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Effects of Aqueous Extract of Neem Leaves (*Azadirachta indica*) on Light Microscopic Features of Hepatic Lobule of Adult Albino Rats

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ABSTRACT

Introduction: Azadirachta indica or Neem is a vital restorative plant which is generally utilized throughout the world as a house hold cure in hypertensive and diabetic patients and furthermore for the cure of different skin illnesses. **Aims and Objective:** To evaluate the histological effects of aqueous extract of neem leaves on the, hepatic lobules, hepatocytes and centrilobular veins of adult albino rats. **Place and Duration of Study:** The study was conducted in PGMI, Shaikh Zayed Hospital Lahore for 21 days**. Material and methods:** 45 Albino rats of both genders were used and they were randomly divided into three groups of 15 animals each, group A (Control), group B (low dosage) and group C (high dosage). The rats of group A received distilled water, while group B and C received 40 mg/kg and 100mg/kg of aqueous extract of neem for 20 days through the orogastric tube. At the end of complete dosing schedule, rats were sacrificed and the livers were dissected out for histological examination. **Results:** The livers of both groups showed deranged hepatocellular architecture with shrinkage, atrophy, hemorrhage and coagulative necrosis of hepatocyte along with dilatation and congestion of centrilobular veins. All these changes were focal affecting one lobule more than the other. These alterations were more marked in high dosage group than the low dosage p- value< 0.001. **Conclusion:** The present research work demonstrates that the higher doses (100mg/kg) of neem extract induce remarkable histological effects on liver.

Key words: Azadirachta indica, Albino rat, hepatocellular architecture, congestion, centrilobular veins

INTRODUCTION

Azadirachta indica A. Juss (Syn: Melia Azadirachta) commonly known as neem is a standout amongst the most valuable therapeutic plants harvested throughout the sub-landmass. It is local to tropical and sub tropical parts of India, Burma and Sri Lanka on deep sandy soils, however it shows an abundant distribution in Asia, Africa, Australia and Pacific Islands.[1,2]

Neem has been used for curing a wide variety of ailments in customary medicinal arrangement of India (Ayurveda, Tibetan and Unani) since old times. Its ongoing practice as a solution goes back to more than 4500 BC.[3, 4] Moreover, all constituents of the tree (root, gum, bark, leaves, blooms and seeds) are used for different therapeutic purposes, however the leaves have been widely utilized for restorative and corrective purposes.[5,6,7] It contains around 140 bioactive ingredients including proteins. It is bitter in taste because due to complex mixes called "Triterpenes" and its tree contains around 40 distinct kinds of dynamic operators known as Tetranoterpernoids or Limonoids.[8,9] Several compound constituents have been isolated from neem leaves utilizing diverse solvents like water (hot or icy), ethanol, methanol, oil, ether and hexane. The most dynamic and best understood substance mixes found in neem are Azadirachtins.[9,10] The exact mode of action of neem is not known but possible pharmacokinetics of neem leaves are involvement with mitochondrial bioenergetics which cause the inhibition of electrochemical proton gradient that is the main energy generator in mitochodria. Neem interacts

with receptors and changes membrane integrity and permeability.[11,12]

An investigation was conducted by Rehman to demonstrate the impacts of pesticide like Vepacide (containing neem) on the biochemicals like aspartate aminotransferase (AST) and alanine amino-transferase (ALT) in serum and diverse tissues of rats of both genders.[13] Biochemical profile proposed that by enhancing the amount of Vepacide caused an increase in ALT and AST compounds in lung, serum and kidneys tissues, while the levels of these proteins were brought down in liver tissues of rats (the two males and females) following 45 and 90 days of treatment.[13]

Besides another investigation was likewise carried out to observe the action of same Vepacide on various proteins like basic phosphatase (ALP) and corrosive phosphatase (AcP) in many tissues of both male and female adult albino rats. It has been documented that the neem leaves at a dose of 200mg/kg exert inflammatory effects.[13,14]

As the neem is widely utilized by individuals as self-pharmaceutical without legitimate therapeutic counsel, the conceivable results might be dangerous. Consequently, the present examination was led with intent to observe the impact of neem leaves aqueous extract on hepatocytes as well as on centrilobular veins in livers of adult albino rats.

MATERIAL AND METHODS

45 adult albino rats of both strains (weighing about 180-200g) were obtained from Veterinary Research Institute, Lahore. They were divided in three groups; A (control), B (experimental low dose) and C (experimental high dose). Each group consisted of 15 rats. The weight of each rat was carefully recorded in a proforma. For identification, the rats were marked with permanent pointer and were placed in different cages for 21 days. A 12 hours light / dark cycle was maintained.[15] The animals were allowed free access to food and water. Aqueous extract of neem leaves was given to the animals by orogastric intubation. The dose schedule was as follows:

Group A: It was the control group containing 15 rats and was not given any extract except for equivalent proportion of distilled water, 20ml per kg body weight of distilled water by orogastric intubation for 20 days.

Group B: It was experimental group containing 15 rats, each of which received 40mg per kg body weight of neem extract by orogastric intubation for 20 days (for example: if the weight of rat was 180g, 7.2 mg dry neem powder dissolved in 2ml of distilled water was given. It means each ml contained 3.6 mg of powder).

Group C: This experimental group contained 15 rats. All the animals of this group were given 100 mg per kg body weight of neem extract by orogastric tube for 20 days (For example: if the weight of rat was 180g, 18 mg dry neem powder was dissolved in 2ml of distilled water. It means each ml contained 9 mg of powder). Hence the dose was adjusted according to weight of the rat.

Dissection and fixation of liver:

At the end of study, the rats of all groups were weighed properly and recorded in proforma. All the rats were euthanized by giving morphine 0.3–0.5mg/kg intraperitoneally, as an analgesic agent. The anaesthetic agent sodium pentobarbital was administered intraperitoneally with dose of 45mg/kg.[16,17]

The animals were put in a supine position with their belly facing up and limbs fixed to the dissection board. A midline incision was made with a pair of scissors from groin to chin and extended laterally. Liver was made free from surrounding structures and placed on a blotting paper to make it free of blood and fluids. After recording the weight of the liver, it was washed with normal saline to remove blood and fixed with 10% formalin for 48 hours in appropriately labelled tissue bottles. Tissue processing was carried out and paraffin tissue blocks of liver were made. Serial 5 micrometer tissue sections were cut by a rotary microtome. These slides were stained with Haematoxylin & Eosin (H & E). Stained sections were studied under light microscope at objectives of x5, x10, x20 and x40. Hepatocytes of group A were taken as reference. All other experimental groups were compared with the control group.

Statistical Analysis:

Data was analyzed by SPSS Version 22. The qualitative data for shape of hepatocyte and necrosis

in hepatocytes, shape of endothelial cells & congestion of centrilobular vein were reported by using frequency and percentage of each group. Comparison among groups was made by using Chi-square.

Quantitative variables like diameter of hepatic lobules, diameter of hepatocytes and diameter of central vein were described by using mean ± S.D. Comparison for this quantitative variable was performed by using ANOVA, Tukey's test for post-hoc analysis was used where required. P-value ≤ to 0.05 was considered statistically significant.

RESULTS

Shape of hepatic lobules:
The livers of group A showed normal architecture of hepatic lobule with normal arrangement of hepatocytes but there was gradual disruption of hepatic lobules from low dose to high dose group and they appeared atrophied and shrunken as compared to control group A. The difference among three groups was statistically significant with p-value <0.001.

Diameter of hepatic lobules:
For the measurement of diameter of hepatic lobules, slides were examined under 10 x objective as mentioned before. The average diameter of hepatic lobules for group A, B and C were 496.0±13.7, 458.7±35.2 and 430.0±45.1 µm respectively. The difference overall was statistically significant with p-value <0.001. The group B and C had significantly lower diameter as compared to group A with p-values 0.012 and <0.001 respectively.

Shape of hepatocytes:
Microscopic examination of hepatocytes of control group A showed normal polygonal shape of hepatocytes but in experimental groups B and C, there was a gradual shrinkage of shape of hepatocyte from lower to high dose that is from 40 mg/kg to 100 mg/kg of neem extract. There was atrophy of hepatocytes in focal areas. The two experimental groups had significantly different shape of hepatocytes as compared to control with p-values <0.001. (Table-1)

Diameter of hepatocytes:
The diameter of hepatocytes for group A was 17.72±0.44 µm, for group B 17.22±0.91 and that for group C was 16.78±0.78 µm. The difference among three groups was statistically significant with p-value 0.005. The decrease in diameters of hepatocytes from low to high dosage group further proved shrinkage and atrophy of hepatocytes of experimental groups. (Table-1)

Necrosis in hepatocytes:
Focal periportal coagulative necrosis was observed in 7 animals of experimental group B with low dose of 40mg/kg and 11 animals of group C with 100mg/kg (fig-3,4). The necrotic areas showed cellular lysis with collapsed stroma, pyknotic nuclei and lymphocytic infiltration. It was absent in hepatocytes for all (100%) animals of group A. The difference between the three groups was statistically significant with p-value <0.001. (Table-2,3)

Shape of endothelial cells:
The shape of endothelial cells lining the central vein was normal showing simple squamous epithelium in all animals of group A. The experimental groups B and C showed dilated and broken margin of endothelial cells with seepage of blood in the surrounding areas of the central vein. (fig-5,6) The difference among three groups was statistically significant with p-value < 0.001. (Table-4)

Diameter of central vein:
For the measurement of diameter of central vein, slides were examined. The central vein for group A had an average diameter of 76.5±8.9 µm and that for group B and C was 110.0±24.6 and 110.3±25.8 µm respectively. Again the difference was statistically highly significant with p-value <0.001. The difference of group B and C was significant from group A with p-values <0.001 and the difference between these two were insignificant with p-value 0.999 (Table-4)

Hematoxylin and Eosinophil (H&E) stained sections of liver showing histological appearance in all three groups are shown in figures 1, 2, 3, 4, 5 and 6 respectively.

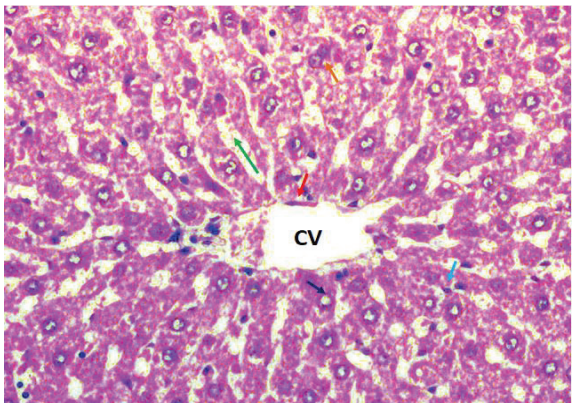

Fig-1: Photomicrograph of liver of adult albino rat of control group A showing a portion of hepatic lobule, having centrilobular vein (CV) lined with endothelial cells (Red arrow), hepatic sinusoids (Green arrow), hepatocytes (Black arrow), binucleated hepatocytes (Orange arrow) and Kupffer cells (Blue arrow). (H&E, 20x).

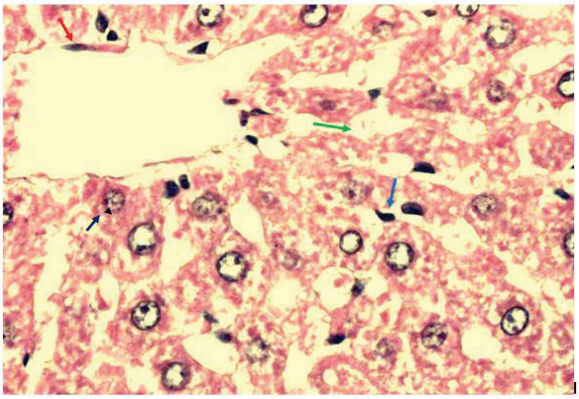

Fig-2: Photomicrograph of liver of adult albino rat of control group A showing polygonal shaped hepatocytes (Black arrow) with intervening hepatic sinusoids (Green arrow) and Kupffer cells (Blue arrow) . (H&E,x 40)

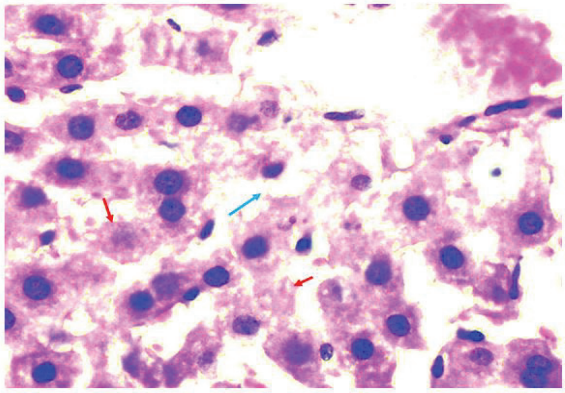

Fig-3: Photomicrograph of liver of adult albino rat of experimental group B showing necrosis of hepatocytes (N), pyknotic nuclei (Red arrow), dilated centrilobular vein (CVd) with dilated and congested sinusoids (Blue arrow). (H& E.x 40)

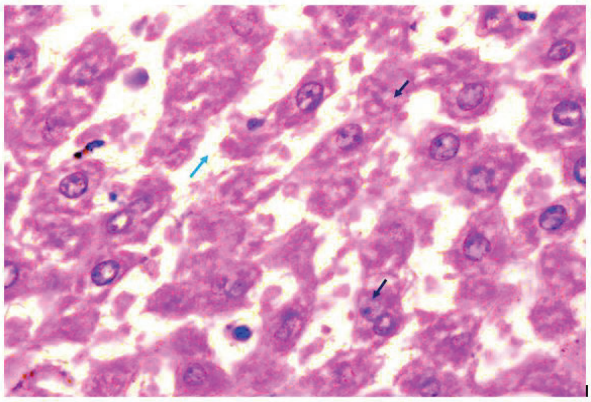

Fig-4: Photomicrograph of liver of adult albino rat of Experimental group C showing necrosis of hepatocytes (N), pyknotic nuclei (Black arrows) with dilated and congested sinusoids. (Blue arrow) (H &E.x 40)

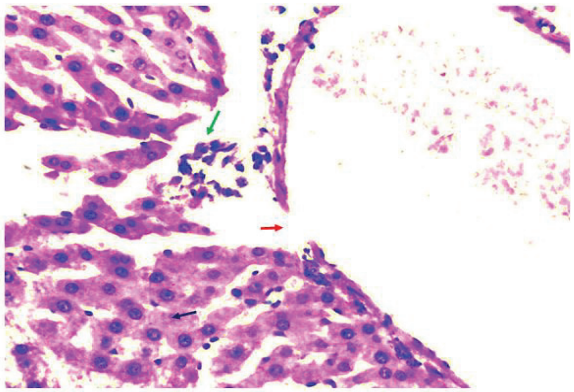

Fig-5: Photomicrograph of liver of adult albino rat of experimental group B showing congested and dilated central centrilobular vein (CVd) with disrupted endothelial margin (Red arrow) (H&E.x20) and dilated sinusoids (Green arrow)

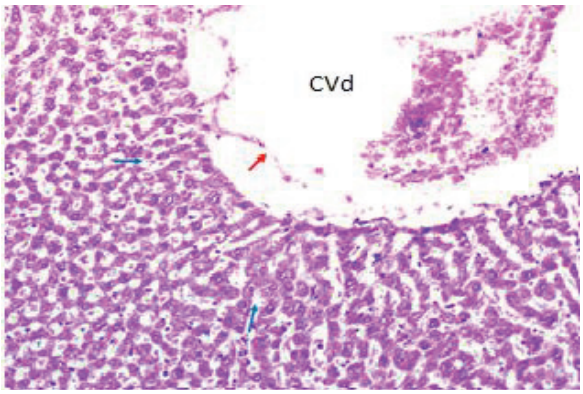

Fig-6: Photomicrograph of liver of adult albino rat of Experimental group C showing dilated centrilobular vein (CVd) with disrupted endothelial margin (Red arrow) and dilated and congested sinusoids (Blue arrow). (H&E x10)

Group	Shape of hepatocytes						Diameter of hepatocytes (µm)			
	Normal		Any abnormality		Total		Mean	SD	Minimum	Maximum
	N	%	N	%	N	%				
Group A	15	100.0	0	0.0	15	100.0	17.72	± 0.44	17.00	18.50
Group B	0	0.0	15	100.0	15	100.0	17.22	± 0.91	15.50	18.50
Group C	0	0.0	15	100.0	15	100.0	16.78	± 0.78	15.50	18.00
Total	15	33.3	30	66.7	45	100.0	-	-	-	-

Table-1: Comparison of shape and diameter of hepatocytes in rats of control and experimental groups after administration of neem extract

Groups	Present		Absent		Total	
	N	%	N	%	N	%
A	0	0.0	15	100.0	15	100.0
B	7	46.7	48	53.3	15	100.0
C	11	73.3	4	26.7	15	100.0
Total	18	44.0	27	60.0	45	100.0

Group 1	Group 2	Chi-Square	Df	P-value
Group A	Group B	9.13	1	0.006*
	Group C	17.37	1	< 0.001**
Group B	Group C	2.22	1	0.264^{+}

Table-2, 3: Comparison of necrosis in hepatocyte of rats in control and experimental group after administration of neem extract

Group	Shape of endothelial cells in central vein						Diameter of the central vein (µm)			
	Normal		Any abnormality		Total		Mean	SD	Minimum	Maximum
	N	%	N	%	N	%	-	-	-	-
Group A	15	100	0	0	15	100	76.5	± 8.9	64.5	92
Group B	0	0	15	100	15	100	110	± 24.6	82	162.5
Group C	0	0	15	100	15	100	110.3	± 25.8	82	172
Total	15	33.3	30	66.7	45	100	-	-	-	-

Chi-square = 45.0 p-value < 0.001

Table-4: Comparison of shape of Endothelial cells & diameter of Central Vein of Rats in control and experimental groups after administration of Neem extract

Based on ANOVA

df Degree of Freedom
F f-test (Ratio of Variances)
** Highly Significant Difference ($p < 0.001$)
A Control Group
B experimental Group
C experimental Group
DF Degree of freedom
++ Highly significant difference. ($P < 0.01$)
+ Non significant difference ($P > 0.05$)

DISCUSSION

Generally, neem has been utilized as a basic piece of our lives for quite a long time as an insecticide, germicide, preventive, antipyretic, antiparasitic, antiarthritic,[18,19] antifungal and hypoglycaemic agent.[19,20] People utilize it as an optional treatment for an assortment of skin problems.[21,22] Neem leaf extract is a basic supplement which is effectively accessible in medical stores and crude leaves are especially utilized by many individuals in our general public. It is normally considered safe in an extensive variety of measurements. Presently it has been demonstrated that it is harmful at higher dosage particularly when utilized for longer time periods. Its destructive impacts on liver, kidneys, lungs and male and female reproductive organs have also been documented.[23,24]

The present work was designed to evaluate the harmful effects of neem on liver as it is the main metabolic organ for neem and its constituents. The detailed histological study of liver of the control group A revealed normal polygonal shape of hepatocytes but in experimental groups B and C, there was a gradual shrinkage of shape of hepatocyte from lower to high dose i-e is from 40 mg/kg to 100 mg/kg of neem extract. There was atrophy of hepatocytes in focal areas. The difference among three groups was significant with p- value 0.001. Similar findings were also observed by Rahman in a study who suggested the increased permeability of membranes, shrinkage of hepatic lobules and hepatocytes along with release of enzymes.[13]

The result of present study showed that the necrosis was absent in hepatocytes for all (100.0%) animals of group but present in 7 (46.7%) animals of group B and 11 animals of group C. They showed mild focal coagulative type of necrosis in hepatocytes. The probable mechanism may be the inhibition of mitochondrial function by dual effect on both beta-oxidation energy productions by inhibiting the synthesis of nicotinamide, flavin adenine dinucleotide and depletion in glutathione that result in decreased ATP production and development of cell necrosis.[25] These same findings were observed by Seth and Jaffery in their research work on pesticides.[26]

The shape of endothelial cells lining the central vein was normal showing simple squamous epithelium in all animals of group A. The experimental groups B and C showed dilated and broken margin of endothelial cells with seepage of blood in areas surrounding the central vein. The difference among the three groups was significant with p- value 0.001. In this research, experimental groups B and C showed marked dilatation of central vein with increased diameter as compared to control group A), (Table-4,5). The cell membrane is the main target of the oxidative damage produced by toxic metabolites. This is mainly due to changes in polyunsaturated fatty acids mainly present in the phospholipids of membranes.[25]

Neem contains Gedunin which is a potent vasodilator as it modulates the production of endothelium derived relaxing factor nitrous oxide.[26] This is in accordance with the findings observed by Fernando and Ferraris who studied the effects of gedunin on articular inflammation and hyper-nociception in mice.[26] They determined that gedunin directly impairs neutrophil and macrophage activation by impairing calcium influx, cell adhesion, chemotaxis and lipid body formation.[26]

CONCLUSION

This research demonstrated that organization of aqueous neem leaf extract in high dosages caused noteworthy negative impact on the histology of hepatocytes and the central vein of adult albino rats. The possible mechanism behind its damaging impact is oxidative stress at cell level. Despite the fact that neem is commonly utilized as non-allopathic drug, its dosage still hasn't been institutionalized. There is a need to evaluate safer dose and duration of usage of neem in general public.

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