Journal of Pharmaceutical Research International

Volume 34, Issue 54B, Page 11-32, 2022; Article no.JPRI.94029
ISSN: 2456-9119
(Past name: British Journal of Pharmaceutical Research, Past ISSN: 2231-2919, NLM ID: 101631759)

Pharmacological Evaluation of *Moringa oleifera* on Collagen-Induced Arthritis in Rats

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Authors' contributions

This work was carried out in collaboration among all authors. All authors read and approved the final manuscript.

Article Information

DOI: 10.9734/JPRI/2022/v34i54B7240

Open Peer Review History:
This journal follows the Advanced Open Peer Review policy. Identity of the Reviewers, Editor(s) and additional Reviewers, peer review comments, different versions of the manuscript, comments of the editors, etc are available here: https://www.sdiarticle5.com/review-history/94029

Original Research Article

Received: 02/10/2022
Accepted: 25/11/2022
Published: 29/11/2022

ABSTRACT

Background and Objective: Arthritis associated with oxidative stress and chronic inflammation has been a major health problem among the people worldwide. The present study was aimed to investigate the potential anti-arthritis activity of the methanolic extract of leaves of *Moringa oleifera* was evaluated on Collagen induced arthritis.
Study Design: *In-vivo* model.
Place and Duration of the Study: Department of pharmacology, Karnataka College of pharmacy, Bangalore India, between January to September 2022.
Methods: The study was to evaluate anti-arthritic activity of *Moringa oleifera* (MO) against auto-immune arthritis in Wistar rats, using collagen-induced arthritis (CIA) model. Effect of the methanol extract of Moringa oleifera in inflammatory response during CIA was studied by measuring CRP, different cytokines in serum and assessment of arthritis index, footpad swelling. Level of CRP, LPO, NO and an enzymatic activity of SOD and CAT was determined to assess the effect of the *Moringa*

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oleifera extract in neutralizing oxidative stress during CIA. Histology of Synovial membrane experiment has been performed to determine whether *Moringa oleifera* has any impact on the changes in synovial tissue during CIA.

Results: Post oral administration of *Moringa oleifera* at 250 and 500 mg/kg body weight doses decreased the arthritic index and footpad swelling. Moringa oleifera administration diminished pro-inflammatory cytokines in serum. The concentrations of pro-inflammatory TNFa, TGF-beta, IFNg, and IL-6 were elevated in the serum of the rat challenged with, type II collagen as compared to the std. and MtEMO-fed groups. Treatment with MtEMO in CIA induced rat significantly decreased these cytokines in serum. Decreased the serum level LPO, NO content, and SOD activity along with concomitant rise in CAT with the treatment of MtEMO. In addition decreased CRP in serum was also observed. Serum concentration of CRP, the most common type of acute phase proteins was tested in our experiments and was found to be significantly attenuated in case of CIA rat treated with MtEMO. Protective effect of MtEMO in CIA group is further supported from histopathological studies which showed improvement during bone damage and also shown less inflamed, and lymphocyte accumulation, cartilage damage decreased and less disruption of the synovial lining cell layer.

Conclusion: Based on the results it can be concluded that the test drug *Moringa oleifera* is capable of regulating oxidative stress during CIA and therefore down regulated local and systemic release of pro-inflammatory mediators.

Keywords: Arthritis; Moringa oleifera; collagen type II-IFA; antioxidants; pro-inflammatory cytokines.

1. INTRODUCTION

Arthritis is an autoimmune disorder affecting about 2% of the world's population. Osteoarthritis and Rheumatic Arthritis, is the propulsion reason over years lived with disability worldwide. Arthritis is related with oxidative stress and chronic inflammation had been a most important health hassle amongst the populace worldwide. Prevalence of Arthritis is more common in female than men, about over 70% women get affected and is used to commonly develops in the fourth and fifth decades of life [1].

Inflammatory Mediators can directly prompt joint nociceptros in accordance with fire action potentials (AP), as per report by Pattison et al., 2019 protons existing among the inflammatory milieu perform spark off a variety over receptors expressed by nociceptors. Secondly, peripheral sensitization can occur, whereby the commencement required for AP generation is reduced, which perform result from changes in the sensitivity and/or expression on ion channels either concerned in transduction over noxious stimuli [2,3,4,5], and in AP generation [6]. Thirdly, a further form of peripheral sensitization includes the provocative environment unmasking previously 'silent' nociceptors reviewed into [7], with recent proof as reported by Prato et al., 2017, figuring out nerve growth factor as existence resolution in conformity with unmasking irresponsive nociceptors to turn out to be mechanically sensitive and consequently furnish greater nociceptive input.

Tumor necrotic factor (TNF) overexpression results in chronic polyarthritis including a one hundred percent incidence [8]. Hyperplasia over the synovium, inflammatory infiltrates within the joint space, pannus formation, and cartilage or skeleton destruction have been observed. Other provocative cytokines may additionally play a function of the induction and upkeep of persistent inflammation into synovial tissue. In this respect the nearly strong cytokines are IL-1βeta or IL-6, as both is able now keep targeted through particular inhibitors. IL-6 has been detected as like an abundant cytokine among washouts about inflamed joints underneath many experimental mannequin conditions. Although its function is pleiotropic, therapy used to be advanced directed at the IL-6 receptor or humanized anti-IL-6 receptor antibodies at present show up efficacious of human RA. In a number of experimental models over the inflammatory state, TNF-alpha, IL-1βeta and IL-6 are expressed at all promptly on and lead accomplishment roles.

Herbal plants an extract and isolated compounds or their derivatives, offer infinite probabilities in conformity with discovery of novel molecules. Use of herbal herbs appears to stand an historical record of human friendly with the nature. Natural herbs known in imitation of keep

old for traditional medicine, as that contain enormous extent on molecules that can be good for infectious ailments as properly as like chronic illness. There were many herbal plants hold maintain the appreciation as like treatments over diabetes mellitus. However, not many keep enticed scientific and clinical scrutiny so the WHO has endorsed remedy of diabetes including herbal plants; that requires intention for scientist in accordance with get the assessment done [9].

Moringa oleifera, belongs to family Moringaceae, is commonly recognized as drumstick tree and horseradish tree is a plant local to northern India that execute additionally grow in other tropical and sub-tropical places, as Asia and Africa. Folk medicinal drug has used the leaves, flowers, seeds, and roots over that plant for centuries [10-12].

Medicinal plants have posed as natural resources of compounds with pharmacological and nutritional properties aiding humans to prevent and treat diseases. Moreover, *M. oleifera* is widely used in water and effluent treatment, for their coagulation, flocculation and sedimentation properties, their ability of improving water quality, by reducing organic matter and microbial load, with special applicability in intensive animal production systems, such as aquaculture. In addition, due to its high nutritional value and several medicinal properties, this tree may act as a nutritional and medical alternative for socially neglected populations. The different parts of Moringa plant back of historically been used as a treatment for certain prerequisites as: Diabetes, Long-lasting inflammation, Bacterial, viral, and fungal infections, Joint pain, Heart health, Cancer.

The research on *M. oleifera* is yet to obtain value in India. It is fundamental up to expectation the nutrients on it wonder tree are exploited for a variety of purposes. The aim of this experiment is to investigate the activities of *Moringa oleifera* fruits on its potential as an anti-arthritic.

2. MATERIALS AND METHODS

2.1 Collection and Preparation of Plant Material

The Leaves of *Moringa oleifera* were brought from Bangalore, Karnataka, India. The plant specimen has been identified and authenticated by department of botany, University of Rajasthan, Jaipur and specimens were kept for the reference with reference number as RUBL 211760. The leaves of *Moringa oleifera* were chopped into small pieces and dried under shade at room temperature for seven days. The dried leaves were powdered and passed through the sieve (Coarse 10/40). The powder was used for the preparation of methanolic extract.

2.2 Extraction of the Plant Material and Sample Preparation

Each 100gm powder was subjected to extraction with 1000ml methanol in a reflux condenser for 3 cycles of 7hrs each till the volume reduced to half. Extract was filtered through Whattman filter paper No.1 and evaporated to dryness to get constant weight. The solvent was removed from the extractor and dried. The extract was then stored in dry airtight bottles for the pharmacological studies. The portion of the extract which is non- soluble remains in the thimble and was discarded [13].

2.3 Experimental Animals

Adult 6-8 weeks old female Wistar rats weighing 150 – 200 g were used for conducting this study. They were acclimatized for one week prior to experiment. Animals were caged in fully ventilated room, were maintained in 12:12 h light and dark cycle and were housed at temperature of 25 ± 2°C. They had free access to a standard chow diet and water *ad libitum*.

2.4 Experimental Design

2.4.1 Acute toxicity studies for dose fixation

Previously reported (Done as per the OECD guidelines no. 425) [13].

2.4.2 Preparation of dose

Dose: 250mg/kg and 500mg/kg were selected for the study.

2.4.3 Induction of collagen type II to induced arthritis in rats

At the end of the treatment, the animals were sacrificed by giving an overdose (100 mg/kg) of pentobarbital sodium salt (on the 45th day of the experiment), and blood and tissue samples collected.

Table 1. 30 Wistar Female rats age 6-8 weeks weighing (150-200g) were divided in to 5 groups, with 6 animals in each group (n=6), in the following manner

01.	Collagen Induced Arthritis in Rat's Model	Group I: Normal Control Group – Vehicle i.e. Normal saline (10 ml/kg bw, p.o.) for 45 days.	6 rats
		Group II: Disease Control - Collagen Type II (Induced method described by Trentham et al, 1997)	6 rats
		Group III: Test Drug Group- Collagen Type II + *Moringa oleifera* 250mg/kg,b.w. p.o [13].	6 rats
		Group IV: Test Drug Group- Collagen Type II + *Moringa oleifera* 500mg/kg,b.w. p.o [13]	6 rats
		Group V: Standard Drug Group - Collagen Type II + Dexamethasone 0.025 mg/kg, b.w. p.o.	6 rats

2.4.3.1 Induction of collagenase type II induced arthritis (CIA) in rat's:51-53

Randomly selected rats (n=6/each group) were immunized with CoII and another group of normal rats had not been immunized and were used as the control. CIA was induced by applying the technique described by Trentham et al. In brief, bovine collagen-II was dissolved in 0.05 M acetic acid at a concentration of 1mg/ml and emulsified together with equal amount of isovolumic incomplete Freund's adjuvant (IFA). A total of 0.2 ml CII-IFA emulsification solution was administered as an intradermal injection at the tail of each rat; the injection site used was~2 cm away from the tail root. An additional 0.1 ml CII-IFA emulsification solution was subcutaneously injected as a booster immunization into the opposite side of the tail 20 days after the initial immunization (Fig. 1).

All rats were observed 2 times a week to assess signs of arthritis. Then after the 25th day of the initial immunization the test drug was administered routinely up to day 41 of the experiment. On day 45 all rats were euthanized and the incidence of arthritis and the clinical score were evaluated.

2.5 Protective Effect of Plant Extract was Assessed by Measuring

A) Arthritis Index or incidence of arthritis and the clinical score &

B) Macroscopic Pathology (Swelling of footpad in mm)

Arthritis was once induced in rats approx. three weeks after initial immunization together with CoII-IFA, i.e. after booster immunization with CoII-IFA. In the course of treatment with test drug, it was observed that with every day administration of the test drug there was appreciably an inhibition of arthritic progression. When arthritis signs were present a semi-quantitative scoring system was used to assess the severity of arthritis as like follows: 0, normal joint; 1, swelling and redness in 1 joint; 2, swelling in >1 joint; 3, whole-paw swelling and 4, joint deformity and/or ankylosis. Accumulated scores for entire four paws of each rat (maximum feasible rating of 16) had been used to determine the severity or progression of the ailment. For the disease incidence, animals were considered after having arthritis if the rating accelerated ≥2 factors compared with the score at the start of the experiment. Footpad swelling was evaluated by measuring the thickness on the two hind paws with an electric digital calliper every other day starting on day 21 after primary immunization (i.e. day four after secondary booster immunization). On day 45 after the preceding immunization, all the rats were euthanized for analysis.

- **Measurement of Pro-Inflammatory Cytokines: [14-18]**

Markers of disease severity; TNF-alpha, IFN-gamma, TGF-Beta, and IL-6 were analysed by Sandwich ELISA Assay (Commercial Available kit, Mercodia, Sweden). Serum was normalized for protein content by Lowry et al. method [19]. Concentrations of pro-inflammatory cytokines were determined by using commercial kits. The serum were used for the estimation of the cytokines was done using antigen capture ELISA and the A450nm was measured by using ELISA reader.

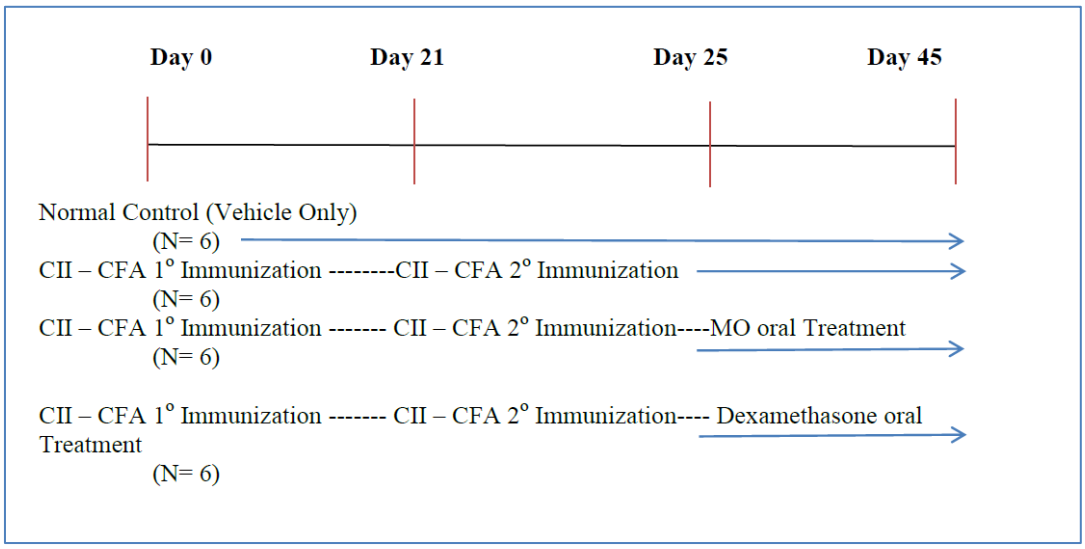


Fig. 1. Model of COII induced arthritis

- **Assessment of Serum C - reactive protein (CRP) Level [20]**

The CRP test is based on the principle of the latex agglutination. When latex particles complexed human anti-CRP is mixed with a serum containing C reactive proteins, a visible agglutination reaction will take place within 2 minutes. CRP ug/ml = 7 x D, where D is the highest dilution of serum showing agglutination and 7 is the sensitivity in ug/ml.

- **Assessment of Serum Antioxidant enzyme study:**

Lipid peroxidation assay (LPO) [21]
Catalase (CAT) assay [22]
Nitric oxide (NO) scavenging activity [23]
Superoxide dismutase (SOD) [24]

- **Histological analysis of Synovial Membrane**

The Synovial membranes were excised from the animals, washed with the normal saline and kept into 10% normal formalin for 12-24 hrs. The synovial membrane then was dehydrated and cleared with ethanol and subsequently with xylene, respectively and then embedding in paraffin wax from which blocks had been prepared. Sections on 5µm thickness were prepared from the blocks by use of a microtome. These were processed in alcohol-xylene series and were stained together with Harris haematoxylin and Eosin (H&E) stain and subjected to histopathological examination [25].

2.6 Statistical Analysis

The results were expressed as Mean ± SEM from n=6 rats in each group. Data was analysed using statistical software Graph Pad Prism version 5. The significance of difference among the groups was assessed using one-way analysis of variance (ANOVA) followed by Tukey's test compared between Normal control (Untreated) vs. all groups $p<0.05$ was considered significant.

3. RESULTS

3.1 Macroscopic Arthritic Scoring and Footpad Swelling in CIA Rat Treated with *Moringa oleifera* Extract

Figs. 2 and 3 Maximal inhibition of arthritic scoring and footpad swelling was performed at the day sixteen after booster immunization among the exclusive groups of rat.

3.2 Assessment of Serum CRP Level

CRP is a sensitive marker of systemic inflammation and is elevated with the Arthritis. A rise in CRP is one of the main hallmarks of inflammatory conditions and High CRP levels can indicate an inflammatory condition like rheumatoid arthritis.

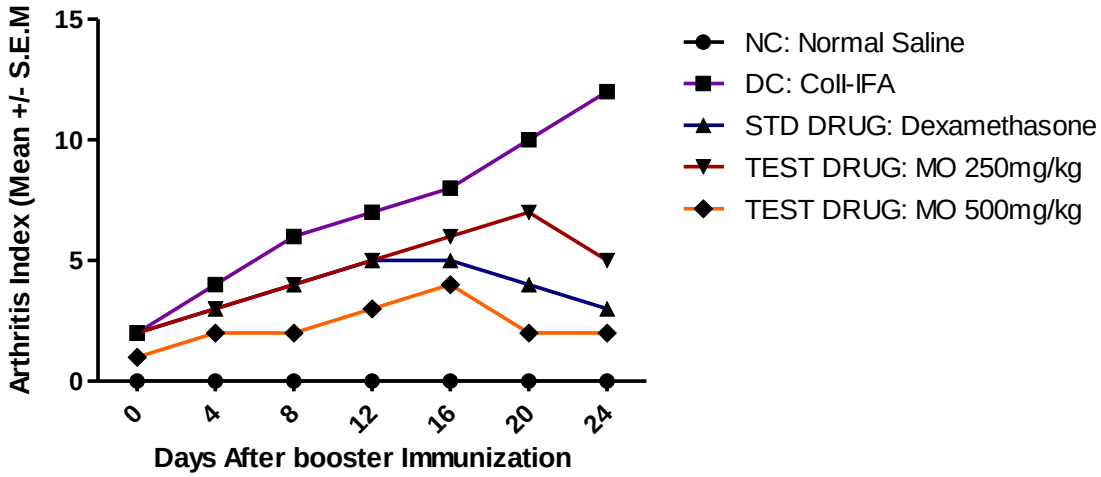


Fig. 2. Macroscopic arthritic scoring in CIA rat treated with Moringa oleifera extract
Values are expressed as Mean ± SEM, (n=6 rats in each group)

Table 2. Statistical comparison test (2 Way ANOVA and Bonferroni post-tests) on Macroscopic arthritic scoring between All the Groups

Row Factor	Difference	P value	Summary
NC: Normal Saline vs DC: CoII-IFA			
0	2.000	P<0.001	***
4	4.000	P<0.001	***
8	6.000	P<0.001	***
12	7.000	P<0.001	***
16	8.000	P<0.001	***
20	10.00	P<0.001	***
24	12.00	P<0.001	***
NC: Normal Saline vs STD DRUG: Dexamethasone			
0	2.000	P<0.001	***
4	3.000	P<0.001	***
8	4.000	P<0.001	***
12	5.000	P<0.001	***
16	5.000	P<0.001	***
20	4.000	P<0.001	***
24	3.000	P<0.001	***
NC: Normal Saline vs TEST DRUG: MO 250mg/kg			
0	2.000	P<0.001	***
4	3.000	P<0.001	***
8	4.000	P<0.001	***
12	5.000	P<0.001	***
16	6.000	P<0.001	***
20	7.000	P<0.001	***
24	5.000	P<0.001	***
NC: Normal Saline vs TEST DRUG: MO 500mg/kg			
0	1.000	P<0.001	***
4	2.000	P<0.001	***
8	2.000	P<0.001	***

Row Factor	Difference	P value	Summary
12	3.000	P<0.001	***
16	4.000	P<0.001	***
20	2.000	P<0.001	***
24	2.000	P<0.001	***
DC: CoII-IFA vs STD DRUG: Dexamethasone			
0	0.0000	P > 0.05	ns
4	-1.000	P<0.001	***
8	-2.000	P<0.001	***
12	-2.000	P<0.001	***
16	-3.000	P<0.001	***
20	-6.000	P<0.001	***
24	-9.000	P<0.001	***
DC: CoII-IFA vs TEST DRUG: MO 250mg/kg			
0	0.0000	P > 0.05	ns
4	-1.000	P<0.001	***
8	-2.000	P<0.001	***
12	-2.000	P<0.001	***
16	-2.000	P<0.001	***
20	-3.000	P<0.001	***
24	-7.000	P<0.001	***
DC: CoII-IFA vs TEST DRUG: MO 500mg/kg			
0	-1.000	P<0.001	***
4	-2.000	P<0.001	***
8	-4.000	P<0.001	***
12	-4.000	P<0.001	***
16	-4.000	P<0.001	***
20	-8.000	P<0.001	***
24	-10.00	P<0.001	***
STD DRUG: Dexamethasone vs TEST DRUG: MO 250mg/kg			
0	0.0000	P > 0.05	ns
4	0.0000	P > 0.05	ns
8	0.0000	P > 0.05	ns
12	0.0000	P > 0.05	ns
16	1.000	P<0.001	***
20	3.000	P<0.001	***
24	2.000	P<0.001	***
STD DRUG: Dexamethasone vs TEST DRUG: MO 500mg/kg			
0	-1.000	P<0.001	***
4	-1.000	P<0.001	***
8	-2.000	P<0.001	***
12	-2.000	P<0.001	***
16	-1.000	P<0.001	***
20	-2.000	P<0.001	***
24	-1.000	P<0.001	***
TEST DRUG: MO 250mg/kg vs TEST DRUG: MO 500mg/kg			
0	-1.000	P<0.001	***
4	-1.000	P<0.001	***
8	-2.000	P<0.001	***
12	-2.000	P<0.001	***
16	-2.000	P<0.001	***
20	-5.000	P<0.001	***
24	-3.000	P<0.001	***

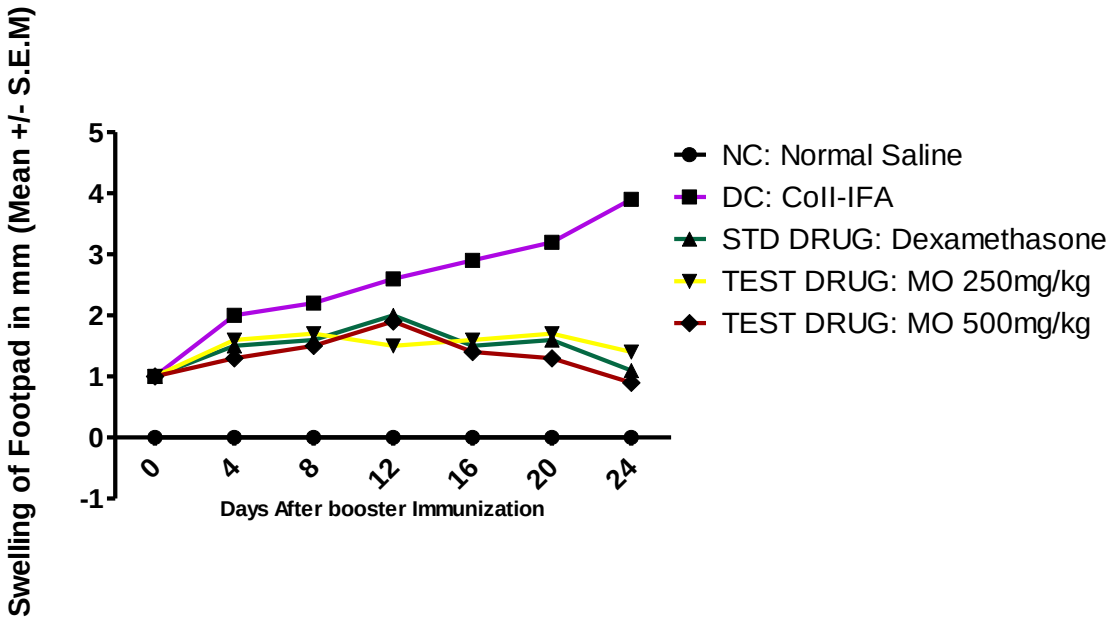


Fig. 3. Macroscopic footpad swelling in CIA rat treated with Moringa oleifera extract
Values are expressed as Mean ± SEM, (n=6 rats in each group).

Table 3. Statistical comparison test (2 Way ANOVA and bonferroni post-tests) on macroscopic footpad swelling between all the groups

Row Factor	Difference	P value	Summary
NC: Normal Saline vs DC: CoII-IFA			
0	1.000	P<0.001	***
4	2.000	P<0.001	***
8	2.200	P<0.001	***
12	2.600	P<0.001	***
16	2.900	P<0.001	***
20	3.200	P<0.001	***
24	3.900	P<0.001	***
NC: Normal Saline vs STD DRUG: Dexamethasone			
0	1.000	P<0.001	***
4	1.500	P<0.001	***
8	1.600	P<0.001	***
12	2.000	P<0.001	***
16	1.500	P<0.001	***
20	1.600	P<0.001	***
24	1.100	P<0.001	***
NC: Normal Saline vs TEST DRUG: MO 250mg/kg			
0	1.000	P<0.001	***
4	1.600	P<0.001	***
8	1.700	P<0.001	***
12	1.500	P<0.001	***
16	1.600	P<0.001	***
20	1.700	P<0.001	***
24	1.400	P<0.001	***
NC: Normal Saline vs TEST DRUG: MO 500mg/kg			
0	1.000	P<0.001	***
4	1.300	P<0.001	***

Row Factor	Difference	P value	Summary
8	1.500	P<0.001	***
12	1.900	P<0.001	***
16	1.400	P<0.001	***
20	1.300	P<0.001	***
24	0.9000	P<0.001	***
DC: CoII-IFA vs STD DRUG: Dexamethasone			
0	0.0000	P > 0.05	ns
4	-0.5000	P<0.001	***
8	-0.6000	P<0.001	***
12	-0.6000	P<0.001	***
16	-1.400	P<0.001	***
20	-1.600	P<0.001	***
24	-2.800	P<0.001	***
DC: CoII-IFA vs TEST DRUG: MO 250mg/kg			
0	0.0000	P > 0.05	ns
4	-0.4000	P<0.001	***
8	-0.5000	P<0.001	***
12	-1.100	P<0.001	***
16	-1.300	P<0.001	***
20	-1.500	P<0.001	***
24	-2.500	P<0.001	***
DC: CoII-IFA vs TEST DRUG: MO 500mg/kg			
0	0.0000	P > 0.05	ns
4	-0.7000	P<0.001	***
8	-0.7000	P<0.001	***
12	-0.7000	P<0.001	***
16	-1.500	P<0.001	***
20	-1.900	P<0.001	***
24	-3.000	P<0.001	***
STD DRUG: Dexamethasone vs TEST DRUG: MO 250mg/kg			
0	0.0000	P > 0.05	ns
4	0.1000	P > 0.05	ns
8	0.1000	P > 0.05	ns
12	-0.5000	P<0.001	***
16	0.1000	P > 0.05	ns
20	0.1000	P > 0.05	ns
24	0.3000	P<0.001	***
STD DRUG: Dexamethasone vs TEST DRUG: MO 500mg/kg			
0	0.0000	P > 0.05	ns
4	-0.2000	P<0.001	***
8	-0.1000	P > 0.05	ns
12	-0.1000	P > 0.05	ns
16	-0.1000	P > 0.05	ns
20	-0.3000	P<0.001	***
24	-0.2000	P<0.001	***
TEST DRUG: MO 250mg/kg vs TEST DRUG: MO 500mg/kg			
0	0.0000	P > 0.05	ns
4	-0.3000	P<0.001	***
8	-0.2000	P<0.001	***
12	0.4000	P<0.001	***
16	-0.2000	P<0.001	***
20	-0.4000	P<0.001	***
24	-0.5000	P<0.001	***

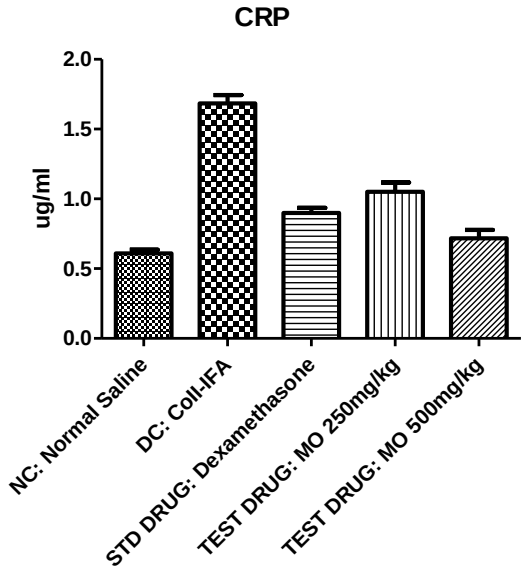


Fig. 4. Serum C - reactive protein level in CIA rat treated with *Moringa oleifera* extract
Values are expressed as Mean ± SEM, (n=6 rats in each group)

Table 4. Statistical comparison test on CRP between all the groups

Tukey's multiple comparison test	Mean Diff.	Significant? P < 0.05?	Summary
NC: Normal Saline vs DC: CoII-IFA	-1.075	Yes	***
NC: Normal Saline vs STD DRUG: Dexamethasone	-0.2917	Yes	**
NC: Normal Saline vs TEST DRUG: MO 250 mg/kg	-0.4417	Yes	***
NC: Normal Saline vs TEST DRUG: MO 500 mg/kg	-0.1083	No	ns
DC: CoII-IFA vs STD DRUG: Dexamethasone	0.7833	Yes	***
DC: CoII-IFA vs TEST DRUG: MO 250 mg/kg	0.6333	Yes	***
DC: CoII-IFA vs TEST DRUG: MO 500 mg/kg	0.9667	Yes	***
STD DRUG: Dexamethasone vs TEST DRUG: MO 250 mg/kg	-0.1500	No	ns
STD DRUG: Dexamethasone vs TEST DRUG: MO 500 mg/kg	0.1833	No	ns
TEST DRUG: MO 250 mg/kg vs TEST DRUG: MO 500 mg/kg	0.3333	Yes	**

3.3 Assessment of Serum Pro-Inflammatory Cytokines Level

Immune suppression or immune activation, have clinical relevance in both a prognostic and diagnostic level. Cytokines including IL6, IFN-G, TNF-A, and TGF-B that can be monitored throughout the course of disease and treatment to help understand the evolving immune environment and can be used as predictive/prognostic biomarkers. The concentrations of pro-inflammatory IL6, IFN-G, TNF-A, and TGF-B had elevated in the serum on the rat challenged with Type II collagen (CIA group) as compared with the STD and the test groups. Treatment with MO among CIA brought about rat appreciably lowered this cytokines into serum. On the other hand, the concentrations on of IL-6 is an important anti-inflammatory cytokine, was once decreased of CIA rat so compared to the STD and MO rat. Treatment with MO significantly restored IFN-G, TNF-A, levels in serum from CIA rat.

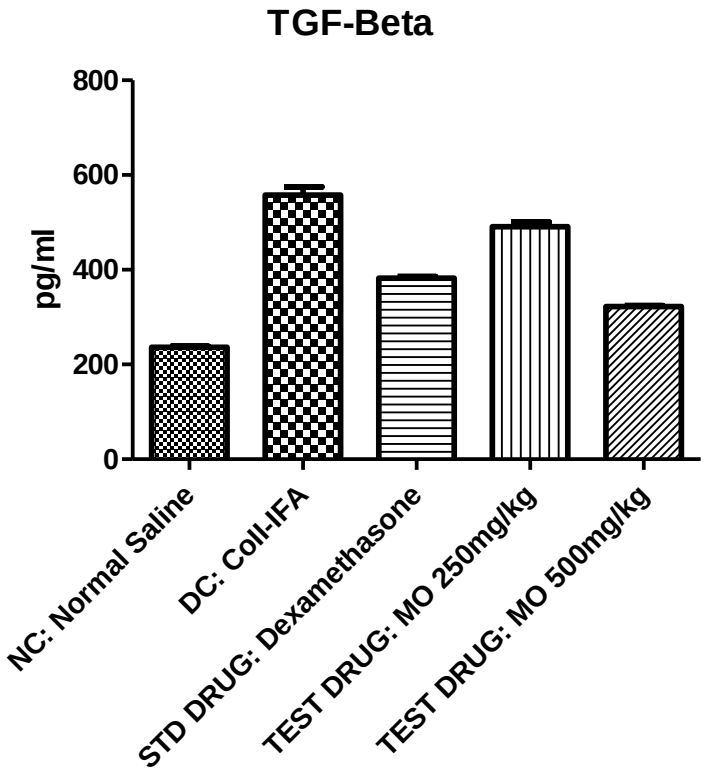


Fig. 5. Serum cytokines assessment; TGF-B Level in CIA rat treated with *Moringa oleifera* extract

Values are expressed as Mean ± SEM, (n=6 rats in each group)

Table 5. Statistical comparison test on TGF-B between all the groups

Tukey's multiple comparison test	Mean Diff.	Significant? P < 0.05?	Summary
NC: Normal Saline vs DC: CoII-IFA	-321.2	Yes	***
NC: Normal Saline vs STD DRUG: Dexamethasone	-146.2	Yes	***
NC: Normal Saline vs TEST DRUG: MO 250 mg/kg	-254.3	Yes	***
NC: Normal Saline vs TEST DRUG: MO 500 mg/kg	-85.50	Yes	***
DC: CoII-IFA vs STD DRUG: Dexamethasone	175.0	Yes	***
DC: CoII-IFA vs TEST DRUG: MO 250 mg/kg	66.83	Yes	***
DC: CoII-IFA vs TEST DRUG: MO 500 mg/kg	235.7	Yes	***
STD DRUG: Dexamethasone vs TEST DRUG: MO 250 mg/kg	-108.2	Yes	***
STD DRUG: Dexamethasone vs TEST DRUG: MO 500 mg/kg	60.67	Yes	***
TEST DRUG: MO 250 mg/kg vs TEST DRUG: MO 500 mg/kg	168.8	Yes	***

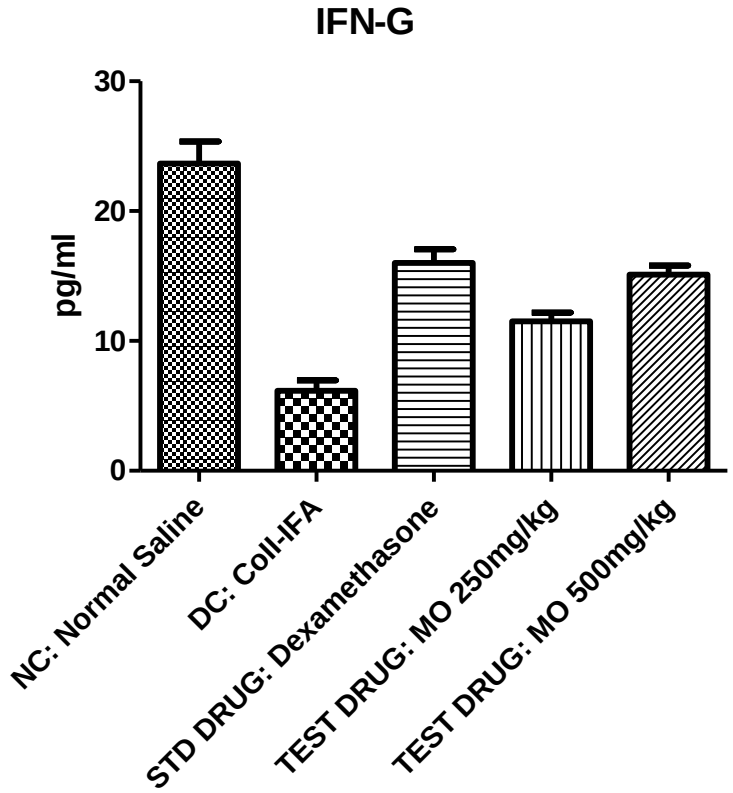


Fig. 6. Serum cytokines assessment; IFN-G Level in CIA rat treated with *Moringa oleifera* extract

Values are expressed as Mean ± SEM, (n=6 rats in each group)

Table 6. Statistical comparison test on IFN-G between all the groups

Tukey's multiple comparison test	Mean Diff.	Significant? P < 0.05?	Summary
NC: Normal Saline vs DC: CoII-IFA	17.50	Yes	***
NC: Normal Saline vs STD DRUG: Dexamethasone	7.667	Yes	***
NC: Normal Saline vs TEST DRUG: MO 250 mg/kg	12.15	Yes	***
NC: Normal Saline vs TEST DRUG: MO 500 mg/kg	8.553	Yes	***
DC: CoII-IFA vs STD DRUG: Dexamethasone	-9.833	Yes	***
DC: CoII-IFA vs TEST DRUG: MO 250 mg/kg	-5.348	Yes	*
DC: CoII-IFA vs TEST DRUG: MO 500 mg/kg	-8.947	Yes	***
STD DRUG: Dexamethasone vs TEST DRUG: MO 250 mg/kg	4.485	Yes	*
STD DRUG: Dexamethasone vs TEST DRUG: MO 500 mg/kg	0.8867	No	ns
TEST DRUG: MO 250 mg/kg vs TEST DRUG: MO 500 mg/kg	-3.598	No	ns

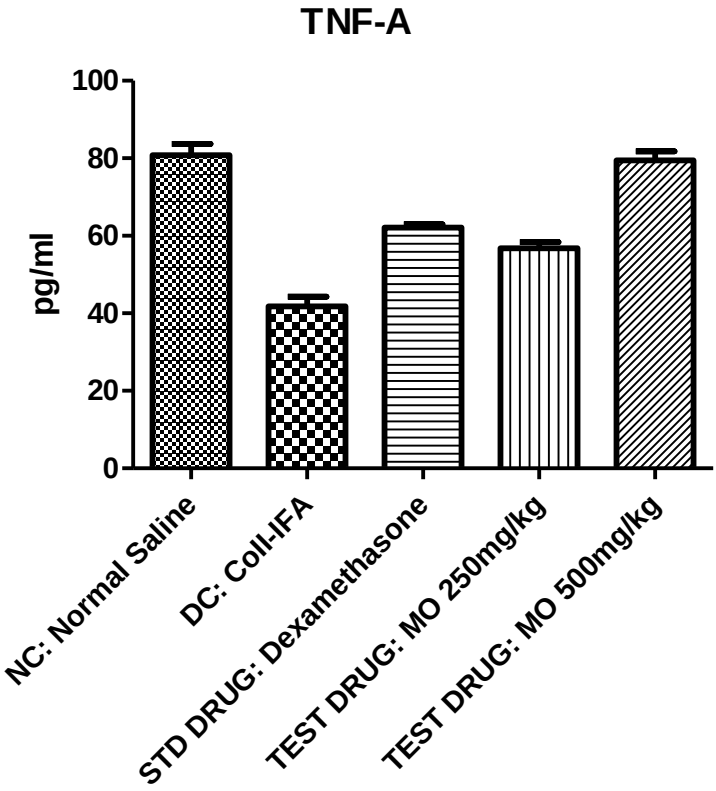


Fig. 7. Serum cytokines assessment; TNF-A Level in CIA rat treated with *Moringa oleifera* extract

Values are expressed as Mean ± SEM, (n=6 rats in each group)

Table 7. Statistical comparison test on TNF-A between all the groups

Tukey's multiple comparison test	Mean Diff.	Significant? P < 0.05?	Summary
NC: Normal Saline vs DC: CoII-IFA	38.99	Yes	***
NC: Normal Saline vs STD DRUG: Dexamethasone	18.59	Yes	***
NC: Normal Saline vs TEST DRUG: MO 250 mg/kg	23.95	Yes	***
NC: Normal Saline vs TEST DRUG: MO 500 mg/kg	1.267	No	ns
DC: CoII-IFA vs STD DRUG: Dexamethasone	-20.40	Yes	***
DC: CoII-IFA vs TEST DRUG: MO 250 mg/kg	-15.04	Yes	***
DC: CoII-IFA vs TEST DRUG: MO 500 mg/kg	-37.72	Yes	***
STD DRUG: Dexamethasone vs TEST DRUG: MO 250 mg/kg	5.360	No	ns
STD DRUG: Dexamethasone vs TEST DRUG: MO 500 mg/kg	-17.32	Yes	***
TEST DRUG: MO 250 mg/kg vs TEST DRUG: MO 500 mg/kg	-22.68	Yes	***

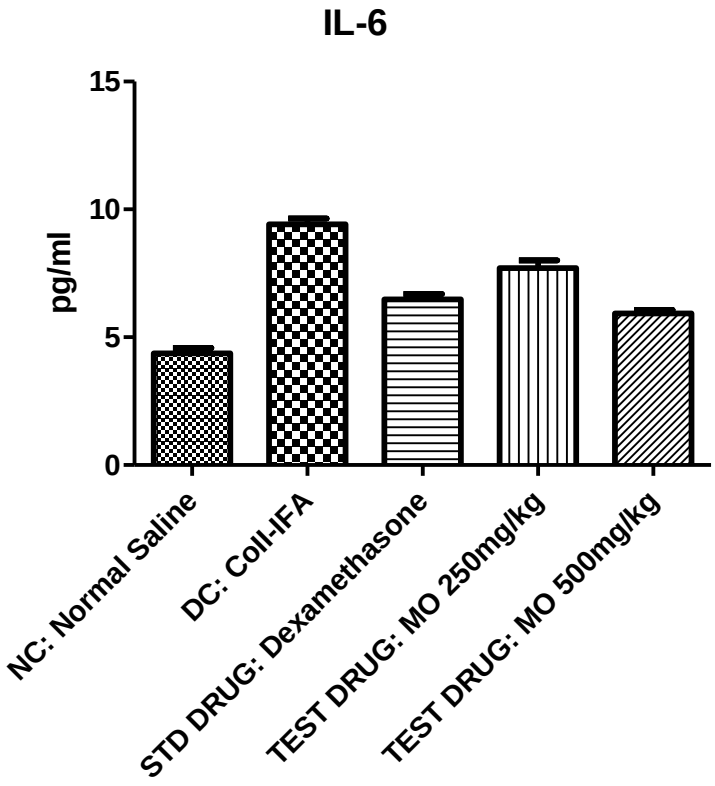


Fig. 8. Serum cytokines assessment; IL-6 Level in CIA rat treated with *Moringa oleifera* extract
Values are expressed as Mean ± SEM, (n=6 rats in each group)

Table 8. Statistical comparison test on IL-6 between all the groups

Tukey's multiple comparison test	Mean Diff.	Significant? P < 0.05?	Summary
NC: Normal Saline vs DC: CoII-IFA	-5.050	Yes	***
NC: Normal Saline vs STD DRUG: Dexamethasone	-2.122	Yes	***
NC: Normal Saline vs TEST DRUG: MO 250 mg/kg	-3.345	Yes	***
NC: Normal Saline vs TEST DRUG: MO 500 mg/kg	-1.570	Yes	***
DC: CoII-IFA vs STD DRUG: Dexamethasone	2.928	Yes	***
DC: CoII-IFA vs TEST DRUG: MO 250 mg/kg	1.705	Yes	***
DC: CoII-IFA vs TEST DRUG: MO 500 mg/kg	3.480	Yes	***
STD DRUG: Dexamethasone vs TEST DRUG: MO 250 mg/kg	-1.223	Yes	**
STD DRUG: Dexamethasone vs TEST DRUG: MO 500 mg/kg	0.5517	No	ns
TEST DRUG: MO 250 mg/kg vs TEST DRUG: MO 500 mg/kg	1.775	Yes	***

3.4 Effect on Antioxidant Enzyme Study Level in CIA Rat Treated with *Moringa oleifera* Extract

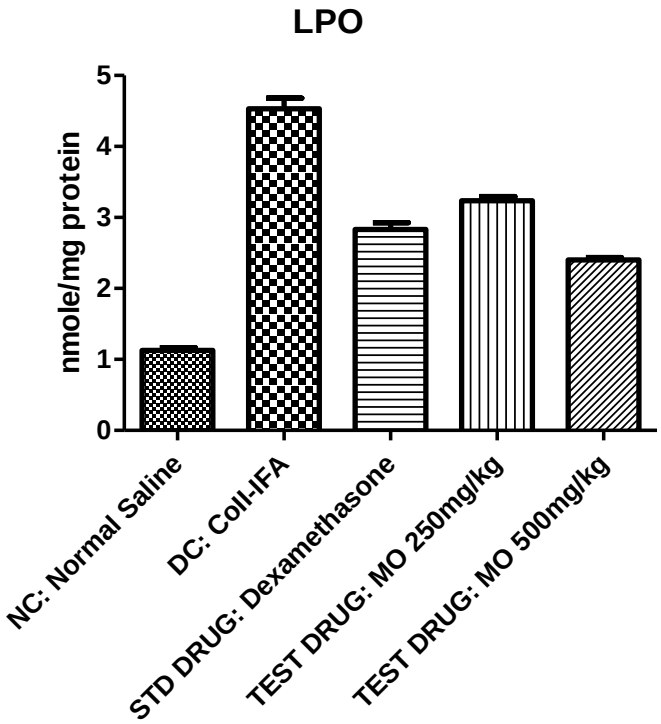


Fig. 9. Serum anti-oxidants assessment; LPO Level in CIA rat treated with *Moringa oleifera* extract

Values are expressed as Mean ± SEM, (n=6 rats in each group)

Table 9. Statistical comparison test on LPO between all the groups

Tukey's multiple comparison test	Mean Diff.	Significant? P < 0.05?	Summary
NC: Normal Saline vs DC: CoII-IFA	-3.405	Yes	***
NC: Normal Saline vs STD DRUG: Dexamethasone	-1.705	Yes	***
NC: Normal Saline vs TEST DRUG: MO 250 mg/kg	-2.105	Yes	***
NC: Normal Saline vs TEST DRUG: MO 500 mg/kg	-1.272	Yes	***
DC: CoII-IFA vs STD DRUG: Dexamethasone	1.700	Yes	***
DC: CoII-IFA vs TEST DRUG: MO 250 mg/kg	1.300	Yes	***
DC: CoII-IFA vs TEST DRUG: MO 500 mg/kg	2.133	Yes	***
STD DRUG: Dexamethasone vs TEST DRUG: MO 250 mg/kg	-0.4000	Yes	*
STD DRUG: Dexamethasone vs TEST DRUG: MO 500 mg/kg	0.4333	Yes	*
TEST DRUG: MO 250 mg/kg vs TEST DRUG: MO 500 mg/kg	0.8333	Yes	***

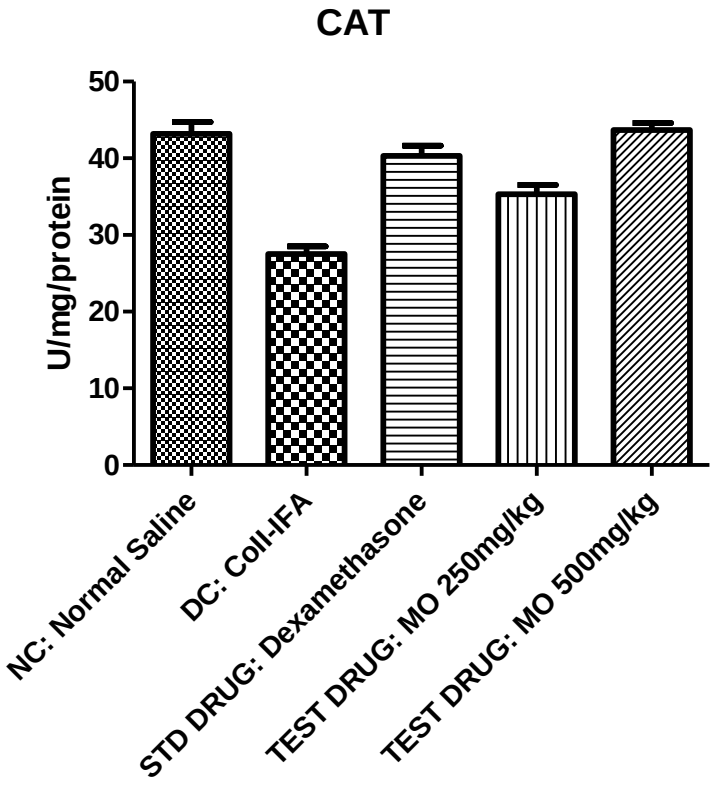


Fig. 10. Serum anti-oxidants assessment; CAT Level in CIA rat treated with *Moringa oleifera* extract

Values are expressed as Mean ± SEM, (n=6 rats in each group)

Table 10. Statistical comparison test on CAT between all the groups

Tukey's multiple comparison test	Mean Diff.	Significant? P < 0.05?	Summary
NC: Normal Saline vs DC: CoII-IFA	15.67	Yes	***
NC: Normal Saline vs STD DRUG: Dexamethasone	2.833	No	ns
NC: Normal Saline vs TEST DRUG: MO 250 mg/kg	7.833	Yes	**
NC: Normal Saline vs TEST DRUG: MO 500 mg/kg	-0.5000	No	ns
DC: CoII-IFA vs STD DRUG: Dexamethasone	-12.83	Yes	***
DC: CoII-IFA vs TEST DRUG: MO 250 mg/kg	-7.833	Yes	**
DC: CoII-IFA vs TEST DRUG: MO 500 mg/kg	-16.17	Yes	***
STD DRUG: Dexamethasone vs TEST DRUG: MO 250 mg/kg	5.000	No	ns
STD DRUG: Dexamethasone vs TEST DRUG: MO 500 mg/kg	-3.333	No	ns
TEST DRUG: MO 250 mg/kg vs TEST DRUG: MO 500 mg/kg	-8.333	Yes	***

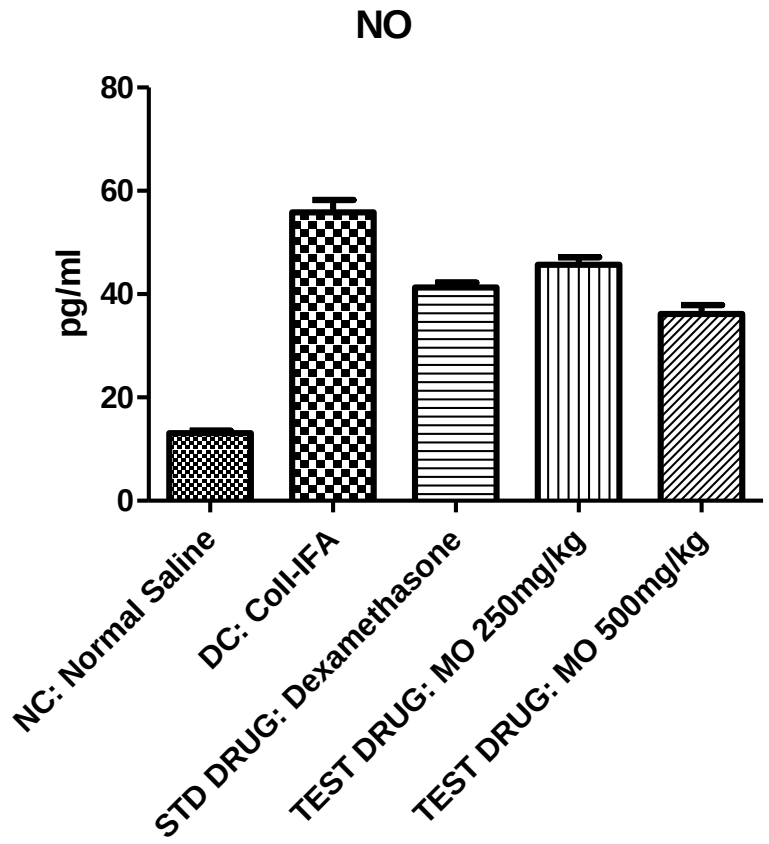


Fig. 11. Serum anti-oxidants assessment; NO Level in CIA rat treated with *Moringa oleifera* extract

Values are expressed as Mean ± SEM, (n=6 rats in each group)

Table 11. Statistical comparison test on NO between all the groups

Tukey's multiple comparison test	Mean Diff.	Significant? P < 0.05?	Summary
NC: Normal Saline vs DC: CoII-IFA	-42.77	Yes	***
NC: Normal Saline vs STD DRUG: Dexamethasone	-28.25	Yes	***
NC: Normal Saline vs TEST DRUG: MO 250 mg/kg	-32.58	Yes	***
NC: Normal Saline vs TEST DRUG: MO 500 mg/kg	-23.08	Yes	***
DC: CoII-IFA vs STD DRUG: Dexamethasone	14.52	Yes	***
DC: CoII-IFA vs TEST DRUG: MO 250 mg/kg	10.18	Yes	***
DC: CoII-IFA vs TEST DRUG: MO 500 mg/kg	19.68	Yes	***
STD DRUG: Dexamethasone vs TEST DRUG: MO 250 mg/kg	-4.333	No	ns
STD DRUG: Dexamethasone vs TEST DRUG: MO 500 mg/kg	5.167	No	ns
TEST DRUG: MO 250 mg/kg vs TEST DRUG: MO 500 mg/kg	9.500	Yes	**

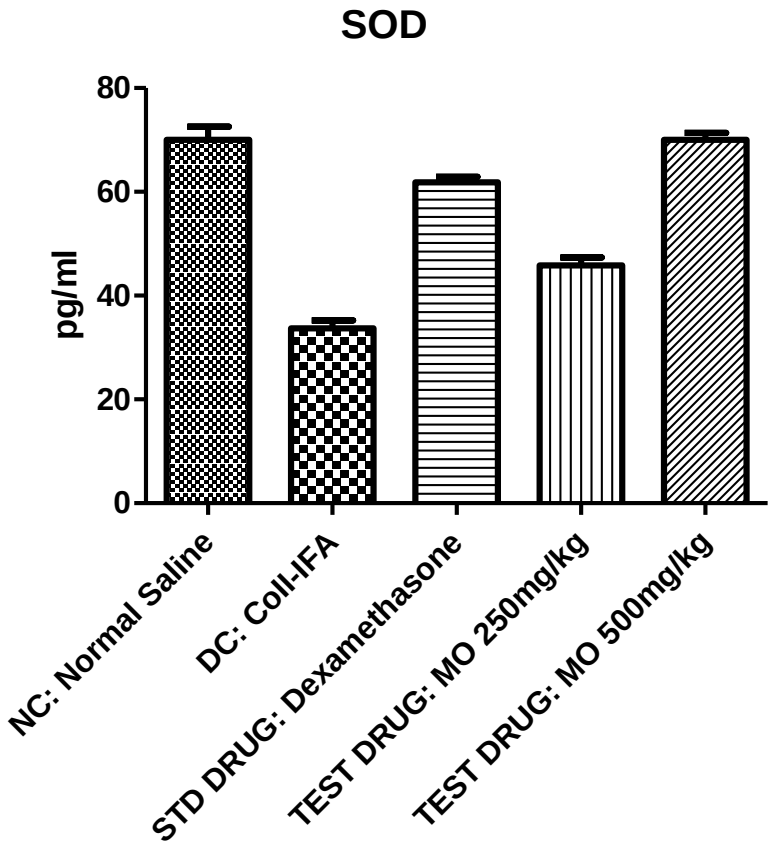


Fig. 12. Serum anti-oxidants assessment; SOD Level in CIA rat treated with *Moringa oleifera* extract

Values are expressed as Mean ± SEM, (n=6 rats in each group)

Table 12. Statistical comparison test on SOD between all the groups

Tukey's Multiple Comparison Test	Mean Diff.	Significant? P < 0.05?	Summary
NC: Normal Saline vs DC: CoII-IFA	36.33	Yes	***
NC: Normal Saline vs STD DRUG: Dexamethasone	8.167	Yes	*
NC: Normal Saline vs TEST DRUG: MO 250 mg/kg	24.17	Yes	***
NC: Normal Saline vs TEST DRUG: MO 500 mg/kg	0.0000	No	ns
DC: CoII-IFA vs STD DRUG: Dexamethasone	-28.17	Yes	***
DC: CoII-IFA vs TEST DRUG: MO 250 mg/kg	-12.17	Yes	***
DC: CoII-IFA vs TEST DRUG: MO 500 mg/kg	-36.33	Yes	***
STD DRUG: Dexamethasone vs TEST DRUG: MO 250 mg/kg	16.00	Yes	***
STD DRUG: Dexamethasone vs TEST DRUG: MO 500 mg/kg	-8.167	Yes	*
TEST DRUG: MO 250 mg/kg vs TEST DRUG: MO 500 mg/kg	-24.17	Yes	***

3.5 Histopathological Study

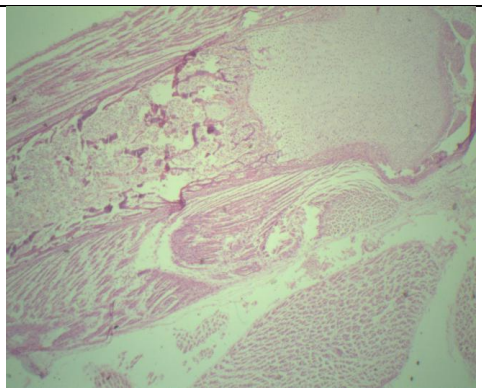
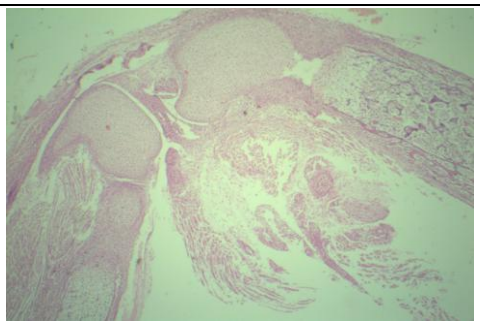
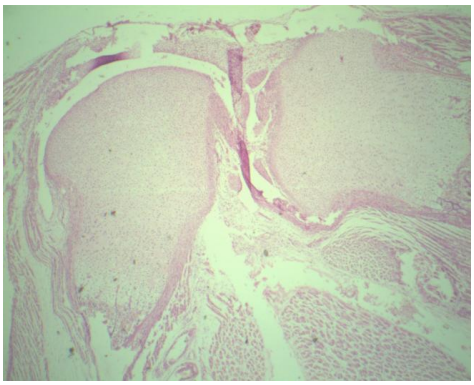
Disease Control - CoII-IFA		**INTERPRETATION** Loss over superficial and extreme layers, and the structural alternate in the articular cartilage showed informal surface layer with fibrillation, swelling and lysis sings in the matrix and the consonant exposure. The synovial lining cells exhibit evidence for widespread apoptosis. **Transverse section, H&E, X200s**
Standard Drug Group - Collagen Type II + Dexamethasone 0.025 mg/kg, b.w. p.o.		**INTERPRETATION** The joints were much less inflamed, and lymphocyte accumulation and cartilage damage decreased. Less disruption of the synovial lining cell layer. **Transverse section, H&E, X200s**
Test Drug Group- Collagen Type II + Moringa oleifera 500mg/kg,b.w. p.o		**INTERPRETATION** There have been losses among the superficial and deep layers into some areas then moderate surface irregularity. Swelling and lysis sings had been less compared with disease control group. Light microscopy showing marked less disruption of the synovial lining cell layer. **Transverse section, H&E, X200s**

Fig. 13. Image of synovial membrane showing the CIA rat treated with *Moringa oleifera* extract

4. DISCUSSION

Many therapeutic agents have been derived from natural sources. A variety of provocative arthritis models have been there for giving relieves from number disease which is associated with it. Although this fashions operate not affect each aspect of chronic pain signs and symptoms commonly associated with human RA, but they endure some key functions on the equal certain as CIA induced model predicting the preclinical efficacy over the therapies, and indicating the inflammatory pathways on arthritis, passive antibody mannequin predicting the exquisite and persistent manifestations on RA while the a number genetic models offering an perception to the genetic basis of the disease. These are into distinction to the CIA models who afford fast onset, predictable development on disease besides animal cost effective. We observed that intradermal injection of native CoII induces an inflammatory arthritis approximately with incomplete Freund's adjuvant. Type II collagen emulsified in oil causes the disease; that modern animal model on arthritis represents a unique instance of experimentally-inducible autoimmunity to a tissue component.

The immune environment, both at a systemic level and at the level of microenvironment plays a major role in the arthritis development and

progression. Cytokines as Prognostic Indicators for inflammation and Cytokines are mediators and are crucial for facilitating the recruitment and activation of specific subsets of leukocytes within the microenvironment of arthritis. Cytokines including IL6, IFN-γ, TNF-α, and TGF- β has been monitored throughout the course of disease and treatment to help understand the evolving immune environment. High IL-6 levels showed significantly poorer inflammation than with low IL-6 levels. TNF-α can act as an endogenous inflammatory promoter. High pre-treatment serum TGF-β levels were associated with poor prognoses arthritis is increased in chronic infections and inflammatory diseases. An increased IFNγ gene signature is correlated with higher overall response rates and longer median progression-free survival. Treatment of *Moringa oleifera* was able to decrease the level of these pro-cytokines levels and increase the levels of IFN-γ. Increase in INF-Gamma levels is indicative of an Immunoprotective and better outcomes. Overall impression was significant improvement in systemic immune suppression and inflammation. Improvement in markers is indicating a reduction in inflammation.

Peroxidation of membrane lipids by reactive oxygen species releases toxic by products such as MDA, [26] which in turn leads to activation of complement cascade, other cytokines, and systemic inflammatory response as a final consequence. MDA is directly associated with tissue injury [27]. In our study, the serum concentration of MDA was significantly elevated in CIA group and treatment appeared to prevent the elevation of MDA activity showing significantly lower serum concentration of MDA. Reported from literature which shows the antioxidant defense consists of SOD and CAT [28]. Reduced antioxidants levels in pancreas is suggestive of oxidative stress at tissue as well as systemic levels. Similarly, the CIA shows a significant decline in SOD and CAT levels, while MtEMO- and Dexamethasone treated groups showed a significantly higher level as compared to CIA group.

Induction of CoII also resulted in a significant raise in nitrate levels. MO treated group showed significantly reduced levels compared to disease group. Treatment with MtEMO dose-dependently reversed the change in nitrate levels. CRP is a sensitive marker of systemic inflammation and is elevated with the Arthritis. Here, we have seen the rise in the CoII-IFA group. A significant increase in serum concentrations of C-reactive protein (CRP) in the CIA rat challenged with type II collagen was observed (indicating acute tissue damage) but it has come down with the treatment of *Moringa oleifera*.

The elevated levels on IL6, IFN-G, TNF-A, and TGF-B has been found in model, eliciting the reality that each of immune-factors bears a primary function into Arthritis. This explains shielding position of MO against the damage or excitant reactions between the joint tissues throughout pathogenesis of CIA. IL6, IFN-G, TNF-A, and TGF-B were also found to be attenuated with MO treatment when measurement of serum rats was done. Inhibition on arthritic scoring and footpad swelling has been submerged with the treatment of MO. Protective impact on MO in Arthritis is further supported from histopathological studies as confirmed improvement in synovial membrane and Light microscopy showing marked less disruption of the synovial lining cell layer in treatment group.

5. CONCLUSION

The present study has demonstrated that the administration of methanolic extract of *Moringa oleifera* leaf improves against chronic inflammation during deleterious progression of Arthritis by down-regulating synovial gene expression and pro-inflammatory mediator release. In-Vivo studies showed that the exhibited anti-arthritis activity against CoII-IFA induced arthritis. The activity was confirmed by significant increment of gain in average body weight, and decrease in the level of arthritis scoring, footpad swelling, CRP, Level of LPO, NO increase in CAT, and SOD enzymatic antioxidants and histopathology of bone tissue of control and experimental animals were observed and shown less inflamed, and lymphocyte accumulation, cartilage damage decreased and less disruption of the synovial lining cell layer. In the study treatment of *Moringa oleifera* was able to decrease the level of pro-cytokines levels and increase the levels of IFN-γ and Improvement in markers indicating a reduction in inflammation. Due to presence of various-phytochemicals it is suspected that *Moringa oleifera* is showing anti-arthritis effect and also due to this it may be used traditionally as an anti-rheumatic drug. It can as well in pharmacological research for future drug discovery and development in the fields of rheumatology.

CONSENT

It is not applicable.

ETHICAL APPROVAL

All the experiments conducted on the animals were in accordance with the standards set for the use of the laboratory animal use and the experimental protocols were duly approved by the IAEC (Institutional Animal Ethical Committee) of Karnataka College of pharmacy, Bangalore. (IAEC Reg. Number: KCP/IAEC/09/21-22/09/18-12-21).

ACKNOWLEDGEMENT

The authors are grateful to Department of Pharmacology, Karnataka college of Pharmacy, Bangalore, India for their support.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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