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EVALUATION OF HEPATOPROTECTIVE ACTIVITY OF POLYHERBAL (HEPIN) AGAINST ALCOHOL INDUCED HEPATOTOXICITY IN WISTAR ALBINO RATS

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ABSTRACT

Objectives: To investigate hepatoprotective activity of polyherbal formulation in alcohol induced hepatotoxicity in rats. **Methods:** Hepatotoxicity was induced in albino Wistar rats by oral administration of 40% ethanol (2ml/100g body weight). Polyherbal formulation "Hepin" was administered at a dose level of 250 mg/kg and 500mg/kg orally for 28 days. On the next day blood was taken by cardiac puncture and used for estimation of biochemical parameters like SGPT, SGOT, ALP, Bilirubin, Total Protein. Animals were sacrificed by cervical dislocation and liver was dissected out and exvivo studies and histopathological analysis of liver was carried out. **Results:** Obtained results revealed that administration of ethanol caused a significant increase in plasma SGPT, SGOT, ALP and Bilirubin compared to the control group. While total protein concentration is significantly declined. Additionally, a significant decrease in the level of hepatic GSH was also observed. However, the treatment of experimental rats with Hepin prevented these alterations and maintained the antioxidant status. The histopathological observations supported the biochemical evidences of hepatoprotection. **Conclusions:** The results of the present investigation indicate that Hepin possesses hepatoprotective activity.

KEYWORDS: Hepatoprotective, Polyherbal, Hepin.

1. INTRODUCTION

The liver plays a major role in food digestion and metabolism. It has a number of important functions including glycogen storage, decomposition of red blood cells and plasma protein synthesis. The liver clears the blood of waste products, drugs, and other poisonous substances, maintains the volume of blood and regulates the factors affecting clotting of

blood. It is an irreplaceable organ and should not be neglected so as to avoid a number of major problems. Liver regeneration remains a fascinating topic, still partly clouded to many as to the exact cellular and molecular mechanisms that bring about this phenomenon. Actually, liver regeneration is a fundamental mechanism by which the liver can withstand injury. Changes in the morphology and physiology of organs and tissues such as the liver might be due to the accumulation of reactive oxygen species (ROS) and reactive nitrogen species (RNS). Because hepatocytes are very rich in mitochondria and have a high respiratory rate, they are exposed to large amounts of ROS and permanent oxidative stress which may be involved in the hepatocyte dysfunction observed in the setting of fulminant hepatic failure (IMAN H. HASAN *et al.*, 2016).

In the absence of reliable liver protective drugs, attention is focused on natural antioxidants such as polyphenol compounds which are found in plants. Global analysis of natural products is an important issue in developing new therapeutic managements for liver disease. Approximately 25% of the drugs prescribed worldwide at present come from plants and 60% of anti-infections drugs already on the market or under clinical investigations are of natural origin (IMAN H. HASAN *et al.*, 2016).

Liver disease has become a global concern worldwide. The principal causative factors are increasing alcohol consumption, infection, malnutrition, anaemia and availability of hepatotoxic drugs over the counter. Moden medicine has very few choices in terms of treatment for liver dieases. The conventional drugs used in the treatment of liver diseases viz., corticosteroids, immunosuppressant agents are sometimes inadequate and may lead to serious adverse effects. Paradoxically, these may themselves cause hepatic damage eg ;- cholestatic jaundice with azathioprine and elevation of transaminases by interferons. More over these drugs are very expensive. It is therefore imperative to search alternative drugs for the treatment of liver disease to replace the currently used drugs of doubtful efficacy and safety (K.R. Subash *et al.*, 2011).

Liver disease is a serious medical problem. Some of the liver injuries are caused by the use and abuse of drugs. Conventional and/or synthetic drugs such as steroids, vaccines, antivirals and other medications can cause serious side effects, even toxic effects on the liver, especially when used for prolonged periods of time. There is a global trend towards the use of traditional herbal preparations for the treatment of liver diseases (Mohamed A Lebda *et al.*, 2013).

In India numerous medicinal plants and their formulations are used for liver disorders in traditional systems of medicine. Some of these plants viz. Silybum marinum, Picrrorhiza kurroa, Andrographis paniculata, Phyllanthus niruri and Eclipta alba are evaluated for their hepatoprotective actions against hepatotoxins K.R. Subash *et al.*, 2011).

This study investigate the hepatoprotective activity of "Hepin" a widely used polyherbal formulation by Nupal Remedies Pvt Ltd for treating liver disorders.

2. METHODOLOGY

2.1 Invitro Mehods

2.1.1 Determination *In* vitro Hepatoprotective Effect of Hepin by MTT Assay Using Chang Liver Cell Line

The hepatoprotective activity of Hepin was assessed by MTT Assay using Chang liver cell line.

Culturing and maintainence of Chang liver cells

HEP G2 (Human Hepatocellular Carcinoma) cell line was purchased from NCCS Pune was maintained in Dulbecco's modified eagles media (HIMEDIA) from National Centre for Cell Sciences (NCCS), Pune, India and maintained Dulbecco's Modified Eagles medium(DMEM) (Sigma Aldrich, USA).

The cell line was cultured in 25 cm^2 tissue culture flask with DMEM supplemented with 10% FBS, L-glutamine, sodium bicarbonate and antibiotic solution containing: Penicillin (100U/ml), Streptomycin (100µg/ml), and Amphoteracin B (2.5µg/ml). Cultured cell lines were kept at 37ºC in a humidified 5% CO_2 incubator (NBS Eppendorf, Germany).

The viability of cells were evaluated by direct observation of cells by Inverted phase contrast microscope and followed by MTT assay method.

Cells seeding in 96 well plate

Two days old confluent monolayer of cells were trypsinized and the cells were suspended in 10% growth medium, 100µl cell suspension ($5x10^4$ cells/well) wasseeded in 96 well tissue culture plate and incubated at 37ºC in a humidified 5% CO_2 incubator.

Preparation of compound stock

1mg of the sample was weighed and completely dissolved in 1mL DMEM using a cyclomixer. The extract solution was filtered through 0.22 µm Millipore syringe filter to ensure the sterility. Alcohol(40%) was added to induce toxicity.

Cytotoxicity Evaluation

After attaining sufficient growth, Alcohol (40%) was added to induce toxicity and incubated for one hour, prepared extracts in 5% DMEM were five times serially diluted by two fold dilution (100µg, 50µg, 25µg, 12.5µg, 6.25µg in 500µl of 5% DMEM) and each concentration of 100µl were added in triplicates to the respective wells and incubated at 37ºC in a humidified 5% CO_2 incubator.

Cytotoxicity Assay by Direct Microscopic observation

Entire plate was observed at an interval of each 24 hours; up to 72 hours in an inverted phase contrast tissue culture microscope (Olympus CKX41 with Optika Pro5 CCD camera) and microscopic observation were recorded as images. Any detectable changes in the morphology of the cells, such as rounding or shrinking of cells, granulation and vacuolization in the cytoplasm of the cells were considered as indicators of cytotoxicity.

Cytotoxicity Assay by MTT Method

Fifteen mg of MTT (Sigma, M-5655) was reconstituted in 3 ml PBS until completely dissolved and sterilized by filter sterilization.

After 24 hours of incubation period, the sample content in wells were removed and 3 0µl of reconstituted MTT solution was added to all test and cell control wells, the plate was gently shaken well, then incubated at 37ºC in a humidified 5% CO_2 incubator for 4 hours. After the incubation period, the supernatant was removed and 100µl of MTT Solubilization Solution (DMSO was added and the wells were mixed gently by pipetting up and down in order to solubilize the formazan crystals. The absorbance values were measured by using microplate reader at a wavelength of 540 nm (Laura B. Talarico et al., 2004).

The percentage of growth inhibition was calculated using the formula:

$$\% \text{ of viability} = \frac{\text{Mean OD Samples}}{\text{Mean OD of control group}} \times 100$$

2.2 *In Vivo* Methods

Selection of animals

Thirty three female wistar albino rats weighing between 150 – 170gm were obtained from Small Animal Breeding House, Mannoothy, Thrissur, Kerala and housed at animal house of Department of pharmaceutical sciences, CPAS, Cheruvandoor, Ettumanoor. The animals were housed in polypropylene cages in room where the congenial temperature 27±1ºC, 30-60% humidity and 12 hours light and dark cycles were maintained.

The animals were allowed to acclimatize to the envioronment for 14 days and randomly divide into 5 groups. They were fed with standard pellet diet. The experiments were carrued out after obataining the permission of Institutional Animal Ethics Committee, Department of Pharmaceutical Scinces, CPAS, Cheruvandoor, Ettumanoor, Kottayam, India under IAEC No:

The hepatoprotective activity was assessed using alcohol induced hepatotoxic model after acute oral toxicity studies. The methods are described as follows:-

2.2.1 Acute Oral Toxicity Studies in Female Wistar Albino Rats

OECD 423 (Acute Toxic Class Method)

- Starting dose level 2000mg/kg body weight p.o given to an overnight fasted rats.
- Food was withheld for a further 3-4 hours after administration.
- Observed for signs for toxicity:

-body weight

-changes in skin, fur and eyes

-motor activity and behavior pattern.

2.2.2 Alcohol Induced Hepatotoxicity In Wistar Albino Rats

Group	Type	Treatment	Route of administration	No of animals
1	Normal Control	1% CMC	P.O	6
2	+ve Control	40% Ethanol (2ml/100g body weight)	P.O	6
3	Hepin Low dose	40% Ethanol + Hepin (250mg/kg body weight)	P.O	6
4	Hepin High dose	40% Ethanol + Hepin (500 mg/kg body weight)	P.O	6
5.	Standard	40% Ethanol + Silymarin(25 mg/kg body weight)	P.O	6

All the above mentioned dosages were given orally once daily for 28 days. On the next day blood was taken by cardiac puncture and used for estimation of biochemical parameters like

SGPT, SGOT, ALP, Bilirubin, Total Protein. Animals were sacrificed by cervical dislocation and liver was dissected out and exvivo studies and histopathological analysis of liver was carried out.

2.2.3 Biochemical Parameters

The biochemical parameters such as SGPT, SGOT, ALP, Bilirubin and Total protein were estimated.

SGPT, SGOT and ALP were estimated as per the standard procedure prescribed by the manufacurer's instruction manual provided in the kit.

Estimation of Bilirubin

Bilirubin reacts with diazotized sulphanilic acid in acidic medium to form a pink coloured azobilirubin with absorbance directly proportional to bilirubin concentration. Direct bilirubin, being water soluble directly react in acidic medium. However, indirect and unconjugated bilirubin is solubalised using a surfactant and then it is reacts similar to direct bilirubin.

Procedure

Addition Sequence	Blank(µl)	Standard	Test
Working reagent	500	1000	1000
Distilled water	20	-	-
Standard	-	25	-
Sample	-	-	25

Mix well. Incubate for 5mnt at 37ºC temperature. Read the absorbance at 546/630 nm against Reagent blank.

Estimation of total protein

The peptide bond proteins react with Cu ions in alkaline solution to form a blue violet complex (Biuret reaction), each copper ion complexing with 5 or 6 peptide bonds. Tartarate is added as stabilizer while iodine is used to prevent auto reduction of alkaline copper complex. The colour formed is proportional to the protein concentration and is measured at 546 nm.

Procedure

Addition Sequence	Blank(µl)	Standard	Test
Working reagent	1000	1000	1000
Distilled water	20	-	-
Standard	-	20	-
Sample	-	-	20

Incubate for 10 min. at 37ºC. Read the absorbance of standard and each sample at 546 nm against reagent blank.

2.2.4 Exvivo Studies

Physical parameters

Determination of wet liver weight: Animals were sacrificed and livers were isolated and washed with saline and weight was determined using an electronic balance. The liver weights were expressed with respect to its body weight.

Determination of wet liver volume: After recording the weight, all the livers were dropped individually in a measuring cylinder containing a fixed volume of distilled water or saline and the volume displaced was recorded.

2.2.5 Hepatic oxidative stress parameters

Preparation of liver homogenate

The liver was quickly removed and perfused immediately with ice-cold saline (0.9% NaCl). A portion of the lover was homogenized in chilled Tris-HCl buffer (0.025M, pH 7.4) using a homogenizer, The homogenate obtained was centrifuged at 500 rpm for 10mnt, supernatant was collected and used for analysis.

Estimation of reduced glutathione (GSH)

0.5 ml tissue homogenate was pippeted out and precipitated with 2.0ml of 5% TCA.1ml supernatant was taken after centrifugation. To the tubes 0.5ml of DTNB and 3ml of phosphate buffer were added. The tubes were capped, mixed by inversion and intensity of yellow colour was measured immediately at 412 nm against blank. Increase in absorbance (OD) is directly proportional to concentration of GSH and vice versa.

Histopathological analysis of liver

Processing of isolated liver

The animals were sacrificed and the liver of each animal was isolated and was cut into small pieces, preserved and fixed in 10% formalin for two days. Then the liver piece was washed in running water for about 12 hours to remove the formalin and was followed by dehydration with isopropyl alcohol of increasing strength (70%, 80% and 90%) for 12 hours each. Dehydration was performed to remove all traces of water. Further alcohol was removed by

using chloroform with two changes for 15 to 20 minutes each. After paraffin infiltration the liver pieces were subjected to automatic tissue processing unit.

Embedding in paraffin vacuum

Hard paraffin was melted and the hot paraffin was poured into L-shaped blocks. The liver pieces were then dropped into the molten paraffin quickly and allowed to cool.

Sectioning

The blocks were cut using microtome. The section were taken on a microslide on which egg albumin i.e., sticking substance was applied. The sections were allowed to remain in an oven at 60 hour. Paraffin melts and egg albumin denatures, thereby fixing to slide.

Staining

Eosin is an acid stain, hence it stains all the cell constituents pink which are basic in nature i.e., cytoplasm. Haemotaxylin, a basic stain which stains all the acidic cell components blue ie DNA in the nucleus.

Statistical Analysis

Graph pad prism version 6.0 was used for statistical analysis. Results were expressed as mean± S.E.M. Data was analysed by one – way ANOVA followed by Dunnett comparisons test.

The P values <0.05 were considered as statistically significant.

3. RESULTS

Table No. 1: Acute Oral Toxicity Studies.

Acute Oral Toxicity Studies

Parameter	Result
Preterminal Death	Nil
Body Weight	No specific change
Motor Activity	Normal
Tremors and convulsions	Absent
Unusual vocalization	Absent
Sedation	Absent
Body Temperature	Normal
Diarrhea	Absent
Skin Colour	Normal
Restlessness	Absent

1. MTT Assay

Table No. 2: Effect of Hepin on Percentage Viability in Chang Liver Cell Lines.

Sl.No	Sample	Concentration(µg/ml)	OD (540 nm)	Percentage Viability(%)
1.	Control	-	0.3019± 0.003493	100
2.	Alcohol		0.1418± 0.000603	46.94
3.	Hepin	6.25	0.1763± 0.000569	58.37
4		12.5	0.1836± 0.000709	60.78
5		25	0.2088± 0.000493	63.11
6		50	0.2214± 0.001484	69.15
7		100	0.1906± 0.002402	73.32

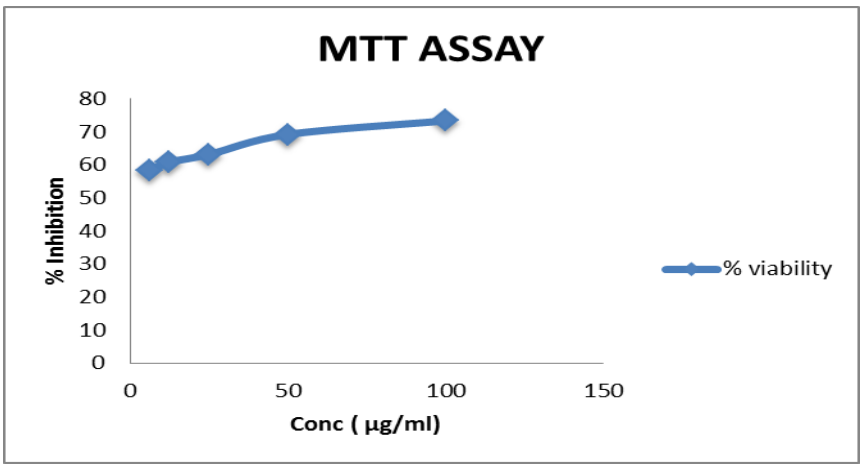


Figure 1: Effect of Hepin on Percentage Viability in Chang Liver Cell Lines.

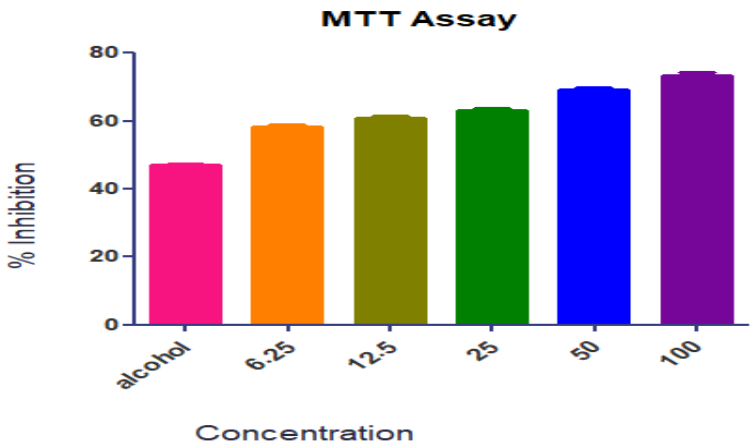


Figure 2: Effect of Hepin on Percentage Viability in Chang Liver Cell Lines.

2. Effect of Hepin on SGPT

Table No. 1: Effect of hepin on SGPT level.

Sl.No.	Group	Treatment	SGPT(U/L)
1.	Normal Control	1% CMC	34.40±1.140
2.	Positive Control	40% Ethanol (2ml/100g body weight)	149.20±1.789
3.	Hepin Low Dose	40% Ethanol + Hepin (250mg/kg body weight)	98.20±0.836
4.	Hepin High Dose	40% Ethanol + Hepin (500 mg/kg body weight)	66.00±1.414
5.	Standard	40% Ethanol + Silymarin(25 mg/kg body weight)	42.60±0.547

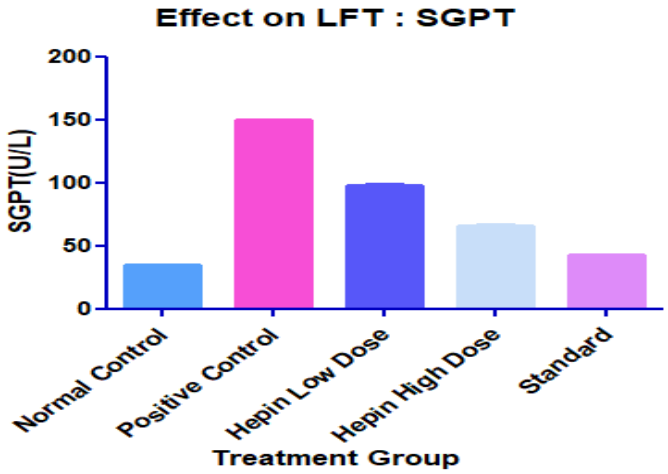
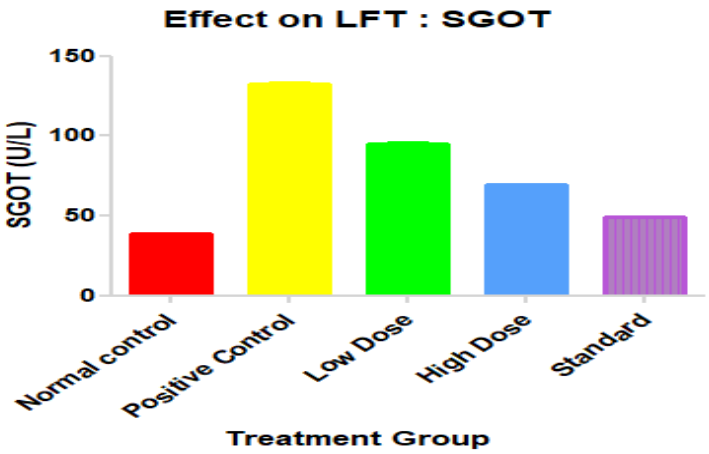


Figure 3: Effect of Hepin on SGPT Level.

2. Effect of Hepin on SGOT Level

Sl.No.	Group	Treatment	SGOT(U/L)
1.	Normal Control	1% CMC	38.50±0.547
2.	Positive Control	40% Ethanol (2ml/100g body weight)	132.8±1.169
3.	Hepin Low Dose	40% Ethanol + Hepin (250mg/kg body weight)	95.17±1.169
4.	Hepin High Dose	40% Ethanol + Hepin (500 mg/kg body weight)	69.50±0.836
5.	Standard	40% Ethanol + Silymarin(25 mg/kg body weight)	49.17±0.752

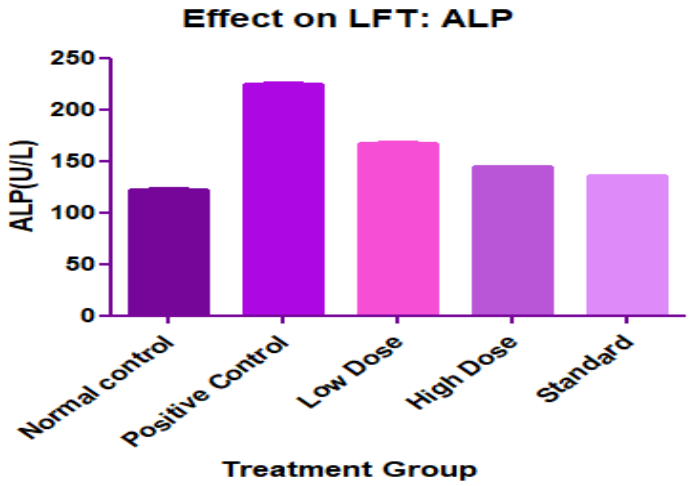
Effect of hepin on SGOT level



4. Effect of Hepin on ALP level

Sl.No.	Group	Treatment	ALP (U/L)
1.	Normal Control	1% CMC	122.5±0.547
2.	Positive Control	40% Ethanol (2ml/100g body weight)	225.2±2.714
3.	Hepin Low Dose	40% Ethanol + Hepin (250mg/kg body weight)	167.3±1.966
4.	Hepin High Dose	40% Ethanol + Hepin (500 mg/kg body weight)	144.3±0.816
5.	Standard	40% Ethanol + Silymarin(25 mg/kg body weight)	135.8±0.752

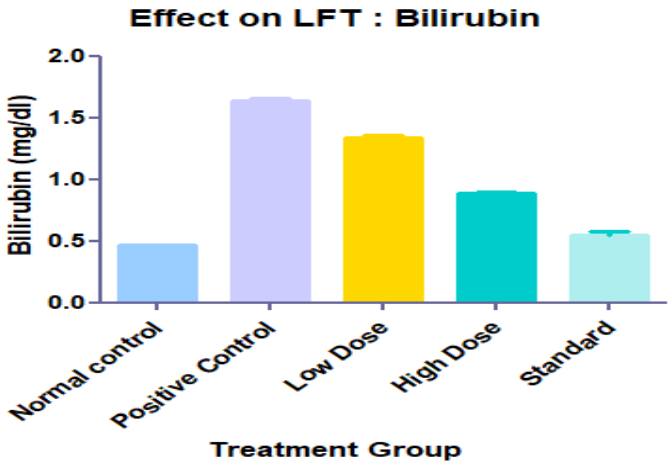
Effect of Hepin on ALP Level.



5. Effect of Hepin on Total Bilirubin Level

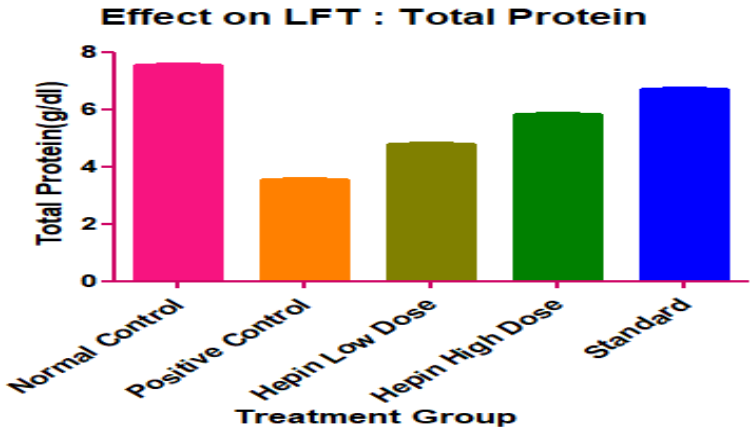
Sl.No.	Group	Treatment	Total Bilrubin(mg/dl)
1.	Normal Control	1% CMC	0.461±0.021
2.	Positive Control	40% Ethanol (2ml/100g body weight)	1.633±0.051
3.	Hepin Low Dose	40% Ethanol + Hepin (250mg/kg body weight)	1.333±0.051
4.	Hepin High Dose	40% Ethanol + Hepin (500 mg/kg body weight)	0.883±0.040
5.	Standard	40% Ethanol + Silymarin(25 mg/kg body weight)	0.550±0.054

Effect of Hepin on Total Bilirubin Level.



6. Effect of Hepin on Total Protein Level

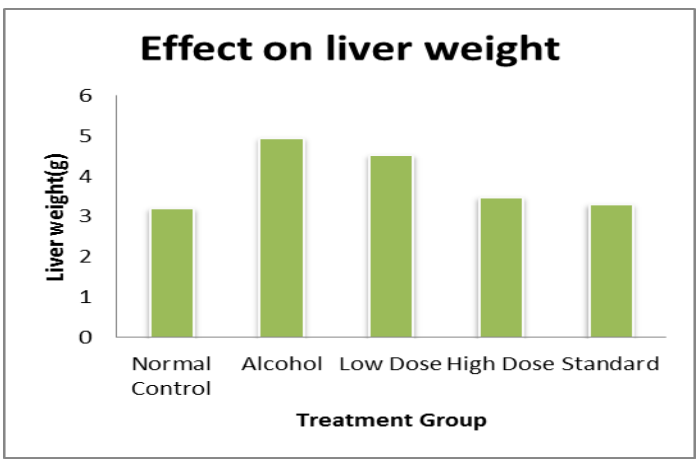
Sl.No.	Group	Treatment	Total Protein (g/dl)
1.	Normal Control	1% CMC	7.567±0.081
2.	Positive Control	40% Ethanol (2ml/100g body weight)	3.567±0.051
3.	Hepin Low Dose	40% Ethanol + Hepin (250mg/kg body weight)	4.783±0.075
4.	Hepin High Dose	40% Ethanol + Hepin (500 mg/kg body weight)	5.833±0.081
5.	Standard	40% Ethanol + Silymarin(25 mg/kg body weight)	6.700±0.089



3. Exvivo Studies

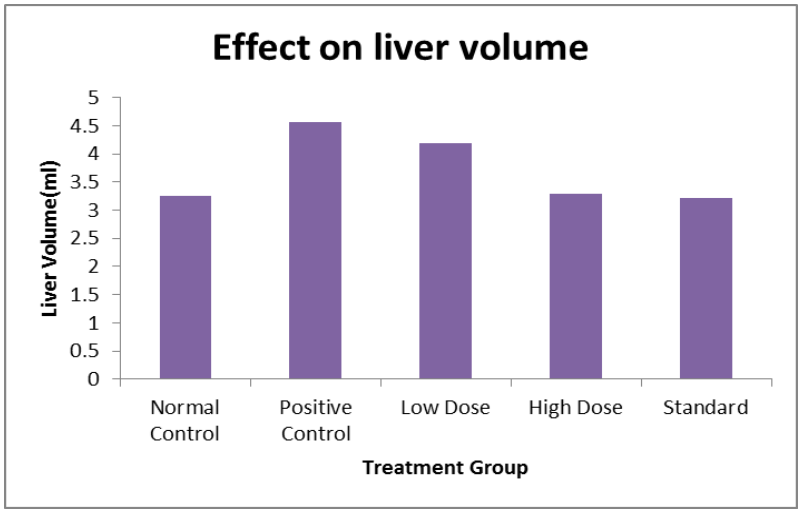
3.1 Effect on Liver Weight

Treatment Group	Liver Weight (gm)
Treatment group	Liver weight (g)
Normal Control	3.207
Positive Control	4.957
Low Dose	4.536
High Dose	3.486
Standard	3.312



3.2 Effect on Liver Volume

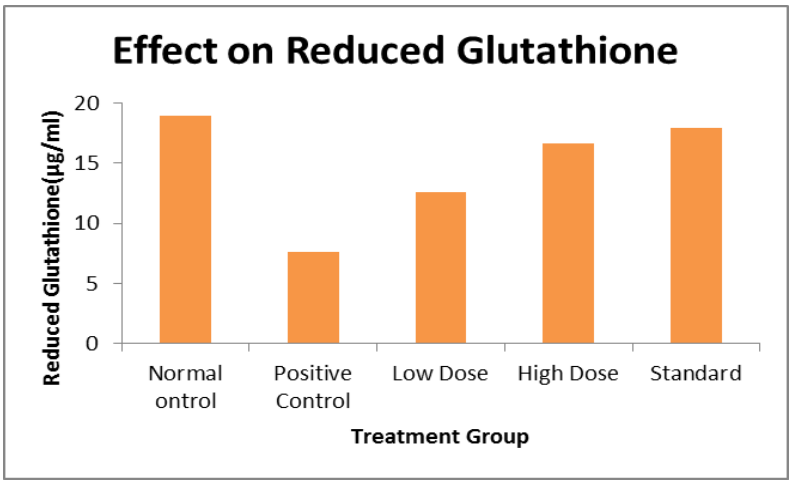
Treatment Group	Liver Volume(ml)
Normal Control	3.26
Positive Control	4.56
Low Dose	4.19
High Dose	3.29
Standard	3.21



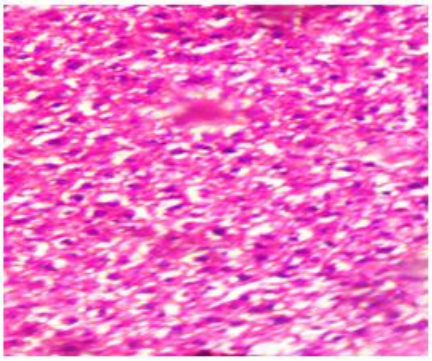
3.3 Effect on hepatic oxidative stress parameters

Determination of Reduced Glutathione

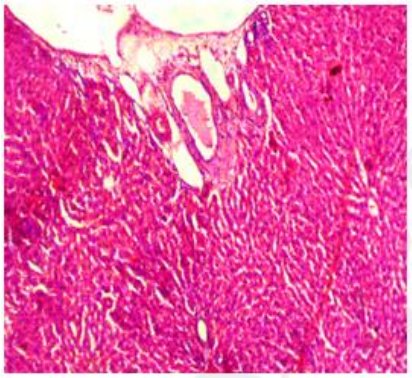
Treatment Group	GSH (µg/ml)
Normal ontrol	18.99
Positive Control	7.65
Low Dose	12.56
High Dose	16.62
Standard	17.95



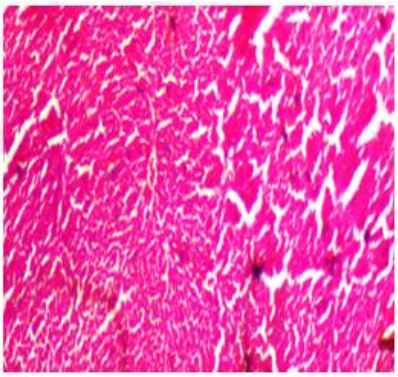
Histopathological Anlysis of Liver



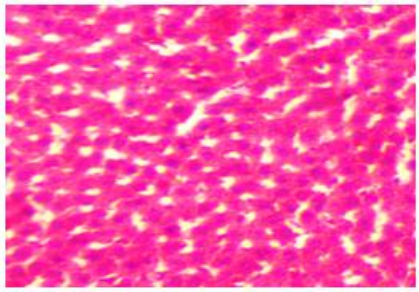
Normal Control Group



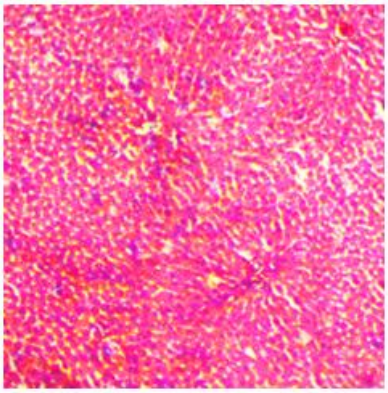
Positive Control Group



Hepin Low Dose Group



Hepin High Dose Group



Standard Group

Normal Control Group: The cytoarchitecture of liver cells were appeared intact. There are no scattered mononuclear inflammatory cells, indicating the absence of liver injury.

Positive Control Group: The architecture of liver parenchyma appeared partly effaced. There are seen degenerating hepatocytes. The infiltration of mixed inflammatory cells comprising of lymphocytes, neutrophils and histocytes were seen. The congestion and dilation of liver sinusoids indicate the presence of liver injury.

Low Dose Group: The cytoarchitecture of liver cells were normal. The infiltration of mixed inflammatory cells comprising of lymphocytes, neutrophils and histocytes were reduced. The congestion and dilation of liver sinusoids were also reduced as compared to positive control group.

High Dose Group: The cytoarchitecture of liver cells were normal. The infiltration of mixed inflammatory cells comprising of lymphocytes, neutrophils and histocytes were reduced. The congestion and dilation of liver sinusoids were also reduced as compared to positive control group.

Standard Group: The cytoarchitecture of liver cells were appeared intact. The morphology of hepatocytes was almost normal.

DISCUSSION

Alcohol-induced liver injury, one of the most common reasons of liver diseases, has been an increasingly important public healthproblem worldwide. Oxidative stress, induced the damage of tis-sue by the imbalance of prooxidant and antioxidant, is thought to play a key role in the pathogenesis of alcohol-induced liver injury. Alcohol mediates oxidative stress in

multiple ways involving the release of liver enzymes such as AST, ALT and ALP in serum, lipid peroxidation, depletion of cytoprotective antioxidant functions, and the generation of reactive oxygen species (ROS). ROS, one kind of pro-oxidants including superoxide radical, hydrogen peroxide, nitric oxide and hydroxyl radical, are generated naturally by cells in the biological systems. It is very important for keeping the biological systems from damage to rapidly clear the normally produced ROS by antioxidants. SOD and GSH-Px, can scavenge ROS and terminate the free-radical chain reaction, are critical antioxidants, and play important roles in hepatoprotection. Lack of SOD and GSH-Px is concerned with the redundantly accumulation of ROS, which could cause lipid peroxidation, tissues and organs injury, diseases and aging. MDA, the stable metabolite of lipid peroxidation products, is associated with free radical effect in biological system. GSH can protect thiol group (SH) of enzymes, adjust the synthesis of ribonucleotide and neutralize free radicals.

Obtained results revealed that administration of ethanol caused a significant increase in plasma SGPT, SGOT, ALP and Bilirubin compared to the control group. While total protein concentration is significantly declined. Additionally, a significant decrease in the level of hepatic GSH was also observed. However, the treatment of experimental rats with Hepin prevented these alterations and maintained the antioxidant status. The histopathological observations supported the biochemical evidences of hepatoprotection.

The results of the present investigation indicate that Hepin possesses hepatoprotective activity.

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