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Research Article

PHARMACOLOGICAL EVALUATION OF STANDARDIZED
EXTRACT OF *AZADIRACHTA INDICA* ON DICLOFENAC-
INDUCED HEPATOCELLULAR DEATH IN
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Pharata, Ta - Shirur Dist. Pune-412211**Abstract:**

Background: Diclofenac is a widely used non-steroidal anti-inflammatory drug that may produce hepatic injury associated with oxidative stress, altered hepatic biochemical function, and apoptotic changes. The present study investigated the protective activity of a standardized extract of *Azadirachta indica* against diclofenac-induced hepatocellular injury in experimental rats.

Methods: Animals were randomly divided into six groups of six rats each. Diclofenac-induced hepatic injury was produced by administration of diclofenac (50 mg/kg, i.p.) on the 14th and 15th days. The standardized *A. indica* extract was administered orally at doses of 100, 200, and 400 mg/kg for 15 days. Silymarin was used as the reference treatment. Body weight, liver weight, serum AST, ALT, ALP, albumin, total bilirubin, and direct bilirubin were evaluated. Hepatic tissue was assessed for total protein, SOD, GSH, MDA, NO, and caspase-3 levels. Histopathological examination of liver sections was also performed.

Results: Diclofenac administration produced significant hepatic alterations, including increased liver weight, elevated AST, ALT, ALP, bilirubin, MDA, NO, and caspase-3 levels, together with reduced body weight, albumin, SOD, and GSH levels. Treatment with *A. indica* significantly attenuated these changes in a dose-dependent manner. The 400 mg/kg dose produced the most pronounced improvement in biochemical and oxidative stress parameters and markedly reduced histopathological abnormalities, including necrosis, congestion, inflammatory-cell infiltration, vesicular fat, and pyknosis.

Conclusion: The standardized *A. indica* extract demonstrated significant hepatoprotective activity against diclofenac-induced hepatic injury. Its protective effect was associated with improvement of hepatic biochemical function, restoration of antioxidant defense, reduction of oxidative/nitrosative stress, attenuation of caspase-3 levels, and preservation of liver architecture.

Keywords: *Azadirachta indica*; diclofenac; hepatoprotection; hepatocellular injury; oxidative stress; antioxidant defense; caspase-3; liver injury; SOD; GSH.

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INTRODCUTION:

Drug-induced liver injury (DILI) is an important adverse effect associated with the use of several therapeutic agents and represents a significant challenge in drug safety and clinical practice. The liver plays a central role in the metabolism and elimination of xenobiotics; consequently, exposure to certain drugs or their reactive metabolites can disturb hepatic cellular homeostasis and produce structural and functional damage. Hepatocellular injury may involve oxidative stress, mitochondrial dysfunction, inflammatory responses, disruption of cellular membranes, and activation of programmed cell-death pathways. Therefore, identification of agents capable of protecting hepatic tissue against drug-induced injury remains an important area of experimental pharmacological research.

Diclofenac is a commonly used non-steroidal anti-inflammatory drug (NSAID) employed for the management of pain and inflammatory disorders. Although it has established therapeutic utility, diclofenac has also been associated with hepatic adverse effects. Diclofenac-induced hepatic injury is characterized by alterations in biochemical markers of liver function and may involve oxidative imbalance and cellular injury. In experimental models, increased serum aminotransferases and alkaline phosphatase, alterations in bilirubin and albumin concentrations, and changes in hepatic antioxidant status are useful indicators of drug-associated hepatic damage. Oxidative stress may contribute to membrane lipid peroxidation and depletion of endogenous

antioxidant defenses, thereby increasing susceptibility of hepatocytes to injury.

The endogenous antioxidant system, including superoxide dismutase (SOD) and reduced glutathione (GSH), plays an important role in maintaining hepatic redox homeostasis. Excessive generation of reactive oxygen and nitrogen species can overwhelm these protective mechanisms, resulting in lipid peroxidation and accumulation of products such as malondialdehyde (MDA). Alterations in nitric oxide (NO) levels may additionally reflect changes in hepatic nitrosative status. Persistent cellular stress can also be associated with activation of apoptotic pathways. Caspase-3 is an important executioner caspase involved in the final stages of apoptosis and therefore provides a useful biochemical indicator for investigating hepatocellular death.

Natural products have attracted considerable interest as potential sources of hepatoprotective agents because of their diverse phytochemical constituents and biological activities. *Azadirachta indica* A. Juss. (family Meliaceae), commonly known as neem, is a medicinal plant traditionally used in various therapeutic applications. Different parts of the plant contain biologically active constituents that have been investigated for antioxidant and other pharmacological properties. The potential of *A. indica* to modulate oxidative processes provides a rationale for evaluating its protective activity against experimentally induced hepatic injury.



Figure 1: *Azadirachta indica* Plant

Despite the established use of diclofenac and the continuing interest in plant-derived hepatoprotective agents, evaluation of a standardized extract of *A. indica* using a combination of biochemical, oxidative stress, apoptotic, and histopathological endpoints provides

a relevant approach for characterizing its protective potential. A standardized extract may provide greater consistency in experimental evaluation than an inadequately characterized plant preparation. Therefore, the present study was designed to investigate the protective activity of a standardized

extract of *Azadirachta indica* against diclofenac-induced hepatocellular death in experimental rats. The study evaluated changes in body and liver weight, serum hepatic biomarkers including AST, ALT, ALP, albumin, total bilirubin and direct bilirubin, and hepatic oxidative stress and antioxidant parameters including SOD, GSH, MDA and NO. Hepatic caspase-3 levels and histopathological alterations were also assessed to further characterize the protective effect of the standardized extract.

MATERIALS AND METHODS:

MATERIALS:

A standardized extract of *Azadirachta indica* was obtained from Natural Remedies Pvt. Ltd., Bengaluru, India (Batch No. AI/08001) and used as the test material. Diclofenac injection (Dynapar®, 75 mg/vial) was procured from Troikaa Pharmaceuticals Ltd., Nani Daman, India (Batch No. D23S163) for induction of hepatocellular injury. Silymarin tablets (Silybon®, 140 mg) obtained from Micro Labs Ltd., Sikkim, India (Batch No. MI24F-0083) were used as the reference hepatoprotective treatment. Analytical-grade chemicals and biochemical reagents, including sodium citrate dihydrate, adenosine triphosphate, potassium hydroxide, sodium hydroxide, sulfuric acid, ammonium molybdate, acetic acid, trichloroacetic acid, potassium dihydrogen phosphate, Folin-Ciocalteu reagent, chloroform, boric acid, epinephrine, Tris-HCl, formaldehyde, hydrochloric acid, polysorbate 80, hydrogen peroxide, bovine serum albumin, Bradford reagent, sodium hydrogen carbonate, ethanol, NADPH, sulphanilamide, phosphoric acid, magnesium sulfate, potassium chloride, EDTA, sodium chloride, Griess reagent, malondialdehyde bis-dimethyl acetal, Triton X-100, potassium phosphate salts, potassium dichromate, 2-thiobarbituric acid, potassium perfusate, sodium phosphate dibasic, aminoguanidine bicarbonate, copper sulfate, 3,4-dihydroxyphenylacetic acid, acetylcholine iodide, and 1,1-diphenyl-2-picrylhydrazyl (DPPH) were obtained from the respective certified suppliers listed in the study records. Commercial biochemical assay kits for alanine aminotransferase (ALT; Cat. No. PD2410), aspartate aminotransferase (AST; Cat. No. PD2380), alkaline phosphatase (ALP; Cat. No. PD2310), albumin (Cat. No. PD2010), total bilirubin (Cat. No. BIL0250), and direct bilirubin (Cat. No. BIL0251) were procured from Pathozone Diagnostics, India. A rat caspase-3 ELISA kit (Cat. No. E-EL-R0160) was obtained from Elabscience, TX, USA. All chemicals and reagents were of analytical grade or equivalent and were used as received unless otherwise specified.

Animals:

Sprague Dawley rats weighing 180-200 gm were purchased Global Bioresearch Solutions Private Limited, H No 251 Nhavi, Tal - Bhore, Dist- Pune Bhore, Pune, Maharashtra-412206, India. The animals were housed in polypropylene cages and maintained under the environmental condition of temperature 25 ± 1 °C and relative humidity of 45-55 % under a 12h light: 12 dark cycles. The animals had free access to food pellets (Nav Maharashtra Chakan oil mills Ltd., Pune) and water ad libitum. The Institutional Animal Ethics Committee (IAEC) of 2168/PO/Re/S/22/CPCSEA approved all the experimental protocols under the Committee for the Purpose of Control and Supervision of Experiment on Animals (CPCSEA). The protocol approval number is 2168/PO/Re/S/22/CPCSEA.

METHODS:

Preparation and Administration of Test and Standard Drugs

The standardized extract of *Azadirachta indica* was freshly prepared in distilled water as the vehicle on each day of treatment. The powdered standardized extract was maintained in a desiccator until use. Following preparation, the drug suspension/solution was transferred to airtight amber-colored containers and maintained at room temperature during the experimental period. The required quantity of the preparation was calculated individually according to the body weight of each animal to ensure administration of the intended dose. The *Azadirachta indica* preparation was administered orally (p.o.) throughout the experimental study.

Silymarin was used as the reference hepatoprotective drug. The required quantity of silymarin was prepared using 1% sodium carboxymethylcellulose as the vehicle. Silymarin tablets were stored under refrigerated conditions ($2-8$ °C), and a fresh preparation was made daily before administration. The dose volume was adjusted according to the individual body weight of the experimental animals. Silymarin was administered orally.

Experimental Design and Induction of Diclofenac-Induced Hepatocellular Injury

The experimental animals were randomly allocated into six groups, with six animals in each group. The normal control group (Group I) received distilled water (10 mg/kg, p.o.) for 15 consecutive days. Animals in the vehicle control/disease control group (Group II) received distilled water (10 mg/kg, p.o.) throughout the 15-day treatment period and were administered diclofenac at a dose of 50 mg/kg intraperitoneally (i.p.) on the 14th and 15th days to induce hepatocellular injury.

Group III received silymarin at a dose of 100 mg/kg orally for 15 days, followed by diclofenac administration at 50 mg/kg i.p. on days 14 and 15.

Groups IV, V, and VI received the standardized *Azadirachta indica* extract at doses of 100, 200, and 400 mg/kg, respectively, by the oral route for 15 consecutive days. Diclofenac (50 mg/kg, i.p.) was administered to these groups on days 14 and 15. The treatment schedule was designed to evaluate the protective effect of the standardized *Azadirachta indica* extract against diclofenac-induced hepatocellular injury.

All treatment volumes were calculated individually based on the body weight of the respective animals. The experimental interventions were performed according to the approved experimental protocol and applicable institutional guidelines for the care and use of laboratory animals.

Assessment of Body Weight

Body weight was recorded daily throughout the experimental period using a calibrated laboratory animal weighing balance. The recorded body weights were used for calculation and adjustment of individual drug administration volumes.

Collection and Preparation of Serum

At the end of the experimental period, blood samples were collected from the animals for biochemical evaluation. The blood was allowed to undergo appropriate separation, and serum was obtained by centrifugation using an Eppendorf cryocentrifuge (Model 5810, Eppendorf, Hamburg, Germany) maintained at 4 °C. Samples were centrifuged at 7000 rpm for 15 min, and the resulting serum was collected for biochemical analysis.

Estimation of Serum Biochemical Parameters

Serum biochemical markers associated with hepatic function, including aspartate aminotransferase (AST), alanine aminotransferase (ALT), alkaline phosphatase (ALP), albumin, total bilirubin, and direct bilirubin, were determined using commercially available diagnostic reagent kits obtained from Pathozyne Diagnostics, India. The assays were performed according to the respective manufacturer's instructions. Absorbance measurements were carried out using a UV-visible spectrophotometer (Jasco V-530, Tokyo, Japan).

Preparation of Liver Tissue Homogenate

At the termination of the experiment, the animals were sacrificed according to the approved experimental procedure, and the liver was rapidly excised. The collected liver tissues were appropriately washed to remove adhering blood and processed for biochemical and histopathological investigations. For biochemical analysis, liver tissue was homogenized in 0.1 M Tris-HCl buffer (pH 7.4). The homogenate was subjected to centrifugation, and the resulting supernatant was used for estimation of total protein,

superoxide dismutase (SOD), reduced glutathione (GSH), malondialdehyde (MDA), and nitric oxide (NO).

Estimation of Lipid Peroxidation

Hepatic lipid peroxidation was assessed by determining malondialdehyde (MDA), a product of lipid peroxidation, using the method of Slater and Sawyer (1971). Briefly, 2.0 mL of liver tissue homogenate supernatant was mixed with 2.0 mL of freshly prepared 10% w/v trichloroacetic acid (TCA) and maintained in an ice bath for 15 min. The mixture was subsequently centrifuged to remove the precipitated material. An aliquot of 2.0 mL of the clear supernatant was then reacted with 2.0 mL of freshly prepared thiobarbituric acid (TBA) reagent. The reaction mixture was heated in a boiling water bath for 10 min and immediately cooled in an ice bath for 5 min. The developed chromogen was measured spectrophotometrically at 532 nm against an appropriate reagent blank. A standard curve was prepared using different concentrations of malondialdehyde (0–23 nM). MDA levels were expressed as nM/mg protein.

Estimation of Superoxide Dismutase Activity

Superoxide dismutase activity in the hepatic tissue homogenate was determined according to the method of Misra and Fridovich (1972). Briefly, 0.5 mL of tissue homogenate was diluted with 0.5 mL of distilled water. Ice-cold ethanol (0.25 mL) and ice-cold chloroform (0.15 mL) were added, and the mixture was thoroughly mixed for 5 min using a cyclomixer. The mixture was centrifuged at 2500 rpm, and 0.5 mL of the resulting supernatant was combined with 1.5 mL of carbonate buffer and 0.5 mL of EDTA solution. The reaction was initiated by addition of epinephrine, and the change in absorbance per minute was recorded at 480 nm against the reagent blank. SOD activity was expressed as units/mg protein. The enzyme unit was defined based on the degree of inhibition of epinephrine oxidation to adrenochrome. A calibration curve was prepared using SOD standards corresponding to 10–125 units.

Estimation of Reduced Glutathione

Reduced glutathione (GSH) content was estimated using the method described by Moron et al. (1979). Equal volumes of tissue homogenate supernatant and 20% TCA were mixed to precipitate proteins. Following centrifugation, 0.25 mL of the clear supernatant was collected and reacted with 2 mL of DTNB reagent. The reaction mixture was made up to a final volume of 3 mL using phosphate buffer. The intensity of the developed color was measured at 412 nm against the reagent blank. A standard calibration curve was generated using glutathione standards in the specified concentration range. The hepatic GSH concentration was expressed as µg/mg

protein.

Estimation of Nitric Oxide

Hepatic nitric oxide (NO) levels were determined indirectly by measuring nitrite using the Griess reaction following reduction of nitrate to nitrite, according to the method described by Miranda et al. (2001). The assay was based on the formation of a colored azo compound following the reaction of nitrite with sulphanilamide and N-(1-naphthyl)ethylenediamine. The resulting pink-colored product exhibited maximum absorbance at 543 nm. A standard calibration curve was prepared using sodium nitrate, and NO levels were expressed as $\mu\text{g}/\text{mg}$ protein.

Estimation of Total Protein

The protein content of the hepatic tissue samples was determined using the method of Lowry et al. (1951), with bovine serum albumin (BSA) used as the reference standard. Briefly, 0.1 mL aliquots of appropriately diluted tissue samples were transferred to test tubes, followed by addition of 0.8 mL of 0.1 M sodium hydroxide and 5.0 mL of Lowry reagent. The reaction mixture was allowed to stand for 15 min. Subsequently, 0.5 mL of Folin–Ciocalteu phenol reagent was added, and the contents were thoroughly mixed. After development of the characteristic color, absorbance was measured at 660 nm against a reagent blank containing distilled water in place of the sample. A calibration curve was prepared using BSA standards in the range of 40–200 μg . Protein concentration was expressed as mg/g of wet liver tissue.

Determination of Hepatic Caspase-3

Hepatic caspase-3 concentration was quantified using a commercially available rat caspase-3 ELISA kit (Elabscience, TX, USA; Cat. No. E-EL-R0160) according to the manufacturer's instructions. The assay was based on a sandwich enzyme immunoassay principle. Microplate wells precoated with a monoclonal antibody specific for rat caspase-3 were incubated with standards, controls, and tissue samples. Caspase-3 present in the samples was captured by the immobilized antibody. Following removal of unbound material by washing, an enzyme-linked polyclonal antibody specific for rat caspase-3 was added. After a further washing step, substrate solution was added, resulting in development of a colored product. The reaction was terminated using the supplied stop solution, producing a color change suitable for

spectrophotometric measurement.

The intensity of the developed color was proportional to the amount of caspase-3 present in the sample. A standard curve was generated using serial dilutions of the recombinant standard on each assay plate, and sample concentrations were calculated from the corresponding standard curve. Hepatic caspase-3 levels were expressed as ng/mg hepatic tissue.

Histopathological Examination

For histopathological evaluation, representative liver tissue samples were collected immediately after sacrifice and fixed in 10% formalin. The fixed tissues were processed through graded isopropyl alcohol and xylene, followed by paraffin embedding. Paraffin blocks were sectioned at approximately 5 μm thickness, and the sections were stained with hematoxylin and eosin (H&E) using a standard staining procedure. The stained sections were examined under a Nikon E200 light microscope at 100 $\times$ magnification. Histological alterations were evaluated qualitatively with particular attention to inflammatory cell infiltration, vascular congestion, edema, hepatocellular degeneration, and necrotic changes.

Statistical Analysis

The experimental data were analyzed using GraphPad Prism version 5.0 (GraphPad Software, San Diego, CA, USA). Results were expressed as mean $\pm$ standard error of the mean (SEM), where applicable. Statistical comparisons were performed between the treatment groups and the vehicle/disease control group. A probability value of $P < 0.05$ was considered indicative of statistical significance.

RESULTS:

Effect of *Azadirachta indica* on Body Weight

Diclofenac administration produced a significant reduction in body weight in the vehicle-control group compared with the normal group ($P < 0.001$). The mean body weight was 237.20 ± 3.72 g in normal animals and decreased to 210.30 ± 1.99 g following diclofenac administration. Treatment with silymarin significantly attenuated the reduction in body weight, with a mean value of 235.20 ± 3.70 g ($P < 0.01$ vs. vehicle control). Among the *Azadirachta indica* treatment groups, the 400 mg/kg dose significantly improved body weight (231.20 ± 3.75 g; $P < 0.01$), whereas the 100 and 200 mg/kg doses did not produce statistically significant changes compared with the vehicle-control group.

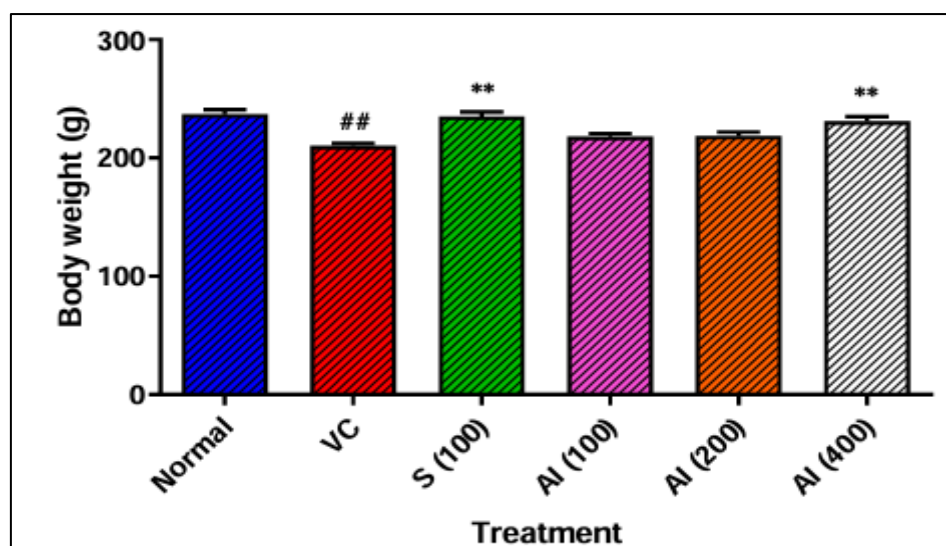


Figure 2: Effect of *Azadirachta indica* on diclofenac-induced alteration in body weight

Data were analyzed by One-Way ANOVA followed by Dunnett's. ###P < 0.001 as compared with normal group and **P < 0.01 as compared with Vehicle Control group on respective days.

Effect on Absolute and Relative Liver Weight

Diclofenac exposure resulted in a marked increase in both absolute and relative liver weights compared with normal animals ($P < 0.001$). Absolute liver weight increased from 4.68 ± 0.20 g in the normal group to 8.15 ± 0.33 g in the vehicle-control group, while the liver-to-body-weight ratio increased from 0.020 ± 0.001 to 0.039 ± 0.002 .

Silymarin significantly reduced both parameters ($P < 0.001$). Administration of the *A. indica* extract produced a dose-related reduction in liver weight. At 200 mg/kg, absolute liver weight decreased to 6.40 ± 0.25 g and the relative liver weight to 0.029 ± 0.001 ($P < 0.01$). The 400 mg/kg dose produced a further reduction to 5.53 ± 0.25 g and 0.024 ± 0.001 , respectively ($P < 0.001$).

Table 1: Effect of standardized *Azadirachta indica* extract on body weight and liver weight

Parameter	Normal	Vehicle control	Silymarin	AI 100 mg/kg	AI 200 mg/kg	AI 400 mg/kg
Body weight (g)	237.20 ± 3.72	210.30 ± 1.99###	235.20 ± 3.70**	218.20 ± 2.39	218.50 ± 3.25	231.20 ± 3.75**
Absolute liver weight (g)	4.68 ± 0.20	8.15 ± 0.33###	4.88 ± 0.29***	7.55 ± 0.23	6.40 ± 0.25**	5.53 ± 0.25***
Relative liver weight (liver weight/body weight)	0.020 ± 0.001	0.039 ± 0.002###	0.021 ± 0.001***	0.035 ± 0.001	0.029 ± 0.001**	0.024 ± 0.001***

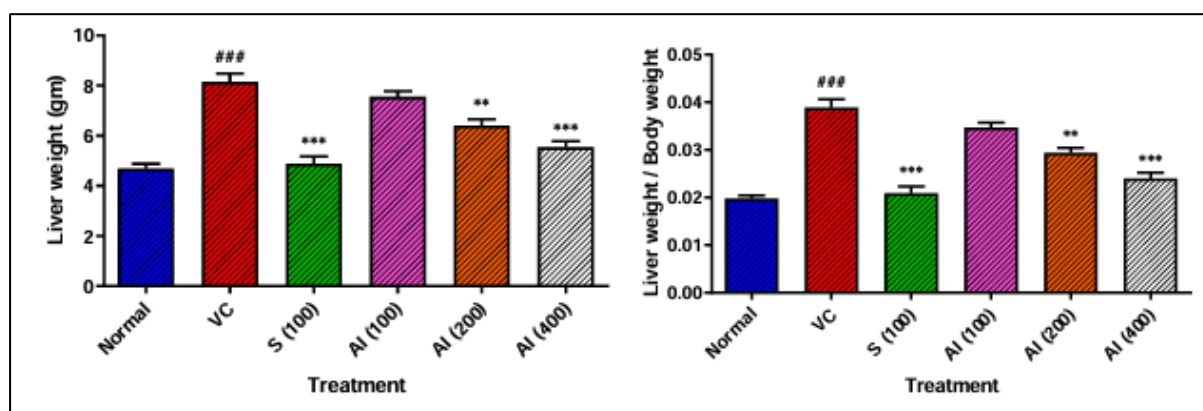


Figure 3: Effect of *Azadirachta indica* on diclofenac-induced alteration in absolute and relative liver weights

Data were analyzed by One-Way ANOVA followed by Dunnett's. ###P < 0.001 as compared with normal group and **P < 0.01, ***P < 0.001 as compared with Vehicle Control group on respective days.

Effect on Serum AST and ALT

Diclofenac challenge significantly elevated serum AST and ALT activities compared with the normal group ($P < 0.001$). AST increased from 79.76 ± 11.98 IU/L in normal rats to 306.00 ± 12.56 IU/L in vehicle-control animals, whereas ALT increased from 33.41 ± 5.13 to 146.20 ± 3.10 IU/L. Silymarin markedly reduced both enzyme activities, yielding AST and ALT values of 118.10 ± 11.05 and 46.46

± 4.47 IU/L, respectively ($P < 0.001$ vs. vehicle control). The *A. indica* extract demonstrated a dose-dependent reduction in transaminase activities. At 200 mg/kg, AST and ALT decreased to 233.00 ± 8.74 and 93.89 ± 7.25 IU/L, respectively ($P < 0.01$), while the 400 mg/kg dose produced greater reductions to 160.40 ± 11.45 and 60.67 ± 7.08 IU/L, respectively ($P < 0.001$).

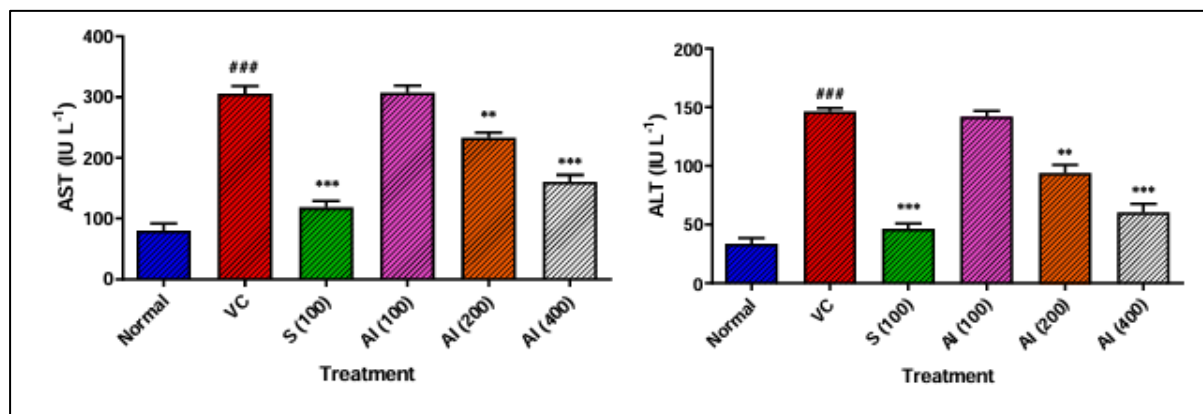


Figure 4: Effect of *Azadirachta indica* on diclofenac-induced alteration in AST and ALT levels

Data were analyzed by One-way ANOVA followed by Dunnett's test. ### $P < 0.001$ as compared with normal group and ** $P < 0.01$, *** $P < 0.001$ as compared with Vehicle Control group.

Effect on Serum Alkaline Phosphatase

Serum ALP activity was substantially increased in diclofenac-treated animals compared with the normal group ($P < 0.001$), increasing from 51.19 ± 3.80 to 390.80 ± 3.85 U/L. Silymarin reduced ALP activity to 93.56 ± 3.68 U/L ($P < 0.001$ vs. vehicle control). Treatment with *A. indica* resulted in a dose-dependent reduction in ALP activity. The 200 mg/kg dose decreased ALP to 328.00 ± 6.16 U/L ($P < 0.01$), whereas the 400 mg/kg dose produced a greater reduction to 258.90 ± 3.72 U/L ($P < 0.001$).

Effect on Serum Albumin

A significant decline in serum albumin was observed following diclofenac administration, with albumin decreasing from 4.25 ± 0.28 g/dL in normal animals to 1.25 ± 0.25 g/dL in the vehicle-control group ($P < 0.001$). Silymarin substantially restored albumin levels to 4.06 ± 0.47 g/dL ($P < 0.001$). Treatment with *A. indica* increased albumin concentrations in a dose-dependent manner.

Albumin levels reached 2.17 ± 0.13 g/dL at 200 mg/kg ($P < 0.01$) and 3.19 ± 0.42 g/dL at 400 mg/kg ($P < 0.001$) compared with vehicle control.

Effect on Total and Direct Bilirubin

Diclofenac administration significantly increased serum total and direct bilirubin concentrations relative to normal animals ($P < 0.001$). Total bilirubin increased from 0.20 ± 0.03 to 0.66 ± 0.03 mg%, while direct bilirubin increased from 0.13 ± 0.03 to 0.32 ± 0.03 mg%. Silymarin significantly reduced total and direct bilirubin to 0.30 ± 0.01 and 0.17 ± 0.03 mg%, respectively ($P < 0.001$). *A. indica* treatment also reduced bilirubin levels in a dose-dependent manner. At 200 mg/kg, total and direct bilirubin decreased to 0.50 ± 0.04 and 0.29 ± 0.03 mg%, respectively ($P < 0.01$). At 400 mg/kg, the corresponding values were 0.43 ± 0.03 and 0.26 ± 0.04 mg%, with significant reductions compared with vehicle control.

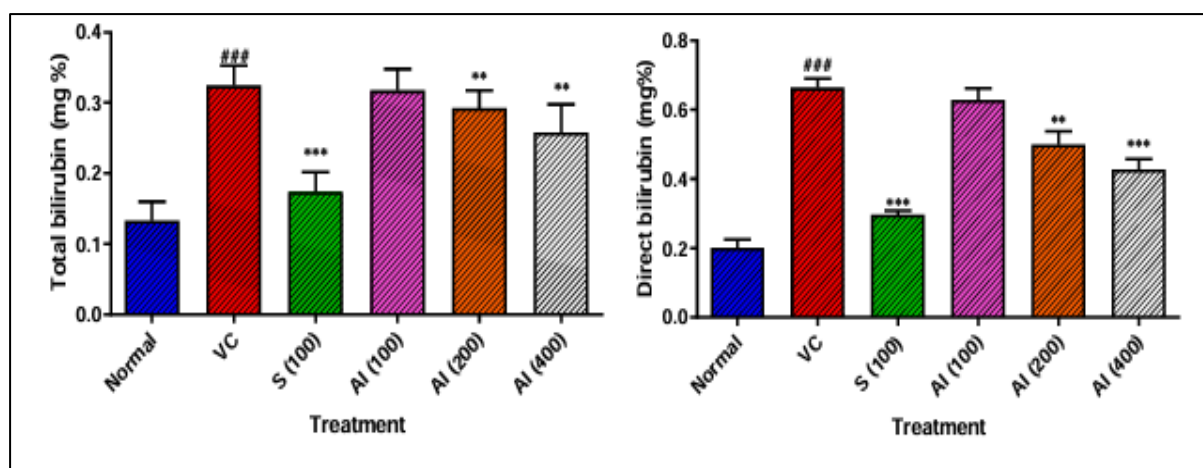


Figure 5: Effect of *Azadirachta indica* on diclofenac-induced alteration in total and direct bilirubin levels
Data were analyzed by One-way ANOVA followed by Dunnett's test. ### $P < 0.001$ as compared with normal group and ** $P < 0.01$, *** $P < 0.001$ as compared with Vehicle Control group.

Effect on Hepatic Total Protein

The vehicle-control group exhibited a significant increase in hepatic total protein compared with the normal group ($P < 0.001$), with values of 85.17 ± 1.68 and 46.36 ± 1.52 mg/g, respectively. Silymarin significantly reduced the elevated protein concentration to 53.52 ± 1.55 mg/g ($P < 0.001$). Similarly, *A. indica* treatment produced a dose-dependent reduction. The 200 mg/kg dose reduced hepatic protein to 73.72 ± 1.57 mg/g ($P < 0.01$), while the 400 mg/kg dose reduced it to 54.21 ± 0.91 mg/g ($P < 0.001$) compared with vehicle control.

Effect on Hepatic SOD and GSH

Diclofenac administration significantly depleted

hepatic antioxidant defenses. SOD activity decreased from 6.96 ± 0.43 U/mg protein in normal animals to 2.25 ± 0.34 U/mg protein in the vehicle-control group ($P < 0.001$). Similarly, GSH decreased from 14.63 ± 0.80 to 5.05 ± 0.83 μ g/mg protein ($P < 0.001$). Silymarin significantly restored both SOD and GSH levels, reaching 5.82 ± 0.45 U/mg protein and 14.30 ± 0.64 μ g/mg protein, respectively ($P < 0.001$). *A. indica* produced a dose-dependent restoration of antioxidant status. At 200 mg/kg, SOD and GSH increased to 3.46 ± 0.48 U/mg protein and 11.25 ± 0.69 μ g/mg protein, respectively ($P < 0.01$). The 400 mg/kg dose produced further increases to 6.10 ± 0.42 U/mg protein and 12.43 ± 0.61 μ g/mg protein ($P < 0.001$).

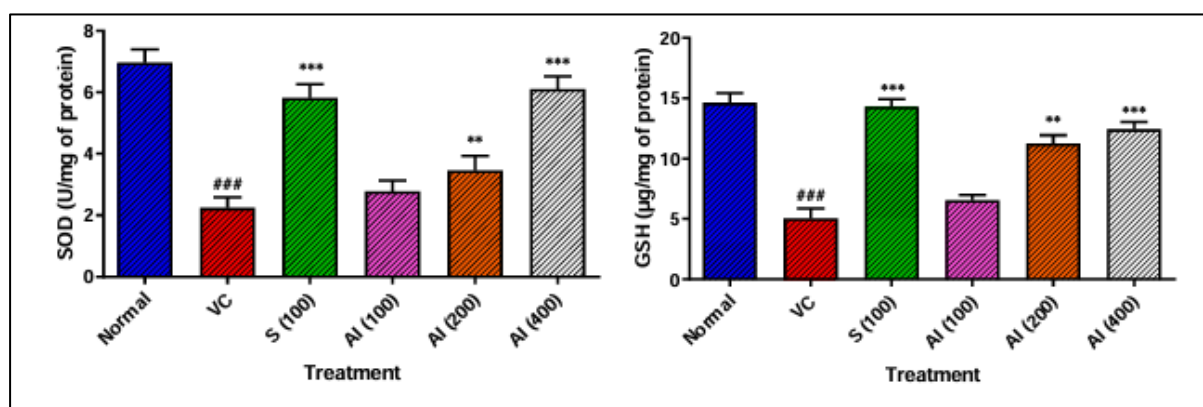


Figure 6: Effect of *Azadirachta indica* on diclofenac-induced alteration in liver SOD and GSH level
Data were analyzed by One-way ANOVA followed by Dunnett's test. ### $P < 0.001$ as compared with normal group and ** $P < 0.01$, *** $P < 0.001$ as compared with Vehicle Control group.

Effect on Hepatic MDA and Nitric Oxide

Diclofenac challenge significantly increased hepatic oxidative and nitrosative stress markers. MDA concentration increased from 0.22 ± 0.04 nM/mg protein in normal animals to 0.82 ± 0.03 nM/mg protein in vehicle-control rats ($P < 0.001$). Similarly, hepatic NO increased from 0.19 ± 0.02

to 0.85 ± 0.05 μ g/mg protein ($P < 0.001$). Silymarin significantly attenuated these elevations, reducing MDA and NO to 0.34 ± 0.03 nM/mg protein and 0.23 ± 0.02 μ g/mg protein, respectively ($P < 0.001$). Treatment with *A. indica* at 200 mg/kg significantly reduced MDA and NO to 0.55 ± 0.04 nM/mg protein and 0.41 ± 0.05 μ g/mg protein,

respectively ($P < 0.01$). The 400 mg/kg dose produced a more pronounced reduction, with MDA and NO values of 0.40 ± 0.04 nM/mg protein and

0.24 ± 0.03 μ g/mg protein, respectively ($P < 0.001$).

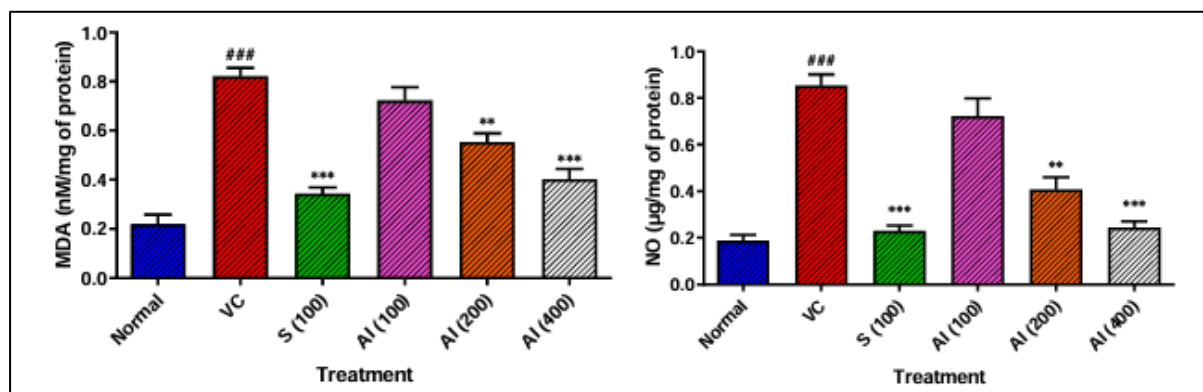


Figure 7: Effect of Azadirachta indica on diclofenac-induced alteration in liver MDA and NO level
Data were analyzed by One-way ANOVA followed by Dunnett's test. ### $P < 0.001$ as compared with normal group and ** $P < 0.01$, *** $P < 0.001$ as compared with Vehicle Control group.

Effect on Hepatic Caspase-3

Hepatic caspase-3 concentration was significantly elevated following diclofenac administration, increasing from 0.47 ± 0.10 ng/mg in normal rats to 1.96 ± 0.10 ng/mg in the vehicle-control group ($P < 0.001$). Silymarin significantly reduced caspase-3 concentration to 0.69 ± 0.19 ng/mg ($P < 0.001$).

Treatment with *A. indica* produced a dose-dependent decline in caspase-3. The 200 mg/kg dose reduced the level to 1.37 ± 0.15 ng/mg ($P < 0.01$), while the 400 mg/kg dose produced a greater reduction to 0.76 ± 0.14 ng/mg ($P < 0.001$) compared with the vehicle-control group.

Table2: Effect of standardized Azadirachta indica extract on hepatic biochemical and oxidative stress parameters

Parameter	Normal	Vehicle control	Silymarin	AI 100 mg/kg	AI 200 mg/kg	AI 400 mg/kg
Total protein (mg/g)	46.36 ± 1.52	85.17 ± 1.68 ###	53.52 ± 1.55 ***	83.00 ± 1.28	73.72 ± 1.57 **	54.21 ± 0.91 ***
SOD (U/mg protein)	6.96 ± 0.43	2.25 ± 0.34 ###	5.82 ± 0.45 ***	2.78 ± 0.36	3.46 ± 0.48 **	6.10 ± 0.42 ***
GSH (μ g/mg protein)	14.63 ± 0.80	5.05 ± 0.83 ###	14.30 ± 0.64 ***	6.55 ± 0.44	11.25 ± 0.69 **	12.43 ± 0.61 ***
MDA (nM/mg protein)	0.22 ± 0.04	0.82 ± 0.03 ###	0.34 ± 0.03 ***	0.72 ± 0.06	0.55 ± 0.04 **	0.40 ± 0.04 ***
NO (μ g/mg protein)	0.19 ± 0.02	0.85 ± 0.05 ###	0.23 ± 0.02 ***	0.72 ± 0.08	0.41 ± 0.05 **	0.24 ± 0.03 ***
Caspase-3 (ng/mg)	0.47 ± 0.10	1.96 ± 0.10 ###	0.69 ± 0.19 ***	1.69 ± 0.12	1.37 ± 0.15 **	0.76 ± 0.14 ***

Histopathological Studies

Histopathological examination demonstrated substantial structural alterations in the liver following diclofenac exposure. Liver sections from normal animals showed largely preserved hepatic architecture with no evident necrosis, congestion, or vesicular fatty changes and only mild inflammatory-cell presence. In contrast, the vehicle-control group exhibited extensive hepatic damage characterized by severe necrosis, congestion, vesicular fat accumulation, inflammatory-cell infiltration, and pyknosis. Silymarin treatment markedly improved the hepatic

architecture, with only mild necrosis, congestion, and vesicular fatty changes and an absence of inflammatory-cell infiltration and pyknosis. Treatment with *A. indica* at 200 mg/kg produced moderate restoration of hepatic architecture, although grade 2 changes in necrosis, congestion, inflammatory-cell infiltration, and pyknosis remained evident. The 400 mg/kg dose produced a more pronounced histological improvement, characterized by only mild necrosis, congestion, vesicular fat, and pyknosis, with no detectable inflammatory-cell infiltration.

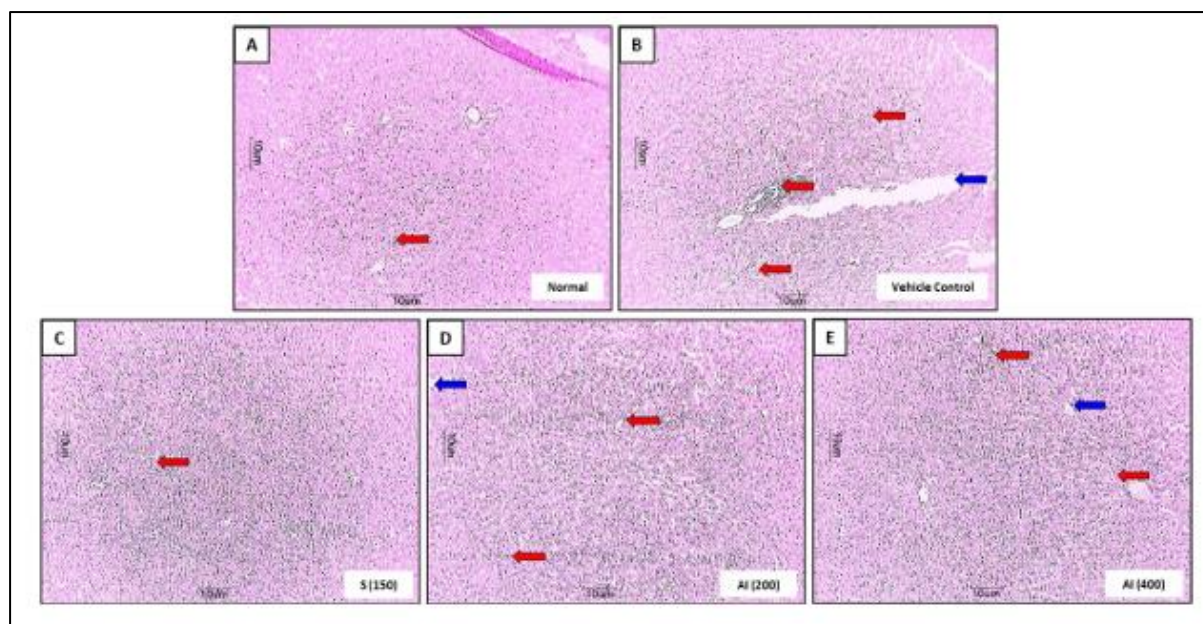


Figure 8: Histopathological representation of liver tissue from normal rats (A), Vehicle Control rats (B), Silymarin (400 mg/kg) treated rats (C), AI (400 mg/kg) treated rats (D). Stained with H&E (at 100 X). Infiltration of neutrophils (red arrow) and necrosis (blue arrow) in liver tissue.

The semiquantitative histopathological assessment further supported the protective effect of the extract. The vehicle-control group showed grade 4 changes in necrosis, congestion, and vesicular fat, together with grade 3 inflammatory-cell infiltration and pyknosis. Silymarin-treated animals showed only grade 1 changes in necrosis, congestion, and vesicular fat, with no inflammatory-cell infiltration or pyknosis. The 200 mg/kg *A. indica* group exhibited grade 2 changes in necrosis, congestion, inflammatory-cell infiltration, and pyknosis, whereas vesicular fat remained at grade 1. The 400 mg/kg group showed grade 1 changes in necrosis, congestion, vesicular fat, and pyknosis, with no inflammatory-cell infiltration.

DISCUSSION:

The present study demonstrated the protective effect of standardized *Azadirachta indica* (AI) extract against diclofenac-induced hepatic injury in rats. Diclofenac administration produced significant alterations in body and liver weight, serum AST, ALT, ALP, albumin, and bilirubin levels, indicating marked hepatic injury. Treatment with AI improved these parameters in a dose-dependent manner, with the 400 mg/kg dose producing the most pronounced response.

Diclofenac also induced oxidative stress, as evidenced by reduced hepatic SOD and GSH and increased MDA and NO levels. AI treatment significantly restored antioxidant status and reduced these oxidative stress markers, particularly at 400 mg/kg.

Hepatic caspase-3 was markedly elevated following diclofenac administration, whereas AI treatment significantly reduced its level in a dose-

dependent manner. Histopathological examination further supported the biochemical findings, showing reduced necrosis, congestion, inflammatory-cell infiltration, and pyknosis following AI treatment. The 400 mg/kg dose produced the greatest improvement in hepatic architecture. Overall, the findings suggest that standardized AI extract attenuates diclofenac-induced hepatic injury through improvement of hepatic biochemical function, antioxidant defense, oxidative stress status, and apoptotic changes.

CONCLUSION:

The present study demonstrated that diclofenac administration produced substantial hepatic alterations in experimental rats, as evidenced by changes in body weight and liver weight, marked elevations in serum AST, ALT, ALP, total bilirubin, and direct bilirubin, together with a reduction in serum albumin. Diclofenac exposure was also associated with depletion of hepatic SOD and GSH, increased MDA and NO levels, elevation of hepatic caspase-3, and pronounced histopathological abnormalities.

Treatment with the standardized *Azadirachta indica* extract attenuated these alterations in a dose-dependent manner. The 200 mg/kg dose produced significant improvement in several biochemical and oxidative stress parameters, whereas the 400 mg/kg dose generally produced the most pronounced protective response. At 400 mg/kg, the extract significantly reduced hepatic enzyme elevations, improved albumin and bilirubin profiles, restored hepatic antioxidant status, reduced MDA and NO levels, and lowered hepatic caspase-3 concentration. These biochemical improvements

were supported by the marked reduction in histopathological abnormalities observed in the liver sections. Taken together, the findings support the hepatoprotective potential of the standardized *A. indica* extract against diclofenac-associated hepatic injury in this experimental model. The observed protection was associated with improvement of serum hepatic biomarkers, preservation of antioxidant defenses, reduction of lipid peroxidation and altered nitric oxide levels, attenuation of caspase-3, and improvement of hepatic tissue architecture. The 400 mg/kg treatment produced the most consistent overall response among the tested extract doses. Further studies involving detailed molecular investigations, characterization of the active phytoconstituents, dose optimization, and mechanistic evaluation of apoptotic and oxidative-stress pathways would be useful for establishing the precise basis of the hepatoprotective activity of standardized *A. indica* extract.

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