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Effect of chronic administration of aqueous extract of Neem (*Azadirachta indica*) leaves on Paracetamol-induced hepatotoxicity in Wistar albino rats

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Abstract

Hepatoprotective drugs are not available for use in modern medicine and different parts of medicinal plants like Neem (*Azadirachta indica*) are used as hepatoprotectants in traditional medicine. Although there are scientific reports of its hepatoprotective activity on acute administration, we found only one study which had evaluated its hepatoprotective effect on chronic administration. **Objectives:** To evaluate the effect of chronic oral administration of Neem on paracetamol-induced hepatotoxicity in Wistar rats. **Methods:** We randomly assigned 72 male and female Wistar albino rats to four groups of 18 animals each and orally administered Distilled water 5ml/kg body weight/day to Groups A (Normal control) and B (Experimental control), 500 mg/kg aqueous Neem leaf extract (Test) to Group C and Silymarin suspension (Standard) 100mg/kg/day to Group D for 30 days. On the 8th day, we induced hepatotoxicity with Paracetamol 2g/kg body weight single dose to groups B, C and D. We performed liver function tests, recorded liver weights and examined liver histology of six rats from each group on 10th, 20th and 30th days. **Results:** We observed significant difference (P<0.05) in Mean ± SEM values of serum bilirubin, ALP, AST, ALT, liver weights, total protein and albumin: globulin in Group B compared to Group A, whereas these changes were significantly less in groups C and D compared to B. Histopathological examination of liver showed hepatic necrosis on 10th day in Groups B, C and D, but on 20th and 30th day in Group B and on 10th day in Groups C and D, we observed decrease in sinusoidal congestion and cloudy swelling along with small areas of regenerative changes. All abnormal histological changes decreased by the 30th day. **Conclusion:** Thus, aqueous extract of *Azadirachta indica* leaves shows hepatoprotective activity on chronic oral administration in Wistar rats.

Key Words: *Azadirachta indica*, Neem, Hepatoprotective, Wistar rats, Paracetamol, Hepatotoxicity

1. Introduction

The liver is a major organ which plays a central role in many essential physiological processes, thus maintaining the metabolic homeostasis of the body. It is also responsible for the metabolism and detoxification of

many endogenous and exogenous compounds (xenobiotics). Hepatic damage may result from primary or secondary liver disease as well as exposure to miscellaneous drugs and environmental chemicals. Although its regenerative capacity is well known, this makes the organ particularly prone to damage from these pharmaceutical and environmental chemicals.[1]

In conditions where liver function is compromised or at risk of toxic effects, hepatoprotective drugs can be administered to the patients. But such medicines are deficient in allopathic medicine. Due to the absence of reliable drugs for the management of liver ailments in modern medicine, plants and natural products are being relied upon as a source of hepatoprotectants. Natural sources of hepatoprotective agents are widely used in traditional medicine. Currently, Silymarin which is derived from Silybum marianum (Milk thistle) is available as a hepatoprotective drug formulation.[2,3] Silymarin is a flavolignan and is a mixture of structural components silybin A, silybin B, isosilybin A isosilybin B, silydianin, silychristin, isosilychristin and taxifolin.[4] Many other phytochemicals present in different parts of plants, like *Allium sativum, Andrographis paniculata, Ocimum sanctum, Solanum nigrum, Phyllanthus niruri, Mangifera indica, Magnolia officinalis, Nigella sativa, Gingko biloba,* etc have been found to have hepatoprotective effect.[5,6]

Neem, known by its botanical name *Azadirachta Indica* A. Juss, belonging to the mahogany family Meliaceae, is an evergreen tree, traditionally cultivated in various parts of South east Asia (Indian subcontinent), Africa but nowadays it is grown in Central and South America also.[7,8] The US National Academy of Sciences published a report in 1992 entitled, "Neem—a tree for solving global problem," underscoring the value of Neem.[9] It is a commonly used traditional medicinal plant in India. Various parts of the Neem tree (leaves, bark, fruit, flowers, oil and gum derivatives) have been reported to possess analgesic, anti-inflammatory, antipyretic, hypoglycaemic, hypolipidaemic, antihypertensive, immunostimulant, hypoglycaemic, antiulcer, antifertility, antioxidant, anxiolytic, antimicrobial, anthelminthic, antimalarial, antiviral, antifilarial and anticarcinogenic, properties.[10-53]

The hepatoprotective effect of different parts of Neem (*Azadirachta indica*), including leaf extract has been reported by previous researchers.[52-76] However, it has been observed by other researchers to be hepatotoxic.[5] Moreover, we found only one study[75] which has evaluated the effect of chronic administration of Neem leaves on the liver.

Thus, our objective was to evaluate the hepatoprotective effect of chronic oral administration of aqueous extract of leaves of Neem (*Azadirachta indica*) on Paracetamol-induced hepatotoxicity in Wistar albino rats

2. Material and Methods

2.1. Collection of Plant material

The study was undertaken after our experimental research protocol was granted permission of Institutional animal ethics committee (IAEC Reg. 634/02/a/CPCSEA).

Fresh leaves *of Azadirachta Indica* (Neem) were collected from local gardens of Dibrugarh, Assam. We collected the leaves once a week on sunny days during spring season. Before collection, the plant was authenticated by Ms. Belinda Lahon, PhD in Botany from North Bengal University, West Bengal, India.

2.2. Drugs and chemicals

Preparation of *Azadirachta indica* aqueous extract (ANLE)

We mixed one Kg of freshly collected, shade dried, powdered leaves of *Azadirachta Indica* (Fig.1) with four liters of distilled water and allowed the suspension to soak overnight. Thereafter, it was centrifuged at 5000 rpm for 20 minutes and filtered through a Whatman's No 1 filter paper. The supernatant fluid was kept in glass petri dishes under tube light to provide heat for

facilitating evaporation and to prevent dampness and avoid contamination by organisms. After drying, we scraped off the powder (total yield 5.5%). We then prepared stock solution by dissolving 500mg of the extract in distilled water.[56]

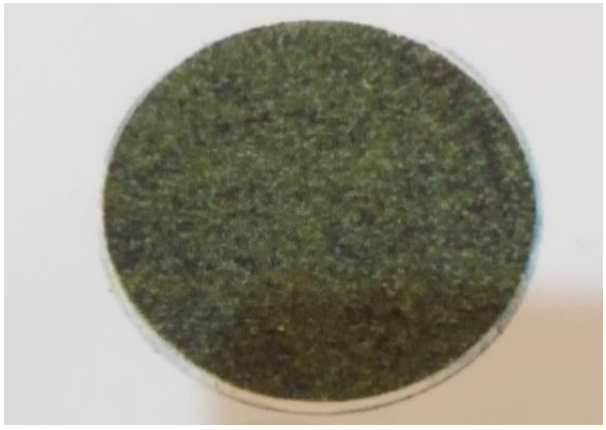

Figure 1. Neem leaf powder

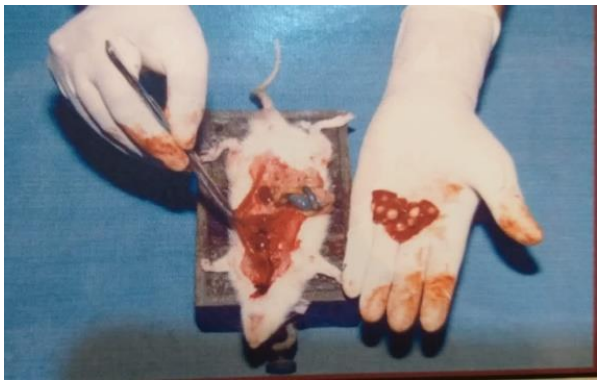

Figure 2. Dissection of rat liver

Silymarin

Silymarin powder was obtained from Micro Labs Ltd., Bangalore, India. The stock solution was prepared by dissolving 100mg of Silymarin powder in 5ml of distilled water and used as a standard drug in the dose of 100mg/Kg body weight/day, following the method of Mansour *et al*.[2]

Paracetamol

Paracetamol powder was obtained from Quality Pharma Pvt. Ltd. (Kolkata, India).

All drugs and chemicals were of analytical grade.

2.3. Experimental animals

Seventy-two healthy adult Wistar albino rats of either sex, weighing 100-150gms were procured from the Central Animal House of Assam Medical College & Hospital, Dibrugarh, Assam. Before starting the experiment, animals were allowed to acclimatize to the laboratory environment for one week. They were housed in a well ventilated room at 23.0±2°C, humidity of 65-70% and 12hr light/dark cycle. They were fed with standard diet along with water in sufficient amount. All animals were looked after ethically, as per the principles of laboratory animal care prescribed by the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA).[77]

2.4. Experimental Design

The experiment was carried out for 30 days. Animals were weighed and their weights were recorded. They were numbered and divided into four groups randomly, each group containing eighteen animals.

Grouping and treatment schedule:

Group A: Normal control received Distilled water 5ml/kg body weight/day X 30 days. Group B: Experimental control received Distilled water 5ml/kg body weight/day X 30 days. Group C: Received Aqueous Neem leaf extract (ANLE) 500mg/kg body weight/day X 30 days.

Group D: Received Silymarin suspension (SILY) 100mg/kg body weight/ day X 30 days. On the 8th day, Paracetamol 2g/kg body weight single dose was given to animals of Groups B, C and D (experimental control, test and standard drug groups).

Dose selection of *Azadirachta indica* (Neem) leaf aqueous extract and standard drug Silymarin was based on previous studies.[56,2] The drugs were administered to the animals orally by an intragastric feeding tube. All animals were observed for any physical and/or behavioural features of toxicity following administration of the extract throughout the experiment.

Six animals from each group were randomly selected for estimation of liver function on the 10th, 20th and 30th day of the experiment. Blood was collected directly by cardiac puncture under light ether anaesthesia, serum was separated and sent for laboratory estimations.

Laboratory parameters for measurement

Liver function tests:

Serum bilirubin, Aspartate aminotransferase (AST), Alanine aminotransferase (ALT), Alkaline phosphatase (ALP), Total serum protein, Albumin:Globulin ratio were estimated by the following methods:

ALT - Modified IFCC/UV kinetic method, AST - Modified IFCC/UV kinetic method, ALP – Total protein - Biuret method, Albumin - BCG Dye binding method.[78-81]

Histopathological examination:

Six animals from each group were humanely sacrificed under Inj. Sodium pentobarbitone 150mg/kg i.p. anaesthesia on 10th, 20th and 30th days respectively to collect the liver specimens for histopathological examination (Fig.2). The gross physical appearance and weight of the livers were recorded and they were preserved in 10% formalin after washing initially with normal saline. Paraffin embedding technique was performed. Liver sections of 5-mm thickness were obtained and stained with hematoxylin and eosin. Thereafter, we examined the liver sections for histopathological changes under a light microscope.[82]

Statistical analysis:

We used GraphPad Instat statistical software for data analysis. The quantitative variables were expressed as Mean ± SEM. Statistical significance of the results of quantitative variables (LFT values) between groups was analysed using one-way ANOVA followed by Bonferroni's multiple comparison test. $P < 0.05$ was considered as statistically significant at 95% confidence level.

3. Results and Discussion

We evaluated the effect of chronic oral administration of aqueous leaf extract of Neem (*Azadirachta indica*) in Wistar albino rats over 30 days.

The animals did not show any physical or behavioural symptoms of toxicity after chronic oral administration of *Azadirachta indica* (Neem) aqueous leaf extract. The non-toxic nature of Neem leaf extract and Neem leaf glycoprotein to mice and rats was reported by Haque *et al.*, Mallick, *et al.* respectively[83,84]

Table 1: Mean ± SEM of LFTs and liver weights of control and drug-treated albino rats

Gr	Serum Bilirubin (mg/dl)	Alkaline Phosphatase (IU/L)	AST (IU/L)	ALT (IU/L)	Serum Protein (g/dl)	Albumin: Globulin	Weight of liver (g) (% B.W)
A (DW 5ml/kg/day)	0.48±0.02	212.00±2.04	36.33±1.28	36.50±1.12	5.58±0.06	1.35±0.01	5.98±0.60
B (DW 5ml/kg/day)	0.57±0.02[a]	529±3.170[a]	216.33±3.07[a]	190.67±3.85[a]	5.32±0.07[a]	1.27±0.01[a]	7.28±0.14[a]
C (ANLE 500 mg/kg/day)	0.42±0.02[b]	315.67±3.20[bd]	78.00±2.48[b]	65.83±2.09[bd]	5.52±0.06[b]	1.39±0.02[bd]	6.50±0.04[b]
D (SILY 100mg/kg/ day)	0.45±0.02[b]	335.33±12.21[b]	82.00±2.44[b]	73.50±1.93[b]	5.50±0.06[b]	1.31±0.01[b]	6.61±0.07[b]

Statistical analysis by One-way ANOVA followed by Bonferroni's test with significance at $P<0.05$. a: $P<0.05$ when compared with Group A; b: $P<0.05$ when compared with Group B; c: $P>0.05$ when compared with Group A; d: $P<0.05$ when compared with Group D; e: $P>0.05$ when compared with Group E. DW = Distilled water, ANLE = Aqueous Neem leaf extract, SILY= Silymarin suspension. Paracetamol 2g/kg single dose administered to groups B,C and D on 8th day. (10th day of experiment; n=6).

Table 2. Mean ± SEM of LFTs and liver weights of control and drug treated albino rats

Gr	Serum Bilirubin (mg/dl)	Alkaline Phosphatase (IU/L)	AST (IU/L)	ALT (IU/L)	Serum Protein (g/dl)	Albumin: Globulin	Weight of liver (g) (% B.W)
A (DW 5ml/kg/day)	0.48±0.02	212.00±2.37	29.33±2.51	32.00±2.25	5.67±0.05	1.29±0.01	6.00±0.07
B (DW 5ml/kg/day)	0.83±0.02[a]	376.67±5.62[a]	171.67±4.05[a]	168.33±3.24[a]	4.97±0.05[a]	1.31±0.02[c]	6.86±0.08[a]
C (ANLE 500 mg/kg/day)	0.36±0.02[bd]	240.67±2.25[bd]	58.50±2.23[bd]	56.33±1.69[b]	5.78±0.06[b]	1.36±0.02[bd]	6.40±0.04[b]
D (SILY 100mg/kg/ day)	0.41±0.01[b]	251.33±5.53[b]	63.67±3.04[b]	59.67±2.44[b]	5.73±0.05[b]	1.32±0.01[e]	6.53±0.04[b]

Statistical analysis by One-way ANOVA followed by Bonferroni's test with significance at $P<0.05$. a: $P<0.05$ when compared with Group A; b: $P<0.05$ when compared with Group B; c: $P>0.05$ when compared with Group A; d: $P<0.05$ when compared with Group D; e: $P>0.05$ when compared with Group E. DW = Distilled water, ANLE = Aqueous Neem leaf extract, SILY= Silymarin suspension. Paracetamol 2g/kg single dose administered to groups B, C and D on 8th day. (20th day of experiment; n=6).

Table 3. Mean ± SEM of LFTs and liver weights of control and drug treated albino rats

Gr	Serum Bilirubin (mg/dl)	Alkaline Phosphatase (IU/L)	AST (IU/L)	ALT (IU/L)	Serum Protein (g/dl)	Albumin: Globulin	Weight of liver (g) (% B.W)
A (DW 5ml/kg/day)	0.47±0.02	169.33±3.04	34.50±0.96	35.00±1.70	5.63±0.04	1.35±0.02	5.99±0.06
B (DW 5ml/kg/day)	0.54±0.02[c]	270.33±2.94[a]	58.67±1.84[a]	52.00±1.55[a]	5.25±0.06[a]	1.32±0.03[a]	6.40±0.06[a]
C (ANLE 500 mg/kg/day)	0.37±0.02[b]	202.00±6.18[b]	28.33±0.95[b d]	38.00±1.15[b]	5.59±0.05[b]	1.40±0.03[bd]	6.04±0.05[bd]
D (SILY 100mg/kg/ day)	0.40±0.02[b]	221.67±4.80[b]	43.00±1.12[b]	39.00±1.98[b]	5.62±0.05[b]	1.36±0.02[b]	6.21±0.07[b]

Statistical analysis by One-way ANOVA followed by Bonferroni's test with significance at $P<0.05$. a: $P<0.05$ when compared with Group A; b: $P<0.05$ when compared with Group B; c: $P>0.05$ when compared with Group A; d: $P<0.05$ when compared with Group D; e: $P>0.05$ when compared with Group E. DW = Distilled water, ANLE = Aqueous Neem leaf extract, SILY= Silymarin suspension. Paracetamol 2g/kg single dose administered to groups B,C and D on 8th day. (30th day of experiment; n=6).

LFT and Liver weights

The results of LFT estimation and liver weights are shown in Tables 1-3.

On 10th day of the experiment, the mean levels of serum bilirubin, ALP, AST, ALT and liver weights were significantly elevated ($P<0.05$) in Group B (Paracetamol treated) when compared with Group A (Normal control) whereas serum protein levels and albumin globulin ratio were significantly decreased ($P<0.05$). The pretreated groups with aqueous Neem leaf extract (Group C) and Silymarin (Group D) however showed a significantly lesser increase ($P<0.05$) in the level of serum bilirubin, alkaline phosphatase, AST, ALT and liver weights and a significantly lesser decrease ($P<0.05$) in total protein level and albumin globulin ratio when compared with Group B (Table 1).

On 20th day of the experiment, mean values of serum bilirubin, ALP, AST, ALT and liver weights decreased and total protein and aalbumin globulin ratio increased compared to values on 10th day (Table 2).

Similar significant differences ($P< 0.05$) were observed in all parameters except for albumin globulin ratio, where we found no significant difference in values between Group A and B and between B and D. However, Group C mean values of LFT and liver weight showed significant difference compared to those of Group B.

On 30th day of experiment, mean values of serum bilirubin, liver enzymes and liver weights decreased and total protein and albumin globulin ratio increased compared to values on 20th day (Table 3). The same pattern of significant differences ($P<0.05$) were observed between Groups A and B except for serum bilirubin values and between C, D compared to B.

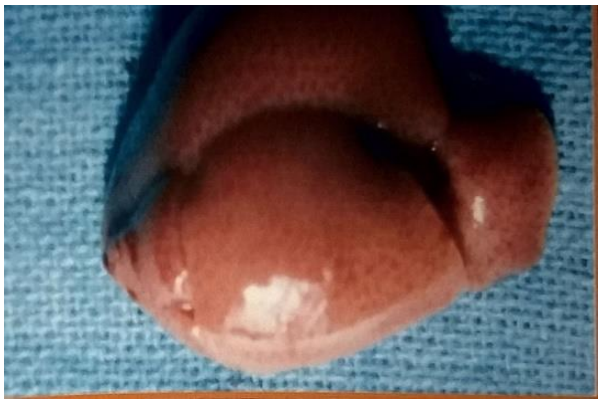

Figure 3. Liver of Normal control group

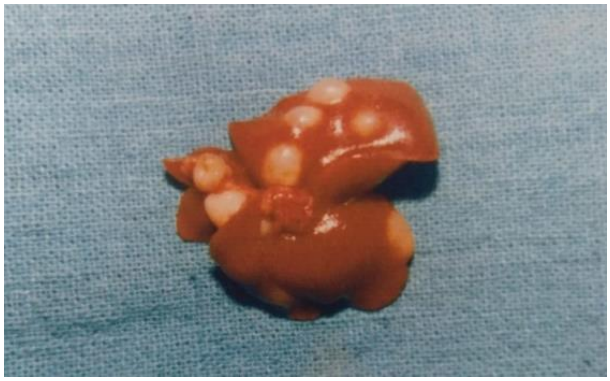

Figure 4. Liver of Paracetamol treated group

Thus, serum bilirubin, ALP, AST, ALT and liver weights were significantly ($P<0.05$) raised in experimental control and total serum protein, albumin globulin ratio (except on 20th day) were significantly decreased ($P<0.05$) compared to normal control on 10th, 20th and 30th days (except serum bilirubin on 30th day) following Paracetamol administration.

In ANLE and Silymarin treated groups also, we observed significant increase ($P<0.05$) in LFT parameters and liver weights and decrease in total protein, albumin globulin ratio on 10th, 20th and 30th days compared to normal control. But the observed increases in liver enzymes and decrease in protein and albumin globulin ratio were less compared to observed values in untreated group (Group B).

Gross appearance of Liver

Gross appearance of the livers of normal control group (Group A) showed normal architecture having red colored smooth regular undersurface (Fig.3). Paracetamol treated (Group B) liver showed multiple white nodules indicating necrotic areas and increase in the weight particularly on the 10th day of experiment. (Fig.4) Thus, gross appearance of liver changed with increase in size and presence of nodules in all Paracetamol treated groups (B,C,D), but by the 30th day, livers of ANLE and Silymarin treated groups showed normal appearance.

Liver Histopathology

Histology of liver from normal control group showed normal arrangement of hepatocyte with clearly brought out nuclei, central vein and portal triad throughout the experimental period (Fig. 5).

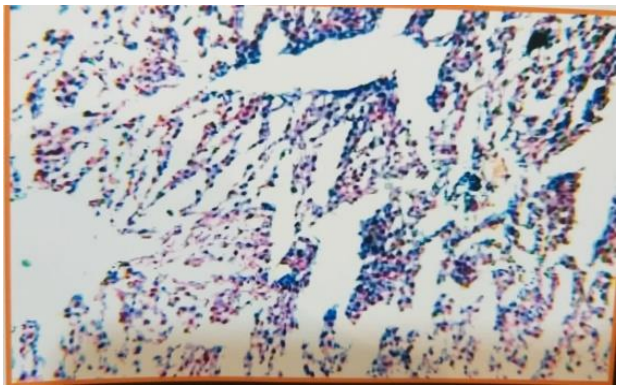

Figure 5. Photomicrograph of normal rat liver (H&E; Low power 10X10)

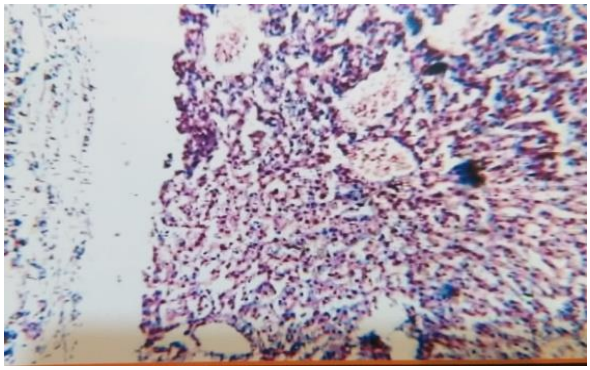

Figure 6. Photomicrograph of liver cell injury by Paracetamol (H&E; Low power 10X10)

The Paracetamol treated group showed congestion of sinusoids, cloudy swelling, central vein congestion, centrilobular fatty changes and necrosis of hepatic cells on the 10th day of experiment (Fig. 6). But on the 20th and 30th day, small areas of focal degeneration and sinusoidal dilatation evidenced the regenerative activity. The groups pretreated with ANLE and Silymarin showed a marked reduction in the congestion of sinusoids and cloudy swelling of liver on the 10th day of experiment (Fig. 7 & 8). Almost all changes were diminished by the 30th day of the experiment.

Thus, histology showed evidence of hepatic necrosis which was partly reversed by ANLE and Silymarin as early as the 10th day, but we observed spontaneus reduction in necrotic areas and appearance of regenerative areas in experimental control group also by the 20th day.

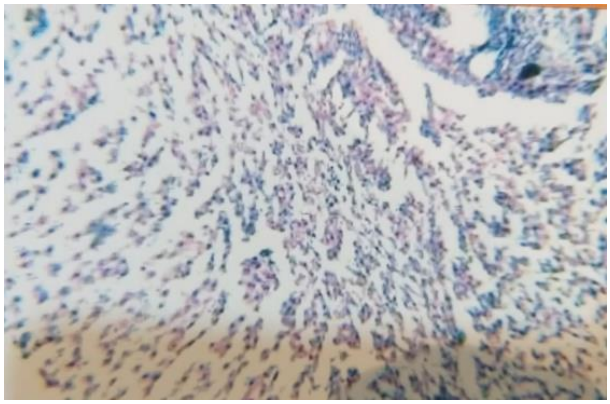

Figure 7. Photomicrograph of regenerative area in liver by Neem leaf extract (H&E; Low power 10X10)

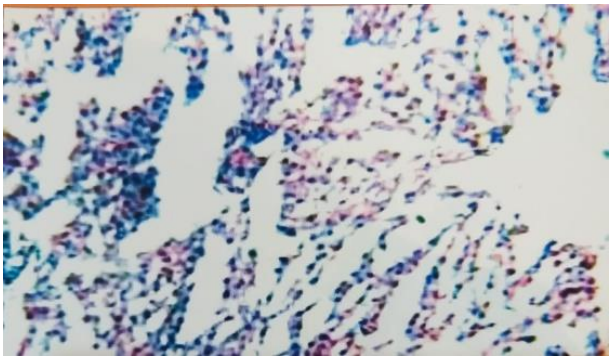

Figure 8. Photomicrograph of regenerative area in liver by Silymarin (H&E; Low power 10X10)

3.1. Hepatotoxicity

The liver can be damaged by disease, infection, during detoxification of endogenous substances or exposure to toxins, which can cause impairment of its function and sometimes, even structural damage at gross and microscopic (histological and ultrastructural) level. Zone 3 of the liver cell acinus is more vulnerable to toxic damage. Liver stem cells are found on the border of the portal system and hepatocyte lobules in zone 3 of liver parenchyma. These stem cells can differentiate into hepatocytes and bile duct cells.[85]

Common biomarkers of liver damage are raised levels of the enzymes AST, ALT and ALP, although ALT is more specific for liver damage. Aminotransferase levels > 75 times the upper reference limit indicate ischemic or toxic liver injury in more than 90% of cases of acute hepatic injury. In hepatocellular damage or death, ALT released from damaged heptocytes increase serum ALT levels.[86] The enzymatic parameters are part of the liver function tests which are more indicative of hepatocyte integrity rather than liver function.[87]Marked release of transaminases into the circulation reflects severe damage to hepatic tissue membranes during paracetamol intoxication.[64]A decrease in serum albumin may indicate decrease in functioning liver mass. Increased protein catabolism in drug - induced hepatitis might have a direct adverse effect on the

synthesis and secretion of albumin. Previous studies have documented hypoalbuminemia during hepatic dysfunction.[64,88-91]
Thus, we can infer that Paracetamol induced hepatotoxicity which led to raised liver enzymes, serum bilirubin and decrease in total serum protein and albumin globulin ratio as well as hepatic inflammation and necrosis.

3.2. Hepatotoxic mechanism of Paracetamol

Paracetamol induced hepatotoxicity results from an unstable toxic metabolite.[92]In normal therapeutic doses, paracetamol is primarily metabolized in the liver by glucuronidation and sulphation; however, a small proportion is converted to N-acetyl-p-benzoquinoimine (NAPQI) after bioactivation by cytochrome P450. This minor metabolite can react with sulphydryl groups such as glutathione[93]and protein thiols to form mercapturic acid.[94] When paracetamol overdose occurs, elevated levels of the toxic NAPQI metabolite are generated, which extensively deplete hepatocellular glutathione (GSH) especially in centrilobular hepatocytes. Thus, NAPQI covalently modifies cellular proteins by binding to their cysteine groups to form 3-(cystein-S-yl) paracetamol adducts; this results in a chain ultimately causing hepatocyte necrosis.[95-97]Cytochrome P450-dependent bio activation of paracetamol is thus the main cause for hepatic necrosis upon administration or intake of lethal doses of paracetamol.[98,99]Hepatotoxic dose of Paracetamol depletes the endogenous glutathione level to below a threshold value (<20% of control), therefore permitting interaction of NAPQI with cell macromolecule.[100]In an adult man, a minimum of 7.5-10 gm of the drug produces hepatic necrosis.[101]But, in alcoholics, as little as 4-8gm Paracetamol /day may produce liver damage. In patients with underlying liver diseases, a much lower dose of Paracetamol can produce liver damage.[102] Gujral, *et al.* reported that mode of hepatocyte cell death in Paracetamol overdose is mostly oncotic necrosis and less apoptosis.[103]

3.3. Silymarin

Silymarin and aqeuous extract of Neem leaf exhibited hepatoprotective activity, which was evidenced by significantly (P<0.05) lower levels of serum bilirubin, alkaline phosphatase, AST, ALT, liver weight and significant rise in total protein and albumin globulin ratio compared to untreated experimental control group. The histopathological examination of Silymarin and Neem leaf extract treated groups revealed hepatoprotection, as shown by marked reduction in congestion of sinusoids, cloudy swelling and congestion of central vein during the experimental period.
Silymarin has multiple mechanisms by which it exerts hepatoprotective effect. Silymarin stabilizes the lipid structures in the hepatocellular structure [102] and has anti-lipid peroxidative effect which helps in preservation of membrane integrity. Silymarin can chelate transition metal ions such as iron and copper, rendering them effective antioxidants. In addition, it increases GSH content in liver and protects it against toxicity of GSH depletors such as paracetamol. Silybinin component of Silymarin inhibits the function of Kupffer cells which are involved in hepatic fibrosis.[104,105]. It also has an inhibitory effect on NF-kB/Rel activity in a human hepatoblastoma-derived cell line and human histiocytic lymphoma cells; this results in partial inhibition of IL- Iß production (anti-inflammatory effect).[64]

3.4. Hepatoprotective activity of Neem (*Azadirachta indica*) leaf extracts

The hepatoprotective activity of Neem was earlier reported by Chattopadhyay, *et al.*, Nahed, *et al.*, Bhanwra, *et al.,* Yanpallewar, *et al.*, Johnson, *et al.*, Nwobodo, *et al.*, when aqueous Neem leaf extract was administered in Paracetamol-induced hepatotoxicity in rats.[53,55,56,58,64,73] Hepatoprotective activity

of aqueous Neem leaf extract and of Azadirachtin-A and Nimbolide compounds (present in Neem leaves) in Carbon-tetrachloride induced hepatotoxicity was also reported by Mukherjee, *et al.*, Kalaivani, *et al.*, Baligar, *et al.,* Idu *et al.*[54,59,66,67] Baligar *et al*. stated that nimbolide hepatoprotective activity was comparable to that of Silymarin.[67] Hepatoprotective effect of aqueous Neem leaf extract was also reported by Kale, *et al.* against anti-tubercular drugs-induced hepatotoxicity, Akinola, *et al.* against Streptozotocin induced hepatotoxicity in diabetic rats, Ezz-din D, *et al.,* Abdel-Moneim, *et al.* and Dkhil, *et al.*, against cisplatin-induced hepatotoxiciy, Essien, *et al.* against Alloxan-induced hepatotoxicity in diabetic rats, Koul, *et al.* against DMBA-induced hepatotoxicity, Sani, *et al* against snake venom induced hepatoxicity, Althaiban against Rifampin induced hepatotoxicity.[57,61,60,67,62,63,65,75]

In an in-vitro model involving *Azadirachta indica* gold nanoparticles (GNP) linked with the anti-HIV drug Azidothymidine, Kesarkar et al. observed that *A.indica* conferred hepatoprotective activity to the gold nanoparticles. The authors made this inference from observing that IL-10 (anti-inflammatory) activities were enhanced, whereas there was controlled secretion of pro-inflammatory IL-6 and downregulation of TNF-alpha in primary co-cultures of rat liver Kupffer cells by ELISA.[68]

Neem comprises of various ingredients such as liminoids, and nimbosterol in different parts of the plants. Important bioactive principles of Neem are flavonoids, alkaloids, tannins, saponins, steroids namely, quercetin, gallic acid, (+)gallocatechin, (-)epicatechin, (+)catechin and epigallocatechin, nimbin, 6-desacetylnimbinene, nimbandiol, 17-hydroxy azadiradione, ascorbic acid, nimbolide, nimbiol n-hexacosanol,7-sdesacetyl-7-benzoylazadiradione,7-sdesacetyl-7-enzoylgedunin, and nimbiol, Azadirachtin, Chlorogenic acid, Kaempferol derivatives, Myricetin, Rutin, Scopoletein, Sigmasterol/Beta-sitosterol.[28,76,85,106,107]Leaves contain mixture of compounds including nimbin, nimbanene, 6-desacetylnimbinene, nimbandiol, nimbolide, ascorbic acid, n-hexacosanol and different amino acids, and nimbiol and several other types of ingredients.[11]Many of these components – Azadirachtin, chlorogenic acid, Kaempferol derivatives, Myricetin flavonoids, Nimbin, Nimbolide, Rutin, Scopoletein, Sigmasterol/Beta sitosterol 6-desacetylnimbinene, quercetin, nimbandiol, 17-hydroxy azadiradione, ascorbic acid, nimbolide, n-hexacosanol,7-sdesacetyl-7-benzoylazadiradione,7-sdesacetyl-7-enzoylgedunin, and nimbiol have anti-inflammatory, immunomodulating and/or antioxidant properties.[28,106,107]

3.5. Mechanism of action of Neem (*Azadirachta indica*) leaf extract as a hepatoprotectant

Terpenoids, alkaloids, flavonoids and glycosides are the major classes of constituents found within the neem leaf extract that in isolation or in combination simultaneously work as antioxidants, f r e e radical scavengers and anti-inflammatory compounds.[28] Chattopadhyay, Chattopadhyay, *et al*., Suhendro, *et al.*, Sithisarn, *et al.*, Manikandan, *et al.*, Al-Hashemi, *et al.* have reported the antioxidant activity of Neem leaf extract.[28,85,108-112])

Exposure to oxidant molecules leads to generation of reactive oxygen species (ROS), like hydrogen peroxide, superoxide, hydroxyl radicals that can readily alter DNA, proteins and/or membrane phospholipids, thus altering their structure and function. Normally, the body's endogenous antioxidants like superoxide dismutase (SOD), catalase (CAT), reduced glutathione (GSH) and glutathione peroxidase (GSH-Px) act against these ROS. But they get depleted in acute oxidative stress leading to ROS accumulation.

Neem leaf extracts reverse this phenomenon and re-establish antioxidant mechanisms.

Neem extract contains phenol and antioxidant effects of phenolic compounds are related to a number of different mechanisms, such as free radical-scavenging, singlet oxygen quenching, metal ion chelation, hydrogen-donation and their action as substrates for free radicals such as superoxide anion and hydroxyl radical.[64]Azadirachtin and nimbolide exhibited concentration-dependent free radical scavenging activity in cancer models.[113]Antioxidants can also elevate the levels of endogenous antioxidant enzymes, such as superoxide dismutase (SOD), catalase (CAT), reduced glutathione (GSH) and glutathione peroxidase (GSH-Px), thus decreasing lipid peroxidation.[64]The antioxidant capacity of Neem leaf extracts were evidenced through reduced H_2O_2 mediated lipid peroxidation and DNA damage.[111]Johnson *et al.* reported that paracetamol administration caused increased lipid peroxidation, reduced SOD, reduced CAT and depletion in GSH activity in the liver. Liver tissue contains relatively high content of polyunsaturated fatty acids (PUFAs), which are sensitive to peroxidative damage. Paracetamol caused increase in lipid peroxidation and due to NAPQI formation, GSH stores decreased, as evidenced by elevated levels of malondialdehyde.[64,73]Abdel Moneim, *et al.* observed that flavonoids in neem possess both antioxidant and anti-inflammatory activities via scavenging free radicals and inhibition of lipid peroxidation.[69] In addition, neem leaves are rich in polyphenolics, which are known for their potent antioxidant and free radical scavenging properties. Suhendro *et al.* proposed that neem leaf extract contains quercetin, flavonoids and beta carotene which have free radical scavenging and, anti-lipid peroxidative properties and also increase Glutathione reductase activity (which protects cell membrane integrity).[85]In paracetamol induced hepatotoxicity, antioxidant mechanisms facilitate good regeneration of hepatocytes. Stem cells outside the necrotic area, particularly on central vein area and the portal system multiply and differentiate to become a hepatocyte and replace the necrotic hepatocytes in the absence of free radicals.[85]NF-κB, a redox-sensitive transcription factor that has been proposed to be the sensor for oxidative stress, was induced by cisplatin induced hepatotoxicity and reduced by neem leaf extract.[62]

Neem leaf extracts have also been reported to inhibit TNF-α triggered induction of NF-κB that is linked to inflammation.[114]Anti-inflammatory effects of chlorogenic acid in neem leaf was observed in lipopolysaccharide (LPS) - stimulated RAW 264.7 cells. Chlorogenic acid significantly inhibited not only NO production butalso the expression of COX-2, nuclear translocation of NF-κB, iNOS along with other cytokines (IL-1β, IL6 and TNF-α) in a dose-dependent manner.[115]Bhanwra *et al.*, proposed that hepatoprotective effect of Neem was probably due to its anti-inflammatory effect. Althaiban also supported the antioxidant and anti-inflammatory basis for the mechanism of action of neem leaf extract.[56,74]

Tables and Figures

4. Conclusion

Paracetamol induced a hepatotoxic effect in Wistar rats which caused increase in LFTs and liver weights. The increase in level of serum bilirubin and liver enzymes AST, ALP, ALT as well as liver weights and decrease in total serum protein and albumin globulin ratio following hepatic injury induced by Paracetamol were less in aqueous Neem leaf extract group compared to experimental control group. Chronic administration of aqueous Neem leaf extract also caused decrease in hepatic necrosis, showing regenerative activity on 10th day of the experiment, compared to such regeneration in untreated animals only on 20th day. All inflammatory and necrotic changes disappeared by the 30th day. Hence, we can conclude that chronic oral administration of *Azadirachta indica* (Neem) aqueous leaf

extract shows hepatoprotective activity in Wistar albino rats.

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Author Contribution

Author 1: Conception, design and development of the protocol, data collection and analysis, initial drafting and reviewing the manuscript and final approval of the prepared manuscript. **Author 2:** Conception, design and development of the protocol, supervision of experiments, data analysis and reviewing the manuscript. **Author 3:** Design and development of the protocol, supervision of experiments, data analysis and reviewing the manuscript. **Author 4:** Supporting role in conducting the experiments, re-drafting and reviewing the manuscript and final approval of the prepared manuscript.

Conflicts of Interest: No

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