Ethanol extract of Ziziphus nummularia ameliorates formaldehyde induced arthritis in rats by regulating oxidative stress biomarkers and hematological profile

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Abstract

Background: Rheumatoid arthritis is an autoimmune inflammatory disorder mainly affects bone and cartilage architecture. The continuous use of NSAIDs and DMARDs is associated with severe toxic effects. Therefore, the current study was designed to scrutinize herb-based therapy for the treatment of RA.

Aim: Evaluation of the anti-arthritic potential of the ethanol extract of *Ziziphus nummularia* (ETZN) in a formaldehyde-induced arthritis model in rats and elucidates the possible mechanism of action.

Material and Method: Anti-arthritic activity of ETZN was studied at three oral doses i.e. 200, 400 and 600 mg/kg. Selected doses were studied using various clinical parameters (viz. paw volume, inflammatory index, motility test, stair test, anti-nociceptive efficacy, walking track analysis and motor activity) from day1 to day10. On last day the animals were sacrificed for the evaluation of hematological parameters, oxidative stress biomarkers, histological and radiographic studies of the hind paw.

Results: Treatment with ETZN at varying doses markedly elicited a significant ($p<0.0001$) reduction in paw volume, inflammatory index, and nociceptive action and increases the latency of pain threshold. The anti-arthritic activity is mainly attributed to a reduction in oxidative stress biomarkers as well as restoration of hematological profile in treated animals. Lastly, the anti-arthritic potential was confirmed from histological and radiological analysis which revealed a marked reduction in inflammatory cells and bone destruction as compared to diseased animals.

Conclusion: The study revealed that ETZN exhibits significant anti-arthritic activity via modulation of oxidative stress biomarkers, restoration of hematological profile and improvement in bone architecture.

1. Introduction

Rheumatoid arthritis is a systemic disorder manifested by synovitis, hyperproliferation of synovial cells and infiltration of inflammatory mediators that cause bone and cartilage destruction. Besides, stimulation of inflammatory cells like neutrophils and macrophages causes marked production of reactive oxygen species that leads to membrane destruction (Paval et al 2009; Lawrence and Helmick. Furthermore, the release of inflammatory molecule such as TNF-α and IL-6 in synovium leads to the destruction of articulate cartilage as well as subchondral bone via the activation of destructive enzymes. Also, elevated level of prostaglandins and leukotrienes are involved in RA progression (McInnes and Schett 2011). NSAIDs (non-steroidal anti-inflammatory drugs) are used primarily for overcoming signs and symptoms of arthritis-like conditions. NSAIDs such as Diclofenac are considered a powerful agent in such cases which acts by inhibiting cyclooxygenase enzyme and thus suppresses the production of prostaglandins (van Vollenhoven 2016; Fries et al 1993). Moreover despite the availability of enormous drugs researchers are desperate to explore better alternative herb-based therapy having fewer side effects and potential therapeutic activity. Therefore, neutraceuticals are widely used nowadays due to their diverse therapeutic properties (Soeken 2003; Haddad 2005).

Ziziphus nummularia (Burm) belongs to the family Rhamnaceae and is a thorn plant commonly known as Jhar Beri (Shah and Tariq 1990). It is cultivated widely in arid and semi-arid areas of India. Various parts of the plant are utilized by locals due to its antioxidant, anti-inflammatory, anti-cough, anti-diarrheal and anti-microbial activity. Nowadays, *Z. nummularia* gains significant importance because of its diverse ethno-medicinal properties (Goyal et al 2011; Rathore 2009). The phytochemical studies revealed the presence of alkaloids, tannins, flavonoids, tannins, steroids and glycosides are active constituents in plants (Bodroth and Das 2012). The active principles of *Z. nummularia* are nummularine and mauritine that might be responsible for the anti-inflammatory and anti-arthritic activity (Suksamrarn et al 2005). The present study aimed to shows the efficacy of the ethanol extract of *Ziziphus nummularia* (ETZN) as an anti-rheumatic agent using formaldehyde-induced arthritis in rats and its comparison with NSAID, Diclofenac for the treatment of arthritis.

2. Material & Methods

2.1 Chemicals:

dithiol-bis-2-nitro benzoic acid (DTNB)ethylene diamine tetra-acetic acid (EDTA), Formaldehyde, Freund's adjuvant complete (CFA), Glycine, Hydrogen Peroxide, Ethanol, nitro blue tetrazolium (NBT), Thiobarbituric acid (TBA) and Trichloroacetic acid (TCA) were purchased. Besides, the above and other common chemicals used were of research grade.

2.2 Animals

The rats of either sex weighing 100–200 gm were procured from Ch. Charan Singh Haryana Agriculture University, Hisar, India. They were placed in stainless steel cages at room temperature 25 ± 2º C with the alternately dark and light cycle of 12 hrs. The animals were allowed to have free access to diet and water *ad libitium* and received all necessary care as per the CPCSEA guidelines. The study was approved by Institutional Animal Ethical Committee (IAEC), Maharshi Dayanand University, Rohtak, Haryana, India (IAEC/69–75/2021). All the experiments were performed as per the ethical guidelines (National Research Council 1996).

2.3 Extraction

The 750 gm of coarse powder of dried leaves of *Z. nummularia* were extracted using ethanol using a Soxhlet apparatus. Ethanol was considered a suitable solvent mainly due to its ability to extract phytoconstituents of the plant which are pharmacologically important and preliminary studies was also conducted to elucidate the most active extract through in vitro studies. After completion exhaustive extraction, the excess solvent was removed using a rotary evaporator and crude solid material was collected (Percentage yield = 8.26%) and preserved in a desiccators for further use (Kotze and Eloff 2004).

2.4 Induction of Formaldehyde induced arthritis and Treatment Protocol

Anti-arthritic activity of *Z. nummularia* was evaluated against formaldehyde-induced arthritis. On day 1, all groups except Group-I, arthritis was induced in rats with 0.1 ml of 2% v/v of formaldehyde solution 30 minutes after the respective administration of the drug. The procedure was repeated on day 3. The respective drug treatment was maintained for 10 days. The degree of arthritis was further evaluated using behavioral, biochemical and histological studies (Manish et al 2013).

Following groups were formed and the rats were divided into 6 groups with n = 6.

Group-I: Normal control rats treated with 5% Tween 20 solution

Group II: Disease/Arthritic control rats treated with 5% Tween 20 solution

Group III: Standard rats treated with 10 mg/kg Diclofenac

Group-IV: ETZN 200 mg/kg

Group-V: ETZN 400 mg/kg

Group-VI: ETZN 600 mg/kg

2.5 Estimation of Clinical Studies:

2.5.1 Paw Volume & Paw Diameter: The degree and duration of arthritis were accessed via multiple behavioral studies. The paw volume was measured on each day for assessing the disease severity and impact of treatment. For this estimation, Plethysmometer was used to quantify the paw volume (Winter et al 1962). Besides, paw diameter was estimated using a digital vernier caliper from day 1 (D1) till day 10 (D10) (Bihani et al 2014).

2.5.2 Body Weight: Furthermore, changes in the body weight of the test compound concerning control animals were also studied to analyze the intensity of arthritis (Bihani et al 2014).

2.5.3 Inflammatory Index: The inflammatory index was measured from day 1 and till day 10. The extent of inflammation was determined using a scale range from 0–4, where 0 = normal paw, 1 = mild swelling of digits, 2 = moderate erythema and swelling of digits, 3 = severe erythema and swelling digits, 4 = gross destruction and unable to use limbs. The score for each rat depicts the inflammatory index (Brahn et al 1998).

2.5.4 Dorsion Flexion Pain: Furthermore, the Dorsion Flexion pain of the experimental paw was scored as per the leg withdrawal latency in response to the flexion of the joint of the rat for five minutes until the toe touches the front of the leg. The score was considered zero when the animal showed no withdrawal latency, score 1 for the rat showed either squeaking or withdrawal latency, or score 2 when both reactions appeared (Wahane and Kumar 2010).

2.5.5 Motility Test & Stair Climbing Test: Motility was accessed for a time interval of 5 minutes and scored 0 = rat walked normally, 1 = walked with difficulty and might not be able to touch toes on the ground and 2 = avoided touching the feet and not able to walk. In the stair climbing test, a staircase was made with steps at 5, 10 and 15 cm with open access to water and food on the second and third stairs.

The score criterion for the test is 0 = not climbing a single stair, 1 = climbing the first step, 2 = climbing the first as well as a second step and 3 = if climbs the entire step (Dief et al 2015).

2.5.6 Assessment of Thermal Analgesia: Hyperalgesic condition in the experimental paw was assessed by using radiant heat apparatus equipped with 40 W lamps. From day 1 the rat paw was placed in front of the lamp apparatus and paw withdrawal latency was evaluated on each day. Cautiously to avoid tissue damage maximum withdrawal latency considered was 15 sec. The values of treated groups for withdrawal latency were compared with the control value.

Eddy's hot plate was used to conduct the anti-nociceptive evaluation. The time between the placing of the animal on a hot plate (55 ± 0.5ºC) and depicting first sign of analgesia like licking the paw, jumping from a heat source, or another abnormal reaction was noticed and compared with the control animals. The maximum cut-off time for showing any reaction was 10 sec. the activity was measured from day 1 and then till day 10.

2.5.7 Measurement of Pain threshold: The pain threshold was measured using Randall Selitto's analgesia meter on day 1 of induction and thereafter till the end of the experiment. For measuring the pain threshold the paw was placed in between the pointer and flat surface, gradually increasing the pressure. The pressure was checked at which the rat shows a stereotypic flinch response. The maximum cut-off pressure was 500 gm (Randall and Selitto 1957).

2.5.8 Rota Rod Apparatus:

An accelerating rota rod apparatus was used to evaluate the motor activity in rats. Rats were placed on a rotating rod with a speed of 25 rpm and the falling latency was measured without interfering with the locomotor activity. The activity was recorded till day 10 from day 1 (Zuo et al 2014).

2.6 Hematological Studies

On the 10th day, the rats from all groups were anesthetized by intraperitoneal administration of ketamine at a dose of 50 mg/kg. The blood samples were collected through cardiac puncture for hematological investigations. The blood samples were preserved for the determination of cell blood count. The red blood cells (RBC), white blood cells (WBC) and platelets were determined using a Neubauer hemocytometer. For the determination of hemoglobin Sahli's apparatus was used and Westergren tubes were used for ESR estimation.

2.7 Determination of Antioxidant Enzymes:

After blood collection, three animals from each group were sacrificed by cervical dislocation and the liver was removed by abdominal incision. The liver was washed with saline phosphate buffer and cut into small pieces. The pieces were homogenized using phosphate buffer and preserved for further analysis.

For SOD estimation, 0.3 ml of supernatant was mixed with 0.1 ml of phenazine methosulfate and 1.2 ml of sodium pyrophosphate. To the reaction mixture, 0.2 ml NADH and 1 ml acetic acid were added to regulate the reaction. The change in the absorbance was estimated at 560 nm using a UV spectrometer and expressed as units/mg protein (Marklund and Marklund 1974).

GSH concentration was estimated by using the DTNB method. Briefly, the reaction mixture is composed of an equal proportion of homogenate and sulfosalicylic acid. The mixture was maintained at 4ºC for 1 hr and centrifuged at 1200 rpm for 20 minutes. To the filtrate, add 0.2 ml of DTNB and 2.5 ml of phosphate buffer pH 7.4. The absorbance was recorded at 412 nm and expressed as n moles of GSH per mg protein (Awasthi et al 1979).

For the determination of CAT activity, the rate of hydrogen peroxide decomposition was measured. The reaction contains 0.1 ml of tissue homogenate, 0.4 ml of 6mM hydrogen peroxide and 2.5 ml of phosphate buffer pH 5.0. The absorbance was recorded after one minute at 240 nm and expressed as units per mg protein (Chance and Herbert 1950).

Lastly, GPx was estimated using a reaction mixture containing 1.5 ml of phosphate buffer pH 7.4, 0.1 ml sodium azide and an equal volume of glutathione reductase and GSH. To the reaction mixture add 0.1 ml of NAPDH and EDTA, 0.01 ml hydrogen peroxide and 0.1 ml of liver homogenate. The activity was recorded at 340 nm and expressed as units per mg of protein (Awasthi et al 1979).

2.8 Determination of Lipid Peroxidation:

Lipid peroxidation was done by following the method of Ohkawa et al. briefly; a reaction mixture contains 0.58 ml of phosphate buffer pH 7.4, 0.2 ml tissue homogenate, 0.2 ml of ascorbic acid and 0.02 ml of ferric chloride. The mixture was incubated at 37ºC for 1 hr afterward equal proportion of trichloroacetic acid and thiobarbituric acid was added and centrifuged at 2500 rpm for 10 minutes. The supernatant was analyzed at 535 nm using a UV spectrophotometer and expressed as n mol of malondialdehyde per mg of protein (Utley et al 1967).

2.9 Determination of NO:

Nitrite concentration was assessed by using a Griess reagent in 5% phosphoric acid. Briefly, the reaction system contains an equal amount of homogenate and Griess reagent and mixes it properly. After 10 minutes measure the change in absorbance at 540 nm using a UV spectrophotometer and expressed it as µM per mg protein. A sodium nitrite standard plot is used to calculate the concentration of nitrite (Ansari et al 2015).

2.10 Histological Studies:

On the 10th day, the rats were sacrificed using cervical dislocation. The articular joints were separated from the hind paw and fixed in a 10% formalin solution. The tissue fixed with formalin was embedded in a paraffin block and trimmed into a small section using a microtone. The sectioned tissue was stained

with hematoxylin and eosin dye. The stain sections were analyzed using a light microscope (Mythilypriya et al 2015).

2.11 Radiological Studies of Joints:

On the last day of the study, the rats were anesthetized with ketamine at a dose of 50 mg/kg and radiographic images of the experimental hind paw were taken using standard protocol for X-ray apparatus. X-ray of each rat was analyzed for soft tissue swelling and bone destruction.

2.12 Statistical Analysis:

All experimental values are expressed as mean ± standard error of the mean. Statistical significant difference from the control, disease and standard group was identified using ANOVA with post hoc Turkey test. All calculations were done using Graph Pad Prism version 8.0.2.

3. Results

3.1 Paw Volume & Paw Diameter:

The paw volume was measured from day 1 till the end of the study followed by the injection of formaldehyde in the left hind paw (Fig-1). The treatment was also started from day 1 and continued till the end of the study. Fig-2 depicts the change in paw volume in each group during the study. The disease control group showed a marked increase in the paw volume till the end of the study. ETZN markedly suppresses the paw volume in a dose-dependent manner as compared to disease control ($p < 0.0001$) animals. ETZN at a dose of 400 mg/kg and 600 mg/kg showed marked improvement in paw volume when compared to Diclofenac (standard) treatment. The improvement in inflammation is depicted in table-1 and percentage inhibition of ETZN 400mg/kg (55.4 ± 1.66) and 600 mg/kg (66.16 ± 1.59) overwhelmed the standard treatment (39.91 ± 0.89) significantly.

Similarly, paw diameter was accessed from day 1 till the end of the study. The marked reduction in paw diameter was observed in treatment groups when compared to the disease control group ($p < 0.0001$). The peak increase in inflammation in treatment groups was seen on day 5, while in the disease control group, the inflammation achieved its peak value on day 6 but the rate of decline was slow when compared to the treatment group. Fig-3 depicts that ETZN in a dose-dependent manner (ETZN 200 mg/kg = 17.57 ± 2.33, ETZN 400 mg/kg = 21.71 ± 1.34 and ETZN 600 mg/kg = 19.93 ± 1.67) markedly suppressed percentage inflammation as compared to standard treatment and disease control group. The maximum percent inhibition was achieved by ETZN 400 mg/kg (23.18 ± 1.54) on day 9 when compared to other treatment groups as revealed by table-2.

Table: 1 Percentage Inhibition of Paw Volume in Formaldehyde Induced Arthritis

Group → / Day ↓	Normal Control	Diclofenac 10mg/kg (Standard)	ETZN200 mg/kg	ETZN400 mg/kg	ETZN600 mg/kg
D1	36.78 ± 2.01	0.69 ± 2.13****	6.88 ± 3.28****	4.55 ± 2.23****	8.54 ± 2.68****
D2	60.52 ± 1.07	2.02 ± 5.22****	12.63 ± 2.28****	11.87 ± 1.14****	18.94 ± 2.68^{****,r}
D3	71.53 ± 0.68	-0.43 ± 4.27****	18.33 ± 4.59^{****,p}	15.56 ± 4.01^{****,r}	23.8 ± 1.27^{****,p}
D4	76.78 ± 0.70	-1.83 ± 4.08****	15.14 ± 1.66^{****,r}	14.13 ± 1.93^{****,r}	17.07 ± 0.96^{****,p}
D5	79.96 ±0.49	5.02 ± 2.49****	18.99 ± 2.55****	16.84 ± 2.23****	21.73 ± 1.02^{****,r}
D6	82.68 ± 0.43	13.17 ± 2.36****	18.18 ± 1.09****	23.7 ± 2.89****	32.32 ± 3.95^{****,p}
D7	85.02 ± 0.29	30.98 ± 2.77****	27.49 ± 1.38****	40.03 ± 2.99****	48.76 ± 3.31^{****,q}
D8	84.06 ± 0.35	35.12 ± 3.01****	32.47 ± 2.01****	46.79 ± 2.98****	57.74 ± 2.52^{****,p}
D9	81.79 ± 0.55	34.31 ± 2.53****	32.09 ± 1.25****	49.02 ± 3.36^{****,s}	60.62 ± 1.87^{****,p}
D10	80.23 ± 0.58	**39.91 ± 0.89******	34.76 ± 1.78****	**55.4 ± 1.66^{****,s}**	**66.16 ± 1.59^{p}**

Values are expressed as Mean ± SEM and Analysed using two way ANNOVA followed by Turkey Test. **** indicates the value of $p < 0.0001$, *** with $p < 0.001$, ** with $p < 0.01$, *with $p < 0.1$ when compared with Normal control. ‘α’ indicates the value of $p < 0.0001$, ‘β’ with $p < 0.001$, ‘γ’ with $p < 0.01$, ‘δ’ with $p < 0.1$ when compared with Disease control. ‘p’ indicates the value of $p < 0.0001$, ‘q’ with $p < 0.001$, ‘r’ with $p < 0.01$, ‘s’ with $p < 0.1$ when compared with Standard group.

Table: 2 Percentage Inhibition of Paw Diameter in Formaldehyde Induced Arthritis

Group → / Day ↓	Normal Control	Diclofenac 10mg/kg (Standard)	ETZN200 mg/kg	ETZN400 mg/kg	ETZN600 mg/kg
D1	24.49 ± 1.59	1.18 ± 3.05****	8.91 ± 2.55****	15.48 ± 1.17^{p}	14.63 ± 1.1^{*,p}
D2	31.46 ± 1.48	0.79 ± 2.62****	8.95 ± 2.07****	14.52 ± 0.75^{****,p}	14.89 ± 1.06^{****,p}
D3	38.34 ± 1.35	3.23 ± 2.37****	10.61 ± 2.18****	17.11 ± 1.01^{****,p}	16.53 ± 0.86^{****,p}
D4	42.53 ± 1.41	3.03 ± 2.16****	8.98 ± 1.37****	15.92 ± 1.35^{****,p}	16.18 ± 1.44^{****,p}
D5	46.04 ± 0.98	4.85 ± 2.22****	8.95 ± 2.09****	14.01 ± 0.98****	14.57 ± 0.95^{****,s}
D6	47.58 ± 1.07	8.89 ± 2.18****	13.34 ± 2.37****	15.41 ± 1.25****	13.8 ± 0.97****
D7	47.38 ± 1.07	13.83 ± 2.18****	19.73 ± 2.29****	22 ± 1.62****	19.43 ± 0.53****
D8	42.83 ± 1.19	10.85 ± 1.91****	20.37 ± 1.73^{****,s}	21.57 ± 1.19^{****,r}	20.75 ± 0.25^{****,s}
D9	38.39 ± 1.29	10.75 ± 2.21****	20.13 ± 1.87****	23.18 ± 1.54^{****,p}	20.71 ± 0.97^{****,s}
D10	32.94 ± 1.46	6.91 ± 3.0****	17.57 ± 2.33^{****,r}	**21.71 ± 1.34^{**,p}**	19.93 ± 1.67^{****,p}

Values are expressed as Mean ± SEM and Analysed using two way ANNOVA followed by Turkey Test. **** indicates the value of $p < 0.0001$, *** with $p < 0.001$, ** with $p < 0.01$, *with $p < 0.1$ when compared with Normal control. ‘α’ indicates the value of $p < 0.0001$, ‘β’ with $p < 0.001$, ‘γ’ with $p < 0.01$, ‘δ’ with $p < 0.1$ when compared with Disease control. ‘p’ indicates the value of $p < 0.0001$, ‘q’ with $p < 0.001$, ‘r’ with $p < 0.01$, ‘s’ with $p < 0.1$ when compared with Standard group.

3.2 Body Weight:

As mentioned in fig-4 ETZN (200, 400 and 600 mg/kg) significantly reduced the extent of weight loss when compared to disease-control animals. The ETZN 400 mg/kg (-4.17 ± 0.83) mg/kg showed marked improvement in body weight as compared to disease control (-9.33 ± 1.09), ETZN 200 mg/kg (-4.33 ± 0.42) and 600 mg/kg (-4.29 ± 0.88) mg/kg groups. The maximum improvement was shown by Diclofenac (-2.0 ± 1.03) when compared to other groups.

3.3 Inflammatory Index:

As depicted in fig-5 the inflammatory index was significantly increased in the disease control group as compared to normal group animals. The maximum score in each group was observed on day 5 in each group but afterward, the treatment group showed marked decline was observed in the treatment group. ETZN 400 mg/kg and Diclofenac 10 mg/kg showed somewhat similar scores when compared to control groups.

3.4 Dorsion Flexion Test:

Fig-5 depicts that in treatment groups marked reduction in pain threshold was observed when compared to the disease control group significantly ($p < 0.0001$). Treatment with ETZN 200, 400 and 600 mg/kg showed a significant reduction in pain response and conduction in a dose-dependent manner. When compared with Diclofenac 10 mg/kg (0.5 ± 0.22), ETZN 200 mg/kg (0.16 ± 0.17) showed better performance and ETZN 600 mg/kg (0.5 ± 0.22) depicts similar activity.

3.5 Stair Test and Motility Test:

Formaldehyde injection into hind paw affected the stair climbing ability as well as motility of animals and significantly reduced in the disease control animals (fig-5). The activity was significantly improved in the treatment group when compared with disease control group ($p < 0.001$). The marked improvement in stair climbing was observed in ETZN 400 (2.5 ± 0.22) and 600 (2.4 ± 0.22) mg/kg treated groups when compared with negative (1.67 ± 0.21), Diclofenac (1.67 ± 0.21) and normal control (3 ± 0) groups as depicted in table-6 on last day of study. Similar results were revealed in motility test where the treatment group showed marked reduction the score as compared to negative group. Animals of ETZN 400 mg/kg showed overlapping results with normal control animals.

3.6 Assessment of Thermal & Mechanical Analgesia:

The reaction time was increased significantly in the treatment group as compared to disease control animals after giving heat stimulus either through eddy's hot plate or radiant heat source. In radiant heat stimuli the reaction time was significantly increased from 4.16 ± 0.31 to 6.0 ± 0.26 sec in Diclofenac treated animals which was similar to animals treated with ETZN 400 and 600 mg/kg i.e. 4.33 ± 0.21 to 5.66 ± 0.33 and 4.16 ± 0.17 to 6.16 ± 0..31 sec respectively upto day 10. Similarly, in eddy's hot plate method the reaction time was also significantly improved in all the treatment groups. But in animals treated with ETZN 400 (6.33 ± 0.21sec) and 600 (6.17 ± 0.31sec) mg/kg marked improvement was observed that surpass the animals treated with Diclofenac (5.17 ± 0.31sec). Besides, ETZN 200 mg/kg treated animals showed similar results to Diclofenac as depicted in Fig-6. As shown in fig-6 the pain withdrawal threshold due to Randall Sellito probe pressure was comparatively higher in treated animals as compared to disease control. ETZN 400 mg/kg (422.5 ± 4.95) showed significant improvement in reduction of nociceptive threshold as compared to standard (397.5 ± 5.88) and normal control (462.5 ± 9.89) animals. Therefore, fig-6 shows that ETZN significant reduced nociceptive activity in both thermal and mechanical analgesia.

3.7 Motor Coordination Activity:

As observed in table-3, ETZN 400 mg/kg showed significant ($p < 0.0001$) improvement in motor activity as compared to other treated groups and negative control (18.33 ± 1.2) groups. A marked improvement in motor activity was observed in the treated group as compared to a negative control group. However ETZN 200 (32.33 ± 1.56) and 400 (42.67 ± 2.03) mg/kg showed dose-dependent response in motor performance and showed better activity than Diclofenac (33.33 ± 1.28).

Table: 3 Effect of ETZN on Motor Coordination Activity in Formaldehyde Induced Arthritis

Group → / Day ↓	Normal Control	Disease Control	Diclofenac 10mg/kg (Standard)	ETZN200 mg/kg	ETZN400 mg/kg	ETZN600 mg/kg
D1	58 ± 0.68	18.8 ± 0.79****	22.3 ± 1.41****	22.7 ± 1.12****	21.7 ± 0.96****	21 ± 1.44****
D2	59.5 ± 0.22	15.5 ± 0.92****	14.2 ± 1.13****	14.7 ± 1.43****	17.3 ± 1.45****	16.3 ± 1.44****
D3	57.3 ± 0.95	15.2 ± 0.91****	14.5 ± 0.81****	13.8 ± 1.08****	14.7 ± 0.56****	15.8 ± 0.91****
D4	58.2 ± 0.71	15 ± 1.13****	14 ± 0.73****	16 ± 1.09****	17.7 ± 1.33****	13.3 ± 1.05****
D5	59.2 ± 0.40	13.6 ± 0.72****	17.3 ± 1.05****	17.7 ± 1.56****	22.5 ± 1.21$^{****,\delta}$	15 ±1.0****
D6	58.8 ± 0.41	14.5 ± 0.85****	21 ± 0.86****	20.3 ± 1.63****	27.3 ± 1.82$^{****,\alpha}$	17.7 ± 1.73****
D7	58.2 ± 0.79	15.8 ± 0.75****	24 ± 0.93****	23.7 ± 2.35****	32.2 ± 1.78$^{****,\alpha}$	23.3 ± 1.38****
D8	59.5 ± 0.34	14.5 ± 0.92****	28.2 ± 1.3$^{****,\alpha}$	27.5 ± 1.73$^{****,\alpha}$	37 ± 2.24$^{****,\alpha,s}$	27.5 ± 0.43$^{****,\alpha}$
D9	59.2 ± 0.31	17.6 ± 0.62****	30.2 ± 2.02$^{****,\alpha}$	31.2 ± 1.35$^{****,\alpha}$	39.2 ± 2.56$^{****,\alpha,s}$	31.5 ± 0.99$^{****,\alpha}$
D10	59.5 ± 0.34	18.3 ± 1.2****	33.3 ±1.28$^{****,\alpha}$	32.3 ± 1.56$^{****,\alpha}$	42.7 ± 2.03$^{****,\alpha,r}$	**35.2 ± 1.01$^{****,\alpha}$**

Values are expressed as Mean ± SEM and Analysed using two way ANNOVA followed by Turkey Test. **** indicates the value of $p < 0.0001$, *** with $p < 0.001$, ** with $p < 0.01$, *with $p < 0.1$ when compared with Normal control. ‘α’ indicates the value of $p < 0.0001$, ‘β’ with $p < 0.001$, ‘γ’ with $p < 0.01$, ‘δ’ with $p < 0.1$ when

compared with Disease control. ‘p’ indicates the value of $p < 0.0001$, ‘q’ with $p < 0.001$, ‘r’ with $p < 0.01$, ‘s’ with $p < 0.1$ when compared with Standard group.

3.8 Hematological Studies:

As shown in table-4, the level of RBC and hemoglobin decreased significantly while WBC and ESR level raises in disease control animals ($p < 0.0001$) as compared to normal control animals which indicate the initiation of the immune response. Treatment with Diclofenac and ETZN showed significant improvement in the hematological profile ($p < 0.0001$-$p < 0.01$). ETZN 400 mg/kg treated groups showed marked elevation in RBC (8.19 ± 0.09) and hemoglobin level (12.98 ± 0.13) as compared to Diclofenac (6.96 ± 0.11, 12.28 ± 0.15) and disease control animals (5.04 ± 0.22, 9.87 ± 0.32). Similarly, WBC and ESR level also declined significantly in ETZN-treated animals as compared to disease-control animals ($p < 0.0001$).

Table: 4 Effect of ETZN on Hematological Parameters in Formaldehyde Induced Arthritis

Parameters → RBC ↓ (10⁶/µl) / Treatment Group	WBC (10⁵/µl)	PLATLETS (10¹/µl)	HAEMOGLOBIN (gm/dl)	ESR (mm/hr)
Normal Control	7.83 ± 0.12	5.05 ± 0.12	3.05 ± 0.04	13.75 ± 0.29	2.83 ± 0.31
Disease Control	5.04 ± 0.22****	8.12 ± 0.04****	5.75 ± 0.25****	9.87 ± 0.32****	14 ± 0.52****
Diclofenac 10mg/kg (Standard)	6.96 ± 0.11$^{\delta}$	6.24 ± 0.13$^{\delta}$	3.66 ± 0.15$^{\gamma}$	12.28 ± 0.15$^{\beta}$	7.17 ± 0.61$^{****,\alpha}$
ETZN200 mg/kg	6.55 ± 0.15	5.86 ± 0.11$^{\gamma}$	4.51 ± 0.12	10.52 ± 0.16^{****,p}	10.5 ± 0.43$^{****,\alpha,p}$
ETZN400 mg/kg	**8.19 ± 0.09$^{\alpha}$**	**5.29 ± 0.09$^{\alpha}$**	3.43 ± 0.12$^{\beta}$	**12.98 ± 0.13$^{\alpha}$**	**6.17 ± 0.40$^{****,\alpha}$**
ETZN600 mg/kg	6.6 ± 0.16	6.27 ± 0.06$^{\delta}$	3.31 ± 0.12$^{\beta}$	12.42 ± 0.19$^{\alpha}$	7.83 ± 0.41$^{****,\alpha}$

Values are expressed as Mean ± SEM and Analysed using two way ANNOVA followed by Turkey Test. **** indicates the value of $p < 0.0001$, *** with $p < 0.001$, ** with $p < 0.01$, *with $p < 0.1$ when compared with Normal control. ‘α’ indicates the value of $p < 0.0001$, ‘β’ with $p < 0.001$, ‘γ’ with $p < 0.01$, ‘δ’ with $p < 0.1$ when compared with Disease control. ‘p’ indicates the value of $p < 0.0001$, ‘q’ with $p < 0.001$, ‘r’ with $p < 0.01$, ‘s’ with $p < 0.1$ when compared with Standard group.

3.9 Antioxidant Activity:

The level of GSH was significantly ($p < 0.0001$) depleted in disease control animals (2.34 ± 0.18) as compared to normal control group (14.21 ± 0.29). But treatment with ETZN (7.41 ± 0.32, 9.55 ± 0.29, 9.09

± 0.17) and Diclofenac (12.46 ± 0.34) enhance the level of GSH in a dose dependent manner as compared to negative control animals. The increased level of GSH reveals the antioxidant potential of ETZN. Further, fig-7 clearly depicts that the level of SOD was significantly increased in diseased animals as compared to normal ($p < 0.0001$). ETZN 400 mg/kg (5.07 ± 0.11) showed moderate decrease in SOD concentration as compared to disease control (6.97 ± 0.07) and Diclofenac (4.16 ± 0.33) treated animals. Lastly, CAT level significantly ($p < 0.001$) increases in disease control animals as compared to treated animals. The ETZN 400 mg/kg (1.57 ± 0.11) showed somewhat comparable results as compared to Diclofenac (1.27 ± 0.11). The ETZN at varying doses regulate the antioxidant status of body to encounter oxidative stress. The level of GPx in disease control animals was significantly ($p < 0.0001$) decreased as compared to normal group. While in ETZN 400 (100.3 ± 1.11) and 600 (99.44 ± 0.97) mg/kg restores the level of GPx in a dose dependent manner as compared to diseased animals (60.9 ± 0.58).

The lipid peroxidation was significantly ($p < 0.0001$) decreased in ETZN treated animals as compared to disease control (22.05 ± 0.18) animals. Treatment with ETZN 400mg/kg (19.06 ± 0.21) restores the level of TBARS in a dose dependent manner as compared to Diclofenac (18.53 ± 0.09) and control animals (17.25 ± 0.35). Similarly, the concentration of nitric oxide was significantly ($p < 0.0001$) increased in negative group (8.16 ± 0.22) as compared to normal animals. Animals treated with ETZN there was significant reduction in reactive nitrogen species as compared to disease control animals. Also, Diclofenac (4.44 ± 0.17) and ETZN 400 mg/kg (4.49 ± 0.05) showed equivalent activity in reducing the nitric oxide concentration (Fig-7).

3.10 Histological Studies:

The microscopic studies showed that extensive infiltration of neutrophils was seen in diseased animals and moderate bone erosion appeared. Fig-8 shows that the synovium of animals treated with either ETZN or Diclofenac has lesser infiltration of PMN cells in a dose-dependent manner. Also, the cartilage destruction was suppressed in treated animals as compared to disease-control animals.

3.11 Radiological Studies:

Radiographic studies of joints as depicted in fig-9 showed that a significant inflammatory reaction was observed in diseased animals as compared to ETZN-treated animals. However, the bone density was sustained in ETZN-treated animals as compared to disease-control animals. The rate of bone erosion was high in diseased animals. The ETZN or Diclofenac-treated animals showed significant recovery as depicted by radiological analysis.

4. Discussion

Rheumatoid arthritis is an autoimmune inflammatory disorder that affects significantly the quality of life and is considered to be more morbid than mortal. Comorbidities such as cardiac disorders, respiratory distress, infection, etc. linked with such inflammatory conditions worsen the condition (Bennett 1978). Currently, the drugs such as NSAIDs (Naproxen, Diclofenac) and DMARDs (Methotrexate, Prednisolone) used for the management of rheumatoid arthritis are considered to be very aggressive as they produce

serious side effects such as gastric intolerance, hepatotoxicity, renotoxicity and ulcer (Scott et al 2010; Aletaha et al 2003). Therefore, it is mandatory to explore potential anti-arthritic agents possessing significant therapeutic potential with fewer side effects. Scientists explore plant-based therapy as a potential alternative for the treatment of such chronic diseases. It has been reported that *Z. nummularia* is widely used for treating inflammatory disorders and the anti-inflammatory potential was also explored through in vitro studies. Furthermore, formaldehyde was used as arthritis inducing agent in rats of either sex as it produces a chronic inflammatory reaction with alteration in an immune response. Formaldehyde induced arthritis model mimics the acute arthritic reaction in humans and due to its high validity, it is used for pre-clinical studies. Thus, the present study assessed the therapeutic potential of ETZN at different oral doses.

The anti-inflammatory potential of the ETZN has been assessed by a decrease in paw volume as well as paw diameter as compared to disease control animals. Various studies report that the reduction of edema and swelling index accounts due to suppression of proinflammatory mediators. Thus reduction in paw volume and diameter indicates that ETZN down-regulates the inflammatory mediators in RA. Besides, in RA due to bone erosion, there is a marked reduction in body weight due to inflammatory cytokine-like tumor necrosis factor. ETZN 400 and 600 mg/kg restores the body weight significantly in a dose-dependent manner. The intensity of inflammation and bone destruction in the hind paw was determined through the inflammatory index, motility, and stair index. As observed during inflammation there is a marked alteration in locomotors and morphological characteristics of the affected area. ETZN 400 mg/kg significantly improves the scores as it suppresses inflammatory processes. The anti-nociceptive efficacy was evaluated by eddy's hot plate, radiant heat apparatus and Randall sellito apparatus. ETZN significantly elucidates the analgesic activity through peripheral as well as central effects. The result revealed that ETZN significantly relieves pain. Lastly, a rota rod test was performed that creates forced motor activity to assess the equilibrium in balance and coordination by measuring the fall-off time or endurance. ETZN increases the fall-off time from the apparatus and thus enhances motor coordination in treated rats as compared to disease rats.

In the current study, an increased count of WBC and ESR in disease rats might be seen due to the involvement of immune response. While the reduction in RBC and hemoglobin levels indicates the anemic condition generally appears in arthritis due to hemolysis. ESR is considered a key determinant for the diagnosis of inflammatory reactions. ETZN 400 mg/kg significantly reverts the altered hematological profile near the normal value via inhibition of cytokines and pro-inflammatory immune response.

The level of endogenous antioxidants such as SOD, CAT, GSH and GPx is generally affected in chronic inflammatory conditions such as RA due to persistent oxidative stress. The antioxidant enzymes act as a first-line defense against reactive oxygen molecules. ROS produces a dual effect on these enzymes i.e. at the low dose the level of antioxidants increases while at the high dose, there is depletion in antioxidant status. As the level of ROS begins to raise in the body the expression of antioxidant enzymes is released to overcome the oxidative stress situation. ETZN markedly restores the level of antioxidant enzymes in the study which reveals its antioxidant potential. Thus ETZN modulates the expression of SOD, CAT, GSH

and GPx by suppressing oxidative stress. Besides, in RA the reactive nitrogen species and lipid peroxidation are also enhanced due to prolonged and aggressive inflammatory reactions mediated mainly by T-cells. ETZN markedly retards the lipid peroxidation process and depletes the level of NO in the body which further confirms its anti-inflammatory and immune regulatory activity.

The radiographic analysis provides a brief about the extent of bone erosion, cartilage damage, osteoclast activity and joint inflammation. ETZN 400 and 600 mg/kg showed around 50–65% improvement in clinical severity with the restoration of bone and cartilage architecture. The results are also validated through histological analysis which reveals the downregulation of inflammatory cells such as PMN cells and the extent of bone damage in ETZN-treated animals as compared to disease animals.

5. Conclusion

This study reveals that ETZN 400 mg/kg exhibits significant anti-inflammatory and antioxidant activity in RA. The exact molecular mechanism through which ETZN exerts action in RA will be included in further studies. While the proposed mechanism for anti-arthritic action includes down-regulation of pro-inflammatory cytokines, modulation of antioxidant enzymes, suppression of ROS and RNS, retards proliferation of osteoclasts and other inflammatory cells. Therefore, the study concluded that ETZN might be considered a potential part of a therapeutic regimen in acute RA attacks due to its diverse actions. Authors are working for the evaluation of chronic activity in rheumatoid arthritis using chronic models.

Declarations

Conflict of Interest:

The authors have no conflict of interest.

Acknowledgement:

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Figures

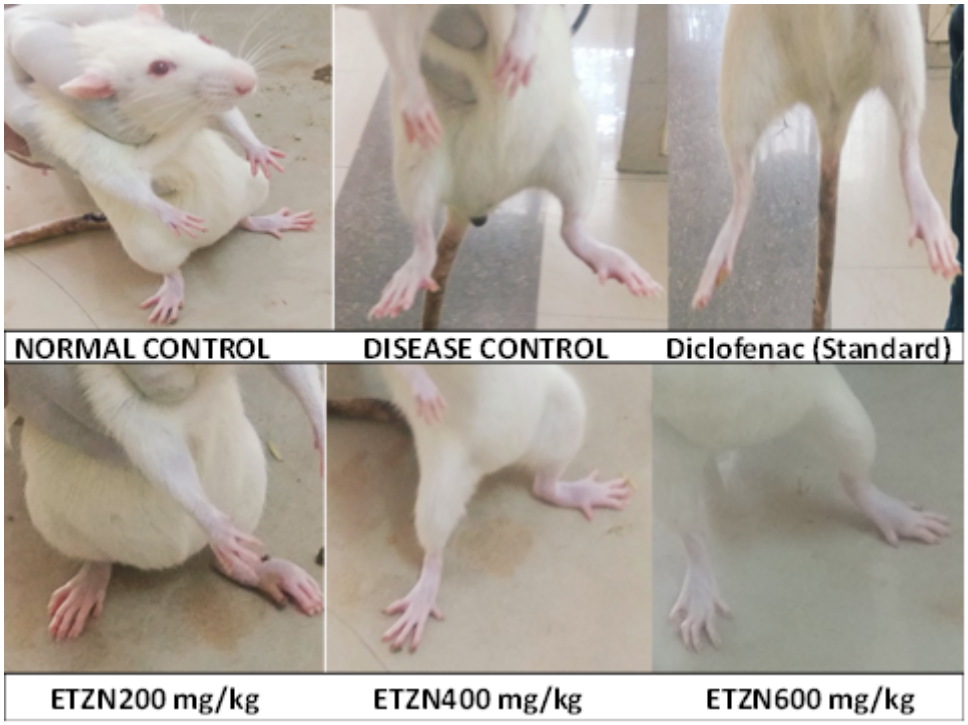

Figure 1

Gross Macroscopy of hind paws in Formaldehyde Induced Arthritis in rats.

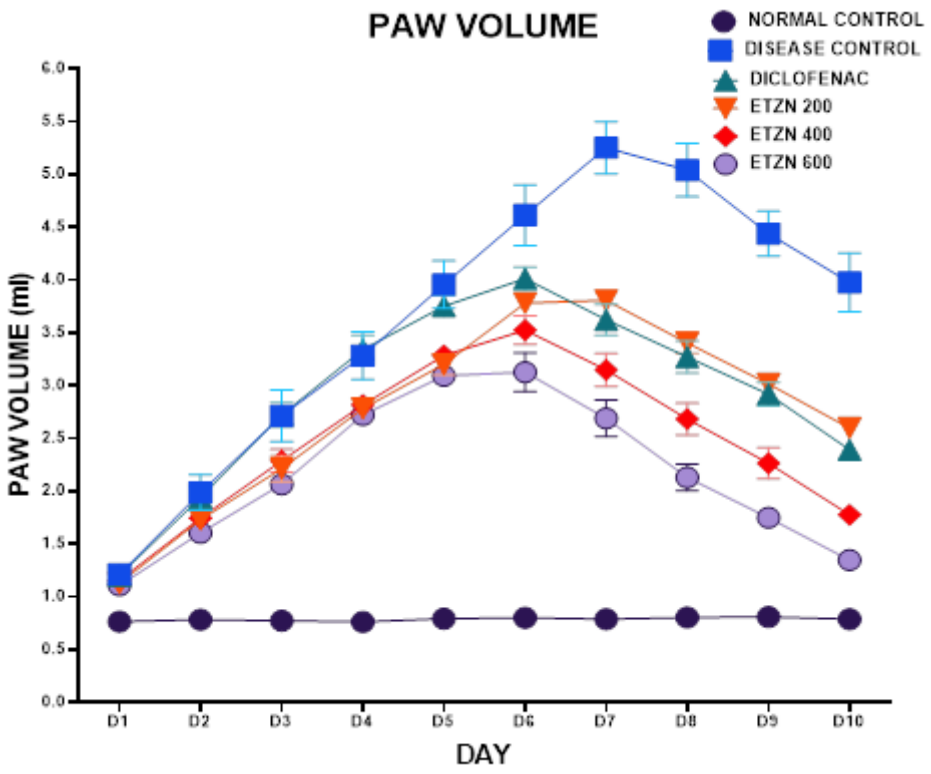

Figure 2

Effect of ETZN on Paw Volume (ml) in Formaldehyde Induced Arthritis. The value is represented as mean ± SEM with n=6.

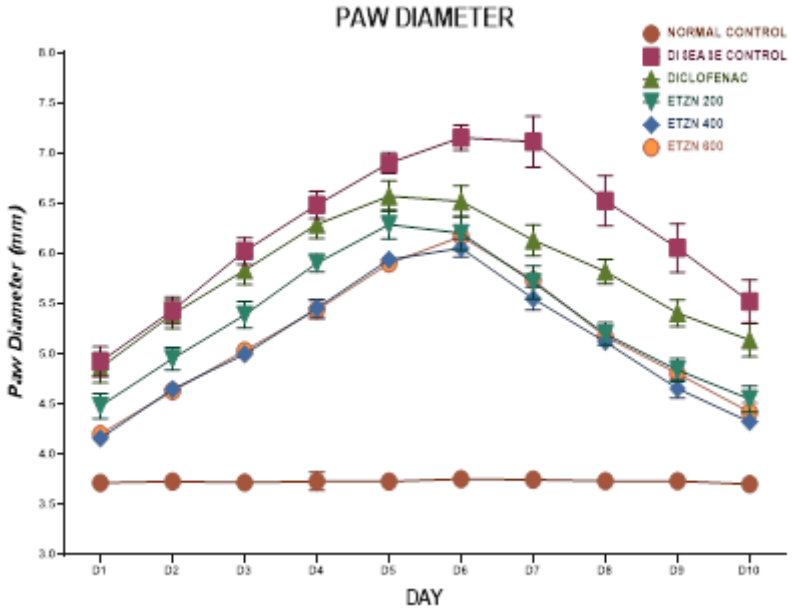

Figure 3

Effect of ETZN on Paw Diameter (mm) in Formaldehyde Induced Arthritis. The value is represented as mean ± SEM with n=6.

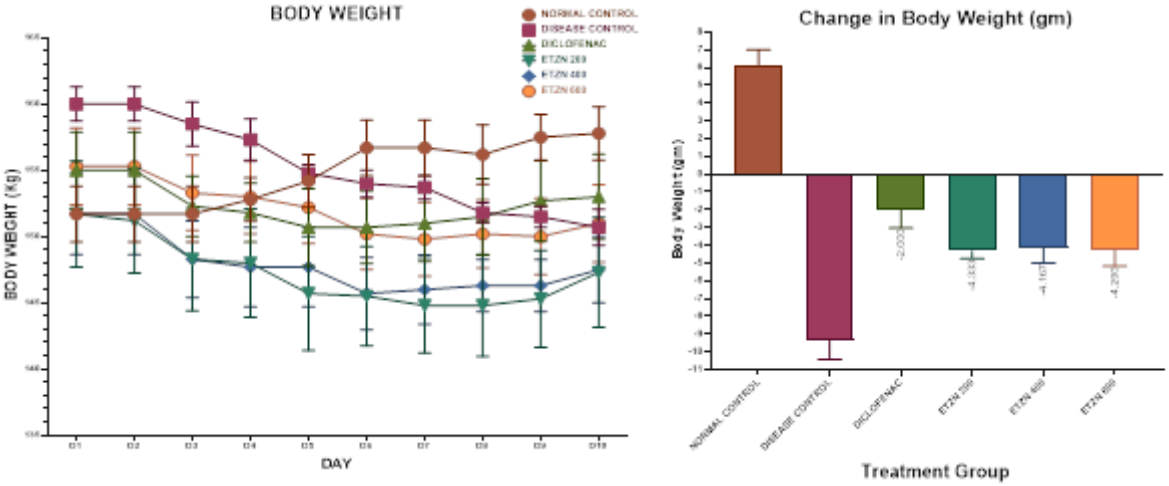

Figure 4

Effect of ETZN on Body Weight (gm)and Change in Body weight in Formaldehyde Induced Arthritis. The value is represented as mean ± SEM with n=6.

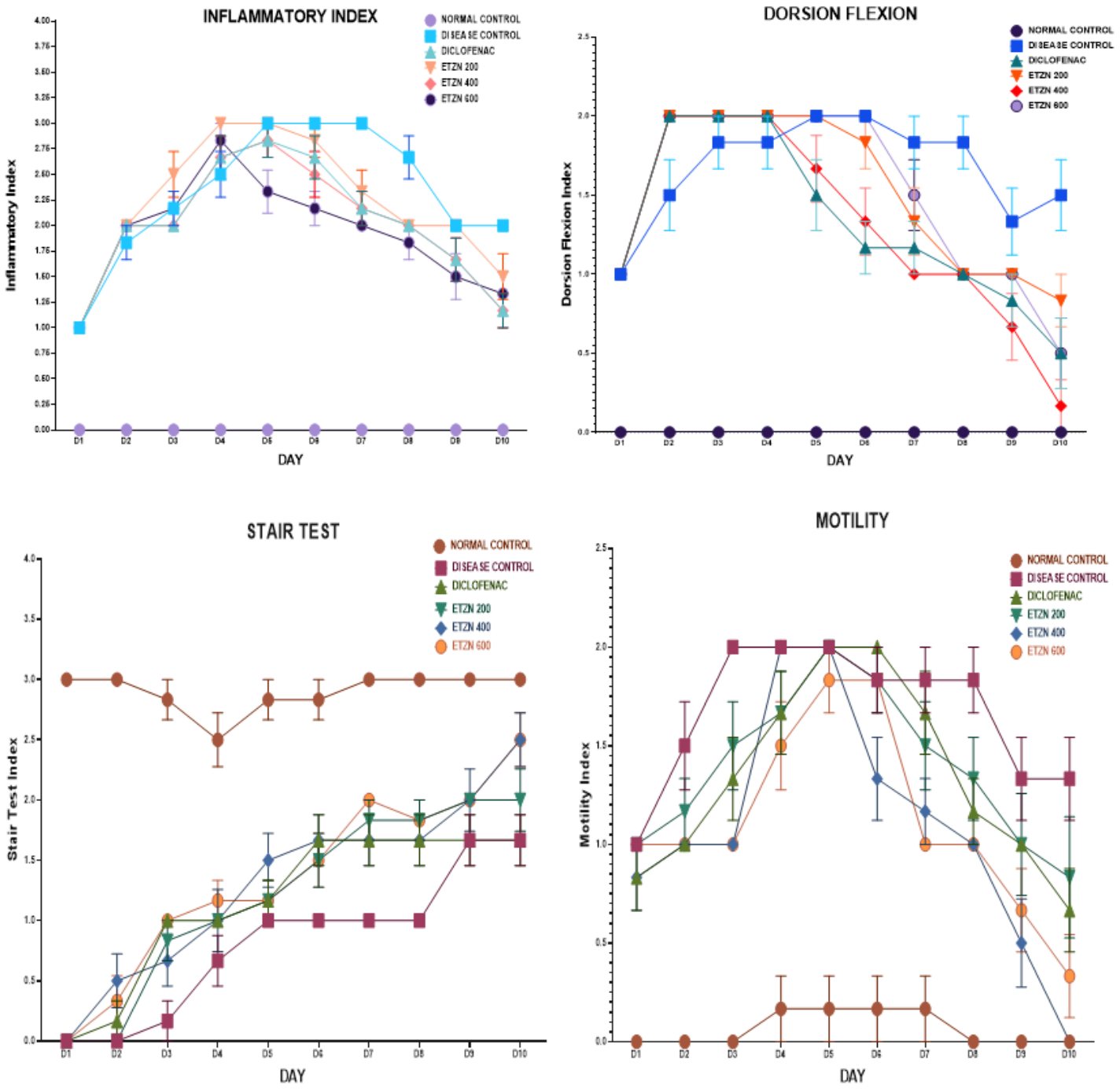

Figure 5

Effect of ETZN on Inflammatory Index, Dorsion Flexion Index, Stair Test and Motility Index in Formaldehyde Induced Arthritis. The value is represented as mean ± SEM with n=6.

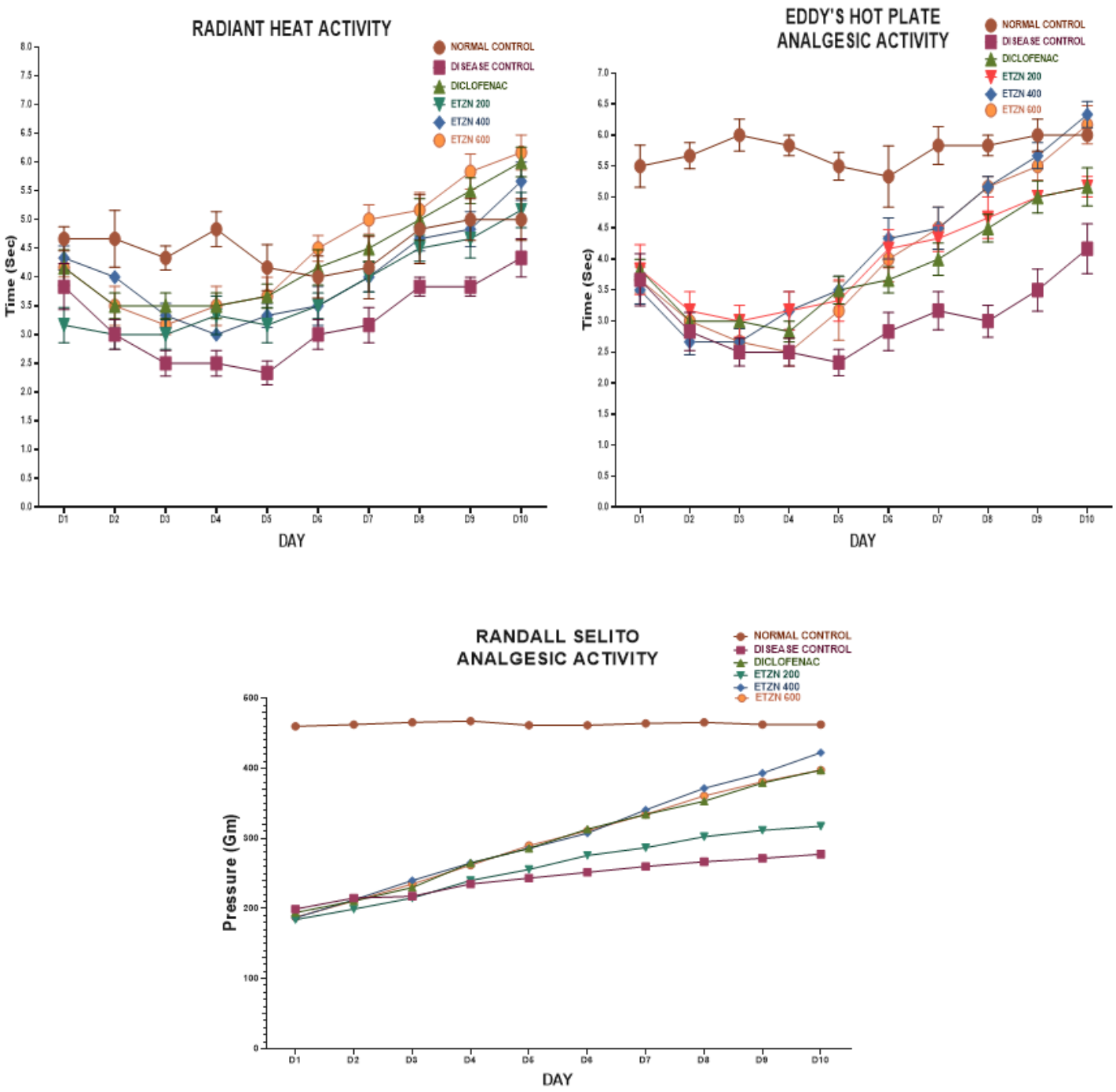

Figure 6

Effect of ETZN on Thermal and Mechanical Analgesia in Formaldehyde Induced Arthritis. The value is represented as mean ± SEM with n=6.

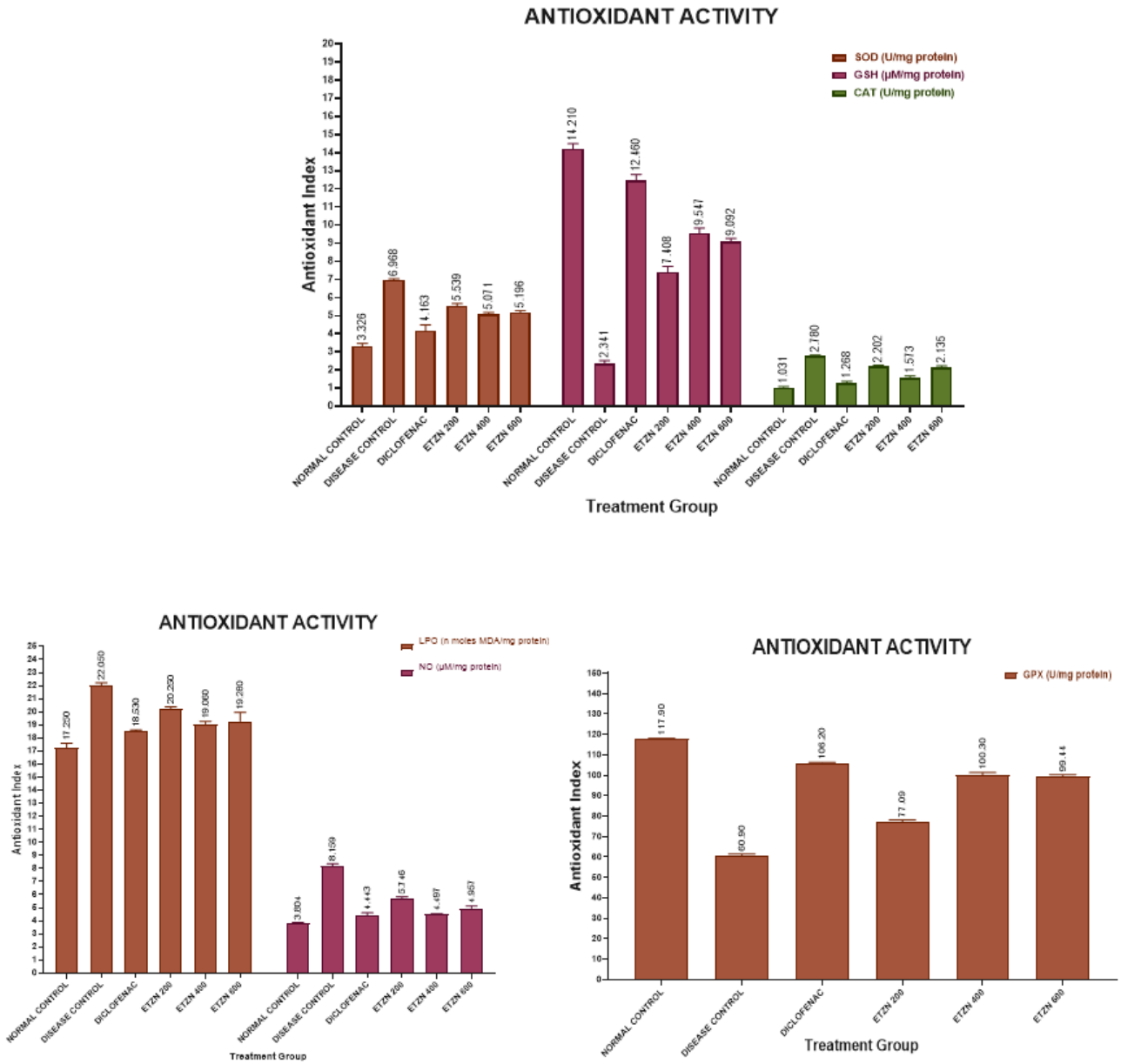

Figure 7

Effect of ETZN on Antioxidants and reactive free radicals in Formaldehyde Induced Arthritis. The value is represented as mean ± SEM with n=3.

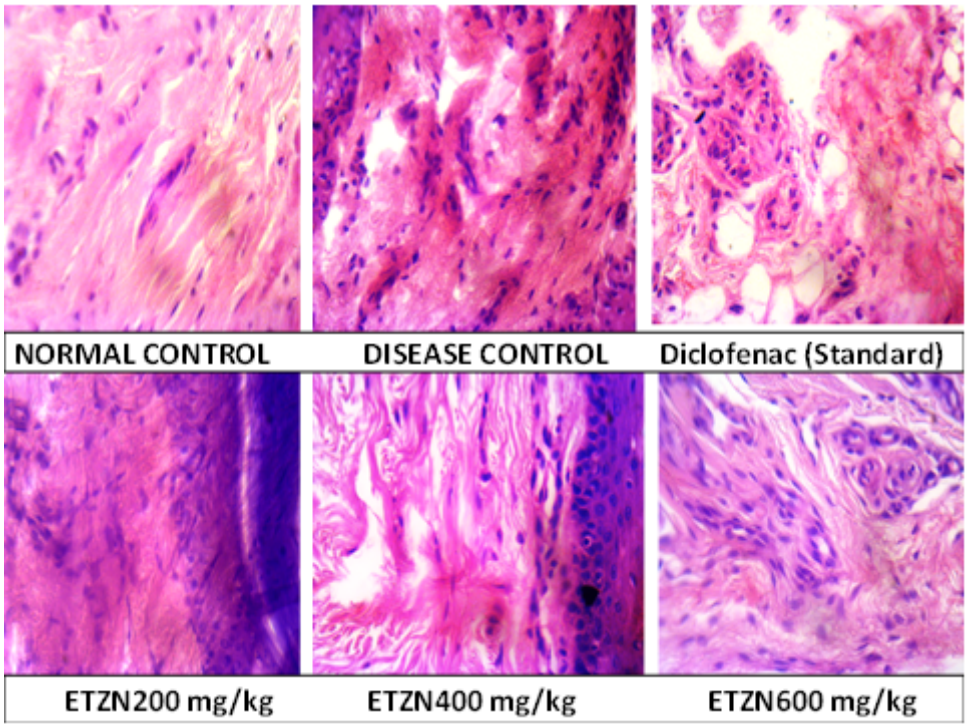

Figure 8

Histological studies indicate that in disease control animals marked infiltration of inflammatory cells along with cartilage destruction. Treatment group shows diminished filtration of neutrophils and lymphocytes in synovium as well as the intensity of cartilage erosion is also reduced.

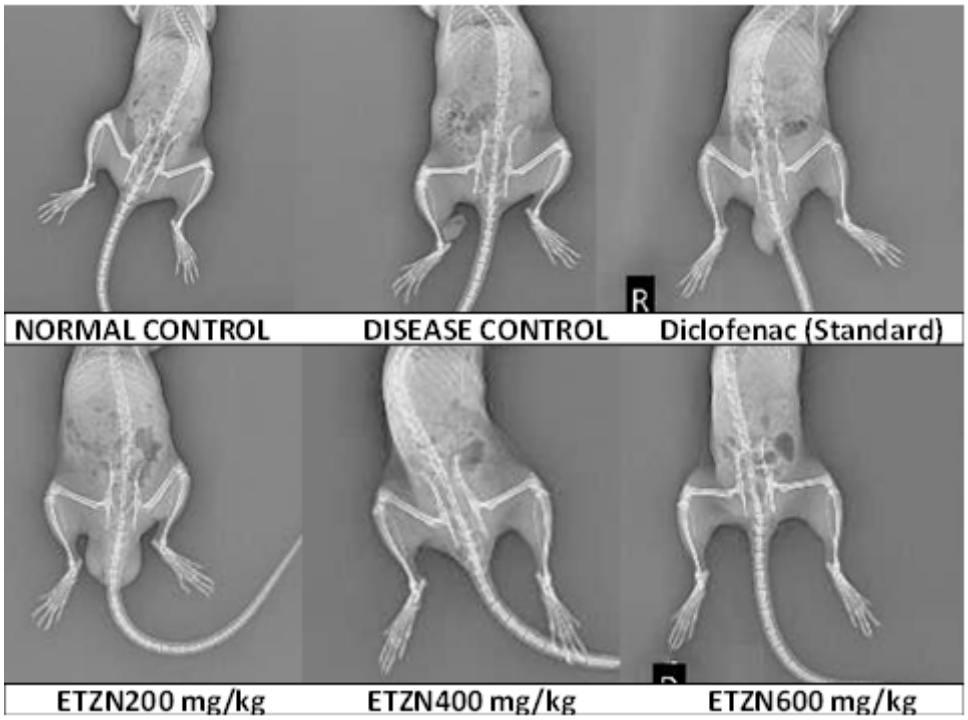

Figure 9

Radiological Assessment reveals that in control group no sign of inflammation and bone erosion was observed. In diseases animals there was marked tissue inflammation and distortion of digits. While animals treated with ETZN negligible digit distortion was observed with insignificant inflammation.