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Endocrine, Metabolic & Immune Disorders - Drug Targets, **2021**, *21*, **1-00**

RESEARCH ARTICLE

Appraisal of Anti-Arthritic and Anti-Inflammatory Potential of Folkloric Medicinal Plant *Peganum Harmala*

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Abstract: ***Background*:** *Peganum harmala* has been traditionally used to manage rheumatoid arthritis (RA) and other inflammatory conditions. However, its use against RA has not been scientifically evaluated. The current study was designed to assess the anti-arthritic and anti-inflammatory activities of the methanolic extract of *P. harmala* leaves by *in vitro* and *in vivo* methods.

***Methods*:** The *in vitro* assays were carried out to determine the effect of plant extract on inhibition of egg albumin denaturation and human red blood cell membrane (HRBC) stabilization. Moreover, 2,2-diphenyl-1-picrylhydrazyl (DPPH) scavenging activity was performed to determine the antioxidant potential. *In vivo* anti-arthritic activity was performed by determining the curative effect against Complete Freund's adjuvant (0.1 ml). The plant extract was administered to rats orally at 200, 400 and 600 mg/kg/day for 21 days.

***Results*:** The values of IC_{50} of plant extract in protein denaturation, stabilization of HRBC and DPPH assays were 77.54 mg/ml, 23.90 mg/ml and 58.09 µg/ml, respectively. Moreover, the plant extract significantly attenuated the poly-arthritis and weight loss, anemia and paw edema. The plant extract restored the level of C-reactive protein, rheumatoid factor, alanine transaminase, aspartate transaminase and alkaline phosphatase in poly-arthritic rats. Moreover, the plant extract restored the immune organs' weight in treated rats. Treatment with *P. harmala* also significantly subdued the oxidative stress by reinstating superoxide dismutase, reduced glutathione, catalase and malondialdehyde in poly-arthritic rats. The plant extract notably restored the prostaglandin-E2 and tumor necrosis factor (TNF)-α in the serum of poly-arthritic rats.

***Conclusion*:** It was concluded that *P. harmala* extract had potential antioxidant, anti-inflammatory and antiarthritic activities, which primarily might be attributed to alkaloids, flavonoids and phenols.

ARTICLE HISTORY

Received: August 03, 2020
Revised: October 06, 2020
Accepted: November 19, 2020

DOI:
10.2174/1871530321666210208211310

Keywords: Rheumatoid arthritis, anti-arthritic, anti-inflammatory, antioxidant, complete Freund's adjuvant, *Peganum harmala*.

1. INTRODUCTION

Rheumatoid Arthritis (RA) is an autoimmune disorder indicated by the persistent inflammation, pain and stiffness of joints. If left untreated, it may result in permanent discomfort, dysfunction and deformation of the joints. The hand, feet and wrist joints are the most commonly affected. About 1% of the human population suffers from RA. The prevalence of RA depends on gender, genetics and ethnicity. Americans are more affected than Asians and females are more susceptible to RA than males. The RA occurs mostly in middle age [1]. The diagnosis of RA is made if the symptoms such as hardness of joints during the first hour of awakening, swelling of the soft tissues and joints of hand, feet

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and wrist, an elevated rheumatoid factor (RF), and joint erosion are present [2].

The confirmed etiology and pathogenesis of this multifaceted syndrome are still unclear. There is an imbalance between pro interleukin (IL)-6, IL-1β and tumor necrosis factor (TNF)-α and anti-inflammatory (IL-4 and IL-10) cytokines. Moreover, the oxidative burden is a crucial disruptor of cytokine signaling pathways, for instance, nuclear factor kappa (NF-κ) B and mitogen-activated protein kinase (MAPK) signaling pathways are implicated in joint and cartilage destruction in RA. The existing treatment options of RA, such as non-steroidal anti-inflammatory drugs (NSAIDs) i.e., piroxicam, diclofenac lessen the associated pain and inflammation of joints. The progression of RA is usually retarding by disease-modifying anti-rheumatic drugs (DMARDs) such as hydroxychloroquine, leflunomide, etc., through suppression of the immunological responses of the body. Corticosteroids are also useful in the pharmacological management of RA [3]. Nevertheless, gastric mucosal disturbances, gastric ulcers, skin rashes, hepatotoxicity, nephrotoxicity and bone marrow suppression are the major limitations of these drugs. Moreover, RA patients are inclined to alternative ways of managing RA due to polypharmacy, expensive therapies and adverse effects [4].

Despite the advancement of medical sciences and the development of new drugs, people are reliant on herbal medicines to cure multiple ailments. *Peganum harmala* (family Zygophyllaceae) is a flowering perennial plant and is commonly named "Wild Rue" "Harmal" and "Esphand". It is used in several regions of the world for the management of rheumatism [5]. It is also utilized as a remedy for cough, asthma, skin infections, rheumatic pains, tapeworm infections, muscle spasms, hypertension and as an abortifacient. It is also used to improve vigor, libido and breast milk production [6, 7]. *P. harmala* contains several known secondary metabolites in its roots, seeds, leaf and flowers, such as alkaloids, anthraquinones, flavonoids and phenols. The several compounds isolated from this plant include Harman, harmaline, harmalol, harmol and harmine [8, 9].

Based on folkloric uses, the extracts of seeds, roots, and leaves of *P. harmala* have been efficiently investigated as a treatment for bacterial, fungal and plasmodial infections [5, 10, 11]. The plant *P. harmala* has also shown significant antidepressant, antihypertensive, hypoglycemic and analgesic activities [12-14]. It is also reported as a potential cure for abdominal spasm, ulcers and diarrhea. Moreover, it exhibited anti-nociceptive, anti-tumor, cytoprotective, anti-diabetic and antioxidant potential [11]. The plant, *P. harmala,* contains several pharmacologically active phytochemicals such as alkaloids, flavonoids and phenolic compounds. The anti-arthritic and anti-inflammatory use of its different parts has mostly been reported and evaluated for inhalation and external applications [15]. However, the low systemic bioavailability of several active ingredients may be a limiting factor in ameliorating the systemic inflammation associated with RA. Therefore, the present study was designed to evaluate the *P. harmala* extract against inflammation and arthritis by oral route.

2. MATERIAL AND METHODS

2.1. Chemicals

Ascorbic acid, Complete Freund's adjuvant (CFA), Thiobarbituric acid (TBA), 2, 2-diphenyl-1-picrylhydrazyl (DPPH), Trichloroacetic acid (TCA) (Sigma-Aldrich®) and Pyrogallol (Sigma-Life Science®) and Diclofenac sodium (DS) (Sami Pharma, Pakistan) were acquired for the current study. All the other chemicals were of analytical grade.

2.2. Animals

Mature Wistar rats (♂/♀) of body weight 240-270 g were acquired and housed in the animal house under standard laboratory conditions i.e., 12 h light/ dark cycle at 25±3°C with prior acclimatization of two weeks. Standard chow diet and water were given to rats in free access. The prior approval was obtained from the Institutional Ethical Committee of GCUF letter No. 2277.

2.3. Plant Identification

The leaves of *P. harmala* were collected from the Upper Dir, Malakand division, Khyber Pakhtunkhwa, Pakistan. The specimen of plant material was authenticated by an expert taxonomist from the Botany Department, University of Agriculture, Faisalabad, Pakistan. The specimen of leaves of *P. harmala* was deposited in the herbarium of the same university tagged with voucher # 2612-02-2018.

2.4. Preparation of Extract

The *P. harmala* leaves were ground to coarse powder after washing and shade drying. The powdered material was soaked in methanol at 1:10 (powder: solvent) for 14 days with occasional daily shaking. It was then filtered and concentrated on a rotary evaporator at 40°C to get a thick semi-solid paste that was further dried in a hot air oven at 37°C. The percentage yield of dried plant extract was 7.2% and the extract was kept at 2-8°C [16].

2.5. Phytochemical Analysis

The qualitative and quantitative phytochemical analysis was performed on the methanolic extract of *P. harmala*. The standard procedure was adopted to detect the secondary metabolites (i.e. alkaloids, carbohydrates, flavonoids, phenols, and steroids) in the crude extract.

2.6. Total Flavonoid Content (TFC)

In a test tube, 0.3 ml of the sample solution (1 mg/ml), 3.4 ml of 30% v/v methanol, 0.5 ml of 0.5 M sodium nitrite ($NaNO_2$) and 0.5 ml of 0.3 M $AlCl_3$ were thoroughly mixed and incubated for 5 min. Afterward, 1 ml of 1 N NaOH was added and the absorbance was determined at 506 nm by UV-Vis spectrophotometer. The rutin solutions of different

concentrations were used as a reference standard, while methanol served as a blank [17].

2.7. Total Phenolic Content (TPC)

In a test tube, 1 ml of the sample solution (1 mg/ml), 2.5 ml of the 10% Folin- Ciocalteau reagent and 2 ml of the 2% w/v sodium carbonate were thoroughly mixed and incubated at room temperature for 15 min. Gallic acid was used as a standard. The absorbance of these solutions was taken at 765 nm [18].

2.8. *In vitro* Antioxidant Activity

For performing antioxidant activity, a sample solution (1 ml) of *P. harmala* extract at different concentrations (250, 125, 62.5, 31.2. 15.62 and 7.81 µg/ml) was mixed with 3 ml DPPH solution (0.1 mM) in methanol in separate test tubes. Vitamin C was used as a reference drug, while methanol served as a blank. All admixed solutions were incubated for 30 min before determining the absorbance at 517 nm. The percentage scavenging of DPPH was assessed by the following formula [19].

Percentage scavenging activity = [1-(absorbance of test sample/absorbance of control)] ×100

2.9. *In vitro* Anti-Inflammatory Activity

2.9.1. HRBC Membrane Stabilization Assay

Fresh blood was acquired from the healthy person who avoided taking any cyclooxygenase inhibitor for two weeks. An equal volume of sterile Alsever's solution was transferred to the blood and red blood cells (RBCs) were separated by centrifugation at 3000 rpm for 10 min. The RBCs were cleaned thrice with phosphate buffer saline (PBS). The suspension of RBCs (10% v/v) and hypotonic solution (NaCl: 0.36% w/v) were prepared. The test solution was prepared by mixing 0.5 ml of the RBC suspension, 2 ml hypotonic solution, 1 ml PBS (pH 7.4) and 1 ml *P. harmala* solution of 50, 25, 12.5, 6.25 and 3.12 mg/ml concentration. The distilled water (DW) was used as the reference control solution, while diclofenac sodium (DS) was taken as a standard control. Each solution was kept for 10 min at 37°C and thereafter centrifuged at 3000 rpm for 10 min. The supernatant was obtained to determine the absorbance at 560 nm. The percentage protection of HRBC stabilization was calculated by the following formula [20].

% inhibition of membrane stabilization = 100 x $[(OD_1 - OD_2) / OD_1]$

Where OD_1 = absorbance of control solution, OD_2 = absorbance of test solution

2.9.2. Protein Denaturation Inhibition Assay

A 5 ml sample solution was prepared by taking and mixing 0.2 ml hen's egg albumin, 2.8 ml of PBS (pH 6.4) and 2 ml of the plant extract at different concentrations (50, 25, 12.5, 6.25 and 3.12 mg/ml). The reference solution contained 2 ml of DW and the standard solution contained DS in place of the extract solution. The solutions were incubated at 37°C for 15 min followed by heating to 70°C for 5 min on the water bath. All solutions were cooled to determine the absorbance at 660 nm. The percentage inhibition was calculated by the following formula [2].

% inhibition of protein denaturation = 100 X $(V_t / V_c - 1)$

Where, V_t = absorbance of the test solution, V_c = absorbance of the control solution

2.9.3. In vivo Anti-Arthritic Study

Animals were randomly distributed into six groups (n=6). The poly-arthritis was induced by injecting 0.1 ml CFA in the sub-plantar tissue of the left hind paw of all rats except the normal control group. The day of CFA injection was taken as Day 1. Relevant treatments were given to all groups starting from day 8 continuously during the next 21 days. All rats showed swelling in a few hours post-CFA injection, which became severe after day 7. Group-I (normal rats) and Group-II (disease control-treated with CFA) received only 1 ml of DW/day via intragastric tube. Group-III served as a positive control group and was treated with DS (5 mg/kg/day). The remaining groups, IV, V and VI were treated with *P. harmala* extract at 200, 400 and 600 mg/kg/-day, respectively. All treatments were given orally between 10-11 AM daily [21]. The paw diameter and body weight of all animals were weekly recorded by vernier caliper and weighing balance till day 28, respectively. The percentage increase or decrease in the paw diameter and body weight was calculated by the following formula.

% age change in paw size/body weight = [(To – Tt) / To] x 100

Where; Tt = Paw diameter at a particular day, To = Paw diameter at day 0

2.9.4. Biochemical Tests

After 28 days, all rats were given anesthesia with diethyl ether, and blood samples were collected by heart puncture separately from each rat in plain and Ethylene diamine tetraacetic acid (EDTA) tubes. All samples were analyzed for complete blood count (CBC) with Norma hematology analyzer®. The liver and kidney function tests, C-reactive protein (CRP) and rheumatoid factor (RF) tests were performed on serum using a micro analyzer® by adopting kit maker's guidelines (Analyticon AC, Biotechnologies, Germany).

2.9.5. Estimation of Inflammatory Markers

The concentration of TNF-α and Prostaglandin (PG)E2 in the serum of poly-arthritic rats was determined by following the ELISA kit maker's protocol (Elabscience Biotechnology®, China) [3].

2.9.6. Immune Organ Weight

After the 28th day, the abdomen of pre-anesthetized rats was open and the immune organs (spleen and thymus) were

harvested, cleaned with DW and weighed to sort out the role of immune organs in CFA-induced arthritis.

2.9.7. Evaluation of Oxidative Stress Biomarkers

The activities of catalase (CAT) and superoxide dismutase (SOD), along with the levels of reduced glutathione (GSH) and malondialdehyde (MDA) in poly-arthritic rats were carried out on the liver tissue homogenate (10%w/v) by following earlier procedures [22].

10% liver tissue homogenate was prepared for each rat by following an earlier procedure. Briefly, 1g liver tissue was homogenized with 3 ml PBS (0.1M, pH 7.4). The final volume was made to 10 ml followed by centrifugation at 3000 rpm for 15 min (4°C) and the upper layer was used to measure the oxidative stress biomarkers. The protein content in the liver tissue was determined by Lowry's method using bovine serum albumin as standard and absorbance was taken at 660 nm [22].

The CAT activity was performed by adopting a previous procedure [23]. The test solution was prepared by adding 0.05 ml tissue homogenate to 50 mM PBS to make volume up to 2 ml with the subsequent addition of 1 ml of 30 mM H_2O_2. The variations in absorbance were recorded for 30 seconds at 240 nm. It was derived from the mentioned formula.

CAT activity = $\delta_{O.D.}$ / (E × Vol. of Sample (ml) × mg of protein)

Where, $\delta_{O.D.}$ is the measured change in the absorbance; E is the extinction coefficient of H_2O_2 (0.071 mM /cm).

For SOD activity, 3 ml test solution was prepared by taking 2.8 ml PBS (0.1 M) and 0.1 ml liver homogenate solution followed by the addition of 0.1 ml of pyrogallol solution (2.6 mM). The superoxide scavenging ability was assessed by recording a change in the absorbance of the test solution for 5 min at an interval of 30 S at 325 nm using a UV-vis spectrophotometer. A single SOD unit was taken as the concentration of SOD that can inhibit 50% pyrogallol autoxidation at the test conditions. The activity of SOD was determined by a regression line drawn from the different concentrations of SOD [22].

The GSH level was measured by an earlier procedure [24]. The test solution was made up of 1 ml tissue homogenate and 1 ml TCA (10%) solution. The tissue homogenate was precipitated, and its supernatant layer was separated. Then, 4 ml PBS was poured into each ml of supernatant followed by the addition of 0.5 ml 5, 5-dithiobis -(2-nitrobenzoic acid) (DTNB) reagent. The absorbance of the upper layer was recorded at 412 nm wavelength. It was calculated from the following formula.

GSH level = [(Absorbance- 0.00314) / 0.0314] x [Dilution factor /Volume of supernatant]

For MDA level, the test solution was prepared by mixing 1 ml liver homogenate and 3 ml of TBA reagent [25]. The absorbance of the supernatant was read at 532 nm and the results were mentioned as nM/mg of protein. The level of MDA was calculated from the given equation.

Level of MDA = $Abs_{532} \times 100 \times V_T (1.56 \times 10^5) \times W_T \times V_U$

Where; V_U, supernatant volume = 1 ml, W_T, dissected liver weight = 1 g,

V_T, the total mixture's volume = 4 ml, Abs_{532} = measured absorbance at 532 nm,

1.56×10^5 is the molar extinction coefficient

2.9.8. Histopathology of Rat Paw

At the 28th day, all rats were killed by cervical dislocation under anesthesia with diethyl ether. The left hind paw was cut and placed in 5% formic acid (for 1 month) for decalcification, followed by embedding in paraffin wax. A cross-section of 3-4 µm thickness was cut with microtome [26]. This cross-section was subsequently stained with hematoxylin-eosin dye and observed under the inverted light microscope for inflammation, pannus formation and bone erosion.

2.10. Statistical Analysis

The data were analyzed using GraphPad Prism (version 6.1). The results were calculated as mean ± standard deviation. The *in vitro* assays were analyzed by regression analysis to demonstrate the concentration-response relationship. The changes in paw diameter and body weight of rats were analyzed by two-way analysis of variance (ANOVA), while the remaining data were analyzed by one-way ANOVA followed by Bonferroni's post hoc analysis.

3. RESULTS

3.1. Phytochemical Analysis

The crude extract of *P. harmala* leaves showed the presence of alkaloids, carbohydrates, flavonoids, phenols and steroids; with TPC: 4.11 mg of rutin equivalents/g of *P. harmala* extract and TPC: 1.43 mg equivalent of gallic acid/g of *P. harmala* extract.

3.2. Antioxidant Activity

The percentage of free radical scavenging of ascorbic acid (86.4 ± 0.35%) and plant extract (54.5 ± 0.69%) was the highest at 250 µg/ml concentration. The concentrations for the 50% DPPH scavenging of ascorbic acid and plant extract were 34.48 and 58.09 µg/ml, respectively (Fig. **1a**).

3.3. *In vitro* Anti-Inflammatory Activity

The percentage membrane stabilization effect was the highest for DS (71.2 ± 0.23%) and the plant extract (78.0 ± 0.22%) at 50 mg/ml concentration. The concentrations of DS and the plant extract for 50% stabilization of HRBC membrane were estimated to be 16.40 and 23.90 mg/ml, respectively (Fig. **1b**).

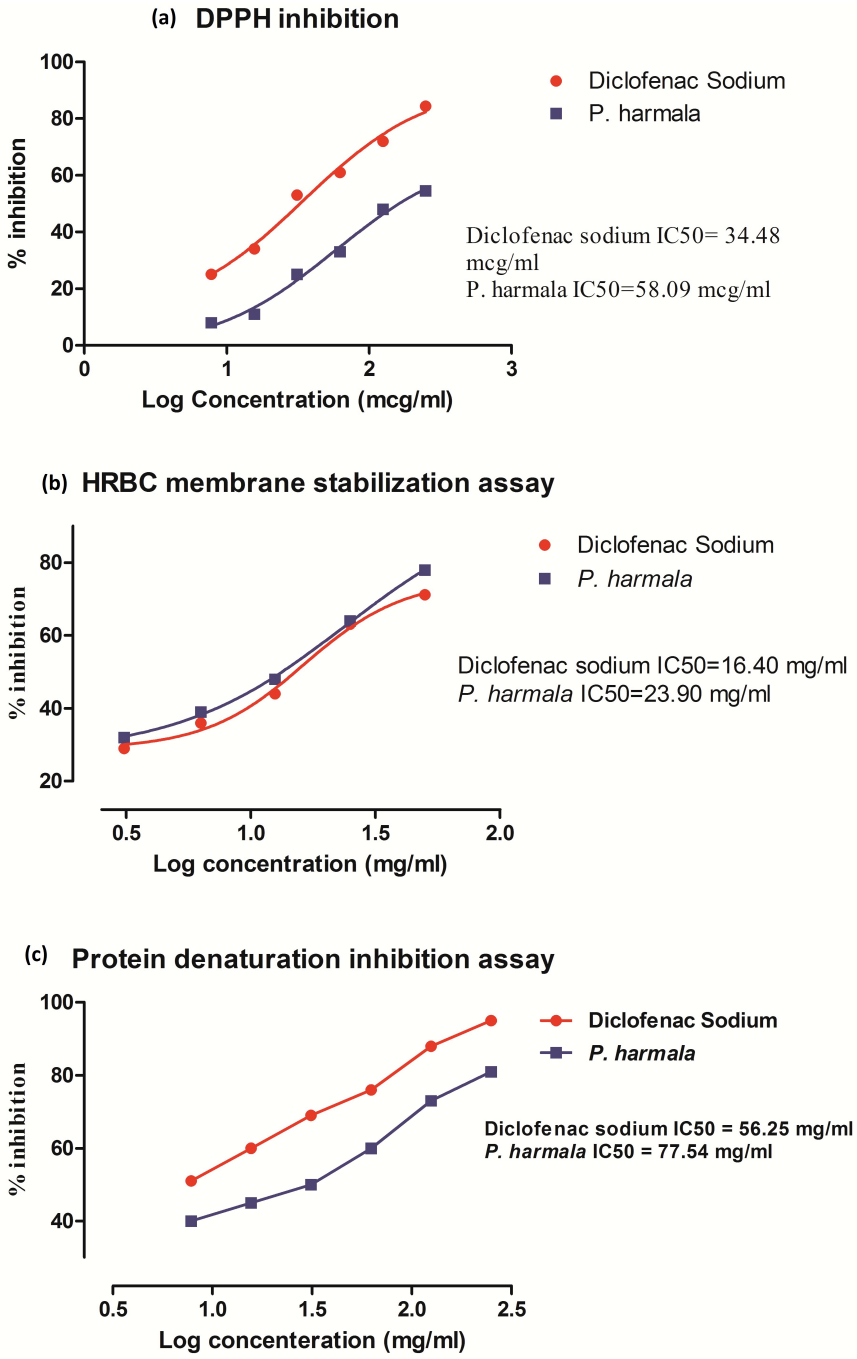

Fig. (1). Effect of *Peganum harmala* extract on (**a**) Free radical scavenging (**b**) stabilization of Red blood cell membrane (**c**) protein denaturation inhibition. *(A higher resolution / colour version of this figure is available in the electronic copy of the article).*

The albumin of hen's egg was used for the protein denaturation inhibition assay. The percentage inhibition was the highest for DS (95.1 ± 0.34%) and the plant extract (81.2 ± 0.23%) at 250 mg/ml concentration. The concentrations of DS and the plant extract for 50% inhibition of protein denaturation (IC_{50} value) were 56.25 and 77.54 mg/ml, respectively (Fig. **1c**).

3.4. *In vivo* Anti-arthritic Activity

3.4.1. Paw Edema of Poly-Arthritic Rats

All the animals had swollen paws within 3 h of CFA injection. The percentage change in paw size of animals treated with the plant extract was compared with the paw size of arthritic rats at the start (day 8th of CFA injection) and the

end of therapy. The paw edema successively increased in disease control rats throughout the study duration as compared to normal control rats. The maximum reduction in paw diameter was recorded in rats treated with *P. harmala* extract 600 mg/kg (19.3± 1.27%) which was significantly ($p<0.05$) different from the disease control group. Similarly, the reduction in paw edema of rats treated with DS (16.80 ± 2.73%), the plant extract 200 mg/kg (14.56 ± 1.99%) and 400 mg/kg (16.82 ± 3.21%) was notably varied from the disease control rats (Table **1**).

3.4.2. Body Weight of Poly-Arthritic Rats

The bodyweight of animals in all groups was also monitored. The bodyweight of untreated poly-arthritic rats was continuously reduced after CFA injection throughout the duration of study. At the end of the study, the bodyweight of the disease control group was noticeably ($p<0.05$) reduced (8±1.39%) as compared to the normal control group (10% ± 0.43%). Therapy with the plant extract and DS notably restored the body weight compared to the disease control rats. The maximum increase in body weight was measured in the plant extract 600 mg/kg (11.8 ± 1.65%) treated group that was significantly varied from the DS (6.3% ± 1.01) and disease control rats on 28th day of study (Table **2**).

3.4.3. Hematological Parameters of Poly-Arthritic Rats

The white blood cells (WBCs), RBCs and platelets (PLTs) counts were determined along with the level of hemo-

Table 1. Effect of *Peganum harmala* extract on the paw edema of the arthritic rats.

Study Group	Paw Size in mm (Percentage Change in Paw Size)		
	Initial Paw Size	Paw Size at the Start of Treatment	Paw Size at the End of Treatment
Disease control	4.08 ± 0.16	7.56 ± 0.17 (↑ 86.05% ± 10.3)	8.12 ± 0.11 (↑ 7.41% ± 1.16) #
Normal control	4.07 ± 0.23	4.27 ± 0.23	5.07 ± 0.28
Diclofenac sodium 5 mg/kg	4.84 ± 0.23	8.3 ± 0.14 (↑ 72.22% ± 7.24)	6.9 ± 0.14 (↓ 16.8% ± 1.04) *
P. harmala 200 mg/kg	4.09 ± 0.17	7.91 ± 0.16 (↑ 81.27% ± 9.22)	6.76 ± 0.19 (↓ 14.56% ± 1.99)*
P. harmala 400 mg/kg	4.73 ± 0.19	8.05 ± 0.18 (↑ 70.3% ± 3.11)	6.68 ± 0.12 (↓ 16.82% ± 3.21) *
P. harmala 600 mg/kg	4.55 ± 0.17	8.08 ± 0.14 (↑ 78.28% ± 9.56)	6.52 ± 0.14 (↓ 19.3% ± 1.27) **

Results presented as Mean±S.D (n=6) and analyzed by two-way ANOVA followed by Bonferroni's Test. ↑ and ↓ indicated increase and decrease, respectively. * and ** showed statistical significant at $p<0.05$ and $p<0.01$ respectively, as compared to the disease control group. # showed statistically significant at $p<0.05$ as compared to the normal control group.

Table 2. Effect on the percentage change in body weight of the arthritic rats.

Study Group	Body Weight in Grams (Percentage Change)		
	Initial Body Weight (Day 1)	Body Weight at the Start of Treatment (Day 7)	Body Weight at the End of Treatment (Day 28)
Disease control	257.33 ± 29.49	254 ± 29.51 (↓1% ± 0.18)	234 ± 29.16 (↓8% ± 1.39)
Normal control	269 ± 14.05	273 ± 12.53 (↑1.5% ± 0.62)	301 ± 15.04 (↑10% ± 0.43) *** #
Diclofenac sodium 5 mg/kg	256.33 ± 29.49	250.67 ± 28.64 (↓2.1% ± 0.13)	266 ± 28.01 (↑6.3% ± 1.01) ***
P. harmala 200 mg/kg	262 ± 32.19	256.67 ± 31.39 (↓2% ± 0.13)	271 ± 31.18 (↑5.8% ± 1.30) ***
P. harmala 400 mg/kg	255.33 ± 29.81	244.67 ± 30.11 (↓4.3% ± 1.25)	264.67 ± 34.09 (↑8% ± 0.93) ***
P. harmala 600 mg/kg	243.67 ± 18.1	236.67 ± 19.22 (↓2.9% ± 0.93)	264 ± 17.78 (↑11.8% ± 1.65) *** ##

Results presented as Mean±S.D (n=6) and analyzed by two-way ANOVA followed by Bonferroni's Test. ↑ and ↓ indicated increase and decrease, respectively. *** showed statistical significant at $p<0.001$ as compared to the disease control group. # and ## showed statistically significant at $p<0.05$ and $p<0.01$ as compared to the normal control group.

globin (Hb) in poly-arthritic rats. The RBCs count was significantly ($p<0.01$) reduced in the disease control group (7.3 ± 0.51 x 10^6 /µl) as compared to the normal control group (11.3 ± 0.67 x 10^6 /µl). The RBCs count was significantly ($p<0.05$) restored in DS (8.4 ± 0.73 x 10^6 /µl), *P. harmala* 400 (10.8 ±0.55 x 10^6 /µl) and 600 mg/kg (11.1± 0.6 x 10^6 /µl) treated poly-arthritic rats as compared to the disease control group. The RBCs counts in *P. harmala* 400 and 600 mg/kg treated rats were insignificantly different from the normal control rats (Fig. **2a**).

The WBCs count was profoundly raised ($p<0.05$) in disease control group (16.2 ± 0.63 x 10^3/µl) as compared to the normal control group (9.28 ± 0.61 x 10^3/µl), The groups treated with DS (11.8 ± 0.74 x 10^3/µl) and *P. harmala* 200 (9.1 ± 1.17 x 10^3/µl), 400 (9.4 ± 0.9 x 10^3/µl) and 600 mg/kg (9.2 ± 0.91 x 10^3/µl) exhibited a significant decrease in WBCs count as compared to the disease control group (Fig. **2b**).

The platelets (PLTs) count was noticeably ($p<0.01$) elevated in the disease control group (769.9 ± 51.91 x 10^3 /µl) in contrast to the normal control group (307.7 ± 49.63 x 10^3 /µl). The PLTs counts were significantly restored in *P. harmala* 200 (508.1 ± 53.31 x 10^3 /µl), 400 (470.8 ± 49.42 x 10^3 /µl) and 600 mg/kg (458.2 ± 48.12 x 10^3 /µl) treated groups as compared to the disease control group. However, PLTs count in animals treated with the plant extract or DS were significantly higher than the normal control ($p<0.01$) (Fig. **2c**).

The Hb level was notably declined in the disease control group (14.8 ± 1.2 g /dl) in contrast to the normal control group (19.1 g /dl ± 1.8 g /dl) that was restored in DS (16.2 ± 1.2 g /dl) and the extract-treated groups, However, the difference among all groups was statistically insignificant (Fig. **2d**).

3.4.4. Effect on Biochemical Parameters of Poly-arthritic Rats

The effect of *P. harmala* extract on the liver functions of arthritic rats was monitored by assessing the level of ALP, AST and ALT in all groups of animals. The ALP level was significantly ($p<0.05$) raised in the disease control group (890 ± 39 U/L) as compared to the normal control group (509 ± 48 U/L) in CFA induced arthritis model. The ALP level was restored in the DS and the *P. harmala* 200, 400 and 600 mg/kg treated groups as compared to the disease

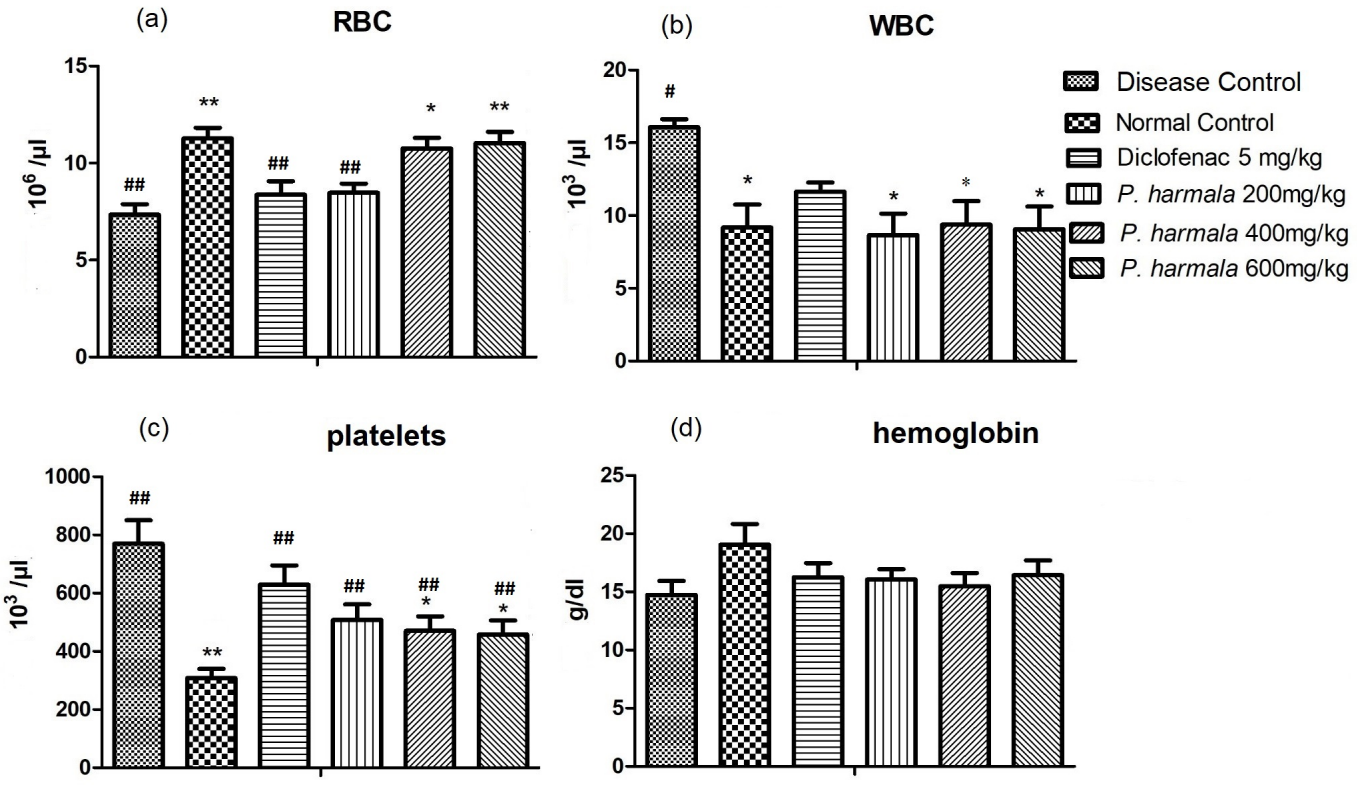

Fig. (2). Effect of treatment with *Peganum harmala* extract on hematology of poly-arthritic rats. Results presented as Mean±S.D (n=6) and analyzed by one-way ANOVA followed by Bonferroni's Test. * and ** showed statistical significant at $p<0.05$, $p<0.01$ respectively as compared to the disease control group. # and ## showed statistically significant at $p<0.05$ and $p<0.01$ as compared to the normal control group. *(A higher resolution / colour version of this figure is available in the electronic copy of the article).*

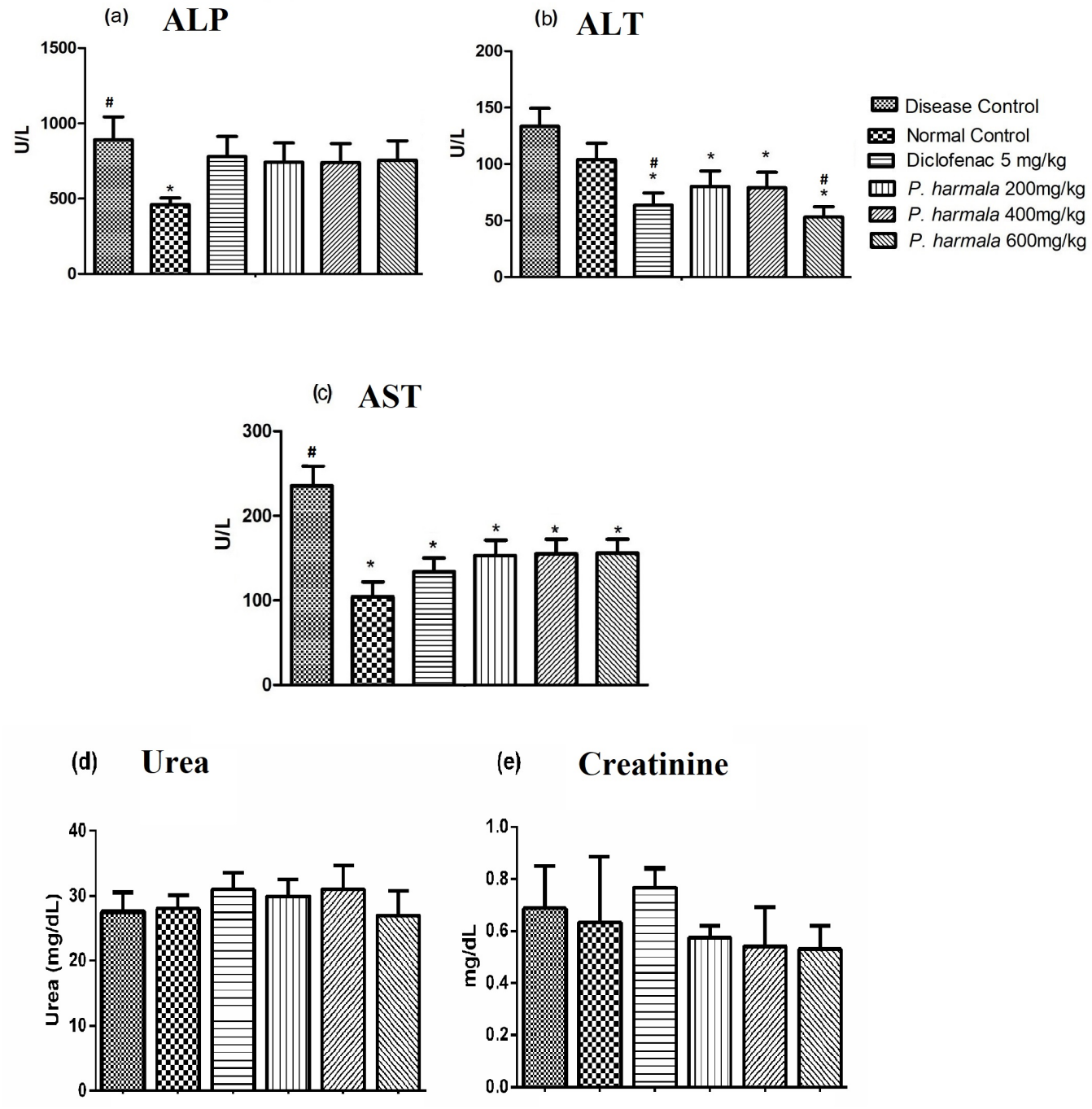

Fig. (3). Effect of *Peganum harmala* extract on liver and kidney function of poly-arthritic rats. Results presented as Mean±S.D (n=6) and analyzed by one-way ANOVA followed by Bonferroni's Test. * showed statistical significant at $p<0.05$ as compared to disease control group. # showed statistically significant at $p<0.05$ as compared to normal control group. *(A higher resolution / colour version of this figure is available in the electronic copy of the article).*

control group (Fig. **3a**). The ALT level was notably raised in the disease control group (133.3 ± 15.3 U/L) as compared to the normal control group (46.16 ± 13.4 U/L) that was significantly ($p<0.05$) restored in DS and the *P. harmala* 200 (80.23 ± 13.74 U/L), 400 (79.16 ± 13.5 U/L) and 600 mg/kg (53.13 ± 9.1 U/L) treated group as compared to the disease control group (Fig. **3b**). The AST level was profoundly ($p<0.05$) increased in the disease control group (236 U/L ± 9 U/L) in contrast to the normal control group (169 ± 9.1 U/L). At the same time, it was restored notably ($p<0.05$) in

DS (176 ± 9.5 U/L) and the plant extract 200 (180 ± 9.2 U/L), 400 (177 ± 9.5 U/L) and 600 mg/kg (174 ± 8.4 U/L) treated groups as compared to the disease control rats (Fig. **3c**).

There was no significant impact of therapy and CFA-induced arthritis on urea and creatinine level in all the arthritic rats as compared to the normal and disease control rats (Fig. **3d** and **e**).

3.4.5. Effect on the Serum Concentration of TNF-α and PGE2

There was a noticeable ($p<0.0001$) upsurge in the quantity (pg/ml) of TNF-α (467.65±20.56) and PGE2 (878.28±38.50) in the serum of non-treated arthritic rats in contrary to the healthy rats TNF-α (44.27±14.23) and PGE2 (98.76±20.20) as mentioned in Fig. (**4a** and **b**). Treatment with the plant extract significantly ($p<0.0001$) declined the level of both inflammatory markers in contrast to the disease control rats. The *P. harmala* extract restored the level of TNF-α and PGE2 in a dose-dependent manner while higher activity exhibited by the *P. harmala* 600 mg/kg [TNF-α (109.41±13.18) and PGE2 (126.89±25.81)].

The RF level raised noticeably ($p<0.0001$) in the disease control rats (53.67±3.51 IU/L) as compared to the normal control rats (12.33±2.51 IU/L) while therapy with DS (16.32±1.53 IU/L) and the plant extract significantly ($p<0.0001$) restored the RF level in treated rats in contrary to the disease control rats as depicted in Fig. (**4c**).

The CRP is a marker of inflammation. There was a significant ($p<0.01$) elevated CRP level was noticed in the disease control group (8.60 mg/dl ± 0.68) as compared to the normal control group (4.17 mg/dl ± 0.61). All the treatment groups of the plant extract at 200 (5.81 mg/dl ± 0.57), 400 (5.44 mg/dl ± 0.54) and 600 mg/kg (.27 mg/dl ± 0.60) and DS (6.17 mg/dl ± 0.61) had substantially ($p<0.05$-0.01) restored the CRP level when compared to the disease control group (Fig. **4d**).

3.4.6. Effect on Immune Organ Weight

The weight (g) of the spleen (1.47±0.08) and thymus (0.67±0.34) was notably ($p<0.0001$) raised in the disease control rats in contrast to the normal rats [spleen (0.44±0.03): thymus (0.24±0.12)]. The therapy with plant extract at all dosage levels and DS remarkably ($p<0.0001$) restored the immune organ weight, mainly at 600 mg/kg dose of the extract (spleen: 0.48±0.24: thymus: 0.298±0.03) as depicted in Fig. (**5a** and **b**).

3.4.7. Amelioration of Oxidative Stress

The CAT activity was noticeably ($p<0.05$) reduced in the CFA control (8.80 ± 0.56 nMol/min/mg) as compared to the normal control group (12.89 ± 0.55 nMol/min/mg). The

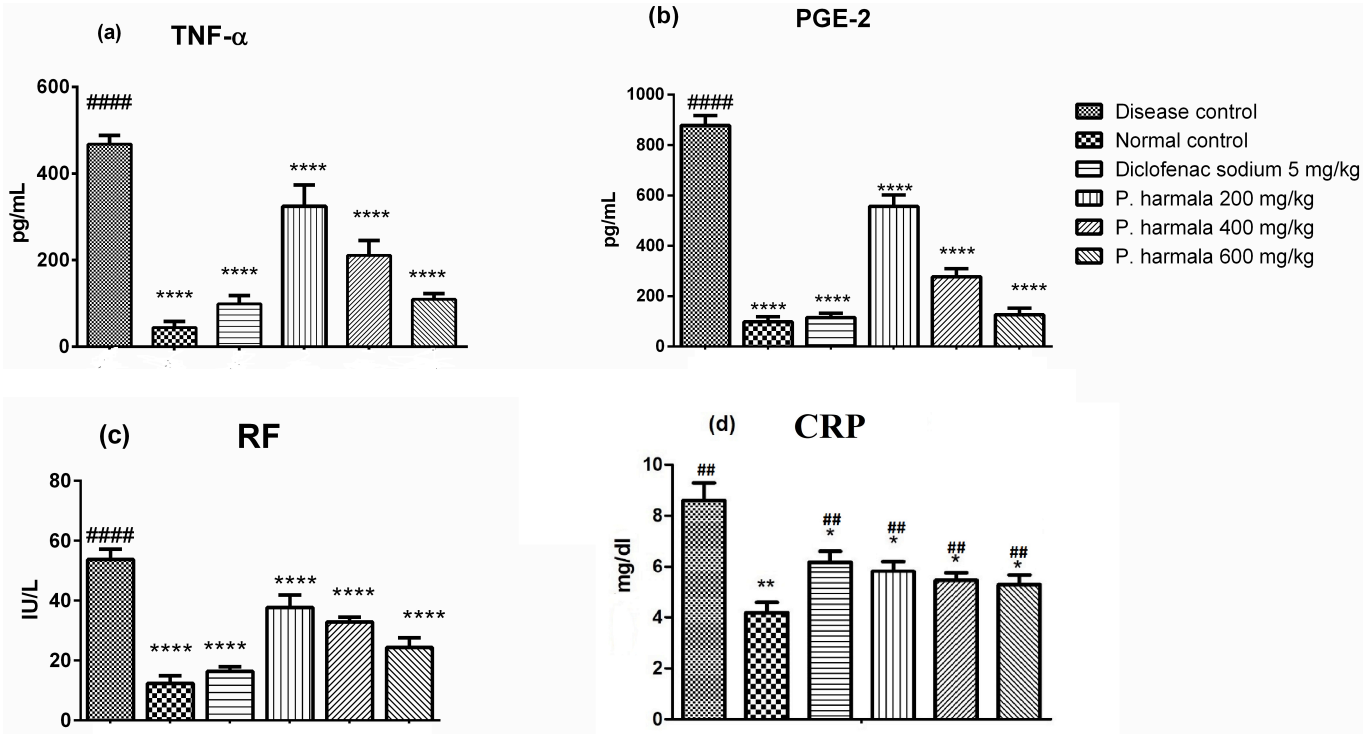

Fig. (4). Effect of *Peganum harmala* extract on inflammatory biomarkers in poly-arthritic rats. Results presented as Mean±S.D (n=6) and analyzed by one-way ANOVA followed by Bonferroni's Test. *, ** and **** showed statistical significant at $p<0.05$, $p<0.01$ and $p<0.0001$ respectively, as compared to the disease control group. ## and #### showed statistically significant at $p<0.01$ and $p<0.0001$ as compared to the normal control group. *(A higher resolution / colour version of this figure is available in the electronic copy of the article).*

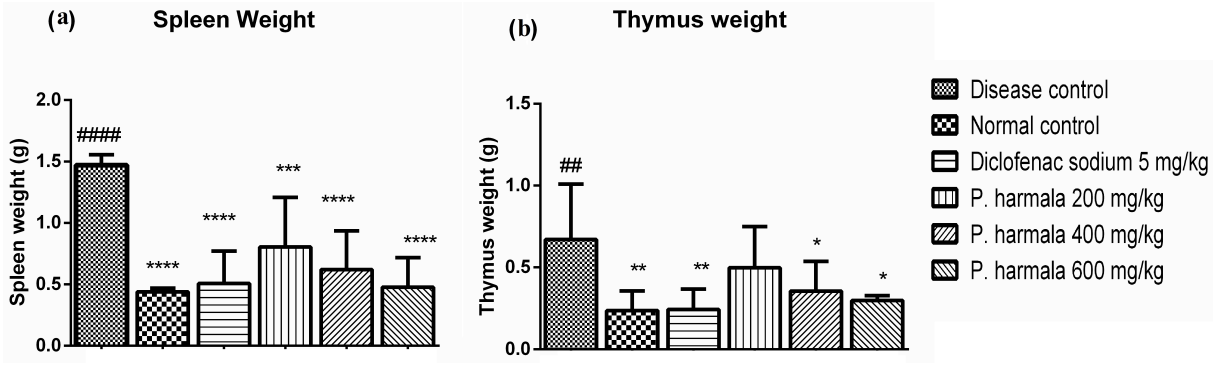

Fig. (5). Effect of *Peganum harmala* extract on immune organs weight of poly-arthritic rats. Results presented as Mean±S.D (n=6) and analyzed by one-way ANOVA followed by Bonferroni's Test. *,**,*** and **** showed statistical significant at p<0.05, p<0.01, p<0.001 and p<0.0001 respectively, as compared to the disease control group. ## and #### showed statistically significant at p<0.01 and p<0.0001 as compared to normal control group. *(A higher resolution / colour version of this figure is available in the electronic copy of the article).*

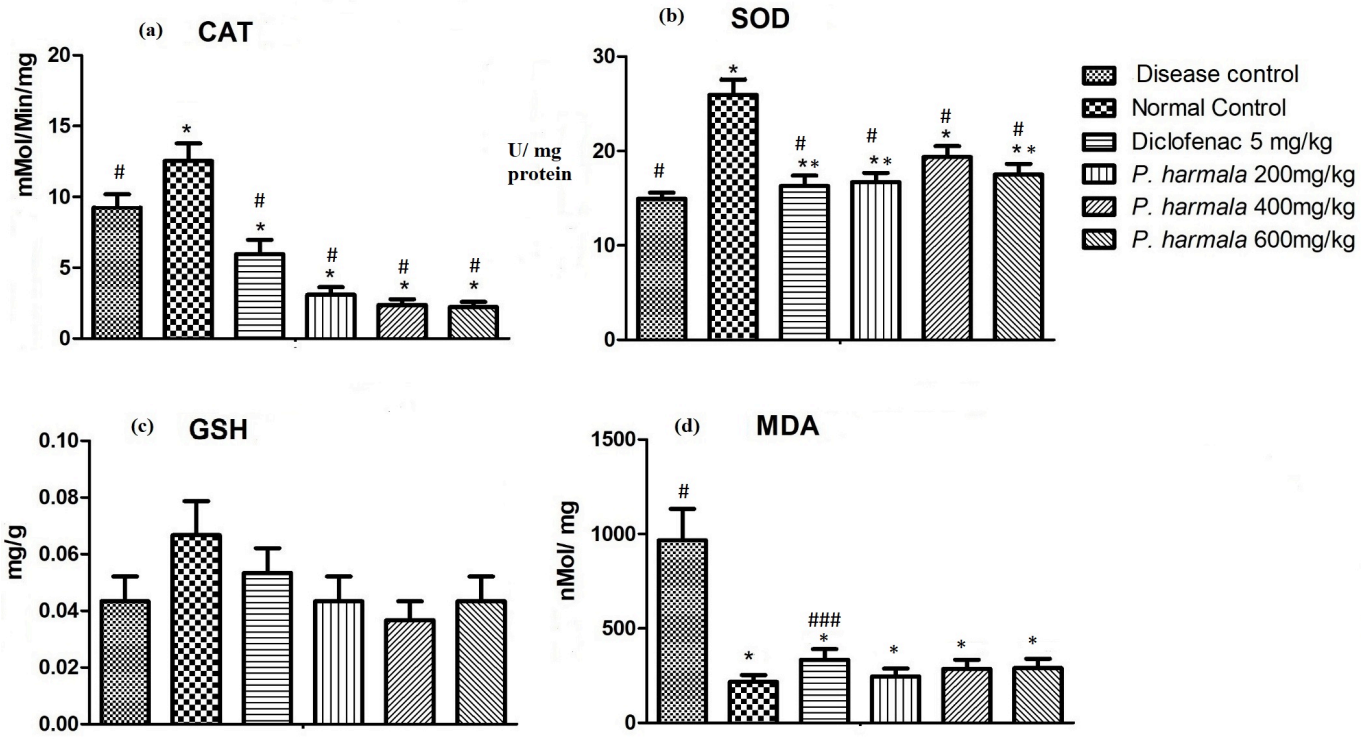

Fig. (6). Effect of Peganum harmala extract on liver oxidative status of poly-arthritic rats. Results presented as Mean±S.D (n=6) and analyzed by one-way ANOVA followed by Bonferroni's Test. * and ** showed statistical significant at p<0.05 and p<0.01 respectively, as compared to the disease control group. ## and ### showed statistically significant at p<0.01 and p<0.001 as compared to the normal control group. *(A higher resolution / colour version of this figure is available in the electronic copy of the article).*

DS (5 mg/kg) treated group had CAT activity (5.86 ± 0.41 nMol/min/mg) that was notably varied from the normal and disease control groups. Whereas the CAT activity in *P. harmala* 200 (4.06 ± 0.42 nMol/min/mg), 400 (3.73 ± 0.38 nMol/min/mg) and 600 mg/kg (3.23 ± 0.38 nMol/min/mg) treated groups was significantly increased as compared to the disease control group (Fig. **6a**).

The SOD activity was significantly (p<0.05) declined (13.89 ± 1.1 U/mg of protein) in the disease control group in contrast to the normal control group (25.93 ± 1.6 U/mg of protein).The SOD activity was remarkably restored in DS (16.28 ± 1.1 U/mg of protein), *P. harmala* 200 mg/kg (16.71 ± 1.0 U/mg of protein), 400 mg/kg (17.51 ± 1.1 U/mg of protein) and 600 mg/kg (19.35 ± 1.2 U/ mg of protein) treated

groups as compared to the disease control rats (Fig. **6b**). The GSH level was declined substantially ($p<0.05$) in CFA control group (0.04 ± 0.01 mg/g) in contrast to the normal group rats (0.07 ± 0.01 mg/g). Treatment with the plant extract and DS notably elevated the GSH level in arthritic rats in contrast to the disease control rats (Fig. **6c**).

The MDA level was remarkably escalated ($p<0.05$) in the disease control group (966.91 ± 51.58 nM/mg) in comparison of the normal control group (216.35 ± 37.07 nM/mg). While treatment with DS (333.88 ± 57.21 nM/mg), and the plant extract at 200 (280.02 ± 49.69 nM/mg), 400 mg/kg (285.8 ± 48.97 nM/mg) and 600mg/kg (295.74 ± 42.11 nM/mg) substantially ($p<0.001$) restored the MDA level in treated arthritic rats in contrast to the disease control rats (Fig. **6d**).

3.4.8. Histopathology of Arthritic Rat Paw

The histopathological slides for all the groups of animals were observed for bone erosion, pannus formation and infiltration with mononuclear cells under a light microscope at 40 and 100x (Fig. **7**). The disease control group exhibited chronic infiltration with mononuclear cells, bone erosion, and pannus formation. The normal control group exhibited the normal synovial membrane while bone erosion and pannus formation were absent in it. The DS group exhibited intact bone with no pannus formation and minimal infiltration and synovial hyperplasia. The *P. harmala* 200 mg/kg group exhibited synovial hyperplasia and moderate infiltration with mononuclear cell, while pannus formation and bone erosion were also present. The *P. harmala* 400 mg/kg treated rats exhibited minimum infiltration with mononuclear cells, less pannus formation and intact bone. The *P. harmala* 600 mg/kg exhibited mild infiltration with mononuclear cells and its joint histology did not exhibit bone erosion and pannus formation (Fig. **7**).

4. DISCUSSION

The use of *P. harmala* leaves is a popular remedy in the folkloric system of medicine for the management of arthritis. The numerous scientific evidences have been reported in the literature on *P. harmala* leaves for the management of different diseases, but its utilization in arthritis still needs scientific evaluation. Qualitative as well as quantitative analysis of the *P. harmala* leaves extract indicated that the leaves are rich in various secondary metabolites, including alkaloids, flavonoids and phenols.

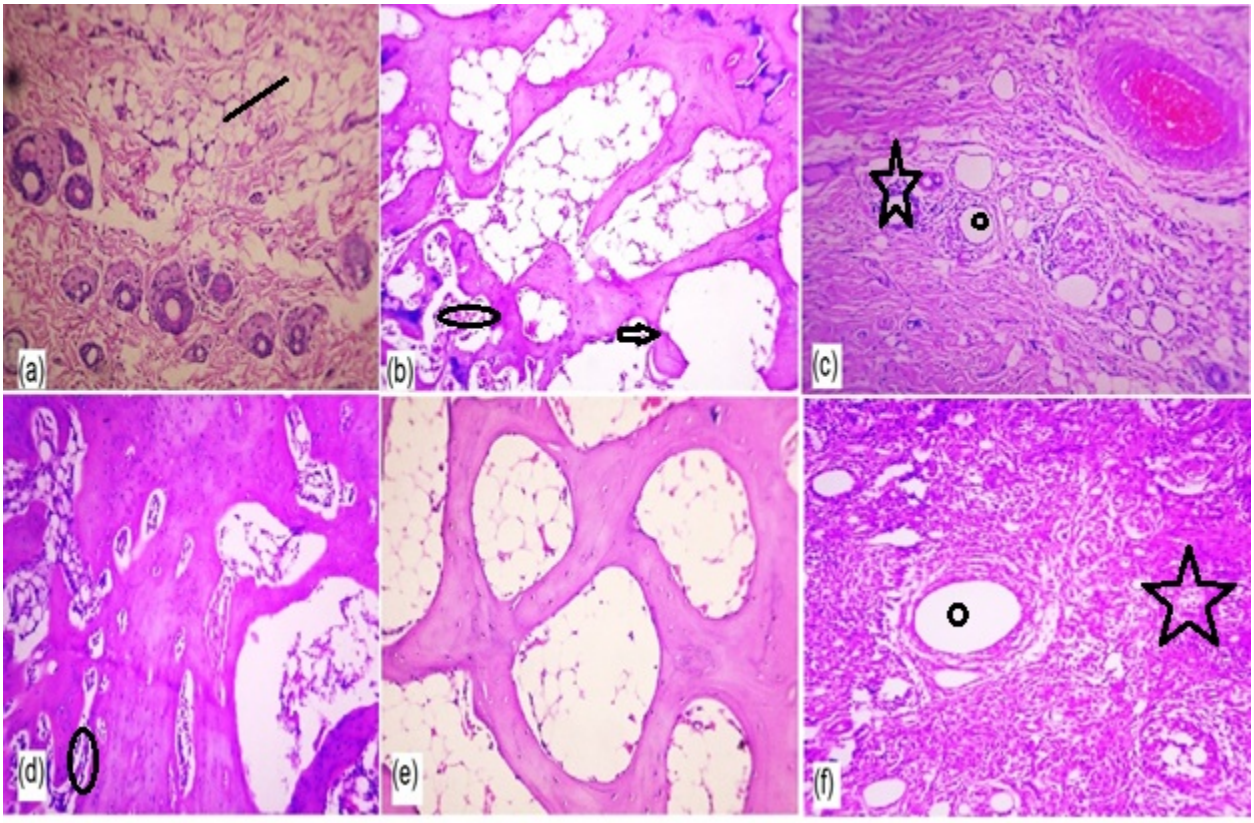

Fig. (7). Effect of Peganum harmala extract treatment on histopathology of joints of polyarthritic rats at 40 and 100X (**a**) normal control (**b**) disease control (**c**) diclofenac sodium (**d**) *P. harmala* 200 mg/kg (**e**) *P. harmala* 400 mg/kg (**f**) *P. harmala* 600 mg/kg. Oblong shape: pannus formation, straight line: fat cells, circle: blood vessel, star: inflammation with monocytes, arrow: bone erosion. *(A higher resolution / colour version of this figure is available in the electronic copy of the article).*

Anti-arthritic drugs and phytochemical constituents of herbal remedies, especially flavonoids, phenols have two possible mechanisms to fight against arthritis: (i) membrane stabilization (ii) prevention of denaturation of cellular proteins. Arthritis aggravates hydrolytic enzymatic action. Hydrolytic enzymes released from the lysosomes induce acute as well as chronic inflammation. Reported secondary metabolites, for instance, phenol, flavonoids and alkaloid in *P. harmala* leaves extract might be involved in the stabilization of lysosomal membrane and prevent the inflammatory processes by inhibiting the action of hydrolytic enzymes from the lysosomes [27]. The HRBC membrane is similar to lysosomal membranes and any constituent involved in the stabilization of HRBC membrane may have the ability to stabilize lysosomal membrane, so this HRBC membrane stabilization activity is used for the assessment of anti-inflammatory activity of any drug [28]. The *P. harmala* leaves extract stabilized the HRBC membrane comparable to that of the standard drug (DS). In RA, auto-antigen is also produced as a result of protein denaturation, which is due to an alteration in bonding [29]. Mostly, NSAIDs inhibit protein denaturation and serve as anti-rheumatic agents [30]. The *P. harmala* leaves extract was found to prevent heat-induced protein denaturation in a dose-dependent fashion in comparison to the DS. In addition, antioxidants of the plant extract scavenged the free radicals and contributed to reduce the inflammatory processes [31]. Therefore, it was established that *P. harmala* leaves extract possessed anti-inflammatory, antioxidants and anti-arthritic activity and can limit the disease progression in RA.

The CFA injection was used to immunize rats with polyarthritis that is similar in pathogenesis to the development of arthritis in humans, and that is why, it is the most recommended method for the study of antiarthritic potential of testing substance in rats. The CFA inoculation caused the irritant effect and initial edema and thickening of tissues may be due to its irritant effect, which further lead to immunological responses and subsequently the development of progressive arthritis. Any drug that suppresses the immunological response can reduce the development of arthritis as well as edema of paws and joints [32]. The *P. harmala* extract reduced the paw edema of arthritic rats in a dose-dependent manner that was probably due to suppression of immunological responses of adjuvant.

The variation in body weight is a useful parameter for assessing the disease progress and effect of treatments on RA. During arthritis, the absorbance of nutrients, including glucose and amino acid (leucine), from the rat's intestine is reduced, which possibly leads to a reduction of the body weight [32, 33]. Inflammation and pain in joints limit the movement of animals and the disease process also transforms the behavior of rats with respect to food that may also contribute to the reduction of their body weight. In the current study, the improved bodyweight of arthritic rats after the treatment with the plant extract (200, 400 and 600 mg/kg) and DS (5mg/kg) indicated the improvement in nutrient absorption from the small intestine.

The CRP is the systemic biomarker of tissue damage and inflammation. It is a reactive protein that is released from the liver stimulated by IL-6 and TNF-α in response to infection, injury and inflammation. The raised levels of CRP and RF attribute to the elevated levels of IL-6 and TNF-α that contribute to accelerate the synovitis and tissue injury as coevidenced from the histology and blood chemistry of disease control rats in contrast to healthy rats. The CRP binds to the invaders and helps macrophages identify and digest them. In the present study, *P. harmala* extract at all dosage levels and DS alleviated the CRP and RF levels in arthritic rats as evidenced from the reduced level of TNF-α in the serum of treated rats as compared to the disease control rats.

An increase in the levels of WBCs and PLT was noticed in the disease control rats that was due to hyperactive immune system [34]. The WBC and PLT counts were significantly restored in the plant extract treated groups as compared to the non-treated arthritic rats. Anemia is the most common feature of RA that is attributed to the reduced level of RBC and Hb [34]. The decreased Hb level in RA is due to the immature destruction of RBCs, non-responsive bone marrow erythropoietin cells and reduction of erythropoietin level [35]. The CFA-induced arthritis results in anemia, as obvious from the reduced level of RBCs and Hb in arthritic rats, while therapy with the plant extract remarkably attenuated adjuvant-induced anemia in treated rats as evidenced from the previous studies.

Serum ALT, AST and ALP are the biomarkers of tissue damage. The level of these biomarkers is raised in RA. The assessment of these biomarkers in serum is a good indicator of the disease stage and anti-arthritic activity of a drug [35]. In the present study, the elevation of liver enzymes in the disease control group was considerably restored in rats taking *P. harmala* extract. The urea and creatinine levels were not altered due to therapy with the plant extract and adjuvant immunization. Thus, it can be assumed that *P. harmala* extract is safe for the kidney. The effect of *P. harmala* extract on liver biomarkers indicated that it possessed the potential to prevent tissue damage and restricting the disease progress in RA.

Various previous studies had investigated free radicals that were involved in many disease pathogenesis such as cancer, arthritis, etc via distressing the normal metabolic processes. A lack of the antioxidant system and an increase in the oxidant system caused a marked elevation of oxidative stress in the liver of CFA-induced arthritic rats [36]. Any drug that possesses antioxidant properties reduces oxidative stress and aids in RA treatment. The oxidative stress biomarkers such as SOD, CAT, MDA and GSH were restored in the plant extract and DS treated arthritic rats in contrast to the disease control rats in CFA-induced arthritis.

The SOD and CAT are biological antioxidant enzymes that provide a defense to the biological system against superoxide and hydrogen peroxide (H_2O_2) free radicals. The SOD catalyzes the conversions of superoxide (reactive) to oxygen and H_2O_2 while CAT catalyzes the conversion of H_2O_2 to water and oxygen [37]. The *P. harmala* extract-treated rats had

exhibited a significant ($P < 0.01$) increase in SOD and CAT activities when compared to disease control rats. The MDA is a biological marker of oxidative stress. It is one of the end products of polyunsaturated fatty acids peroxidation, which is a chain reaction initiated by a free radical [38]. The *P. harmala* extract had lowered the level of MDA in liver tissue homogenate of treated rats as compared to the non-treated arthritic rats. The GSH is another antioxidant biomarker that protects the cell from injury by reactive oxygen species [39]. The decreased GSH level prones the cells to the risk of damage by H_2O_2 and lipid peroxidation [37]. The disease control rats had a decline in GSH level, which was indicative of liver injury. All the treated groups exhibited the elevation of GSH.

The TNF-α is a pro-inflammatory cytokine, that after discharge from macrophage and monocytes, excites the release of TNF-α and other pro-inflammatory cytokines like IL-6, IL-1β and chemokines, which lead to accumulation of cytokines, vasodilation and infiltration at the edema site. The PGE2, a pivotal inflammatory mediator, is responsible for pain and swelling in RA. The inflammatory mediators like TNF-α directly upregulate the cyclooxygenase (COX)-2 enzyme level that subsequently results in enhanced production of PGE2. The elevated level of PGE2 and TNF-α as noted in disease control rats was notably reduced by the DS and the plant extract dose-dependently in treated rats. The overstimulated immune system is a symbolic feature of RA that is characterized by increased immune organ weight. In this study, the plant extract at all tested doses noticeably restored the immune organ weight in poly-arthritic rats that correlate to the polyphenols and alkaloidal contents of the *P. harmala* extract.

In the histopathological studies, the CFA immunization produced pannus formation, bone erosion and infiltration with mononuclear cells in arthritic rats, which were absent in normal control rats [40, 41]. The *P. harmala* extract exhibited antiarthritic potential by restoring arthritic indexes (inflammation, bone erosion and pannus formation) in treated rats as compared to disease control rats as evidenced from the previous findings [22, 42].

Harmine, an important alkaloid isolated from the same plant, significantly inhibited nuclear factor kappa B signaling to treat lipopolysaccharide-induced inflammation [42]. Several other β-carboline alkaloids present in *P. harmala* extract such as harmaline, harmalol, harmol and others might have also contributed to retard the progression of poly-arthritis through inhibition of angiogenesis and inflammation [11].

CONCLUSION

In the current study, *P. harmala* extract exhibited the anti-arthritic action through attenuation of CFA-induced paw edema, weight loss, anemia, oxidative stress and restoring inflammatory mediators (PGE2 and TNF-α) level. It is presumed from findings that the plant leaves extract possessed antioxidant, anti-arthritic and anti-inflammatory activities. The anti-arthritic activity was probably due to free radical scavenging, downregulation of inflammatory mediators (PGE2 and TNF-α), attenuation of oxidative stress and protein denaturation, and stabilization of RBC membrane. The anti-inflammatory, antioxidant and anti-arthritic activities of *P. harmala* might be due to the presence of alkaloids, phenols and flavonoids. This study is a step forward to prompt ethnomedicinal use for the management of inflammation and RA through validation of the oral use of *P. harmala* extract. There is a strong urge to further investigate various molecular mechanisms involved in RA treatment.

LIST OF ABBREVIATIONS

ALT	=	Alanine Transaminase
ALP	=	Alkaline Phosphatase
AST	=	Aspartate Transaminase
CAT	=	Catalase
CFA	=	Complete Freund's Adjuvant
CRP	=	C reactive Protein
DMARD	=	Disease Modifying Anti-Rheumatic Drugs
DS	=	Diclofenac Sodium
DPPH	=	2,2-diphenyl-1-picrylhydrazyl
GSH	=	Reduced Glutathione
HB	=	Hemoglobin
HRBC	=	Human Red Blood Cell Membrane
MDA	=	Malondialdehyde
NSAIDs	=	Non-Steroidal Anti-Inflammatory Drugs
PGE2	=	Prostaglandin E2
RA	=	Rheumatoid Arthritis
RBC	=	Red Blood Cells
RF	=	Rheumatoid Factor
SOD	=	Superoxide Dismutase
TBA	=	Thiobarbituric Acid
TNF-α	=	Tumor Necrosis Factor
WBC	=	White Blood Cells

AUTHORS' CONTRIBUTION

MFA, SAR, MMFAB, AS, ZJ, US and KS performed most of the experimental work and participated in data interpretation. MFA, AS, AR, AS and IH participated in the data analysis and interpretation. AS, IH, MFA and US, conceived the study, carried out the experimental design and, prepared and revised the manuscript. All authors read and approved the final manuscript.

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

The study was approved by the Institutional Ethical Com-

mittee of GCUF (Reference No.: GCUF/ERC/2277), Government College University Faisalabad, Pakistan.

HUMAN AND ANIMAL RIGHTS

No humans were involved in the study. All animal procedures were performed in accordance with the guidelines of the National Institute of Health for the care of laboratory animals.

CONSENT FOR PUBLICATION

Not applicable.

AVAILABILITY OF DATA AND MATERIALS

All data of the current study are included in this published manuscript.

FUNDING

None.

CONFLICT OF INTEREST

The authors declare no conflict of interest, financial or otherwise.

ACKNOWLEDGEMENTS

The authors are grateful to Prof Dr. Mansoor Hameed (taxonomist), the University of Agriculture Faisalabad, for identification and authenticating the plant material.

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