Paederia foetida Linn. Inhibit adjuvant induced arthritis by suppression on PGE$_2$ and COX-2 expression via nuclear factor-κB.

Vikas Kumar[1*], F. A. Al-Abbasi[2], Danish Ahmed[1], Amita Verma[1], Mohd. Mujeeb[3], Firoz Anwar[2*]

[1]Department of Pharmaceutical Sciences, Faculty of Health Sciences, Sam Higginbottom Institute of Agriculture, Technology & Sciences, Allahabad, Uttar Pradesh, India - 211007

[2]Department of Biochemistry, Faculty of Science, King Abdulaziz University-Jeddah, Kingdom of Saudi Arabia. 80200

[3]Department of Pharmacognosy & Phytochemistry, Faculty of Pharmacy, Jamia Hamdard, New Delhi, India - 110062

Abstract

The current investigation was confronted to epitomize the anti-inflammatory and antioxidant effect of *Paederia foetida* Linn. (*PF*) along with mechanism of action implicated in tissue protection. HPTLC use to identification of quercetin compound. While the *in vitro* analysis expressed that the *PF* confirmed the significance antioxidant and anti-inflammatory action. We initially demonstrated that *in vivo*, anti-inflammatory effect of *PF* was evaluated against the variety of phlogistic agents as well as turpentine oil, prostaglandin and arachidonic acid. Groups of rats, fasted overnight received Group I: normal control (vehicle), Group II: *PF* (100 mg/kg), Group III: Arthritic control (CFA only, 0.05 ml), Group IV, V, VI: CFA (0.05 ml) + *PF* (25, 50 and 100 mg/kg) and Group VII: CFA (0.05 ml) + indomethacin (10 mg/kg, b.w.). *PF* significantly confined against paw edema, arthritic index and body weight alteration induced by Complete Fruend's Adjuvant (CFA). Another observation like histological and macroscopically changes was observed in CFA induced inflammation in knee joints. Subcutaneously administration of CFA was accompanied by proinflammatory cytokines status appraised by an amplification of interlukin-2 (IL-2), interlukin-1β (IL-1β) and tumor necrosis factor (TNF- α); an status of oxidative stress was estimated by enhance level of lipid peroxidation (LPO), a depletion of superoxide dismutase (SOD), glutathione peroxidase (GPx) and glutathione (GSH). Pre-treatment with *PF* significantly (P<0.001) sheltered against CFA induced oxidative stress and proinflammatory cytokines. More prominently, CFA administration augmented tissue and plasma superoxide (O$_2$) and hydrogen peroxide (H$_2$O$_2$), while the *PF* pre-treatment significantly (P<0.001) inverted all CFA induced intracellular interruption. Following CFA induced arthritis,

PF were tested for their free radical scavenging activity against DPPH, ABTS radicals and inhibitory proficiency against COX-1 and COX-2 *in vitro*. Considering the above, the current research confirmed momentous protection against CFA induced arthritis, which could be endorsed to their anti-inflammatory and pro-oxidant nature.

Keywords: *Paederia foetida* Linn, Prostaglandin, Cyclooxygenase, Complete Fruend's Adjuvant.

Corresponding Author

*Vikas Kumar

Department of Pharmaceutical Sciences,

Faculty of Health Sciences,

Sam Higginbottom Institute of Agriculture, Technology & Sciences,

Allahabad, Uttar Pradesh, India – 211007

Email: phvikas@gmail.com

*Prof. Firoz Anwar

Department of Biochemistry,

Faculty of Science,

King Abdulaziz University-Jeddah, Kingdom of Saudi Arabia. 80200

Email: firoz_anwar2000@yahoo.com

Introduction

Rheumatoid arthritis (RA) is a chronic, systematic and typical inflammatory disease that influences the 1% of young population globally. It is exemplify that a lot of pathological process involved in the arthritis like swelling in joints, destruction of gristle and progressive ruination of subchondral bone and cartilage.[1] Pro-inflammatory cytokines such as (TNF-α), (IL-6) and (IL-1β) are known to be the primary mediators, which play an imperative function in the pathophysiology of RA, and have been targeted to discover the new drugs for the protection of RA. Pro-inflamatory cytokines induces inflammation through the angiogenesis, tissue ruination and leucocytes linkage in RA.[2] Another pathogenesis of RA, free radicals especially reactive oxygen species (ROS) participate an crucial role to induce the cartilage ruination either activation of MMPs or degradative effect on matrix components.[3] Adjuvant induced arthritis in rats which shares the similar RA feature like the human RA including oxidative stress, weight loss and inflammatory infiltration with joint ruination.[4] CFA containing the 1 mg/ml of dry heat-killing tubercle bacillus (*Mycobacterium tuberculosis)* per sterile paraffin oil. CFA produced the inflammation through the liberation of blood cells, mastocyte activation, cytokines and formation of free radicals.[5] Clinically, several non-steroidal anti-inflammatory drugs (NSAIDs), TNF-α inhibitors, selective cyclooxygenease inhibitors (COX-2), immunosuppressive and antioxidant drugs are used to treat chronic inflammatory, pain and bone destruction during the arthritis.[6] But above treatment having the limitation in the treatment of RA due to their undesirable effects like less effectiveness with chronic use, myelosuppression, gastrointestinal disorder (mainly ulcer), neutropenia, cardiovascular disorder, hemorrhage and high in cost.[7] With these complexities, the RA research field has been improvised towards plant based therapies that are thought of safe and effective altogether elevating chronic pain related to arthritis. RA is that most often occurs arthritis, being classified treated with the antioxidant and anti-inflammatory drugs.[8]

The utilization of indigenous medicine has been a dependable source of antioxidant and anti-inflammatory therapy for decades. Therefore, most of the researcher has been paying consideration to find effectual therapies that have antioxidant/anti-inflammatory effect with less or no side effect. It is therefore important that we prolong search the herbal pharmacopeia for antioxidant and anti-inflammatory potential of plant which have the protection against the inflammatory arthritis such as RA. The current investigation is the foremost to appraise the

antioxidant and anti-inflammatory potential of *Paederia foetida* Linn. in adjuvant induced arthritic rats.

Paederia foetida Linn. belonging to Rubiaceae family; native to India and China. The plant of *PF* grows wild throughout in deciduous, evergreen forests of India and China at an altitude 1800 meter.[9,10] The plant possesses a spectrum of the therapeutic activity starting from anti-inflammatory,[11] antiviral,[12] antinocietive,[13] antidiarrheal,[14] antitussive activity,[15] antibacterial,[16] antithrombolytic and antidiabetic properties,[17] therefore, used in the piles, ulcers, skin diseases, asthma and laxative etc.

MATERIAL AND METHODS

Chemical

Turpentine oil (Arora Pharmaceutical, New Delhi), 2, 2 Diphenyl picrazile, 2, 6 dichloro indophenols (SD fine, Mumbai), Ascorbic acid 98 % (SD fine, Mumbai) and all other solvents, chemicals used for experiment were purchased from reputed vendor, Allahabad, India. The entire reagent utilized for the experimental purpose which were analytical grade.

Animals

Swiss albino (Wistar strain) rats weight between 140-190 gm (8 Weeks old, both male and female) used for the current study. All animal were housed under the quality laboratory conditions (temperature 25-27°C) and maintain 12 hour light, 12 hour dark cycle. The animals were received standard food pellets (fortified with vitamins and minerals) and water *ad libitum*. The experimentation animals were maintains as per the Institutional Animal Ethical Committee constituted by Siddhartha Institute of Pharmacy (1435/PO/a/11/CPCSEA).

Preparation of *Paederia foetida* Linn methanolic extract

Paederia foetida Linn. Leaves were cultivated from the region of Dibrugarh (Assam) and identified by Botanical Survey of India, Shilong, India (Ref. No.DU/PSc/HRB-2/08). The collected leaves were afterward desiccated in an incubator till 72 hours at 50°C and powdered the plant material using electric blender. The plant material was prepared in methanol, under soxhlet apparatus during 24 hours; lyophilized the extract and stored at -80°C for further use.[19]

High performance thin layer chromatography (HPTLC) method

Test sample and reference compound were applied on the pre-coated silica gel plate (60F$_{254}$) with band width of 6 mm band. Camag 100 microliter taster syringe used for injecting the

sample with a Linomat 5 applicator under low flow of N_2 gas. Hexane: ethyl acetate: formic acid (5:4:1) used as a mobile phase to carried out the development of chromatogram in linear ascending method using Camag glass twin tough chamber (20X10 cm). For getting the good resolution of tested compounds, we optimized the TLC chamber (20 min) and total run time was about 25 min at room temperature (27±2°C) and relative humidity (50±2%). After run the TLC plate in mobile phase and hair drier used for drying the TLC plate and different wavelength used for TLC image optimization.

Total flavonoids content

The estimation of the flavonoids content of *PF* was evaluated using the aluminum chloride spectroscopic method with minor modification. Quercetin (10 mg) was suspended in methanol (10 mL) and then diluted into different concentrations (20 - 100 μg/mL). Distilled water (2.8 mL), 1 M potassium acetate (0.1 ml) and 0.5 mL of diluted standard solution of quercetin were added and incubated at room temperature for 30 min. The absorbance of incubated solution was estimated at 415 nm using the Shimadzu UV-1700 (Japan) spectrophotometer. 10% of aluminum chloride was substituted by the equal quantity of distilled water. Aluminum chloride and *PF* extract (1 mg/mL) was reacted for estimation the flavonoids quantity as expressed above.[20]

Free radical-scavenging activity on 2, 2-diphenyl-1-picrylhydrazyl (DPPH)

The antioxidant activity of *PF* was evaluated by following the DPPH free radical scavenging method with minor modification.[20] Briefly, prepared the 0.1 mM solution of DPPH in 4 mL of methanol; prepared solution of DPPH was added to 1 mL of sample solution at various concentration of *PF* (5, 10, 25, 50, 100, 200 μg/mL) and incubated at 27°C during 30 min. After thirty minutes, the optical density of the sample was enumerated at 517 nm. The free scavenging activity of *PF* was described as a percentage and calculated by following formula.

$$Radical\ Scavenging\ Activity\ (\%) = (Control - Test)x100/Control$$

The same concentration of reference molecule (Quercetin and ascorbic acid) was used as the test extract. To scavenge 50% of the DPPH radical, the effective concentration 50 (EC50) was calculated.

Free radical scavenging activity on 2, 2'-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) radicals

The radical scavenging activity of *PF* was evaluated using $ABTS^+$ free radical scavenging method as previously described method with minor modification.[21] Briefly, ABTS solution (7 mM) was prepared by reacting with 2.45 potassium persulfate (4.95 mM) and incubated the

solution at room temperature for 12-16 hr; after 16 hr blue green colour persist at the end. Further, the solution was diluted with ethanol to yield an absorbance of 0.7 ± 0.02 at 734 nm. Then, sample (0.1 mL) mixed with working solution (3.9 mL) and incubated at room temperature for 10 min. The percentage of inhibition and EC_{50} value was calculated using the procedure described for DPPH method.

Acute toxicity study

PF extract was tested for acute oral toxicity study in Swiss albino Wistar rats (weighing about 140-200 gm, both male and female). The Wistar rats divided into different groups (each group contains 6 animal) and each groups received the different doses of *PF* (2000, 1000 and 500 mg/kg, b.w.). All group animals were observed for water intake, food consumption, mortality and general behavior till 48 hr following the oral administration of *PF* extract.[22,23,24]

Short term sub acute toxicity study

To investigate the sub acute toxicity study, Swiss albino Wistar rats (weighing 140-190 gm, both male and female) divided into following group; Group I-Normal control (1 ml/kg); Group II-*PF* (2000 mg/kg, b.w.); Group III-*PF* (1000 mg/kg, b.w.); Group IV-*PF* (500 mg/kg, b.w.) and each group contains the 3 male and 3 female rats. The all group rats received the pre-determined treatment orally once per day using oral needle. Rats were monitored for mortality, clinical symptoms, adverse reactions and any other effects till the 28 days. The food consumption, water intake and body weight were estimated at regular intervals. All group rats blood sample was withdrawn by tail vein and used for the estimation of hematological parameters like white blood cells (WBCs), red blood cells (RBCs), hemoglobin (Hb), erythrocytes sedimentation rate (ESR), neutrophils; serum biochemistry like alkaline phosphatase (ALP), aspartate Aminotransferase (AST), bilirubin, creatinine, urea, triglyceride, cholesterol, high density lipoprotein (HDL), low density lipoprotein (LDL), very low density lipoprotein (VLDL) . The all group animals were scarified on day 29 using the excess of anesthesia. The vital organs like kidney, liver and heart were removed and performed the morphology and histopathology study.

Turpentine oil-induced joint edema in rat

All the animals were distributed into following groups and each group contains the 6 rats. The entire groups of rats starved overnight (12 h) before the experiment carried out and rats were divided into following groups; Group I: normal control; Group II: inflammatory control; Group

III: *PF* (25 mg/kg, b.w.); Group IV: *PF* (50 mg/kg, b.w.); Group V: *PF* (100 mg/kg, b.w.); Group VI: Indomethacin (10 mg/kg, b.w.), respectively. Acute inflammation was induced in the paw by injecting the 0.02 ml turpentine oils into the synovial cavity of knee joint.[25,26] The joint diameter of all group rats was monitored at regular intervals till 6 hours using the screw gauge micrometer.[27] The percentage inhibition of paw edema was estimated by using the following formula

$$\% \, Inhibition = \frac{(VC - VT)x100}{VC}$$

Where VC= Paw edema of inflammatory control, VT= paw edema of the test group.

Inflammatory mediator induced inflammation

The potency of odemogens, time of determination the edema and volume injected are shown in the parenthesis; Arachidonic acid (AA) [0.1 mL (0.5% in 0.2 M carbonate buffer at pH 8)], Prostaglandin (PGE_2) (0.1 mL) and histamine (0.1 mL).[28] The edema of the injected paw was estimated in the hind paw of animals after the 1 hr of orally administration of tested and standard drugs (different standard drug used in different models).

In vitro COX and LOX estimation

The *PF* was evaluated against the COX-1, COX-2 and LOX inhibitory test for explicating the possible anti-inflammatory mechanism. The estimation of the COX and LOX was estimated using the standard kits, following the manufacture instructions.

Complete freund's adjuvant (CFA) induced arthritis

Wistar rats fasted overnight (12 h) before the experimentation. The rats were divided into following groups and each group contains 6 rats. Group I: normal control; Group II: normal control + *PF* (100 mg/kg, b.w.); Group III: arthritic control; Group IV: *PF* (25 mg/kg, b.w.); Group V: *PF* (50 mg/kg, b.w.); Group VI: *PF* (100 mg/kg, b.w.); Group VII: Indomethacin (10 mg/kg, b.w.), respectively. CFA containing the heat killed *Mycobacterium tuberculosis* (10 mg) per milliliter sterile in paraffin oil [25]. 1 day before the CFA injection and predetermined treatment continued for 28 days and edema of left paw was evaluated at regular intervals. Arthritis was assessed by measuring the mean increase in paw diameter over a period of 28 days using a micrometer screw gauge.[27] Body weight was measured at regular intervals. The percentage of inhibition was expressed using the following formula

$$\% \, inhibition = (Control - Test)x100/Control$$

Arthritis index in CFA rats

Arthritis assessment in the CFA induced arthritis rat evaluated by visual scoring system. The symptom of arthritic was divided into the score like without any change: score 0, swelling and erythema: score1, erythema of limb and mild swelling: score 2, erythema of limb and gross swelling, inability of limb and gross deformity. The score record was maximum 16 and also the score was evaluated at regular intervals.[29]

Biochemical Analysis

For estimation of the biochemical parameters, blood samples were withdrawn from puncturing the retro-orbital under mild anesthesia condition. Blood samples were collected into the anticoagulation agent containing tubes, centrifuged blood sample and also the supernatant serum was collected.

Pro-inflammatory cytokines estimation

The pro-inflammatory cytokines like interlukin-2 (IL-2), interlukin-1β (IL-1β) and tumor necrosis factor (TNF- α) was estimated at the end of the study, all group animals were sacrificed at end of the study and blood samples were collected into the EDA coated tubes and centrifuged the samples at 2000Xg for 10 min at room temperature to get the clear plasma. The pro-inflammatory cytokines were estimated using the radioimmunoassay kits following the manufacture instructions.

Estimation of Antioxidant markers

The level of the Lipid peroxidation (LPO) was evaluated from the concentration of malondialdehyde (MDA), which is the indicator of the lipid peroxidation. The endogenous antioxidant marker such as superoxide dismutase (SOD) was estimated to evaluate the capacity to restrain the auto-oxidation of pyrogallol method.[30] Glutathione peroxidase (GPx) activity was calculated by method of Nam et al.,[30] by estimation the consumption of NADPH. Glutathione (GSH) activity was measured by the method of campo et al.,[31] on the reaction with Tris-EDTA. The estimation of the lipid peroxidation (LPO) was evaluated by using the method of.[32]

Histopathology study

The end of experimental study, all group animals were sacrificed with excess of diethyl ether and total knee joints were expunged. The expunged knee joints were fixed in the 40% formaldehyde for 4 days followed by decalcification in formic acid. The decalcified knee joints were fixed and embedded in paraffin. 5 μm samples of each knee joints were cut and stained with eosin and

haemotoxylin. The prepared section of knee joints were scrutinized with microscope for evaluation the damage to articular cartilage facing the femoral head.

Statistical analysis

The statistical analyses were performed on the Dunnett's multiple comparison, one way ANOVA, and Graph Pad Prism version 5.0. The values were expressed as the mean S.E.M. to showed variation in all treatment groups. $P<0.05$ was considered statistically significant.

Result:

HPTLC study

HPTLC chromatogram presences of the quercetin in the plant extract (Figure 1) which was confirmed by the comparison of the standard molecule quercetin HPTLC chromatogram (Figure 2).

Antiradical activity and estimation the polyphenol content

We have found quercetin equivalent (QUE) content 21.02 ± 1.83 mg g^{-1} in the *PF* extract. Concerning the free radical scavenging capacity, the figure revealed that the radical scavenging activity of *PF* and quercetin against ABTS and DPPH radical increased significantly in a dose dependently (figure 3, 4). The EC_{50} values DPPH radical was estimated from the graph exhibited a higher rate of RSA for *PF* (EC_{50} = 50 μ g/mL) and quercetin (EC_{50} = 15 μ g/mL) well known antioxidant molecules (DPPH). The EC_{50} value for ABTS radical was estimated form the graph exhibited a higher rate of RSA for *PF* (EC_{50} = 92.43 μ g/mL) and quercetin (EC_{50} = 27 μ g/mL) well known antioxidant molecules.

Acute oral toxicity study

Oral administration of *PF* extract (200 to 500 mg/kg b.w.) did not demonstrate any adverse reactions, mortality and change in clinical/behavioural during the toxic dose study. The water, food intake and body weight of the rats were unchanged at end of the study. Sub acute toxicity study of *PF* (2000, 1500 and 500 mg/kg b.w.) also did not produce any signs and symptoms of neurological, behavioural, mortality and abnormal clinical changes as compare to control group of animals. The data showed that the safety of *PF* extract upon the continuous administration for 28 days, irrespective of sex of animals. The sub acute toxicity study (28 days), the relative body weight of the diverse organs of normal control like liver (4.34±0.32), kidney (0.83±0.06) and heart (2.31±0.08) was found in the male animals; and liver (2.98±0.26), kidney (0.67±0.09) and heart (1.96±0.04) respectively in female animals. The relative vital organ weight noted was liver

(4.43±0.48), kidney (0.82±0.08) and heart (2.64±0.07) in male animals; liver (3.03±0.52), kidney (0.68±0.07) and heart (2.10±0.09) in female animals. The data of the normal control and *PF* (2000 mg/kg b.w.) almost similar. Even the data of *PF* (1000 and 500 mg/kg b.w.) did not confirm any deviation in the relative organ weight. The body weight of the normal control male animals increased from 152±8.93 to 222.8±14.34 with a growth gain rate of 2.52±0.43 per day and the female animal body weight was 132±7.73 to 191.8±12.73 with a gain rate 2.53±0.18 respectively. The body weight of *PF* (2000 mg/kg b.w.) was increased from 155.8±11.83 to 232.8±16.5 with a gain rate 2.75±0.98 per day in male animals and female animals increased body weight 136±8.93 to 204.2±13.43 with gain rate 2.43±0.42 respectively. *PF* (1000 mg/kg b.w.) increased the body weight from 158.4±9.73 to 214.4±14.2 with gain rate 2±0.19 per day in male animals and female rats increased the body weight 142.3±10.23 to 195.6±12.34 with gain rate 1.90±0.17 respectively. Another dose *PF* (500 mg/kg b.w.) increased body weight from 159.5±12.74 to 208.2±15.32 with gain rate 1.73±0.13 per day and female animals increased body weight form 143.8±7.83 to 188.4±14.54 with gain rate 1.59±0.11 respectively (Table 1.1).

The data of the acute study did not demonstrate any momentous changes in the biochemical and haematological parameters of *PF* treated rats (Table 1.2 & Table 1.3). *PF* treated rats showed normal range of RBC, WBC, platelet counts, haemoglobin and different counts as compared to the normal control. *PF* treated rats showed the normal range of renal and hepatic value as compared to the normal control. Another parameters lipid profile like triglyceride, cholesterol, LDL, HDL and VLDL also unchanged at end of the study (Table 1.2).

The histopathology study of the normal control and *PF* treated animals confirmed the normal appearance for various tissue and organs (Figure 5). The normal and treated animals did not demonstrate the any significant changes in the relative weight and organs (liver, kidney and heart) of male and female rats.

Effect of *PF* on turpentine oil and proinflammatory induced paw edema

Subplanter injection of turpentine oil induced the paw edema that was continued during the complete scrutiny period (Table 2). The reduction of paw edema followed by oral administration of *PF* and standard drug (indomethacin) was contrasted at regular intervals after the post injection of turpentine oil. Oral administration of *PF* inhibited the paw edema at dose

dependently as compared to turpentine oil induced control group rats; with a significant (P<0.001) reduction of paw edema 11.21, 23.97 and 45% respectively for 25, 50 and 100 mg/kg b.w. doses of *PF,* when the paw edema was evaluated after 6 hours of post injection. On contrary, indomethacin treated group rats showed the relative inhibition of 44.83%. The *PF* (100 mg/kg b.w.) also restrained arachidonic acid induced inflammation (P<0.001) considerably. In PGE$_2$ induced edema, the *PF* (100 mg/kg b.w.) manifested inhibition of edema (Table 3). Thus, *PF* (100 mg/kg b.w.) significantly restrained the AA, PGE$_2$ and histamine induced inflammatory reactions.

Effect of *PF* on CFA induced arthritis

Subcutaneous injection of CFA caused serve arthritis throughout the rat hind paw (Figure 6). CFA induced rats treated with *PF* significantly (P<0.001) inhibited the paw edema a dose dependently (Table 4). While the oral administration of *PF* (25 mg/kg b.w.), restrained the paw edema 1.158 with 28.61% inhibition, the paw edema followed by 50 mg/kg b.w. was 0.982 with 39.46 % inhibition and that at 100 mg/kg b.w. was only 0.65 with 59.93% inhibition. The paw edema inhibition with the indomethacin (10 mg/kg b.w.) was 0.71 with 56.23% inhibition (Table 4).

In-vitro examinations of the anti-inflammatory effect of *PF* and its inhibitory potential on COX-1 and COX-2 enzymes was measured with significant analysis in order to further elucidate the possible mechanistic link (Table 5).

Treatment with *PF* showed the significant inhibition of proinflammatory cytokine production on day 28; whereas *PF* (100 mg/kg) exhibited the inhibition of IL-2, IL-1β, TNF- α generation, in comparison to adjuvant controls (Table 6).

Effect of *PF* on body weight

The body weight effect of *PF* extract in CFA induced arthritic rats illustrated in figure 7. We observed the no change in the body weight in first two weeks. After 2 weeks, we observed the significant loss of body weight in CFA induced arthritic rats as compared to control rats, which was persistence till the end of experimental study. *PF* ameliorated the curtail body weight of the adjuvant treated rats.

Effect on arthritis index

Regular scrutinizing the paw edema of arthritic rat is essential in appraising the effectiveness of treatment. The visual evidence of arthritic score in arthritic rats was obtained by examined each

paw, which was a biphasic response (figure 8). Adjuvant induced the swelling in the injected paw joints started to show and rigidity around 10-12 days and maximum arthritic score was observed on day 20. *PF* administration produced a dose dependent decrease to 6.4±0.21 (p<0.05) at 25 mg/kg   4±0.45 at 50 mg/kg (p<0.01) and to a maximum 1.2±0.12 at 100 mg/kg b.w. (p<0.001). The indomethacin at a dose 10 mg/kg also decreased the arthritic score 1.3±0.41 (p<0.001) to which was slightly higher to *PF* 100 mg/kg dosage.

Effect of *PF* on antioxidant marker

To scrutinize the implication of oxidative stress in the arthritic effect of *Paederia foetida* Linn, articular tissue was firstly appraised for LPO determination. We observed that CFA administration significantly increased MDA level. More importantly, *PF* treated rats significantly and dose dependently inhibited the arthritic induced by adjuvant (Figure 9). We also calculated the superoxide dismutase in articular cartilage. As expected, adjuvant treatment significantly declined the articular cartilage antioxidant activity as SOD. CFA treated received the *PF* significantly improved the level of SOD as dose dependently (Figure 10). Arthritic rats declined the level of glutathione peroxidase. Glutathione peroxidase, which cleaves hydrogen peroxide (H_2O_2) and lipid peroxides, due to cleavage of the hydrogen peroxide (H_2O_2) and lipid peroxides it, is considered as free radical scavenging enzyme. Upon administration of *PF* extract to normal control rat produce the level of glutathione peroxidase unchanged (Figure 11). CFA treated rat received oral administration of *PF* extract significantly augmented the level of glutathione peroxidase. Figure showed the effect of *PF* on endogenous glutathione, we administered *PF* to normal as well as in arthritic rats. Glutathione (GSH) estimated the endogenous defense against hydrogen peroxide (H_2O_2) radical formation. Oral administration of *PF* 100 mg/kg, b.w. in normal control rat did not show any changes in the level of glutathione. Different doses of *PF* administration to arthritic rats showed exponential increased level of glutathione. CFA induced arthritic rat treated *PF* 100 mg/kg b.w., showed the maximum augmentation in the level of glutathione as compared to other *PF* received groups rat (Figure 12).

Effect of *PF* on histopathology

The histopathology section of the normal control rats shows the normal articular surface with formation of one to four layers of synovial cells. The surface morphology of the knee joint is

very smooth with the arrangement of loose connective tissue below the synovial membrane (Figure 13A). The histological transverse section of knee joint reports confirm that the formation of pannus of bone and cartilage and serve bone erosion, deformity with the presence of neutrophil infiltration in arthritic control group (Figure 13B). *PF* treated rats revealed the pannus formation of bone and cartilage with inhibition of neutrophil infiltration at dose dependent manner (Figure 13C,D,E). Indomethacin treated rats showed the smooth articular surface as well as less pannus formation of cartilage and bone (Figure 13F).

Discussion

Paederia foetida Linn is the extensive use drugs in varied Ayurvedic and Unani system for the treatment of varied disorders. The phytochemical screening of plant extract showed that the plant is rich source of quercetin, ascorbic acid, phenolic resin compound, tannin, sterol, gum and mucilage. Since the plant derived flavonoids have already shown to be capable to afford fortification against various oxidative stress arbitrated diseases, including diabetes, cardiovascular, gastrointestinal etc. The existing study was undertaken to appraise the suspicious effect of *Paederia foetida* Linn. methanolic extract on CFA induced arthritic. We also assessed the oxidative stress as well as inflammation protecting mechanism in this eventual arthritic defense. The plant material was subjected to prepare the methanolic extract and HPTLC analysis revealed the existence of quercetin in the plant extract.

In vivo, we firstly demonstrated that acute TO administration evidently induced acute inflammation, including increase the vascular permeability and vasodilatation. TO arbitrates a nonspecific inflammation described by triphasic discrete stage of mediator release including serotonin and histamine in foremost, second stage kinin and later stage prostaglandin, whereas AA is a precursor of the PGs, generated through the LOX and COX pathway.[33,34] The result depicted a noticeable effect of on turpentine oil induced inflammation, portentous the potential anti-inflammatory probable of the same. Indomethacin significantly ($P<0.001$) reduced the swelling in all phases of turpentine oil.[35] The result proved that marked inhibition of *PF* (100 mg/kg b.w.) of TO and AA induced paw edema, respectively (table). The data suggest the anti-inflammatory effect of *PF*, which could be attributed through the inhibition of inflammatory mediators like serotonin, histamine, kinin and prostaglandin release in all phases.

This model has been widely exploited to scrutinize and appraise the newly developed anti-arthritic drugs. Captivating description the anti-inflammatory activity of *PF* and to appraise

the long term anti-inflammatory effect of *PF* was further subjected for investigate against adjuvant induced immunological chronic inflammation, which is more analogue to clinical, pathological situations, namely CFA induced arthritis.[36] adjuvant gets aggravated the phagocyte activation and secretion of inflammatory mediators that take an essential part in tissue destruction, vascular and fibrosis over a period of time.[5] After injecting the CFA into hind paw, progressively increased the paw swelling (which persist for weeks that is the primary reactions).[27] After few days, the adjuvant starts the deposition into soft tissue and developed the nodules in ear as well as tail, front paw as well as contra-lateral paw (later systemic response).[37,38,39] *PF* appreciably diminished humoral immune reaction, actually due to its capacity to reduced acute inflammation by inhibiting vascular permeability and reducing the other inflammatory mediators like PGE_2. The secondary reaction of adjuvant induced was developed after 12th day, was considerably inhibited by *PF* as effectual as indomethacin. The adjuvant induced secondary lesions have been exposed to be apparently due to a delayed hypersensitivity reaction and *PF* created an apparent consequence on this as well, and claim the antiarthritic effect.[40]

In this current investigation, in order to investigate the effect of compounds on development of adjuvant induced arthritis, body weight was considered as an indirect index. The decline in the weight was observed in arthritic rats during throughout the study period are due to alteration in the metabolic activity and enhanced severity of arthritis in diseases rats.[41,42] Adjuvant induced arthritic rats showed the loss of body weight which was improved by the *PF* and supported the antiarthritic effect.

It is already prove that reactive oxygen species/free radical play an imperative role to destroy the DNA, membrane lipid proteins and cartilage. The endogenous antioxidant defense mechanism endorsed with aerobic cells to neutralize the damaging of ROS.[43] During arthritic condition, increase the production of free radical mainly superoxide (O_2), hydroxyl radical (OH), hypochlorous acid (HOCl) and hydrogen peroxide radicals (H_2O_2) due to increase level of macrophages and granulocytes inflammatory mediators.[27] SOD is the first line most important antioxidant enzyme which catalase the dismutation of O_2 to H_2O_2 by the help of GPx. Another antioxidant GSH, fights against lipid peroxidation, it take part in the diminution of hydroperoxides. Another action of GSH mainly in the protection of detoxification of xenobiotics by acting as a co- substrate (nucleophilic) to glutathione thransferases. GR play a crucible role to

maintain the cellular GSH through GSH redox cycle. Adjuvant rats diminish the level of SOD and CAT along with GSH activity as an effect of their supersaturation and consumption during the cellular lysis as well as oxidative stress, which effect on the lysosomal destruction and lipid peroxidation.[44,45] Lipid peroxidation may be a vital mechanism occurred due to enhance free radical production and disjoint the tissue during the RA condition. The present investigation demonstrated the enhance level of LPO (maker of toxicity and cellular integrity) as well as hepatic markers (ALP and ALAT) in arthritic rats, which deplicate the decline lysosomal stability of adjuvant treated rats. Arthritic rats showed the elevated level of ALP start the secretion into circulation in the bone formation and resorbtion, which is may be due to increase in peroarticular ostopenia and bone erosion.[46] The significant alteration in antioxidant system (SOD, GSH, CAT and GPx) and cellular toxicity markers (LPO, ALP and ALAT) respectively, had shown the organ protection and bone loss effect of *PF* in arthritic rats through the free radicals scavenging effect.

TNF-α (pleiotropic cytokine), which play a critical role in acute and chronic inflammation by helps inflammation cell in penetration by encouraging bond of lymphocytes and neutrophil to endothelial cells.[47] Its persuade the generation of IL-1β, macrophage chemotactic protein-1 (MCP-1) and IL-6, which spread the inflammation. TNF-α is present in the synovial and sera fluid of RA patient and used as the clinical and laboratory markers for confirmation RA diseases.[48] When the production of TNF-α is blockage, the severity of inflammation is inhibited. Macrophages squirt the IL-1β, which enhance the effect of TNF-α and spreading of inflammatory reaction. Both proinflammatory cytokines Macrophage containing TNF-α and IL-1β were activated by the nuclear factor and osteoclasts were differentiated which further cause the reabsroptio and destruction of bone.[3] Monocytes, fibroblasts and T-cells start the production of IL-6, which stimulate the MCP-1 and induces the osteoclast discrimination. Proinflammatory cytokines TNF-α, IL-6 and IL-1β are involved in increasing the clinical sign & symptoms in adjuvant treated rats as well as blood neutrocytosis, joint swelling, arthritic index and body weight loss.[49]

The *PF* leave extract rich source of the quercetin, ascorbic acid, polyphenol etc. Modern science has exposed that quercetin restrain the inflammation by inhibit the monocytes to endothelial cells via reducing the generation of cell surface adhesion molecules. QE also inhibit the nuclear factor-κB, which lead to restrain the cyclooxygenease-2 (COX-2), proinflammatory

cytokines (IL-1β & TNF-α) and chemokines (MCP-1) as well as osteoclastogenesis. The COX-2 plays a crucial role in the conversion of arachidonic acid into prostaglandin (like PGE$_2$), it's profusely expressed in the synovial explants of RA by IL-1β & TNF α. PGE$_2$ and COX-2 provoked the expansion of synovial fibroblasts, which associated with pannus formation by inhibiting the apoptosis.[50,51] Therefore, restrain of PGE$_2$ and COX-2 level by QE has an obvious impact on the comfort of RA development. Our result revealed the anti-inflammatory effect of *PF* shown in the study. The *PF* extract demonstrated the anti-inflammatory effect due to presence of quercetin which inhibits the COX-2 and PGE$_2$ mediators.

The current investigation shown the antioxidant and anti-inflammatory effect of *PF* which was more efficacious that that of indomethacin (P<0.001). The *PF* (quercetin) have pharmacological effect to inhibit the inflammation by inhibiting the proinflammatory mediator (PGE$_2$ and COX-2) in whole human leucocytes and perform as dual acting NSAIDs.[52,53] The current data signify that *PF* act as restrains of COX (COX-1 and COX-2) enzymes, but the *PF* having higher selectivity toward COX-2. On the other hand, indomethacin diminish swelling, arthritic pain and inflammation by inhibiting the COX-1 and COX-2 over liopxygenase and overproduction/or synthesis of PG. But the inhibition of COX-1 caused the adverse effect like gastrointestinal and renal injury.[54]

Conclusion:

Thus, it can be concluded that *PF* demonstrated the safety and efficacy against complete fruend's adjuvant induced chronic inflammation. *PF* exhibited the significantly anti-inflammatory effect mediated through the inhibition of the AA pathway. However, further investigations are needed to recognize and isolate the probable phytoconstituents involved in the anti-arthritic activity. Which would facilitate the use of *PF* in inflammatory disease?

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

VK premeditated and carried out the extraction of the *Paederia foetida* Linn leaves. and carried out the antiarthritic activity and biochemical estimations. MM characterize the chemical compound present in the plant extract. FA, DA and AV analyses the statistical data. All the authors are involved in the critical evaluation of the manuscript.

Acknowledgement

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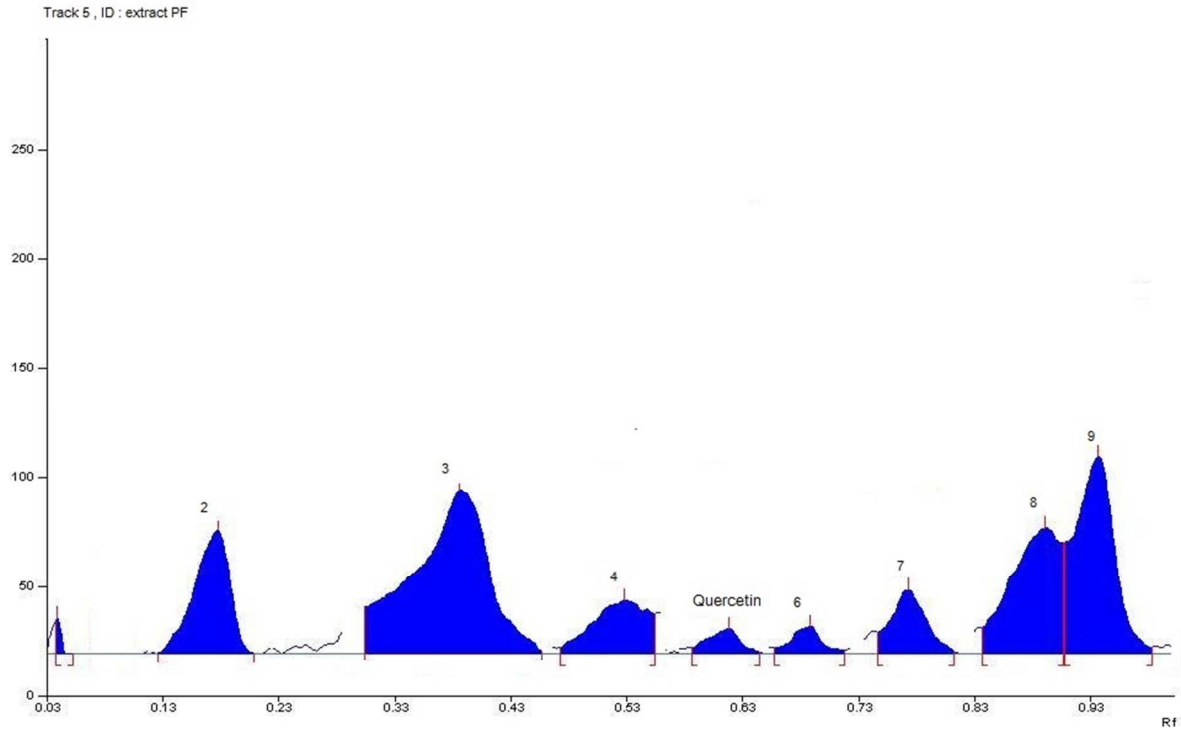
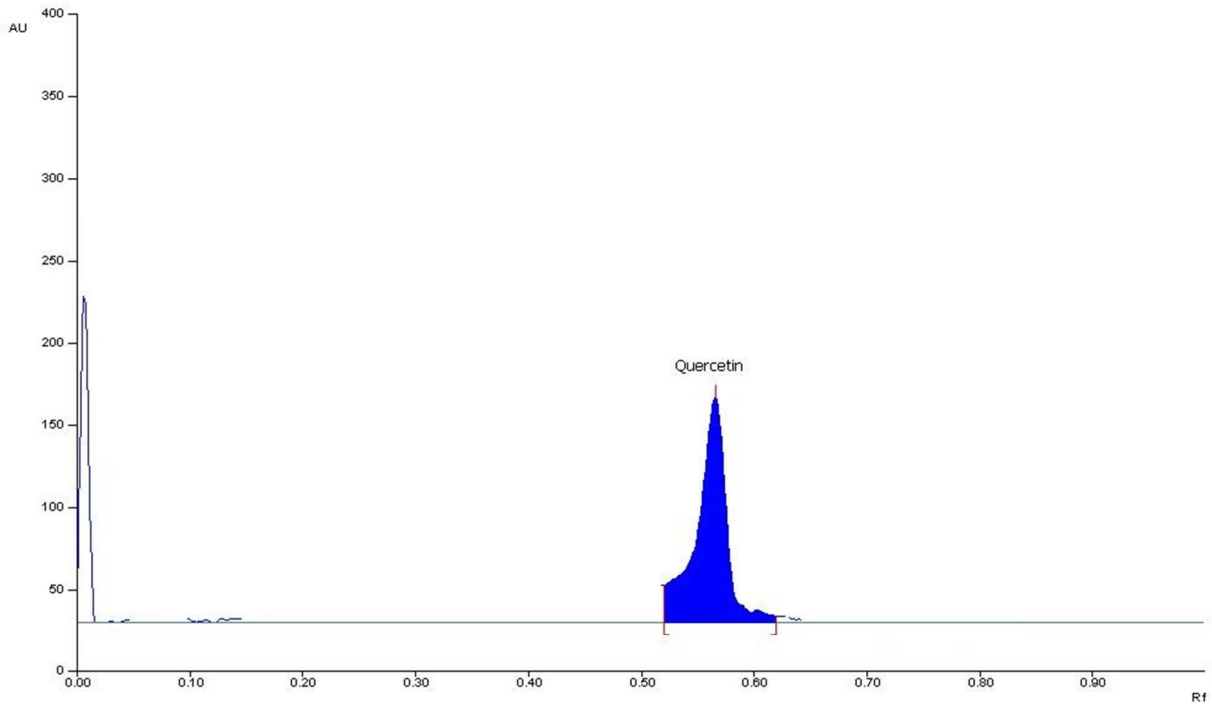**Figure1:** HPTLC profile of *paederia foetida* Linn. extract.

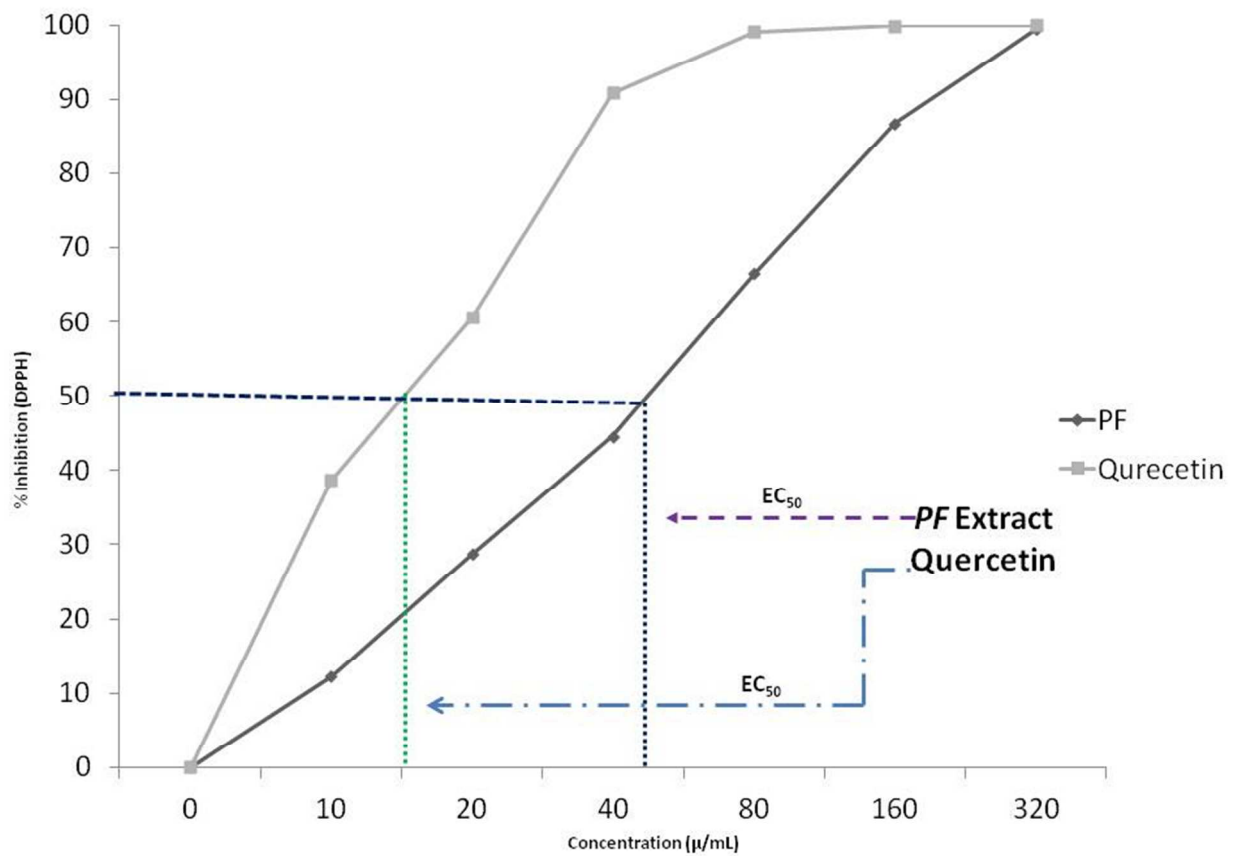**Figure2:** HPTLC profile of quercetin.

Figure 3: Free radical-scavenging activity of *Paederia foetida* Linn. methanolic extract (*PF*) and quercetin on 2,2-diphenyl-1-picrylhydrazyl (DPPH).
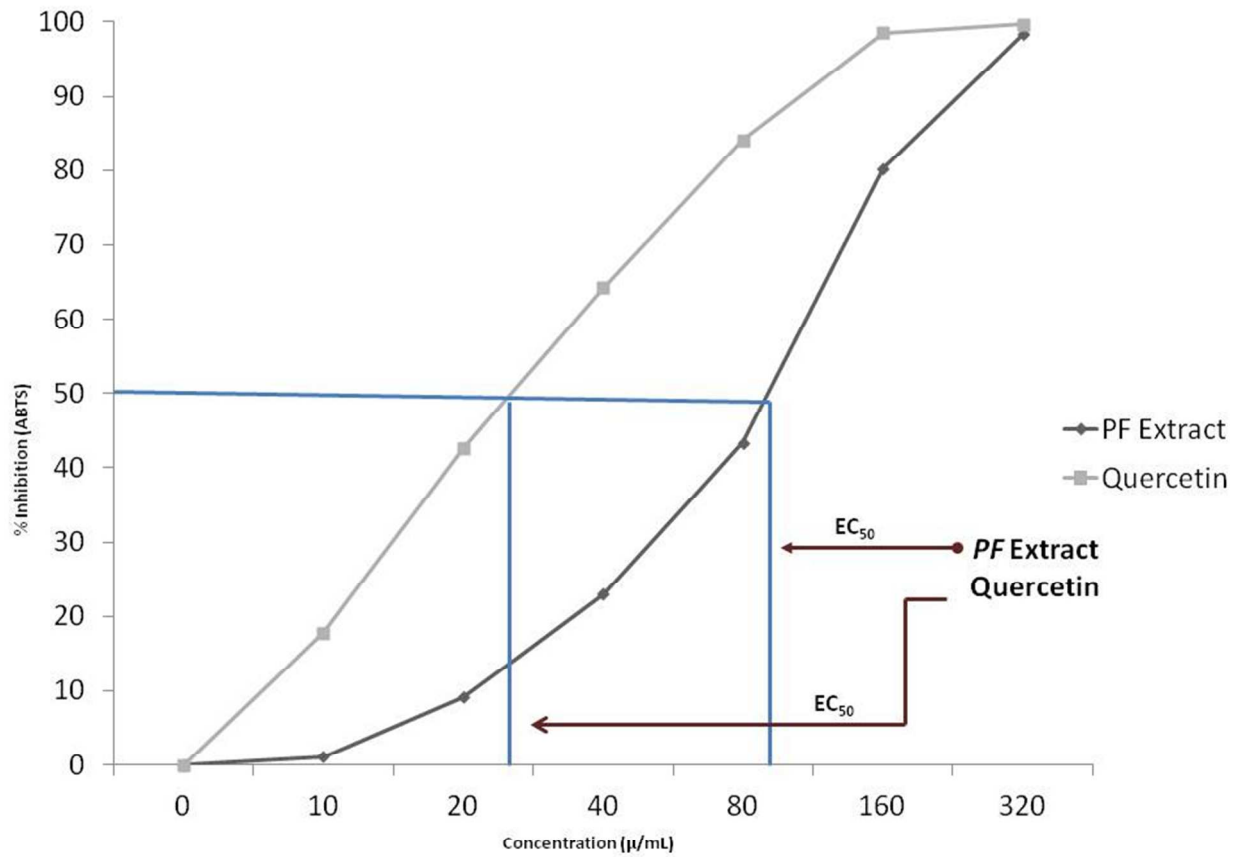
Figure 4: Free radical-scavenging activity of *Paederia foetida* Linn. methanolic extract (*PF*) and quercetin on 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS).
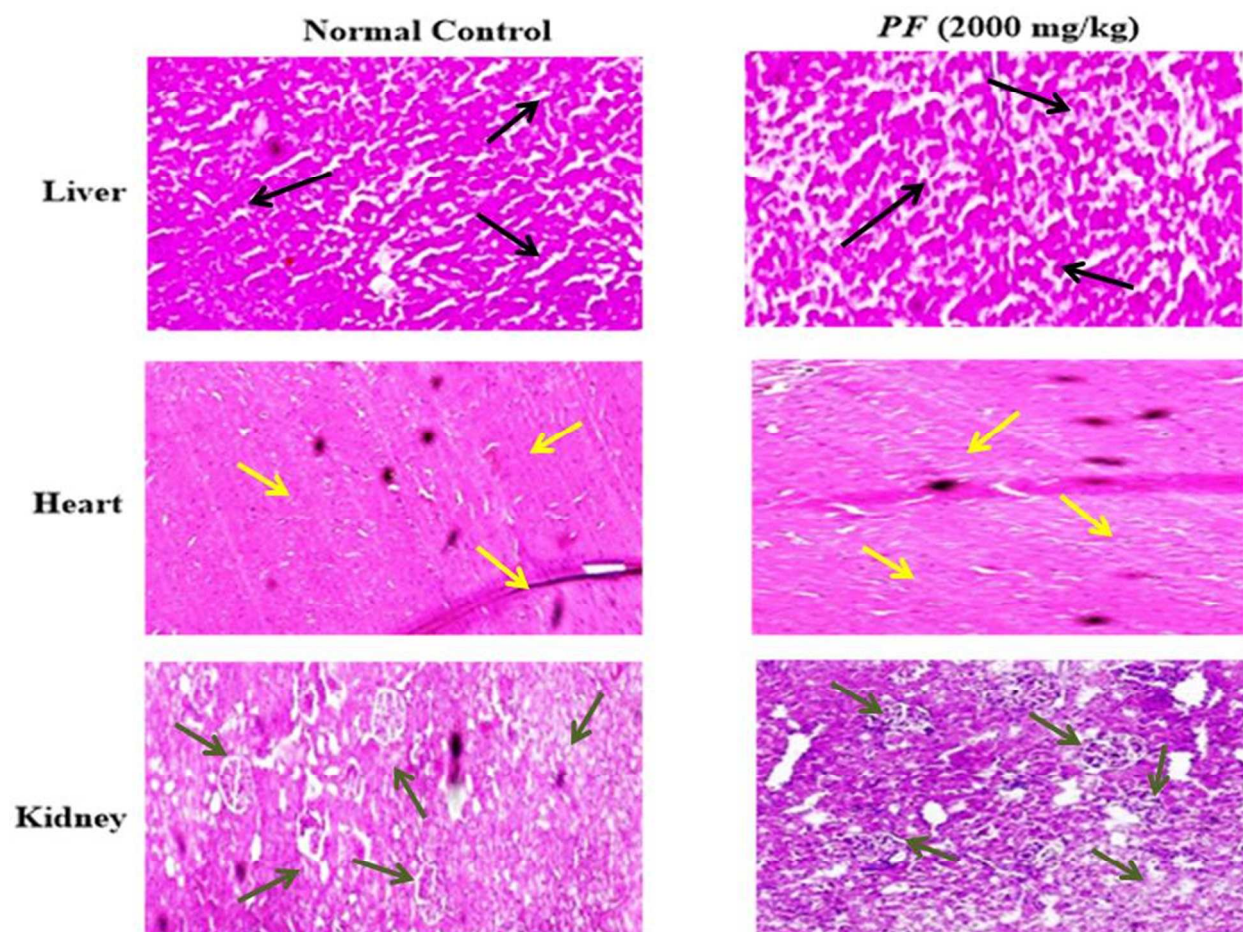
Figure 5: Histopathological observation of vital organ of sub acute toxicity study rats of the normal control and *Paederia foetida* Linn. (*PF*) treated group. Liver (Normal control): Black arrow shows normal structure of portal triad and central vein of the normal hepatocytes. Liver (PF, Normal control): Black arrow depicts normal structure of portal triad and normal central vein. Heart (Normal Control): Yellow arrow indicates normal intercalated disc. Heart (PF, Normal control): Yellow arrow depicts normal intercalated disc. Kidney (Normal control): Green arrows indicates normal glomerulus and tubules. Kidney (PF, Normal control): Green arrow shows normal structure of renal tubules.

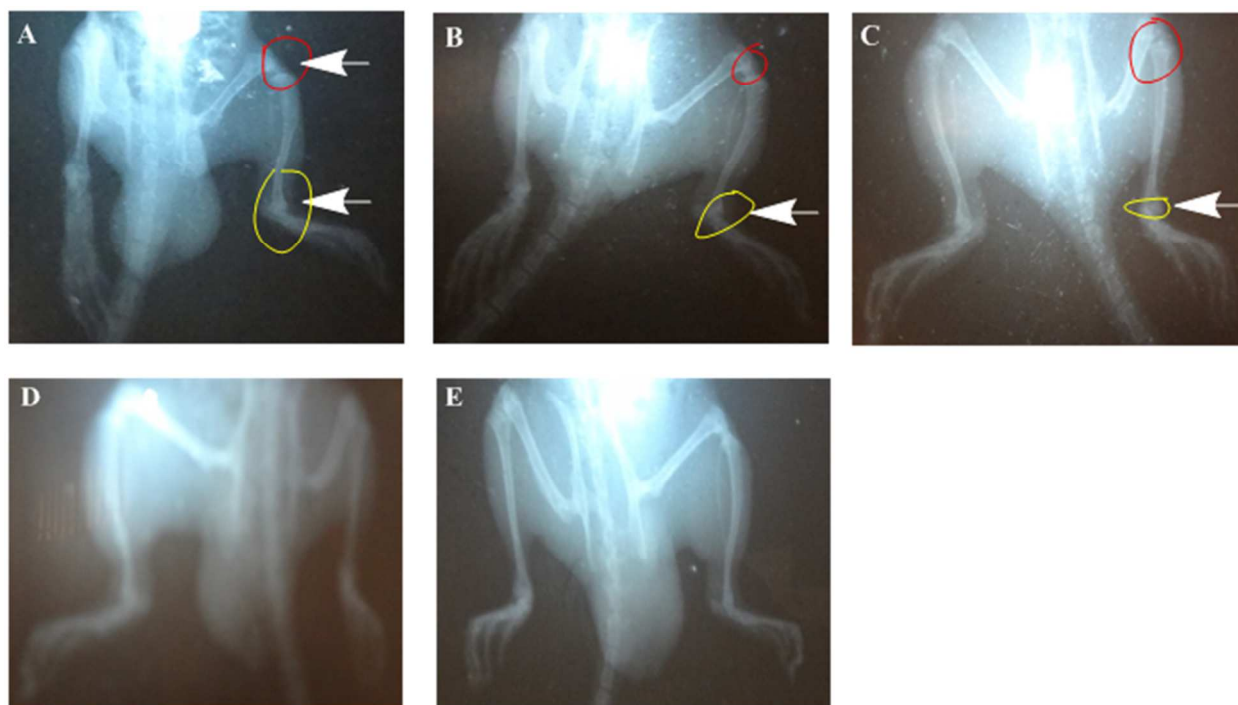**Figure 6**: Effect of *Paederia foetida* Linn. (*PF*) in rats (X-rays photograph). **A:** Arthritic Control; **B:** *PF* 25 mg/kg; **C:** *PF* 50 mg/kg **D:** *PF* 100 mg/kg; **E:** Indomethacin 10 mg/kg.
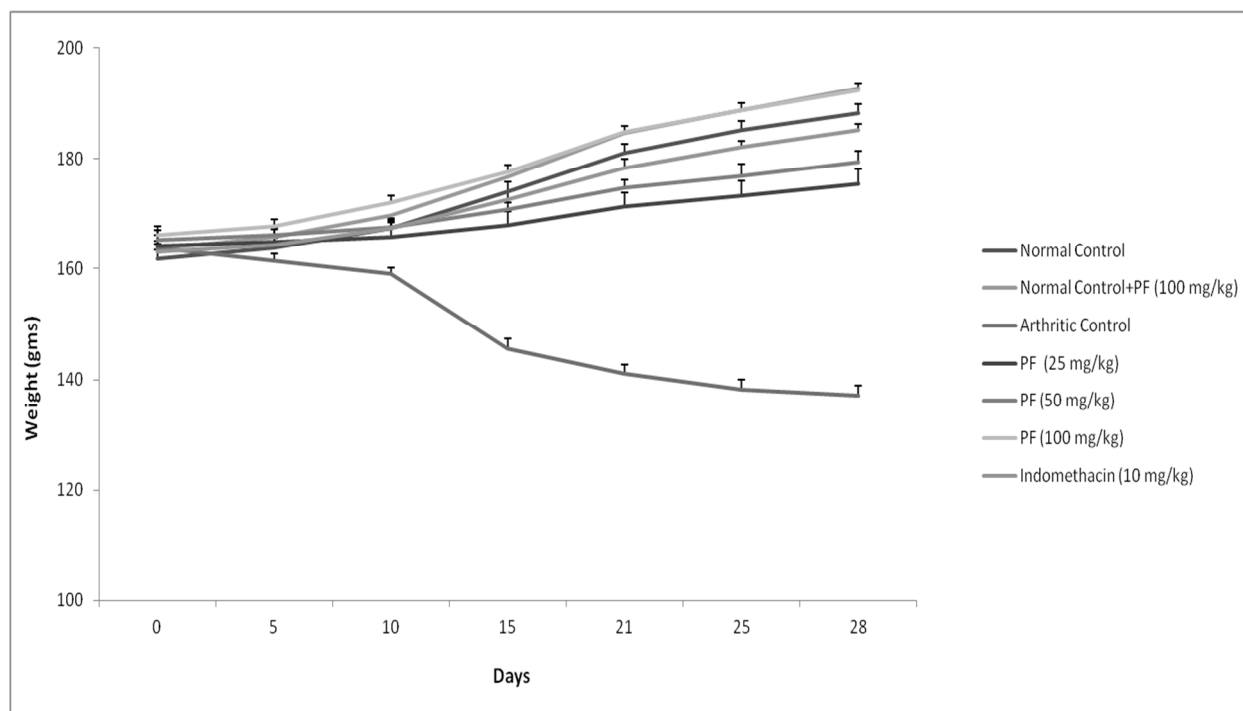
Figure 7: Effect of *Paederia foetida* Linn. (*PF*) on body weight effect of CFA induced arthritic rats.
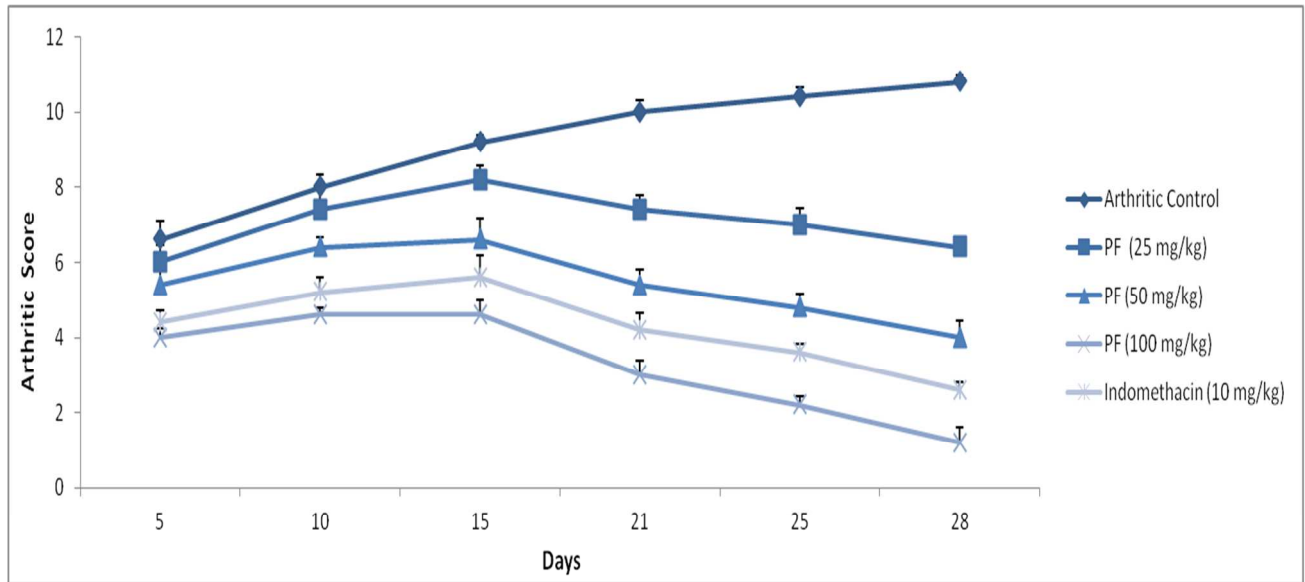
Figure 8: Effect of *Paederia foetida* Linn. (*PF*) on the arthritic score of CFA induced arthritic rats.

Figure 9: Effect of *Paederia foetida* Linn. extract on malonaldehyde antioxidant marker in CFA-induced arthritic rats. Values are presented as mean±SEM, n=6. *=P<0.01, # P<0.05 compared with positive control group.
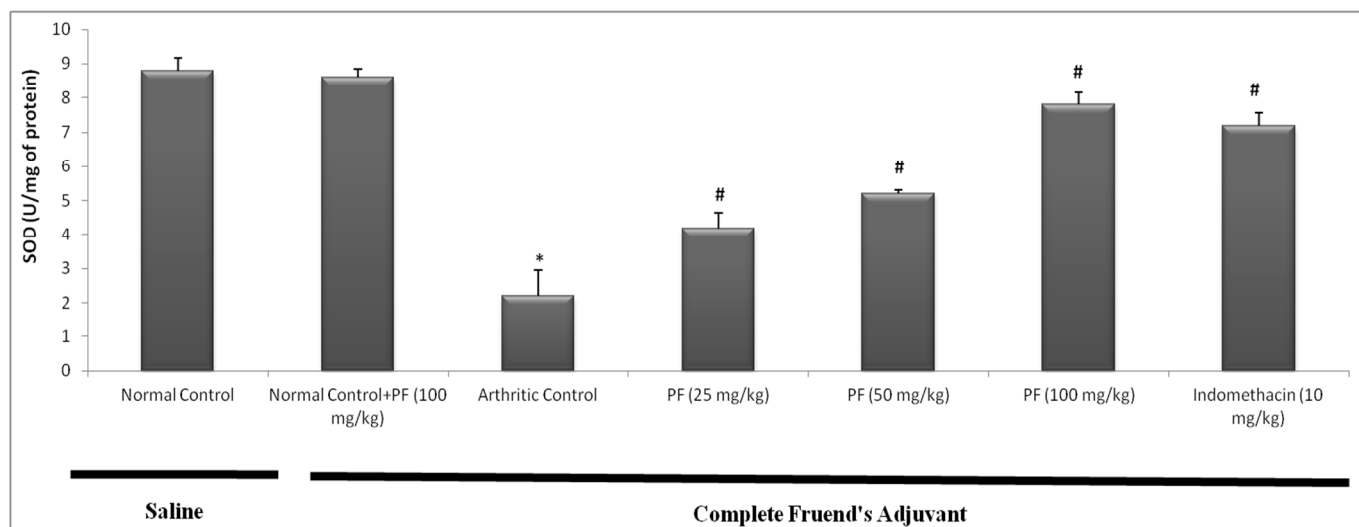
Figure 10: Effect of *Paederia foetida* Linn. extract on superoxide dismutase antioxidant marker in CFA-induced arthritic rats. Values are presented as mean±SEM, n=6. *=P<0.01, # P<0.05 compared with positive control group.
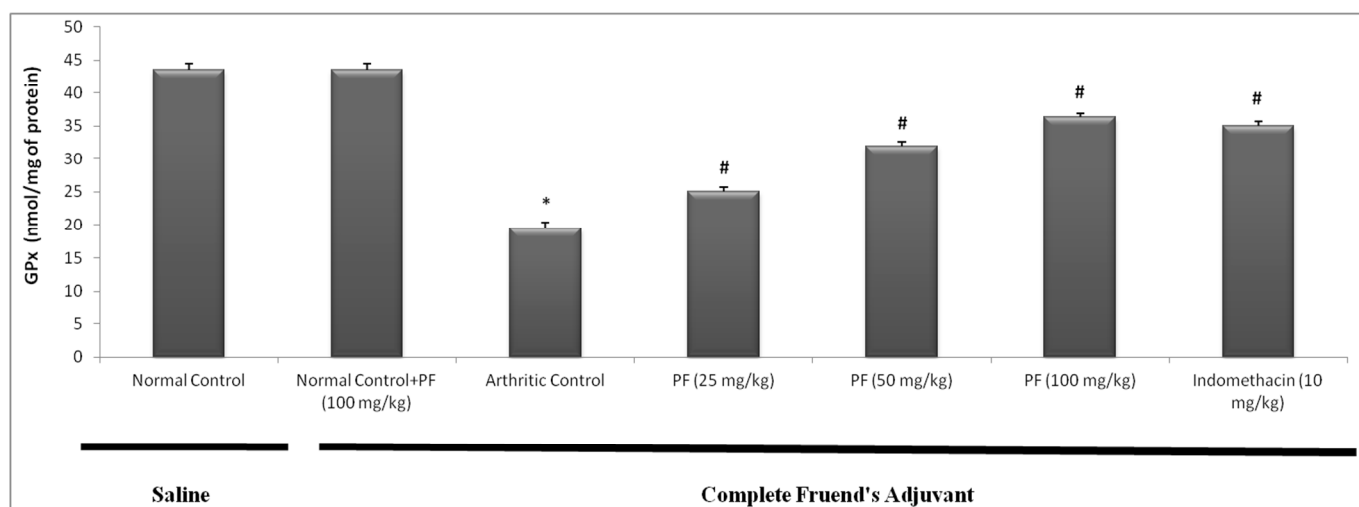
Figure 11: Effect of *Paederia foetida* Linn. extract on glutathione peroxidase antioxidant marker in CFA-induced arthritic rats. Values are presented as mean±SEM, n=6. *=P<0.01, # P<0.05 compared with positive control group.
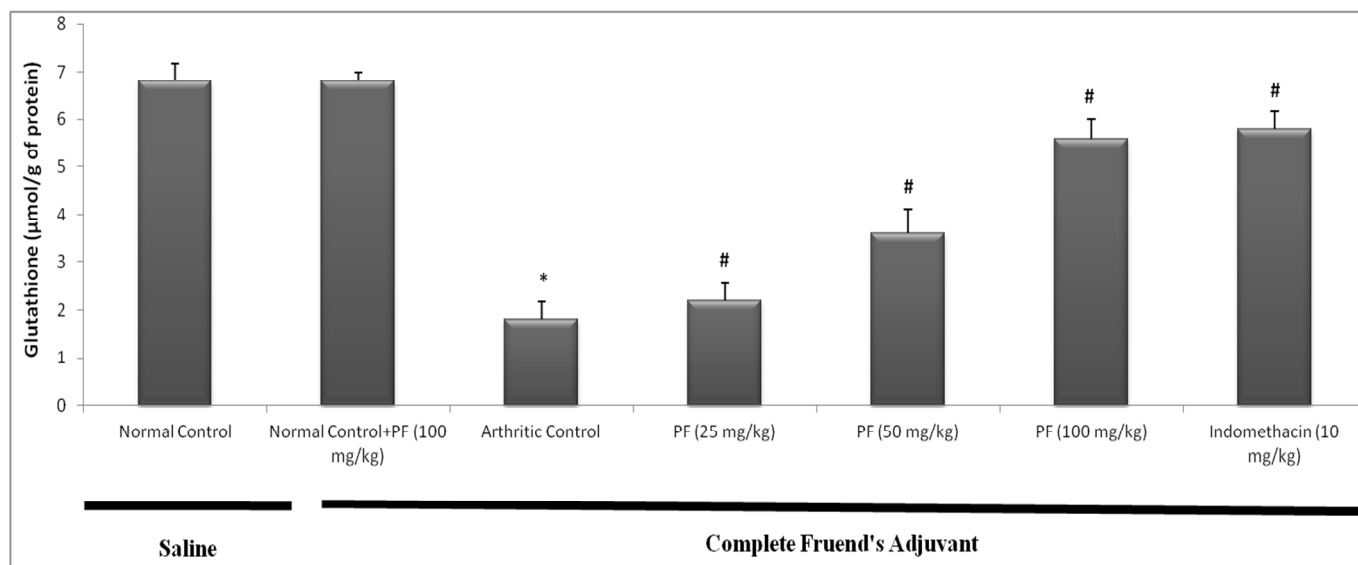
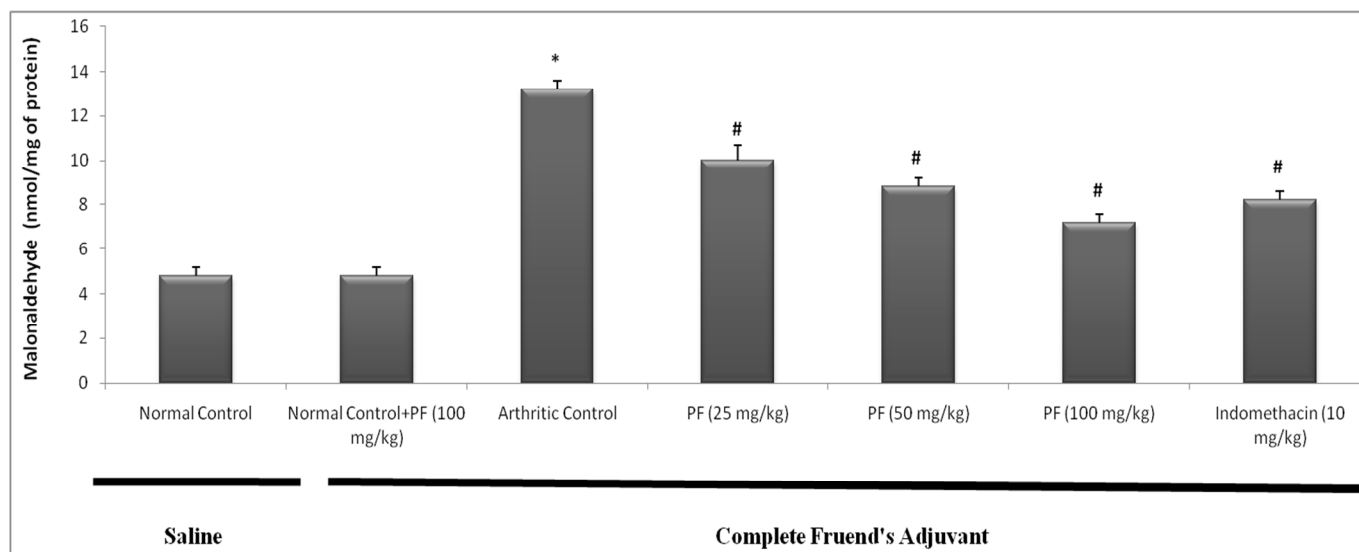**Figure 12:** Effect of *Paederia foetida* Linn. extract on glutathione antioxidant marker in CFA-induced arthritic rats. Values are presented as mean±SEM, n=6. *=P<0.01, # P<0.05 compared with positive control group.
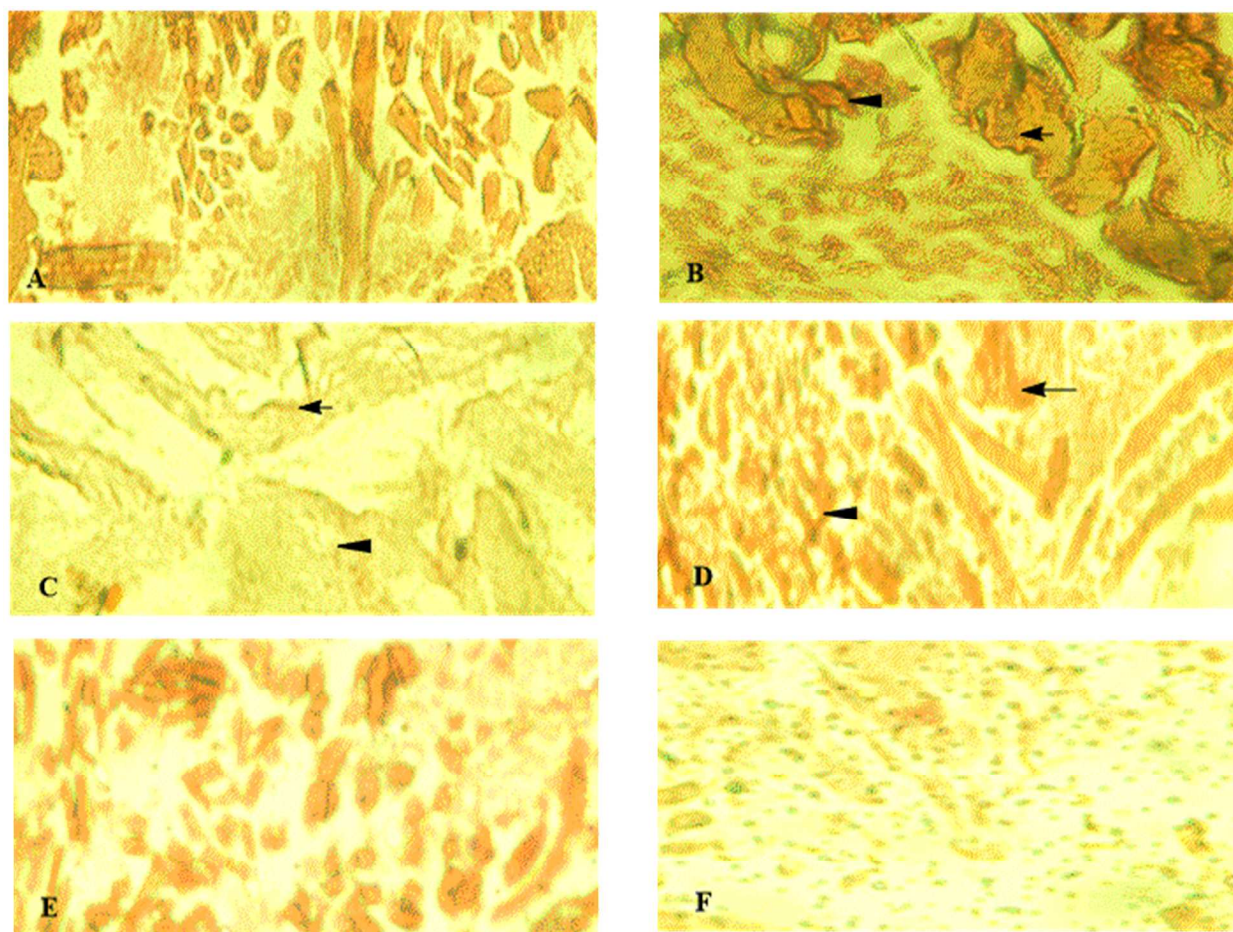
**Figure 13:** Histopathological effect of *Paederia foetida* Linn. on CFA induced arthritic rats. **A:** Normal Control: displayed the normal architecture of articular cartilage; **B:** CFA control: demonstrated the mononuclear cell infiltration, serve bone erosion, deformity with the presence of leukocytes infiltration (arrow) and synovitis (arrowhead); **C:** CFA control + *PF* (25 mg/kg b.w.) leukocytes infiltration (arrow) and synovitis (arrowhead); **D:** CFA control + *PF* (50 mg/kg b.w.) leukocytes infiltration (arrow) and synovitis (arrowhead) ; **E:** CFA control + *PF* (100 mg/kg b.w.) showed the normal architecture with less deformity of the bone and cartilage; **F:** CFA control + Indomethacin (10 mg/kg b.w.): treatment rats showed the improved histopathological profile towards normal architecture with less deformity of the bone and cartilage.

Table 1.1: Effect of administration of *PF* (28 days) on general effect in Wistar rats (Male and Female).

S. No	Parameters	Normal Control		*PF* (2000 mg/kg, b.w.)		*PF* (1000 mg/kg, b.w.		*PF* (500 mg/kg, b.w.)	
		Male	Female	Male	Female	Male	Female	Male	Female
1	**Body Weight (gm)**	222.8±14.34	191.8±12.73	232.8±16.53	204.2±13.43	214.5±14.23	195.6±12.34	208.2±15.32	188.4±14.54
2	**Water Intake (mL)**	112	75	117	82	113	78	109	73
3	**Food Intake (gm)**	68	42	67	44	69	41	66	40

Table 1.2: Effect of administration of *PF* (28 days) on biochemical parameters in Wistar rats (Male and Female).

S. No	Parameters	Normal Control		*PF* (2000 mg/kg, b.w.)		*PF* (1000 mg/kg, b.w.		*PF* (500 mg/kg, b.w.)	
		Male	Female	Male	Female	Male	Female	Male	Female
1	**ALP (UL^{-1})**	265.8±23.72	243.1±22.9	299.8±31.37	278.3±24.9	313.2±42.32	293.3±30.8	342.3±35.89	307.4±32.3
2	**ALT (UL^{-1})**	71.7±3.48	60.43±2.43	72.3±2.83	59.43±3.45	70.45±3.45	64.45±3.87	67.98±2.34	67.34±4.54
3	**AST (UL^{-1})**	117.4±9.81	90.3±9.84	116±10.98	93.83±12.34	121.4±9.43	101.2±12.54	125±13.45	107.3±12.34
4	**Bilirubin (mg dL^{-1})**	0.53±0.08	0.38±0.04	0.54±0.09	0.39±0.08	0.59±0.06	0.43±0.06	0.65±0.07	0.44±0.09
5	**Creatinine (mg dL^{-1})**	0.75±0.21	0.56±0.05	0.76±0.32	0.55±0.08	0.81±0.65	0.59±0.09	0.87±0.98	0.64±0.12
6	**Urea (mg dL^{-1})**	39±3.74	31±2.24	36.3±4.39	32.1±2.83	42.8±3.98	33.8±3.02	43.8±4.59	36.1±3.72
7	**Cholesterol(mg dL^{-1})**	70.20±1.56	75.20±1.72	79.60±1.21	79.60±1.12	82.60±2.02	81.40±1.29	84.80±2.85	87.20±2.04
8	**Triglyceride (mg dL^{-1})**	73.80±1.59	92±1.41	84.40±1.50	91.8±1.43	90.40±1.66	91.6±1.12	93.60±1.63	93.8±1.16
9	**LDL (mg dL^{-1})**	36.56±2.21	54.52±2.35	52.77±1.03	58.89±1.77	56.99±1.16	59.89±1.71	58.89±3.16	65.01±1.51
10	**HDL (mg dL^{-1})**	25.6±1.03	21±1.23	22.6±0.93	20.4±1.75	23.2±1.16	20.6±0.93	26±0.89	21.6±1.42
11	**VLDL (mg dL^{-1})**	14.76±0.32	18.4±0.28	16.88±0.32	18.36±0.16	18.08±0.34	18.32±0.22	18.72±0.43	18.76±0.23

Table 1.3: Effect of administration of *PF* (28 days) on hematological parameters in Wistar rats (Male and Female).

S. No	Parameters	Normal Control		*PF* (2000 mg/kg, b.w.)		*PF* (1000 mg/kg, b.w.		*PF* (500 mg/kg, b.w.)	
		Male	Female	Male	Female	Male	Female	Male	Female
1	**RBC (10^6/cu mm)**	6.98±1.09	5.23±0.98	8.43±2.01	6.12±0.73	7.12±1.93	5.45±1.04	6.69±1.98	5.01±1.98
2	**WBC (10^3/cu mm^3)**	9.12±1.18	7.86±1.93	9.95±1.82	7.94±1.54	10.25±1.83	8.74±1.87	11±1.98	9.12±1.73
3	**Hb (g dL^{-1})**	13.27±1.83	12.54±1.73	14.34±1.90	12.83±1.99	13.34±2.03	12.34±1.63	12.73±2.34	12±1.98
4	**Platelet (10^5/cu mm)**	4.11±0.64	4.01±0.44	4.12±0.83	4.09±0.83	5.43±1.03	5.01±1.37	6.2±1.83	5.97±1.73
5	**Eosinophils (mm^3)**	812±384	532±175	743±373	501±273	701±384	475±192	612±384	412±243
6	**Lymphocyte (mm^3)**	4110±830	3174±984	4293±1037	3283±983	4484±1193	3384±1083	4623±1148	3511±1094
7	**Neutrophils (mm^3)**	1678±785	1489±409	1589±874	1364±593	1681±894	1434±623	1714±974	1573±841

Table 2: Effect of *Paederia foetida* Linn. leaves extract on turpentine oil induced rat paw edema.

S. No	Treatment	Increase in joint diameter						% inhibition
		1h	2h	3h	4h	5h	6h	
1	Inflammatory Control	0.98 ± 0.012	1.13 ± 0.019	1.44 ± 0.017	1.35 ± 0.019	1.23 ± 0.018	1.16 ± 0.014	-
2	*PF* I (25 mg/kg)	0.98 ± 0.019^{ns}	1.19 ± 0.018^{ns}	$1.31\pm0.017^{*}$	$1.24\pm0.019^{*}$	$1.12\pm0.017^{**}$	$1.03\pm0.015^{***}$	11.02
3	*PF* II (50 mg/kg)	0.99 ± 0.012^{ns}	$1.16\pm0.013^{*}$	$1.25\pm0.008^{*}$	$1.11\pm0.007^{**}$	$0.97\pm0.005^{***}$	$0.88\pm0.009^{***}$	24.13
4	*PF* III (100 mg/kg)	0.82 ± 0.008^{ns}	$0.93\pm0.005^{**}$	$0.99\pm0.006^{**}$	$0.89\pm0.012^{***}$	$0.73\pm0.015^{***}$	$0.63\pm0.008^{***}$	45.68
5	Indomethacin (10 mg/kg)	0.82 ± 0.006^{ns}	$0.95\pm0.007^{**}$	$1.02\pm0.005^{**}$	$0.92\pm0.011^{***}$	$0.82\pm0.007^{***}$	$0.64\pm0.006^{***}$	44.83

The data are expressed in mean ± SEM) (n = number of animals in each group = 6). The comparisons were made by ANOVA followed by Dunnett's test .ns-non-significant; *PF- Paederia foetida* Linn.

*P < 0.05 is considered as significant.

**P < 0.01 is considered as very significant.

***P < 0.001 is considered as extremely significant.

Table 3: Effect of *Paederia foetida* Linn. leaves extract on inflammatory mediator induced paw edema.

S. No	Treatment	Inflammatory mediators		
		Arachidonic acid	Prostaglandin	Histamine
1	**Inflammatory Control**	0.77 ± 0.08	0.47 ± 0.09	0.58 ± 0.09
2	*PF* **I (25 mg/kg)**	$0.69\pm0.07^{**}$	0.46 ± 0.08^{ns}	$0.51\pm0.02^{*}$
3	*PF* **II (50 mg/kg)**	$0.45\pm0.04^{***}$	$0.39\pm0.05^{*}$	$0.43\pm0.04^{**}$
4	*PF* **III (100 mg/kg)**	$0.32\pm0.02^{***}$	$0.24\pm0.02^{***}$	$0.3\pm0.08^{***}$
5	**Indomethacin (10 mg/kg)**	$0.34\pm0.03^{***}$	-	$0.33\pm0.07^{***}$
6.	**Ibuprofen (10 mg/kg)**	-	$0.26\pm0.05^{***}$	-

The data are expressed in mean ± SEM) (n = number of animals in each group = 6). The comparisons were made by ANOVA followed by Dunnett's test.ns-non-significant; *PF- Paederia foetida* Linn.

*$P < 0.05$ is considered as significant.

**$P < 0.01$ is considered as very significant.

***$P < 0.001$ is considered as extremely significant.

Table 4: Effect of *Paederia foetida* Linn. leaves extract on CFA induced rat paw edema.

S.No	Treatment	Increase in joint diameter						% Inhibition
		Day 5	Day 10	Day 15	Day 20	Day 25	Day 28	
1	Arthritic Control	1.22 ± 0.021^{ns}	1.44 ± 0.017	1.57 ± 0.019	1.63 ± 0.016	1.65 ± 0.015	1.71 ± 0.018	-
2	*PF* I (25 mg/kg)	1.21 ± 0.063^{ns}	$1.41\pm0.011^{*}$	$1.38\pm0.012^{**}$	$1.32\pm0.013^{***}$	$1.25\pm0.011^{***}$	$1.16\pm0.018^{***}$	32.16
3	*PF* II (50 mg/kg)	1.31 ± 0.025^{ns}	$1.31\pm0.015^{*}$	$1.24\pm0.016^{***}$	$1.17\pm0.019^{***}$	$1.07\pm0.013^{***}$	$0.98\pm0.017^{***}$	42.69
4	*PF* III (100 mg/kg)	1.07 ± 0.053^{ns}	$1.1\pm0.022^{**}$	$0.93\pm0.016^{***}$	$0.82\pm0.019^{***}$	$0.74\pm0.011^{***}$	$0.65\pm0.014^{***}$	61.99
5	Indomethacin (10 mg/kg)	1.09 ± 0.073^{ns}	$1.15\pm0.014^{**}$	$0.99\pm0.0112^{***}$	$0.91\pm0.013^{***}$	$0.84\pm0.011^{***}$	$0.71\pm0.015^{***}$	58.48

The data are expressed in mean ± SEM) (n = number of animals in each group = 6). The comparisons were made by ANOVA followed by Dunnett's test.

ns-non-significant; UFD-Umbelliferone diglucoside CFA- Complete Fruend's Arthritic

*P < 0.05 is considered as significant.

**P < 0.01 is considered as very significant.

***P < 0.001 is considered as extremely significant

Table 5: Effect of *Paederia foetida* Linn. leaves extract on COX-1 and COX-2

S. No	Treatment	Inhibition percentage	
		COX-1	COX-2
1	*PF* (30 µg/mL)	72.23±4.53	82.84±4.85
2	Quercetin (30 µg/mL)	34.21±2.65	28.35±2.84
3	Indomethacin (30 µg/mL)	82.84±6.54	93.48±5.98

Values are mean ± SEM; experiment was performed in triplicate

Table 6: Effect of *Paederia foetida* Linn. leaves extract on proinflammatory cytokines.
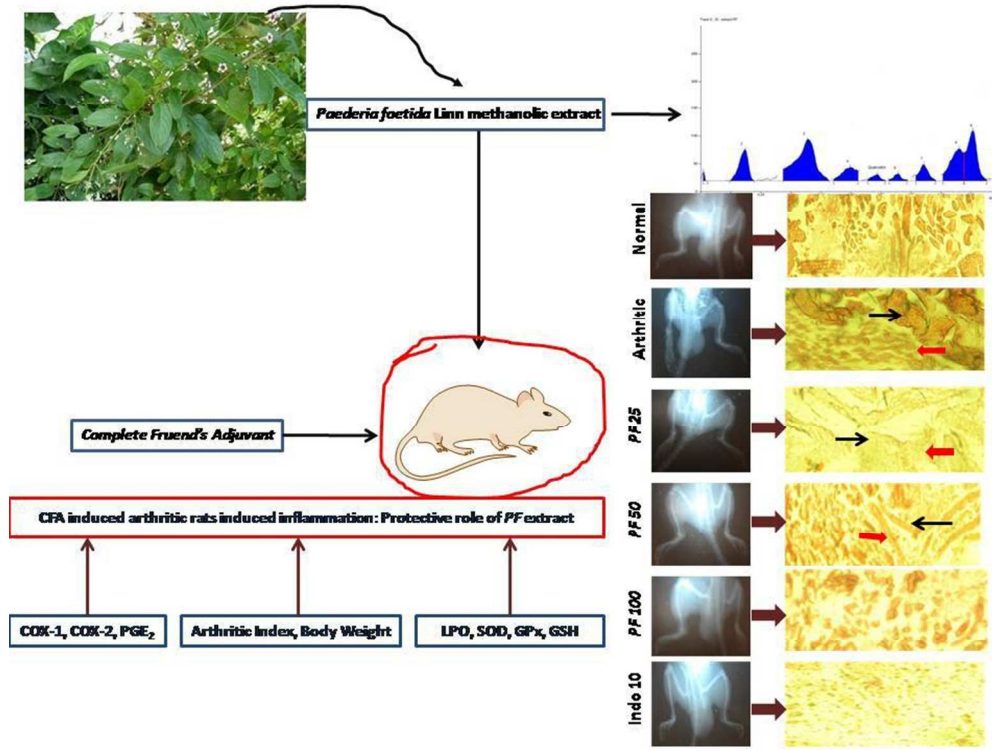
S. No	Treatment	Cytokine (pg/mg protein)		
		TNF-α	IL-1β	IL-6
1	**Arthritic Control**	85.3±11.30	90.82±12.83	66.43±12.83
2	*PF* I (25 mg/kg)	78.6±12.61*	82.3±10.92*	65.43±11.45^{ns}
3	*PF* II (50 mg/kg)	59±10.92**	58.23±9.83**	48.54±9.69*
4	*PF* III (100 mg/kg)	39.8±7.93***	29.7±7.93***	32.73±7.83***
5	**Indomethacin (10 mg/kg)**	40.32±9.03***	30.21±8.83***	34.3±6.94***

The data are expressed in mean ± SEM) (n = number of animals in each group = 6). The comparisons were made by ANOVA followed by Dunnett's test.

ns-non-significant; *PF- Paederia foetida* Linn. CFA- Complete Fruend's Arthritic

*P < 0.05 is considered as significant.

**P < 0.01 is considered as very significant.

***P < 0.001 is considered as extremely significant

254x190mm (96 x 96 DPI)