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Research Article

Anti-Arthritic Activity of *Annona Reticulata* Leaves Methanolic Extract on Adjuvant Induced Arthritis in Rats

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Abstract

The research is expected to assess the effect of *Annona Reticulata* Methanolic leaves Extract (ARME) on progression of adjuvant induced arthritis in *Wistar* rats. ARME was obtained through bioactivity guided fraction by 5-Lipooxygenase inhibitory activity. ARME was administrated at 100 and 200mg/kg body weight via oral route for a period of 35 days to the experimental models. On day 8, via a single intra-plantar injection of 0.1mL suspension of heat killed Mycobacterium tuberculosis (100µg/animal) Arthritis was induced, in incomplete Freund's adjuvant of left foot pads of *Wistar* rats (female). The paw volume was observed by recording the hematological and biochemical parameters, hind limb paw volume, pathological and radiography changes to evaluate the effect of the extract. On day 35, levels of pro-inflammatory cytokines (TNF-α, IL-1β) and inflammatory mediators (PGE_2, LTB_4) was measured in serum samples. On the day of sacrifice, the liver was collected to record the stabilizing ability of lipid peroxide, activities of enzymatic antioxidants catalase, Superoxide Dismutase (SOD) and non-enzymatic antioxidants reduced glutathione (GSH) levels. ARME treated groups; 100 and 200mg/kg and prednisolone 10mg/kg significantly decreased hind paw volume, reduced serum levels of TNF-α, IL-1β, PGE_2 and LTB_4, reduced MDA levels and increased levels of catalase, SOD and GSH in the liver. The promising results of serum biochemistry, haematology, histopathology and radiographic changes suggest that the administration of ARME was able to effectively modulate the inflammatory response and conquer the advancement of arthritis in the experimental animal model. Results obtained from the acute oral toxicity limit test establish that ARME (LD_{50}>2000mg/kg) was safe in accordance to the OECD guideline 423. These findings may help to overcome some hurdles in the treatment of rheumatoid arthritis.

Keywords: Rheumatoid arthritis; Annona reticulata leaves; Methanolic extract (ARME); Inflammation; 5-Lipooxygenase; TNF-α; IL-1β; PGE_2; LTB_4

Introduction

Annona reticulata, most commonly known as ramphal in the Asian subcontinent in Southern Asia belongs to the family of Annonaceae. Most species of this family are known to contain medicinal properties which play a key role in metabolism. The extracts obtained from each part of the plant range through various medical potential. Annonaceous acetogenins have been known to have potential as anticancer agents. The extracts were obtained from the stems as well as the barks of *Annona reticulata* [1-4]. The role of herbal alternatives to manufactured medicines is gaining momentum in today's world more specifically, the function in the treatment of arthritis.

The human body has various mechanisms in place to prevent infections/disease. One such protective mechanism is inflammation; which is a defense mechanism against toxins, foreign substances and microbes invading the human vessel, disrupting homeostasis [5]. Rheumatoid Arthritis (RA) is an autoimmune disorder where in, deregulated white blood cell i.e., neutrophil activation leads to RA [6]. One of many mechanisms involved in RA is the release of ROS and other proteolytic enzymes which involves both; the innate and acquired immune systems [2]. RA is an inflammatory condition which affects ~1% of adults, which may lead to premature death or physical disability if not

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addressed in time [7]. RA has a higher occurrence in females as compared to males at an age group above 50. Various pathways within the human body are involved in the inflammatory process. Arachidonic Acid (AA) metabolism plays a major role in the same. Most manufactured drugs block the Cyclooxygenase (COX) and Lipoxygenase (LOX) pathways. As any other drug, the anti-inflammatory drugs display many side-effects which range from gastrointestinal irritation, ulcers to hypertension and cardiac abnormalities [8, 9].

Many intermediary mediators such as PGE_2, LTB_4 play a role in the COX and LOX pathways and thus can be the potential target to prevent inflammation [10]. Hence, herbal sourced products are more reliable and cause fewer side effects to those of manufactured drugs. They were used as 5-LOX inhibitors to treat inflammatory disorders such as arthritis [11].

Annona reticulata leaf extracts were tested for its various effects on the human body such as: antioxidant, anti-hypersensitive, anti-diabetic, anti-microbial, anti-inflammatory activities. The role of the leaf leaf extract was not explored in inflammatory and arthritis models to test its efficacy. Hence, the properties of the methanolic extract from the leaves of *Annona reticulata* were tested for 5-lipoxygenase inhibition and its role in treatment of arthritis.

Materials and Methods

Plant Material and Preparation of Extract

Annona reticulata plants' leaves were obtained from the forest area of Kondapalli, Andhra Pradesh, India, in January. The authentication of the plant was done in Department of Botany from Acharya Nagarjuna University by Prof. Dr. S. M. Khasim.

The initiation of the experiment was done by drying the leaves in the absence of sunlight followed by severing the leaves into small pieces. The pieces were ground into a coarse powder. Using the Soxhlet extraction method, the powdered material was extracted using: Hexane, Ethyl Acetate, Methanol and water. The raw material of the leaves was then extracted using the non-polar to polar grade above solvents (hexane, ethyl acetate, methanol, aqueous methanol, and water) using soxhlet apparatus.The extracts were concentrated and later dried using a rotary evaporator under limited pressure. 5-LOX; potent inhibitory fraction was obtained through bioactivity fractionation was often used for *in vivo* efficacy and toxicity studies. The most potent 5-LOX inhibitor was that of the methanolic extract (ARME).

Experimental Animals

Strain of Female *Wistar* rats weighing from a range of 180g to 220g were housed in a group of 3 per cage. Cages were made of polypropylene and stainless steel with the bedding of paddy husk and standard rodent diet of Nutrilab manufactured in Provimi Pvt. Ltd. Water used was UV sterlised supplied ad libitum to all cages. The temperature was maintained at 22±3°C, humidity of 30% - 70% and light (12h light and 12h dark). All experiments involving animals were performed in line with the standard protocol and guidelines of the Animal Ethical Committee, Govt. of India Reg.No. 1629/PO/a/12/CPCSEA, after proper approval.

In vitro 5-Lipoxygenase (5-LOX) enzyme inhibitory assay 5-LOX enzyme inhibitory activity of AMME was measured using the method of [12,13]. The assay mixture contained 80M linoleic acid and 10 µL potato 5-lipoxygenase enzyme in 50 mM phosphate buffer (pH 6.3). The reaction was initiated by addition of the enzyme buffer mix to linoleic acid (substrate) and its activity was observed as increase in absorbance at 234 nm.

The reaction was observed in UV-Kinetic mode on Varian Cary-50 UV- Vis spectrophotometer for 2 minutes. Various test substances were used to observe the inhibitory potential at different concentrations of the test substances, for 120 seconds

Table 1: FCA induced Arthritis - Body weights.

Test item	Dose (mg/kg)	Body weight (g)					
		Day 0	Day 7	Day 14	Day 21	Day 28	Day 35
Control	-	164.33 ± 8.33	182.67 ± 9.67	191.00 ± 8.49	198.33 ± 8.78	205.33 ± 10.39	195.17 ± 10.40
ARME	100	167.17 ± 7.55	186.00 ± 7.72	199.33 ± 8.36	211.00 ± 7.80	222.50 ± 7.71	234.67 ± 8.07
	200	167.33 ± 8.87	186.00 ± 9.38	200.00 ± 9.27	211.17 ± 9.83	221.50 ± 10.84	233.33 ± 11.31
Prednisolone	10	166.17 ± 6.82	181.33 ± 7.28	190.83 ± 7.91	198.83 ± 9.35	202.00 ± 9.94	211.33 ± 9.91

Values are expressed as Mean ± S.D, n=6 and S.D: Standard Deviation

Table 2: FCA induced Arthritis - Paw oedema.

Test item	Dose (mg/kg)	Paw oedema (mL) after treatment with extract			
		Day 14	Day 21	Day 28	Day 35
Control	-	1.82 ± 0.09	2.71 ± 0.38	3.07 ± 0.31	3.34 ± 0.41***
ARME	100	1.81 ± 0.11	2.39 ± 0.15	2.28 ± 0.13***	2.18 ± 0.11***
	200	1.81 ± 0.10	2.08 ± 0.11***	1.78 ± 0.05***	1.61 ± 0.07***
Prednisolone	10	1.31 ± 0.15***	1.41 ± 0.19***	1.10 ± 0.24***	0.76 ± 0.44***

Values are expressed as Mean ± S.D, n=6 and ***$P \leq 0.001$ versus control, S.D: Standard Deviation

Table 3: FCA induced Arthritis - Biochemical Analysis on day-35.

Biochemical parameter	AST/SGOT (IU/L)	ALT/SGPT (IU/L)	ALP (IU/L)	Total Bilirubin (IU/L)	Total Protein (g/dL)	Albumine (mg/dL)	Cholesterol (mg/dL)	Tgl (mg/dL)	Creatinine (mg/dL)	Urea (mg/dL)	CRP (mg/L)
Control	151.17 ± 13.03	110.50 ± 9.29	198.83 ± 16.55	0.73 ± 0.08	10.41 ± 0.66	4.59 ± 0.33	90.50 ± 5.09	98.50 ± 10.09	1.63 ± 0.12	42.77 ± 2.04	7.18 ± 0.49
ARME 100 mg/kg	126.50 ± 8.76***	68.33 ± 4.84***	153.17 ± 10.98***	0.39 ± 0.05***	7.13 ± 0.66***	3.69 ± 0.42***	83.00 ± 6.29	80.50 ± 2.43***	0.82 ± 0.06***	28.65 ± 1.25***	4.11 ± 0.22***
ARME 200 mg/kg	127.00 ± 6.99***	67.33 ± 5.65***	153.17 ± 11.92***	0.32 ± 0.06***	7.28 ± 0.56***	3.64 ± 0.51***	84.33 ± 8.26	80.00 ± 0.89***	0.87 ± 0.07***	27.56 ± 2.98***	3.87 ± 0.21***
Prednisolone	136.50 ± 6.57*	105.17 ± 2.48	179.33 ± 11.98	0.30 ± 0.08***	7.12 ± 0.21***	3.37 ± 0.35***	83.67 ± 8.38	92.67 ± 4.97	1.47 ± 0.11*	27.50 ± 1.56***	3.49 ± 0.29***

Values are expressed as Mean ± S.D, n=6 and *$P \leq 0.05$, ** $P \leq 0.01$ and, *** $P \leq 0.001$ versus control, S.D: Standard Deviation

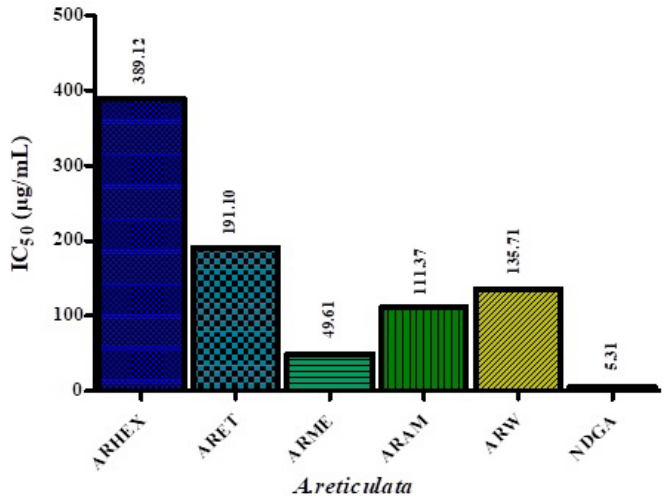

Figure 1: 5-Lipoxygenase Inhibitory Activity.

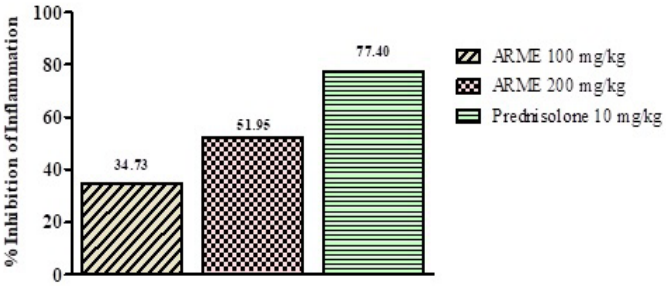

Figure 2: FCA induced Arthritis –Percentage Inhibition of Paw oedema.

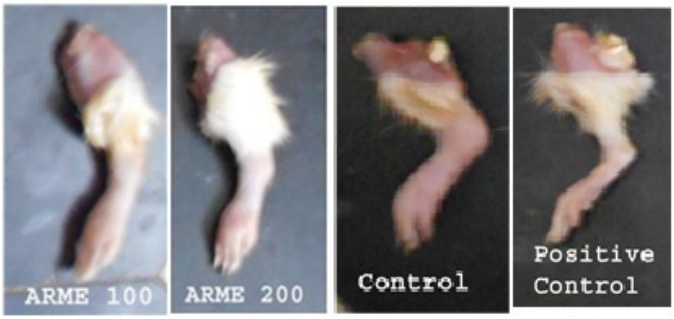

Figure 3: FCA induced Arthritis – Paw Swelling.

before addition of the substrate. Assays were done in triplicates and an average was used for calculation. Inhibition percentage was calculated by comparing increase of absorbance to control of enzyme activity. The activity of plantextracts was compared with the standard positive control Nordihydroguaiaretic acid (NDGA).

Acute Toxicity Study

The acute toxicity of ARME was determined as per the OECD 423 guideline (Acute oral toxicity class method) [14]. Six animals (Step I and II) were used for screening of acute oral toxicity. The animals were observed for clinical signs for a period of 2 weeks/14 days after a single oral dose of ARME was administered at 2000mg/kg body weight.

Adjuvant Induced Arthritis

In vitro screened potent bioactive fraction was used to test its efficacy *in vivo* for chronic inflammation using Freund's Complete Adjuvant [FCA] Induced Arthritis in Wistar rats as the experimental model. Wistar rats were divided into 4 groups of 6 animals per group. Group I animals served as arthritic control and received 0.5% CMC. The required quantity of test substance was weighed using an analytical balance and transferred into a mortar and pestle for further breakdown. The required volume of CMC was added and triturated to obtain a final concentration of 10 and 20mg/mL of homogenized test substance. For positive control, standard drug Prednisolone 10mg/kg in 0.5% CMC was used. From day 0 to day 35, animals were administered their respective dose (dose volume calculated based on measured body weights recorded on a weekly basis) based on the group, via the oral route. After dose administration, the rats were immediately transferred to the respective cages. The treatment regimen was as follows; Group I- arthritic animals without drug treatment, Group II- arthritic animals treated with ARME (100mg/kg), Group III- arthritic animals treated with ARME (200mg/kg) and Group IV was arthritic animals treated with Prednisolone (10mg/kg).

Arthritis was induced in all groups of *Wistar* rats on day 8. This was done using a single intra-plantar injection of 0.1mL suspension of heat killed M. tuberculosis (100µg/animal) incomplete Feund's adjuvant (Sigma-Aldrich, USA), into the sub plantar region of left hind limb. [FCA Preparation: The required quantity of heat killed M. tuberculosis H37Ra (Difco laboratories) was suspended in Freund (100µg/animal) in incomplete Feund's adjuvant (Sigma-Aldrich, USA), into the sub plantar region of left hind limb. [FCA Preparation: The required quantity of heat killed Mycobacterium tuberculosis H37Ra (Difco laboratories) was suspended in Freund's Incomplete Adjuvant and triturated using a mortar and pestle to obtain a FCA suspension containing 1mg/mL of Mycobacterium tuberculosis H37Ra].

Animals were observed for clinical signs once day, everyday throughout the duration of the study. Before administration of dose body weights were recorded and weekly once there after during the course of the study and prior to the termination of the study. Paw volume was measured using a plethysmometer (UGO basile) on days 0 (prior to treatment), 7 (prior to AIA induction), 20, 24, 28 and 35. Oedema volume was calculated as the difference between the final and initial paw volume. The percent inhibition of oedema volume was calculated through the formula;

$$\{(Vc-Vt / Vc)\} X 100$$

Where,

V0 - Oedema volume of normal control group

Vc- Oedema volume of AIA control group

Vt - Oedema volume of treatment group

Blood Analysis

On day 35, blood was collect through a small puncture in the retro-orbital plexus, under isoflurane anesthesia. Using a Humstar 600 automated biochemistry analyser blood was centrifuged at a speed of 1500 rpm for a short span of 15 minutes total bilirubin, total protein, albumin, cholestrol, triglycerides, creatinine, urea and CRP AST/SGOT, ALT/SGPT and to separate serum from the sample. On day 35, the rats were sacrificed and blood was collected in 1% EDTA sample tubes for hematological analysis i.e., RBC, WBC, heamoglobin (Hb), Lymphocytes and PCV using an Automated Hematology Analyzer (Humacount 60ts, HUMAN and Germany). ESR (Erythrocyte Sedimentation Rate) is an assay used to screen for an active disease (presence or absence) and measured using modified Westgren Method in mm/hr.

Table 4: FCA induced Arthritis - Hematology on day-35.

Test item	Dose (mg/kg)	Hematology Analysis						
		ESR (mm/hr)	RBC count ($x10^6$ Cells/mL)	WBC count ($x10^3$ Cells/mL)	Haemoglobin (g/dL)	Platelet count (Laks/mL)	Lymphocytes%	PCV %
Control	-	13.80±0.36	5.85±0.22	12.12±0.55	10.37±0.38	9.78±0.64	68.71±1.28	26.25±0.85
ARME	100	10.02±0.30***	7.44±0.39***	6.78±0.54***	12.22±0.88**	9.07±0.37	57.43±0.44***	27.92±0.81**
	200	8.67±0.38***	7.27±0.23***	6.32±0.66***	12.62±0.57***	8.82±0.48**	47.35±0.58***	28.03±0.58**
Predniso-lone	10	6.95±0.70***	7.02±0.27***	6.08±0.46***	12.80±1.53***	8.24±0.26***	56.50±0.18***	28.82±0.50***

Values are expressed as Mean ± S.D, n=6 and **p≤0.01 and, ***p≤0.001 versus control, S.D: Standard deviation

Table 5: FCA induced Arthritis - Inflammatory Biomarkers in Serum on Day-35.

Test item	Dose (mg/kg)	Inflammatory Biomarkers			
		TNF-α (pg/mL)	IL-β(pg/mL)	PGE_2 (pg/mL)	LTB_4 (pg/mL)
Control	-	60.36±1.67	173.64±0.91	206.20±0.60	221.74±2.45
ARME	100	28.86±0.51***	128.68±1.06***	160.11±0.85***	170.66±1.73***
	200	26.36±0.46***	108.18±0.99***	141.56±1.34***	143.87±1.98***
Prednisolone	10	13.20±0.82***	64.12±0.91***	92.98±0.43***	103.70±1.74***

Values are expressed as Mean ± S.D, n=6 and ***p≤0.001 versus control, S.D: Standard deviation

Table 6: FCA induced Arthritis - In vivo antioxidant Activity in Liver tissue on Day-35.

Test item	Dose (mg/kg)	Antioxidant Activity			
		MDA (nM/mg protein)	Catalase (U/mg protein)	SOD (U/mg protein)	GSH (µg/mg protein)
Control	-	2.22±0.57	67.37±6.61	1.34±0.19	2.00±0.42
ARME	100	0.77±0.31***	80.60±3.54**	3.03±0.18***	2.79±0.53*
	200	0.60±0.34***	84.98±7.93***	4.17±0.27***	3.34±0.53***
Prednisolone	10	0.29±0.05***	77.95±5.34*	3.89±0.34***	3.51±0.23***

Cytokines and Inflammatory Mediators

Blood was collected by retro-orbital plexus puncture on day 35 under isoflurane anaesthesia. The serum was separated by centrifugation at 1500rpm for 15 minutes and the samples were analysed for pro-inflammatory cytokines (TNF-α, IL-1β inflammatory mediators PGE_2 and LTB_4) levels in serum was estimated using ELISA assay kit (R&D systems) according to manufacturer's protocol. Lastly, the specific chromophore reactions were read at a wavelength of 540nm in a micro well plate ELISA reader (BioRad, USA).

Antioxidant Studies

All experimental rats were euthanized through an overdose of ketamine followed by necropsy after termination of the study. The liver was collected and washed with cold saline, preserved at a temperature of -20°C until antioxidant analysis. Bradford Assay was performed for protein estimation in liver samples (using 20% liver homogenate), for the different groups of rats.

The enzymatic antioxidant activities like catalase estimated by Sinha 1972, Superoxide Dismutase (SOD) estimated by Kakkar and the non-enzymatic antioxidant activities like reduced glutathione (GSH) estimated by Ellman.

Histopathology and Radiology of Knee Joint

The tibio-tarsal joint of all animals were dissected, fixed using 10 % buffered formalin and later decalcified in a 1:1 formic acid and distilled water for 7 days, after termination of the study. Dehydration of tissue samples at various concentrations of isopropyl alcohol i.e. 70, 80, 90 and 100 % was performed for a time period of two hours. It was then processed for paraffin embedding and sectioned into a thickness of 3-5 µm. The slides were examined under light microscope for histopathological lesions after staining using Hematoxylin and Eosin (H&E) and Toluidine Blue (TB). The ankle joints were fixed in 10% buffered formalin and was subjected to radiography on Siemens triodors (100 MA at 100cm focal distance, 40 KV and 2 mAs exposure). The lateral radiographs were retained and exposure was recorded.

Statistical Analysis

Analysis of data was performed using one-way ANOVA as the primary test followed by Dunnett's test in graph pad prism software. All results were expressed in the form of Mean ± S.D and considered significant only if P>0.05.

Results

5-lipoxygenase Inhibitory Activity

5-Lipoxygenase enzyme inhibitory activity of *Annona reticulata* extracts ARHEX, ARET, ARME, ARAM and ARW exhibited inhibitory activity 5-Lipoxygenase enzyme by showing IC_{50} values as 389.12, 191.10, 49.61, 111.37 and 135.71µg/mL respectively, Standard NDGA showed an IC_{50} value of 5.31 µg/mL.5-Lipoxygenase enzyme inhibitory action of *Annona reticulata* was depicted Figure 1.

Acute Oral Toxicity

The experiment revealed the administration of ARME is safe and median lethal dose was found >2000mg/kg. Hence1/20th (100mg/kg) and 1/10^{th} (200mg/kg) of this dose were selected for further study.

Adjuvant Induced Arthritis

Administration of FCA (100µg/animal) produced an increase in the paw volume of all the animals, which was persistent throughout the observation period. Maximum joint swelling was observed between days 21 to 28, after which there was a gradual decrease except in the control group wherein paw

swelling was maintained till day 35 (Figure 3). Oral administration of ARME (100 & 200mg/kg) and prednisolone (10mg/kg) were efficacious in reducing the paw oedema significantly as compared to control group. The paw oedema showed the dose dependent and significant (P 0.001) reduction in *Annona Reticulata* Methanolic Extract animals (ARME 100 and 200mg/kg). Percent inhibition of paw oedema observed in AMME 100mg/kg treated animals was 0.82%, 11.74%, 25.90% and 34.73%, whereas in ARME 200mg/kg treated inhibition observed as 0.82%, 23.48%, 41.97% and 51.95% on day 14, 21, 28 and 35 respectively as compared to adjuvant induced arthritis (AIA) control. Table 2 and Figure 2 shows the study results obtained for FCA induced arthritis model. All animals were apparently healthy and normal throughout the study duration and no clinical signs of toxicity observed. No morbidity and mortality was observed during the study period (Table 1). No significant differences in body weights were observed in any of the test items treated groups as compared to normal control. However noticeable decrease in body weights was observed in Prednisolone treated group animals.

Biochemical Studies

Blood was collected by retro-orbital plexus puncture on day 35 and centrifuged for serum at 1500rpm for 15 minutes and subjected to biochemistry analysis. The increased serum SGOT/AST, SGPT/ALT, ALP, total bilirubin, total protein, albumin, creatinine, Urea, triglycerides and CRP levels was observed in AIA control group on day 35,whereas treatment with ARME (100 and 200mg/kg) and prednisolone (10mg/kg) caused a highly significant (P≤0.001) reduction in AST, ALT, ALP, total bilirubin, total protein, albumin, creatinine, urea, triglycerides and CRP levels when compared to AIA control on day-35. Whereas serum cholesterol levels of AIA control group showed an increased activity in day 35 serum sample but treatment with ARME (100mg/kg) and prednisolone (10mg/kg) did not caused a significant reduction in cholesterol level when compared to AIA control. All the serum biochemistry results were depicted in (Table 3).

Hematological Analysis

Blood was collected by retro-orbital plexus puncture in EDTA tubes from each animal on day 35 for the analysis of hematological parameters. High levels of ESR, WBC, platelets and lymphocytes percentage was observed in AIA control group, whereas in ARME (100 and 200mg/kg) and prednisolone (10mg/kg) treated groups it was significantly recovered to normal levels. The reduced levels of RBC, Haemoglobin and percentage packed cell volume was observed in AIA control group, whereas in ARME (100 and 200mg/kg) and prednisolone (10mg/kg) treated groups it was significantly increased. All the hematology results were depicted in (Table 4).

Inflammatory Biomarkers

Serum TNF-α, IL-1β, PGE2 and LTB4 Profile

There was an increase in serum pro-inflammatory cytokines such as TNF-α and IL-β levels observed in AIA control group on day 35. Whereas treatment with ARME (100 and 200mg/kg) and prednisolone (10mg/kg) caused a significant (p≤0.001) reduction in TNF-α and IL-β level when compared to AIA control on day-35.

There was an increase in serum inflammatory mediators such as PEG2and LTB4 levels observed in AIA control group when compared to normal control on day 35. However treatment with ARME (100 and 200mg/kg) and prednisolone (10 mg/kg) caused a significant (p≤0.001) reduction in PEG_2 and LTB_4 level when compared to AIA control on day-35 (Table 5).

Antioxidant Profile

MDA and Antioxidants Profile in Liver Lysates

MDA level as measured as TBARS was elevated in liver lysates of AIA control group. However the treatment with ARME (100 and 200mg/kg) and prednisolone (10mg/kg) significantly (p≤0.001) reduced the levels of MDA in liver lysates as compared to AIA control group. The enzymatic antioxidants catalase, SOD and reduced non-enzymatic antioxidant GSH levels in liver lysates of AIA control animals were found depleted. Treatment with ARME (100 and 200mg/kg) and prednisolone (10 mg/kg) significantly (p≤0.001) restored the catalase and SOD levels (Table 6).

Histopathology

Histopathology of tibio-tarsal joint of all group animals were screened in hematoxylin and eosin stained slides. Severe neutrophil infiltration, synovial hyperplasia, ankyloses, pannus formation, rough cartilage surface, small cracks and clumps of cartilage tissue and bone erosion was seen in knee joints of Arthritic control rats, whereas in ARME (100 and 200mg/kg) and prednisolone (10mg/kg) treated rats there were significant reduction in neutrophil infiltration, pannus formation, bone erosion, fibrosis and decreased cartilage degradation as shown in Figure 4. The results are comparable with reference standard drug Prednisolone.

Radiographic Changes

The clinical analysis of Rheumatoid Arthritis allows therapeutic monitoring which remains the standard method (Radiograph or X-Ray) for evaluating the disease progress. The loss of articular cartilage leads to diminished joint space, which may be brought about a variety of pathological mechanism. The degree of bone resorption, diminished joint space and tissue swelling was markedly reduced with ARME (100 and 200mg/kg) and prednisolone (10mg/kg) (Figure 5).

Discussion

In the present study, we have used one chronic inflammatory model by a chronic inflammatory agent freund's complete adjuvant for evaluating the anti-arthritic activity of the selected *Annona reticulata* methanol extract. Earlier studies have indicated that the different solvent extracts of the selected plant have shown a significant anti diabetic, cytotoxicity, analgesic, anti-inflammatory activity and anti-ulcer activity *Annona reticulata* [15]. The standard drug used for positive control is prednisolone, which is a corticosteroid useful for the treatment of inflammation and auto-immune conditions such as rheumatoid arthritis [16].

Generally the term inflammation is considered as a natural and primary physiologic defense mechanism that helps the body to protect itself against infection, burns, toxic chemical substances, allergens, pathogenic microorganisms and other noxious stimuli and has been implicated in the pathogens of arthritis, cancer, and stroke as well as in neurodegenerative and cardiovascular disease [17]. Chronic inflammation may be developed following acute inflammation and may last for weeks or months and in some instance for years. According to medical experts, an uncontrolled and prolonged persistent inflamma-

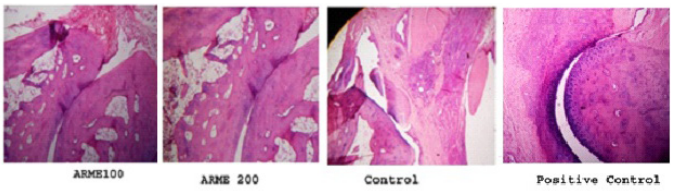

Figure 4: FCA induced Arthritis-Histopathology.

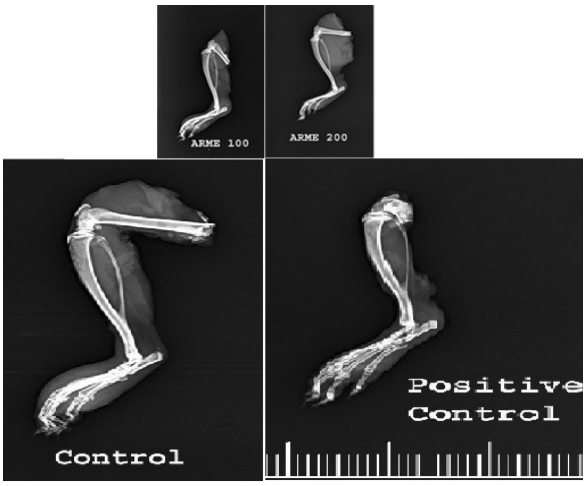

Figure 5: FCA induced Arthritis – Radiography.

tion may be one of the reasons for many of the chronic inflammatory diseases like rheumatoid arthritis [18].

This alternative therapeutic approaches able to modulate the immune system and disrupt the pro-inflammatory cascade through a variety of mechanisms, including antioxidant effects, alterations in cell signalling, cytokines and pro-inflammatory mediators. In a recent study, dietary supplements along with thyme and rosemary essential oils have shown a significant anti-inflammatory effect [19].

Rheumatoid arthritis is described by synovial inflammation, destruction of cartilage and bone, severe joint pain and finally deprives the function of limbs. The current study was conducted to assess the therapeutic effects of ARME at dose levels of 100 and 200mg/kg against an adjuvant induced arthritis rat model. This model is well known for studying regarding the pathogenesis of autoimmune arthritis as well as for the screening of anti-rheumatic drugs as this model has various similarities to human rheumatoid arthritis [20]. Administration of selected plants extracts significantly inhibited the arthritic paw oedema. The disease onset in rats commenced from day 14 and reached maximum on day 35 in the adjuvant induced arthritic rats (Control group). The most important index for evaluation of arthritis is the food pad swelling, these extracts showed the ability to inhibit the paw oedema. The test item treated groups effectively inhibited the progression of arthritis with significant reduction of the paw oedema.

The levels of AST, ALT and ALP measurement provide an excellent and simple tool to assess the anti-arthritic activity of tested compounds. CRP is prominent marker for inflammation was significantly reduced in ARME treated groups [21]. The serum biochemistry levels of different parameters showed highly significant reduction in plant extract treated animals when compared to control group animals, which is comparable with the positive control treated animals. This indicates the safety of compound without any side effects on liver and kidney.

Erythrocyte Sedimentation Rate (ESR) is an indirect measure of acute phase response to determine activity of rheumatoid arthritis [22]. Haemoglobin (Hb), fibrinogen, rheumatoid factor and immunoglobulins influence the ESR. The increased activity of ESR in arthritic rats provides information reflects the chronic nature and severity of disease. The increased level of ESR was significantly restored by ARME (100 and 200mg/Kg). The reduction in RBC, haemoglobin and Packed Cell Volume (PCV) in the adjuvant induced arthritic control rats denotes the anemic condition of rats; this represents the irregular storage of iron in the reticuloendothelial system and synovial tissue. Anemia is the common haemtological abnormality observed in rheumatoid arthritis [23]. These decreased levels of RBC, haemoglobin and PCV was significantly increased by ARME (100 and 200mg/Kg). The increased levels of WBC and platelet count in the adjuvant induced arthritic control rats due to the stimulation of immune system against the invading pathogens [24]. These increased levels of WBC and platelet count was significantly decreased by ARME (100 and 200mg/Kg). The increased levels of lymphocytes indicates incidences of active inflammation, lymphocytes are the highly responsible to the initiation and maintenance of immune response [25]. The concentration of lymphocytes in control group is high, whereas this concentration of lymphocytes in ARME (100 and 200mg/Kg).

The inflammatory cytokines TNF-α and IL-1β were analyzed in serum of all the experimental rats on day 35. The pro-inflammatory cytokines TNF-α and IL-1β produced from macrophages and other immune cells plays an important role in the pathogenesis of rheumatoid arthritis. These cytokines in turn supports the progression of inflammation, articular destruction, bone erosion, cartilage degradation and the comorbidities associated with rheumatoid arthritis. The inflammatory mediators PGE_2 and LTB_4 are the key inflammatory biomarkers, which are derived from the COX and LOX pathways of Arachidonic acid mechanism. They are involved in controlling the intensity and duration of inflammatory reactions, have cell and stimulus specific sources and recurrently have divergent effects. PGE_2 involved in the enhancing vascular permeability, vasodilation, blood flow and local pyrexia and potentiation of pain caused by other agents and it also encourages the production of MMPs and stimulates bone resorption. LTB_4 augments vascular permeability, local blood flow, leucocytes action and prompts release of lysosomal enzymes. This also enhances the release of ROS (Reactive Oxygen Species) and pro-inflammatory cytokines like TNF-α, IL-6 and IL-1β etc. The literature reveals high levels of PGE_2 and LTB_4 are found in serum and joint tissues in arthritis induced rat models. The compounds which are able reduce the activity of PGE_2 and LTB_4 are considered as a potential anti-inflammatory agents. The imbalance between pro-inflammatory and inflammatory cytokine activities aids the induction of autoimmunity, chronic inflammation and joint damage in arthritis. In the present study ARME 100 and 200mg/kg treated animal serum samples screened for TNF-α, IL-1β, PGE_2 and LTB_4 on day 35; the compound showed dose dependent and significant reduction. However the arthritic control showed increase in TNF-α, IL-1β, PGE_2 and LTB_4 levels in serum. The results can be comparable with positive control. Hence the plant extract exhibited potent significant activity in suppressing the pro-inflammatory cytokines and inflammatory mediators in adjuvant induced arthritis. As these TNF-α, IL-1β, PGE_2 and LTB_4 levels are key in the human rheumatoid arthritis, the effect of plant extracts may be beneficial in contribution to ameliorating the rheumatoid arthritis.

The macrophages and lymphocytes are activated in the adjuvant induced arthritis due to the structural similarity between

mycobacterium and rat cartilage proteoglycans. Therefore large amount of reactive oxygen species are produced by activated inflammatory cells and followed by inhibitory activity of anti-oxidant enzymes leads to the production of lipid peroxide. In the present study the lipid peroxide in liver samples was significantly increased in arthritic control rats, this increased level of lipid peroxide in liver samples was significantly reduced by the ARME (100 and 200mg/Kg) treated arthritic rats. This effect might be due to the presence of flavonoids in these methanolic extracts and their free radical scavenging power. Superoxide reacts with nitric oxide and forms highly toxic peroxynitrite, this peroxynitrites plays vital role in pathogenesis of inflammation. The Superoxide dismutase and catalase enzymes are key enzymes in obstructing superoxide radical activity. The Super-Oxide Dismutase (SOD) enzyme catalyzes the toxic Superoxide molecule into oxygen and water by dismutation. Catalase (CAT) enzyme decomposes hydrogen peroxide into oxygen and water. As the consequence of these enzymes actions decreases lipid peroxidase levels and protect against the oxidative damage of cells. Reduced glutathione (GSH) substrate augment the activity of Glutathione peroxidase (GPx) and Glutathione S-Transferase (GST) in catalyzing the hydrogen peroxide into oxygen and water. The reduced glutathione has the ability to reduce the oxidized glutathione, catalyzed by Glutathione Reductase (GR). Hence, reduced glutathione influences the activity of Glutathione Peroxidase (GPx), Glutathione Reductase (GR) and Glutathione S-Transferase (GST). The results in the present study shows that arthritic rats showed decreased activity of liver SOD, catalaseand GSH, these decreased activity of liver SOD and catalase was significantly restored by ARME (100 and 200mg/Kg).

Inflammatory cellular infiltration and prominent synovial hyperplasia were observed in Hematoxylin and Eosin tissue sections of arthritic control animals. In contrast, the histopathological changes were restored in ARME treated groups at 100 and 200mg/kg. The radiological analysis of the tibio tarsal joint in control and extracts treated animals further supports and confirms the potent anti-arthritic effect of *Annona reticulata* a dose dependent manner and it suppress the pathological changes such as pannus formation, bone destruction, degree of bone resorption and diminished joint space.

Conclusion

The present study results reveals good inhibitory activity in both acute and chronic inflammatory process similar to COX inhibitors and hence the methanolic extract of selected plants were selected basing on 5 LOX inhibition and antioxidant property can be used in amelioration of arthritis without

any side effects like mutagenic, hepatotoxic and nephrotoxic effects as generally we come across with the marketed COX inhibitors. This research work opens new path for the treatment of chronic inflammatory diseases like rheumatoid arthritis by inhibiting the 5 LOX pathway. The antioxidant activity exerted by ARME may be a part of their mechanism in preventing inflammation and arthritis. Even though animal models appeared to show effective and promising results in anti-inflammatory and anti-arthritic it is necessary for a detailed molecular study for the better understanding of the mechanism of action as well as specific compound involved.

Author Statements

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