentration of the byproducts of lipid peroxidation, thiobarbituric acid reactive substances (TBARS) was determined by the method of (Niehaus et al. 1968).

Measurement of GSH/GSSG

The cells were seeded at densities of $1X10^5$ per well in six-well plates in a 3ml complete medium. After incubation with additives, the cells were rinsed with PBS twice. The cells were collected, then 250 µl of ice-cold 10% TCA and 0.01 N HCl were added to the cell pellets. The tubes were instantaneously agitated with a vortex mixer, kept on ice for 15 min and centrifuged at 12,000 ×g for 20 min at 4°C. The resulting supernatants were removed six times through diethyl ether to remove TCA. The aqueous phase was divided into two parts and used for total GSH and GSSG measurement (Tietze 1969).

Comet assay

Comet assay was accomplished to evaluate DNA strand breaks, according to by the technique of (Singh 2000). Frosted microscopic slides were shielded with 200 µL of 1% normal melting agarose in PBS at 65°C and the second layer of 100 µl of 1% low melting agarose encompassing approximately 10^5 cells was added immediately with a cover slip placed over a frozen ice pack for 5 min. After solidification of the low melting agarose, coverslips were

detached and the slides were immersed in ice-cold lysis solution such as 2.5 M NaCl, 100 mM EDTA and 1% Triton X-100 for 16 h at 4°C. After eliminating from the lysis solution, the slides were placed horizontally in an electrophoretic tank filled with alkaline buffer (300mM NaOH, 1mM EDTA, pH >13.00). The slides were endorsed to stand in the electrophoretic tank for 20 min after which electrophoresis was carried out at 0.8 V/cm for 15 min. After electrophoresis, the slides were washed with distilled water stained with 50 µl ethidium bromide and observed beneath a Nikon fluorescence microscope equipped with a 365 nm excitation filter and a 435 nm barrier filters. The extent of DNA damage was calculated by % head DNA, tail length, tail movement, and olive tail movement (OTM). Comet images were examined using Comet Assay Software Programme (CASP).

Immunoblotting

Chang liver cells were harvested, washed with PBS and the lysate was prepared in RIPA buffer (25mM Tris (pH 7.6), 150 mM NaCl, 1% NP-40, 1% sodium deoxycholate and 0.1% SDS). Approximately, 30 µg lysate was resolved in 10–12% SDS-PAGE and reassigned onto the PVDF membrane (Millipore, USA). The PVDF membrane was blocked and incubated with the respective primary antibodies [TNF- α (1: 300), Mitochondrial and cytosol cytochrome c (1:300), Bax (1:300), caspase -3,-8,-9 (1:400), and Bcl-2 (1:300)], at 4°C overnight. Blots were washed, incubated with HRP-conjugated secondary antibodies and bands were identified by treating the membranes with 3, 3'-diaminobenzidine tetrahydrochloride (western blot detection reagent, Sigma USA) and densitometry was done using 'Image J' analysis software.

Statistical analysis

Values are expressed as mean ± SD. The data were statistically investigated by one-way analysis of variance (ANOVA) followed by Duncan's multiple range test (DMRT) using a statistical package program (SPSS 10.0 for windows). $P < 0.05$ were considered statistically significant.

Results

Effect of azelaic acid on ethanol-induced cytotoxicity in Chang liver cells

The cytotoxic consequence of ethanol and azelaic acid in Chang liver cells were examined by MTT assay. Fig. 1shows that 30 mM concentration ethanol initiated significant cell death and it resulted in oxidative stress. Further, the nontoxic concentration of azelaic acid shows upto 1000 µM concentration of azelaic acid did not cause significant cytotoxicity in Chang liver cells. Hence, the minimum concentration of azelaic acid (500 µM) was selected against ethanol- induced toxicity of this study. Moreover, we found that azelaic acid (500 µM) significantly prevented ethanol (30 mM) induced cytotoxicity in Chang liver cells.

Effect of azelaic acid on ethanol-induced lactate dehydrogenase (LDH) activity in Chang liver cells

Lactate dehydrogenase (LDH) activity was increased, indicating leakage of the cytosolic enzyme across the cytoplasm in ethanol-treated cells, whereas ethanol plus azelaic acid treated cells showed rejuvenated LDH enzyme activity (Table 1)

Effect of azelaic acid on ethanol-induced lipid peroxidation and antioxidant status

Chang liver cells treated with ethanol (30 mM) for 48 h showed a significant increase in the levels of TBARS and a significant decrease in the levels of antioxidants such as GSH as compared to the control cells, (Table 1). However azelaic acid treatment of ethanol-treated Chang liver cells for 72h showed elevated levels of antioxidants and reduced levels of lipid peroxidation.

Effect of azelaic acid on ethanol-induced reactive oxygen species

Overproduction of ROS involved in oxidative stress. In this study, ethanol-treated Chang liver revealed a significant increase in the ROS production when compared to the control cells. In Fig 2, ethanol (30 mm) treated Chang liver cells exposed to azelaic acid for 48h showed significantly decreased the intracellular ROS levels when compared to ethanol (30 mM) alone group.

Effect of azelaic acid on ethanol-induced mitochondrial membrane potential

Alteration of mitochondrial membrane potential has been considered as the earliest sign of apoptosis. Mitochondrial membrane potential (MMP) was assessed by staining with the mitochondria-specific dye rhodamine 123. On ethanol (30 mM) exposure for 48 h, the mitochondrial membrane potential was significantly decreased as compared to the control. However, treatment with azelaic acid (500 µM) attenuated ethanol-induced mitochondrial membrane depolarization, as evidenced by an increase in the fluorescence intensity (Fig. 3).

Effect of azelaic acid on ethanol-induced nuclear apoptosis

Nuclear fragmentation and condensation main event for conforming apoptosis. Azelaic acid on ethanol-induced nuclear fragmentation was assessed by Hoechst staining. In this study, ethanol (30 mM) showed increased nuclear condensation a treated Chang liver cells as experimental by a fluorescent microscope (Fig. 3). Azelaic acid (500 µM) treatment significantly reduced the nuclear condensation and fragmentation in ethanol-exposed Chang liver cells.

Effect of azelaic acid on ethanol -induced DNA damage

Ethanol-induced oxidative stress-mediated DNA damage was studied by using comet assay in differentiated Chang liver cells (Fig. 4). Ethanol (30 mM) treated cells significantly increased tail moment, tail length, % head DNA and olive tail moment, In contrast, azelaic reduced the DNA damage attributes in ethanol exposed cells.

Effect of azelaic acid on ethanol-induced inflammatory and apoptotic marker expressions

TNF-α expression was upregulated in ethanol-treated Chang liver cells, whereas treatment with azelaic acid reduced the pro-inflammatory cytokine TNF-α expression. The Bcl-2 and Bax are Bcl-2 family members involved in apoptosis, but with opponent effects. Bcl-2 has been implicated in inhibiting apoptosis, whereas Bax promotes apoptosis. Western blot analysis showed that azelaic acid treatment significantly upregulated the expression of Bcl-2 and slightly downregulated the expression of Bax (Fig 5). Ethanol treatment to Chang liver cells downregulated the expression of the antiapoptotic protein Bcl-2 and upregulated the expression of the proapoptotic protein Bax. However, cytochrome c is an important mitochondrial outer membrane protein released from the mitochondria into the cytosol during apoptosis, which in turn activates downstream caspases causing cell death. Our results documented that ethanol treatment significantly increased the release of cytochrome c and the expressions of active caspases -3,-8 and -9. Treatment with azelaic acid distinctly regressed the ethanol toxicity by diminishing the release of cytochrome c into the cytosol and the expressions of the key executioner apoptotic proteins caspases-8, -9 and -3.

Discussion

Alcoholism is one of the utmost major public disputes in the world (Rehm et al. 2009). In an effort to interpret the protective functions of azelaic acid on ethanol-induced toxicity we examined its dose-dependent effects on Chang liver cells. Cytotoxicity studies on Chang liver cells show that high concentrations of ethanol (30 mM) produced a significant loss of cell viability after 24 h treatment (Kaviarasan et al.2007). Similarly, (Neuman et al. 1993) observed significant dose-dependent cytotoxicity in hepatoblastoma cells (HepG2) exposed to 10-80 mM ethanol for 24h. Further, in this present study, different

concentration of azelaic acid (15.625µM -2000 µM) was observed in Chang liver cells and the IC_{50} concentration of azelaic acid at 72h was observed to be 500 µM.

The cells can readily metabolize ethanol by ADH, ALDH, and CYP2E1 and produce the potent acetaldehyde. Metabolism of ethanol by alcohol dehydrogenase causes an increase in NADH/NAD^+ ratio thus impairing the redox state. Further, the acetaldehyde formed during ethanol metabolism elicits microsomal NADPH-dependent pathway (microsomal acetaldehyde - oxidizing system). Induction of CYP2E1 can facilitate superoxide production, resulting in oxidative stress and cell injury. Ethanol treatment increased the levels of TBARS indicating the excessive the formation of free radicals and thereby damage to the Chang liver cells. Hall et al. 1999 have also documented that during ethanol metabolism, there is increased formation of 1-hydroxyethyl radical and thus lipid peroxidation

Moreover, chronic ethanol exposure results in mitochondrial ROS generation leading to single and double strand breaks of the cellular DNA. Purines and pyrimidines are altered to their hydroxyl derivatives, such as 8-hydroxyguanine. Comet assay showed increased DNA damage and enhanced tail moment in 30 mM ethanol-treated Chang liver cells, whereas significant DNA damage did not occur in the azelaic acid-treated cells. Similarly, dichlorodihydrofluorescein diacetate (DCFH-DA) staining revealed that ethanol treatment induced ROS generation which could be suppressed by 500 µM azelaic acid treatment. In this context, Navasumrit et al. 2000 have also documented free radical production and hepatic DNA strand breaks in rats on chronic ethanol consumption. Muthulakshmi et al. 2003 have also shown the protecting effects of azelaic acid in contradiction of high-fat diet prompted oxidative stress in the liver, kidney, and heart of mice.

GSH is one of the most imperative antioxidants in the cells, which plays a leading role in the defense mechanism against alcohol-induced stress and cell injury. Acetaldehyde the product of ethanol oxidation depletes the cellular mitochondrial GSH and thus contributes to ROS accumulation and liver damage (Lieber 2000). Azelaic acid may help to prevent the oxidative stress and decrease the generation of ROS, and maintain the intracellular GSH levels in the Chang liver cells in the presence of ethanol.

TNF-α is a pro-inflammatory cytokine. A variability of effects that are facilitated through TNFR1 and R2 (tumor necrosis factor receptor) which initiates the TRADD, an adaptor molecule hired by the death domain protein complex I, which also contain TRAF2. This complex then disassociates from a different complex in the cytosol called complex II. Mitochondrial dysfunction is recognized to induce the apoptotic pathway amplified by caspases -8 activations. An opening of the mitochondrial permeability transition pore has been revealed to induce depolarization of the transmembrane potential ($\Delta\psi m$), a discharge of apoptogenic factors and loss of oxidative phosphorylation. Mitochondrial depolarization and oxidative stress consequently result in cytochrome c release from the mitochondria into the cytosol, activation of caspases followed by apoptosis. Initiator caspases recruit and stimulate the death-inducing signals through the cell surface death receptors or from the mitochondria. Caspase 8 activation clues to proteolytic cleavage of Bid to abridged Bid leading to downstream mitochondrial permeabilization via stimulation of Bax. The anti-apoptotic Bcl-2 family proteins, such as Bcl-2 and Bcl-XL, obstruct the mitochondrial death pathway, whereas pro-apoptotic Bcl-2 family proteins, Bax promotes apoptosis (Yin 2000). The execution phase of apoptosis excites the effector caspases, which cleaves cellular proteins prominent to morphological changes and

destruction of the nucleus, DNA fragmentation, chromatin condensation, and cell shrinkage.

In our results ethanol treated Chang liver cells showed down-regulated Bcl-2 expression and upregulated Bax expression leading to the activation of caspases, culminating in apoptosis. Deshmuhk et al. 1998 have shown that ethanol induces mitochondrial dysfunction foremost to the release of the cytochrome c from the mitochondria. However azelaic acid treatment suppressed apoptosis not only by reducing the release of cytochrome c but also by obstructing caspases -8,-9 and -3 and simultaneously increasing Bcl-2 and decreasing Bax expressions as well. Thus, azelaic acid due to its inherent ability to diminish oxidative stress prevents ethanol-induced apoptosis. In this context, Matsubara et al. 2007 reported that azelaic acid also overwhelms the production of ROS such as the superoxide anion and hydroxyl radicals by human neutrophils, although it significantly did not influence ROS generation in a cell-free system.

Conclusion

Our data shows that azelaic acid markedly protects the cells from ethanol-induced toxicity. These effects appear to be related to its ability to reduce oxidative stress, maintain the mitochondrial membrane potential and regulate the expressions of Bcl-2, Bax, and caspases. Further *in vivo* studies could throw light on the role of azelaic acid on ethanol-induced hepatotoxicity.

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Legend

Figure 1: Mitochondrial function as assessed by MTT metabolism in Chang liver cells. (A) The cells were treated with different concentrations of ethanol (10 mM-35 mM) for 24h. (B) The cells were treated with different concentrations of azelaic acid (15.625-2000 µM) for 24h.

(C) Cells were incubated for 24 h with 30 mM ethanol alone or in combination with an azelaic acid. Data are expressed as mean ± SD of four independent experiments. *Significantly different from control ($P< 0.05$). **Significantly different from ethanol-treated cells ($P< 0.05$)

Figure 2 (A): Effect of azelaic acid on ethanol-induced ROS generation. Chang liver cells treated with ethanol (30mM) for 24 h then treated with azelaic acid (500 µM) for 48 h. Intracellular ROS accumulation was measured using the fluorescence probe DCFH-DA. Control cells show diminished green fluorescence indicating reduced ROS levels. Ethanol-treated cells show markedly increased green fluorescence. Ethanol + azelaic acid treated cells show decreased green fluorescence. Azelaic acid treated cells show diminished green fluorescence similar to that of control cells.

(B) Percentage of ROS generation was detected by spectrofluorimeter. Results are expressed as mean ± SD of four experiments in each group. *P < 0.05 compared to control and #P < 0.05 as compared to ethanol group (DMRT). Magnification =40X

Figure 3(A): Mitochondrial membrane potential of ethanol and azelaic acid treated Chang liver cells stained with Rh 123 and monitored by fluorescent microscope. Control cells show increased fluorescence intensity. Ethanol-treated cells show decreased fluorescence. Ethanol plus azelaic acid treated (24 h and 48 h) cells show increased fluorescence as compared to ethanol alone treated cells. Azelaic acid treated cells show increased fluorescence as compared to the control cells. Nuclear morphology of ethanol and azelaic acid treated Chang liver cells stained with Hoechst 33258. Apoptotic features in Chang liver cells treated with ethanol (30 mM) and azelaic acid (500 µM) for 72 h. Nuclear condensation and /or fragmentation are indicative of apoptosis. Control cells showing normal nuclei. Ethanol-treated cells show nuclear swelling condensed chromatin, fragmented nuclei, and apoptotic bodies. Ethanol plus azelaic acid treated cells show less condensed chromatin, fragmented nuclei, and apoptotic bodies. Azelaic acid treated cells show normal nuclei as compared to that of control cells. Magnification =40X

(B) Fluorescence intensity was detected by a spectrofluorimeter. The experiments were repeated three times independently. Results are expressed as mean ± SD of four experiments in each group. *P < 0.05 compared to control and #P < 0.05 compared to ethanol group (DMRT).

Figure 4 (A): Fluorescence microscopic images of oxidative DNA damage (comet assay) Control cells showing round and intact DNA. Ethanol-treated cells show well-defined comet tail. Ethanol + azelaic acid treated cells show reduced tail moment. Azelaic acid treated cells show intact DNA as compared to the control cells.

(B) Bar chart illustrating changes in the levels of % tail DNA, % of tail length, tail moment, and olive tail moment. Results are expressed as mean ± SD of four experiments in each group. *P <0.05 compared to control and #P 0.05 compared to ethanol group (DMRT).

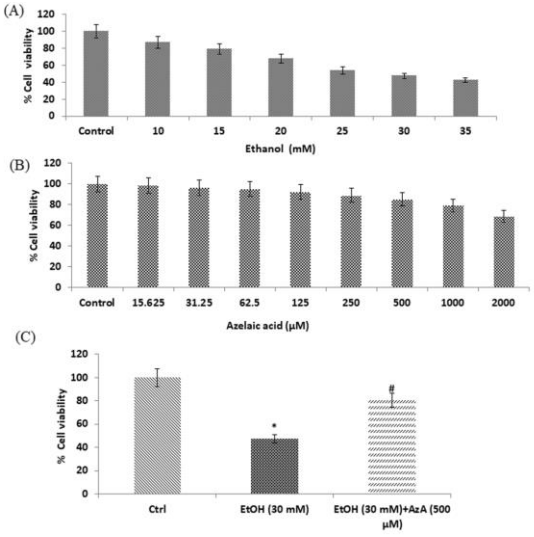
Figure 5 (A): Western blot analysis of TNF-α, Bax, Bcl-2, mitochondrial and cytosolic cytochrome c and caspases -8, -9 and -3, expressions in Chang liver cells. Ethanol (30 mM) significantly enhanced the expressions of TNF- α, mitochondrial and cytosolic cytochrome c, caspases -8,-9 and -3 while azelaic acid (500 µM) significantly diminished the expressions of TNF- α, mitochondrial and cytosolic cytochrome c, caspases -3,-8 and -9 in ethanol-treated Chang liver cells.

(B) The representative graph shows the relative protein expression of fold changes in western blots of TNF-α, Bax, Bcl-2.

(C) The western blots analysis of cytochrome c of mitochondria and cytosol, caspases of -8,-9, and -3, β-

actin as an internal control. Values are expressed as mean ± SD (Standard deviation) of four experiments in each group. *P < 0.05 compared to four experiments in each group #P < 0.05 compared to alcohol group (DMRT).

Fig :1



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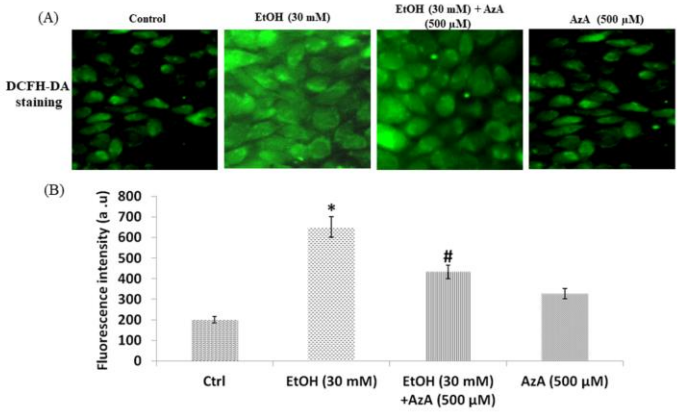

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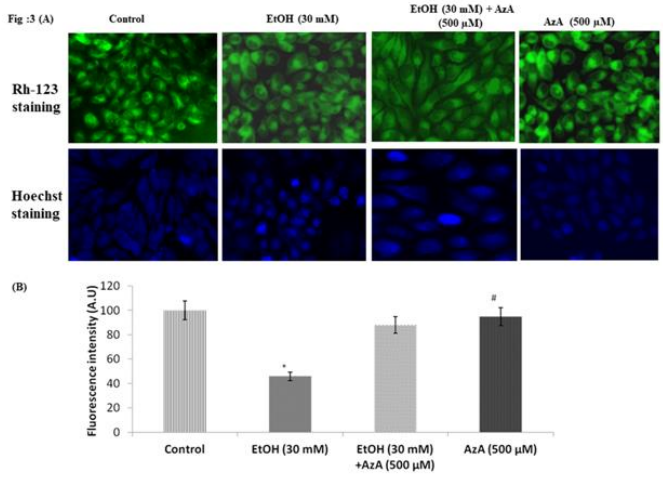

Fig :4

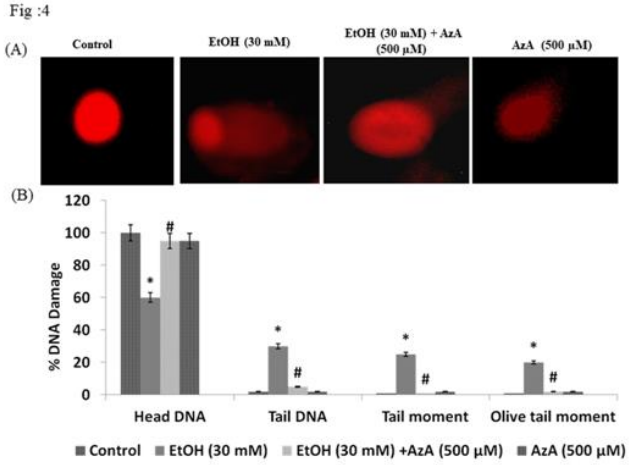

Fig :5

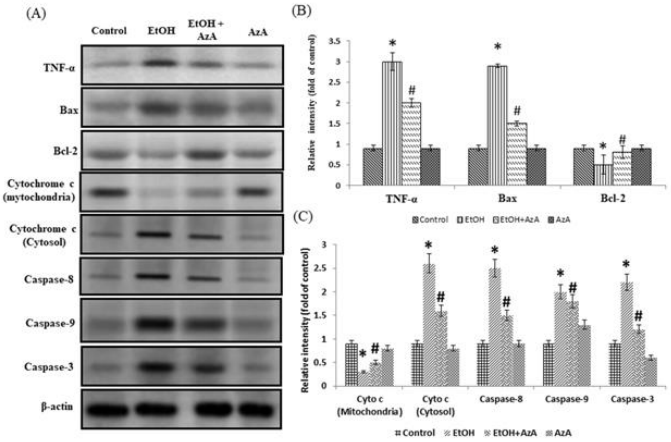

Table 1: Effect of azelaic acid on ethanol induced alteration in antioxidant level, thiobarbituric acid reactive substances and lactate dehydrogenase.

Parameters	Control	Ethanol	Ethanol+ Azelaic acid	Azelaic acid
GSH (nmol/mg Protein)	48.6 ±3.70	30.10±2.29 *	49.50±3.77†‡	48.10±3.68‡
GSSG (nmol/mg Protein)	5.62±0.43	12.06±0.92*	8.50±0.65†‡	4.12±0.31‡
GSH/GSSG ratio	8.64±0.66	4.49±0.34 *	8.63±0.66 †‡	11.72±0.89‡
TBARS (nmol/mg protein)	2.8±0.21	7.2±0.55*	3.10±0.24 †‡	2.52±0.19‡
LDH leakage (%)	9.52±0.73	56.09±4.27 *	18.72±1.43†‡	10.72±0.82‡

Values are expressed as means ± S.D. of three independent experiments.*Significantly different from control ($p< 0.05$). †Significantly different from Ethanol-treated cells ($p< 0.05$). ‡Not significantly different from control.