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RESEARCH ARTICLE

D-Galactosamine/Lipopolysaccharide Induced Hepatoprotective Activity of *Boswellia ovalifoliolata*

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Abstract

Present study provides focus on hepatoprotective activity of the roots in methanol and hexane extracts by D-Galactosamine/Lipopolysaccharide induced hepatotoxicity model. Physical parameters, liver functioning, antioxidant levels and histopathological study of the liver were conducted to assess the hepatoprotective action. Treatment with *Boswellia ovalifoliolata* root extracts has protected liver from induced hepatotoxicity. This was demonstrated by reducing the elevated levels of biochemical markers and additional histopathological observations have shown that there is an improvement in the structural design of liver due to induced hepatotoxicity. Present study is a contribution to the literature about hepatoprotective action of *B. ovalifoliolata* roots.

Keywords: Hepatoprotective activity, histopathology, *Boswellia ovalifoliolata*, biochemical markers.

Introduction

Medicinal plants survive even before human being made their life on the earth. Animals' survival on this earth has been made possible only because of the crucial role played by plants. Plants and animals coexist together and their addiction on each other gives importance to living life (Kokate *et al*., 1996). *Boswellia ovalifoliolata* (Burseraceae) is a narrow endemic and endangered deciduous tree species (Savithramma *et al*., 2010). Liver is a vital organ of the human body. It plays very important role in protein synthesis, nutrient storage, detoxification of toxic substances and production of bile. Any impairment in liver functioning costs high to living legendry. Yet many of health practitioners rely on natural resources to cure liver impairments. *Boswellia ovalifoliolata* roots lack literature regarding hepatoprotective activity. Present study analyses methanolic and hexane extract of *Boswellia ovalifoliolata* for the hepatoprotective action by D-Galactosamine/Lipopolysaccharide induced hepatotoxicity model.

Materials and methods

Collection and authentification of plant: The roots of *Boswellia ovalifoliolata* was collected from Ananthagiri forest region, Vishakhapatnam District, AP, India. The plant species were authenticated by Dr. Madhava Chetty, Taxonomist, Dept. of Botany, Sri Venkateswara University, Tirupati, AP, India. The voucher specimen number is 2231 for *Boswellia ovalifoliolata* Bal and Henry.

Hepatoprotective activity: The protocol is accepted by institutional ethical committee (769/2011/CPCSEA). In the dose response experiment, albino rats were randomly assigned into 9 groups of 6 individuals each. The animals were divided into 9 groups of six animals each. Group I served as vehicle control and was administered with Tween-80 in saline. Group II rats served as positive control received distilled water for 6 days. For group III, IV, V, VI, VII, VIII and IX were given silymarin 100 mg/kg, MEBO, HEBO 200, 400 and 600 mg/kg as above. On 6th day, a single intraperitoneal injections of D-GalN and LPS (300 mg/kg BW) and 30 µg/kg BW) administered 18 h before the experiment to group II to IX. The rats were anesthetized and sacrificed after the experimental period by cervical decapitation. The blood so collected was centrifuged immediately to get clear serum and was subjected to various biochemical studies (Kanchi, 2006).

Group I: Animals (-ve control) were administered Tween-80 in saline (1 mL/kg, p.o) for 6 days.
Group II: Animals (+ve control) were administered distilled water (1 mL/kg, p.o) for 6 days.
Group III: Animals were administered with Silymarin 100 mg/kg p.o. for 6 days.
Group IV: Animals were administered with MEBO 200 mg/kg p.o. for 6 days.
Group V: Animals were administered with MEBO 400 mg/kg p.o. for 6 days.
Group VI: Animals were administered with MEBO 600 mg/kg p.o. for 6 days.

Group VII: Animals were administered with HEBO 200 mg/kg p.o. for 6 days.
Group VIII: Animals were administered with HEBO 400 mg/kg p.o. for 6 days.
Group IX: Animals were administered with HEBO 600 mg/kg p.o. for 6 days.

Physical parameters
Determination of wet liver weight: Animals were sacrificed and livers were isolated and washed with saline and weights determined by using an electronic balance. The liver weights were expressed with respect to its body weight i.e. g/100 g.

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.

Estimation of biochemical markers to assess liver functions: Serum glutamate pyruvate transaminase (SGPT/ALT), Serum glutamate oxaloacetate transaminase (SGOT/AST), Serum alkaline phosphatase (ALP), Serum total bilirubin, Serum direct bilirubin, Serum cholesterol (CHL), Serum total protein (TPTN), Serum albumin (ALB) (Green *et al.,* 1982; Marcocci, 1994; Blois, 1958; Maria, 1979; George, 1959; Saunders, 1986; Goodwin, 1965; Jocobs and Van Denmark, 1960; Trinder, 1969; Allain *et al.,* 1974).

Estimation of serum SGPT (UV- Kinetic method): SGPT estimation kit was obtained from Coral clinical systems, Verna Goa, India. Pipetted out 1000 µL of working reagent with 100 µL of sample, mixed well and read the initial absorbance A_0 after 1 min and repeated the absorbance reading after every 1, 2 and 3 min. Calculated the mean absorbance change per min (A/min).

Estimation of serum SGOT (UV-Kinetic method): SGOT estimation kit was obtained from Coral clinical systems, Verna Goa, India. Pipetted out 1000 µL of working reagent with 100 µL of sample, mixed well and read the initial absorbance A_0 after 1 min and repeated the absorbance reading after every 1, 2 and 3 min. Calculated the mean absorbance change per min (A/min).

Estimation of serum Alkaline Phosphatase (ALP): ALP estimation kit was obtained from Coral clinical systems, Verna Goa, India. Pipetted out 1000 µL of working reagent with 20 µL of sample, mixed well and read the initial absorbance A_0 after 1 min and repeated the absorbance reading after every 1, 2 and 3 min. Calculated the mean absorbance change per min (A/min).

ALP activity in U/L = A/min × 2754

Estimation of serum bilirubin: The serum bilirubin estimation kit was obtained from Beacon Diagnostics Pvt. Ltd, India. For direct bilirubin, added 1 mL of direct bilirubin reagent with 0.1 mL of sample and for total bilirubin, added 1 mL of direct bilirubin reagent, 0.05 mL direct nitrate reagent and 0.2 mL sample. Mixed well, incubated for 5 min at room temperature for direct bilirubin and 10 min for total bilirubin. Read absorbance at 546 nm against reagent blank.

Total or direct bilirubin in mg/dL = Abs. T × 13

Estimation of serum total proteins: Serum total protein estimation kit was obtained from Erba Diagnostics, India. Pipetted out 1000 µL of reagent in 3 test tubes each, added 20 µL distilled water in first test tube and consider it as blank. Added 20 µL of standard in second test tube and consider it as standard. Added 20 µL of test sample in third test tube and consider it as test. Mixed well, incubated for 10 min at 37°C. Read absorbance of the standard and each test at 546 nm (520-560 nm) against reagent blank. Calculate total proteins as:

$$\text{Total Protein} = \frac{\text{Absorbance of Test}}{\text{Absorbance of Standard}} \times 6.0\ \text{g/d}$$

Estimation of serum cholesterol: Serum cholesterol estimation kit was obtained from Beacon Diagnostics Pvt. Ltd, India. Pipetted out 1000 µL of enzyme reagent in 3 test tubes each, added 10 µL blank reagents in first test tube and consider it as blank. Added 10 µL of standard in second test tube and considered it as standard. Added 10 µL of test sample in third test tube and considered it as test. Mixed well and incubated for 5 min at 37°C. Measured the absorbance of standard and test against the reagent blank at 505 nm (490-530). Calculated the cholesterol concentration as:

$$\text{Cholesterol conc. (mg/dL)} = \frac{\text{O. D. Sample}}{\text{O.D. Standard}} \times 200$$

Estimation of antioxidant levels (Trinder, 1969):
Catalase: The liver homogenates contain 5 µg total protein was mixed with 700 µL, 5 Mm hydrogen peroxide and incubated at 37°C. The disappearance of peroxide was observed at 240 nm for 15 min. One unit of catalase activity is that which reduces 1 µmol of hydrogen peroxide per min. Checked absorbance at time interval of (0, 15, 30, 45, 60, 75, 90, 105 and 120 sec).

CAT = T × dilution factor × 100 / (OD at 0 sec × mg protein/mL)
Unit: µmole of H_2O_2/sec/mg protein/mL

Superoxide dismutase: About 2.8 mL of sodium carbonate buffer (0.05 mM) and 0.1 mL of tissue homogenate or sucrose (blank) was incubated at 30°C for 45 min. Then, the absorbance was adjusted to 0 to sample. Thereafter, the reaction was initiated by adding 10 µL of adrenaline solution (9 mM). The change in absorbance was recorded at 480 nm for 8-12 min.

Table 1. Effect of MEBO and HEBO on wet liver weight and wet liver volumes in D-GalN/LPS induced hepatotoxic rats.

Groups	Wet liver weight (g/100 g)	Wet liver volume (mL/100 g)
Group I	3.145±0.108	3.197±0.084
Group II	4.338±0.201	4.447±0.174
Group III	3.173±0.148***	3.253±170***
Group IV	3.920±0.138^{ns}	4.103±0.116^{ns}
Group V	3.483±0.143**	3.650±0.160**
Group VI	3.245±0.1338***	3.340±0.121***
Group VII	4.133±0.191^{ns}	4.235±0.130^{ns}
Group VIII	3.672±0.152*	3.802±0.127 ^{ns}
Group IX	3.318±0.165***	3.398±0.149***

Values are Mean±SEM (n=6), one way ANOVA followed by Tukey-Karmer's test. Where ***P<0.001, **P<0.01, *P<0.05 and ns represents Not significant. All the values are compared to D-GalN/LPS treated group.

Throughout the assay, the temperature was maintained at 30°C. Similarly, SOD calibration curve was prepared by taking 10 U/mL as standard solution. One unit of SOD produces approximately 50% of inhibition of auto-oxidation of adrenaline. The results are expressed as unit (U) of SOD activity per mg of tissue.

SOD = (C × total volume × 1000)/(50 × sample volume × mg protein per mL)

Histopathological studies (Luna, 1986): Deparaffinized the sections by washing with chloroform for about 15 min, hydrated the sections by washing in isopropyl alcohol of decreasing strength (100, 90, 80 and 70%). Finally washed with water and stained with haematoxylin for 15 min. Rinsed in tap water, differentiated in 1% acid alcohol by 10 quick dips, checked the differentiation with a microscope. Nuclei were distinct and the background was very light (or colorless), washed in tap water and dipped in (Lithium carbonate) until sections become bright blue (3-5 dips). Washed in running tap water for 10 to 20 min, if washing is inadequate, eosin will not stain evenly. For even staining results, dipped slides several times before allowing them to set in the eosin for the desired time, dehydrated in 95% isopropyl and absolute isopropyl alcohol until excess eosin was removed, 2 changes of 2 min each (checked under microscope) followed by absolute isopropyl alcohol 2 changes of 3 min each and chloroform 2 changes of 2 min each, mounted in DPX (Desterene dibutyl phthalate xylene). Nuclei was blue colored and cytoplasm was with various shades of pink identifying different tissue components. All the sections of the tissues were examined under microscope for analyzing the altered architecture of the pancreas tissue due to D-GalN/LPS treatment and improved pancreas architecture due to pretreatment with test extracts and standard drug.

Results and discussion

D-GalN/LPS treatment in rats resulted in enlargement of liver which was evident by increase in the wet liver weight and volume. The groups were treated with silymarin and MEBO, HEBO showed significant restoration of wet liver weight and wet liver volume nearer to normal. The results are summarized in Table 1.

There was a marked increase in SGPT levels observed in D-GalN/LPS treated group. However, the SGPT levels were decreased by MEBO and HEBO dose dependently. In addition, the standard silymarin has restored the SGPT levels significantly. Serum SGOT levels have been also elevated in the D-GalN/LPS treated groups. Treatment with standard silymarin has brought back the SGOT levels to the near normal levels. However, treatment with the MEBO and HEBO has decreased the SGOT levels in a dose dependent manner, which is statistically significant. In case of total and direct bilirubin there is a noticeable rise in serum levels on D-GalN/LPS treatment observed. Treatment with MEBO and HEBO has reversed the total and direct bilirubin serum levels by dose dependent manner, which is statistically significant when compared with D-GalN/LPS treated group. The reversal treatment with standard silymarin was also significant. Rise in ALP serum levels observed in D-GalN/LPS treated group and was remarkable, decreased significantly by MEBO and HEBO by dose dependent manner and standard silymarin treatment. The results are summarized in Table 2. D-GalN/LPS treatment considerably reduced serum total protein levels. Pretreatment with silymarin and MEBO, HEBO showed a significant increase in total protein levels as compared with toxicant control group. The results are summarized in Table 3.

The lipid profile was evaluated by estimating triglycerides (TG), total cholesterol (TC), HDL-Cholesterol (HDL-C), LDL-Cholesterol (HDL-C), VLDL-Cholesterol (VLDL-C) in normal and D-GalN/LPS induced hepatotoxic rats. The D-GalN/LPS induced hepatotoxic rats showed a significant increase in the TG, TC, LDL-C and VLDL-C levels and suppression of HDL-C levels compared to control group (Table 4). But after treatment with the 200, 400, 600 mg/kg p.o dose of MEBO, HEBO and silymarin, D-GalN/LPS induced hepatotoxic rats showed decrease in the TG, TC, LDL-C and VLDL-C levels and increased HDL-C levels compared to untreated D-GalN/LPS induced hepatotoxic rats. D-GalN/LPS induced hepatotoxic rats exhibited significant lower catalase (90.97±2.178) activity as compared to those of negative control rats (26.66±1.865) treatment with the plant extract significantly elevated the reduced catalase levels.

Table 2. Effect of MEBO and HEBO on biochemical markers in D-GalN/LPS induced hepatotoxic rats.

Groups	SGPT (U/L)	SGOT (U/L)	Total bilirubin (mg/dL)	Direct bilirubin (mg/dL)	ALP (U/L)
Group I	56.63±3.127	83.52±3.109	0.910±0.035	0.347±0.032	121.4±3.532
Group II	321.4±5.698	402.3±6.233	3.712±0.229	0.906±0.058	310.5±5.560
Group III	71.86±2.178***	109.4±2.963***	1.138±0.108***	0.395±0.024***	139.7±4.075***
Group IV	205.6±5.029***	245.1±4.603***	2.102±0.150***	0.702±0.047**	181.6±4.376***
Group V	138.3±3.247***	176.5±4.038***	1.527±0.183***	0.568±0.029***	158.1±3.240***
Group VI	82.57±3.086***	119.0±3.256***	1.176±0.096***	0.415±0.073***	142.7±3.022***
Group VII	242.6±4.960***	268.3±5.137***	2.364±0.275***	0.835± 0.090^{ns}	227.3±5.034***
Group VIII	163.1±4.183***	192.8±4.248***	2.045±0.208***	0.683±0.043***	183.3±3.429***
Group IX	103.5±3.637***	136.3±3.156***	1.523±0.146***	0.502±0.028***	160.2± 3.167***

Values are Mean±SEM (n=6), one way ANOVA followed by Tukey-Karmer's test. Where ***P<0.001, **P<0.01, *P<0.05 and ns represents Not significant. All the values are compared to D-GalN/LPS treated group.

Table 3. Effect of MEBO and HEBO on serum total protein levels in D-GalN/LPS induced hepatotoxic rats.

Groups	Total protein (g/dL)
Group I	7.953±0.178
Group II	3.462±0.143
Group III	7.518±0.189***
Group IV	6.298±0.201***
Group V	7.217±0.215***
Group VI	7.497±0.273***
Group VII	5.942±0.240***
Group VIII	6.650±0.252***
Group IX	7.050±0.329***

Table 4. Effect of MEBO and HEBO on lipid profile in D-GalN/LPS induced hepatotoxic rats.

Groups	TC	TG	HDL-C	LDL-C	VLDL-C
Group I	116.4±4.236	103.4±3.126	41.23±2.785	54.49±0.825	20.68±0.625
Group II	193.2±4.986	204.2±4.536	24.56±1.124	127.8±2.954	40.84±0.907
Group III	128.6±3.056***	116.3±2.832***	40.7 4±2.237***	64.60±0.252***	23.26±0.566***
Group IV	157.2±4.135***	166.2±3.237***	33.86±1.217**	90.10±2.270**	33.24±0.647***
Group V	138.4±3.283***	148.0±3.713***	36.14±1.690***	72.70±0.850***	27.68±0.742***
Group VI	129.8±3.056***	118.7±3.075***	39.73±1.701***	66.33±0.740***	23.74±0.615***
Group VII	164.6±3.964***	158.3±4.135***	33.15±2.031**	99.79±1.106**	31.66±0.827***
Group VIII	144.3±2.982***	139.4±3.532***	36.08±1.470***	80.34±0.805***	27.88±0.706***
Group IX	126.8±4.056***	120.7±3.065***	38.73±2.651***	65.36±01.240***	23.54±0.675***

Table 5. Effect of MEBO and HEBO on catalase levels in D-GalN/LPS induced hepatotoxic rats.

Groups	Absorbance
Group I	90.97±2.178
Group II	26.66±1.865
Group III	82.16±2.853***
Group IV	56.25±5.283***
Group V	71.96±2.707***
Group VI	80.05±2.173***
Group VII	48.89±3.636***
Group VIII	59.25±3.108***
Group IX	72.67±2.869***

Table 6. Effect of MEBO and HEBO on SOD levels in D-GalN/LPS induced hepatotoxic rats.

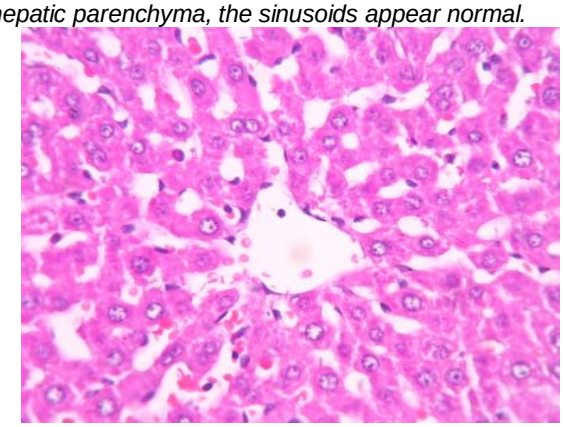
Groups	Absorbance
Group I	15.52±0.530
Group II	5.125±0.237
Group III	14.25±0.391***
Group IV	10.64±0.420***
Group V	13.19±0.380***
Group VI	14.06±0.405***
Group VII	8.570±0.450***
Group VIII	11.60±0.311***
Group IX	13.43±0.451***

The 400, 600 mg/kg p.o dose of MEBO, HEBO and silymarin showed a marked increase in the catalase levels (P<0.001) compared to the positive control. The results are summarized in Table 5. D-GalN/LPS induced hepatotoxic rats exhibited significant lower SOD (4.963±0.236) activity as compared to those of negative control rats (14.83±0.400) treatment with the plant extract significantly elevated the reduced SOD levels. MEBO, HEBO and silymarin showed a marked increase in the SOD levels (P<0.001) compared to the positive control (Table 6).

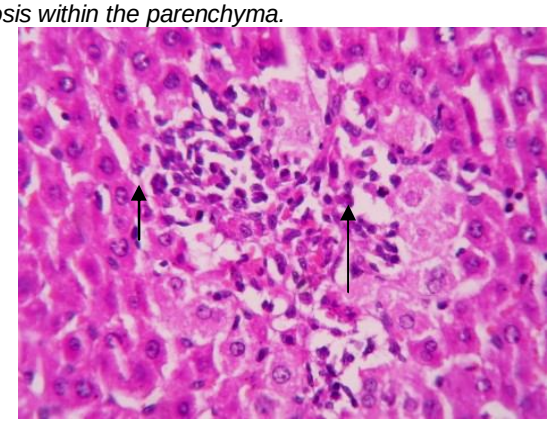
Histopathological studies: All the sections of the tissues were examined under microscope for analyzing the altered architecture of the pancreas tissue due to D-GalN/LPS treatment and improved pancreas architecture due to pretreatment with test extracts and standard drug (Fig. 1a-i).

Fig. 1a-i. Histopathological studies of the rat liver in D-GalN/LPS induced hepatotoxicity.

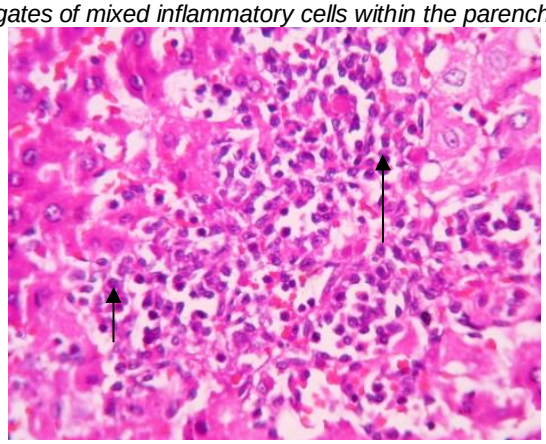
a. Group I: Section studied shows liver parenchyma with intact architecture. Most of the perivenular hepatocytes, periportal hepatocytes and midzonal hepatocytes appear normal. Within the hepatic parenchyma, the sinusoids appear normal.



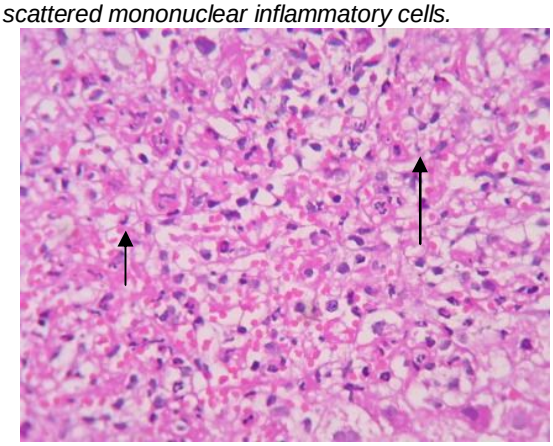
b. Group II: Section studied shows liver parenchyma with effaced architecture. Most of the sinusoids are disrupted by acute inflammatory cells. The hepatocytes show degenerative changes due to inflammatory infiltration. There are seen perivenular aggregates of acute inflammatory cells along with necrosis within the parenchyma.



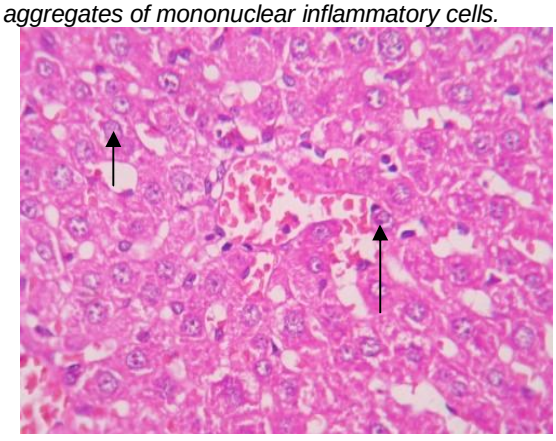
c. Group III: Section studied shows liver parenchyma with partially effaced architecture. Most of the sinusoids and hepatocytes are replaced by mixed inflammatory cells. Some of the hepatocytes show degenerative changes. Also seen are aggregates of mixed inflammatory cells within the parenchyma.



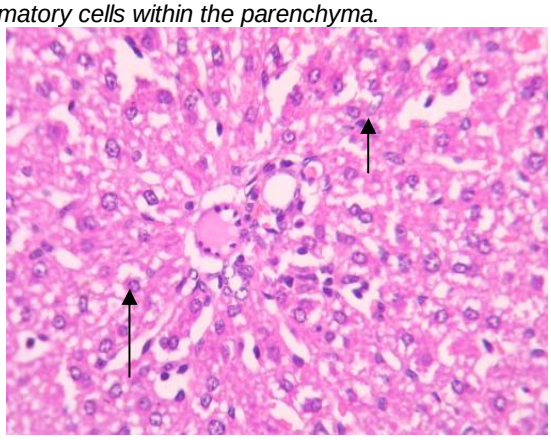
d. Group IV: Section studied shows liver parenchyma with effaced architecture. Most of the hepatocytes show microsteatosis and macrosteatosis. Also seen are scattered degenerative hepatocytes. Intervening the hepatocytes are seen scattered mononuclear inflammatory cells.



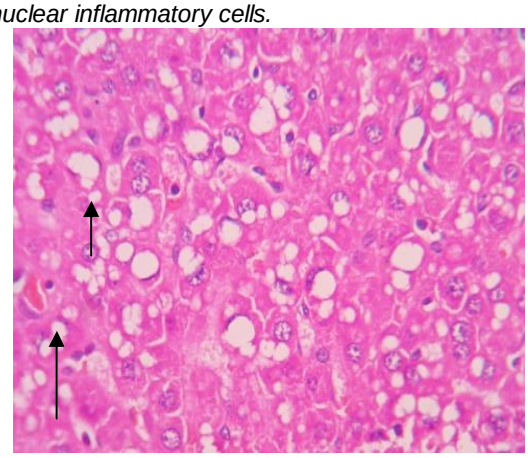
e. Group V: Section studied shows liver parenchyma with partially effaced architecture. Most of the hepatocytes show microsteatosis. Also seen are scattered degenerative and regenerative hepatocytes. Intervening the hepatocytes are seen aggregates of mononuclear inflammatory cells.



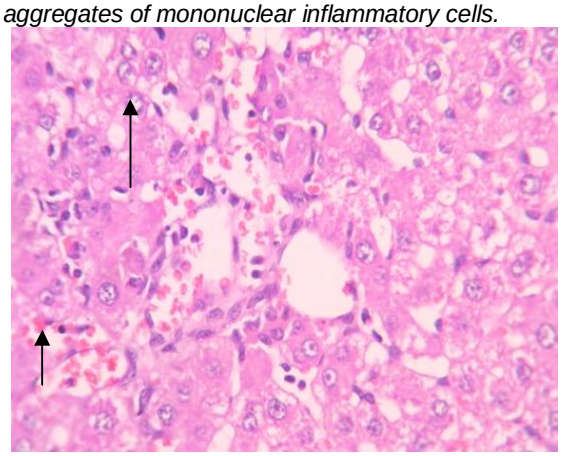
f. Group VI: Section studied shows liver parenchyma with intact architecture. Few of the sinusoids and central veins appear congested. Also seen are scattered regenerative hepatocytes. Intervening the hepatocytes are seen scattered mononuclear inflammatory cells within the parenchyma.



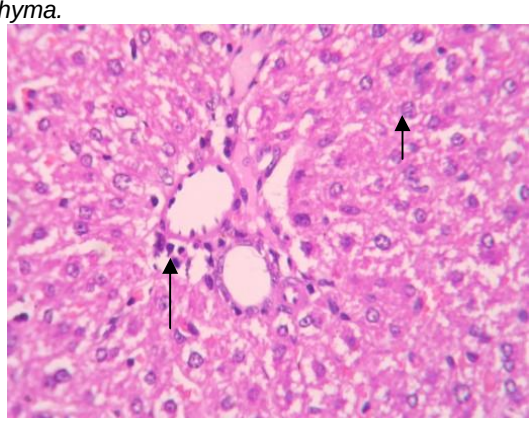
g. Group VII: Section studied shows liver parenchyma with effaced architecture. Most of the hepatocytes show microsteatosis. Also seen are scattered degenerative hepatocytes. Intervening the hepatocytes are seen scattered mononuclear inflammatory cells.



h. Group VIII: Section studied shows liver parenchyma with partially effaced architecture. Most of the hepatocytes show microsteatosis. Also seen are scattered degenerative and regenerative hepatocytes. Intervening the hepatocytes are seen aggregates of mononuclear inflammatory cells.



i. Group IX: Section studied shows liver parenchyma with intact architecture. Few of the sinusoids and central veins show congestio. Also seen are regenerative hepatocytes. Intervening the hepatocytes and periportal region, are seen focal aggregates of mononuclear inflammatory cells within the parenchyma.



Conclusion

Treatment with MEBO, HEBO has protected liver from induced hepatotoxicity. This was demonstrated by reducing the elevated levels of biochemical markers like SGPT, SGOT, ALP, total protein, total and direct bilirubin, triglycerides, total cholesterol, HDL-Cholesterol, LDL-Cholesterol and VLDL-Cholesterol. In addition, histopathological observations have shown that there is an improvement in the structural design of liver due to induced hepatotoxicity model. Thus, study can be further elaborated by one more hepatoprotective model before clinical trials in human.

Abbreviations

Methanolic extract of *Boswellia ovalifoliolata* (MEBO), D-Galactosamine/Lipopolysaccharide (D-GalN/LPS), n-Hexane extract of *Boswellia ovalifoliolata* (HEBO), Serum glutamate pyruvate transaminase (SGPT/ALT), Serum glutamate oxaloacetate transaminase (SGOT/AST), Serum alkaline phosphatase (ALP), Serum cholesterol (CHL), Serum total protein (TPTN), Serum albumin (ALB), Triglycerides (TG), Total cholesterol (TC), High density lipoprotein-Cholesterol (HDL-C), Low density lipoprotein-Cholesterol (LDL-C), Very low density lipoprotein-Cholesterol (VLDL-C).

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