INTERNATIONAL JOURNAL FOR INNOVATIVE RESEARCH IN MULTIDISCIPLINARY FIELD
ISSN(O): 2455-0620 [Impact Factor: 7.581]
Monthly, Peer-Reviewed, Refereed, Indexed Journal with IC Value : 86.87
Volume - 9, Issue - 4, April - 2023 Publication Date: 30/04/2023

DOIs:10.2015/IJIRMF/202304023 --:-- Research Paper / Article / Review

Hepatoprotective Role of Hydro-Ethanolic Extract of *Alocasia indica* Tubers against Ethanol Induced Oxidative Stress

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Abstract: *Alocasia indica* is widely used in Indian folklore medicine and has its mention in Ayurveda. Especially in Ayurveda, it is used to treat tumors, inflammations, hemorrhages, hepato-splenopathies, amenorrhea, dysmenorrhea, fatigue and general debility. The current study investigates the hepatoprotective effect of *Alocasia indica* against chronic ethanol induced oxidative stress and tissue damage. The rats were segregated into seven groups which included normal control, ethanol treated (40%*w/v*) 2gm/kg body weight /day, ethanol+silymarin (100mg/kg), ethanol+*AI* (250mg/kg), ethanol+*AI* (500mg/kg), only *AI* (250mg/kg) and only *AI* (500mg/kg) for 30 days to evaluate the hepatoprotective effect against ethanol toxicity. Hepatic antioxidant enzymes, liver markers and pro-inflammatory cytokines were assayed biochemically. Histomorphological alterations were detected by H and E stain. Besides, degree of hepatic glycogen, DNA content, fibrosis and apoptotic cell death were examined by PAS, Feulgen, Picro Sirius stain and TUNEL assay technique respectively. Liver marker enzymes ALT, AST and ALP, pro-inflammatory cytokines, tissue TBARS and activity of glutathione metabolizing enzymes GPx and GST were significantly ($P<0.001$) elevated whereas cytosolic and mitochondrial SOD, catalase and levels of reduced glutathione were significantly ($P<0.001$) decreased in the ethanol treated group compared to control. However, hydro-ethanolic extract of *Alocasia indica* (*AI*) supplementation to the ethanol treated rats reversed these effects to normal. Furthermore, degenerative changes in hepatocytes, with ethanol treatment were minimized to near normal architecture by *AI* supplementation. Glycogen and DNA depletion along with excess collagen deposition and increased apoptotic cell number were also restricted by *AI* supplementation; with the higher dose being more promising. Thus, ethanol induced hepatotoxicity was attenuated by *AI* treatment. Hence it can be used as a regular nutrient or therapeutic agent to protect the hepatic cells.

Key Words: *Apoptosis; Fibrosis; Hepatotoxicity; Oxidative Stress; Phytonutrients; Pro-inflammatory cytokines.*

1. INTRODUCTION:

Alcoholic liver diseases (ALD) are the most challenging current health problems worldwide. Over the period of time, alcohol (ethanol) has evolved as one of the socially accepted addictive drugs worldwide (Frazier et al. 2010).The risk of alcohol-induced liver disease (ALD) increases dose- and time-dependently with consumption of alcohol (Arteel 2010). Progression of ALD is well characterized and is actually a spectrum of liver diseases which ranges initially from simple steatosis, to inflammation and necrosis (steatohepatitis), to fibrosis and cirrhosis (Diehl 2002) thus contributing to the alcoholism-related high morbidity and mortality.

Alcohol acts through numerous pathways to affect the liver and other organs and to lead to the development of alcoholic liver disease (Tsukamoto and Lu 2001). Many mechanisms act in concert, reflecting the spectrum of the organism's response to a myriad of direct and indirect actions of alcohol. Firstly, alcohol oxidation by alcohol dehydrogenase and aldehyde dehydrogenase leads to increase in the levels of alcohol metabolites that result in metabolic stress and the development of a fatty liver. Secondly, the ethanol-induced microsomal ethanol oxidation system (MEOS)

contributes to ethanol metabolism thereby enhancing production of reactive oxygen species (ROS) by its component enzyme, CYP2E1 (cytochrome P450). The later described pathway plays a central role in alcohol–induced damage by excessive generation of molecules called free radicals, which can result in a state called oxidative stress (Pearson 1986, Dey and Cederbaum 2006).

Several reports reveal a basal level of reactive oxygen species (ROS) generation during normal cellular metabolism. Because ROS generation is a naturally occurring process in the human body, a variety of enzymatic and non-enzymatic mechanisms have evolved to protect cells from ROS. However, cells exposed to additional toxins or free radical generators produce vast amounts of ROS that leads to impairment of those mechanisms (Kehrer and Klotz 2015). Reports of previous studies demonstrate that oxidative stress plays a crucial role in the pathogenesis of ethanol-induced liver damage which induces endogenous antioxidant depletion, lipid peroxidation, protein degradation, DNA damage, necrosis and apoptosis (Jaeschke 2002).

The liver regulates many important metabolic functions, and liver damage can distort these metabolic functions (Shaker et al. 2010).Despite the fact that acute and chronic liver diseases represent a global concern; modern medical treatments often have limited efficiency. In view of the scarce treatment options and significant adverse effects incurred by conventional chemical agents, novel prophylactic and therapeutic agents against chronic liver disease are urgently needed. As oxidative stress is a key mechanism underlying alcohol-mediated hepatotoxicity, antioxidant therapy for alcoholic liver disease is particularly important. Over the past three to four decades, mounting evidence has shown that dietary phytochemicals are efficacious in preventing oxidative stress-related liver diseases and protecting cells from toxic insult.

From the ancient past in India, several medicinal plants have been extensively used for the management of liver disorder. *Alocasia indica* is an indigenous herb belonging to family Araceae, traditionally used in inflammation and in diseases of abdomen and spleen (Baman Das Basu and Kanhoba Ranchoddas Kirtikar 1987). The juice of leaves of the plant is used as digestive, laxative, diuretic, and astringent and for the treatment of rheumatic arthritis (Nadkarni 1994). The leaves of *A. indica* showed antioxidant, antinociceptive, and anti-inflammatory activities and hepatoprotective activity against CCl_4 induced liver damage model (Mulla et al. 2010, Mulla et al. 2009). It was also reported that the leaves of the plant showed anthelmintic (Mulla et al. 2010), antimicrobial, antidiarrheal, and *in vitro* antiprotozoal activities (Mulla et al. 2011). But interestingly the medicinal property of the tuber is not explored so far. In our previous study, we have demonstrated the *in vitro* free radical scavenging activity against DDPH, superoxide, hydroxyl and lipid peroxyl radicals (Basu et al. 2012). Simultaneously we have also conducted quantitative assessment of the proximate components, mineral, antioxidant vitamins and anti-nutrient contents. Besides, the GCMS analysis of the hydro-ethanolic extract of *A. indica* tuber showed presence of several bioactive components like phytosterols, antioxidant vitamins, flavanoid fractions and polyphenols in the extract (Basu et al. 2014). To the best of our knowledge, no other report was available using the tuber of the plant as hepatoprotectant against alcohol-induced liver damage. The objective of this study was to assess the hepatoprotective effects of hydro-ethanol extracted *A. indica* tuber extracts on the alcohol-induced liver damage rat model. This study also aimed to establish the molecular mechanism underlying ethanol induced hepatic damage and the ameliorative effect of the hydro-ethanolic extract in view of its antioxidative, antiapoptotic, anti-inflammatory and anti-fibrogenic activity.

2. MATERIALS AND METHODS:

Procurement of plant material

Fresh tubers of *Alocasia indica* (Roxb.) Schott. were purchased from the farmers of Santragachhi village of Howrah district, West Bengal, India, from the month of May to October. Authentication was done by the Central National Herbarium of Botanical Survey of India (BSI), Howrah, West Bengal, India. Herbarium of the specimen is maintained in the institute library bearing the number RMC/PHY/SB/03/14.

Preparation of hydro-ethanolic extract of *Alocasia indica* tuber

Raw fresh tubers after procurement were cut into thin slices and washed under running tap water to remove any impurity and mud. After proper cleaning the wet materials were soaked in blotting paper and then dried in hot air oven at 50°Celsius, until materials became crispy in texture. These dried materials were grinded into coarse powder form and stored at -4° Celsius in air tight Tarson jars until further use.

For the preparation of the hydro-ethanolic extract, 20 gm of the powdered dried samples of *AI* were taken in the thimble of Soxhlet and extracted with 250 ml of ethanol (70%) in the round bottomed flask continuously for a week. The extracts were then filtered through muslin cloth, centrifuged and the collected filtrates were concentrated by evaporation to dryness using rotary evaporator (M/s B.C. Chatterjee & Co., Kolkata, West Bengal, India) at 50° Celsius.

The dried samples, hydro-ethanolic extracts of and *Alocasia indica* (*AI*) were collected and stored in air tight plastic vials at -4° Celsius for future use. The dried sample was dissolved in normal mammalian saline (0.9 gm% NaCl) at concentrations of 250 and 500 mg/kg body wt for administration to experimental animal groups.

Animal selection, care and maintenance

Wistar strain male Albino rats weighing 170-200 g body weight were procured from authorized breeders (Kolkata, India). The rats were housed in autoclavable polypropylene cages and maintained under temperature controlled room (25˚±2˚C) with 12:12 hour L:D photoperiod. Rats were given standard pellet diet (Lipton, Rat Feed, Ltd., Pune, India) and water *ad libitum* throughout the experimental period. All experiments were in accordance with the guidelines of the Committee for the Purpose of Control and Supervision of Experiments on Animal (CPCSEA), Government of India [1795/PO/ERe/S14CPCSEA].

Acute toxicity study of ethanolic extract of *Alocasia indica* tuber

Acute oral toxicity was conducted according to the Guidelines for Testing of Chemicals adopted by the Organization for Economic Co-operation and Development (*Guideline, OECD, 2001*). The median lethal dose (LD50) of the plant extract was determined by method of (Lorke 1983) using twelve rats weighing between 150 – 190g. In the first phase, eight rats were divided into four groups of two rats each and were treated with the hydro-ethanolic tuber extract of the plants (*AI*) at dosages of 1600 and 2000mg/kg body weight intra-peritoneally. They were observed for 24 hours for signs of toxicity. In the second phase, twenty eight rats were again divided into forteen groups of two rats each and were also treated with the hydro-ethanolic extract of *Alocasia indica* at dosages of 3000, 3500, 4000, 4500, 5000, 5500 and 6000 mg/kg body weight intra-peritoneally.

Experimental design

The animals were divided into the following groups:-
Group I: Animals in this group were treated with equal volume of normal saline (0.9gm% NaCl).
Group II: Standard diet + Standardized doses of ethanol (2gm/kg body weight).
Group III: Standard diet + Standardized doses of ethanol (2gm/kg body weight) + Standardized dose of standard hepatoprotective drug Silymarin (100mg/kg body weight)
Group IV: Standard diet + Standardized doses of ethanol (2gm/kg body weight) + Standardized dose of ethanolic extract of *Alocasia indica* (*250* mg/kg body weight)
Group V: Standard diet + Standardized doses of ethanol (2gm/kg body weight) + Standardized dose of ethanolic extract of *Alocasia indica* (*500* mg/kg body weight)
Group VI: Standard diet + Standardized dose of ethanolic extract of *Alocasia indica* (*250* mg/kg body weight)
Group VII: Standard diet + Standardized dose of ethanolic extract of *Alocasia indica* (*500* mg/kg body weight)

Collection of sample

After thirty days of continuous treatment, the animals were kept fasted overnight without any treatment. The day following, the animals were sacrificed after being anaesthetized with an intraperitoneal injection of a combination of 100mg/kg of ketamine and 10mg/kg Xyalazine (Mitra et al. 2014). Blood samples were collected by cardiac puncture. The collected blood was allowed to stand for some time to separate out the serum. Clear serum was obtained after centrifugation of collected blood specimens at 3500 rpm for 20 minutes using mini centrifuge (M/s Remi Laboratory Instruments, Model R-303). Liver from different experimental groups were excised, trimmed of connective tissues, rinsed with ice cold saline to eliminate blood contamination, dried by blotting with filter paper and weighed to obtain the organ weight. Part of the excised livers was used for homogenate preparation and the other part was transferred to fixative for preparation of histological sections.

Measurement of liver weight to body weight ratio:

Body weight of all animals at the end of the treatment period, along with their respective liver weight on the day of sacrifice was noted to determine the liver weight to body weight ratio of animals in each group.

Assessment of hepato-protective activity

Preparation of tissue homogenate

A 10% w/v liver tissue homogenate was prepared in ice cold Phosphate buffer saline (PBS) containing 1mM EDTA (pH 7.4), using tissue homogenizer. The homogenate was then centrifuged at 10,000 rpm for 30 min, at 4 °C. The supernatant thus obtained was used further, for the following biochemical assay of tissue oxidative stress markers.

Preparation of mitochondrial fraction

For mitochondrial preparation, the liver homogenate was first centrifuged at 2000 rpm for 5 min. The supernatant was collected and centrifuged again at 10,000 rpm for 20 min. The supernatant was discarded and the pellet was suspended in Tris-HCl buffer (pH 7.4).

Serum analysis

Liver function enzymes

Liver function was assessed by estimating serum levels of liver marker enzymes, such as Alanine Transaminase (ALT), Aspartate Transaminase (AST) by the method of Reitman and Frankel (Reitman and Frankel 1957), and Alkaline Phosphatase (ALP) by the method of Kind and King (Kind and King 1954), using commercially available kits. The results were expressed in U/L.

Cytokine assessment

Assessment of pro-inflammatory cytokines, viz., tumour necrotic factor alpha (TNF-α) and interleukin 6 (IL-6) was also carried out using commercially available ELISA kit and expressed in picogram per milliliter (pg/mL).

Estimation of serum and tissue protein

Serum and tissue protein were estimated according to the method of Lowry et al. (Lowry et al. 1951) using BSA as standard.

Endogenous Antioxidant Assessment

Lipid peroxidation was estimated by measuring thiobarbituric acid reactive substance (TBARS), according to the method of Ohkawa et al., (Ohkawa et al. 1979). Cu-Zn SOD activity was measured according to the method of Marklund and Marklund (MARKLUND and MARKLUND 1974). Catalase activity was determined by the decomposition of H_2O_2 at 240 nm, according to the method of Aebi (Aebi, 1984). Reduced glutathione content was estimated according to the method of Ellman (Ellman 1959). Glutathione Peroxidase activity was measured according to the method of Rotruck et al., (Rotruck et al. 1973). Glutathione-S-Transferase was estimated according to the method of Habig et al. (Habig et al. 1974), by observing the conjugation of CDNB with GSH at 340 nm. Glutathione reductase was estimated according to the method of Racker (Racker 1955), based on amount of NADPH utilized to convert GSSG to GSH. Glucose-6-Phosphate Dehydrogenase was estimated according to the method of Lee (Lee, 1982). All absorbance at different wavelengths were carried out using UV–VIS spectrophotometer (Systronics 118) and ELISA micro-plate reader (Alere, AM 2100).

Histological and histochemical studies

A part of the rat liver was immediately fixed in 10% formalin buffer and embedded in paraffin after routine clearing and dehydration. 5 μm thick sections was then prepared from the tissue blocks using rotary microtome. The sections were then stained with hematoxylin-eosin (HE) stain, periodic-schiff (PAS) stain, feulgen and Sirius red (SR) stain. The tissue sections were viewed under microscope (Magnus, MLXi) and images were captured using a digital camera Olympus BX51 (Olympus Corporation, Tokyo, Japan) attached to it. The images were further analyzed using image analysis system (Image J, NIH Software, Bethesda, MI) and glycogen, DNA and collagen content were respectively expressed as % fraction. Fragmented DNA content was estimated by TUNEL assay using TUNEL assay kit for both light and confocal microscopy. The TUN

EL stained sections for confocal microscopy were viewed under laser scanning confocal microscope (Leica SP8, Germany) and the stacked images through multiple slices were also captured. The images were further analyzed using image analysis system (Image J, NIH Software, Bethesda, MI) to note down the extent of DNA fragmentation and calculate fragmented DNA amount/mm^2.

Statistical Analysis:

Each experiment was repeated at least three times. Data are presented as means ± S.D. Significance of mean values of different parameters between the treated groups were analyzed using one way post hoc tests (Tukey's HSD test) of analysis of variances (ANOVA) after ascertaining the homogeneity of variances between the treatment groups. Statistical tests were performed using SPSS software version 20.0. A value of $p < 0.05$ was considered statistically significant.

3. RESULT:

The results in table 1 show that a single dose of ethanol (2gm/kg body weight/per day) for thirty consecutive days develops severe hepatotoxicity as reflected in the significant (P<0.001) elevated levels of ALT, AST and ALP in comparison to control. Such elevation of liver markers can be prevented when co-administered with the hydro-ethanolic extract of *AI* at 250 and 500 mg/kg body weight significantly (P<0.001) lower the levels ALT, AST and ALP compared to the ethanol treated group. Such lowering of liver markers by the extract is comparable with the standard hepatoprotective drug silymarin, a group maintained in this study to compare the hepatoprotective activity of the extracts with that of standard hepato-guard dr ugs commercially available. Dose dependent variation is observed for the hydro-ethanolic extract of *AI*. The ALT, AST and ALP levels for the higher doses of *AI* is found to be significantly lower (P<0.05) when compared with the lower dose. Moreover, this study also shows that ethanol administration significantly lowered (P<0.001) serum protein level as compared to control. But co-administration with ethanolic extract of *AI* significantly (P<0.001) prevented such decline in protein level when compared to only ethanol treated group. It is noteworthy that the only extract groups show no significant alterations in the levels of liver markers and serum protein when compared to control indicating the absence of non-hepatotoxic action when administered alone.

Table 1: Effect of hydro-ethanolic extract of *ai* on liver marker enzymes and serum protein in the control and experimental rats.

Groups	ALT (IU/L)	AST (IU/L)	ALP (IU/L)	Total Protein (gm%)
Control	50.79 ± 6.12	192.59 ± 5.02	185.64 ± 7.12	7.5 ± 0.58
Only Ethanol	109.19 ± 5.02*	379.35 ± 6.12*	417.92 ± 6.02*	5.3 ± 0.21*
Ethanol+Silymarin	61.48 ± 7.90**	212.49 ± 7.16**	243.68 ± 7.22**	7.3 ± 0.35**
Ethanol+*AI* (250mg/kg BW)	82.92 ± 5.80$^{**/\#}$	274.33 ± 7.42$^{**/\#}$	355.32 ± 10.90$^{**/\#}$	6.8 ± 0.44**
Ethanol+*AI* (500mg/kg BW)	70.16 ± 4.09**	238.09 ± 6.14**	280.59 ± 6.64**	7.2 ± 0.38**
Only *AI* (250mg/kg BW)	63.52 ± 7.42^{**ns}	227.96 ± 3.84 ^{**ns}	214.88 ± 5.56 ^{**ns}	7.0 ± 1.28 ^{**ns}
Only *AI* (500 mg/kg BW)	68.96 ± 6.09^{**ns}	200.04 ± 6.49 ^{**ns}	192.45 ± 6.87 ^{**ns}	7.4 ± 0.21 ^{**ns}

All values are expressed as MEAN ± SD, of 6 animals in each group. Data are analyzed by one way analysis of variance (ANOVA) followed by Tukey's Kramer post hock analysis. * P<0.001 vs. control. ns P>0.05 vs. control, **P<0.001 vs. only ethanol, $^{\#}$P<0.05 vs. ethanol+ *AI* (500mg/kg BW)

Table 2 represents the levels of various non-enzymatic, enzymatic and lipid peroxidation products in different experimental animal groups. In this study, level of hepatic GSH, Cu/Zn and Mn-SOD, catalase and TBARS in the control group receiving mammalian normal saline is considered the basal level. It is observed that upon administration of ethanol for thirty consecutive days the levels of GSH, catalase, Cu/Zn and Mn-SOD significantly (P<0.001) decreases in chronic ethanol treated group while TBARS, one of the final products of polyunsaturated fatty acid peroxidation in cells get significantly elevated (P<0.001) in chronic ethanol treated group as compared to the basal respectively. However, co-administration with the extract of *AI* (250 and 500 mg/kg body wt) along with ethanol significantly (P<0.001) attenuated this depletion in GSH, catalase, Cu/Zn and Mn-SOD content as compared to the ethanol treated group. On the other hand for TBARS content, extract co-administration along with ethanol significantly (P<0.001) prevented the elevation of TBARS content as compared to the only ethanol treated group. Among the two doses a significant (P<0.05) dose dependent variation is observed for *AI* tubers in case of all the parameters. The higher doses show more prominent effect than the lower dose in all the respective cases. The only extract treated groups show no significant (P>0.05) alteration in the discussed parameters as compared to control group indicating no hepatotoxic effect of the tubers itself. It is worth mentioning that the results of the tuber co-administered groups are comparable with that of the standard hepatoprotective drug, silymarin treated group. The tuber extracts efficiently maintain the non-enzymatic antioxidant GSH store, the TBARS content and enzymatic antioxidant i.e. Cu/Zn and Mn-SOD and CAT activities like the standard hepatoprotective drug silymarin.

Table 2: Effect of hydro-ethanolic extracts of *ai* on endogenous antioxidants and lipid peroxidation products in the control and experimental rats.

Groups	GSH (mg/100gm)	Cu/Zn SOD U/min/mg	Mn-SOD U/min/mg	CAT µmoles H_2O_2 consumed/ min/mg	TBARS µmoles/100 gm
Control	81.22 ± 6.45	5.60 ± 0.56	7.21 ± 1.20	211.41 ± 8.56	24.65 ± 3.54
Only Ethanol	46.84 ± 5.42*	2.61 ± 0.42*	3.55 ± 0.94*	164.04 ± 10.25*	52.71 ± 4.56*
Ethanol+Silymarin	77.18 ±9.24**	4.65 ± 1.05**	6.57 ± 1.08**	204.27 ± 10.96**	26.22 ± 4.22**
Ethanol+*AI* (250mg/kg BW)	60.85 ± 6.12**	3.02 ± 0.95**	5.04 ± 0.90**	177.25 ± 7.45**	40.72 ± 5.52**
Ethanol+*AI* (500mg/kg BW)	73.88 ± 6.87$^{**/\#}$	4.89 ± 0.85$^{**/\#}$	5.9 ± 0.68$^{**/\#}$	195.03 ± 8.65$^{**/\#}$	35.71 ± 5.12$^{**/\#}$
Only *AI* (250mg/kg BW)	75.88 ± 7.85^{ns}	5.05 ± 0.65^{ns}	7.02 ± 1.03^{ns}	193.18 ± 10.54^{ns}	35.5 ± 4.96^{ns}
Only *AI* (500 mg/kg BW)	70.67 ± 7.45^{ns}	4.65 ± 0.80^{ns}	6.59 ± 0.86^{ns}	199.35 ± 11.45^{ns}	29.36 ± 4.85^{ns}

All values are expressed as MEAN ± SD, of 6 animals in each group. Data are analyzed by one way analysis of variance (ANOVA) followed by Tukey's Kramer post hock analysis. * P<0.001 vs. control. ns P>0.05 vs. control, **P<0.001 vs. only ethanol, $^{\#}$P<0.05 vs. ethanol+ *AI* (500mg/kg BW)

Table 3 represents the variation in activities of different enzymes responsible for maintaining the endogenous reduced glutathione store in the experimental groups. It is observed that the activities of hepatic glutathione utilizing enzymes, glutathione peroxidase and glutathione-S-transferase show significant elevated (P<0.001) levels of activities in the ethanol treated group of animals as compared to control. Remarkably when co-administered with the standard hepatoprotective drug silymarin and the tuber extracts of *AI* attenuation in both the enzyme activity at different significant levels is observed. The lower dose shows significance at P<0.05 while the higher dose shows significance at P<0.001 level. Moreover, the extract also exhibit a dose dependent variation in enzyme activity between the higher and lower dose at P<0.05 level. The only extract treated groups show no significant alteration in enzyme activity (P>0.05) as compared to control.

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Table 3: Effect of hydro-ethanolic extract of *ai* on endogenous reduced glutathione balance maintaining enzymes in the control and experimental rats.

Groups	GPx µmoles GSH consumed/min/mg	GST µmoles CDNB-GSH conjugate formed/ min/mg	GR µmoles NADPH consumed/min/mg	G-6-PD U/min/mg
Control	7.97 ± 1.25	29.26 ± 2.56	97.5 ± 5.23	1.84 ± 0.25
Only Ethanol	23.44 ± 0.95*	51.09 ± 6.42*	43.77 ± 4.26*	0.69 ± 0.36*
Ethanol+Silymarin	10.88 ± 1.33**	31.88 ± 6.05**	91.28 ± 6.42**	1.63 ± 0.28**
Ethanol+*AI* (250mg/kg BW)	19.73 ± 0.57$^{\#}$	44.79 ± 4.95$^{\#}$	58.67 ± 5.95**	1.08 ± 0.06**

Ethanol+*AI* (500mg/kg BW)	$14.99 \pm 1.02^{**/\#\#}$	$36.42 \pm 5.85^{**/\#\#}$	$72.46 \pm 4.88^{**/\#\#}$	$1.35 \pm 0.18^{**/\#\#}$
Only *AI* (250mg/kg BW)	12.71 ± 0.84^{ns}	31.64 ± 4.65^{ns}	85.75 ± 6.97^{ns}	1.66 ± 0.20^{ns}
Only *AI* (500 mg/kg BW)	10.14 ± 0.75^{ns}	29.33 ± 5.80^{ns}	95.84 ± 7.45^{ns}	1.75 ± 0.08^{ns}

All values are expressed as MEAN ± SD, of 6 animals in each group. Data are analyzed by one way analysis of variance (ANOVA) followed by Tukey's Kramer post hock analysis. * P<0.001 vs. control. ns P>0.05 vs. control, **P<0.001 vs. only ethanol, $^{\#\#}$P<0.05 vs. ethanol+ *AI* (500mg/kg BW), $^{\#}$P<0.05 vs. ethanol

Table 4 represents the serum cytokine levels in chronic ethanol treatment as well as in extract supplemented groups. The results indicate that chronic ethanol treatment significantly elevates (P<0.001) both serum TNF-α and IL-6 levels compared to control. However, such elevation in pro-inflammatory cytokine levels in serum is restricted when the rats were treated simultaneously with hepatoprotective drug, silymarin. Similar protection is also observed as significant decreased (P<0.001) levels of TNF-α and IL-6 in the extract of *AI* treated groups when compared to only ethanol receiving group. This indicates the significant protection that the extract offers against ethanol toxicity. Noteworthy, the only extract treated groups showed no significant alteration (P>0.05) in the levels of TNF-α and IL-6 as compared to control.

Table 4: Effect of hydro-ethanolic extract of *AI* on TNF-α and IL-6 levels in the control and experimental rats.

Groups	**TNF-α (pg/ml)**	**IL-6 (pg/ml)**
Control	162.96 ± 7.25	91.66 ± 4.56
Only Ethanol	$364.43 \pm 8.95^{*}$	$173.5 \pm 8.42^{*}$
Ethanol+Silymarin	$188.63 \pm 7.33^{**}$	$106.15 \pm 6.05^{**}$
Ethanol+*AI* (250mg/kg BW)	$271.83 \pm 10.57^{**}$	$131.21 \pm 9.95^{**}$
Ethanol+*AI* (500mg/kg BW)	$206.81 \pm 8.02^{**}$	$106.10 \pm 7.85^{**}$
Only *AI* (250mg/kg BW)	180.38 ± 9.84^{ns}	110.38 ± 8.65^{ns}
Only *AI* (500 mg/kg BW)	172.66 ± 8.75^{ns}	99.33 ± 5.80^{ns}

All values are expressed as MEAN ± SD, of 6 animals in each group. Data are analyzed by one way analysis of variance (ANOVA) followed by Tukey's Kramer post hock analysis. * P<0.001 vs. control. ns P>0.05 vs. control, **P<0.001 vs. only ethanol

Figure 1 represents H & E, PAS and Feulgen stained sections from different experimental groups to detect morphological changes, glycogen content and DNA content respectively. The liver section from the control group show normal lobular architecture with central veins and radiating hepatocytes. Liver from rats treated with only ethanolic extract of *AI* at 250 and 500 mg/kg body weight and silymarin also exhibits near normal architecture indicating their non-toxic effect and hepatoprotective nature respectively. However, rats treated with ethanol alone shows marked alteration in the architecture and clear signs of liver injury indicated by severe dilatation and congestion of blood sinusoids, necrosis and phagocytic cell infiltration surrounding the central vein. Using the ethanolic extract of *AI* as protective agent against ethanol induced toxicity, a remarkable effect is observed in terms of decreased portal congestion, dilatation and cellular infiltration, as comparable to silymarin, the standard hepatoprotective drug treated group and control.

Examination of liver sections from ethanol treated rats show massive diminution of glycogen content as evidenced from the faded intensity of red or magenta colour. However, such depletion of glycogen content is prevented when rats receiving ethanol are also simultaneously supplemented with the extracts of *AI* at dose 250 and 500mg/kg body weight. The protection offered at the higher dose of the extract is comparable with the hepatoprotective drug silymarin. Similarly, the liver sections from only extract treated groups also show glycogen content like that of control rats. For demonstrating DNA content, sections are stained with Schiff's reagent for Feulgen reaction to demonstrate DNA as magenta color. Sections of liver from control and only extract treated groups show normal amount of DNA content. But treatment of rats with ethanol show reduced DNA content as explained by moderate magenta colour in the hepatocytes. Such reduction in DNA content due to ethanol treatment is prevented when the rats are simultaneously treated with the two different doses of extract of *AI* just like that of silymarin, the standard hepatoprotective drug.

Figure 3a and 3b shows the amount of glycogen and DNA content respectively as observed by the image analysis software. It is observed that in ethanol treated animals there is significantly lower (P<0.001) glycogen and DNA content than control group. However, co-administration with silymarin and extract of *AI* significantly (P<0.001) prevent the depletion of glycogen and DNA amount. It is to be mentioned that the extract of *AI* at 250mg/kg body wt however show less potency in preventing glycogen and DNA depletion when compared to ethanol treated group, the values are significantly different at P<0.05 level i.e. at a lower level of significance. Thus, it can be said that *AI* at higher dose i.e. 500mg/kg body wt is more effective as hepatoprotective agent. The only extract treated groups in this study show no significant alteration in glycogen and DNA depletion as compared to control indicating their non-hepatotoxic effect.

Figure 2 represents light and confocal microscopy images of collagen deposition and DNA fragmentation by picrosirius stain and TUNEL assay respectively in different experimental groups. It is observed that amount of collagen deposition and DNA fragmentation is remarkably high in ethanol treated animals as compared to control indicating high prevalence of fibrosis and apoptosis respectively in liver tissue exposed to chronic ethanol. However, it is observed that co-administration of silymarin and ethanolic extracts of *AI* along with ethanol attenuates both the degree of fibrosis and DNA fragmentation. Figure 3c and 3d represents the volume of collagen deposition and fragmented DNA content respectively as analyzed by image analysis software. It is observed that both collagen deposition and DNA fragmentation is significantly (P<0.001) higher in the ethanol treated group when compared to control. This elevation of collagen deposition and DNA fragmentation is not observed when the animals were co-administered with extracts of *AI* at the two different doses. Instead there was significant (P<0.001) lowering of collagen deposition and DNA fragmentation like that of the silymarin treated group. Thus the extract offers hepatoprotection against ethanol by preventing fibrosis and apoptosis in hepatic tissue.

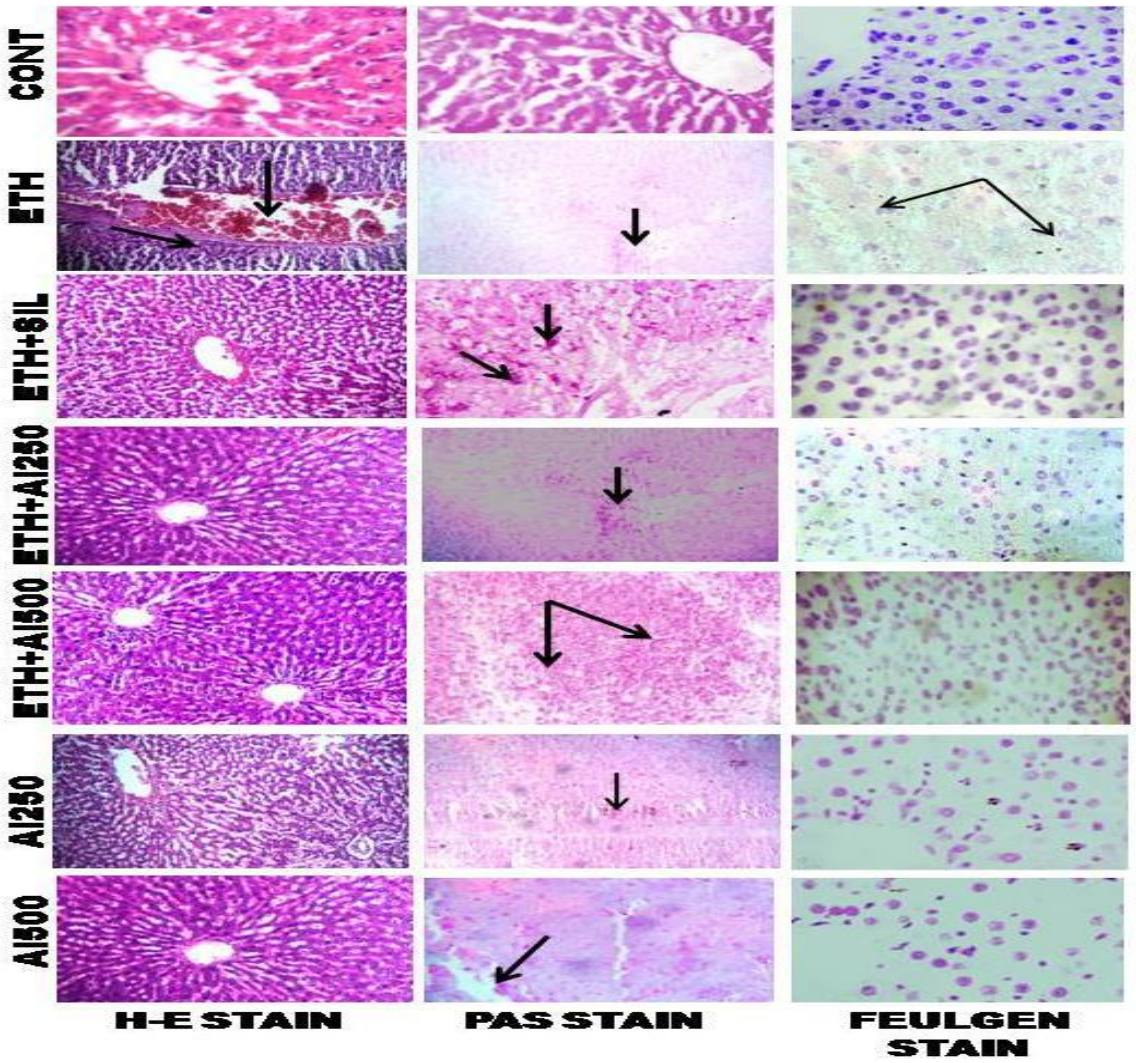

Figure 1: Represents H &E (100X magnification), PAS (100Xmagnification) and Feulgen stained (400X magnification) sections of liver tissue from different experimental groups

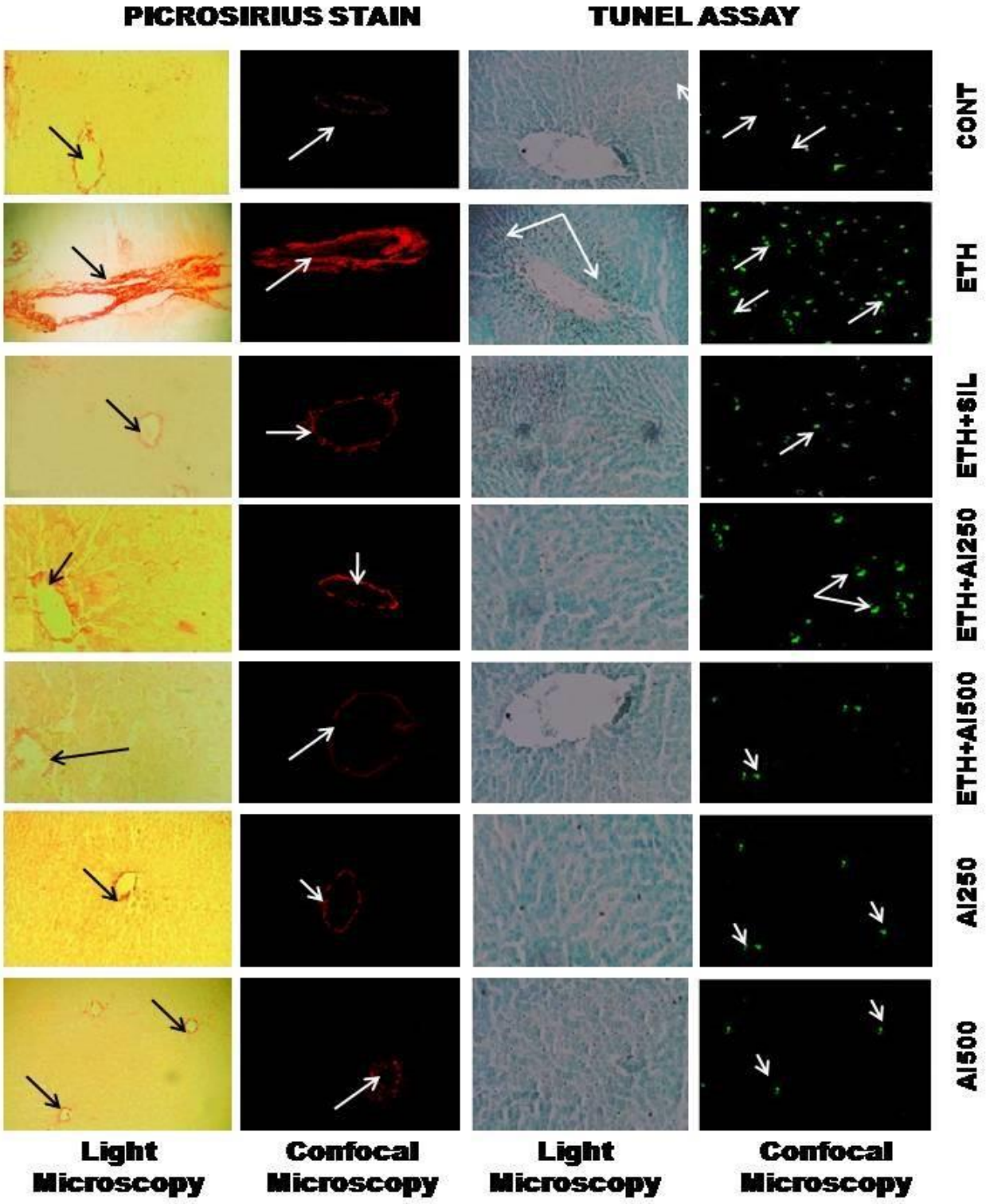

Figure 2: Represents light and confocal microscopic images of Picrosirius Red (100X magnification) and TUNEL assay (400X magnification) of liver tissue from different experimental groups

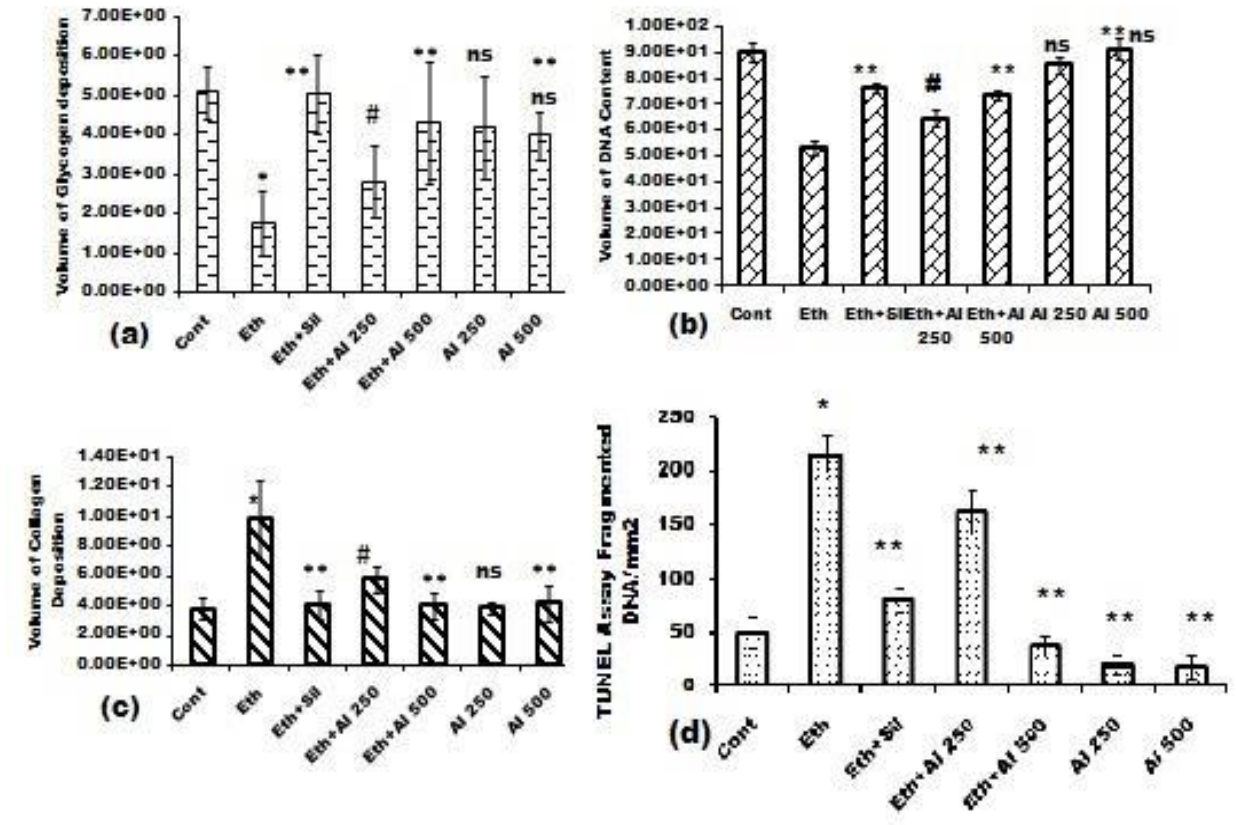

Figure 3a, 3b, 3c and 3d depicts graphical presentation of glycogen deposition, DNA content, Collagen deposition and DNA fragmentation respectively from different experimental groups. * P<0.001 vs. control. ns P>0.05 vs. control, **P<0.001 vs. only ethanol, #P<0.05 vs. ethanol

4. DISCUSSION :

Alcohol is one of the main causes of end-stage liver diseases. It acts through numerous pathways to affect the liver and other organs and to lead to the development of alcoholic liver disease (ALD) (Cederbaum 2001). In this study, liver is on focus which is the major organ affected in chronic alcohol abuse. Liver, being the major site for detoxification of xenobiotics from intestinal absorption in the blood, gets exposed to high concentrations of toxicants and toxic metabolites and thus particularly susceptible to chemical induced injury (Gu and Manautou 2012).

In this study a single dose of 40% ethanol (2gm/kg body wt) for thirty consecutive days led to hepatic damage as indicated by changes in biochemical markers in the ethanol treated group from that of normal control group. Liver injury is primarily assessed by measuring the serum markers such as AST, ALT, ALP, and total protein in an *in vivo* study in alcohol intoxicated rats. Ethanol consumption is notably associated with hepatic damage and the prominent sign of hepatic injury is the leakage of these cellular enzymes into serum, and measurement of the activity levels of enzymes in the body fluids is a useful monitor of a disease state. In the current study, initial hepatic injury in ethanol intoxicated rats is manifested by severe leakage of hepatic marker enzymes into serum as indicated by their elevated activity levels due to increased membrane permeability, cellular damage and/or necrosis of hepatocytes About 90% of total hepatocyte enzymes are represented by these transaminases and among them high level of ALT is a better index of liver injury as it resides only in hepatocytes rather than AST which is inherent in liver parenchymal cells, blood and cardiac/muscle cells. ALP resides in cells lining biliary duct of livers, bone and placental tissue is also elevated due to cholestasis and increased biliary pressure and this coincides with our results and refer to liver damage caused by ethanol (Siddique and Kowdley 2012). However, the hepatoprotective nature of the extract at two different doses (250mg/kg body wt) and (500mg/kg body wt) show restored and diminished hepatic enzyme activities in serum like that of control and standard hepatoprotective drug silymarin (100mg/kg body wt) treated groups. Moreover, decline in total serum protein content can also be deemed as a useful index of the severity of cellular dysfunction in chronic liver disease. It is to mention that besides restoring the values of ALP, ALT and AST, the extract at its two selected doses also revert back the total protein content by stimulating protein synthesis in the extract treated group. This is an important feature of the hepatoprotective nature of the extract that accelerates the regeneration process and production of new liver cells (Fahmy

2011). Besides biochemical changes hepatic damage is also highlighted histomorphologically where the H and E stained sections of liver slices indicate prominent changes in hepatic architecture after chronic ethanol administration. Visual changes indicate central vein inflammation and leukocyte infiltration, enlargement of central vein diameter, congestion, pyknotic nucleus especially in necrotic hepatocytes and fatty infiltration. However such changes are restricted when the animals received the extracts of *AI* as hepato-protective agent. Liver slices show morphology similar to that of control animals. Apart from architectural deformations, ethanol treated liver slices histochemically show diminished glycogen store by PAS staining which is in agreement with the findings of other investigations (Udoh et al. 2015). Altered metabolic function of hepatocytes in ethanol treatment is reflected in the incapability of hepatocytes to maintain normal glycogen store. Altered enzyme activity of glycolysis, TCA cycle and gluconeogenesis may be responsible for such diminished glycogen store (Ebaid et al. 2007). The study reveals that the extracts prevent glycogen exhaustion from hepatocytes under chronic ethanol stress.

Besides, this study focuses on the tissue oxidant-antioxidant microenvironment. Ethanol is a potent stressor and a well known ROS generator. Most of the deleterious effects of ethanol are attributed to its ability to generate free radicals and disrupt the *in vivo* redox balance homeostasis. In this study the alteration in antioxidant tissue markers are examined after chronic ethanol exposure. The generation of oxygen metabolites such as superoxide, hydrogen peroxide and hydroxyl radical during ethanol metabolism is believed to be the main cause in the pathogenesis of alcoholic liver injury (Zima et al. 2001). Increased generation of free radicals results in the loss of membrane integrity and function via lipid peroxidation. Free radical scavenging enzymes such as SOD and CAT are the first line of defense against oxidative injury (Wen et al. 2013) while GPx and GST is thought as second line of defense during oxidative stress. SOD scavenges superoxide ions generated in response to redox balance disruption in chronic ethanol treatment (Kondeti Ramudu et al. 2011). It is a ubiquitous chain breaking antioxidant, and plays an important role in protection against deleterious effects of lipid peroxidation. Not only so, catalase also plays a vital role in decomposition of hydrogen peroxide to water and oxygen, thus protecting the cellular components from oxidative damage by hydrogen peroxide (Saravanan et al. 2003). The GSTs are a multigene family of isozymes that catalyze the conjugation of GSH to a variety of electrophilic compounds, and thereby exert a critical role in cellular protection against ROS (Damodara Reddy et al. 2010). GSH is a major non-protein thiol plays a central role in coordinating the antioxidant defense process. It is involved in the maintenance of normal cell structure and function through its redox and detoxification reactions (Townsend et al. 2003). GSH in association with GPx metabolizes hydrogen peroxide to water, thereby protecting mammalian cells against oxidative damage. GSH depletion is considered to be the chief factor leading to lipid peroxidation (Bhatia and Jain 2004). Earlier report revealed that alcohol was capable of generating ROS by glutathione synthesis inhibitor, producing glutathione loss from the tissue, and increasing MDA levels (Yanardag et al. 2007). In our experiment, decreased activities of first line of defence - CAT and SOD, were observed in chronic ethanol treated rats. Besides, increased lipid peroxidation products, equivalent to thiobarbituric acid reactive substances (TBARS) signifies enhanced degree of lipid peroxidation along with decreased GSH content in the said group. Supplementation of the extract (250 and 500mg/kg body wt) surprisingly elevated and restored the activities of SOD and CAT enzymes and GSH levels to near normal with concomitant lowered lipid peroxidation products.

On the other hand cellular activities of GPx and GST gets elevated when SOD and CAT becomes exhausted in order to neutralize the generated peroxides. Utilization of GSH by these enzymes is also another reason for the depleted levels of tissue GSH as mentioned earlier. GR is the only enzyme that participates in GSH recycling by reducing GSSG to GSH at the expense of NADPH, thereby forming a redox cycle (Parke and Piotrowski, 1996). In this study, it is observed that GPx and GST activity is elevated but that for GR, which reduces oxidized glutathione (GSSG) back to reduced form with the availability of NADPH as cofactor is lowered. Availability of NADPH in turn depends on the activity of G6PD, which maintains cellular reducing equivalents by participating in the dehydrogenation reaction of pentose phosphate pathway, where glucose-6-phosphate is converted to 6-phosphoglucono-δ-lactone (Brzóska et al. 2001). Here the study reveals that activity of G-6-PD is also lowered upon ethanol treatment and this may be a reason for non-availability of sufficient NADPH which can be utilized by GR to regenerate the GSH store. The study demonstrates that chronic ethanol exposure supersedes GSH utilization compared to replenishment. This can be a reason accounting for low GSH content after prolonged ethanol treatment as observed in this study. Thus, we see that the glutathione peroxidase/reductase cycle acts like that in severe oxidative stress condition. The changes brought about by only ethanol administration is however reversed back to control when the rats were supplemented with standard drug silymarin and test extract. Such results are clear indicative of the fact that the hydro-ethanolic extract of *A. indica* tubers successfully meets up the antioxidant challenge by chronic ethanol administration.

This study also extends an insight to the molecular mechanism underlying pathogenesis of liver tissue in response to ethanol administration. Free radicals are potent activators of hepatic Kupffer cells that are involved in the pathogenesis of liver injury mainly by production of inflammatory cytokines. Numerous reports have demonstrated that

TNF-α plays a pivotal role in the ethanol-induced liver pathology (Honchel et al. 1992). In the liver, TNF-*α* is mainly produced by Kupffer cells and TNF-*α* is also an important mediator in various physiological processes, such as inflammation, cell proliferation, and apoptosis. In alcoholic hepatitis (AH), inflammatory cytokines, such as TNF-*α* or IL-6, induce liver injury (Ji et al. 2004). Serum TNF-*α* is increased in patients with ALD and correlates with mortality. Administration of excessive ethanol to TNF-*α* knockout mice does not cause liver injury. Thus TNF-*α* is thought to be the main cytokine of inflammation. In our study an alarming level of both TNF-α and IL-6 have been observed in ethanol treated rats. The role of IL-6 in ALD is complex and not well understood. It appears to have some beneficial effects on the liver. IL-6 may protect against hepatocyte apoptosis and participate in mitochondrial DNA repair after alcoholic liver injury (McClain 1991, Hong et al. 2002). Elevated IL-6 is found in chronic alcohol-fed animals and in alcoholics, with or without liver disease (Latvala et al. 2005). On the other hand, IL-6 knockout mice fed chronic alcohol showed increased liver fat accumulation, lipid peroxidation, mitochondrial DNA damage, and sensitization of hepatocytes to TNF-*α* induced apoptosis, which was prevented by the administration of recombinant IL-6 (El-Assal et al, 2004, Zhang et al. 2010). These findings suggest that IL-6 has a protective effect at the early phase of ALD. Our study has also focused on the extent of apoptosis in the injured hepatocytes in connection with elevated inflammatory cytokines. Indication of apoptosis by TUNEL assay is morphologically characterized by some cellular changes, including DNA fragmentation and the appearance of cytoplasmic apoptotic bodies. In our study, the cell death in ethanol treated group was detected by an increase in the apoptotic cell number in chronic alcohol exposed rat livers by Feulgen and TUNEL assay that quantifies cellular DNA and fragmented DNA respectively. Lesser DNA damage and subsequent decrease in apoptotic cell number was also observed following administration of the test extract (250 and 500mg/kg body wt). These findings positively correlate the elevated TNF- *α* levels with greater apoptosis and an elevated IL-6 as a responsive effect to rescue the cells from apoptosis in ethanol administered group.

Liver fibrosis is the result of an exacerbated wound-healing process after chronic hepatic damage and is characterized by the activation of hepatic stellate cells (HSC) and excess production of extracellular matrix (ECM) components by these cells. In our study we have assessed the degree of hepatic fibrosis in terms of collagen deposition in hepatic tissue using histochemical staining with Direct Red-*80* that binds collagen type I and type II fibers post ethanol treatment. The results show increased extent of fibrosis in ethanol group while a remarkable heal up following administration of the extract *(250 and 500mg/kg body wt).* Low collagen content indicates absence of fibrosis in the extract treated groups. It can be concluded that molecular pathways activated by ROS signaling are all attenuated by the extract of *AI*. The extract exerts its hepatoprotective effect mainly by preventing alteration of the cellular redox balance which ethanol tends to hamper.

Acknowledgements:

The first author acknowledges Department of Science and Technology, Govt. of India INSPIRE programme, for the INSPIRE fellowship. The second author highly acknowledges the grant received from UGC Major Research Project [F. No. 42-625/2013 (SR)], Govt. of India. All authors greatly acknowledge Dr. Pranabes Nath, faculty of Rammohan College for providing constant co-operation and guidance throughout the study. The authorities of Rammohan College and Bose Institute, Kolkata are also acknowledged for providing necessary facility to carry out the study. Special thanks are also due to Sri Sumanto Ghosal for providing technical assistance.

Conflict of Interests:

The authors declared no conflict of interest.

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