IOSR Journal of Pharmacy and Biological Sciences (IOSR-JPBS)
e-ISSN: 2278-3008, p-ISSN:2319-7676. Volume 10, Issue 5 Ver. II (Sep - Oct. 2015), PP 15-22
www.iosrjournals.org

Sclerocarya birrea stem bark extract modulates tumour necrosis factor-alpha (TNF-α) and antioxidants levels in ethanol-induced hepatotoxicity in Wistar rats

Balogun, S.U.[1, 4*], Balogun, J.B.[2], Attah, M.O.[3], Akpulu, P.[4], Ella, E.E.[5], Kwanashie, H.O.[6], Alawa, J.N.[4]

[1](Department of Human Anatomy, College of Health Sciences, Kogi State University, P.M.B 1008, Anyigba, Kogi State, Nigeria)
[2](Department of Biological Sciences, Federal University, Dutse, P.M.B 7156, Dutse, Jigawa State Nigeria)
[3](Department of Human Anatomy, College of Medical Sciences, University of Maiduguri, P.M.B. 1069, Maiduguri, Borno State, Nigeria)
[4](Department of Human Anatomy, Ahmadu Bello University, Zaria, Kaduna State, Nigeria)
[5](Department of Microbiology, Ahmadu Bello University, Zaria, Kaduna State Nigeria)
[6](Department of Pharmacology and Clinical Therapeutics, Ahmadu Bello University, Zaria, Kaduna State, Nigeria)

***Abstract**: The study was aimed at evaluating the effect of S. birrea stem bark extract on antioxidants and TNF-α level in ethanol-induced hepatotoxicity in Wistar rats. Five groups of male Wistar albino rats were studied as A, B, C, D and E respectively. Group A and C recieved 30 mg/kg distilled water and extract respectively for four weeks, group B recieved 20 ml of 40 % ethanol (vol/kg body weight) for four weeks, group D and E recieved 20 ml of 40 % ethanol (v/v) for three weeks followed by 15 mg/kg and 30 mg/kg stem-bark extract respectively for one week. At the end of the experiment, blood was collected via cardiac puncture for serum analysis of liver enzymes and antioxidants while liver tissues were harvested for determination of TNF-α levels, genomic DNA and histopathological analysis. Results showed that S. birrea extract decreased levels of liver enzymes AST, ALT and TNF-α while increasing levels of SOD and GSH though not to significant levels. It also improved DNA yields and purity while reversing cytoarchitectural change in the liver tissue. Hence, the study demonstrated that S. birrea modulates TNF-α by decreasing its levels in the liver tissue while increasing levels of antioxidants.*

***Key words:** hepatotoxicity, tumour necrosis factor, antioxidants, cytotoxicity*

I. Introduction

TNF-α is a proinflammatory cytokine produced by macrophages and acts by inducing immune and inflammatory response [1]. Its biological actions being attached to the inflammatory intracellular signaling pathway and the apoptotic pathway via the death domain of tumor necrosis factor- receptor 1 (TNF-R1 i.e. p55 TNFR) [2]. Various pathways have been reported to play a role in ethanol-induced tissue injury such as changes in cellular nicotinamide adenine dinucleotide, induction of CYP2E1, formation of 1-hydroxyethyl radicals, ethanol-mediated mitochondrial damage, endotoxin-derived activation of kupffer cells and the subsequent production of tumor necrosis factor-α [3]. Under normal circumstances, hepatocytes are resistant to TNF-α induced killing. However, previous studies have shown that primary hepatocytes from rats chronically fed alcohol have increased TNF-α cytotoxicity [4].

Sclerocarya birrea (A. Rich) Hochst., subspecies Caffra (family:Anacardiaceae) is an important ethnomedicinal plant in Africa [5]. The tree is commonly found in semi-arid deciduous and savanna regions of sub-Saharan Africa [6]. It grows in wooded grasslands, riverine woodland areas and bushlands and is frequently associated with rocky hills. Its geographical distribution stretches from Gambia in West Africa across Nigeria and Cameroon in Central Africa, to Ethiopia and Sudan in East Africa [5]. Phytochemical screening of *S. birrea* stem-bark has been reported to contain gallotannins, flavonoids, alkaloids, steriods (i.e. β-sitosterol), coumarins, tritepenoids, sesquiterpene, hydrocarbons, ascorbic acid, oleic acid, myristic, stearic and amino acids with a predominance of glutamic acid and arginine [5]. The stem-bark, roots and leaves have been reported to possess medicinal and other properties in addition to the nutritional values of the fruit and seeds of the plant [7]. *Sclerocarya birrea* is widely used in traditional medicine in Africa against hypertension, stomach or gastro-enteritis, cough and as an antihyperglycemic agent [8]. Although the crude extract of *S. birrea* stem bark has been used traditionally in the management of a variety of ailments including liver disease [9, 10], a clear understanding of its mode of action in ethanol-induced hepatotoxicity is not fully known. The study was

therefore aimed at evaluating the effect of *S. birrea* stem bark extract on TNF-α levels following ethanol-induced hepatotoxicity in Wistar rats.

II. Materials And Method

2.1 Experimental animals

Forty-eight young male adult Wistar rats (approx weight of 120 g per group) were obtained from the Department of Physiology, Ahmadu Bello University, Zaria, Kaduna State, Nigeria. They were kept in plastic cages and maintained under laboratory conditions of temperature, hummidity and light with free access to food (standard pellet diet, Grand Cereal Ltd, Jos Pleateau State) and water. The experimental animals were acclimatized for two weeks after which they were divided into five groups of eight animals each. The experimental animals were kept and handled in accordance with the guidelines for experimental animal care and handling of the Department of Human Anatomy, Ahmadu Bello University, Zaria, Nigeria.

2.2 Plant material

Pieces of fresh *S. birrea* stem bark were harvested from the Federal Polytechnic Staff Quarters, Bauchi, Bauchi State, Nigeria. This was identified and authenticated at the Department of Biological Sciences, Ahmadu Bello University, Zaria Herbarium by U.S. Gallah with a Voucher specimen number 1071.

2.3 Chemicals

Abcam TNF-alpha rat ELISA kit, Abcam, USA; ZR Genomic DNA-Tissue MINI prep (ZYMO research, USA).

2.4 Preparation of extract

The fresh stem-bark of *S. birrea* was air dried, minced and powdered using laboratory mortar. 50 g of stem back powder was extracted in 1.5 L of distilled water using a Soxhlet extractor. This was filtered using a Whatman's filter paper (24 cm). The filtrate was dried in a laboratory drier at 35^{o}C and a total yield of 8.1 g was obtained. For each series of experiment, 10 mg of the extract was weighed and dissolved in 1 ml of distilled water to obtain the stock solution.

2.5 Experimental design

Twenty five adult Wistar rats were used for the study. The experimental animals were blocked by weight into 5 groups of 5 animals each i.e A (water control), B (ethanol control group), C (extract control group), D and E (low and high dose therapeutic treatment groups respectively). The experiment lasted a period of 4 weeks during which group A and C recieved 30 mg/kg distilled water and extract respectively for four weeks, group B recieved 20 ml of 40 % ethanol (vol/kg body weight) for four weeks, group D and E recieved 20 mL of 40 % ethanol (v/v) for three weeks followed by 15 mg/kg and 30 mg/kg stem-bark extract respectively for one week. At the end of the experiment, blood was collected via cardiac puncture for packed cell volume (PCV) analysis, serum analysis of liver enzymes and antioxidants while liver tissue were harvested for DNA analysis, enzyme linked immunosorbent assay of TNF-α levels and histopathological assessment of liver damage using haematoxylin and eosin stain. The drugs were administered orally via gavage based on established methods i.e intragastric enteral protocol [11].

2.6 Biochemical studies

Blood was collected at sacrifice via cardiac puncture into plain sterilized centrifuge tubes and allowed to clot. The clotted blood samples were centrifuged and the serum for liver enzymes alkaline aminotransaminase (ALT), aspartate aminotransaminase (AST), alkaline phosphatase (ALP) from which the ALT: AST ratio was determined. This was done using AC-114 digital colorimeter and Agapae diagnostic reagents according to the manufacturer's instructions.

2.7 Detection of antioxidants in the serum

***2.7.1 Reduced glutathione (GSH)*:** Reduced glutathione levels were determined using Northwest Life Science Specialities (LLC) GSH01 Spectrophotometer (cuvette) Protocol. 40 µL of calibrator/sample was added into test tubes respectively. To these were added 400 µL of assay buffer, 50 µL of DTNB and 50 µL of glutathione reductase enzyme. This was incubated for 2-3 mins after which 50 µL of NADPH was added and vortexed briefly. The absorbance recorded at 412 nm for 3 mins at <2.25 mins intervals. The calibration curve was plotted and the GSH concentrations obtained from the curve.

***2.7.2 Superoxide dismutase (SOD)*:** SOD levels were determined using Northwest Life Science Specialities (LLC) superoxide dismutase activity assay kit. 929 µL of assay buffer was added to each cuvette for the assay. To this was added 40 µL of assay buffer and 40 µL of samples respectively and incubated for 2 minutes. After

this, 40 µL of haematoxylin reagent was added to initiate the auto-oxidation reaction. This was mixed quickly and the absorbance recorded immediately at 560 nm every ten seconds for 5 mins.

2.8 Detection of Tumor necrosis factor – alpha (TNF-α)

The levels of tumor necrosis factor – alpha (TNF-α) in liver tissue homogenate was determined using Abcam TNF alpha rat Enzyme Linked Immunosorbent Assay kit (USA). Fifty milligrammes of liver tissue was homogenized to release the cell content. This was then collected in micropipette tubes. 20 µL of tissue lysate and serum were diluted with 80 µL 1x sample diluent buffer in a tissue culture multi well plate with cover (Linbro Scientific, Inc.) All reagents and samples were brought to room temperature. 100 µL of each standard and sample were added into appropriate wells in the TNF-alpha micro plate coated with anti-Rat TNF-alpha. The wells were covered with clean film and incubated for 2.5 hours at room temperature with gentle shaking. The solution was discarded and washed four times. After the last wash, the micro plates were inverted and blotted with clean paper towels to remove every trace of the wash buffer. 100 µL of 1x prepared biotinylated antibody was added to each well and incubated for one hour at room temperature with gentle shaking. The solution was discarded and washed as above. 100 µL of prepared streptavidin solution and incubated for 45 minutes at room temperature with gentle shaking. The solution was discarded and plate washed again as above. 100 µL of 3,3',5,5'-tetramethylbenzidine (TMB) substrate reagent to each well. This was incubated for 30 minutes at room temperature in the dark with gentle shaking. After this, 50 µL of Stop Solution was added to each well and the absorbance read at 450 nm wavelength immediately using GF-M3000 micro plate reader.

2.9 DNA extraction and quantification

DNA was isolated from the hepatic tissue using ZR Genomic DNA^{TM} – Tissue Miniprep kit. 20 mg of fresh liver tissue was placed in a microcentrifuge tube and a solution comprising water (95 µL), 2X digestion buffer (95 µL) and Proteinase K (10 µL) was added. This was mixed and incubated at 55^0 for 2 hours, 45 minutes. 700 µL of genomic lysis buffer was added to the tube, vortexed and centrifuged at 10,000 x g for one minute to remove insoluble debris. The supernatant was then transferred to a Zymo - Spin^{TM} IIC column in a collection tube and centrifuged at 10,000 x g for one minute. 200 µl DNA pre - wash buffer was added to the spin column in a new collection tube and centrifuged at 10,000 x g for one minute. To this, 400 µl genomic-DNA wash buffer was added and centrifuged at 10,000 x g for one minute. The spin colunm was then transferred into a clean micro-centrifuge tube and 50 µL DNA elution buffer was added to the spin column, incubated for 2-5 minutes at room temperature and then centrifuged at top speed for 30 seconds to elute the DNA. Genomic DNA was quantified using an eppendorf biophotometer and the absorbance were measured at 260 nm (for DNA) and 280 nm (for protein) and the 260/280 ratio obtained.

2.10 Histopathological analysis

Liver tissue was fixed in 10 % buffered formalin and subsequently embedded in paraffin, sectioned and stained with haematoxylin and eosin for general hepatic architecture using routine histological techniques. The sections were viewed under the light microscope and photomicrographs were obtained using a microscope eyepiece attached to a computer monitor.

2.11Statistical analysis

The results obtained were analyzed using Sigmaplot statistical package (SPW 11.0) and the results were expressed as the mean value ± standard error of mean. The differences among the mean values were determined using one way analysis of variance (ANOVA) and a value of $P<0.05$ was considered as statistically significant. Dunnet's post hoc test was used to determine where the level of significance lay.

III. Results

3.1Liver enzymes

Results expressed in Table 1 showed that the ethanol control group expressed high levels of AST and ALT. *S. birrea* extract caused a decrease in the levels of AST and ALT in the serum of the extract-treated groups though not to significant levels. Besides, the ethanol control group had insignificantly higher AST/ALT ratio.

3.2Antioxidants

Results shown in Table 2 indicates that administration of *S. birrea* extract following ethanol hepatotoxicity caused increase in the levels of SOD and GSH as compared to low levels observed in the ethanol control group though not to significant levels. A decrease in SOD and GSH levels was also observed in the ethanol control group compared to the water control group. High levels of SOD and GSH were observed in the

extract control group as well as the other treatment groups. Further more, the rising levels of SOD and GSH in the treatment groups was also found to be in a dose-dependent manner being highest at 30 mg/kg.

3.3 TNF-α

Levels of TNF- α were found to be significantly high in the ethanol control group and the extract control group as compared to the water control group ($P<0.05$) while a decrease in TNF-α concentrations in the liver tissue lysate was observed following treatment with *S. birrea* extract in a dose-dependent manner as compared to the ethanol and extract control groups respectively as seen in Fig. 1.

Table 1: Biochemical analysis of liver enzymes

Group	Weeks				AST (U/L)	ALT (U/L)	ALP (U/L)	AST/ALT ratio
	1	2	3	4				
A	W	W	W	W	16.2 ± 2.20	26.6 ± 0.87	73.4 ± 6.88	0.60 ± 0.07
B	E	E	E	E	22.5 ± 1.71	33.3 ± 5.36	65.0 ± 6.89	0.72 ± 0.11
C	W	W	W	*Sb* 30	20.4 ± 1.17	31.8 ± 2.90	71.7 ± 3.90	0.58 ± 0.06
D	E	E	E	*Sb* 15	19.6 ± 1.17	31.4 ± 2.09	70.6 ± 0.93	0.63 ± 0.03
E	E	E	E	*Sb* 30	18.4 ± 1.75	29.6 ± 1.17	68.6 ± 1.36	0.63 ± 0.70

W = Distilled water (30 ml/kg), E = Ethanol (20 ml/kg of 40% ethanol), *Sb* 15 = *S. birrea* (15 mg/kg), *Sb* 30 = *S. birrea* (30 mg/kg)

Table 2: Serum levels of superoxide dismutase (SOD) and reduced glutathione (GSH)

Group	Weeks				SOD (U/ml)	GSH (nmol/mg)
	1	2	3	4		
A	W	W	W	W	1.70 ± 0.06	66.8 ± 5.98
B	E	E	E	E	1.62 ± 0.19	60.0 ± 11.11
C	W	W	W	*Sb* 30	1.84 ± 0.17	68.0 ± 2.26
D	E	E	E	*Sb* 15	1.74 ± 0.14	71.4 ± 1.57
E	E	E	E	*Sb* 30	1.82 ± 0.14	81.8 ± 4.29

W = Distilled water (30 ml/kg), E = Ethanol (20 ml/kg of 40% ethanol)
Sb 15 = *S. birrea* (15 mg/kg), *Sb* 30 = *S. birrea* (30 mg/kg)

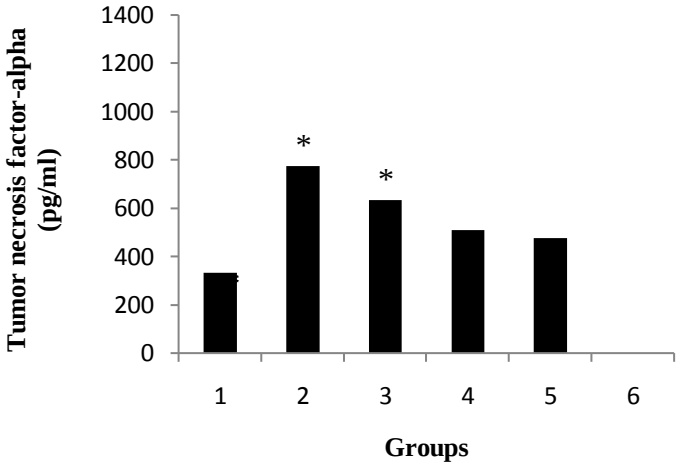

Fig. 1: Concentration of TNF-α in the liver tissue lysate of the different experimental groups.
Key: 1 = Water control group, 2 = Ethanol control group, 3 = Extract control group (*S. birrea* 30 mg/kg), 4 = Treatment group I (*S. birrea* 15 mg/kg), 5 = Treatment group II (*S. birrea* 15 mg/kg). *= $P<0.05$ significant (1,2; 1,3)

DNA analysis

Results in Table 3 showed low DNA yields in the ethanol control group and this was reversed following treatment with *S. birrea*, with the low therapeutic dose showing a better presentation than the high dose group. DNA yield was not affected by treatment with the plant extract alone. High levels of protein contamination was observed in the DNA of the ethanol control group as compared to the water control group

thus, indicating low DNA purity as revealed by the 260 nm/280 nm ratio. However, treatment with *S. birrea* improved DNA purity when used therapeutically being more effective at the high dose.

Table 3: mean genomic DNA yields (%) in hepatocytes

Group	Weeks				Weight of liver tissue (mg)	DNA yield (µg/ml)	DNA yield (%)
	1	2	3	4			
A	W	W	W	W	26.4 ± 0.46	48.6 ± 11.21	8.95
B	E	E	E	E	22.3 ± 1.88	33.2 ± 1.67	4.94
C	W	W	W	*Sb* 30	28.4 ± 1.17	45.5 ± 12.52	7.29
D	E	E	E	*Sb* 15	30.1 ± 1.43	47.4 ± 8.87	7.46
E	E	E	E	*Sb* 30	27.5 ± 1.56	38.5 ± 6.32	5.40

W = Distilled water (30 ml/kg), E = Ethanol (20 ml/kg of 40% ethanol), *Sb* 15 = *S. birrea* (15 mg/kg), *Sb* 30 = *S. birrea* (30 mg/kg)

Table 4: DNA absorbance at 260 nm, 280 nm and the 260/280 ratio.

Group	Weeks				DNA A260 (nm)	DNA A280 (nm)	A260/A280 ratio
	1	2	3	4			
A	W	W	W	W	1.04 ± 0.19	1.00 ± 0.15	1.44 ± 0.09
B	E	E	E	E	0.66 ± 0.03	0.47 ± 0.03	1.35 ± 0.09
C	W	W	W	*Sb* 30	0.93 ± 0.21	0.70 ± 0.15	1.67 ± 0.15
D	E	E	E	*Sb* 15	0.80 ± 0.13	0.72 ± 0.10	1.39 ± 0.09
E	E	E	E	*Sb* 30	0.99 ± 0.13	0.81 ± 0.13	1.51 ± 0.09

W = Distilled water (30 ml/kg), E = Ethanol (20 ml/kg of 40% ethanol), *Sb* 15 = *S. birrea* (15 mg/kg), *Sb* 30 = *S. birrea* (30 mg/kg)

3.4 Histopathology

Liver sections of water control group (Plate I) showed a normal hepatic architecture with normal hepatic cells having preserved cytoplasm, prominent nucleus and nucleolus, and well brought out central vein while liver sections of the ethanol control group (Plate II) showed areas of fatty degeneration and loss of cellular boundaries. Liver sections of rats from the extract control group (Plate III) showed liver parenchyma comparable to that of the water control group with normal hepatic cells having preserved cytoplasm, prominent nucleus and nucleolus, and well brought out central vein. The effect of ethanol on the hepatic architecture was seen to be reversed following *S. birrea* extract administration in a dose dependent manner, being more effective at 30 mg/kg (Plate IV and V).

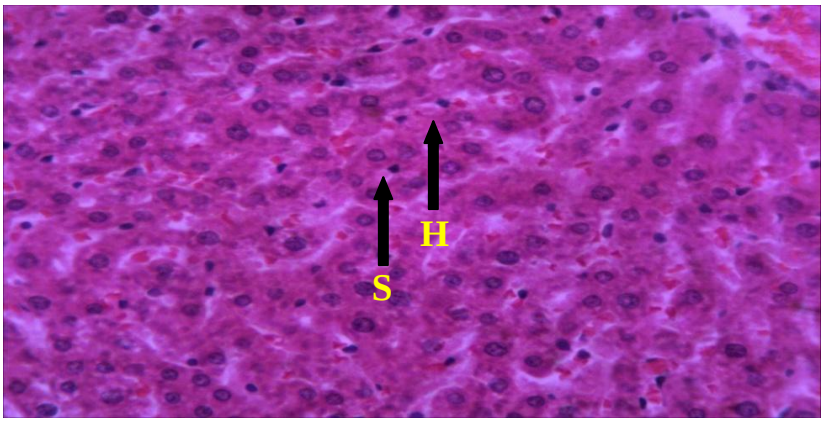

Plate I (Distilled water control)

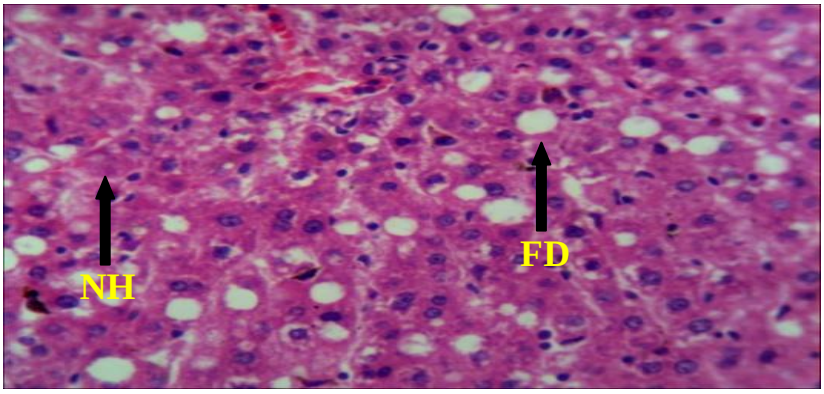

Plate II (Ethanol control)

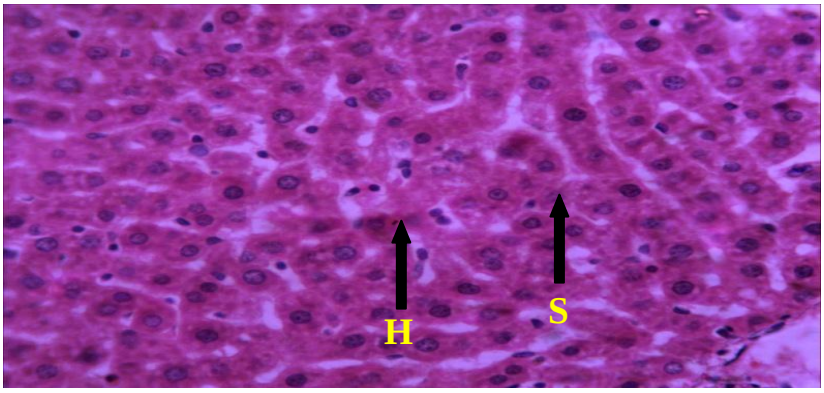

Plate III (Extract control)

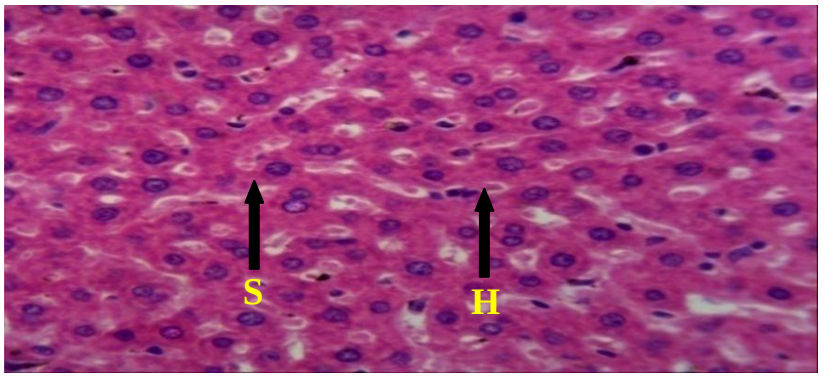

Plate IV (ethanol + 15 mg/kg extract)

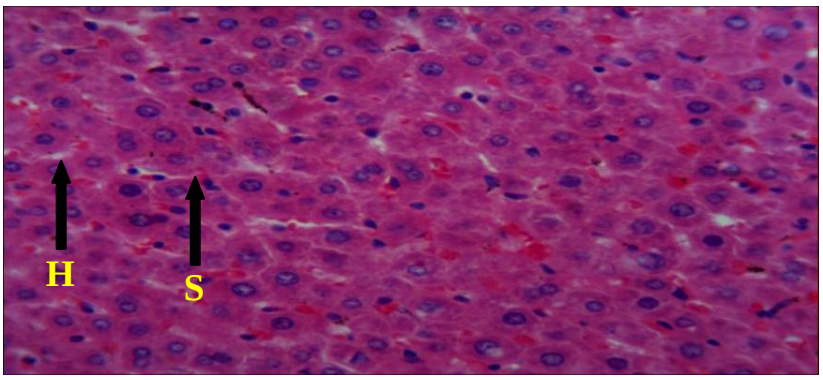

Plate V (ethanol + 30 mg/kg extract)

H=Normal hepatocytes, S=Liver sinusoids, FD=Fatty degeneration, NH=Necrotic hepatocytes. Haematoxylin and Eosin Stain, X400

IV. Discussion

Liver enzymes Alanine aminotransferase (ALT) and aspartate aminotransferase (AST) are present in high concentrations in the hepatocytes and these enzymes leak into circulation when hepatocytes or their cell memebrane are damaged [2, 12]. ALT is more specific for liver damage as compared to AST [13] and elevations in ALT activity persist longer than those of AST activity [14]. The elevated levels of these enzymes may be attributed to the presence of injury to the hepatocytes resulting from ethanol consumption [2, 12]. The levels were higher in the ethanol control group as compared to the other experimental groups. The fall in the levels of these enzymes following *S. birrea* administration suggests that *S. birrea* stem-bark extract possesses therapeutic properties against ethanol induced hepatotoxicity and this may be due to the presence of flavonoids which are antioxidants in the plant.

Superoxide dismutase (SOD) belongs to a family of antioxidant enzymes that catalize the dismutation of superoxide to yield hydrogen peroxide and oxygen [15]. SOD is considered as the first line of defence against the deleterious effects of oxygen radicals in the cells and it scavenges ROS by catalizing the dismutation of superoxide to hydrogen peroxide [16]. Results obtained from this study showing a decrease in the activity of SOD in the ethanol control group as compared to the water control group while high levels of SOD were observed in the extract control group and other treatment groups suggests that *S. birrea* has the ability to stimulate SOD production hence, possessing an antioxidant property. Furthermore, the increased levels of SOD in the treatment groups was dose-dependent being highest at 30 mg/kg demonstrating that *S. birrea* has good anti-oxidant properties which may be the source of its hepatoprotective mechanism. GSH is an antioxidant and a powerful nucleophile critical for cellular protection such as detoxification of reactive oxygen species (ROS), conjugation and excretion of toxic molecules and control of the inflammatory cascade [17]. Depletion of GSH in tissues results in impairment of cellular defence against ROS leading to peroxidative injury [3]. GSH is a critical cellular antioxidant which is important in limiting the toxicity of ethanol as well as many other toxic chemicals. Ethanol is known to deplete GSH levels via generation of oxidants as well as by inhibiting the mitochondrial glutathione transporter [18, 2]. GSH in the mitochondria is the major defence available to metabolize hydrogen peroxide. Results from this present study showing a decrease in GSH levels in the ethanol control group as compared to the other treatment groups indicate that *S. birrea* extract had a dose dependent modulatory effect on GSH levels.

The production of TNF-α has been reported to be one of the earliest events in many types of liver injury. This triggers the production of other cytokines that recruit inflammatory cells, kill hepatocytes and initiate a healing response such as fibrogenesis [2]. Chronic ethanol consumption leads to cell injury in virtually every tissue and TNF- α constitute a major factor in the development of alcohol-induced liver injury [19]. Results obtained from the study showing significantly high levels of TNF- α in the tissue of the ethanol control group as compared with those in the other treatment groups indicating the presence of liver injury as reported by Das and Vasudevan [2] who suggested that higher levels of TNF- α in the serum were associated with liver injury. Results from the study also showed significantly high levels of TNF- α in the extract control group as compared to the water control group though not as high as the ethanol control group showing that the extract had the ability to induce production of TNF- α in cells, suggesting a possible anti-tumoural effect for malignant cells. Though TNF- α level in the extract control group was high, the extract still possessed the ability to lower its levels in the other treatment groups and this reduction was found to be dose dependent being lower at 30 mg/kg as compared to that at 15 mg/kg.

The low mean DNA yields in the ethanol control group suggests deleterious effect of ethanol on the hepatic DNA while higher and dose dependent yields in the treatment control group as well as the other treatment groups respectively also indicate that *S. birrea* may possess DNA preservative and restorative properties. The DNA repair/restoration could be due to the cumulative effect of *S. birrea* throughout the study indicative that *S. birrea* had a hepatoprotective effect against ethanol-induced hepatotoxicity. *S. birrea* also restored GSH and SOD levels in the extract treated groups and this could be attributed to the presence of antioxidants such as flavonoids, polyphenols etc which could participate in the protection against free radical production [20]. Furthermore, studies have shown that TNF alpha may be involved in the regulation of certain aspects of liver homeostasis [21]. In studies carried out on mice devoid of TNF-alpha receptor type 1, reduced liver regeneration was observed while antibodies to TNF alpha inhibited liver regeneration following hepatectomy [22, 23]. This result provided a basis for the results obtained from the DNA absorbance which showed lower absorbance in the ethanol control group as compared to the other experimental groups.

The findings obtained in the histopathological studies showed that *S. birrea* when given therapeutically to rats attenuated the histopathological changes associated with ethanol toxicity by reducing fatty degeneration, necrosis and vacuolation of the hepatocytes and restoring normal architecture of the liver. Furthermore, the extract control group showed liver parenchyma comparable to those of the water control group though TNF-α level in the extract control group was significantly higher. This could be due to the homeostatic regulatory role TNF-α plays in the liver [21]. These findings confirmed data obtained in the biochemical, immunological and

molecular studies that ethanol-induced liver damage is ameliorated to some extent by *S. birrea* stem bark extract.

V. Conclusion

Ethanol-induced hepatotoxicity characterized by high levels of TNF alpha was attenuated following administration of *S. birrea* stem bark extract in the treatment groups indicating a possible mode of action of the extract in its hepatoprotective action on the liver. *S. birrea* also modulated levels of antioxidants (SOD and GSH) thus, reducing oxidative stress, apoptosis and DNA damage observed in ethanol-induced hepatotoxicity. Further study is recommended using higher doses of the extract in the treatment groups.

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