

Research Article

Hepatoprotective activity of herbal extract mixture (HEM) on ethanol induced hepatotoxicity in rats

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Abstract: Ayurveda an ancient Indian system of medicine claims to have remedy for disorders likes' jaundice and liver cirrhosis, which is inadequate with present allopathy. The present study was conducted to find out the effectiveness of some herbal extracts mixture (HEM) against ethanol induced liver damage in experimental animal models. An attempt is made to prepare formulation with herbal extracts that possess membrane stabilizing activity and inhibition of lipid peroxidation. The selected aqueous herbal extracts include *Phyllanthus amarus*, *Terminalia chebula*, *Ricinus communis*, *Cichorium intybus*, *Vitex negundo*, *Aloe vera* of various parts use in the formulation. In Prophylactic *in vivo* studies with the ethanol induced hepatotoxicity in rats, the herbal formulation showed dose dependent effect at the doses of 300 and 600 mg/kg body weight and compared to 100 mg/kg dose of silymarin in preventing the rise in the levels of ALP, SGOT, SGPT and BIT compared to matching controls. The presence of flavonoids, terpenoids, tannins, saponins and polyphenolic compounds present in the herbal extracts might be responsible for the antioxidant and hepatoprotective activities of herbal formulation.

Keywords: Hepatoprotective, herbal formulation, ethanol, antioxidants & silymarin.

INTRODUCTION

Alcohol abuse can affect almost all organs of the body [1]. However, the liver is particularly susceptible to injury because it is the site responsible for majority of ethanol oxidation [2]. Chronic alcohol intake provokes susceptible hepatic changes consisting of steatosis (fatty liver), fibrosis, alcoholic hepatitis and cirrhosis. The alcoholic liver injury appears to be generated by the effect of alcohol metabolism and the toxic effect of acetaldehyde, which may be mediated by immune response to alcohol, or acetaldehyde altered proteins [3].

Alcohol is the third leading cause of preventable mortality in India and also worldwide. In the US, it contributes about 1 lakh deaths annually. The economic burden of alcoholism on the US economy, in great part due to health care expenditures, rose to a great extent [4]. Despite the fact that the population in general is well aware of the adverse and often fatal consequences of alcohol consumption, it is estimated that more than 7% of individuals 18 years and over have problems with drinking. Alcohol liver disease (ALD) is one of the major drinking related health problems and primary cause of liver disease among Caucasians.

Histopathological features of alcoholic liver disease include fat accumulation and hepatitis followed by fibrosis and cirrhosis. A major breakthrough in understanding the molecular mechanisms of these pathological changes was the development of an enteral animal model of alcoholic liver disease in the rat by Tsukamoto [5]. In the rat liver, when ethanol is delivered continuously, pathological changes reflective of human alcoholic liver disease occur (i.e., fat accumulation, inflammation and fibrosis). One hypothesis to account for the mechanism of alcohol induced liver injury is that CYP2E1, induced predominantly in hepatocytes by ethanol, increases production of free radicals. Indeed, ethanol causes formation of α -hydroxyethyl radical in the Tsukamoto-French rat model [6].

On the other hand, evidence has been presented in support of the hypothesis that Kupffer cells, the resident hepatic macrophages, play a key role in alcohol induced liver damage [7]. Specifically, it is known that alcohol increases gut permeability for Gram negative bacterial endotoxin. Endotoxin is a potent activator of Kupffer cells, which release toxic cytokines and inflammatory mediators (e.g., TNF- α) as well as reactive oxygen species. Kupffer cells may be the source of oxidants [8]. NADPH oxidase is a major

oxidant generating enzyme in activated macrophages [9].

Based on the above mentioned factors responsible for liver necrosis the present study was designed to evaluate the hepatoprotective activity of HEM in the alcohol induced hepatotoxicity in rats.

MATERIALS AND METHODS

Plant extracts used in HEM include PA, TC, RC, CI, VN and AV were supplied by Laila Impex, Vijayawada, A.P., India as gift samples. Silymarin was a gift sample from Microlabs, Bangalore, and Karnataka, India, and ethanol was purchased from Changshu Yangyuan Chemicals, Mumbai. Animal feed was supplied by Rayon biotech Ltd, Hyderabad. Kits for estimation of

selected biochemical parameters such as SGPT, SGOT, ALP and BIT were purchased from Medsource ozone Ltd., Faridabad, and U.P, INDIA. Oral feeding needle was purchased from BIK Instruments Ltd., Mumbai

Preparation of herbal extract mixture (HEM):

Based on the in vitro antioxidant activity, those extracts which have shown better superoxide and hydroxyl radical scavenging activity were selected and included in HEM and tested against ethanol induced hepatotoxicity as it is believed to cause hepatic necrosis through generation of free radicals [10]. The HEM was prepared as per the composition given in table No 1. The doses of HEM used were 300 and 600 mg/kg bd. wt. They were administered orally as aqueous solutions.

Table 1: Composition of HEM

Plant Name	Part Used	Composition mg / 10ml
<i>Phyllanthus amarus</i> (PA)	Whole herb	500
<i>Terminalia chebula</i> (TC)	Seed	500
<i>Ricinus communis</i> (RC)	Leaf	500
<i>Cichorium intybus</i> (CI)	Root	500
<i>Vitex negundo</i> (VN)	Leaf	500
<i>Aloe vera</i> (AV)	Mucilage	500
Total		3000 mg

Oral administration:

For administration of vehicle/ toxicant/ HEM/ drug, an oral feeding needle attached to a syringe was used. The needle was curved and round tipped. The animals were positioned securely by holding the backside skin of the neck with left hand and the oral feeding needle was introduced through intradental space right into the oesophagus and the substances were administered to the respective groups by pushing the plunger of the syringe. Then the needle was withdrawn slowly and smoothly.

Prophylactic Study Procedure:

In this method male albino Wistar rats weighing between 150-250g were used for the study. The rats were housed individually under standard conditions of constant temperature and lighting (12 hours light/dark cycle). They had access to standard pellet diet (Rayon Biotech limited, Hyderabad) and water *ad libitum*. The institutional Ethics committee of A.U college of Pharmaceutical sciences, Andhra University, India is approved by Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA) with registration number 516/01/a /CPCSEA. The rats were selected and divided into 5 groups each containing six animals. Silymarin was dissolved 2% gum acacia suspension and herbal mixture was dissolved in water. The treatment protocol was planned to study the effect of herbal mixture in preventive aspect of ethanol induced hepatotoxicity⁽¹⁰⁾. The doses of HEM selected were 300 and 600 mg/kg body weight of each extract. The dose of silymarin used was 100 mg/kg bd. wt. The treatment protocol is summarized and given below.

Group 1- Normal Control: 2% w/v gum acacia p.o 1 ml/kg once daily for 25 days

Group 2- Toxicant 40% ETH (Ethanol) 3.76 g/kg twice daily, p.o for 25 days

Group 3- HEM (Herbal extract mixture) 300 mg/kg p.o. 30 min later ETH 3.76 g/kg bd. wt. p.o for 25 days

Group 4- HEM 600 mg/kg p.o. 30 min later ETH 3.76 g/kg bd. wt. p.o for 25 days

Group 5- SIL (Silymarin) 100 mg/kg p.o. 30 min later ETH 3.76 g/kg bd. wt. p.o for 25 days

On 0 day and 26th day blood was collected from all animals by retroorbital puncture. Serum was separated by centrifugation (3000 rpm for 15 min) and subjected for estimation of biochemical parameters. Then the animals were sacrificed and the livers were isolated and washed with fresh saline and processed with 10% formalin. The slides were prepared for histopathological study.

The biochemical parameters studied include:

Serum glutamate pyruvate transaminase (SGPT)
 Serum glutamate oxaloacetate transaminase (SGOT)
 Serum alkaline phosphatase (ALP)
 Serum total bilirubin (BIT)

Histopathological examination:

Small portions from the right and left lobes of liver were quickly dissected out from the animals after autopsy and processed for routine microtomy. Sections were made about 4-6 µm in thickness. They were

stained with hematoxylin and eosin and observed with a microscope. The sections were later photographed.

Statistical analysis

Results were expressed as mean ± SD. The difference among means was analysed by unpaired Student’s t-test.

RESULTS

1. Alanine aminotransferase levels (ALT or SGPT):

The dose of 3.76 g/kg body weight of ethanol induced significant increase in serum SGPT levels with an increase of 389.96 % (41.61 IU/L to 204.17 IU/L) compared to normal control where the increase was 0 % (41.17 IU/L to 41.17 IU/L). Ethanol induced serum rise of SGPT was protected by 300 and 600 mg/kg bd. wt. doses of HEM and 100 mg/kg dose of silymarin. The rise was only 10.90 % (45.83 IU/L to 50.83 IU/L) and 1.93 % (43.33 IU/L to 44.17 IU/L) and 9.81% (42.50 IU/L to 46.67 IU/L) respectively after 25 days treatment. The results were given in table No. 2 & 3 and Fig. No.1 & 2.

2. Aspartate aminotrasferase levels (AST or SGOT):

The dose of 3.76 g/kg body weight of ethanol induced significant increase in serum SGOT levels with an increase of 419.02 % (83.33 IU/L to 432.50 IU/L) compared to normal control where the increase was 2.90 % (85.83 IU/L to 83.33 IU/L). Ethanol induced serum rise of SGOT was protected by 300 and 600 mg/kg bd. wt. doses of HEM and 100 mg/kg dose of silymarin. The rise was only 12.12 % (82.50 IU/L to 92.50 IU/L) and 2.30 % (85.83 IU/L to 87.50 IU/L) and 6.80 % (85.83 IU/L to 91.67 IU/L) respectively after 25 days treatment. The results were given in table Nos. 2 & 3 and Fig. Nos.1 & 3.

3. Alkaline phosphatase levels (ALP):

The dose of 3.76 g/kg body weight of ethanol induced significant increase in serum ALP levels with an increase of 186.93 % (204.17 IU/L to 585.83 IU/L) compared to normal control where the increase was 1.20 % (208.33 IU/L to 205.83 IU/L). Ethanol induced serum rise of ALP was protected by 300 and 600 mg/kg bd. wt. doses of HEM and 100 mg/kg dose of silymarin. The rise was only 10.65 % (203.33 IU/L to 225 IU/L) and 0.82 % (201.67 IU/L to 203.33 IU/L) and 4.42 % (207.50 IU/L to 216.67 IU/L) respectively after 25 days of treatment. The results were given in table No. 2 & 3 and Fig. No.1 & 4.

4. Bilirubin total levels (BIT):

The dose of 3.76 g/kg body weight of ethanol induced significant increase in serum BIT levels with an increase of 198.85 % (0.87 mg/dL to 2.60 mg/dL) compared to normal control where the increase was 1.28 % (0.78 mg/dL to 0.77 mg/dL). Ethanol induced serum rise of BIT was protected by 300 and 600 mg/kg bd.wt. doses of HEM and 100 mg/kg dose of silymarin. The rise was only 23.52 % (0.85 mg/dL to 1.05 mg/dL) and 2.50 % (0.80 mg/dL to 0.82 mg/dL) and 9.33 % (0.75 mg/dL to 0.82 mg/dL) respectively after 25 days of treatment. The results were given in table No. 2 & 3 and Fig. No.1 & 5.

5. Histopathological studies:

The hepatic architecture was present in normal control group with features of polygonal nucleus with nucleolus, abundant cytoplasm and bilobed nucleus (Fig. No: 6). These normal structures were absent in toxic control group i.e group 2 in which there were macrovesicular steotosis, hepatic necrosis and ballooning degeneration (Fig. No: 7). In HEM/ SIL treated groups i.e group 3, 4 and 5 where it was found that the protection of cells showed bilobed nucleus and pleomorphic hepatocytes and abundant cytoplasm etc (Fig. No. 8, 9 & 10).

Table 2: Influence of herbal extract mixture (HEM) on selected serum biochemical parameters in ethanol treated rats (Prophylactic study)

Group	Treatment	SGPT (IU/L)		SGOT (IU/L)		ALP (IU/L)		BIT (mg/dl)	
		0 day	26 th day	0 day	26 th day	0 day	26 th day	0 day	26 th day
1.	Normal gum acacia 1 ml/kg	44.17 ±3.76	44.17 ±7.36	85.83 ±5.84	83.33 ±8.76	208.33 ±12.90	205.83 ±13.19	0.78 ±0.19	0.77 ±0.16
2.	ETH 3.76 g/kg	41.67 ±5.16	204.17 ±10.20***	83.33 ±9.83	432.50 ±15.73***	204.17 ±12.41	585.83 ±64.45***	0.87 ±0.15	2.60 ±0.38***
3.	ETH 3.76 g/kg + HEM 300 mg/kg	45.83 ±7.35	50.83 ±7.36***	82.50 ±5.24	92.50 ±5.24***	203.33 ±10.80	225 ±8.36***	0.85 ±0.22	1.05 ±0.20***
4.	ETH3.76g/kg + HEM 600 mg/kg	43.33 ±5.16	44.17 ±4.91***	85.83 ±7.35	87.50 ±5.24***	201.67 ±13.66	203.33 ±11.69***	0.80 ±0.17	0.82 ±0.14***
5.	ETH3.76 g/kg + SIL100 mg/kg	42.50 ±5.24	46.67 ±4.08***	85.83 ±7.35	91.67 ±6.05***	207.50 ±15.08	216.67 ±14.02***	0.75 ±0.10	0.82 ±0.11***

Values indicate mean ± SD for 6 animals in each group.

*** P < 0.001 significant when compared Group 1 with group 2 and group 2 with group 3, 4 and 5

ETH - Ethanol, HEM- Herbal extract mixture

Table 3: Average percentage change in selected serum biochemical parameters in ETH induced hepatotoxicity (Prophylactic Study)

Groups	Treatment	SGPT	SGOT	ALP	BIT
1.	Gum acasia 2ml/kg	0.00 ± 0.00	2.90±0.06	1.20±0.08	1.28±0.07
2.	ETH 3.76 g/kg	389.96±10.84***	419.02±12.78***	186.93±8.64***	198.85±7.38***
3.	ETH 3.76 g/kg +HEM 300mg/kg	10.90±0.92***	12.12±1.04***	10.65±0.82***	23.52±1.86***
4.	ETH 3.76 g/kg +HEM 600mg/kg	1.93±0.08***	2.30±0.12***	0.82±0.04***	2.50±0.14***
5.	ETH3.76 g/kg +SIL 100mg/kg	9.81±1.08***	6.80±0.96***	4.42±0.86***	9.33±1.04***

*** P < 0.001 significant when compared Group 1 with group 2 and group 2 with group 3, 4 and 5. ETH - Ethanol, HEM- Herbal extract mixture, SIL - Silymarin

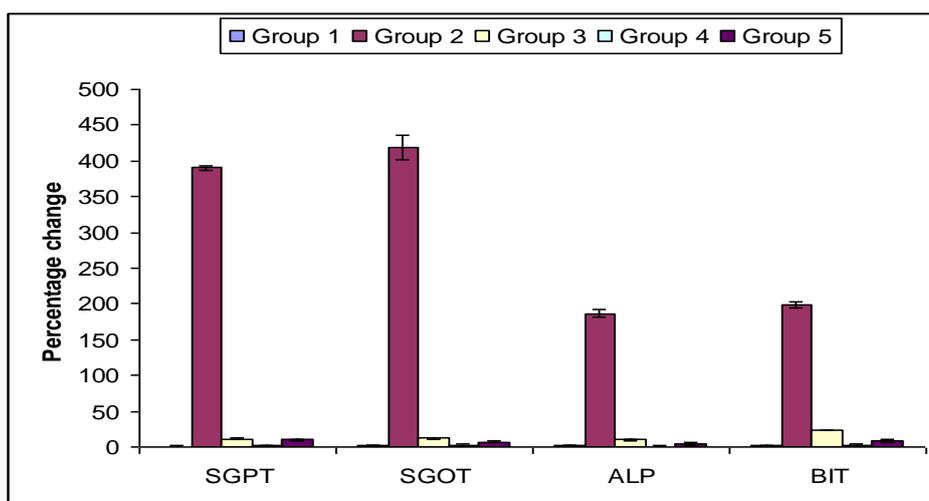


Fig. 1. Average percentage change in selected serum biochemical parameters in PCM-induced hepatotoxicity in rats (Prophylactic study)

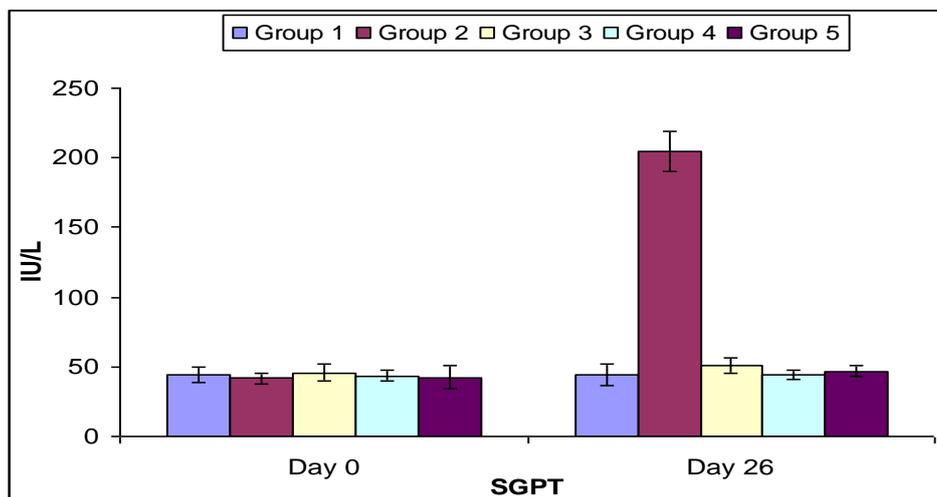


Fig. 2. Influence of HEM on SGPT in ETH induced hepatotoxicity in rats (Prophylactic study)

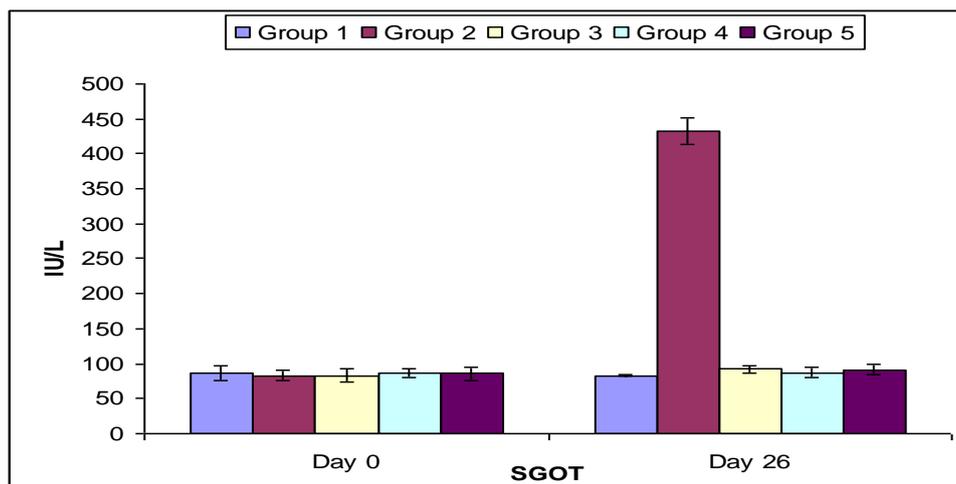


Fig. 3. Influence of HEM on SGOT in ETH induced hepatotoxicity in rats (Prophylactic study)

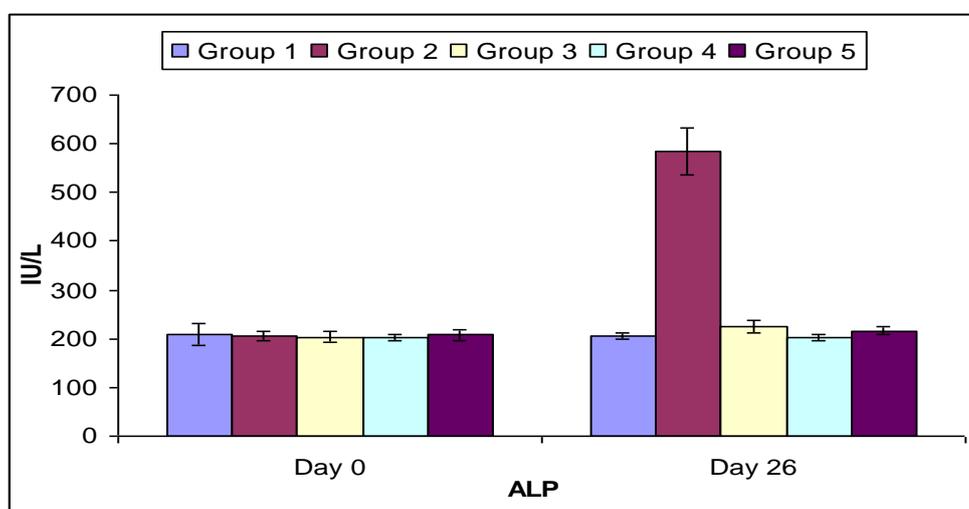


Fig. 4. Influence of HEM on ALP in ETH induced hepatotoxicity in rats (Prophylactic study)

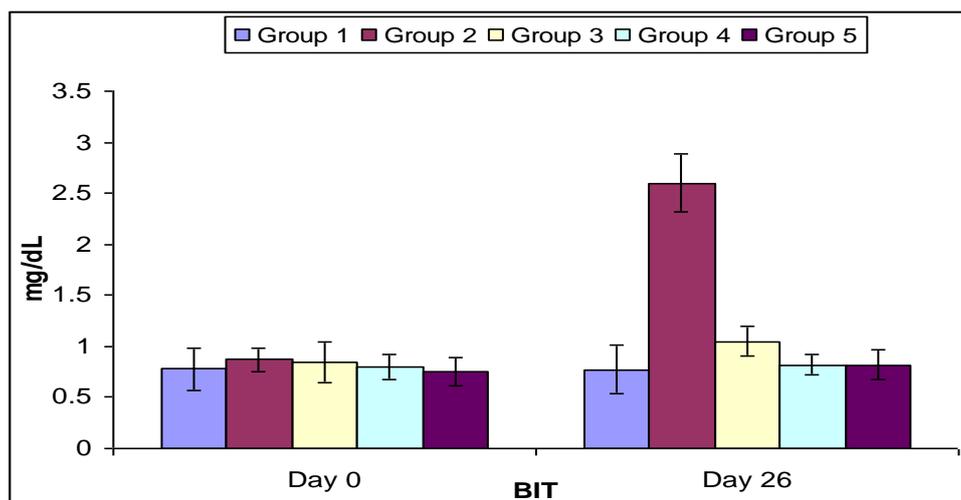


Fig. 5. Influence of HEM on BIT in ETH induced hepatotoxicity in rats (Prophylactic study)

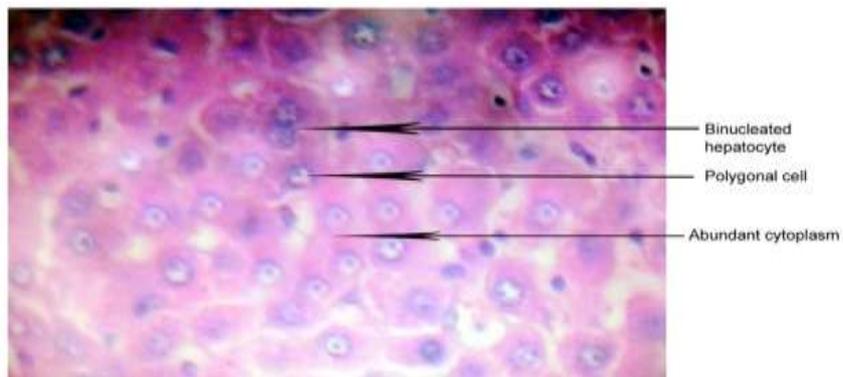


Fig-6: Normal control features of hepatocytes in group-1 (400X)

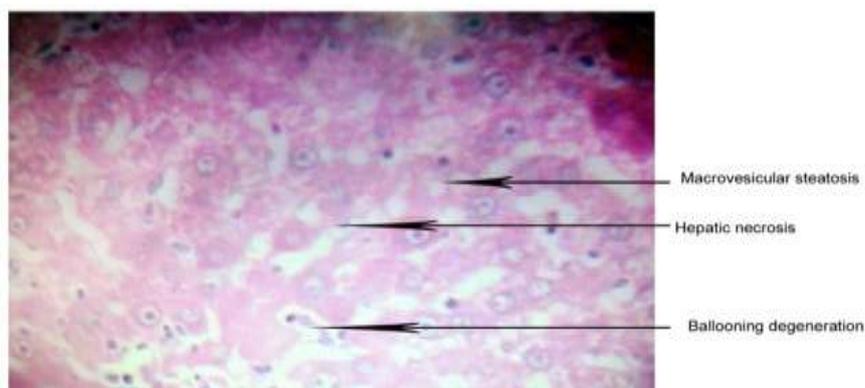


Fig-7: Treatment with ETH 3.76 g/kg (Toxic Control) for 25 days (400X)

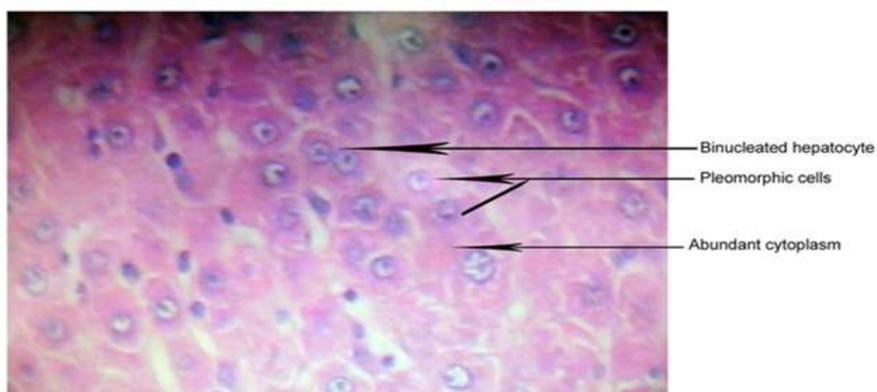


Fig-8: Treatment with ETH 3.76 g/kg + HEM 300 mg/kg for 25 days in prophylactic (400X)

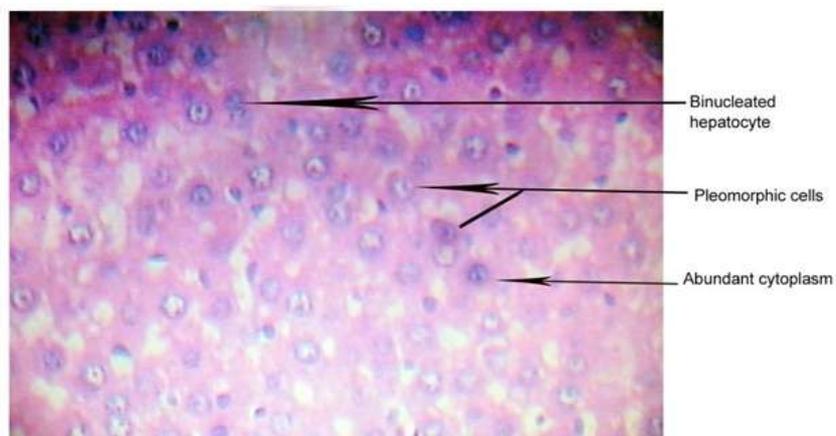


Fig-9: Treatment with ETH 3.76 g/kg + HEM 600 mg/kg for 25 days in prophylactic (400X)

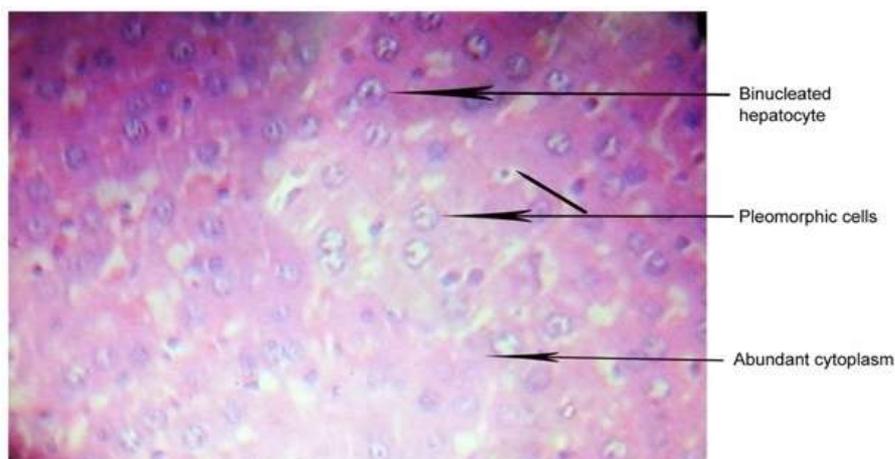


Fig-10: Treatment with ETH 3.76 g/kg + SIL 100 mg/kg for 25 days in prophylactic (400X)

DISCUSSION:

Recent studies indicate that oxidative stress is involved in the pathogenesis of liver diseases including drug induced hepatic damage, alcoholic hepatitis and viral hepatitis or ischaemic liver injury [11,22]. The incidence of deaths due to liver disorders appears to be increasing year by year. The chronic alcoholism is the third leading cause of the total deaths in many countries [13]. The major reason offered is the toxicity caused by chronic alcoholism in the form of fat accumulation (steatosis), hepatitis followed by fibrosis and cirrhosis.

Ethanol induced hepatotoxicity was used by several workers as a model for screening hepatoprotective activity of drugs. The dose used for induction of hepatotoxicity by different workers was found to vary. The dose of ethanol used was 3.76 g/kg body weight [14,15] in albino Wistar rats of either sex while Das S.K. *et al.*, 2006 used 1.6 g/kg bd. wt. in male rats. In the present study 3.76 g/kg bd. wt. was used (the highest dose reported) in male albino Wistar rats to ensure hepatic damage and also to ensure hepatoprotective activity of the formulation against a definite toxic dose of ethanol.

Alcoholic liver disease is one of the major drinking related health problems and a primary cause of liver disease in humans. In acute toxicity alcohol increases lipid peroxidation and in chronic toxicity it causes free radical generation [16]. Histopathological features of alcoholic liver disease include fat accumulation and hepatitis, followed by fibrosis and cirrhosis. One hypothesis to account for the mechanism of alcohol induced liver injury is that CYP 2E1, induced predominantly in hepatocytes by ethanol, increases production of free radicals.

It is well known that animals with alcoholic liver disease (ALD) exhibit impaired liver regeneration [17]. It is reported that acetaldehyde, a potent toxic metabolite of ethanol, induces liver injury via its covalent binding to structural or functional proteins of

the cells [18]. One of the proposed mechanisms of chronic ethanol induced toxicity resulted in a significant decrease in glutathione peroxidase (GPx) activity in liver may be due to either free radical dependent inactivation of enzyme or depletion of its co-substrates i.e., GSH and NADPH in ethanol treated rats [10,17]. Glutathione s-transferase (GST) plays an essential role in liver by eliminating toxic compound acetaldehyde by conjugating it with glutathione. Increased GST activity and decreased glutathione reductase activity, followed by thiol depletion are important factors sustaining a pathogenic role for oxidative stress [19].

In the assessment of liver damage by ethanol the serum levels of SGOT, SGPT and BIT are largely used [20]. Necrosis or membrane damage releases them into circulation and hence can be measured in serum. It is reported that SGPT is more specific marker of liver damage while SGOT & BIT indicate damage of liver and other tissues like muscle and kidney [21], where as increased level of BIT indicate the damage of liver and in haemolytic anaemia. The raised level of serum ALP on the other hand is related to the hepatic cell and bone (Table no 1 and 2). Our reports are similar to earlier studies [14].

In the present study the hepatoprotective activity of HEM was evaluated in ethanol induced liver toxicity. Chronic administration of ethanol for 25 days produced elevation of the serum levels of these markers in treated animals (Group II) compared to that of the control group (Group I). Treatment with HEM at dose of 300 and 600 mg/kg bd. wt. produced dose dependent reduction in ETH induced rise of the parameters. Silymarin at 100 mg/kg dose significantly prevented such rise in prophylactic study. The effect of silymarin was found to be in between the effect of selected doses of HEM. The protective effect of silymarin was well established in several models of hepatotoxicity and was reported to be due to its antioxidant and membrane stabilizing activities [22,23].

From the results it can be concluded that ethanol induced stress can be prevented by HEM supplementation. The HEM formulation was particularly prepared by combining the selected plant extracts that exhibited better free radical scavenging activity (PA, TC, RC, CI, VN and AV) such as superoxide and hydroxyl radical scavenging activity. The hepatic damage associated with ethanol is due to release of toxic metabolite acetaldehyde and its free radical generation. Antioxidants represent a potential group of therapeutic agents for ALD. They are likely to provide beneficial effects on hepatocytes via desensitization against oxidant stress [24,25]. The extracts also possess lipid peroxidation inhibition activity apart from free radical scavenging activity. Hence, lipid peroxidation inhibition may also contribute for the hepatoprotection [10].

It is reported earlier that, the plant extracts contain flavonoids, terpenoids, polyphenols, tannins and saponins etc. and their antioxidant activity appears to be responsible for protection. Flavonoids include kaempferol 3-O-beta-D-rutinoside and kaempferol-3-O-beta-D-xylopyranoid, 5 hydroxy 6,7,8,3',4' pentamethoxy flavone, gardenin A, carymbosin IV, Casticin, chryso splenol D and isoorientin and other flavonoids are present in RC, VN, AV and CI[26-32]. Lignans like Phyllanthin, hypophyllanthin, diarylbutane, aryltetrahydronaphthalene are present in PA [33]. Tannins like Phyllanthusin D, amarilin, amarulone and amarilic acid are present in PA [34]. Quercetin and rutin etc are present in TC [35]. Hence their combined antioxidant action may contribute for the hepatoprotection. The marketed formulations contain mixtures of herbal extracts, which may or may not be scientifically evaluated. The polyherbal formulation HEM is proved to be effective against ethanol toxicity model, which is also known to produce hepatic toxicity in humans. The formulation at the dose of 600 mg/kg bd. wt. was found to be superior to 100 mg/kg bd. wt. of silymarin.

The histopathological study also supported the biochemical evidence for the hepatoprotection shown by HEM. The normal hepatic cell is a polygonal cell and binucleated with nucleolus and abundant eosinophilic cytoplasm [36]. The above features were found in normal control group (Fig. No: 6). In ethanol treated group i.e group 2 all the above mentioned structures were modified and there was macrovesicular steatosis, necrosis and degeneration indicating hepatic damage (Fig. No: 7). In the HEM / SIL treated groups the normal structures were protected in prophylactic study (Fig. No: 8 to 10). Dose dependent protection / regeneration were observed in HEM & SIL treated groups.

CONCLUSION

The herbal extract mixture (HEM) is proved to be effective against ethanol, which is known to produce

hepatic toxicity in humans. The HEM at the dose of about 600 mg/kg bd. wt. was found to be more effective than 300 mg/kg bd. wt. and standard silymarin 100mg/kg.wt. doses. Thus the presence of flavonoids, terpenoids, saponins, tannins and polyphenolic compounds in the herbal extracts might be responsible for the hepatoprotective activity.

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